

# **Toxicity of Lunar and Martian Dust Simulants to Alveolar Macrophages Isolated from Human Volunteers**

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**NASA is planning to build a habitat on the Moon and use the Moon as a stepping stone to Mars. JSC-1, an Arizona volcanic ash that has mineral properties similar to lunar soil, is used to produce lunar environments for instrument and equipment testing. NASA is concerned about potential health risks to workers exposed to these fine dusts in test facilities. The potential toxicity of JSC-1 and a Martian soil simulant (JSC-Mars-1, a Hawaiian volcanic ash) was evaluated using human alveolar macrophages (HAM) isolated from volunteers; titanium dioxide and quartz were used as reference dusts. This investigation is a prerequisite to studies of actual lunar dust. HAM were treated in vitro with these test dusts for 24 h; assays of cell viability and apoptosis showed that JSC-1 and TiO<sub>2</sub> were comparable, and more toxic than saline control, but less toxic than quartz. HAM treated with JSC-1 or JSC-Mars 1 showed a dose-dependent increase in cytotoxicity. To elucidate the mechanism by which these dusts induce apoptosis, we investigated the involvement of the scavenger receptor (SR). Pretreatment of cells with polyinosinic acid, an SR blocker, significantly inhibited both apoptosis and necrosis. These results suggest HAM cytotoxicity may be initiated by interaction of the dust particles with SR. Besides being cytotoxic, silica is known to induce shifting of HAM phenotypes to an immune active status. The immunomodulatory effect of the simulants was investigated. Treatment of**

1 **HAM with either simulant caused preferential damage to the suppressor macrophage**  
2 **subpopulation, leading to a net increase in the ratio of activator (RFD1<sup>+</sup>) to suppressor**  
3 **(RFD1<sup>+7+</sup>) macrophages, a result similar to treatment with silica. It is recommended that**  
4 **appropriate precautions be used to minimize exposure to these fine dusts in large-scale**  
5 **engineering applications.**

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16 Running Head: TOXICITY OF LUNAR AND MARTIAN DUST SIMULANTS

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**INTRODUCTION**

NASA has begun preparing to return to the Moon for permanent occupancy and research. Successful lunar habitation will be used as a stepping stone to a Mars mission. The projected lunar tour of duty will be 180 days, during which NASA will use the lunar soil in situ for various purposes, including extracting life-essential resources such as oxygen and fuel. JSC-1, an Arizona volcanic ash that has mineral properties similar to those of lunar soil (McKay et al., 1994), is used for various ground-based experiments on lunar soil utilization and for simulating lunar environments in which to test instruments and other hardware. This research includes environmental dust control, spacesuit durability, agriculture, and oxygen-production (Wagner, 2004). During these activities, workers at NASA and lunar project scientists and engineers at academic institutions could be exposed to this lunar soil simulant about which there is little toxicity data.

JSC-1 is a glass-rich basaltic ash derived from the San Francisco volcanic field near Flagstaff, Arizona (Glaser, 1992). It is very similar to lunar mare soil with respect to chemical composition (rich in silica and low in titanium), mineralogy (plagioclase, pyroxene, and olivine), and mechanical properties (specific gravity, angle of internal friction, and cohesion). These similarities have made it ideal for large-scale soil utilization studies involving agriculture and oxygen extraction for life support. Both lunar soil and the simulant, JSC-1, contain about 50%

1 SiO<sub>2</sub>; other common soil oxides (Al<sub>2</sub>O<sub>3</sub>, FeO, MgO, and CaO) account for another 42-45%. No  
2 trace or heavy metals were found in either lunar soil or JSC-1 (McKay et al., 1994).

3  
4 The lunar soil simulant, like lunar soil collected on the Apollo missions, contains a fraction of  
5 fine dust particles in the respirable size range. NASA is concerned about the potential health risk  
6 to workers who are exposed to this dust in Earth-based test facilities. In the study reported here,  
7 the interaction of lunar dust simulant with alveolar macrophages obtained from human  
8 volunteers and toxicity of the stimulant to the macrophages were assessed; titanium dioxide  
9 (TiO<sub>2</sub>), a low-toxicity dust, and quartz dust (SiO<sub>2</sub>), which is fibrogenic in the lungs, were  
10 included as reference dusts. A Martian soil simulant was also included in this study. JSC-Mars  
11 1 is a silica-containing volcanic ash derived from a cinder cone, Mauna Kea, in Hawaii. It is  
12 analogous to the bright regions of Mars based on reflectance spectral data obtained by Viking I  
13 and II and Pathfinder Landers. The simulant is comparable to Martian soil in mineralogy; both  
14 are rich in feldspar and Ti-magnetite [15-22% TiO<sub>2</sub>], with minor olivine and pyroxene in a  
15 glassy matrix. Furthermore, JSC-Mars 1 is high in ferric oxide, as is Martian soil (Allen et al.,  
16 1998).

17  
18 Both lunar and Martian soil simulants contain silica. Silica, especially in the crystalline form,  
19 causes lung inflammation and fibrosis on prolonged exposure. Macrophages are the first cell  
20 type to come into contact with dust in the lung. Silica and silicates in the lung cause macrophage  
21 injury leading to necrosis or apoptosis (Iyer et al., 1996). The alveolar macrophage is important  
22 in nonspecific host defense of the lung and is a central cell in regulating the inflammatory  
23 response in the lung. Necrosis involves rupture of the cell membrane with spillage of

1 intracellular contents. Apoptosis, which can be followed by necrosis, is a “programmed” cell  
2 death that does not involve rupture of cell membranes, but does involve an orderly series of  
3 morphological and biochemical changes in the cell cytoplasm, nucleus, and membrane. Changes  
4 that can be visualized by light microscopy include cytoplasmic shrinkage, chromatin clumping,  
5 membrane blebbing, and apoptotic body formation (Kimbell, 2007).

6

7 The mechanisms, by which particulates such as silica initiate alveolar macrophage injury have  
8 not been fully elucidated, but seem to involve scavenger receptors (SR). Scavenger receptors  
9 Class A I/II (SRA) and macrophage receptor with collagenous structure (MARCO) have been  
10 implicated in the mechanism of silica-induced toxicity to the alveolar macrophage (Hamilton et  
11 al., 2000; Hamilton et al., 2006). Silica-induced apoptosis has been blocked by pretreatment of  
12 the cells with polyinosinic acid, an SRA inhibitor (Iyer et al., 1996; Hamilton et al, 2000), and  
13 the requirement for SRA has been confirmed using Chinese hamster ovary cells transfected with  
14 the murine SRA (Hamilton et al, 2000). Here we report the results of a similar study with the  
15 lunar and Martian dust simulants.

