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Macrophage fusion caused by particle instillation

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ABSTRACT

Background: Multinucleated giant cells (MGC) are formed by fusion of macrophages in pathological conditions. These are often studied in the context of the foreign body response to biomaterial implants, but MGC formation is rarely assessed in response to inorganic particles in the lungs. Therefore, a major objective of this study was to quantitatively compare *in vivo* macrophage fusion resulting from exposure to a spectrum of micron- and nano-sized particles from both environmental and engineered origin, including crystalline silica, multiwalled carbon nanotubes, titanium nanobelts, and crocidolite asbestos.

Methods: Groups of C57Bl/6 mice were instilled with inorganic particles or PBS control. Lung cells were collected by lavage after one week for cell differentials, quantification of macrophage fusion, and microscopic observation of particle uptake.

Results: MGC were present in lungs of all mice exposed to particles; no MGC were found in control mice. Asbestos exposure resulted in significant macrophage fusion, which coincided with significantly increased total lavage cells and percent neutrophils. Microscopic observations show particle internalization in MGC and a unique case of potential heterotypic fusion of macrophages with neutrophils.

Conclusion: MGC can form in the lungs of mice within a relatively short one-week time period after particle exposure. The number of MGC was sufficient for quantification and statistical analysis, indicating that MGC formation was more than simply a rare chance occurrence. Observations of particles within MGC warrants further investigation of MGC involvement in inflammation and particle clearance.

1. Introduction

Multinucleated giant cells (MGC) are macrophage syncytia associated with granulomas in various tissues. In the lung, they are found in interstitial lung diseases caused by unknown (idiopathic) reasons, infection, or inhaled substances (Trout et al., 2016). One of these inhaled substances is inorganic particles from environmental or occupational exposures. Increased MGC have been observed in bronchoalveolar lavage from humans with pneumoconiosis after inhalation of asbestos (Kern et al., 2003; Takemura et al., 1989), silica (Takemura et al., 1989), coal (Takemura et al., 1989), and hard metals (Davison et al., 1983; Tanaka et al., 2014). Among these small numbers of studies assessing MGC formation in response to inorganic particle inhalation, some include only a limited number of case examples. Other challenges with human studies are that they rely on environmental exposures with unknown doses, durations, confounding co-exposures (*e.g.* smoking), and other inter-individual variabilities.

Despite thousands of lung toxicology studies of laboratory animals exposed to environmental and anthropogenic inorganic particles, the potential for these particles to cause macrophage fusion into MGC is rarely assessed. We identified only seven *in vivo* studies that quantitatively assessed MGC formation in lungs in response to inorganic particle exposure, all of which showed an increase in MGC. The particles of interest and animals used in these studies are: amosite asbestos in rats (Beno et al., 2005; Kamp et al., 1995), chrysotile asbestos in rats (Lemaire, 1991; Lemaire, 1985), crocidolite asbestos in mice (Prieditis and Adamson, 1996), multiwalled carbon nanotubes in mice (Murphy et al., 2012), ultrafine titanium dioxide in rats (Warheit et al., 2010), and silica in mice (Prieditis and Adamson, 1996) and rats (Warheit et al., 2010). Among these studies, it is challenging to compare relative effects of different particles on MGC formation due to nonstandard quantification methods and varying experimental conditions (*e.g.* exposure timelines). The chrysotile studies by Lemaire were the only ones to quantify MGC using fusion index, which is a commonly used metric to normalize MGC in relation to macrophages while also considering MGC size in terms of nuclei number (Helming and Gordon, 2009; Trout and Holian, 2019). Therefore, the *in vivo* fusion rates caused by

Abbreviations: MGC, multinucleated giant cell;; PBS, phosphate-buffered saline; SiO_2, crystalline silica; CNT, multiwalled carbon nanotube; TNB, titanium nanobelt; Asb, crocidolite asbestos.

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engineered nanomaterials relative to asbestos or other nanomaterials remain unknown.

If lung exposure to certain inorganic particles causes MGC formation, a logical next step is to determine whether MGC directly interact with the particles. Microscopic images of MGC containing particles have been used as evidence of phagocytosis in lung cells from mice exposed to multiwalled carbon nanotubes (Kinaret et al., 2017; Porter et al., 2010) and humans with occupational exposure to coal (Takemura et al., 1989) or hard metals (Davison et al., 1983; Moriyama et al., 2007). To our knowledge, there are no published reports containing images with discernable MGC uptake of asbestos, silica, or other inorganic particles.

