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Toxicity of lunar dust assessed in inhalation-exposed rats

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Abstract

Humans will again set foot on the moon. The moon is covered by a layer of fine dust, which can pose a respiratory hazard. We investigated the pulmonary toxicity of lunar dust in rats exposed to 0, 2.1, 6.8, 20.8 and 60.6 mg/m^3 of respirable-size lunar dust for 4 weeks (6 h/day, 5 days/week); the aerosols in the nose-only exposure chambers were generated from a jet-mill ground preparation of a lunar soil collected during the Apollo 14 mission. After 4 weeks of exposure to air or lunar dust, groups of five rats were euthanized 1 day, 1 week, 4 weeks or 13 weeks after the last exposure for assessment of pulmonary toxicity. Biomarkers of toxicity assessed in bronchoalveolar fluids showed concentration-dependent changes; biomarkers that showed treatment effects were total cell and neutrophil counts, total protein concentrations and cellular enzymes (lactate dehydrogenase, glutamyl transferase and aspartate transaminase). No statistically significant differences in these biomarkers were detected between rats exposed to air and those exposed to the two low concentrations of lunar dust. Dose-dependent histopathology, including inflammation, septal thickening, fibrosis and granulomas, in the lung was observed at the two

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Declaration of interest

The employment affiliations or associations of the authors are shown on the cover page. This work was conducted during the normal course of the authors' employment. Three of the authors (KED, DEG and ROM) served as members of the LADTAG and received reimbursement for their travel expenses and modest honorarium in some cases. The analyses and opinion presented in this article are exclusively those of the authors and do not represent NASA policy or the policy of the employers of the authors, including NIOSH. The proposed exposure limits are not NASA official exposure standards for lunar dust.

higher exposure concentrations. No lesions were detected in rats exposed to ≤6.8 mg/m^3. This 4-week exposure study in rats showed that 6.8 mg/m^3 was the highest no-observable-adverse-effect level (NOAEL). These results will be useful for assessing the health risk to humans of exposure to lunar dust, establishing human exposure limits and guiding the design of dust mitigation systems in lunar landers or habitats.

Keywords

Lunar dust toxicity; lung lavage biomarkers; moon dust; nose-only exposure

Introduction

The brief lunar missions of the Apollo Program have left many Americans with a desire to return to the moon for long-duration exploration and research (Bilder, 2009; Schmitt, 2005); President Bush in 2004 called upon NASA to use the moon as a stepping-stone to Mars. Even though long-duration exploration of the moon is not NASA's current primary objective, returning to the moon is still a future option. Other spacefaring nations, including European countries, Russia, China, Japan and India, are planning to send humans to the moon for the first time (Hindu, 2009; MoonDaily, 2006; Rianovosti, 2007; Wikipedia, 2013; Xinhua, 2012) and subsequently would explore the possibility of lunar mining to extract helium-3 as fuel for thermonuclear fusion to generate safe and clean energy (Bilder, 2009; Schmitt, 2005). The surface of the moon is covered by a layer of fine dust, which is very sticky; as John Young of the Apollo 16 mission noted, after returning from outside into the Lunar Module, "… our feet and hands and our arms were all full of dust…." Apollo crewmembers were exposed to moon dust that had adhered to spacesuits, become dislodged and subsequently become airborne in the Lunar Module or Service Module; indeed, some astronauts reported eye and throat irritation during the brief lunar dust exposures (Wagner, 2006). A flight surgeon who inhaled some moon dust during unpacking of the spacesuits from stowage experienced respiratory immunological symptoms, which progressively worsened after exposure following the two subsequent missions (Scheuring et al., 2008). The projected duration of future human lunar habitation is longer than that of the Apollo astronauts, and the living quarters could be contaminated with dust brought inside on spacesuits or hardware during each outside activity. Humans will set foot on the moon again and be exposed to lunar dust; therefore, information about the toxicity of lunar dust is essential for assessing the health risks of human exposure, setting permissible exposure limits and designing appropriate dust decontamination systems for a lunar habitat or landing vehicle. The present study was carried out to acquire toxicity information on airborne lunar dust for these purposes.

The surface of the moon has been bombarded by micrometeoroids and radiation for more than 4 billion years (NASA, 2009). Micrometeoroids, typically 50 μm (0.002 in) in diameter or less, strike the moon at high speeds, resulting in impact forces that generate temperatures reaching 2000 °C or higher, and crushing, melting and/or partially vaporizing surface particles. The lofted molten particles drop back to the surface and weld surrounding grains together into glassy agglutinates, which are pulverized to fine dust particles upon subsequent

impacts (Park et al., 2008). Cosmic and solar radiations impart electrical charges to lunar surface material (NASA, 2009); charges build up to levels high enough that submicron particles (0.1 μm) are repelled as high as 100 km (62 miles or ~330 000 ft) and could remain above the low-gravity and airless lunar surface for long periods (Stubbs et al., 2005). These processes cause continuous migration and mixing of fine dust particles on the lunar surface. The constant micrometeoroid bombardments and dust electrostatic levitation during the 4-billion-year geological history of the moon have caused the lunar surface to be covered with a blanket of fine dust. As Alan Bean of Apollo 12 noted, "The entire lunar surface was covered with this mantle of broken-up material, fine dust of varying depth. As a result, everything looked pretty much the same …" (Wagner, 2006).

The lunar dust for the present toxicity investigation derived from a regolith (surface soil) sample (14003, 96) collected during the Apollo 14 mission from the Fra Mauro Formation near Cone Crater in the Imbrium Basin, located near the lunar equator. On the basis of the mineral properties of lunar regolith samples collected from different Apollo mission landing sites, Apollo 14 regolith samples have been considered as good representatives of fine mare surface materials (low-Ti basalt) on the moon, except for lunar highlands with high Ti-Ca anorthosite content. The mineral properties of this lunar soil are similar to those of some terrestrial volcanic ashes. In fact, an ash from the San Francisco Volcano (near Flagstaff, AZ), designated as JSC-1 (McKay et al., 1994) or JSC-1A (new batch) (Carpenter et al., 2006; Gustafson et al., 2006), which has geologic and mineral properties similar to those of lunar regolith, has been used by NASA and European Space Agency (ESA) engineers and scientists as a lunar dust simulant for various lunar-related tests including our previous toxicity studies (Lam et al., 2002a,b; Latch et al., 2008). ESA is using this lunar soil simulant (JSC-1A) as a surrogate to study the possibility of using lunar soil for shielding astronauts from cosmic radiation during deep space exploration (ESA, 2012). Regolith sample 14163, collected during this Apollo 14 mission and for which JSC-1 is a simulant, has also served as the yardstick for lunar simulants for other countries including China (CAS-1) and Japan (FJS-1) (Kanamori et al., 1998; Zheng et al., 2009).

In general, lunar regolith contains about 20% dust (≤20 μm diameter) by mass, and 1–2% fine dust (≤3 μm) (Park et al., 2008). To obtain enough lunar dust (>20 g) of respirable size (mass median aerodynamic diameter [MMAD] of ≤3 μm, mass median diameter [MMD] ≤2 μm) for a 4-week nose-only inhalation study, we would need to aerodynamically isolate the fine dust from several kilograms of precious lunar soil, for which we would exhaust all or a major portion of the samples collected during the Apollo 14 mission. We decided to grind (by jet mill) an aliquot of the coarse fraction of lunar dust that was retained in a cyclone during our early effort to collect a small amount of very fine (respirable) dust for an intratracheal instillation (ITI) study (Lam et al., 2013). In our ITI study, we found that the respirable lunar dusts isolated from ground (either by jet mill or ball mill) and unground lunar soil samples produced comparable pulmonary toxicity and concluded that the ground lunar dust is a good surrogate for the unground parent sample. The current toxicity study was conducted in Fischer 344 rats exposed to lunar dust in Jaeger-NYU nose-only chambers, and the respirable dust was aerodynamically generated from a jet-mill ground sample.

Materials and methods

Animals and animal care

Specific-pathogen-free Fischer 344 adult male rats (150–250 g; ~8–10 weeks old at arrival) were purchased from Charles River Laboratories (Raleigh, NC or Portage, MI). Rats were housed in an animal facility at NASA Johnson Space Center (Houston, TX), where this study was conducted. The animals were housed two per cage; the cages were ventilated with HEPA-filtered air. The rats had free access to water and food (Harlan 7912 Irradiated Global 18% Protein Rodent Diet, Harlan Laboratories, Houston, TX). The animals were allowed to acclimate for 1 week before they were used in experiments. The guidelines of the NASA Johnson Space Center (JSC) Institutional Animal Care and Use Committee (IACUC) and IACUC-approved test protocols were followed.

Animal acclimatization and exposure to lunar dust in nose-only inhalation chambers

After they were acclimated to the vivarium environment at the JSC animal facility for 1 week, the animals were placed in Battelle rat restraint tubes (CH Technologies, Inc., Westwood, NJ) for stress acclimation. They were acclimated to the tubes over a period of 5 days, 1 h on the first day and 1 h added each day. Rats that showed signs of stress, like restlessness and diarrhea, were excluded from the inhalation study; also excluded from the study were rats at both ends of the weight spectrum. Sixty-six male Fischer rats were randomly divided into three groups; each rat was color-coded, with a number written on its tail, and weighed. One group of 22 rats (including 2 extra) was exposed to filtered house air; the second group was exposed to a low concentration of lunar dust and the remaining group was exposed to a high concentration of lunar dust. Each rat was placed inside a Battelle restraint tube, which was then connected to one of the 24 nose-ports of a Jaeger-NYU nose-only chamber (CH Technologies) (Figure 1A). All animals were exposed 6 h daily, 5 days a week for 4 weeks for a total of 120 h. For the groups exposed to dust, each rat was exposed in a nose-port in the upper layer or the lower layer in daily alternation. After each daily 6-h exposure, the animals were returned to the vivarium, housed in pairs and observed for clinical signs. Body weights were recorded weekly or biweekly.

