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Investigating palygorskite's role in the development of mesothelioma in southern Nevada: Insights into fiber-induced carcinogenicity

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ABSTRACT

Similar to asbestos fibers, nonregulated mineral fibers can cause malignant mesothelioma (MM). Recently, increased proportions of women and young individuals with MM were identified in southern Nevada, suggesting that environmental exposure to carcinogenic fibers was causing the development of MM. Palygorskite, a fibrous silicate mineral with a history of possible carcinogenicity, is abundant in southern Nevada. In this study, our aim was to determine whether palygorskite was contributing to the development of MM in southern Nevada. While palygorskite, in vitro, displayed some cytotoxicity toward primary human mesothelial (HM) cells and reduced their viability, the effects were roughly half of those observed when using similar amounts of crocidolite asbestos. No Balb/*c* (0/19) or MexTAg (0/18) mice injected with palygorskite developed MM, while 3/16 Balb/*c* and 13/14 MexTAg mice injected with crocidolite did. Lack of MM development was associated with a decreased acute inflammatory response, as injection of palygorskite resulted in lower percentages of macrophages ($p = .006$) and neutrophils ($p = .02$) in the peritoneal cavity 3 d after exposure compared to injection of crocidolite. Additionally, compared to mice injected with crocidolite, palygorskite-injected mice had lower percentages of M2 (tumor-promoting) macrophages ($p = .008$) in their peritoneal cavities when exposed to fiber for several weeks. Our study indicates that palygorskite found in the environment in southern Nevada does not cause MM in mice, seemingly because palygorskite, in vivo, fails to elicit inflammation that is associated with MM development. Therefore, palygorskite is not a likely contributor to the MM cases observed in southern Nevada.

Background

Experimental and epidemiological studies have demonstrated that the inhalation of carcinogenic mineral fibers is the primary cause of malignant mesothelioma (MM) and other respiratory diseases such as asbestosis (Omenn et al. 1986; Gibbs 1990), and an important cause of lung and other cancers (Omenn et al. 1986; Gibbs 1990; Boffetta 2004). BAP1 germline mutations (Carbone et al. 2013), radiation, and SV40 infection have also been linked to MM (Carbone 1999; Carbone, Rizzo, and Pass 2000; Gazdar and Carbone 2003). MM is an aggressive cancer with a very poor prognosis, although certain factors such as peritoneal localization, early stage (I)

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and age (<50 yr) of diagnosis, and the female sex have been associated with prolonged survival (Flores 2009; Miller and Hassan 2012; Rusch et al. 2012; Pass et al. 2014; Cao et al. 2015). Current treatments including surgery, chemotherapy, and novel molecular therapies are not curative (Pinton et al. 2013). Most MM studies have focused on carcinogenesis due to occupational exposure to the six regulated industrial mineral fibers collectively called "asbestos" (Baumann, Ambrosi, and Carbone 2013; Baumann et al. 2015). Besides this family of six regulated asbestos minerals, there are other nonregulated fibers that possess physical and chemical structures that are similar to industrial asbestos, and that may also be carcinogenic (Baumann, Ambrosi, and Carbone 2013; Baumann et al. 2015). Recent studies have begun addressing this hypothesis and have demonstrated that natural asbestiform fibers elicit cancer-promoting inflammation and/or cause MM (Carbone et al. 2011; Carbone and Yang 2012). Moreover, epidemiological studies in New Caledonia and Turkey have already proven a link between some of these fibers and MM development in humans (Baris et al. 1978; Dogan 2003; Dogan et al. 2006; Baumann et al. 2011; Carbone et al. 2011; Baumann, Ambrosi, and Carbone 2013)

In areas where deposits of fibrous minerals are present, erosive processes or human activities can disturb soils, releasing the mineral fibers into the air (Baumann et al. 2015). Once fibers are dispersed in the environment and become breathable, individuals can be exposed, threatening human health. In the United States, and particularly in the more arid portions of Nevada and California, both natural dust emissions and anthropogenic activities (e.g., such as off-road-vehicle [ORV] use, construction, etc.) have significantly increased emissions of mineral fiber-containing dust (Goossens and Buck 2009; Goossens and Buck 2011; Goossens, Buck, and McLaurin 2012), and increased the risk of asbestos-related diseases. Therefore, it is important to determine the carcinogenic potential of asbestiform fibers present in the environment.

Chronic inflammation contributes to the initiation and progression of MM and of other cancers (Hillegass et al. 2010; Carbone and Yang 2012; Noy and Pollard 2014). Within days of exposure to carcinogenic mineral fibers, a vigorous proinflammatory immune response is elicited. An influx of macrophages occurs as they try to engulf and clear asbestos fibers but cannot, and therefore undergo a process of frustrated phagocytosis (Miller and Shukla 2012; Hillegass et al. 2013). This leads to the release of proinflammatory cytokines and chemotactic factors resulting in the recruitment of more macrophages and neutrophils (Hillegass et al. 2010; Miller and Shukla 2012; Hillegass et al. 2013). Recruited macrophages and neutrophils release reactive oxygen species (ROS) and reactive nitrogen species (RNS), which are mutagenic (Hillegass et al. 2010; Carbone and Yang 2012; Kagan 2013). Other factors released during this inflammatory response, such as high-mobility group box 1 (HMGB1) and tumor necrosis factor (TNF)-α, promote the survival of damaged mesothelial cells, increasing the odds that a malignant cell will develop (Yang et al. 2006; Yang et al. 2010; Carbone and Yang 2012).

Inflammatory responses to fibers are classically implicated in the promotion of MM; however, immune responses can also participate in immune-mediated tumor cell killing (Igney and Krammer 2002). While this immune surveillance relies on a variety of cells, macrophages are thought to be key participants in the antitumor response. Alternatively activated macrophages, also called M2 macrophages, release growth factors that can promote tumor growth and development (Zhang et al. 2014) and express surface receptors and cytokines that can inhibit the antitumor effect of other immune cells (Noy and Pollard 2014). Moreover, we recently reported that higher levels of M2 macrophages were associated with increased MM development in BAP1 heterozygous mice (Napolitano et al. 2016) and increased levels of M2 macrophages in tumors have been associated with a poorer prognosis in MM patients (Linton, van Zandwijk, et al. 2012).

Palygorskite is a nonregulated, asbestiform mineral fiber. It is a fibrous, hydrated, high-magnesium phyllosilicate mineral (Wagner, Griffiths, and Munday 1987; Singer 1989; Bish and Guthrie 1993) that forms in arid soils, alkaline groundwater environments, hydrothermal systems, and alkaline lake or marine environments (Callen 1984; Singer 1989; Gibbs et al. 1993; García-Romero et al. 2007; Robins, Brock-Hon, and Buck 2012). A similar fibrous mineral, sepiolite, often occurs together with palygorskite as intergrowths with a continuous compositional

series between the two minerals (Suárez and García-Romero 2011). They typically are found as aggregates of needlelike and asbestiform structures (Huggins, Shell, and Denny 1962), which can be separated into discrete fibers if placed in polar fluids or by using sonication (Wagner, Griffiths, and Munday 1987; Galan 1996). Because of these properties, they are sometimes used as substitutes for asbestos in commercial products (Álvarez et al. 2011). While "palygorskite" is the term used in the field of mineralogy, "attapulgite" has served as the term used in most of the toxicology literature (IARC 1997).