The objective of the current study was to demonstrate that significant numbers of macrophages fuse into MGC in mouse lungs within a relatively short time (7 days) after exposure to inorganic particles. Particles for these experiments were selected to represent a wide variety of micron- and nano-sized particles of both environmental and anthropogenic origin. They include crystalline silica (SiO_2), multiwalled carbon nanotubes (CNT), titanium nanobelts (TNB), and crocidolite asbestos (Asb). Each particle type was hypothesized to result in quantitatively different macrophage fusion rates. Particles were observed to be engulfed by MGC, suggesting an active role in uptake. Finally, we present a case of potential macrophage heterotypic fusion with neutrophils, a unique phenomenon that has not yet been described in response to inorganic particle exposure.

2. Materials and methods

2.1. Particles

The particles for this study were selected to represent a wide variety of micron- and nano-sized particles of both environmental and anthropogenic origin. The selected particles have been shown previously by our laboratory group to be pro-inflammatory, insoluble, and biopersistent in mice. SiO_2, CNT, and Asb particles were obtained from Pennsylvania Glass Sand Company (Pittsburgh, PA), Sun Nano (Fremont, CA), and Research Triangle Institute (Research Triangle Park, NC), respectively. During previous studies, SiO_2 was washed in 1 M HCl (Biswas et al., 2017) and TNB was synthesized (Hamilton et al., 2009). Particle characteristics are shown in Table 1, including references for further detail. Endotoxin levels were determined to be negligible by Limulus Amebocyte Lysate assay (Cambrex, Walkersville, MD).

2.2. Particle suspension

Particles were prepared into homogenous dispersions in phosphate-buffered saline (PBS, pH 7.4) immediately prior to each *in vivo* exposure. The following were added to PBS for adequate dispersion of engineered nanomaterials (CNT and TNB): 5.5 mM D-glucose, 0.6 mg/ml mouse serum albumin, and 0.01 mg/ml 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (Sigma-Aldrich, St. Louis, MO). This dispersion medium has previously been shown to not significantly alter pulmonary responses compared to PBS alone (Porter et al., 2008). TNB were suspended by mechanical stirring for 1 h to avoid potential fracture by sonication (Xia et al., 2013). Remaining particles were sonicated for 1 min with a 500 W, 20 kHz Qsonica Q500 (Newtown, CT) cup-horn system at 30% amplitude pulse.

2.3. Mice

Male and female C57Bl/6 mice (Jackson Laboratories, Bar Harbor, ME) aged 8 to 12 weeks were used for all experiments. Mice were housed in microisolator cages with *ad libitum* access to food and water in a specific-pathogen-free (SPF) facility maintained at 22 ± 2 °C, 30–40% humidity, and 12-hour light/12-hour dark cycle. Euthanasia was performed by intraperitoneal injection of sodium pentobarbital. Experimental protocols were approved by the University of Montana Institutional Animal Care and Use Committee (IACUC).

2.4. In vivo experiments

Mice were anesthetized by isoflurane inhalation and exposed to 30 μl of particle suspension or sterile PBS control by oropharyngeal aspiration. Doses were 1 mg/mouse for SiO_2 or 50 μg/mouse for other particles. These doses were selected to induce similar ranges of inflammatory responses as observed during previous dose escalation experiments in our laboratory and to enable comparisons with our existing published data (Biswas et al., 2017; Jessop et al., 2017; Girtsman et al., 2014). Mice were euthanized after 7 days, then cells were collected by 3 × 1 ml lung lavages with PBS, centrifuged, and resuspended for counting with a Beckman Coulter (Indianapolis, IN) Z2 cell counter. Cytocentrifugation of cells was performed at approximately RCF_{avg} 250 ×*g* for 5 min. Slides were stained using a Hematek 2000 autostainer (Bayer Diagnostics, Dublin, Ireland) with a modified Wright-Giemsa (PROTOCOL™; Fisher Scientific, Kalamazoo, MI).

2.5. Microscopy analysis

Differential cell counting was completed by manual morphological evaluation. Multinucleated giant cells (MGC) were defined as containing three or more nuclei. Macrophage fusion into MGC was quantified as previously described (Trout and Holian, 2019) by counting MGC and macrophage nuclei in at least five independent (non-overlapping), random 100× to 200× magnified image fields per treatment. MGC quantification results are expressed as percent fusion by dividing the number of nuclei within MGC by the total macrophage and MGC nuclei, then multiplying by 100. Images showing particle uptake were acquired using an Axioskop microscope with AxioCamMR3 camera (Carl Zeiss, Jena, Germany). The image of potential heterotypic cell fusion was acquired using an Eclipse E800 (Nikon, Melville, NY) microscope with DP26 (Olympus, Waltham, MA) camera.