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17 Lunar soil particles are powdery and have a great tendency to adhere to surfaces, including  
18 spacesuits. When these particles were transported on spacesuits into the cabin of Apollo  
19 spacecraft after extravehicular activities, they caused visibility problems on resumption of  
20 weightlessness. Some mechanical systems aboard the spacecraft were damaged due to dust  
21 contamination. Furthermore, astronauts inhaled the dust particles and reported nasal and eye  
22 irritation (Wagner, 2004). At least one Apollo astronaut reported symptoms resembling “hay  
23 fever” when exposed to lunar dust. Spectral data obtained from robotic Martian missions

1 indicate that Martian surface soil is both oxidative and reactive. Exposure to reactive Martian  
2 dust will create an even greater concern for the astronauts' health and the reliability of  
3 mechanical systems (Wagner, 2004).

4  
5 Silica is associated with immunological impairment in exposed workers (ICMR, 2001; Steenland  
6 et al., 2001). The immunomodulatory effects of silica have been previously reported.

7 Crystalline silica induces a shift of human alveolar macrophage (HAM) phenotypes to an  
8 immune active status; the treatment led to preferential damage to the suppressor (RFD1<sup>+</sup>7<sup>+</sup>)  
9 macrophage subpopulation, resulting in a net increase in the ratio of activator (RFD1<sup>+</sup>) to  
10 suppressor (RFD1<sup>+</sup>7<sup>+</sup>) macrophages (Holian et al., 1997). We hypothesize that the lunar and  
11 Martian soil simulants, both silicates, affect macrophage subpopulations similarly. Such a shift  
12 toward a more activated immune status could result in lung inflammation and increased risk for  
13 pulmonary and cardiovascular disease (Holian et al., 1998).

14  
15 The pulmonary toxicity of lunar dust simulant was previously investigated in mice exposed to  
16 the dust by intratracheal instillation; the results showed that the dust simulant is relatively low in  
17 acute toxic toxicity (Lam et al., 2002a), but high dose (1 mg/mouse) and prolong exposure (90-d)  
18 can cause fibrosis (Lam et al., 2002b). The present study with alveolar macrophages isolated  
19 from human volunteers complements the animal studies in revealing the toxicity of the lunar soil  
20 simulant and its mechanism of toxicity. NASA is planning to conduct toxicity studies with  
21 actual lunar dust. One goal of the present study, like that of the animal studies, is to establish  
22 experiment protocols that could be applied to the study of lunar dust. NASA requires that  
23 investigators conduct successful studies using the lunar dust simulant before they can receive

1 precious lunar soil for proposed studies, and our HAM study using the lunar soil simulant serves  
2 as a prerequisite to a toxicity study of real lunar dust as well as an investigation of the effect of  
3 the lunar and Martian dust simulants on human macrophages.

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5

## 6 **METHODS**

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### 8 **Test Dusts**

9

10 *Lunar and Martian Dust Simulants.* Samples of raw Arizona and Hawaii volcanic ashes  
11 [designated as JSC-1 (McKay, 1994) and JSC-Mars-1 (Allen et al., 1998)], respectively, were  
12 provided by the NASA Astromaterials Curator; the soil samples were provided to Lovelace  
13 Respiratory Research Institute for size fractionation. The fine particles from the second (50%  
14 cutoff aerodynamic diameter 1.95  $\mu\text{m}$ ) and third stages (0.28  $\mu\text{m}$ ) of the cascade impactors, and  
15 the backup filter were collected and pooled for the present study. Thus, the mass median  
16 aerodynamic diameter (MMAD) of both dusts was expected to be less than 5  $\mu\text{m}$ . Analysis by  
17 Microtrac Inc. (Montgomeryville, PA) showed that the respirable fraction of the lunar dust  
18 simulant had a mass median diameter (MMD) of 3  $\mu\text{m}$  or less and contained 5% large particles  
19 with an MMD of 81  $\mu\text{m}$ ; on ultrasonication, all of the large particles disappeared and the  
20 resulting suspension showed a bimodal distribution, with MMDs of 1.05  $\mu\text{m}$  (44%) and 2.99  $\mu\text{m}$   
21 (56%). The Martian dust simulant, which was recently analyzed by Particle Technology Labs  
22 (Downers Grove, IL) using TSI Aerosizer LD 8050 (TSI Incorporated, Shoreview, MN), showed  
23 the aerodynamic MMD of 0.93  $\mu\text{m}$  and particles with sizes  $>1.8 \mu\text{m}$  accounting for 5% mass.

1            *Titanium Dioxide and Crystalline Silica.* The titanium dioxide sample, a product of  
2 Particle Information Services (Kingston, MA), had an average particle diameter of 0.45  $\mu\text{m}$ .  
3 Crystalline silica (quartz) (acid-washed Min-U-Sil-5), which has a mass median diameter of 1.7  
4  $\mu\text{m}$  and in which 97% of the particles are less than 5  $\mu\text{m}$  was obtained from Pennsylvania Glass  
5 and Sand (Pittsburgh, PA). These particle-size specifications were from the respective vendors.

### 7 **Isolation of Alveolar Macrophages from Human Volunteers**

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9            HAM were obtained by bronchoalveolar lavage of healthy adult volunteers of both sexes  
10 as described previously (Dauber et al., 1979). This protocol was approved by the University of  
11 Texas Committee for the Protection of Human Subjects. Briefly, for each volunteer subject, the  
12 lung was washed 2 or 3 times with sterile saline (each instillation contained 240 to 300 ml) to  
13 collect about 200 to 600 ml of lavage fluid; the fluid was stored at 4°C until cells were isolated  
14 by centrifugation at 1500 revolutions/minute (rpm). The saline supernatant was aspirated and  
15 discarded, and cells were resuspended in a small volume (1 to 5 ml) of N-2-  
16 hydroxyethylpiperazine-N'-2-ethanesulfonic acid-buffered medium 199 (GIBCO-BRL,  
17 Bethesda, MD) with 10% heat-inactivated fetal calf serum (Sigma, St. Louis, MO) and  
18 antibiotics (50 U/ml penicillin, 50  $\mu\text{g}/\text{ml}$  gentamicin, and 50  $\mu\text{g}/\text{ml}$  streptomycin [GIBCO-BRL,  
19 Bethesda, MD]). Cells were counted using a ZBI Coulter Counter (Coulter Electronics, Hialeah,  
20 FL). Lavages yielded an average of  $2 \times 10^7$  cells that were > 92% HAM, as verified by  
21 Leukostat staining (Fisher Scientific, Houston, TX). Viability was > 90% as determined by  
22 trypan blue exclusion.