Monitoring chamber CO_2 concentration during animal exposure

Portable CO_2 monitors, which were designed for monitoring Space Shuttle and International Space Station CO_2 concentrations (Industrial Scientific Corp., Pittsburgh, PA), were used to measure chamber CO_2 concentrations. Each CO_2 monitor had an internal pump with a flow of ~300 ml/min, which is close to that of the ventilation rate of a rat (200–240 min/ml). During an inhalation exposure, one of the upper nose-ports of each chamber was connected to a decommissioned Space Shuttle/International Space Station CO_2 monitor, and CO_2 level was continuously monitored during the exposure; for the chambers that had dust, the aerosol stream was pulled through a Casella dust monitor (see below) to measure the dust concentration, and then the dust was trapped in a filter before entering the CO_2 monitor. Each monitor had a visible and audible alarm set at 1% CO_2. The internal volume of a two-layer NYU-Jaeger chamber is very small (~1.5 l); if the air flow to a chamber were disrupted for a few minutes, the alarm would be triggered and the animals would show signs of distress due to the rapid increase of CO_2. The use of CO_2 monitors with an audible alarm is

crucial with the nose-only chambers to prevent animal death due to a rapid buildup of CO_2 from an equipment malfunction or blockage or disconnection of the connecting tubing. Chamber CO_2 concentrations generally did not exceed 600 ppm.

Preparation of ground lunar dust for inhalation study

The major mineral components of the parent Apollo 14 lunar regolith sample (14003,96) are SiO_2 (amorphous silica), Al_2O_3, CaO, Fe_2O_3 and MgO, which account for 48%, 20%, 12%, 8% and 7% of the dust by weight, respectively (McKay et al., 1994). The lunar soil does not contain heavy metal oxides or crystalline silica at levels that pose a toxicity concern. Two hundred grams of this lunar regolith was placed in an inhouse-built fluidized bed, in which ultrapure nitrogen percolated from the bottom of the bulk sample. The aerosol stream was allowed to pass through a cyclone (CH Technologies). About 2.5 g of respirable dust was collected on the filter paper for the ITI study (Lam et al., 2013). A portion of the coarser dust fraction that was captured in the cyclone was ground by a jet mill. The coarse particles in the jet mill, which was connected to a tank of ultrapure nitrogen, were subjected to a high-pressure N_2 stream; the violent collisions of the particles led to formation of finer dust particles. The sample aliquots were weighed and stored in N_2 until they were used. All these dust preparation procedures were carried out in a tightly sealed N_2-filled hood.

Air generation and air supply to dust-generation systems and expsoure chambers

Filtered and dehumidified house air was generated by a Jun-Air Compressor (Model OF302-4MD2, Benton Harbor, MI); the head pressure of the compressed air was regulated to maintain a pressure of 25–30 psi. Air from this generator was fed into all the nose-only chambers including the one for air-exposed rats. When dust generation was started, the air pressure was gradually increased to avoid a pressure surge to the dry-dust generators, cyclones and associated connectors to prevent dust concentration spikes. The flow rate of air to each chamber was set at 8.0–8.5 l/min. This flow rate was chosen for the optimal performance of the cyclone, and to provide enough air for animal breathing, as well as to conserve the precious lunar dust. The flow rate to the air-only exposure chamber was regulated with a flowing stainless-steel ball regulator. The air mass flow rate to each of the Vilnius dust-generation systems, which were connected to a nose-only exposure chamber, was controlled with an Alicat mass flow controller (Alicat Scientific, Incorporated, Tucson, AZ). To ensure that the target airflow was delivered to the chamber, the air flow rate at the outlet end of the cyclone was verified with a Gilan Gilibrator air flow calibrator (a very low back-pressure airflow bubble meter; Sensidyne, St. Petersburg, FL) several times during the 4-week exposure.

Dust generation systems

The lunar dust aerosol stream in each nose-only chamber for rat exposure was generated by a Vilnius Aerosol Generator (VAG; CH Technologies). The output of the VAG was controlled by a controller. The concentrations in each chamber were photometrically monitored and recorded by a Casella MicroDust Pro (CasellaUSA, Buffalo, NY), which was placed very close to the chamber with the light path less than 4 inches from the chamber. The concentration signals of the Casella monitor were fed back to the VAG controller to maintain the target concentration. After several pilot test runs, we obtained the ratios

between the concentrations determined gravimetrically (G) and the concentrations photometrically (P) recorded by the Casella dust monitor. Because the Casella instruments were calibrated by the manufacturer against a dust containing only ~10% respirable dust (ISO 12103-1, A2 Fine Test Dust, MMD ~10 μm [PTI, 2013]) in a wind tunnel (Casella Measure, 2013), the concentrations the MicrodustPro recorded photometrically were not the true concentrations of the respirable lunar dust in the chambers. In our pilot studies, we obtained the profiles of G/P ratios, which increased with decreases in chamber concentrations, to guide us for setting the VAG controllers for target chamber concentrations. The aerosol generated by the VAG passed through a cyclone, allowing only respirable-size particles to enter the exposure chamber. The cyclones were specially made by the vendor to be used with the nose-only inhalation chambers for optimal flow rates of ≤10 l/min (CH technologies).

Monitoring of dust concentrations in nose-only exposure chambers

We conducted the first 4-week lunar dust inhalation exposure study at target concentrations of 0, 20 and 60 mg/m^3. Since 20 mg/m^3 caused some adverse effects, we then carried out a second study at target concentrations of 0, 2.0–2.5 and 6.0–7.5 mg/m^3. The dust concentrations in each chamber were continuously recorded by a computer. The data collected for each exposure session allowed us to determine the average chamber dust concentration for the 6-h daily exposure.

A short (~2 in) 3/8″-diameter copper tube situated in the center of a rubber stopper was inserted into one of the nose ports of a dust chamber; the other end of the copper tube was connected (epoxy-glued) to the pore (drilled by us) of a 47-mm conductive polypropylene cassette (SKC Inc. Houston, TX) containing a pre-weighed mixed cellulose ester (MCE) filter. The cassette was then tightly assembled and sealed with tape. The chamber aerosol stream was drawn through the filter at 0.5–1.0 l/min using a Universal pump (SKC Inc.). The collection time and flow rate (calibrated each time) depended on the chamber concentrations. Dusts on filters were collected from both chambers each day except for a few times when the port was used to connect to a Mercer Cascade Impactor (InTox Inc., Albuquerque, NM) for particle size determination. Dust particles that adhered to the inside of the collecting cassette were recovered with preweighed polyester tipped applicators, or swabs, and the weight of dust was added to that found in the MCE filter. The duration of dust collection on filters ranged from 2 to 6 h depending on chamber dust concentrations. If a dust sample was collected for only 3 h, the G_3/P_3 ratio was calculated (refer to G/P ratios in the preceding section, “Dust generation systems”). The gravimetric concentration for the 6-h chamber exposure concentration (G_6) for that day was estimated as follows: $G_6 = P_6 \times G_3/P_3$. Because the chamber concentrations of a particular day were very stable (Figure 1B), the ratios of G_3/P_3 and G_6/P_6 were very close. For those few days when no gravimetric samples were taken, the G_6 concentration was estimated from P_6 of that day using the following equation: $G_6 = P_6 \times G_{avg}/P_{avg}$, where G_{avg} = the average gravimetric concentration of all the days when samples were taken, and P_{avg} = the average photometric concentration for those days. The average exposure concentrations of lunar dust that were reported for each chamber were based on gravimetric determinations.

Characterization of particle size distributions in exposure chambers

The particle size distribution in the chamber was determined by a Quartz Crystal Microbalance (QCM) Cascade Impactor (California Measurement, Inc., Los Angeles, CA), with a recommended sampling flow rate of 250 cc/min. This real-time particle-size monitor has 10 stages (QCM) that sort particles by weight into 10 bins and record the weight-distribution of the particle versus the aerodynamic diameters of the particles; when dust particles land on the vibrating quartz crystal, they reduce the vibration, which reflects the amount of dust collected on that stage. To draw the aerosol into the QCM monitor, a rat with its restraining tube was briefly (a few minutes) removed from the exposure port, allowing the copper tube (15 in long; OD: 1/8 in, positioned in the middle of a rubber stopper) connected to the QCM monitor to be inserted into the chamber port. The particle-size profile from each chamber was determined at least once daily. Measurements were taken alternately from the upper and lower levels of the chamber. No difference in profiles was noticed between the two levels, and very little daily variation in the particle size profile was noted.

Like the QCM Cascade Impactor, the Aerodynamic Particle Sizer (APS 3321, TSI Inc., Shoreview, MN) was used for real-time determination of the particle-size profile. The TSI APS 3321 coupled with a TSI 3302A Aerosol Dilutor allowed us to measure high aerosol concentrations in the chambers. To use the TSI 3321-3302A system, a rat with its tube was removed briefly (a few minutes) from the exposure port, allowing the insertion of the front end of a special plastic tube (12″ long; OD: 1″; ID: 0.5″; a specialty sampling tube made by TSI), the back end of which was connected to a TSI dilutor-sizer, which was also placed within 8 in from a chamber nose port. The particle-size profile in each chamber was determined at least once daily. Measurements were taken alternately from the upper and lower levels of the chamber; no difference in profiles was noticed.

Mass median aerodynamic diameters (MMADs) of the aerosols in the lunar dust exposure chambers were determined by a seven-stage Mercer Cascade Impactor (InTox Inc., Albuquerque, NM) with a manufacture-recommended flow-rate of 0.5 l/min; the impactor's inlet protruded through a rubber stopper and inserted directly in a nose-port. An aerosol stream was drawn into the impactor, which contained seven preweighed and greased stainless-steel discs and a pre-weighed filter on its outlet. Aerosol sampling time (45 min to 6 h) depended on the chamber dust concentrations; the long sampling time was needed for study of exposure to the lowest concentration because we do not have a microbalance. The weights of the dust collected in the discs and filter were entered into a computer program provided by InTox, for calculating the MMAD of the aerosols collected. MMADs for each chamber, except the one for the lowest concentration, were assessed a few times during the 4-week exposure; the chamber for the lowest exposure was assessed twice.