Conflicting data are found in the literature concerning the carcinogenic potential of palygorskite. Palygorskite has behaved much like a carcinogenic fiber in some in vitro experiments, but not in others. For example, it was shown to induce hemolysis (Oscarson, Van Scoyoc, and Ahlrichs 1986; Nadeau et al. 1987) and cytotoxicity in mouse peritoneal macrophages, rat and rabbit alveolar macrophages (Chamberlain et al. 1982; Jaurand et al. 1987; Nadeau et al. 1987), and both bovine and human endothelial cells (Garcia, Dodson, and Callahan 1989). Additionally, palygorskite affected colony formation of Chinese hamster lung fibroblasts (Chamberlain et al. 1982). However, in other studies, palygorskite showed little to no toxicity to human embryonic intestinal cells (Reiss, Millette, and Williams 1980), and it displayed low cytotoxicity to rat pleural mesothelial cells (Jaurand et al. 1987) and did not alter their growth (Renier et al. 1989).

In vivo studies conducted to determine whether palygorskite can cause MM have also been inconclusive. In fact, the incidence of experimental MM induced by palygorskite in rat models varied between 2.5% and 94% across several studies performed more than two decades ago (Pott, Huth, and Friedrichs 1974; Pott et al. 1987; Rodelsperger et al. 1987; Wagner, Griffiths, and Munday 1987). Others observed no development of MM in rats receiving palygorskite (Stanton et al. 1981; Jaurand et al. 1987; Pott et al. 1987; Rodelsperger et al. 1987). Differences in palygorskite fiber length and purity (i.e., presence of other carcinogenic mineral fibers) may have been responsible for the disparate results observed in those experiments.

Recently, increased proportions of women and young individuals among MM cases were found in Clark County, in southern Nevada, suggesting that the development of MM in that area may be due to an environmental exposure to mineral fibers (Baumann et al. 2015). This finding has generated significant concern among residents and has led to considerable media attention and the involvement of government health agencies. While some deposits of different fibrous minerals, including asbestos and erionite, are present in the natural environment and may cause these environmentally related MM (Buck et al. 2013; Baumann et al. 2015; Metcalf and Buck 2015), palygorskite mineral fibers are very abundant in this region. In southern Nevada, palygorskite is primarily found in arid soils (Post 1978; Reheis et al. 1992; Brock and Buck 2005; Brock 2007; Brock and Buck 2009; Robins 2010; Brock-Hon, Robins, and Buck 2012; Robins, Brock-Hon, and Buck 2012; Soukup et al. 2012), but one deposit formed in an alkaline lake (Papke 1972; Post 1978; Miles 2011), and one is likely due to hydrothermal alteration (Post 1978). Because this mineral is so common in southern Nevada, and because these arid soils can be highly emissive for dust, populations in southern Nevada are exposed to palygorskite fibers. Additionally, many individuals have been inadvertently exposed to palygorskite, as there have been more than 80 known industrial uses for palygorskite (IARC 1997). Considering (1) the lack of clarity concerning the carcinogenic potential of palygorskite mineral fibers and (2) that in areas where palygorskite is present, MM occurs in increased proportions of females and young adults, a finding suggestive of environmental exposure, the aim of our study was to determine whether these fibers may contribute to the development of MM.

Results

Palygorskite Deposits Are Localized in Regions of Southern Nevada, Where Environmental MM Cases Are Suggested

Known fibrous palygorskite deposits are concentrated in southern Nevada (Figure 1A). In these areas, a higher proportion of female and young MM cases was reported, suggesting an environmental etiology (Baumann et al. 2015).

The vast majority of palygorskite occurs and forms in alkaline, calcareous soils (Figure 1A, yellow stars), through pedogenic processes influenced

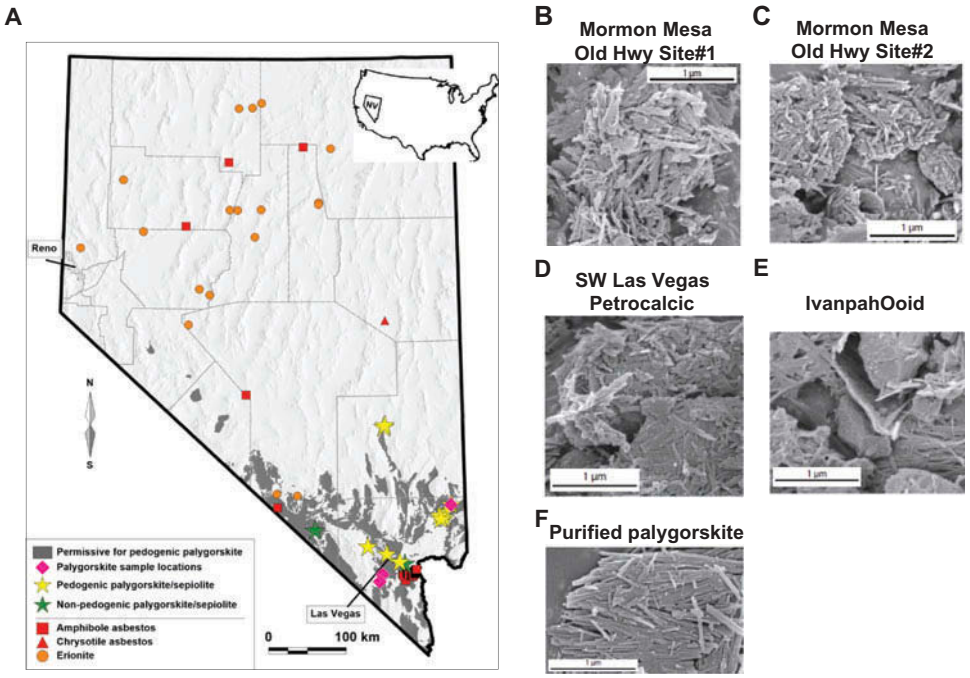

Figure 1. Palygorskite deposits are localized in regions of southern Nevada, where environmental MM cases are suggested. Map of Nevada shows (A) four sample locations for pedogenic palygorskite analyzed in this study (pink diamonds), published locations for pedogenic palygorskite/sepiolote (yellow stars) (Brock and Buck 2009; Brock-Hon, Robins, and Buck 2012; Jube et al. 2012; Reheis et al. 1992; Robins, Brock-Hon, and Buck 2012), published locations for nonpedogenic palygorskite/sepiolite (green stars) (Miles 2011; Papke 1972; Post 1978), and surface units predicted to be permissive for pedogenic palygorskite to be present (gray shading) using PRISM climatological data (PRISM Climate Group n.d.). SEM photographs of palygorskite samples collected from Mormon Mesa Old Highway site 1 (B), Mormon Mesa Old Highway site 2 (C), southwest Las Vegas petrocalcic region (D), Ivanpah ooid region (E), and Source Clays Repository palygorskite standard (F).

by temperature and precipitation (Robins, Brock-Hon, and Buck 2012). Using known pedogenic processes of palygorskite formation (Graham and O'Geen 2010), we modeled the likely occurrence of soil palygorskite in Nevada by combining data on mean annual temperatures and low mean annual precipitation. The parameters used were (1) areas of Miocene to Quaternary age playa and alluvial deposits from the geographic information system (GIS) database of the Nevada state geological map (Ludington et al. 2005), and (2) mean annual temperature >13°C and mean annual precipitation <20 cm averaged over the period 1981–2010 (PRISM Climate Group n.d.). The resulting areas are geologically permissive for pedogenic palygorskite (Figure 1A, gray areas).