2.6. Statistics

Multiple comparisons of means from cell differential counts were completed by one-way ANOVA followed by Tukey's HSD test. The MGC % fusion dataset contained sample groups with many zero values (*e.g.* in PBS group), violating ANOVA assumptions of normality and homogeneity of variance. The nonparametric Kruskal-Wallis test was not appropriate because the shape of each sample distribution was very different. Therefore, a one-sample sign-test with one-sided alternative was selected to determine whether % fusion of each treatment group was significantly different from zero. The Holm-Bonferroni correction was applied to p-values to counteract increased type I error due to multiple comparisons. Statistical significance

Table 1
Particle characteristics. Properties of particles used in this study have been previously described by our laboratory group and collaborators: SiO_2 (Thakur et al., 2009), CNT (Hamilton Jr et al., 2012), TNB (Xia et al., 2013), and Asb (Blake et al., 2007). Densities were provided by suppliers or the National Toxicology Program (Keith and Walters, 1992; National Toxicology Program, 1988). The estimated true density is shown for CNT.

Abbr	Particle	Identifier	Shape	Diameter (nm)	Length (μm)	Density (g/cm^3)
SiO_2	Crystalline silica	MIN-U-SIL5	Sphere, irregular	200 to 2500	N/A	2.65
CNT	Carbon nanotube	FA21	Tube, multiwall	27	5 to 15	~2.1
TNB	Titanium nanobelt	NB-2 Long	Belt, anatase	10 × 200	7	3.9 to 4.2
Asb	Crocidolite asbestos	N/A	Fiber, amphibole	160	5	3.3

was defined as a probability of type I error occurring at < 5%. Graphs display mean and standard error. The number of independent replicate mice in each group were 4 (PBS), 4 (SiO_2), 9 (CNT), 7 (TNB), and 8 (Asb). All analysis was completed in R v3.4.0 statistical software.

3. Results

3.1. MGC in lavage

An objective was to determine whether macrophages fuse into MGC within a relatively short, 7-day time period after exposure to inorganic particles. C57Bl/6 mice were instilled with a representative selection of micron- and nano-sized particles of both environmental and anthropogenic origin, including crystalline silica (SiO_2), multiwalled carbon nanotubes (CNT), titanium nanobelts (TNB), and crocidolite asbestos (Asb). As expected, MGC were not found in the lung lavage when mice were not exposed to particles (Fig. 1; PBS control). Measurable numbers of MGC appeared in all particle-treated mice. MGC formation was quantified by % fusion, which reflects both MGC size and abundance relative to macrophages. Asbestos exposure resulted in the highest number of MGC, with statistically significant % fusion (Fig. 1).

3.2. Cell differentials in lavage

Lavage cell differentials were evaluated 7 days after particle exposure in order to provide a more complete assessment and explore potential correlations with MGC formation (Fig. 2). The total number of cells per mouse was significantly higher in response to asbestos exposure compared to control. This increase in total cells was largely attributed to neutrophil influx. The percentage of neutrophils in the lavage from mice exposed to asbestos was significantly increased compared to control. Lung cell differentials from mice exposed to other particles had similar increasing trends in total cells and neutrophils, though not statistically significant.

3.3. Particle uptake

MGC appeared to actively interact with particles in the lung. Larger particles and nanomaterial aggregates became visible in the interior of MGC cells at increased magnification (630×) with careful adjustment of microscope focus. MGC were found to contain particles of all types examined (Fig. 3).

3.4. Possible heterotypic fusion

A unique cell was discovered in the lung lavage from a mouse exposed to asbestos. This was a multinucleated cell containing nuclei from both macrophages and neutrophils within a common cytoplasm (Fig. 4). No visible membranes surround the neutrophil nuclei, suggesting this large cell was formed by heterotypic fusion rather than MGC engulfing whole neutrophil cells. Other cells of this type may have been present in the lung lavage from asbestos-treated mice, but they unfortunately could not be identified with confidence due to less clear staining and overlapping clumps of nuclei.