Collection of bronchoalveolar lavage fluid (BALF) samples

To study biomarkers of toxicity in the BALF, a rat was weighed and deeply anesthetized with a lethal dose (~0.5 ml) of Euthasol® (containing pentobarbital sodium and phenytoin sodium, Abbott, North Chicago, IL) at 1 day, 1 week, 4 weeks or 13 weeks after the termination of exposure. The legs and the incisors were secured on a flat platform. After the abdominal cavity was opened, blood samples were collected from the vena cava for serum

chemistry, cytokine and gene assays. The chest cavity was then opened; the left lung was tied. The neck skin was cut open to expose the trachea for insertion of a catheter. After the catheter was tied to the trachea, the right lung lobes were lavaged with 4 ml of phosphate-buffered saline (PBS), and then further washed four times more each with 5 ml of PBS. The first lavage was centrifuged, and its supernatant was used for measuring the acellular BAL biomarkers. The cell pellets of the first and subsequent lavages were combined and suspended in 1 ml of HEPES-buffered solution for assessment of cell numbers and cell differentials.

Assessment of acellular components of the BALF

The cell-free BALF and serum samples were measured for enzymes and proteins by the NASA JSC clinical laboratory using an AU480 Chemistry System (Beckman Coulter, Inc., Brea, CA). The Beckman spectrophotometric method uses the reagents pyrogallol red and molybdate. The red complex reaction product binds basic amino groups of protein molecules, which results in a blue-purple complex with maximum absorbance of 600 nm. The lactate dehydrogenase (LDH) assay uses a modification of the method based on the conversion of lactate in the presence of NAD^+ to pyruvate and NADH. The concentration of NADH can be spectrophotometrically measured at 340 nm. Other enzymes were measured according to Beckman's standard protocols. Assessment of BALF cytokines and cells by receptor-phenotyping followed by flow cytometry will be reported elsewhere, as well as methods and results of measuring chemiluminescence in BAL cells (Lam et al., 2013).

Assessment of BAL cells

The total numbers of BAL cells were counted using a laboratory cell counter (Coulter Multisizer 3, Coulter Electronics, Hialeah, FL). Cytospin microscopic slides of BAL cells were prepared in a Cytospin centrifuge (Shandon Cytospin II, Shandon Inc., Pittsburgh, PA) and stained with Wright-Giemsa dye solution (Hema-Tec 2000, Bayer Corp., Elkhart, IN). Cell differentials were performed by visually counting 300 cells; the numbers of macrophages, neutrophils and other leukocytes were recorded.

Assessment of blood chemistry and hematology profiles

The serum chemistry and hematology profiles were assessed by the NASA clinical laboratory at the JSC using instruments that were dedicated for human blood or urine samples. No efforts were made to acquire instruments specifically for rat studies. Neither the serum chemistry nor hematology profiles of rats instilled with quartz or exposed to lunar dust showed treatment-dependent or consistent changes. Overall results showed that lunar dust in the lung did not produce toxicological changes that could be detected in the blood in rats exposed to two high concentrations of lunar dust; no blood samples were collected from the rats exposed to two lower concentrations for chemistry and hematology assays.

Rats instilled with quartz served as positive controls for biomarkers of toxicity measurement

Rats that were excluded from the inhalation exposure study were used as positive controls for toxicity biomarker assays; some additional rats were ordered specifically for this

purpose. One week (±1 day) before the designated time for animal euthanization for the pulmonary toxicity study, a group of five rats was instilled with 2.5 mg quartz (Min-U-Sil 5) that has an MMD of 1.6 μm (U.S. Silica, Berkeley Springs, WV). These animals were assessed for toxicity biomarker profiles concurrently with the inhalation-exposed rats. The weights of the quartz-control rats varied greatly; no efforts were made to match the weights of positive-control rats to those of the inhalation-exposed rats sacrificed on the same day of the biomarker assay.

Left lung collection and histopathological examination

After the right lung lobes were lavaged to collect BALF samples for biomarker study, as described above, the ligature on the left lungs was removed. The animal with the platform was then placed on an inclined position (~70°); formalin (10% in neutral phosphate buffer) was allowed to drip by gravity (from a 25-ml syringe barrel hanging about 1.5 ft above the neck) through the lavage catheter into the lung. The lung was then isolated from the chest cavity and placed in the same fixative. The left tracheobronchial and parathymic lymph nodes were removed and placed in a histology microcassette. The lung and the lymph nodes from each rat were placed in a container filled with about 60 ml of the same fixative, and were fixed for at least 1 week before they were processed further. The left lung lobe was first cut longitudinally (horizontally) into two halves, and one-half was placed in a cassette for paraffin embedding. A second cassette contained two lymph nodes. The paraffin-embedded lungs were thin-sectioned and mounted on glass microscope slides according to standard histopathological techniques. A section of each lymph node and a left lung tissue slice of a given rat were mounted on the same slide; duplicate slides were prepared from each rat. Tissue sections were stained with hematoxylin–eosin. For the high exposure concentrations study, a second set of tissue sections were stained with Mason's trichrome blue for visualization of connective tissues for signs of fibrosis (trichrome blue staining was not carried out on the second study, in which rats were exposed to low lunar dust concentrations). The slides were diagnosed by pathologists who have extensive experience assessing pulmonary toxicity of dusts, and the lesions of each rat and the diagnosis were entered on a histopathology score sheet.

Statistics

The data were first tested for normality, using the Shapiro–Wilk test, and for homogeneity of variance (Bartlett's test and then Levine's test), before testing for differences between means. If the data passed these tests, the means of the various treatment groups were tested for differences by one-way analysis of variance (ANOVA). If differences were detected, *post hoc* testing by the method of Bonferroni was used to identify pairs that differed significantly. If the data were not normally distributed or variance was nonhomogeneous, then the nonparametric Kruskal–Wallis test was performed and a modified Bonferroni test was used to identify pairs that differed significantly. Statistical significance was established when $p<0.05$ in all cases except the modified Bonferroni testing. In that case, the threshold for statistical significance was set at 0.05 divided by the number of pairwise rank sum tests that had been performed after a Kruskal–Wallis test had indicated a statistically significant difference between means of the treatment groups.

Results

Chamber exposure concentrations

The dust concentrations in each chamber were monitored by a Casella Microdust Pro real-time monitor continuously throughout the exposure (Figure 1). Dust samples were collected on filter paper for every exposure day except a few when the sampling port was needed for particle collection by the Mercer Cascade Impactor. The animal exposure concentrations, determined on the basis of gravimetric samples (see Methods for details), were 2.1 ± 0.4, 6.8 ± 0.9, 20.8 ± 2.5 and 60.6 ± 8.1 mg/m^3 (Table 1). The daily average chamber concentrations recorded photometrically and determined gravimetrically for the 4-week (20 × 6-h) exposures are shown in Figure 2.

Chamber aerosol characteristics

Chamber particle size profiles were obtained in real time using a Quartz Crystal Microbalance Cascade Impactor and a TSI Aerodynamic Particle Sizer. The instruments were calibrated by the manufacturers using particle densities of 2.0 g/cc and 1.0 g/cc (styrene) respectively. Lunar dust has a density of 2.7 g/cc, which is similar to that of silica. Most of the modes of lunar dust particle distributions for all four chamber exposures recorded by the QCM were 1.6 μm; the MMADs of aerosols in each chamber would also be close to 1.6 μm (Figure 1C). There was little difference between aerosols in high- and low-concentration exposure chambers and no difference between samples taken from nose ports in the upper and lower levels or on different days, measured by the QCM. APS is a light scattering spectrometer for measuring particles on the basis of time of flight; MMADs measured by APS were slightly sensitive to the particle concentrations. MMADs recorded in the chambers with higher dust concentrations were higher. The MMADs of the aerosol chambers with the two lower concentrations were mostly 2.0–2.4 μm (geometric standard deviation [gSD] ~1.6), whereas the MMADs of aerosols in the chamber with the highest concentration of dust were mostly 2.4–2.8 μm. We used the Mercer impactor several times for each chamber for MMAD determination; the MMAD data showed no great difference between these chamber exposures, with MMADs for the four exposure regimes being 2.3–2.5 μm with gSD of ~1.3 (Table 1). Data from all these instruments showed that the sizes of the particles were in the respirable range. The pathologist who made note of the particle sizes of lunar dust in the lung parenchyma reported that the particles were mostly below 1 μm (which can be converted to an MMAD of 1.8 μm by assuming particles are spherical and uniformly sized).

Assessment of biomarkers in bronchoalveolar lavage fluids for pulmonary inflammation and injury

Three groups of 22 rats (2 for spares) each were exposed nose-only to air, or to lunar dust concentrations of 20.8 ± 2.5 and 60.6 ± 8.1 mg/m^3 for 4 weeks (6 h/day, 5 days/week). Results for BALF biomarkers of toxicity and lung histopathology showed that both of these exposure concentrations produced some mild to moderate toxicity in the lung. To determine a NOAEL, a second 4-week lunar dust study was conducted in which rats were exposed to air and lunar dust concentrations of 2.1 ± 0.4 and 6.8 ± 0.9 mg/m^3. In both studies, groups of five rats were assessed for pulmonary toxicity at 1 days, 1 week, 4 weeks and 13 weeks after

the termination of inhalation exposure; the right lungs were lavaged for assessment of biomarkers of toxicity in BALF while the left lungs (unlavaged) were microscopically examined for lung lesions.

Lunar dust exposures produced concentration-dependent increases in the total number of cells, neutrophils and lymphocytes in the BALF samples from all post-exposure rats (Figure 3). However, neither the total number of cells in the BALF nor the percentage of neutrophils was significantly different between the air-exposed group and the groups exposed to the two lower lunar dust concentrations (2.1 and 6.8 mg/m^3) for all time points (Figures 3 and 4) except for a slight increase in neutrophils in the BALF from rats exposed to 6.8 mg/m^3 and assessed 13 weeks after the exposure. The number of cells for the low-concentration exposure study (0, 2.1 and 6.8 mg/m^3) plotted together with the number of cells for the high-concentration study were too small to visualize in Figure 3; therefore the data for the low-concentration study are plotted separately in Figure 4 so the results for rats exposed to 2.1 or 6.8 mg/m^3 can be visually compared with those for the concurrent air-exposed group.

Besides assessing cellular components in the BALF as indices of inflammation, we also measured total protein concentration, an index of the integrity of the capillary wall, and the levels or activity of cellular enzymes including LDH, aspartate transaminase (AST or SGOT) and glutamyl transferase (γGT). As with the BALF cellular biomarkers, we also observed concentration-dependent increases in BALF protein concentrations and the levels of these cellular enzymes (Figure 5); no differences were detected between the results for rats exposed to two lower exposure concentrations and those for the air-exposed controls.