1 **Culture of Human Alveolar Macrophages**

2

3 Stock suspensions of test dusts were freshly prepared in buffered saline followed by brief  
4 sonication in a water bath just before they were added to HAM culture. Unless otherwise  
5 indicated, cells were cultured at  $1 \times 10^6$  cells/ml in the presence or absence of a test dust at a  
6 designated concentration for 24 h at 37°C and 5% CO<sub>2</sub> in a water-jacketed incubator (Queue,  
7 Parkersburg, WV). Cell suspension was maintained by end-over-end tumbling (Labquake  
8 Shakers, Labindustries, Berkley, CA) in sterile polypropylene tubes. Samples were not pooled,  
9 so that each dose response was conducted on cells derived from an individual volunteer.

10

11 **Cell Morphological Study**

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13 Immediately after cell culture,  $3 \times 10^4$  cells were incubated with phosphate-buffered  
14 saline (PBS, pH 7.2) and centrifuged for 5 minutes at 1,500 rpm onto positively charged glass  
15 slides (Probe On Plus, Fisher Scientific, Houston, TX) using a Shandon Cytospin 2 (Shandon  
16 Upshaw, Pittsburgh, PA). Slides were kept at 25°C until Leukostat fixation and staining were  
17 performed. After they were fixed in methanol for 90 seconds, the cells on the slides were stained  
18 in Leukostat eosin stain (Fisher Scientific, Houston, TX) for 90 seconds, and then in Leukostat  
19 methylene blue (Hema 3, Fisher Scientific, Houston, TX) for 4 seconds. Slides were air-dried  
20 and examined by light microscopy with a 600× dry objective (Zeiss Axioskop, Thornwood, NY).

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## 1 **Cell Viability Assay (Trypan Blue Exclusion)**

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3 To determine viability, cells were mixed with trypan blue dye (0.04% in PBS, Sigma, St.  
4 Louis, MO), placed on a hemocytometer, and examined under light microscopy at 600× dry  
5 objective (Zeiss Axioskop, Thornwood, NY). Viable cells exclude this dye and remain  
6 unstained.

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## 8 **Apoptosis Assay (Cell Death Enzyme-Linked Immunosorbent Assay)**

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10 Intra-nucleosomal DNA cleavage that occurs late in the process of apoptosis can be  
11 assessed by cell death enzyme-linked immunosorbent assay (ELISA), which determines  
12 cytosolic histone-bound DNA fragments. For this assay of apoptosis, cells from control and dust  
13 treatments were processed and analyzed for cytosolic histone-bound DNA fragments determined  
14 using the Cell Death Detection ELISA™ kit (Boehringer Mannheim, Indianapolis, IN) according  
15 to the manufacturer's protocol. The reaction of the Boehringer reagents with histone-bound DNA  
16 fragments released into the cytosol produced a colored product that can be spectrometrically  
17 measured at 405 nm.

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## 19 **Assessment of the Interaction of Test Dusts with Scavenger Receptors**

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21 To determine if scavenger receptors were involved in cytotoxicity, HAM were  
22 preincubated with or without polyinosinic acid (100 µg/ml, Sigma, St. Louis, MO) for 15  
23 minutes at room temperature. Cells were cultured at  $1 \times 10^6$  cells/ml in the presence or absence

1 of lunar or Martian dust simulant (250 µg/ml) under conditions described above. The upper limit  
2 of the suspension concentration was determined by the relative cytotoxicity of these dusts in a  
3 24-h cell culture; at concentrations > 250 µg/ml, both types of particles significantly decreased  
4 cell viability. Cells were assayed for apoptosis by Cell Death Detection ELISA™ and  
5 morphology and for necrosis by trypan blue exclusion, as described above.

6

### 7 **Immunomodulation Assay of Dust-Treated HAM**

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9 RFD1 and RFD7 surface markers on HAM subpopulations were analyzed by flow  
10 cytometry after the procedures described previously (Holian et al., 1997, 1998) were performed.  
11 Briefly, at the termination of the culture, cells were centrifuged, medium was aspirated, and the  
12 cell pellet ( $1 \times 10^6$  cells) was resuspended in 500 µl PBS with 3.5% bovine serum albumin  
13 (BSA, Sigma, St. Louis, MO). Monoclonal antibodies to RFD1<sup>+</sup> (murine IgM) and RFD7<sup>+</sup>  
14 (murine IgG1) surface antigens (Serotec, Kidlington, Oxford, England) were both added at a  
15 1:200 dilution (2.5 µg in 500 µl). The mixture was incubated 30 minutes at room temperature,  
16 and the reaction was then terminated by centrifugation and aspiration. The cell pellet was  
17 washed 3 times with PBS and resuspended in PBS/BSA, and fluorescein anti-mouse IgM (Vector  
18 Labs, Burlingame, CA) and R-phycoerythrin anti-mouse IgG (Vector Labs, Burlingame, CA)  
19 were added concurrently at a 1:100 dilution (5 µg in 500 µl) and incubated 30 minutes at room  
20 temperature. The incubation was terminated as described above and washed 3 times in PBS.  
21 The cells were then suspended in 1% formaldehyde (phosphate buffered) and stored at 4°C  
22 before flow cytometric analysis. Flow cytometry was performed on a Coulter EPICS Elite flow  
23 cytometer (Coulter, Miami, FL) using the Elite software. Using forward and side scatter of the

1 total cell population; gates were drawn to include macrophages on the basis of the size and  
2 granularity of the cells. The instrument was calibrated with beads coated with fluorescein  
3 isothiocyanate and phycoerythrin to compensate for any overlap within the green and red  
4 wavelengths. Cells stained without the inclusion of primary antibodies had no significant  
5 staining. Controls included unstained cells, cells stained with secondary antibodies only  
6 (negative control), and cells stained for only one of these surface markers (positive control).

7

## 8 **Statistical Analysis**

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10 The experiment results were statistically analyzed by analysis of variance (ANOVA)  
11 followed by a Student-Newman-Keuls or Tukey's multiple comparison test. The number "N" in  
12 the figure legends denotes the number of HAM cultures given the treatment shown.

1 **RESULTS**

2

3 **Comparison of the Toxicity of Lunar and Martian Dust Simulants with Titanium**  
4 **Dioxide and Crystalline Silica in HAM**

5

6 HAM in cultures were treated with lunar and Martian dust simulants, TiO<sub>2</sub>, or quartz (SiO<sub>2</sub>) at a  
7 concentration of 100 µg/ml for 24 h; cell viability was determined by trypan blue exclusion. In  
8 the apoptosis assay, HAM were treated similarly and the extent of apoptosis was determined by  
9 assessing spectrometrically the amount of histone-bound DNA fragments released into the  
10 cytosol. The results of cell viability and apoptotic index analyses are shown in Figures 1A and  
11 1B. Statistical tests were performed to compare the simulants with TiO<sub>2</sub> or quartz, and the  
12 results show the lunar dust and TiO<sub>2</sub> were comparable, and more toxic than saline control, but  
13 less toxic than quartz (Tables 1A and 1B).