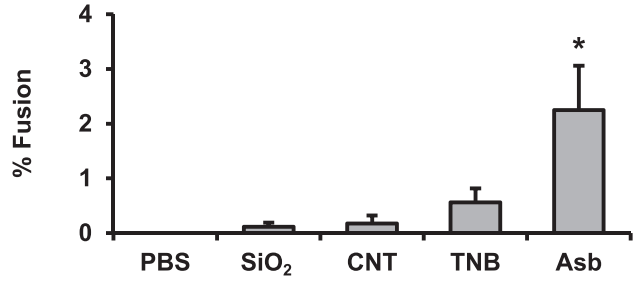

Fig. 1. Fusion *in vivo*. Quantification of MGC in mouse lung lavage 7 days after *in vivo* exposure to specified inorganic particles. The % fusion of asbestos-treated group was significantly greater than zero. *p < 0.05.

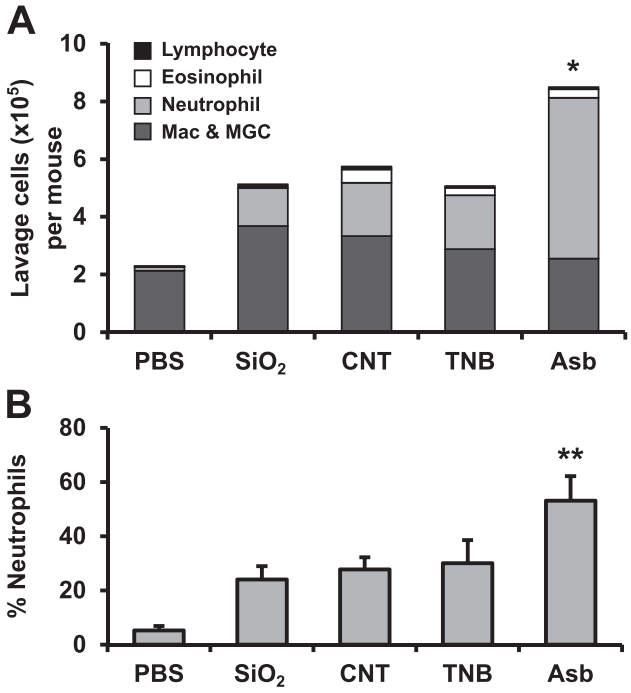

Fig. 2. Cell differential. (A) Lavage cell differentials and (B) % neutrophils in mouse lung lavage 7 days after *in vivo* exposure to specified inorganic particles. The total cells per mouse ($x10^5$) and % neutrophils of asbestos-treated group were significantly greater than PBS control. *p < 0.05, **p < 0.01.

4. Discussion

Most particle and fiber toxicology publications neglect to assess macrophage fusion into MGC and, consequently, their potential significance. When MGC formation is reported, fusion is often quantified by nonstandard methods or not at all. This makes it challenging to compare fusion relative to other exposures or granulomatous conditions, such as sarcoidosis or tuberculosis. One objective of this study was to compare MGC formation in response to different particles using appropriate quantification methods.

Results indicate that measurable numbers of MGC appeared in the lungs of mice exposed to all four inorganic particles within a short, 7-day time period. Crocidolite asbestos caused the highest % fusion. This could be a direct consequence of particle physical properties (*e.g.* size or aspect ratio) or an indirect consequence of asbestos influencing other inflammatory processes. The % fusion quantification method allows for more universal comparisons to other conditions where MGC are found, which is useful for generating hypotheses relating to etiology and mechanisms. For example, the fusion in response to particles in this study was lower than fusion observed surrounding biomaterial implants after 7–14 days in mice (Yang et al., 2014; Padmanabhan et al., 2016). It would be valuable for future studies to explore whether this is a result of differences in MGC formation mechanisms, tissue locations, material size, or cell proximity and attachment to the material.

Macrophage fusion tended to correlate with neutrophil influx. Similar soluble factors in the inflammatory lung environment that stimulate neutrophilia may also contribute to fusion. For example, stimulation of Mac-1 (CD11b/CD18; integrin αMβ2) is important for both neutrophil extravasation (Sumagin et al., 2010) and macrophage fusion (Podolnikova et al., 2016). Alternatively, because neutrophils are primarily the first cells recruited to an inflammatory site, it is also possible that they contribute to MGC formation. For example, neutrophils release matrix