Palygorskite samples were obtained from four different locations in southern Nevada, and scanning electron microscopy (SEM) analysis showed that all samples exhibited fibrous physical features similar to those of asbestos minerals (Figures 1B–1F). All samples contained some fibers with dimensional aspect ratios >10:1. These physical characteristics allow for palygorskite to become readily dispersed in the air and respirable (Table 1). The lengths and widths of these palygorskite samples were also nearly identical to a pure palygorskite standard obtained from Source Clays Repository (Table 1), making the pure standard an excellent representative of palygorskite deposits found in southern Nevada.

Because of the consistent presence of palygorskite deposits in areas with reported environmental MM, we investigated whether palygorskite fibers had carcinogenic properties.

Table 1. Physical characteristics of palygorskite samples.

Sample	Length, minimum/maximum (μm)	Thickness, minimum/maximum (μm)
Source Clays Repository palygorskite	0.16/4.0	0.015/0.04
Mormon Mesa Old Hwy Site #1	0.2/0.7	0.02/0.06
Mormon Mesa Old Hwy Site #2	0.22/1.1	0.04/0.1
SW Las Vegas petrocalcic	0.1/2.6	0.03/0.09
Ivanpah ooid	0.4/3.0	0.01/0.04

Palygorskite Is Cytotoxic and Reduces Cell Viability In Human Mesothelial Cells

Carcinogenic fibers exhibit cytotoxicity toward both rat and human mesothelial cells (HM) in vitro (Yegles et al. 1995; Qi et al. 2013). To assess palygorskite's cytotoxicity, HM cells were exposed to increasing concentrations of pure palygorskite. Glass beads, which do not cause MM, were used as a negative control and crocidolite, a known carcinogenic fiber (Hillegass et al. 2013), was used as a positive control. As shown in Figure 2, all concentrations of palygorskite caused significantly more lactate dehydrogenase (LDH) to be released into culture supernatant than glass beads, demonstrating that palygorskite is capable of causing cellular damage to HM cells (Figure 2A). Accordingly, a significant reduction in cell viability was observed using both the MTS and Alamar blue assays (Figures 2B and 2C). These effects on HM cells increased as more palygorskite was added, indicating a clear dose effect.

However, when compared to crocidolite, palygorskite was significantly less cytotoxic in the same culture conditions (Figures 2A–2C). Four-parameter curves were constructed using the mean values of palygorskite dose-response curves, and interpolated to determine the concentration of palygorskite required to induce effects equivalent to a 5-μg/cm^2 dose of crocidolite. Using this calculation for the LDH assay, 5.9 μg/cm^2 of palygorskite was found capable of generating levels of cytotoxicity similar to those generated by 5 μg/cm^2 crocidolite (Supplemental Figure 1A). For the Alamar blue assay, 11.7 μg/cm^2 was determined to be the concentration necessary to reduce viability of HM cells to the same degree as 5 μg/cm^2 crocidolite (Supplemental Figure 1B). Finally, 12.4 μg/cm^2 was the concentration of palygorskite calculated that would reduce viability as much as 5 μg/cm^2 crocidolite using the MTS assay (Supplemental Figure 1C). These results indicate that pure palygorskite was able to induce cytotoxicity in HM cells, but to a lesser extent compared to crocidolite.

Palygorskite Does Not Cause MM In Vivo

To test whether palygorskite was carcinogenic in vivo, Balb/*c* mice were injected intraperitoneally with either palygorskite, or glass as a negative control, or crocidolite as a positive control, and monitored for signs of disease for up to 1 yr after fiber administration. Intraperitoneal injection of carcinogenic fibers, such as crocidolite, has been previously reported, by us and others, to induce MM in rodent species (Kroczynska et al. 2006; Robinson et al. 2011). We observed the development of ascites in more than 30% of mice and the development of MM in 19% of mice exposed to crocidolite (Table 2 and Figures 3A and 3B). On the contrary, glass and palygorskite failed to induce ascites or MM in Balb/*c* mice (Table 2).

MexTAg transgenic mice express the SV40 large T antigen in mesothelial cells and are therefore much more susceptible to asbestos carcinogenesis (Robinson et al. 2006). Thus, these mice are an excellent model to interrogate the carcinogenic potential of fibers, even fibers that may have low carcinogenic

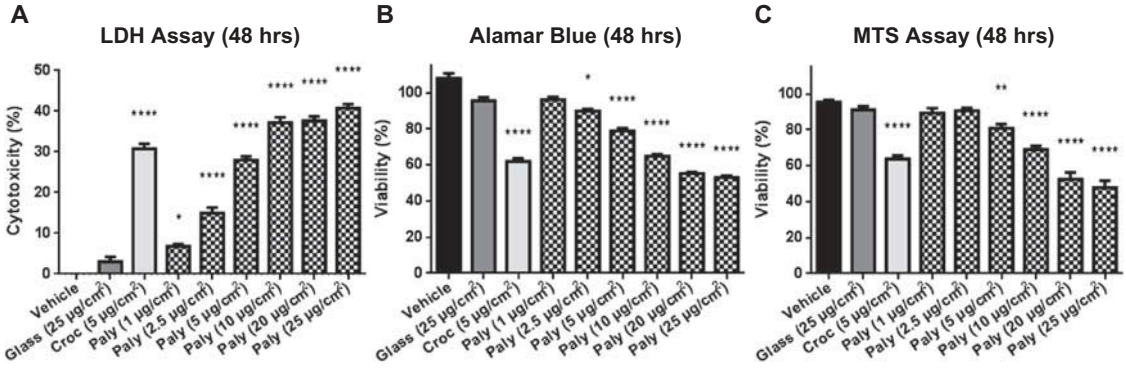

Figure 2. Palygorskite is cytotoxic and reduces cell viability in human mesothelial cells. Lactate dehydrogenase (LDH) assay performed by exposing HM cells to increasing concentrations of palygorskite for 48 h (A). Reductions in the viability of HM cells 48 h after adding palygorskite demonstrated by using the Alamar blue assay (B) and MTS assay (C). HM cells from at least 2 different patients and 12 or more total replicates. Mean ± SEM values shown (*$p < .05$, **$p < .01$, ****$p < .0001$).

Table 2. The development of ascites, mesothelioma, and hyperplasia in Balb/*c* and MexTAg mice.

Group	Ascites	Mesothelioma	Hyperplasia
Balb/*c*–glass (2 × 1 mg)*	0/3 (0%)	0/3 (0%)	N/D
Balb/*c*–palygorskite (2 × 1 mg)*	0/19 (0%)	0/19 (0%)	N/D
Balb/*c*–crocidolite (2 × 1 mg)*	5/16 (31.3%)	3/16 (18.8%)	N/D
MexTAg–glass (2 × 6 mg)[P]	0/15 (0%)	0/15 (0%)	0/15 (0%)
MexTAg–palygorskite (2 × 6 mg)[P]	0/18 (0%)	0/18 (0%)	0/18 (0%)
MexTAg–crocidolite (2 × 3 mg)[P]	14/14 (100%)	13/14 (92.9%)	14/14 (100%)

Note. N/D, not determined.
*In mice exposed to glass or fiber for up to 1 yr.
[P]In mice exposed to glass or fiber for 6 mo or more.