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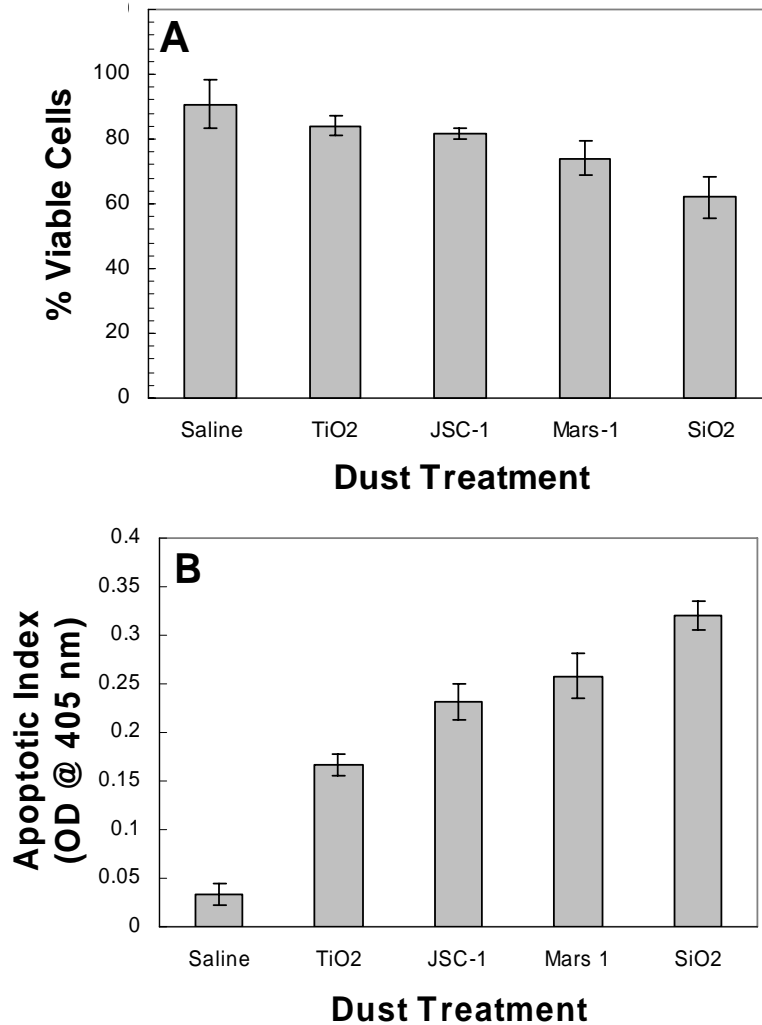


FIG. 1. A: Viability of human alveolar macrophages (HAM) in cultures treated with test dusts (100 $\mu$ g/mL) for 24 h. Each bar illustrates the mean  $\pm$  SEM percent viable cells. B: HAM in cultures were treated with test dusts for 24 h and then assessed for apoptosis by spectrometrically measuring the release of cytoplasmic histone-bound DNA fragments into the cytosol. Each bar on the graph illustrates the mean  $\pm$  SEM value of optical density at 405 nm. Statistical tests were performed on these data, and the results are shown in Tables 1A and 1B (N= 6).

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TABLE 1 A

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Results of cell viability measurements in HAM cultures treated with test dusts for 24 h

Test dust	% viable cells	Effect due to treatment <sup>1</sup>	Test vs. saline	Test vs. TiO <sub>2</sub>	Test vs. SiO <sub>2</sub>
Saline	91	---	---	$\rho = 0.23$	$*\rho < 0.001$
Titanium dioxide	84	7.7	$\rho = 0.23$	---	$*\rho = 0.04$
Lunar dust simulant	82	9.9	$\rho = 0.11$	$\rho = 0.12$	$*\rho = 0.03$
Martian dust simulant	74	18.7	$*\rho = 0.04$	$*\rho = 0.01$	$\rho = 0.16$
Crystalline silica	62	32.9	$*\rho = 0.01$	$*\rho = 0.04$	---

4

\* Indicates significance at  $p < 0.05$  compared with the control by ANOVA, followed by Tukey's procedure.

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<sup>1</sup>The difference obtained by minus the result of dust-treated cultures from that of saline controls.

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TABLE 1 B

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Results of the apoptotic assays of HAM cultures treated with test dusts for 24 h

Test dust	Apoptotic index	Effect due to treatment <sup>1</sup>	Test vs. saline	Test vs. TiO <sub>2</sub>	Test vs. SiO <sub>2</sub>
Saline	0.033	---	---	$*\rho < 0.001$	$*\rho < 0.001$
Titanium dioxide	0.167	0.134	$*\rho < 0.001$	---	$*\rho < 0.001$
Lunar dust simulant	0.231	0.198	$*\rho < 0.001$	$\rho = 0.10$	$*\rho = 0.005$
Martian dust simulant	0.258	0.225	$*\rho < 0.001$	$*\rho = 0.04$	$*\rho = 0.03$
Crystalline silica	0.320	0.287	$*\rho < 0.001$	$*\rho < 0.001$	---

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\* Indicates significance at  $p < 0.05$  compared with the control by ANOVA, followed by Tukey's procedure.

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<sup>1</sup>The difference obtained by minus the result of dust-treated cultures from that of saline controls.

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## 2 **Effects of Lunar and Martian Dust Simulants on HAM Viability**

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4 To determine the dose response of cytotoxicity to HAM in cultures treated with lunar and

5 Martian dust simulants, the cultures were treated with simulants at 0, 100, 250, or 500  $\mu\text{g/ml}$  for

6 24 h and cell viability was measured. The results (Figure 2) indicated that both simulants

7 induced a dose-dependent decrease in viability.

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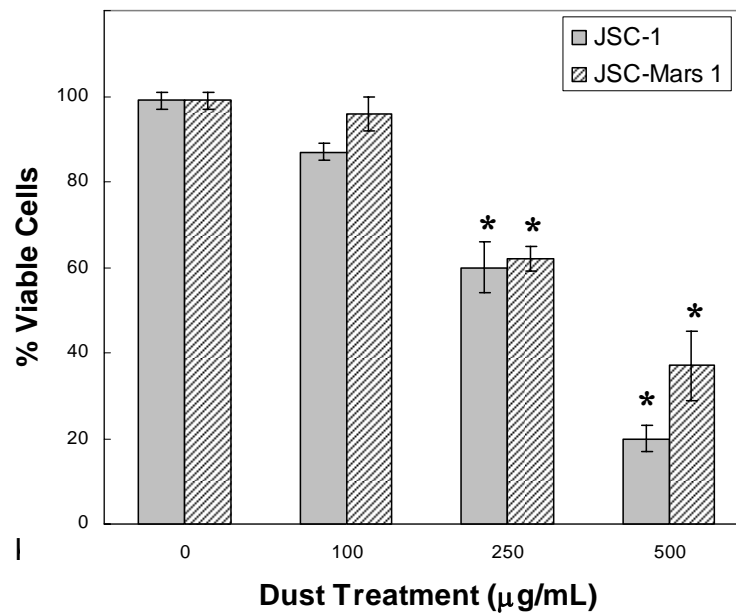
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18 FIG. 2. Viability of human alveolar macrophages in cultures treated with lunar or Martian dust simulant at 0, 100,

19 250, and 500  $\mu\text{g/ml}$  for 24 h. Each bar represents the mean  $\pm$  SEM percent viable cells. \* indicates significance at  $p$

20  $< 0.05$  compared with the control (0  $\mu\text{g/ml}$ ) by the Student-Newman-Keuls procedure (N = 6).