Rats instilled with quartz served as positive controls for the measurement of biomarkers of toxicity

In both inhalation studies and at each scheduled necropsy day, designated as 1-day, 1-week, 4-week or 13-week post-exposure, five rats were instilled with quartz at 2.5 mg/rat 1 week (±1 day) before the necropsy day of inhalation-exposed rats. The inclusion of the quartz-ITI rats serving as positive controls concurrent with the inhalation-exposed rats for BALF toxicity biomarker assessment should ensure that, if no effects of an inflammation or cytotoxic marker were observed, experimental error would not be suspected as the cause. Results showed that quartz produced the expected lung inflammation and cytotoxicity. As described in Methods, the rats used as quartz controls included those that were excluded from the inhalation studies because they were at one end or the other of the weight spectrum, the weights of these rats varied greatly. The quartz treatment was by ITI and only the right lung of each rat was lavaged. These factors (animal weight variations and uneven spread of instilled dust suspensions in the lung) contribute to greater variations of the biomarker values seen in the instilled rats than in rats exposed to lunar dust by inhalation (Figures 3–5).

Microscopic examination of lesions for pulmonary inflammation and injury

Results from rats exposed to 0, 20.8 and 60.6 mg/m^3—After the 4-week exposure, rats were necropsied and their lungs were examined 1 day, 1 week, 4 weeks or 13 weeks after the last exposure; the data are summarized in Table 2 and Figure 6. Dust deposited in the lung was visible as individual particles diffusely scattered throughout the lung

parenchyma, free in alveoli or within alveolar macrophages (Figure 7A and B). The numbers of macrophages in alveoli were clearly increased relative to those of controls in rats necropsied 1 day after the last dust exposure. Relative to those on the alveolar surface, the numbers of particles and macrophages were slightly decreased in the subpleural alveoli, and fewer particles and macrophages were present in the interstitium. A mild diffuse neutrophil infiltrate was associated with the macrophages and particles. Numbers of visible dust particles, dust-free macrophages and inflammatory cells in the lungs decreased with time whereas a time-dependent increase occurred in the numbers of particles within macrophages relative to particles that were free in alveoli. The particles were mostly <1 μm in diameter. The clearly diffuse pattern of particles and macrophages seen shortly after the termination of exposure progressed into many small microfoci; this pattern continued to change to variably sized foci of accumulated particles, mostly within macrophages and accompanied by a predominantly neutrophilic infiltrate, frequently in areas surrounding blood vessels, as observed in lungs of rats necropsied 13-week post-exposure. Perivascular accumulations of lymphocytes were clearly present in lungs in the two higher exposure groups necropsied after 4 weeks of exposure; perivascular accumulations of lymphocytes and macrophages were even more prominent in rats necropsied after 13 weeks of exposure (Figure 7C). Although categorized as vasculitis, these lesions did not directly involve vessel walls but rather were aggregates of inflammatory cells closely surrounding vessels.

Fibrosis of lung parenchyma or lymph nodes was not evident, even in tissue slides stained with trichrome blue; areas of increased cellularity or septal thickening, visible in sections stained with H&E, did not clearly indicate fibrosis. The only evidence suggesting a progressive lesion that might lead to eventual fibrotic changes was the presence of foci in the lungs that were classified as granuloma/nodules (Figure 7C). Trichrome blue staining of tissues revealed a mild increase in collagen deposition in areas of thickened septa (Figure 7D). The predominant perivascular location of many inflammatory cells in lung sections from the rats necropsied later in the post-exposure process very likely reflects the role of the lymphatic drainage system in removal of foreign materials. Lymphatic vessels (not visible with light microscopy) lie parallel to the pulmonary vasculature. The general inflammation responses were concentration-dependent.

A minimal accumulation of visible dust particles was diffusely scattered through the lymph nodes 1 day after the 4-week exposure. Accumulation of particle-laden macrophages within lymph nodes clearly increased 4 weeks after exposure, and even more when lymph nodes were examined 13 weeks after the exposure. Enlarged paracortical areas containing many well-formed foreign-body-types of small granulomas with many particles were observed in the lymph nodes of all rats exposed to the highest concentration and one rat exposed to the next lower level (Figures 6 and 8). There was a minimal to mild diffuse lymphoid hyperplasia 1 day after the exposure; an increase in lymphoid hyperplasia was observed 4 weeks after the exposure. The degree of lymphoid hyperplasia progressed with time, but did not change perceptibly between 4- and 13-week post-exposure (Figure 8 and Table 2).

Results from rats exposed to 0, 2.1 and 6.8 mg/m^3—Rats exposed for 4 weeks to these lunar dust concentrations showed normal lung tissues (Figure 7E and F) with deposition of particles. Lungs of rats examined at high magnification 1 day or 1 week after

the last exposure showed deposited dust visible as individual particles free in alveoli or within alveolar macrophages diffusely scattered throughout the lung parenchyma. In animals necropsied 4 weeks after last exposure, the vast majority of dust particles were within macrophages and some dust-containing macrophages were in groups of several larger macrophages containing particulate material. Clustering of dust-containing macrophages was even more evident in the lungs of rats necropsied 13 weeks after the last dust exposure.

In a "blinded" examination of lung sections without knowledge of exposure or control group, the numbers of macrophages in alveoli were not clearly increased relative to concurrent controls in any dust-exposed group at any interval after dust exposure (Table 3). Focal or multifocal minimal aggregates of macrophages were present in the lungs of variable numbers of rats in dust-exposed or control groups, and these small aggregates had no clear relationship to dust exposure. Numbers of inflammatory cells (neutrophils, lymphocytes, eosinophils, basophils/mast cells) in lung parenchyma and surrounding connective tissue were similar in dust-exposed and concurrent control groups at all necropsy intervals (Table 3).

The microscopic appearance of lymph nodes in rats of these low-concentration groups necropsied 1 day after the last exposure was not different from those of concurrent controls. Minimal increases in the numbers of visible particles were observed at high magnification in macrophages in the medullary sinuses of lymph nodes of 4/5 rats in the high dose group and 3/5 in the low dose group necropsied 1 or 4 weeks after the last exposure and 1/5 in high dose and low dose groups 13 weeks after the last exposure.

Discussion

In an effort to thoroughly investigate the toxicity of lunar dust, we first conducted an intratracheal instillation study and then the nose-only inhalation toxicity studies reported here. The ITI study was conducted to find out how toxic this extraterrestrial dust is by comparing its toxicity with those of two well-studied terrestrial reference dusts, TiO_2 and quartz, and to obtain toxicity information needed for the follow-up inhalation toxicity studies. The results of the ITI study showed that lunar dust was moderately toxic; it was more toxic than TiO_2 but less toxic than quartz (James et al., 2013; Lam et al., 2013). Using the toxicity data obtained from rats instilled with lunar dust at 1, 2.5 and 7.5 mg/rat in the ITI study, and with the limitation of only two chamber systems available for the dust exposures, we selected the target concentrations of 20 and 60 mg/m^3 for our first 4-week inhalation study intending to produce some slight effects in rats exposed to 20 mg/m^3 and mild to moderate pulmonary toxicity in the 60 mg/m^3 exposed group. We succeeded in meeting our overall objective, although 20 mg/m^3 group showed mild effects, which were somewhat greater than anticipated. We then carried out a second inhalation study (conducted 1 year after the first) at target concentrations of 2.0–2.5 and 6.0–7.5 mg/m^3 in an effort to find an exposure concentration(s) to be considered a NOAEL.

The overall results of the combined lunar dust inhalation studies showed concentration-dependent increases in inflammation biomarkers in BALF cellular components (total cell counts, and neutrophil and lymphocyte counts), cytotoxic biomarkers (cellular enzymes

[LDH, γGT and AST]), and a marker for capillary wall integrity (total protein concentration). No statistically significant differences were observed in these biomarkers between lungs of rats exposed to air and lungs of rats exposed to 2.1 or 6.8 mg/m^3 lunar dust at any post-exposure time of assessment, except for a slight increase of neutrophil counts in BALF assessed in rats exposed to 6.8 mg/m^3 and only in those necropsied 13 weeks after the exposure. The percentage of BAL cells identified as neutrophils in this group of five rats was 4.3% ± 1.3%. The increase in neutrophil number in this group was very small compared with that in the groups exposed to 20.1 mg/m^3 (see Figure 3). In a 2-year rat inhalation study with toner and pigment-sized titanium dioxide, neutrophil percentages in the range of 4% were judged to be non-adverse (Bellmann et al., 1991; Muhle et al., 1991). Bermudez et al. (2002) reported ~3.5% of cells in BALF were neutrophils in rats exposed to 10 mg/m^3 of TiO_2, but no lung lesions were detected. Consistent with these reports, the pathologists in our study detected no increases in neutrophils or lesions in lung tissues of the groups exposed to 6.8 mg/m^3. Besides observing no histopathology in rats exposed to 6.8 mg/m^3, we also detected no cytotoxicity or cell injury induced by lunar dust, based on the results of BALF assays; therefore, 6.8 mg/m^3 is regarded as a NOAEL.

Microscopic examination of the left lung showed that dust particles were evenly distributed in the pulmonary parenchyma shortly after the exposure was terminated; the clearly diffuse pattern of particles and macrophages progressed into many small microfoci. This pattern continued to change to variable-sized foci of accumulated particles, mostly within macrophages and accompanied by a predominantly neutrophilic infiltrate. Gradual relocation of the dust-laden macrophages and accompanying inflammatory cell infiltrates from a diffuse distribution to a more multifocal and microfocal distribution indicates that the macrophage-lymphatic-inflammatory cell system was working to remove the dust from the lung parenchyma into the draining lymph nodes and presumably to the gastrointestinal tract by way of the mucociliary elevator system.

Areas of increased cellularity or septal thickening, visible in H&E sections, did not clearly indicate fibrosis. The presence of progressive foci of granuloma/nodules in the lungs of rats exposed to the high concentrations of lunar dust suggests fibrotic potential. However, these relatively small uncompact foci of granulomas/nodules could eventually be resolved by the macrophage phagocytosis process. All these mild degrees of neutrophilic and lymphocytic inflammation, septal thickening, and granulomatous reactions were concentration-dependent, with no inflammation or lesions detected in the two lower exposure concentrations. These results also identified 6.8 mg/m^3 as the NOAEL, which is consistent with the findings of the biomarker study.