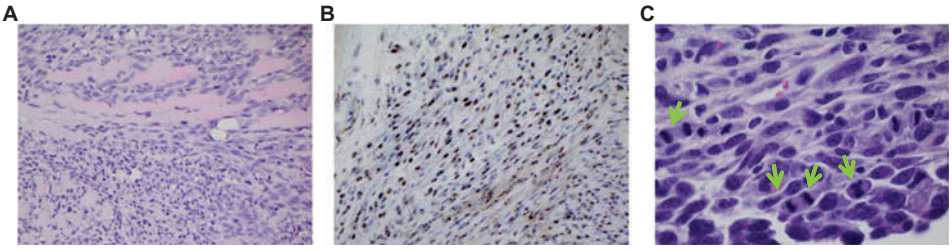

Figure 3. Palygorskite does not cause MM in vivo. MM was diagnosed in tissues taken from Balb/*c* mice after they were injected twice, 1 wk apart, with 1 mg glass, palygorskite, or crocidolite, or from MexTAg mice after they were injected twice, 1 mo apart, with 6 mg glass or palygorskite, or 3 mg crocidolite. MM visible in hematoxylin and eosin (H&E)-stained diaphragm tissue from Balb/*c* mouse exposed to crocidolite for approximately 8 mo (A). Presence of MM was confirmed by positive nuclear WT1 staining in a serial tissue section (B). Under higher magnification, aberrant mitosis (green arrows) was observed in MM cells infiltrating the diaphragm of MexTAg mouse exposed to crocidolite for 6 mo or more (C). Magnification 400× for (A) and (B), and 1000× for (C).

potential. While only approximately 20–30% of wild type mice injected intraperitoneally with asbestos fiber develop MM, ~100% of MexTAg mice become stricken with this cancer under the same experimental conditions (Robinson et al. 2011).

To determine whether palygorskite was carcinogenic in vivo, MexTAg mice were injected in the peritoneal cavity as previously described (Robinson et al. 2011). Crocidolite and glass were used as positive and negative controls, respectively. Mice were injected twice, 1 mo apart, with 6 mg palygorskite, double the dose of crocidolite, because in vitro, palygorskite was found to be roughly half as effective at inducing cytotoxicity in HM cells as crocidolite (Figures 2A–2C and Supplemental Figure 1). Mice that developed maladies were euthanized and tissues were assessed histologically for the presence of hyperplasia and tumors. In glass, or palygorskite-injected mice, no ascites was present and no hyperplasia was observed in the peritoneum (or any other organ in the peritoneal cavity), while crocidolite-injected mice developed ascites and their tissues had marked hyperplasia (Table 2). Importantly, none of the MexTAg mice injected with palygorskite or glass developed MM (Table 2). However, 93% of MexTAg mice injected with crocidolite developed MM (Table 2 and Figure 3C). Positive nuclear staining with WT1 antibody confirmed the histological diagnosis of MM in both MexTAg and Balb/c mice (Figure 3B).

Balb/*c* and MexTAg mice given glass or palygorskite showed little to no inflammation in tissues, other than foamy macrophage collections observed in palygorskite-injected animals (Supplemental Figures 2A and 2B), whereas mice given crocidolite had areas of foreign body giant cell type inflammatory reactions (not shown).

Overall, these data indicate that palygorskite, despite its in vitro cytotoxicity, has no carcinogenic potential in vivo since no MM could be found in any of the tissues examined from palygorskite-exposed mice.

Palygorskite Induces Significantly Less Inflammation in Mice Compared to Crocidolite

To investigate why palygorskite was not carcinogenic in vivo, inflammation in an additional set of fiber-exposed mice was examined. Peritoneal lavages were performed and peritoneal cells were collected from mice 3 d after the administration of glass or fibers, and cell type frequencies were

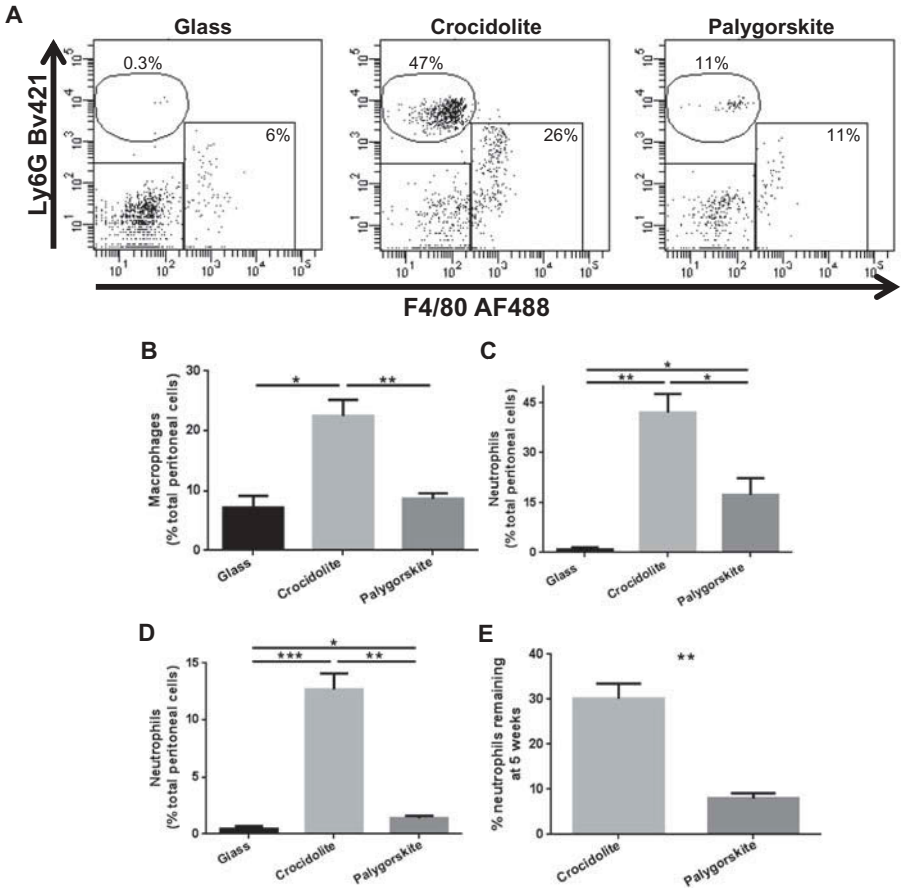

Figure 4. Palygorskite induces significantly less inflammation in mice compared to crocidolite. Representative flow cytometry dot plots showing the identification of macrophages (F4/80 + cells) and neutrophils (Ly6G + cells) from the peritoneal cavity of Balb/*c* mice (A). Percentages of macrophages (B) and neutrophils (C) in the peritoneal cavity of mice 3 d after intraperitoneal injection of 1 mg of glass, crocidolite, or palygorskite. Percentage of neutrophils in the peritoneal cavity 5 wk after injecting Balb/*c* mice twice (1 wk apart) with 1 mg of glass, crocidolite, or palygorskite (D). Sustainability of inflammation determined by dividing the percentage of neutrophils in the peritoneal cavity of Balb/*c* mice at 5 wk by the average percentage of neutrophils measured in the peritoneal cavity 3 d after fiber exposure (E). Mean ± SEM values displayed from 4 to 8 mice per group (*$p < .05$, **$p < .01$, ****$p < .0001$).