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# 1 Apoptosis in HAM Induced by Lunar and Martian Dust Simulants

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3 The ability of the lunar and Martian dust simulants to induce HAM to undergo apoptosis in  
4 cultures treated with the simulants at 0, 100, 250, or 500  $\mu\text{g/ml}$  for 24 h was determined. The  
5 results of the apoptosis assay (Figure 3) show that dust treatment produced a general dose-  
6 dependent increase in the apoptotic index. However, at the highest dose (500  $\mu\text{g/ml}$ ), the  
7 apoptosis induced by lunar dust simulant was less than that produced by the second highest dose  
8 (250  $\mu\text{g/ml}$ ); the apoptosis induced by the highest dose of Martian dust simulant did not increase  
9 over that produced by the second highest dose. It is noteworthy that the highest dose of either  
10 test dust produced the highest percent of cell death (Figure 2); it may be that at the highest doses,  
11 a greater proportion of the cells undergo necrosis, which cannot be detected by the apoptotic

12 assay (Figure 3)

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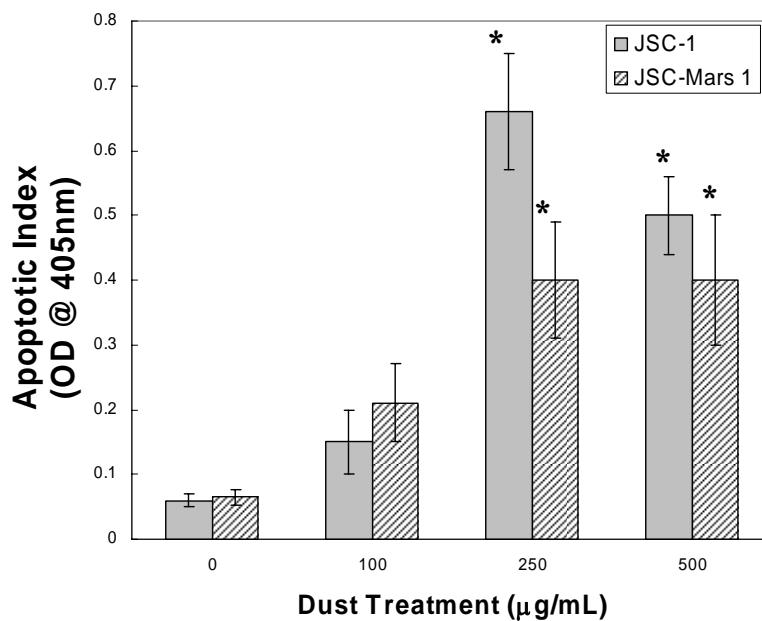
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21 FIG. 3. Human alveolar macrophages in cultures treated with lunar or Martian dust simulant for 24 h were assessed  
22 for apoptosis by spectrometrically measuring the release of cytoplasmic histone-bound DNA fragments into the  
23 cytosol. Each bar illustrates the mean  $\pm$  SEM value of optical density measured at 405 nm. \* indicates significance  
24 at  $p < 0.05$  compared with the control (0  $\mu\text{g/ml}$ ) by the Student-Newman-Keuls procedure (N = 6).

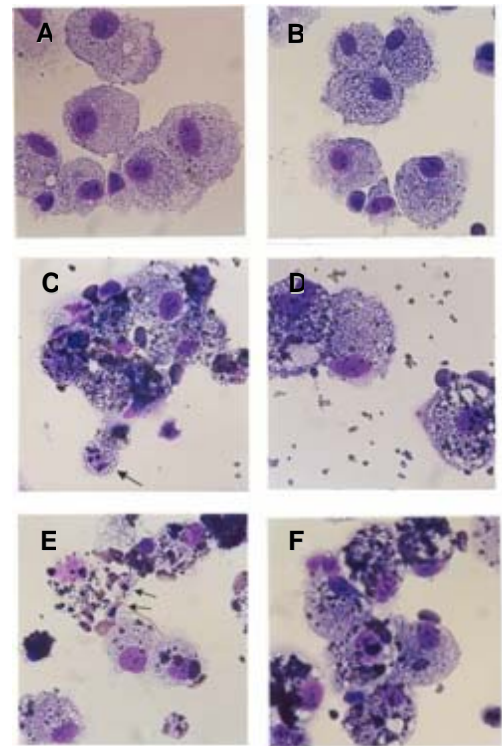
## 1 Morphology of HAM Treated with Martian or Lunar Dust Simulants

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3 To confirm that these dust simulants induce apoptosis in HAM, cells were cultured with or  
4 without a 15-minute pretreatment with polyinosinic acid (poly I), exposed to lunar or Martian  
5 dust simulant (250  $\mu\text{g}/\text{ml}$ ), and stained for morphology assessment. Untreated HAM are intact  
6 cells with large, distinct nuclei (Figure 4A). Poly I had no effect on control cells (Figure 4B).  
7 Cells exposed to lunar dust (Figure 4C) had dark, shrunken, sometimes segmented nuclei  
8 characteristic of nuclear condensation common in apoptosis. Pretreatment with poly I protected  
9 the cells against apoptosis induced by the dust (Figure 4D). Nuclear disintegration, another  
10 feature of apoptosis, was evident in some of the treated cells. Cells treated with Martian dust  
11 simulant also had dark, shrunken nuclei (Figure 4E), and pretreatment with poly I also protected  
12 them against apoptosis (Figure 4F).