Rats have been shown to be the most sensitive rodent species to the toxic effects of poorly soluble particles in the lungs (Mauderly 1997). It is noteworthy that the threshold limit value (TLV) for nuisance dusts or low toxicity dusts, like TiO_2, is 5 mg/m^3 (time-weighted average) set for industrial workers' life-time exposure of 40 h/week. At the relatively low exposure concentration and short duration of 6.8 mg/m^3 for 120 h, the dust is unlikely to impair macrophages' dust removal capacity and is unlikely to cause inflammation or cytotoxicity in the lungs of exposed humans. Of dusts in exposure studies that have an MMAD of ~2.5 μm, which was what we found lunar dust to have in our study, the fraction

of respirable dust deposited in the pulmonary or alveolar region was 8% in rats exposed to aluminosilica (Raabe et al., 1988); the pulmonary deposition was ~25% obtained from experimental data and predicted models for human subjects (Lippmann et al., 1980). Bellmann et al. (1991) reported a pulmonary clearance half-life of 67–87 days in rats exposed to iron oxide (MMAD 3.5 μm), while Cohen et al. (1979) observed a clearance $T_{1/2}$ of 70 days in humans exposed to iron oxide (MMAD 2.8 μm). These data show rats and humans have similar elimination kinetics for this dust. It is noteworthy that unanesthetized Sprague Dawley rats of 300 g inhale ~210 cc/min (minute ventilation rate [*MV*] or 0.7 l/min/kg) (Parent, 1991). *MV* for a resting human (70 kg) is 7.5 l/min and for light exercise is 20 l/min (NRC, 1992); if we assume the 24-h average *MV* ([7.5 +20] ÷ 2) is 14 l/min/70 kg or 0.2 l/min/kg, rats breathe in ~3.5 times more air on a body weight basis than humans and their lungs are exposed to proportionally more dust. Rats inhale 3.5 times more than do humans, but the fraction of deposition of respirable particles in pulmonary regions is ~3 times higher in humans than in rats. NASA selected a conservative approach, deciding on a species extrapolation factor of 3. Therefore the NOAEL for humans is assumed to be 1/3 of the study NOAEL of 6.8 mg/m^3, that is, a human NOAEL of 2.3 mg/m^3 for 1-month exposure (6 h/day; 5 days/week).

One of the main NASA objectives for conducting these lunar dust toxicity studies is to obtain data for estimating safe exposure limits for astronauts living and working in a lunar habitat, which could be as long as 180 days. We recognize that, to obtain data to establish an exposure limit for 180 days, a rodent inhalation exposure of 26 weeks, rather than 4 weeks, would have been ideal, but the limited availability of lunar dust and the prolonged stress to the rats in restraint tubes would not favor long exposures. Therefore, we have to use the data that we obtained from this 4-week inhalation exposure study for estimating safe exposure concentrations to lunar dust for 180 days.

Insoluble dusts are eliminated slowly from the lung, and the lung burden would depend on exposure time and concentration. Pulmonary toxicity would depend on the lung burden of dust. If we ignore dust elimination, Haber's rule ($Conc. \times Time = C \times T = k$) appears to be our best approach for setting safe human exposure limits for lunar dust using the animal NOAEL obtained in our 1-month study. As pointed out by Gaylor (2000) of the U.S. Food and Drug Administration, "Haber's rule is appropriate for extrapolation to different durations of exposure for conditions where dose rate is not the determining factor and only dose dictates the biological effect". Haber's rule was used by the Subcommittee on Military Smoke and Obscurants of the National Research Council to establish exposure guidance levels for brass flakes (a smoke obscurant) for the military (NRC, 1999) and by U.S. Environmental Protection Agency (EPA) on inhaled poorly soluble particles (Jarabek et al., 2005). To calculate a human equivalent exposure concentration for particulates from animal data, EPA's default approach is, "a time adjustment is applied to account for the correction from the noncontinuous inhalation regimen in laboratory animal studies to an assumed continuous, lifelong exposure of 70 years. that is used as the target human exposure by: $NOAEL_{ADJ} = NOAEL \times (H \div 24) \times (D \div 7)$, where H, D and $NOAEL_{ADJ}$ designate hours per day, days per week, and the NOAEL that is duration-adjusted from an intermittent exposure regimen to a continuous exposure level, respectively. The default duration

adjustment is based on the premise that the product of the exposure concentration and duration of time ("C × t" product) produces the same level of effect for a given endpoint (Haber, 1924)" (Jarabek et al., 2005).

Using EPA's default approach, a human 1-month NOAEL, and a time-of-exposure extrapolation factor of 6 for 1 to 6 months would yield an exposure limit of 0.4 mg/m^3 (or 2.3 mg/m^3 ÷ 6) of lunar dust. This 6-month exposure scenario (6 h/day, 5 days/week) could be envisioned as crewmembers being exposed to lunar dust only after returning from outside activities on the lunar surface, for no more than 6 h/day as the dust is scrubbed from the atmosphere, and crewmembers are not exposed at all to dust at other times. However, if the dust concentration is monitored 24 h daily, the recommended exposure limits would be 0.06 mg/m^3 (or 2.3 mg/m^3 × 120 h/4320 h) as a time-weighted average. Other exposure scenarios could be envisioned and the exposure limits can be similarly estimated using Haber's rule; these are presented in Table 4. The selection of which exposure limit to apply depends on how the dust monitoring plan is implemented.

Our safe-exposure estimates have important limitations. One is that any safe-exposure estimate based on a NOAEL is highly dependent on the design of the study, the choice of endpoints and the concentration intervals; therefore, we are in the process of performing benchmark dose modeling of the inhalation data with the expectation that a higher point of departure than the NOAEL of 6.8 mg/m^3 may be identified. The safe exposure estimate from the ITI study was in the range from 0.5 to 1.0 mg/m^3 (James et al., 2013), which is comparable to the estimate of 0.4 mg/m^3 from the data of the present inhalation study. Because of our conservative approach of using an animal-to-human species extrapolation factor of three, we estimate the 180-day TWA of 0.06 mg/m^3 of lunar dust, which is lower than the Permissible Exposure Limit of 0.1 mg/m^3 of quartz established by OSHA for industrial workers' life-time exposure (40 h/week). Given that we found that quartz was much more toxic than lunar dust in our ITI study, it is likely that the species extrapolation factor of 3 may not be needed. In establishing the human exposure guidance limit on TiO_2, NRC (1999) pointed out that " … the uncertainty factor was reduced to 1 because the rat is a more sensitive species than the human to the effects of poorly soluble particles in the lung."

Conclusions

Rats were exposed nose-only for 4 weeks to air and four concentrations of ground lunar dust ranging from 2.1 to 61 mg/m^3. A large spectrum of toxicological endpoints was studied from 1 day to 13 weeks after the end of exposures. The endpoints included biochemical parameters in lavage fluid, cellular markers of toxicity in lavage fluid, and histopathology of lungs and lymph nodes. The NOAEL was found to be 6.8 mg/m^3, which allows us to estimate a human NOAEL of 2.3 mg/m^3. These data allow us to estimate intermittent and continuous lunar dust exposure limits for 180 days of 0.4 and 0.06 mg/m^3, respectively. The toxicity results and the estimated exposure limits will be useful for assessing the health risk of human exposure to lunar dust, and guiding the design of dust mitigation systems in lunar landers or habitats.

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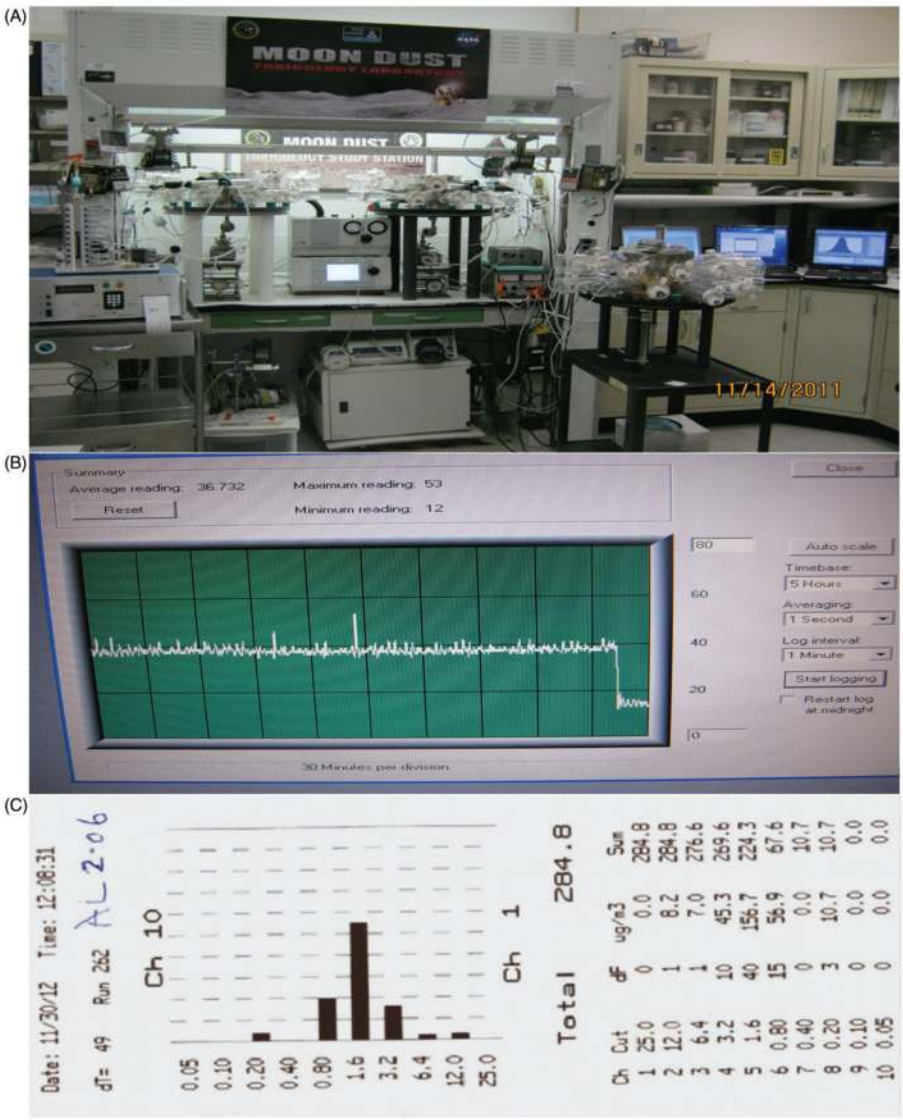

Figure 1.