analyzed by flow cytometry (Supplemental Figure 3). Glass-injected animals had low percentages of macrophages and neutrophils in their peritoneal cavities (Figure 4A, left panel). Acute inflammation elicited by injecting crocidolite was markedly increased (Figure 4A, middle panel) compared to glass, as higher percentages of macrophages ($p = .016$, Figure 4B) and neutrophils ($p = .004$, Figure 4C) were observed. Palygorskite injection had little to no effect on levels of macrophages in the peritoneal cavity, but did slightly increase percentages of neutrophils ($p = .03$; Figures 4A right panel, 4B, 4C). Nonetheless, crocidolite-injected mice had significantly higher levels of both macrophages ($p = .006$) and neutrophils ($p = .02$) in the peritoneal cavity compared to palygorskite-injected mice (Figures 4B and 4C). In addition to differences in cell frequencies, alterations in cell numbers were also observed. Total leukocytes in the peritoneal cavity of mice increased dramatically after crocidolite exposure ($p = .004$; Supplemental Figure 4A). Crocidolite-injected animals also had significantly higher numbers of macrophages (Supplemental Figure 4B), neutrophils (Supplemental Figure 4C), and mast cells (Supplemental Figure 4D) in their peritoneal cavities than either glass or palygorskite-injected animals. However, only neutrophil numbers were elevated in palygorskite-injected mice compared to glass controls ($p = .03$), and this increase was still much lower than that observed in crocidolite-exposed mice ($p = .004$, Supplemental Figure 4C). Levels of the DAMP HMGB1, which can become elevated

following tissue damage and asbestos exposure (Yang et al. 2010), were measured in the peritoneal lavage supernatant from mice exposed to glass or fiber for 3 d. The amounts of HMGB1 measured from mice exposed to glass or palygorskite were similar. However, levels of HMGB1 were significantly higher from mice exposed to crocidolite when compared to mice exposed to glass (p = .049) or palygorskite (p = .049, Supplemental Figure 5).

These results indicate that palygorskite induced a weaker acute inflammatory response compared to crocidolite, as crocidolite elicited more HMGB1 and recruited an increased number of granulocytes and an increased number of macrophages.

Next, we investigated the possible persistence of the inflammation caused by palygorskite. To this aim, mice were exposed to fibers or glass and analyzed for the presence of inflammatory cells in the peritoneal cavity 5 wk after injections. Few to no neutrophils were found in the peritoneal cavity of glass-receiving mice. Significantly higher percentages of neutrophils were found in the peritoneal cavities of mice that received crocidolite compared to glass (p = .001). A low percentage of neutrophils was measured in the peritoneal cavities of palygorskite-injected mice (Figure 4D), which was significantly lower than that of crocidolite-injected mice (p = .001). Of note, no mesothelial hyperplasia was present in the peritoneal tissues of glass or palygorskite-injected mice, while diffuse mesothelial hyperplasia was consistently observed in peritoneal tissues from crocidolite-injected mice (not shown). When we compared the percentage of neutrophils 5 wk after crocidolite and palygorskite injection with the percentage of neutrophils we observed 3 d after injection, we found that a drop in the levels of neutrophils occurred more precipitously in palygorskite-injected mice than in crocidolite-injected mice (p = .007, Figure 4E).

Together, these findings suggest that the lack of carcinogenicity in vivo correlates with the inability of palygorskite to elicit a chronic inflammatory response, which is important since chronic inflammation appears to play a key promoting role in the development of MM following asbestos exposure (Carbone and Yang 2012).

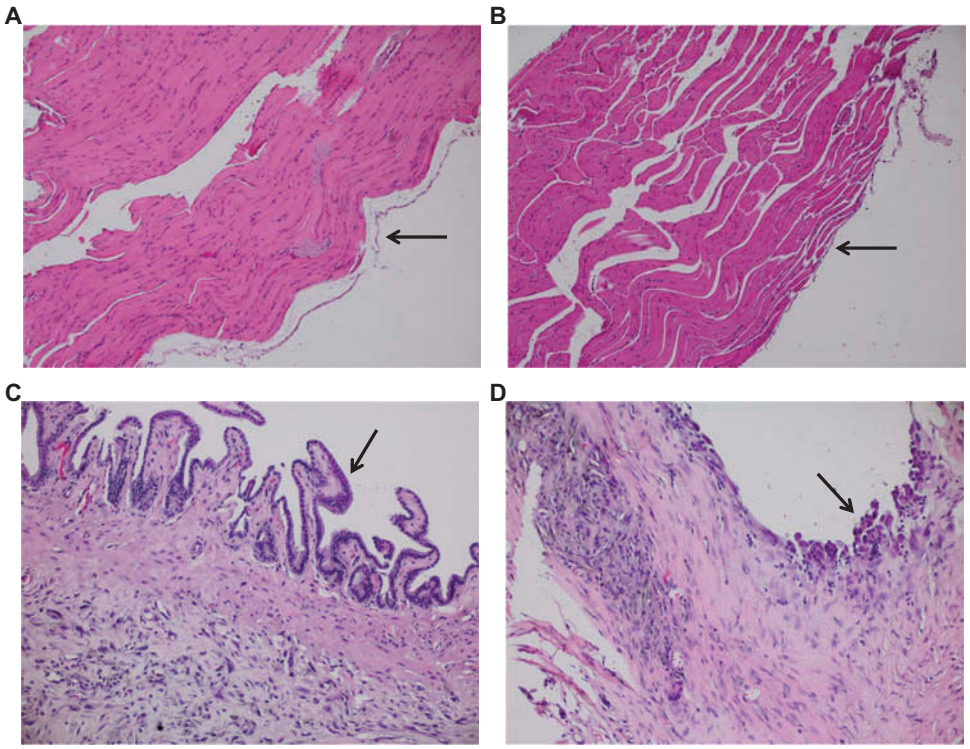

Figure 5. Sustained exposure to palygorskite does not lead to the development of hyperplasia in mice. Diaphragm tissue obtained from Balb/*c* mice 5 wk after receiving 5 biweekly injections with 0.5 mg of glass (A), palygorskite (B), or crocidolite (C, D) and stained with H&E. Normal mesothelium was observed after glass and palygorskite exposure (A, B, black arrows). Mesothelial hyperplasia observed after exposure to crocidolite (C, D, black arrows). Magnification 200×.

Sustained Exposure to Palygorskite Does Not Lead to the Development of Hyperplasia in Mice

Since poor persistence of inflammation induced by palygorskite could account for the lack of mesothelial hyperplasia and MM observed in our initial long-term study, we designed a new injection protocol where mice received fibers more frequently and for a longer period of time. Balb/*c* mice were injected intraperitoneally twice weekly for 5 wk with fibers or glass beads, euthanized 5 wk later, and tissues in the peritoneal cavity were collected and assessed for the presence of hyperplasia.

No mesothelial hyperplasia was found in any peritoneal tissues of glass or palygorskite-exposed mice (Figures 5A and 5B), while it was always observed in peritoneal tissues of mice injected with crocidolite (Figures 5C and 5D). Much like the mice from our long-term study, there was little to no inflammation in the peritoneal tissues of glass- and palygorskite-injected mice, other than occasional foamy macrophage accumulations, which at times reached considerable size (up to about 0.5 cm in diameter), whereas crocidolite-injected mice showed diffuse mesothelial hyperplasia in areas near asbestos deposits, which were also characterized by foreign body giant cell type inflammatory reactions. These data suggest that the limited persistence of inflammation caused by palygorskite, which may be related to the fiber's biopersistance, may not be the primary reason why palygorskite did not induce tumors.