13

14 FIG. 4. Morphology of human alveolar macrophages 24 h after  
15 exposure to lunar and Martian dust simulants. A, control macrophages  
16 cultured for 24 h; (B): Macrophages preincubated with 100  $\mu\text{g}/\text{ml}$   
17 polyinosinic acid and cultured for 24 h; (C): Macrophages incubated  
18 with lunar dust simulant (250  $\mu\text{g}/\text{ml}$ ) for 24 h; (D): macrophages  
19 incubated with lunar dust simulant (250  $\mu\text{g}/\text{ml}$ ) for 24 h after a 15-  
20 minute preincubation with polyinosinic acid (100  $\mu\text{g}/\text{ml}$ ); (E):  
21 Macrophages incubated with Martian dust simulant (250  $\mu\text{g}/\text{ml}$ ) for 24  
22 h; (F), macrophages incubated with Martian dust simulant (250  $\mu\text{g}/\text{ml}$ )  
23 for 24 h after a 15-minute preincubation with polyinosinic acid  
24 (100 $\mu\text{g}/\text{ml}$ ). Arrows indicate morphology consistent with macrophage  
25 apoptosis (photomicrographs 600X, dry objective).



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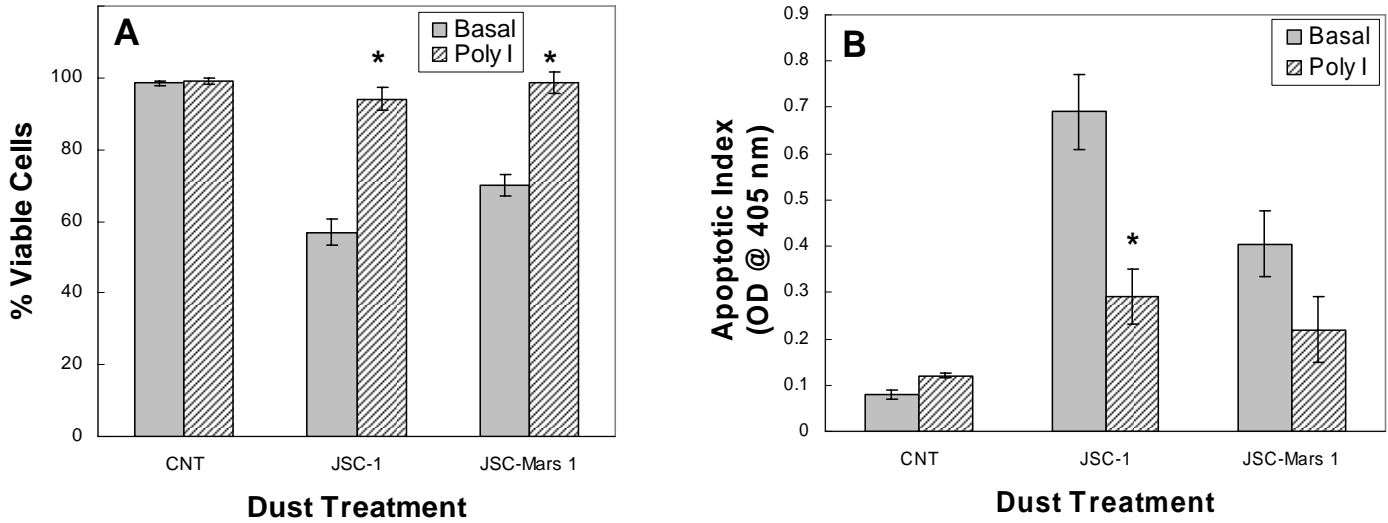
**Assessment of a Mechanism by Which Lunar and Martian Dust Simulants Might Induce Cytotoxicity in HAM**

Scavenger receptor class A I/II (SRA) has been implicated in the mechanism of silica-induced toxicity of the alveolar macrophage (Hamilton, 2000). It was speculated that the toxic effects of silica resulted from the interaction between the macrophage surface SRA and silica. Silica-induced apoptosis was blocked by pretreatment of cells with polyinosinic acid (poly I), the SRA-inhibitor (Iyer et al., 1996; Hamilton et al., 2000). To test whether the HAM cytotoxicity induced by the dust simulants involved SRA, cells in culture were preincubated with or without polyinosinic acid, and then exposed to 250 µg/ml lunar or Martian dust simulant for 24 h. Cells were assessed for viability by trypan blue exclusion (Figure 5A) or for apoptosis by cell death ELISA (Figure 5B). These results demonstrate that pretreatment of the cells with polyinosinic acid protected against both dust-induced apoptosis and necrosis of HAM.

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13 FIG. 5. Effect of the scavenger receptor antagonist, polyinosinic acid, on dust-induced cytotoxicity in human alveolar  
14 macrophages. Polyinosinic acid (100  $\mu\text{g/ml}$ ) treated or untreated HAM cells were exposed *in vitro* to lunar or Martian  
15 dust simulants (250  $\mu\text{g/ml}$ ) for 24 h. Cell viability and apoptosis were determined. In (A), bars represent the mean  $\pm$   
16 SEM percent viable cells; in (B), bars represent the mean  $\pm$  SEM apoptotic index (optical density at 405 nm). \*  
17 indicates significance at  $p < 0.05$  compared with appropriate control by Student-Newman-Keuls procedure (N = 3).

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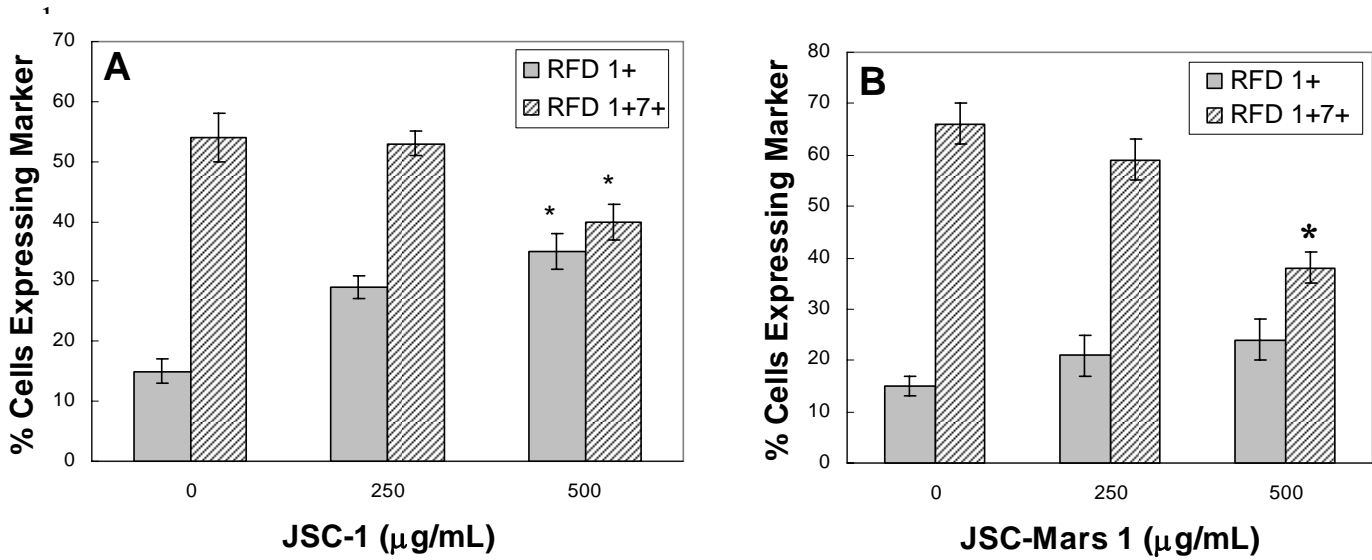
## 1 **Immunomodulation of HAM by Lunar and Martian Dust Simulants**