(A) Nose-only inhalation chamber systems and the associated dust generators, cyclones, dust monitors and data acquistion computers. (B) A typical 6-h chamber concentration recorded by the Casella Microdust Pro. (C) An example of a particle size profile recorded by the Quartz Crystal Microbalance Cascade Impactor.

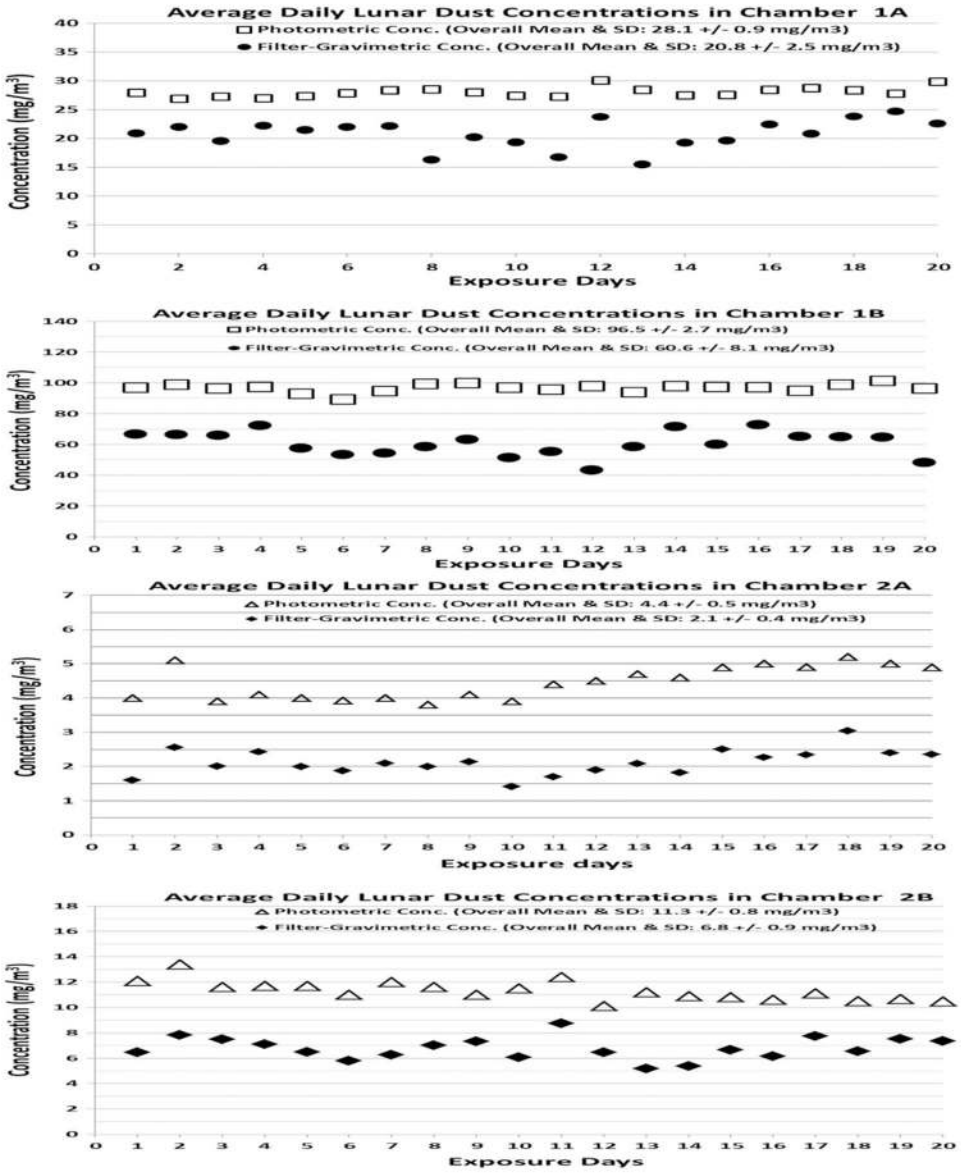

Figure 2.

Lunar dust concentrations in each chamber were photometrically recorded by a Casella Microdust Pro real-time monitor continuously throughout the exposure period. Dust samples were collected on filter papers and the exposure concentrations were estimated on the basis of gravimetric determination. Plotted are average daily dust concentrations in each chamber for the two 4-week studies (20 exposures); Chambers 1A and 1B for 1st Study and Chambers 2A and 2B for 2nd Study.

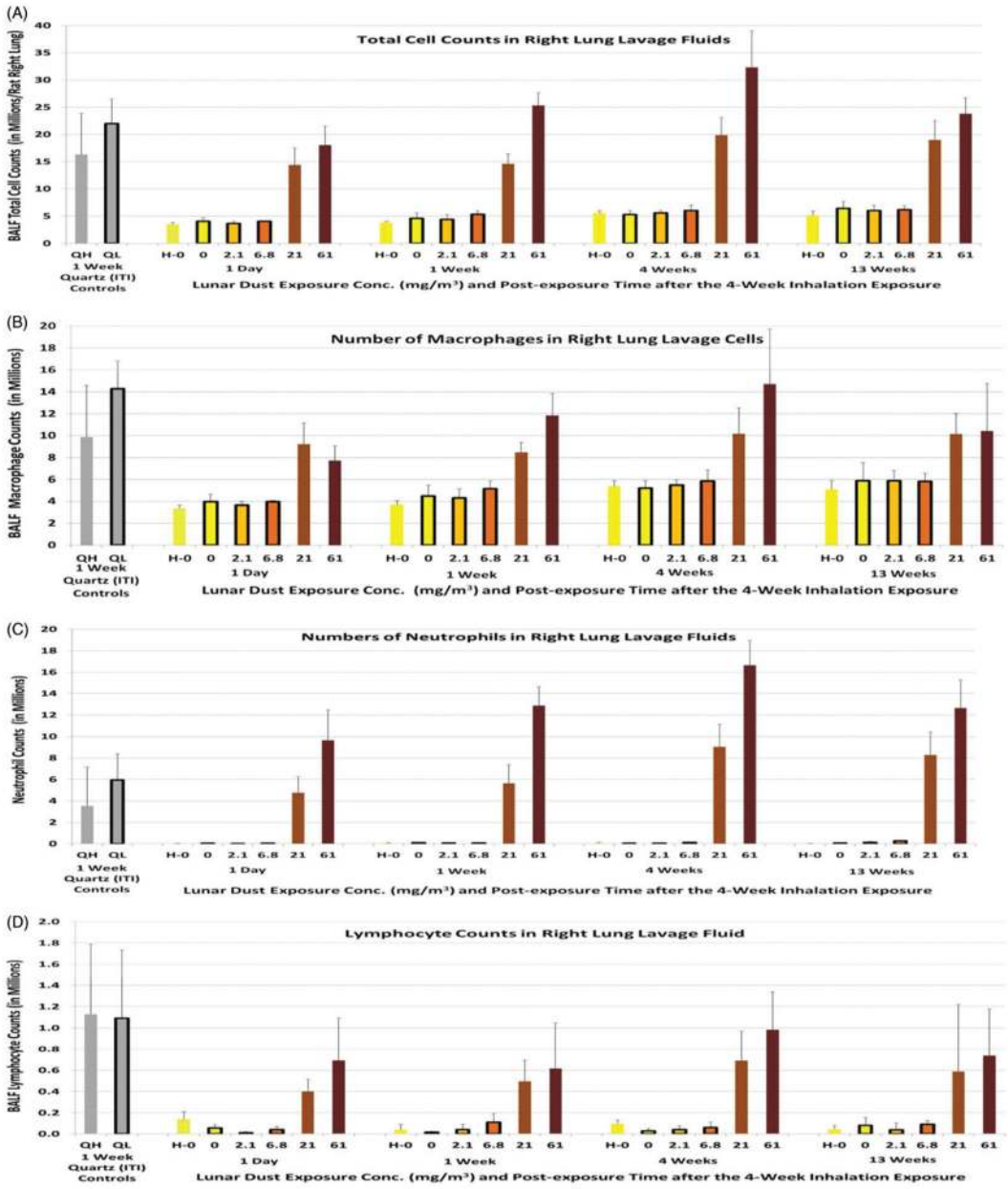

Figure 3.

Cellular components in right-lung lavage fluids of lunar dust (LD) inhalation-exposed rats: total cell counts (A), macrophage counts (B), neutrophil counts (C) and lymphocyte counts (D). Control groups H-0 and 0, were exposed to air at the same time as other groups of rats were exposed to high concentrations (21 or 61 mg/m^3) or low concentrations (2.1 or 6.8 mg/m^3) of lunar dust, respectively. Groups of five rats each were instilled with 2.5 mg quartz 1 week before the day of lung lavage serving as positive controls for biomarker assays; QH (4 × 5 rats) and QL (4 × 5 rats) were lavaged on the same days as the groups exposed to high-concentrations or low concentrations of lunar dust, respectively. All the

values from the groups exposed to 21 and 61 mg/m^3 were significantly different from H-0 at the same time point.