M2 Macrophages Increase in the Peritoneal Cavity of Mice After Sustained Exposure to Crocidolite but Not to Glass and Palygorskite

To determine whether palygorskite fiber exposure promoted a protumoral, or M2 phenotype, peritoneal macrophages (F4/80+ and CD11b+ cells, Figure 6A) found in mice after continuous exposure to fibers were further characterized. Expression of CD206, found on the surface of M2 or M2-like macrophages, was assessed in macrophages using flow cytometry. Mice injected with glass had macrophages with little to no CD206 expression (Figure 6B left panel, Figure 6C). Instead, mice injected with crocidolite had substantial increases in macrophage CD206 expression (Figure 6B middle panel, Figure 6C). Macrophages from mice that received palygorskite had only a slight increase in macrophage CD206 expression compared to macrophages from mice injected with glass (Figure 6B right panel, Figure 6C). Increased CD206 expression resulted in higher percentages of M2 macrophages (identified as CD206+, CD86– cells) in the peritoneal cavities of mice injected with crocidolite compared to mice injected with glass (p = .008) or palygorskite (p = .008, Figure 6D).

In summary, these data indicate that mice injected with crocidolite had a significantly increased amount of M2, or protumoral, macrophages compared to mice injected with glass or palygorskite. These results correlate with the lack of carcinogenicity of palygorskite in vivo.

Discussion

Increasing evidence underscores the important role of environmental exposure to natural asbestiform fibers in the etiology of MM (Linton, Vardy, et al. 2012; Baumann et al. 2015). Determining what causes cancer is a complex process through which data from molecular and genetic findings, in vitro experiments, animal studies, and epidemiological studies are synthesized to form a coherent picture of carcinogenicity (Carbone et al. 2004). Recently, a significant increase in the proportions of females and young adults among MM mortality in southern Nevada has raised attention to possible environmental exposure to carcinogenic fibers in those areas (Baumann et al. 2015). Because occupational exposure is classically associated with the onset of disease at a later age, and with a male to female ratio of 4–8 to 1, an increased proportion of female and young individuals with MM is an indicator of a possible environmental etiology. In individuals who are environmentally exposed to carcinogenic fibers, MM occurs at a younger age because they are usually exposed since birth, compared to MM that develops in workers exposed to asbestos where exposure begins 20 yr or so later (Baumann et al. 2011; Carbone et al. 2011). While erionite and amphibole deposits have been found in southern Nevada (Figure 1), potentially implicating them as the probable cause of "environmental" MMs, the overwhelming presence of palygorskite deposits in this geographical area provided compelling reasons to suspect that palygorskite might have carcinogenic potential and contribute to some MMs in Nevada.

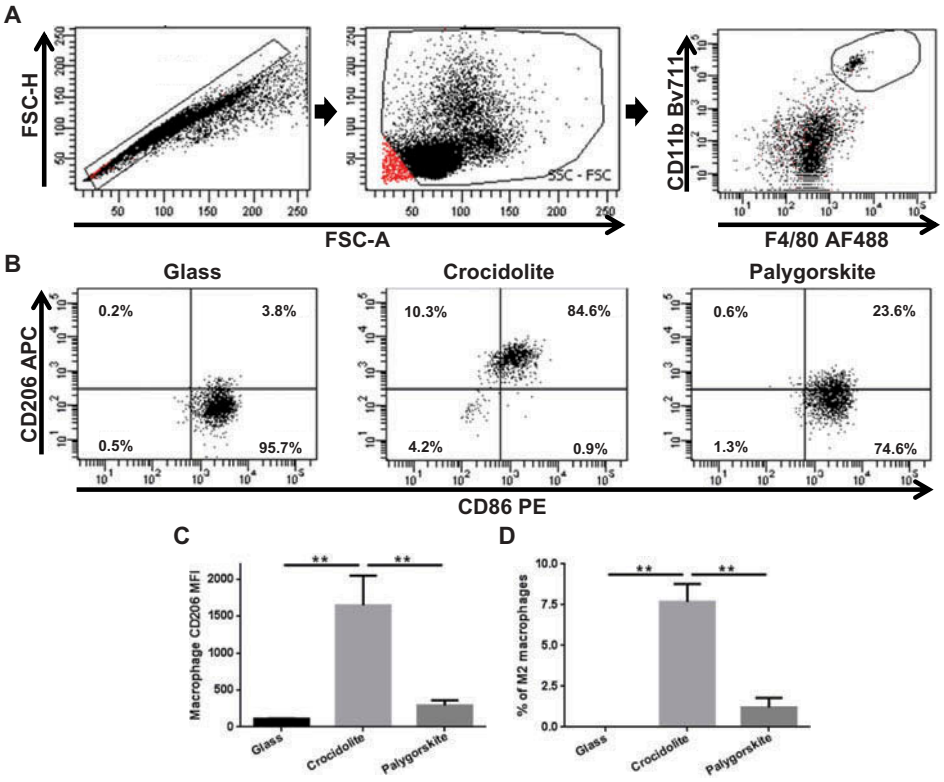

Figure 6. M2 macrophages increase in the peritoneal cavity of mice after sustained exposure to crocidolite but not to glass and palygorskite. After excluding doublet cells (A, left panel) and debris (A, middle panel), peritoneal macrophages were identified using flow cytometry by gating on $CD11b^+$ and $F4/80^+$ cells (A, right panel). Representative flow cytometry dot plots demonstrating changes in peritoneal macrophage CD206 expression in Balb/*c* mice 5 wk after receiving 5 biweekly injections of 0.5 mg glass (B, left panel), crocidolite (B, middle panel), or palygorskite (B, right panel). Peritoneal macrophage CD206 mean fluorescence intensity (MFI) from Balb/*c* mice exposed to glass, crocidolite, or palygorskite (C). Percentage of peritoneal macrophages from Balb/*c* mice expressing CD206, but not CD86 and identified as M2 macrophages (D). Mean ± SEM values displayed from five mice per group (**$p < .01$).

Due to its fibrous nature, palygorskite had been proposed as carcinogenic in studies performed decades ago (Pott, Huth, and Friedrichs 1974; Pott et al. 1987; Rodelsperger et al. 1987; Wagner, Griffiths, and Munday 1987; Pott and Heinrich 1990). However, the data published to date are not consistent and are, therefore, inconclusive. This has led the International Agency for Research on Cancer (IARC) to declare that palygorskite fibers >5 μm are possibly carcinogenic to humans (Group 2B) and that for fibers <5 μm, there is inadequate evidence and carcinogenicity to humans is inconclusive and not classifiable (Group 3) (IARC 1997). The fact that the carcinogenicity of palygorskite fibers <5 μm is currently considered inconclusive, and the fact that there is evidence that short mineral fibers may be capable of inducing mesothelioma, prevented us from ruling out palygorskite as a potential cause of MM in Nevada even though all of the environmental samples of palygorskite we collected were close to, but did not exceed, 5 μm in length. The biological significance of the 5 μm length is questionable. For example, a study that measured the length of fibers in the lungs of individuals with MM found that the geometric mean length of amosite fibers was 3.9 μm and the geometric mean length of crocidolite fibers was 2.8 μm (Churg and Wiggs 1984). Additionally, erionite fibers measured from air samples in Turkey, where MM was occurring, had mean lengths of 3.6 μm (Carbone et al. 2011). In this study, we report a comprehensive examination of the properties and potential activities of palygorskite in both in vitro and in vivo experiments. Based on our results, we conclude that palygorskite mineral fibers found in southern Nevada are not carcinogenic.