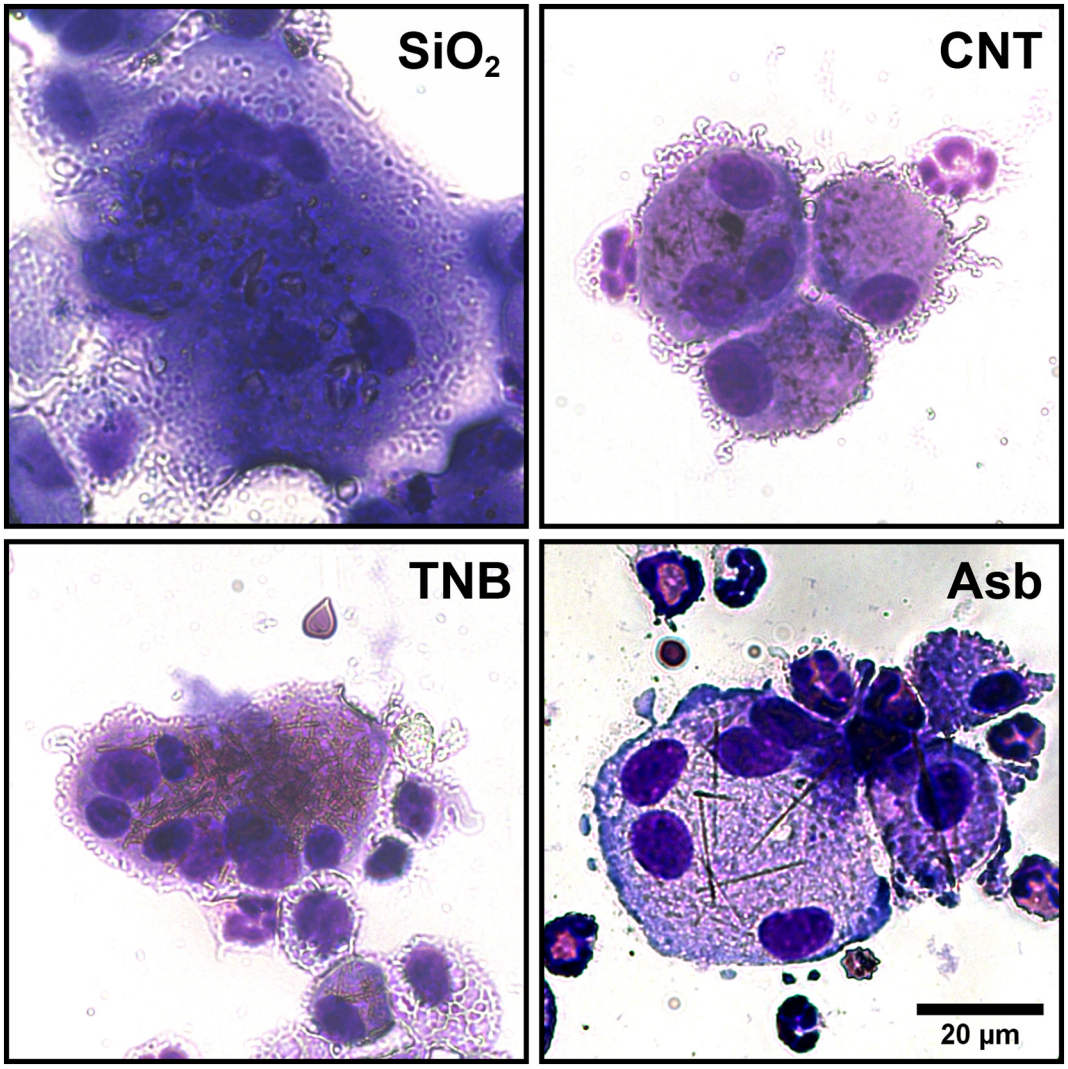

Fig. 3. Particle uptake. MGC observed 7 days after *in vivo* exposure to particles specified in image labels. Scale equal for all images, bar 20 μm.

metalloproteinase 9 (MMP-9; gelatinase B) (Xu et al., 2011), which has been shown to increase MGC (MacLauchlan et al., 2009).

An interesting case of potential heterotypic fusion of macrophages and neutrophils was observed. Another publication (Whiteley et al., 2017) claimed that *Burkholderia thailandensis* caused heterotypic fusion of RAW264.7 macrophage cell line with neutrophils from human blood *in vitro*. It is unclear in these images whether it is a clump of cells or a common cytoplasm is shared. It is also likely that bacteria cause fusion by a separate mechanism. To our knowledge, our study shows the first case of macrophage and neutrophil fusion *in vivo* and in response to inorganic particles. It is important to note that only one obvious cell of this type was observed, so additional confirmatory studies would be important. Other cells types have been considered for potential heterotypic fusion with macrophages as well, including T lymphocytes in response to HIV-1 (Bracq et al., 2017) and somatic cells in tumor pathogenesis (Aguilar et al., 2013).