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3 To determine if the dust simulants had immunomodulating effects on HAM, the HAM  
4 phenotypes were assessed in cultures treated with the lunar dust simulant (0, 100, or 250  $\mu\text{g/ml}$ )  
5 or the Martian dust simulant (0, 250, or 500  $\mu\text{g/ml}$ ). Both dusts induced a small concentration-  
6 dependent increase in the percentage of immune activator (RFD1<sup>+</sup>) HAM (Figures 6A and 6B).

7 In addition, a concentration-dependent decrease occurred in the percentage of suppressor  
8 (RFD1<sup>7+</sup>) HAM (Figures 6A and 6B) compared to controls. These data indicate that a

9 significant shift occurred in HAM subpopulations toward an immune active phenotype on  
10 exposure to either dust simulant.



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11 FIG. 6. A: Effects of lunar dust simulant on human alveolar macrophage phenotype distributions. Each bar  
 12 represents the mean  $\pm$  SEM percent activator macrophages (RFD1<sup>+</sup>) and suppressor macrophages (RFD1<sup>+</sup>7<sup>+</sup>) after  
 13 24 h of culture with lunar dust simulant (0, 100, and 250 µg/ml). \* indicates significance at  $p < 0.05$  compared with  
 14 appropriate control (0 µg/ml) by the Student-Newman-Keuls procedure (N = 3). B: Effects of Martian dust simulant  
 15 on human alveolar macrophage phenotype distributions. Each bar represents the mean  $\pm$  SEM percent activator  
 16 macrophages (RFD1<sup>+</sup>) and suppressor macrophages (RFD1<sup>+</sup>7<sup>+</sup>) after 24 h of culture with Martian dust simulant (0,  
 17 250, and 500 µg/ml). \* indicates significance at  $p < 0.05$  compared with appropriate control (0 µg/ml) by the  
 18 Student-Newman-Keuls procedure (N = 3).

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## DISCUSSION

The present study was conducted to address NASA's concern about its workers being exposed to a lunar dust simulant for which little toxicity information is available. Thus, the potential toxicity and immunomodulatory effects of this simulant (JSC-1) and a Martian regolith simulant (JSC-Mars 1) were evaluated. A previous study from our laboratory showed that silica at a dose of 66 or 133 μg/ml in cultures cause apoptosis in HAM, with the results of the high dose approached a plateau value; TiO<sub>2</sub> at 100, or 200 μg/ml produced little apoptosis (Iyer et al., 1996). Based on these findings, a dose of 100 μg/ml was chosen for our present comparative study to examine the apoptosis and cytotoxicity induced by the test dusts in HAM isolated from human volunteers. In this study, the HAM were treated with the test dusts *in vitro* for 24 hours; the cells were examined for viability by trypan blue exclusion and for apoptosis by morphology and Cell Death Detection ELISA™ assay. The findings, which are consistent with the results of our previous study (Lam et al., 2000a) on biomarkers of toxicity in lung alveolar lavage fluid from intratracheally-instilled mice, showed that the lunar dust and TiO<sub>2</sub> were comparable, and more toxic than saline control, but less toxic than quartz. These results showed that both simulants are relatively low in acute toxicity.

Even both dust simulants are relatively low in toxicity when compared with silica, they did cause significant necrosis and apoptosis of HAM in a concentration-dependent manner. Since both simulants consist of more than 40% silica, we had hypothesized that their bioactivity on HAM would be similar to that observed with crystalline silica, but reduced. To elucidate a possible

1 mechanism by which these fine dust simulants are cytotoxic, we investigated the role of SR. SR  
2 has been reported to mediate silica-induced cytotoxicity of HAM (Iyer et al, 1996). The class A  
3 type II scavenger receptor was specifically implicated in silica-induced cytotoxicity in studies  
4 with a Chinese hamster ovary cell line stably transfected with the murine gene for this receptor  
5 (Hamilton et al., 2000). However, recent studies demonstrated that MARCO may be more  
6 important in mediating binding and toxicity of silica particles (Hamilton et al., 2006). The  
7 results of the present study also implicate scavenger receptors as the mediator of cytotoxicity  
8 induced by both lunar and Martian dust simulants, since a 15-minute pretreatment with  
9 polyinosinic acid (poly I), a SR antagonist, significantly inhibited both apoptosis and necrosis.  
10  
11 Finally, the impact of these dust simulants on macrophage subpopulations was examined using  
12 flow cytometry to classify treated HAM as activator (RFD1<sup>+</sup>) or suppressor (RFD1<sup>+7+</sup>)  
13 subpopulations based on expression of these surface antigens. Silica was previously shown to be  
14 more toxic to suppressor than to activator HAM phenotypes (Spiteri and Poulter, 1991; Holian et  
15 al., 1997, 1998). In addition, phenotype shifts were observed in HAM treated with the fibrogenic  
16 particles silica or asbestos but not with non-fibrogenic particles, such as titanium dioxide or  
17 wollastonite (Hamilton et al., 1996; Iyer et al., 1996; Iyer and Holian, 1997). In the current  
18 study, both lunar and Martian dust simulants caused an increase in the ratio of RFD1<sup>+</sup> (immune  
19 active) to RFD1<sup>+7+</sup> (immune suppressor) HAM. Thus, HAM subpopulations were significantly  
20 shifted toward an immune active phenotype on exposure to either of these dust simulants. If  
21 similar changes occur *in vivo*, chronic lung inflammation could develop, precipitating fibrosis.  
22 In fact, inflammation and fibrosis were observed microscopically in mice 7-d and 90-d after



1 intratracheally exposed to bolus doses of lunar and Martian dust simulants, but not with  
2 TiO<sub>2</sub>(Lam et al., 2002b).