Palygorskite is a common mineral worldwide, especially in arid environments (Galàn and Singer 2011; Robins, Brock-Hon, and Buck 2012). Thus, our findings are of general relevance, although we

cannot completely rule out the possibility that palygorskite with different physical characteristics may exist and may cause MM.

Our study should alleviate the concerns that individuals exposed to palygorskite may be at increased risk of developing MM. Since we demonstrated that palygorskite from these locations does not cause MM, our study suggests that amphibole asbestos and/or erionite is likely responsible for any possible environmental cases of MM observed in Nevada, since, with the exception of the ubiquitous palygorskite, they are the primary other mineral fibers that have been found in that area and they are known to be carcinogenic.

In our in vitro studies, we found that palygorskite was cytotoxic to HM cells and reduced their viability in vitro. However, palygorskite displayed less toxicity than crocidolite toward HM cells, as nearly double amounts of palygorskite compared to crocidolite were required to induce similar changes in HM. Besides palygorskite's relatively weak effects on HM viability and growth in vitro, no mouse given palygorskite developed MM. A similar lack of correlation between in vitro cytotoxicity and MM after intrapleural inoculation of fibers has been reported (Yegles et al. 1995), highlighting the relevance of in vivo experiments to accurately determine the carcinogenic potential of mineral fibers. While we did not evaluate the effect fibers had on mouse mesothelial cells in vitro, in all likelihood our results would be the same. Past studies have shown that fibers exhibit cytotoxicity toward mesothelial cells from other rodent species (Jaurand et al. 1987; Renier et al. 1989), and levels of cytotoxicity in those studies are similar to what we observed using HM cells.

Our study gives insights as to why some mineral fibers are not carcinogenic despite their similarities to other carcinogenic fibers. Indeed, our data suggests that the inflammatory nature of mineral fibers is linked to their carcinogenic potential. The in vivo inflammatory response generated following palygorskite exposure was greatly attenuated compared to that mounted after crocidolite exposure. For example, the acute inflammatory response, based on neutrophil influx into the peritoneal cavity 3 d after fiber exposure, was 141% greater in crocidolite-injected mice compared to palygorskite-injected mice. The ability of palygorskite to cause sustained inflammation was also significantly reduced compared to crocidolite. However, mice continuously exposed to palygorskite did not develop mesothelial hyperplasia 5 wk after exposure ended. Whereas, mice injected with crocidolite, using the same injection protocol, consistently developed atypical mesothelial hyperplasia, suggesting that reduced inflammation, and not its inability to sustain the minimal inflammation it elicits, was the reason for palygorskite's lack of carcinogenicity in vivo.

Of note, we observed changes in macrophage phenotypes following continuous exposure to crocidolite versus palygorskite. Continuous exposure to crocidolite resulted in increased levels of M2 macrophages compared to mice exposed continuously to palygorskite fibers. M2s are alternatively activated macrophages that release factors such as EGF, FGF, and VEGF that can promote the growth and development of tumors (Zhang et al. 2014). M2 macrophages also express surface receptors and cytokines that are immunosuppressive and inhibit the cytotoxic abilities of T cells and NK cells (Noy and Pollard 2014). In fact, an immunohistochemical analysis of MM tumor samples showed high levels of M2 macrophages were negatively correlated with survival (Linton, van Zandwijk, et al. 2012), presumably due to their contribution to the immunosuppressive tumor microenvironment. We have previously reported that the increase of M2 macrophages contributes to MM development in BAP1 heterozygous mice (Napolitano et al 2015). Our study replicates this finding, suggesting that early development of an M2 macrophage phenotype response appears to be related to MM pathogenesis. Development of M2 macrophages upon exposure to other carcinogens, and before actual tumor development, has been shown to occur in other cancers (Redente et al. 2010; Zaynagetdinov et al. 2011), demonstrating that this phenomenon is not restricted to MM and may be involved in cancer development in general.

Conclusions

Our study shows that palygorskite does not induce MM and therefore that not all mineral fibers are carcinogenic. We found that palygorskite fibers do not cause MM, presumably because they elicit much reduced inflammation compared to carcinogenic crocidolite fibers. Here we describe specific

inflammatory changes that occur after exposure to carcinogenic fibers, which may be useful when interrogating the carcinogenic potential of other mineral fibers. In particular, the ability of inducing an M2 macrophage response appears related to the potent tumor-inducing capacity of carcinogenic fibers.

In addition, our findings indicate that those MM in southern Nevada that appear related to exposure to carcinogenic fibers present in the environment, are not due to exposure to ubiquitous palygorskite fibers located there. Instead, these MMs are more likely related to exposure to asbestos and erionite present in the environment in more limited geographical areas in that region.

Methods

Geological Sampling

Four petrocalcic horizons were sampled in southern Nevada (Figure 1). At all four sites, the petrocalcic horizons are exposed at the surface, where weathering and erosion can release the fibers so that they can become airborne. Petrocalcic horizon samples were gently broken into smaller fragments and were placed in a buffered pH 5 sodium acetate (NaOAc) solution, which is a commonly used method to extract silicate clays (Robins et al. 2014). We altered this method by not heating the solution in order to carefully dissolve calcite while not damaging the palygorskite fibers (Robins et al. 2014). After carbonate dissolution samples were thoroughly rinsed (Soukup, Buck, and Harris 2008) with ASTM Type I (electric resistivity 18.2 MΩ-cm) water. Due to the difficulties of purifying palygorskite from environmental samples, a pure palygorskite standard obtained from Source Clays Repository was used in all experiments.

Mineralogical Analysis and Micromorphology Measurements

Samples were dried for 1 d at 60°C and maintained in a desiccator prior to mounting on a specimen holder with a double coated carbon conductive tape before sputter deposition of an Au thin film (±10 nm). Fifty fibers of each sample were measured using a scanning electron microscope (SEM; Philips SFEG XL30 and Hitachi-S4800) equipped with a high-resolution field emission gun (CP2M Facility at Aix-Marseille University, France, and at the Central Microscopy Research Facility at the University of Iowa, Iowa City, IA). Images were obtained at a low accelerating voltage (1.5 to 2.0 kV) to prevent surface charging and measurements were achieved using the built-in SEM software at different magnifications based on the sample's micromorphology. A second analysis was performed by an independent mineralogist where 100 fibers of each sample were measured in order to confirm initial results.

Fiber Preparation

Crocidolite asbestos (SPI-Chem) was used as a positive control for experiments and was previously characterized by our lab to have an average length of 13.60 ± 20.24 μm (~0.5–250 μm min/max) and an average width of 0.60 ± 0.45 μm (~0.2–5 μm min/max) (Qi et al. 2013). Due to reports of glass fibers causing tumors in rodents (Stanton et al. 1977), glass beads (Polysciences, Inc.) were used as a negative control for experiments. According to company specifications, glass beads had diameter ranges of 3–10 μm. Glass beads, crocidolite, and palygorskite (Source Clays Repository) were prepared as previously described (Jube et al. 2012; Qi et al. 2013). Briefly, fibers were baked for 18 h at 150°C, added to 1× phosphate-buffered saline (PBS), and fiber bundles were disaggregated by passing through a 22-gauge needle 10 times.