Microscopic observations of particle uptake were evidence of MGC interaction with particles in the lung after *in vivo* exposure to all types examined (SiO_2, CNT, TNB, and Asb). It is unknown whether phagocytosis occurred before or after macrophage fusion into MGC since this would be challenging to assess *in vivo*. Researchers have hypothesized that macrophages fuse as a result of attempting to phagocytose larger objects, which could explain why larger particles such as asbestos cause more MGC formation. *In vitro* studies using polystyrene beads have shown that MGC are capable of phagocytosis and can internalize larger particles than macrophages (Nakanishi-Matsui et al., 2012; Milde et al., 2015; Moreno et al., 2007). Some other potential reasons for increased MGC may include particle shape, durability, and biopersistence. For example, long titanium nanobelts (Porter et al., 2013) and asbestos (Musselman et al., 1994; Roggli et al., 1987) have been shown to have relatively long retention times in the lung and do not change.

Future studies aiming to correlate particle types with pathology outcomes or to translate MGC observations to human hazard and biomarker assessments should include comprehensive particle dosimetry evaluation. It is expected that MGC formation would correlate with dosing and that comparisons among particles may vary depending on whether dosing was completed according to mass, surface area, volume, or other metrics. In the current study, single doses were selected based on their ability to elicit a biological response in previously published research. This is an important consideration when attempting to draw conclusions regarding relative particle toxicity, which was not the main objective of this study. Rather, the aim was to demonstrate that macrophage fusion occurs in response to a variety of particles to an extent that can be quantified.

Overall, these results demonstrate the value of assessing MGC in response to particle exposures. We expect these initial observations of significant macrophage fusion in the lung will stimulate interest and encourage future studies. Additional particle types could be compared if appropriate MGC quantification methods are used. This would aid further investigations into reasons why certain particles cause more fusion than others. One example study is to determine whether cell-free lung lavage fluid

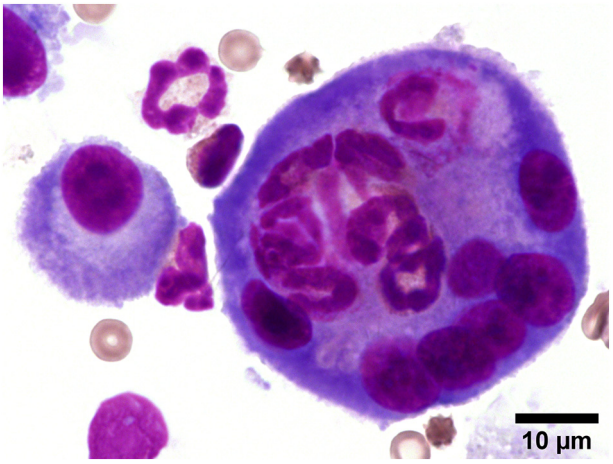

Fig. 4. Heterotypic fusion. Unique case of potential macrophage heterotypic fusion with neutrophils. This cell was observed 7 days after *in vivo* exposure to asbestos. Scale bar 10 µm.

from mice exposed to asbestos or other particles stimulates fusion of bone marrow-derived macrophages *in vitro*, then assess relative importance of each soluble factor (*i.e.* cytokines or chemokines). Another valuable future study would be to compare macrophage and MGC phagocytic capacity for various environmental and engineered particles, as well as expression of surface receptors related to phagocytosis. Finally, it would be important to evaluate macrophage fusion kinetics by particle exposure time courses. Factors such as particle deposition and clearance may influence MGC formation.

5. Conclusions

This research shows that MGC can form in the lung within only 7 days after exposure to inorganic particles, with significant macrophage fusion after exposure to asbestos. Asbestos also resulted in significant increases in total lavage cells and percent neutrophils. Nano- and micron-sized particles of both environmental and anthropogenic origin were observed to be engulfed by MGC. Therefore, it is important for inhalation toxicology researchers to be familiar with MGC in order to identify them while completing routine cell differentials, as well as appropriately report quantification of fusion. Further investigations are warranted to determine the role of MGC in the inflammatory response and particle clearance.

CRediT authorship contribution statement

Kevin L. Trout: Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Writing - original draft. **Andrij Holian:** Conceptualization, Funding acquisition, Writing - review & editing.

Declaration of competing interest

The authors report no conflict of interest.

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