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4 The present study with alveolar macrophages isolated from human volunteers complements our  
5 previous animal studies in revealing potential toxicity of JSC-1 and JSC-Mars 1. The overall  
6 results of these three studies showed that these dusts, especially the JSC-1, are relatively low in  
7 toxicity, but are more toxic than titanium dioxide, whose toxicity has been equated with that of  
8 nuisance dusts; taken together, the above results indicate that individuals inhaling moderate to  
9 large amounts of these dusts for prolong periods could suffer adverse health effects. The results  
10 of this study, therefore, are relevant to human health here on Earth, since these soil simulants are  
11 used for large-scale engineering applications at NASA. It is reasonable to speculate that lunar  
12 dust would be more toxic than its surrogate. Lunar dust resides in near-vacuum conditions and is  
13 constantly bombarded by solar winds and cosmic rays (Stubbs et al., 2005); on the Moon, the  
14 chemical bonds on the grain surfaces are not pacified by moisture, thus making them very  
15 reactive (Stubbs et al., 2007). The results of the present study support NASA's directive that the  
16 lunar dust needs to be tested toxicologically.

17  
18 The current investigation supports NASA's requirement to develop experiment protocols using  
19 dust simulants before conducting toxicity studies with extraterrestrial dusts (Wagner, 2004).  
20 Furthermore, this study is consistent with the National Research Council's recent  
21 recommendation to utilize *in vitro* toxicity testing methods to evaluate toxicological effects on  
22 cells, cell lines, or cellular components, preferably of human origin (NAS, 2007). Using *in vitro*  
23 methods such as these would allow investigators to conduct toxicology studies of extraterrestrial

1 dusts with minute quantities of these precious materials. Therefore, the design and methodology  
2 of the current study could be adopted for pulmonary and immunotoxicity studies of actual lunar  
3 and Martian dusts.

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6 **ACKNOWLEDGMENTS**

7

8 The authors gratefully acknowledge Dr. Carlton Allen, Astromaterials Curator of the NASA  
9 Johnson Space Center, for providing the raw simulated lunar and Martian soils, and Dr. Yung-  
10 Sung Cheng of Lovelace Respiratory Research Institute (Albuquerque, NM) for preparing the  
11 fine dust samples from these raw materials. Furthermore, the authors thank Dr. Jane Krauhs of  
12 Wyle for her assistance with technical writing and Dr. Tatyana Gots of the University of Texas  
13 Health Science Center, Houston (TX) for her technical assistance. This work was supported by  
14 NASA's Graduate Student Researchers Program (GSRP) Grant #NGT5-50084 (JNL) and by  
15 NIH Clinical Research Center Grant M01-RR-02558 (AH).

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### 33 **FIGURE LEGENDS**

34 FIG 1. A: Viability of human alveolar macrophages (HAM) in cultures treated with test dusts  
35 (100µg/mL) for 24 h. Each bar illustrates the mean ± SEM percent viable cells. B: HAM in  
36 cultures were treated with test dusts for 24 h and then assessed for apoptosis by spectrometrically  
37 measuring the release of cytoplasmic histone-bound DNA fragments into the cytosol. Each bar  
38 on the graph illustrates the mean ± SEM value of optical density at 405 nm. Statistical tests were  
39 performed on these data, and the results are shown in Tables 1A and 1B (N= 6).  
40

41 FIG. 2. Viability of human alveolar macrophages in cultures treated with lunar or Martian dust  
42 simulant at 0, 100, 250, and 500 µg/ml for 24 h. Each bar represents the mean ± SEM percent  
43 viable cells. \* indicates significance at  $p < 0.05$  compared with the control (0 µg/ml) by the  
44 Student-Newman-Keuls procedure (N = 6).  
45  
46

1 FIG. 3. Human alveolar macrophages in cultures treated with lunar or Martian dust simulant for  
2 24 h were then assessed for apoptosis by spectrometrically measuring the release of cytoplasmic  
3 histone-bound DNA fragments into the cytosol. Each bar illustrates the mean  $\pm$  SEM value of  
4 optical density measured at 405 nm. \* indicates significance at  $p < 0.05$  compared with the  
5 control (0  $\mu\text{g/ml}$ ) by the Student-Newman-Keuls procedure (N = 6).

6

7 FIG. 4. Morphology of human alveolar macrophages 24 h after exposure to lunar and Martian  
8 dust simulants. A: Control macrophages cultured for 24 h. B: Macrophages preincubated with  
9 100  $\mu\text{g/ml}$  polyinosinic acid and cultured for 24 h. C: Macrophages incubated with lunar dust  
10 simulant (250  $\mu\text{g/ml}$ ) for 24 h. D: Macrophages incubated with lunar dust simulant (250  $\mu\text{g/ml}$ )  
11 for 24 h after a 15-minute preincubation with polyinosinic acid (100  $\mu\text{g/ml}$ ). E: Macrophages  
12 incubated with Martian dust simulant (250  $\mu\text{g/ml}$ ) for 24 h. F: Macrophages incubated with  
13 Martian dust simulant (250  $\mu\text{g/ml}$ ) for 24 h after a 15-minute preincubation with polyinosinic  
14 acid (100 $\mu\text{g/ml}$ ). Arrows indicate morphology consistent with macrophage apoptosis  
15 (photomicrographs 600 $\times$ , dry objective).

16

17 FIG. 5. Effect of the scavenger receptor antagonist, polyinosinic acid, on dust-induced  
18 cytotoxicity in human alveolar macrophages. Polyinosinic acid (100  $\mu\text{g/ml}$ ) treated or untreated  
19 HAM cells were exposed *in vitro* to lunar or Martian dust simulants (250  $\mu\text{g/ml}$ ) for 24 h. Cell  
20 viability and apoptosis were determined. In (A), bars represent the mean  $\pm$  SEM percent viable  
21 cells; in (B), bars represent the mean  $\pm$  SEM apoptotic index (optical density at 405 nm). \*  
22 indicates significance at  $p < 0.05$  compared with the appropriate control by Student-Newman-  
23 Keuls procedure (N = 3).

24

25 FIG. 6. A: Effects of lunar dust simulant on human alveolar macrophage phenotype distributions.  
26 Each bar represents the mean  $\pm$  SEM percent activator macrophages (RFD1<sup>+</sup>) and suppressor  
27 macrophages (RFD1<sup>7+</sup>) after 24 h of culture with lunar dust simulant (0, 100, and 250  $\mu\text{g/ml}$ ). \*  
28 indicates significance at  $p < 0.05$  compared with the appropriate control (0  $\mu\text{g/ml}$ ) by the  
29 Student-Newman-Keuls procedure (N = 3). B: Effects of Martian dust simulant on human  
30 alveolar macrophage phenotype distributions. Each bar represents the mean  $\pm$  SEM percent  
31 activator macrophages (RFD1<sup>+</sup>) and suppressor macrophages (RFD1<sup>7+</sup>) after a 24-h culture  
32 with Martian dust simulant (0, 250, and 500  $\mu\text{g/ml}$ ). \* indicates significance at  $p < 0.05$   
33 compared with the appropriate control (0  $\mu\text{g/ml}$ ) by the Student-Newman-Keuls procedure (N =  
34 3).