HM Cell Culture

Primary human mesothelial (HM) cells were obtained from the pleural fluids of patients with nonmalignant medical ailments, in accordance with the institutional review board (IRB) protocol, and cultured in Dulbecco's modified Eagles's medium (DMEM; Corning, Cellgro) supplemented with 20% FBS (Gibco, Life Technologies), as previously described (Qi et al. 2013). HM cells used for assays were between passage 2 and 4 and were characterized morphologically and by positive immunostaining for calretinin and cytokeratin and negative staining for SV40 and carcinoembryonic antigen.

Mice and Injections

All experiments with mice had the approval of the University of Hawaii Institutional Animal Care and Use Committee (IACUC).

Long-term experiments performed to assess palygorskite's ability to cause MM were conducted using Balb/*c* (Taconic) and MexTAg (kindly provided by Cleo Robinson) mice. Balb/*c* mice were injected twice intraperitoneally, 1 wk apart, with 1 mg glass, crocidolite, or palygorskite. Using a specific protocol developed for MexTAg mice, MexTAg mice were injected twice, 1 mo apart, with 3 mg crocidolite or 6 mg glass or palygorskite (Robinson et al. 2011). Tissues were taken from Balb/*c* mice exposed to glass or fiber for ≤1 yr and from MexTAg mice exposed for ≥6 mo. Tissues were preserved in formalin and examined under the microscope by a trained pathologist for the presence of MM.

Short-term, 3-d-exposure experiments were conducted in Balb/*c* (Taconic) mice by injecting them with 1 mg glass, crocidolite, or palygorskite in order to measure inflammatory responses elicited by fibers. The development of hyperplasia and how well inflammatory responses were maintained were assessed in longer term exposure experiments in separate Balb/*c* mice injected intraperitoneally twice, 1 wk apart, with 1 mg glass, crocidolite, or palygorskite. Peritoneal cells were collected 3 d (for short-term experiment) and 5 wk (for longer term exposure experiments) after fiber injection and inflammation was measured. In additional Balb/*c* mice, the development of hyperplasia was assessed and peritoneal macrophage subtypes were determined after injecting mice twice weekly, for 5 wk, with 0.5 mg glass, crocidolite, or palygorskite. Peritoneal cells and tissues were collected 5 wk after the last injection.

Peritoneal Lavage

Peritoneal cells were collected as previously described (Larson and Mitre 2012). Briefly, 5 ml of 1× HBSS was injected into the peritoneal cavity of mice and the abdomen was manually agitated for at least 1 min. Cells were removed from the peritoneal cavity by collecting lavage fluid using a transfer pipet. Lavage fluid was measured and leukocytes were counted using a hemocytometer. Total cell counts from each mouse were corrected to account for any HBSS not recovered. Numbers of each cell type were determined by multiplying the total corrected number of peritoneal cells by the cell type percentages that were determined by flow cytometry. No peritoneal lavages were performed on mice in the long-term, tumor development study.

Viability and Cytotoxicity Assays

HM cells (5000–10,000 per well in 96-well plates) were incubated with fiber or glass beads in 1% DMEM for 48 h in 96-well plates. MTS (Promega) and Alamar blue (AbD Serotec) assays were used to assess cell viability and the LDH assay (Roche) was used to measure cytotoxicity. LDH values were determined by first subtracting absorbance value of a well containing media with no cells, then using the following formula: (absorbance value of experimental sample – absorbance value of cells incubated with media)/(absorbance value of total cell lysis – absorbance value of cells incubated with media) × 100. Values for viability were calculated by first subtracting absorbance value of well with only media and then dividing absorbance value of experimental sample by the absorbance value of cells incubated with media and multiplying by 100.

HMGB1 ELISA

HMGB1 levels were measured in peritoneal lavage supernatant from 3-d glass or fiber-exposed mice using an HMGB1 enzyme-linked immunosorbent assay (ELISA) kit (IBL International).

Flow Cytometry

Peritoneal cells were pelleted, supernatant was removed, and fixative from whole blood lysing reagent (Beckman Coulter) was added to cells. Cells were washed twice with 1× PBS and resuspended with 200 µl 1% bovine serum albumin (BSA), fraction V (Calbiochem) and stored at 4°C until surface staining was performed. Immediately before staining, Fc receptors were blocked by using TruStain fcX (anti-mouse CD16/32) antibody (Biolegend) in 1% BSA for 20 min on ice. To determine peritoneal cell

phenotypes, 7×10^5 peritoneal cells were centrifuged and resuspended with the following antibodies: CD117 (2B8) BV711 (BD Biosciences), F4/80 (CI:A3-1) AF488 (AbD Serotec), Ly-6G (1A8) BV421 (BD Biosciences), CD3 (17A2) APC (eBioscience), and B220 (RA3-6B2) PE for 30 minutes at 4°C. For macrophage subtype analysis, a separate tube of cells was stained with CD11b (M1/70), F4/80 (CI:A3-1) AF488 (AbD Serotec), CD206 (CO68C2) APC (BD Biosciences), CD86 (GL1) PE (BD Biosciences). After staining, cells were washed twice with 1× PBS and cellular fluorescence was measured using an LSRFortessa flow cytometer (BD Biosciences) and FACSDiva v6.2 software (BD Biosciences).

For all flow cytometry experiments, antibody concentrations were determined by titration experiments and BD CompBeads (BD Biosciences) were used to perform compensation. Cutoff gates for positivity were established using the fluorescence-minus-one (FMO) technique (Baumgarth and Roederer 2000).

Pathology and Immunohistochemistry

Kidney, stomach, diaphragm, peritoneum, spleen, intestines, liver, and reproductive tissues in the peritoneal cavity were collected from experimental animals, immediately placed in 10% formalin, and subsequently embedded in paraffin. Slides were made and stained with hematoxylin and eosin (H&E) or WT1 antibody (Abcam). Two different lots of WT1 antibody were used and both lots were first titrated to determine the optimal dilution to use for study samples, which was found to be 1:250 and 1:300. Two board-certified pathologists reviewed H&E and WT1-stained tissues for the presence of hyperplasia and/or MM.

Statistics

Graphs depicting cytotoxicity/viability levels, cell percentages, and cell numbers were displayed as means ± standard error of the mean (SEM) using GraphPad Prism (version 6.0) software. Statistical differences between palygorskite-injected and crocidolite-injected, or glass-injected and either palygorskite- or crocidolite-injected animals were determined using the Mann–Whitney nonparametric test (GraphPad Prism, version 6.0). Differences were considered significant if $p < .05$.

Competing Interests

MC and HY have pending patent applications on BAP1 and HMGB1; M.C. provides consultation for mesothelioma diagnosis. The other authors declare no competing financial interests.

Authors' Contributions

DL wrote the article, designed and performed experiments, and performed data analysis. AP and MC performed and reviewed pathology and immunohistochemistry. J-PA, MD, and UD performed SEM and mineralogical analyses and reviewed the article. MT, AN, EF, LP, and CJ helped performed experiments. FB helped to coordinate parts of the study and contributed to the writing of the article. BB, BM, and DM contributed to the writing of the article and performed geological sampling and mapping of fibers. SP contributed to the writing of the article and critically reviewed experimental results. MC and HY designed and directed the study, critically reviewed experimental results, and contributed to the writing of the article. PM, HP, and CR critically reviewed results.

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