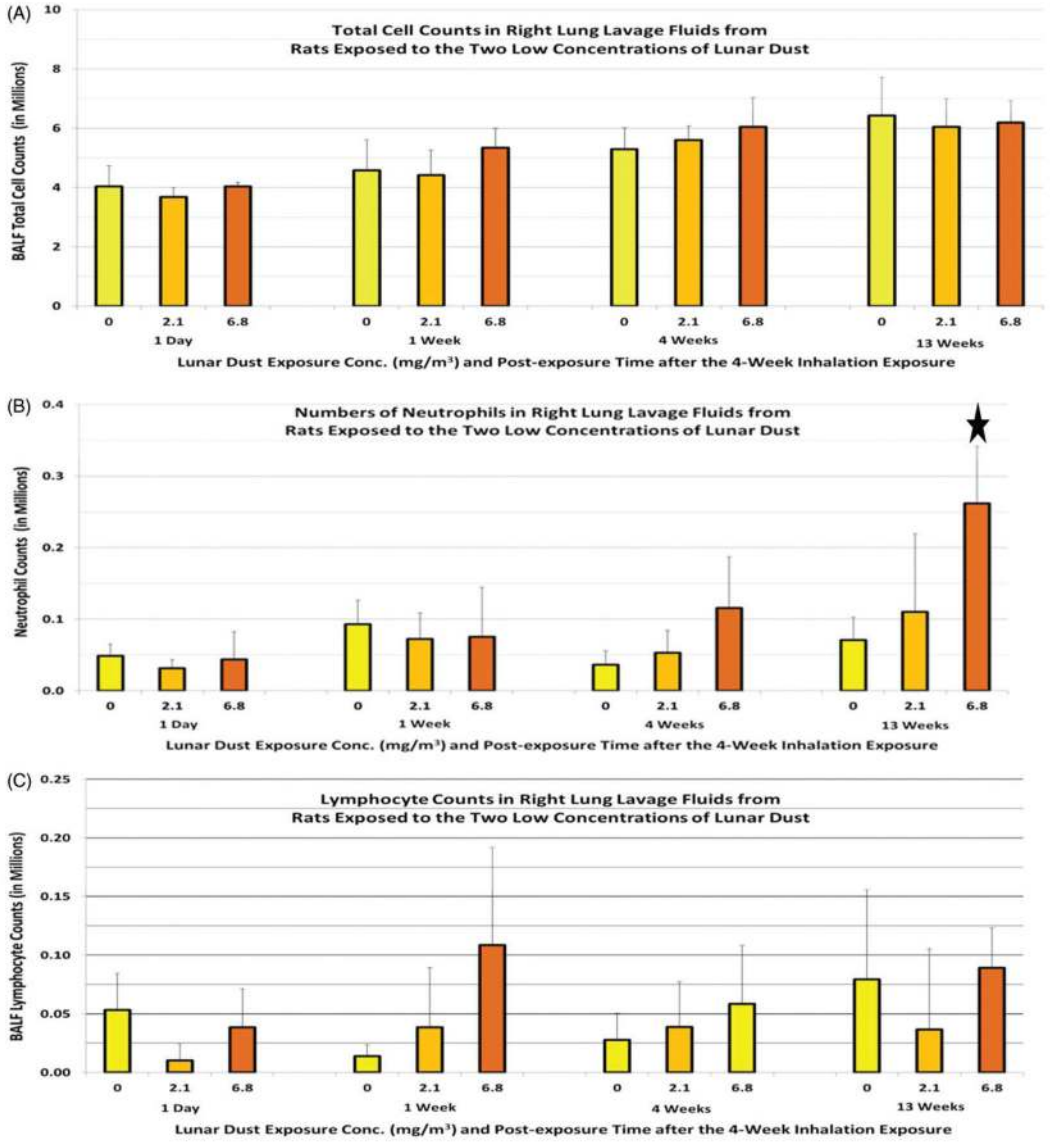

Figure 4.

Three groups of 20 rats each were exposed to 0, 2.1 or 6.8 mg/m^3 of lunar dust for 4 weeks (6 h/day, 5 days/week). Five rats from each group were euthanized on 0- (day 1), 1-, 4- or 13-week post-exposure for assessment of toxicity biomarkers in lavage fluids from right lungs and for lesions in the left lungs. Plotted here are total cell counts (A), neutrophil counts (B), and lymphocyte counts (C) in lavage fluids. *Statistically significantly different from air-exposed group (0) at the same time point.

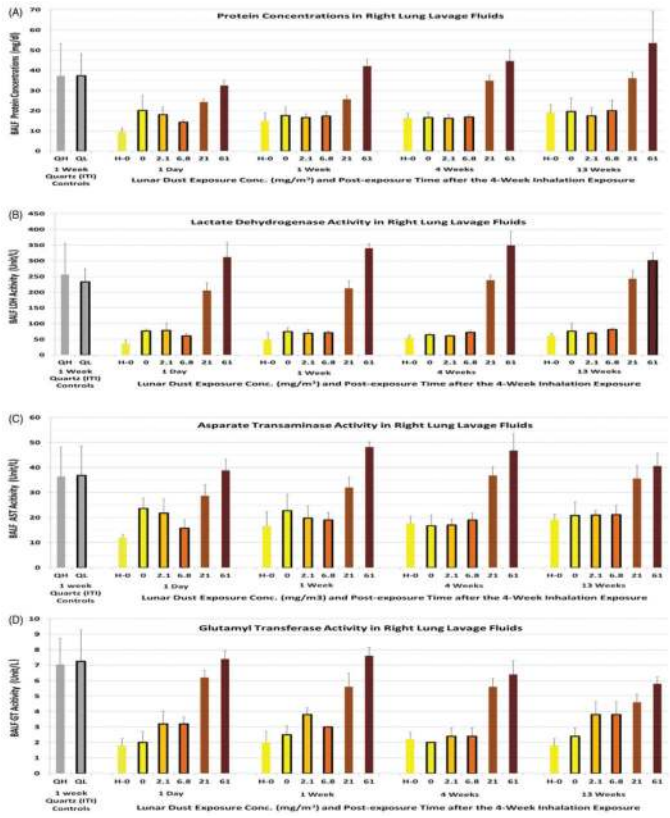

Figure 5.

Acellular biomarkers of toxicity in right-lung lavage fluids of rats exposed by inhalation to lunar dust (LD). Control groups, H-0 and 0, were exposed to air at the same time as other groups of rats were exposed to high concentrations (21 or 61 mg/m^3) or low concentrations (2.1 or 6.8 mg/m^3) of lunar dust, respectively. Groups of five rats each were instilled with 2.5 mg quartz 1 week before the day of lung lavage serving as positive controls for biomarker assays; QH (4 × 5 rats) and QL (4 × 5 rats) were lavaged on the same days as high-concentration groups and low-concentration groups, respectively. LD: $N = 5$ rats. All the values from the groups exposed to 21 and 61 mg/m^3 are significantly different from those of the H-0 groups at the same time point.

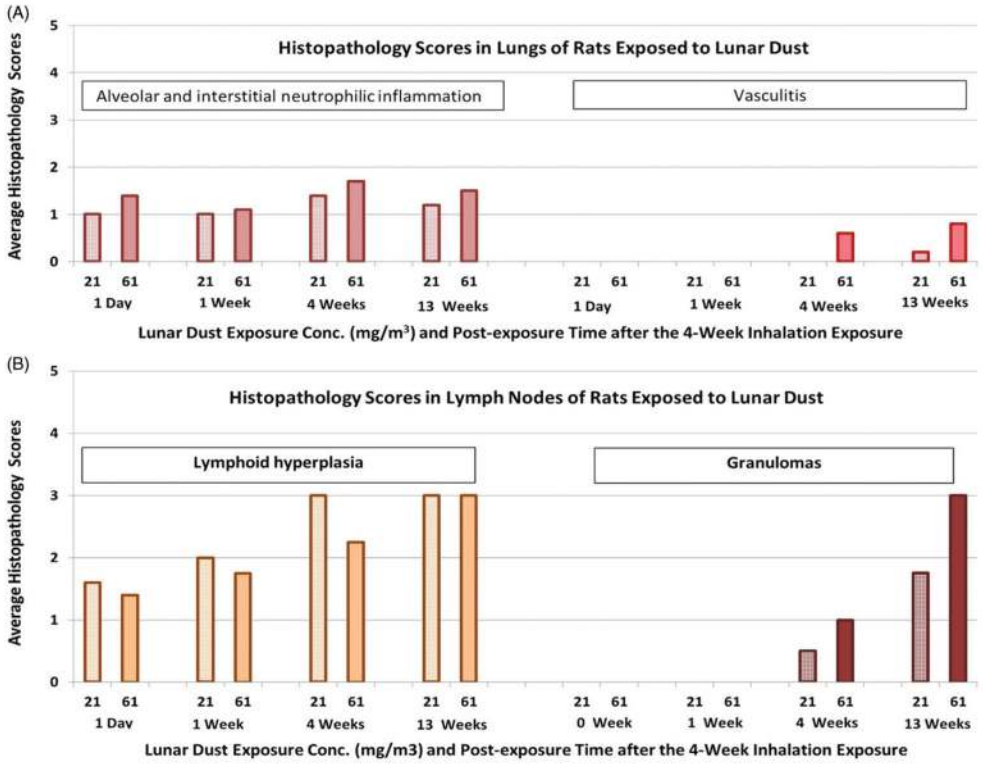

Figure 6.

Among the indices assessed and shown in Table 2, the major histopathology indices with average scores in lungs of rats exposed to lunar dust are shown here. Scores for lungs of rats exposed to air or to 2.1 or 6.8 mg/m^3 lunar dust were minimal (increase of macrophages) or 0 and are not plotted in the graphs. Histopathology scoring scales: 1, minimal; 2, mild; 3, moderate; 4, marked and 5, severe. Each bar is the average score for five rats.

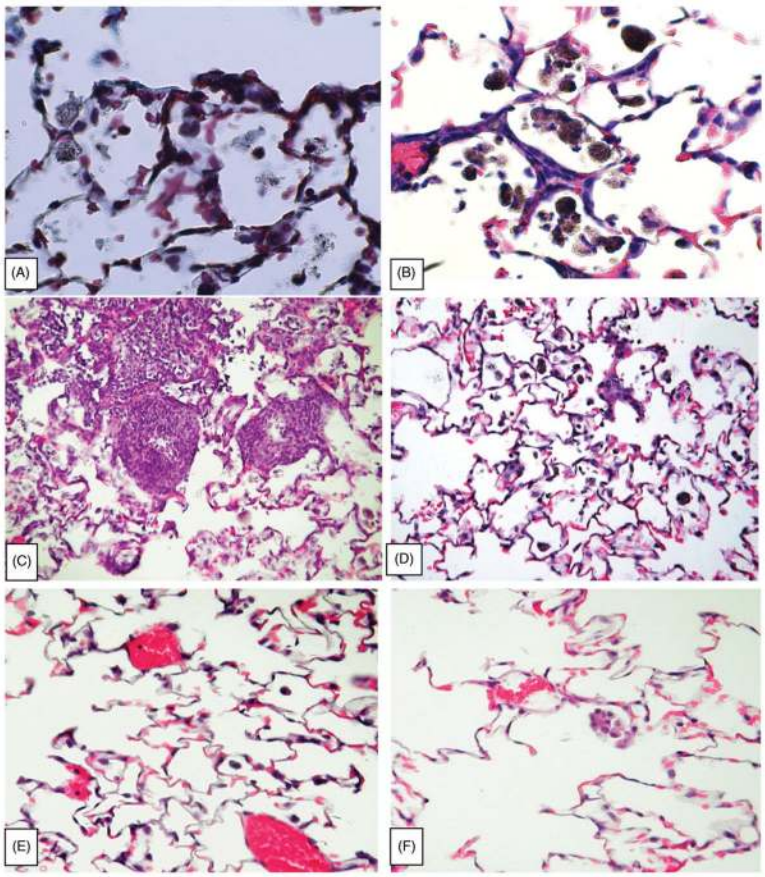

Figure 7.

Lung tissues of rats exposed to lunar dust at 61 (HC) or 6.8 (LC) mg/m^3 for 4 weeks. (A) HC: lunar dust particles visible in alveoli or within macrophages 1-day post-exposure. (B) HC: lung with thickened alveolar septa, macrophages containing particles, 1-week post-exposure. (C) HC: perivascular lymphocytes and macrophages in lung from a rat necropsied 4-week post-exposure. (D) HC: focal septal thickening, macrophages and inflammatory cells in alveoli 13-week post-exposure. (E) LC: lung tissue of a rat exposed to 6.8 mg/m^3 lunar dust for 4 weeks and necropsied 1-week post-exposure showing normal structure like that in air-exposed rats. (F) LC: an alveolus at the center contains a cluster of macrophages containing dust particles. Image magnification: (A) and (B): 40×; (C–F): 20×.

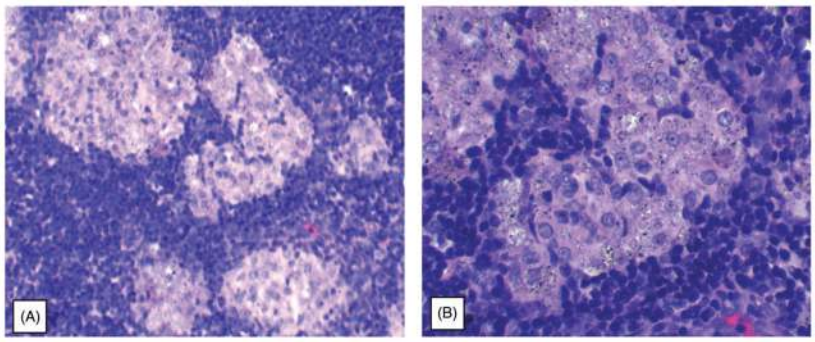

Figure 8.

Tracheobronchial lymph nodes from rats exposed to 61 mg/m^3 for 4 weeks and examined 13 weeks after the last exposure. (A) Macrophages containing lunar dust particles in the lymph node of a rat necropsied 13-week post-exposure. (B) Higher magnification of (A) shows numerous lunar dust particles in granulomas. Image magnification: (A): 20×; (B): 40×.

Table 1

Chamber lunar dust concentrations and particle size distribution*.

Target expo. conc. (mg/m^3)	Gravimetric conc. (mg/m^3)	Photometric conc. (mg/m^3)	MMAD (μm) (Mercer Impactor)	Geometric SD (Mercer Impactor)
0 (Air)	–	–	–	–
2.0–2.5	2.1 ± 0.4	4.4 ± 0.5	2.30, 2.37	1.26
6.0–7.5	6.8 ± 0.9	11.3 ± 0. 8	2.26 ± 0.11	1.25
20	20.8 ± 2.5	28.1 ± 0.9	2.48 ± 0.04	1.22
60	60.6 ± 8.1	96.5 ± 2.7	2.48 ± 0.15	1.27

* Because the Mercer Impactor shared a sample port with the gravimetric sampler, and because, for the lowest concentration chamber, it was needed to collect sample for 6 h in order to have enough dust on the impactor discs for weight determination, we collected only two samples from this chamber for MMAD determinations. For higher concentration exposures, several samples were collected each chamber.

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Table 2

Summary of the histopathology scores of rats exposed to 21 and 61 mg/m^3 of lunar dust*.

Exposure conc. →	20.8 mg/m^3 Lunar dust				60.6 mg/m^3 Lunar dust			
Post-exposure time →	**1 day**	**1 week**	**4 weeks**	**13 weeks**	**1 day**	**1 week**	**4 weeks**	**13 weeks**
End points								
Intra-alveolar tissue								
Macrophages	2.2	2	1.8	1.6	2.6	2.2	2.2	2
Alveolar epithelial changes	0	0	0	0	0	0	0	0
Neutrophils (infiltration)	1.2	1	1.8	1.4	1.8	1.2	2.2	2
Lymphocytes (infiltration)	0	0	0	0.8	0	0	0.8	0.8
Lipoproteinosis	0	0	0	0	0	0	0	0
Interstitium								
Macrophages	1	1	1	1	1	1	1.8	2
Neutrophils (infiltration)	0.8	1	1	1	1	1	1.2	1
Lymphocytes (infiltration)	0	0	0	1	0	0	0.6	1
Fibrosis	0	0	0	0	0.25	0	0	0
Granulomas/nodules	0	0	0	0.2	0	0	0.6	0.8
Vascular tissue								
Vasculitis	0	0	0.8	1.4	0	0	1.6	2
Neutrophils (infiltration)	0	0	0.8	1.4	0	0	1.6	2
Lymphocytes (infiltration)	0	0	0.8	1.2	0	0	1.6	2
Bronchi/bronchioles								
Inflammatory cell infiltrate	0	0	0	0	0	0	0	0
Fibrosis	0	0	0	0	0	0	0	0
Lymph node								
Particle accumulation	1	2	2.5	3.75	1	2	3	4
Inflammation/neutrophilic infiltrate	0	0	0	0	0	0	0	0
Lymphoid hyperplasia	1.6	2	3	3	1.4	1.75	2.25	3
Granulomas/nodules	0	0	0.5	1.75	0	0	1	3
Fibrosis	0	0	0	0	0	0	0	0
Trichrome stain								

Exposure conc. →	20.8 mg/m^3 Lunar dust				60.6 mg/m^3 Lunar dust			
Post-exposure time →	**1 day**	**1 week**	**4 weeks**	**13 weeks**	**1 day**	**1 week**	**4 weeks**	**13 weeks**
Fibrosis	0	0	0	0	0.2	0	0	0
Collagen deposit	0	0	0	0	0	0	0	0
Smooth muscle changes	0	0	0	0	0	0	0	0

Scoring scale: 0 = none/normal, 1 = minimum, 2 = mild, 3 = moderate, 4 = marked, 5 = severe.

*Each histopathology score is the average of five rats.

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Table 3

Summary of the histopathology scores of rats exposed to 2.1 and 6.8 mg/m^3 of lunar dust*.

Exposure conc. →	0 mg/m^3 Air				2.1 mg/m^3 Lunar Dust				6.8 mg/m^3 Lunar Dust			
Post-exposure time →	**1 day**	**1 week**	**4 weeks**	**13 weeks**	**1 day**	**1 week**	**4 weeks**	**13 weeks**	**1 day**	**1 week**	**4 weeks**	**13 weeks**
End points												
Intra-alveolar tissue												
Increased macrophages	0	0.2	0.2	0.4	0.2	0.6	0	0.2	0.2	0.2	0.2	0.4
Dust in macrophage	0	0	0	0	1	1	1	0.4	1	1	1	1
Dust free in alveoli	0	0	0	0	1	1	0	0	1	1	1	0
Neutrophils (infiltration)	0	0	0	0	0	0	0	0	0	0	0	0
Lymphocytes (infiltration)	0	0	0	0	0	0	0	0	0	0	0	0
Lipoproteinosis	0	0	0	0	0	0	0	0	0	0	0	0
Interstitium												
Macrophages	0	0	0	0	0	0	0	0	0	0	0	0
Neutrophils (infiltration)	0	0	0	0	0	0	0	0	0	0	0	0
Lymphocytes (infiltration)	0	0	0	0	0	0	0	0	0	0	0	0
Fibrosis	0	0	0	0	0	0	0	0	0	0	0	0
Granulomas/nodules	0	0	0	0	0	0	0	0	0	0	0	0
Vascular tissue												
Vasculitis	0	0	0	0	0	0	0	0	0	0	0	0
Neutrophils (infiltration)	0	0	0	0	0	0	0	0	0	0	0	0
Lymphocytes (infiltration)	0	0	0	0	0	0	0	0	0	0	0	0
Bronchi/bronchioles												
Inflammatory cell infiltrate	0	0	0	0	0	0	0	0	0	0	0	0
Fibrosis	0	0	0	0	0	0	0	0	0	0	0	0
Lymph node												
Visible particles	0	0	0.3	0	0	0.6	0.6	0.2	0	0.8	0.8	0.2
Neutrophilic infiltrate	0	0	0	0	0	0	0	0	0	0	0	0
Lymphoid hyperplasia	0	0	0	0	0	0	0	0	0	0	0	0
Granulomas/nodules	0	0	0	0	0	0	0	0	0	0	0	0
Fibrosis	0	0	0	0	0	0	0	0	0	0	0	0

Scoring scale: 0 = none/normal, 1 = minimum, 2 = mild, 3 = moderate, 4 = marked, 5 = severe.

*Each histopathology score is the average of five rats.

Table 4

Recommended lunar dust exposure limits for humans.

Days (24 h/day)	Exposure duration (h)	Exposure conc. (mg/m^3)	$C \times T$
Human NOAEL estimated based on rat data	120	2.3	276
7*	168	1.6	267
30*	720	0.4	288
180*	4320	0.06	259
30 h/week for 180 days**	770	0.4	309

* Time-weighted-average for daily 24-h exposure.

** 180 days of 30 h/week in the last row.