Published in final edited form as:

Toxicol Appl Pharmacol. 2016 October 15; 309: 101–110. doi:10.1016/j.taap.2016.08.029.

Autophagy Deficiency in Macrophages Enhances NLRP3 Inflammasome Activity and Chronic Lung Disease Following Silica Exposure

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Abstract

Autophagy is an important metabolic mechanism that can promote cellular survival following injury. The specific contribution of autophagy to silica-induced inflammation and disease is not known. The objective of these studies was to determine the effects of silica exposure on the autophagic pathway in macrophages, as well as the general contribution of autophagy in macrophages to inflammation and disease. Silica exposure enhanced autophagic activity *in vitro* in Bone Marrow derived Macrophages and *in vivo* in Alveolar Macrophages isolated from silica-exposed mice. Impairment of autophagy in myeloid cells *in vivo* using Atg5^{fl/fl}LysM-Cre⁺ mice resulted in enhanced cytotoxicity and inflammation after silica exposure compared to littermate controls, including elevated IL-18 and the alarmin HMGB1 in the whole lavage fluid. Autophagy deficiency caused some spontaneous inflammation and disease. Greater silica-induced acute inflammation in Atg5^{fl/fl}LysM-Cre⁺ mice correlated with increased fibrosis and chronic lung disease. These studies demonstrate a critical role for autophagy in suppressing silica-induced cytotoxicity and inflammation in disease development. Furthermore, this data highlights the importance of basal autophagy in macrophages and other myeloid cells in maintaining lung homeostasis.

Keywords

Autophagy; Atg5; NLRP3 Inflammasome; HMGB1; IL-18; Silicosis

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Declaration of Interests

The authors declare that they have no competing interests

Funding Sources¹

This work was supported by research grants from NIEHS (R01ES023209), Shared Instrument grant (S10RR026325-01), and Institutional Development (IDeA) Awards from NCRR (P20 RR017670), and NIGMS (P30 GM103338). Additionally, Forrest Jessop was supported in part by a PhRMA Foundation Individual Pre-doctoral Fellowship. The content of this manuscript is solely the responsibility of the authors and does not necessarily represent the views of the National Institute of Health or the PhRMA Foundation.

Introduction

Autophagy is critical in maintaining cell homeostasis and is generally considered a pro-survival mechanism. Prolonged or enhanced autophagy has also been implicated in cell death (1). While there are multiple types of autophagy, these studies focus primarily on macroautophagy, which has a fundamental responsibility of sequestering and degrading large macromolecular protein structures and damaged organelles (2). In the autophagic process, cytosolic microtubule-associated protein 1A/1B-light chain 3 (LC3-I) is cleaved to the LC3-II isoform and incorporated into the forming autophagosome membrane. Atg5 is a critical protein required for autophagosome elongation and LC3 lipidation (3).

Macromolecular structures are targeted towards the autophagic pathway through hyper-ubiquitination and the autophagy specific chaperone SQSTM1 (p62) (4). Targeted material and p62 is sequestered within autophagosomes, which fuse with lysosomes in order to degrade and recycle the target and carrier proteins. Autophagy is reported to be a primary mechanism for degradation of the AIM2 and NLRP3 inflammasomes with poly(dA:dT) or ATP and Nigericin stimulation, respectively, in THP-1 and Bone Marrow derived Macrophages (5). Therefore, regulating autophagy could possibly have profound implications in macrophage responses to pro-inflammatory and **cytotoxic particles such as silica**.

Recent reports highlight that silica exposure and the development of pneumoconiosis continues to be a significant health concern (6). There are few studies investigating the contribution of autophagy to particle-induced lung disease, let alone those caused by exposure to hazardous particulates such as silica. Failure of autophagy to degrade inflammasomes and clear bulk damaged organelles and protein aggregates are thought to contribute to the development of chronic lung disease. However, the state of autophagy appears to vary with different types of chronic lung diseases (7). Autophagy is reported to be greatly enhanced in Chronic Obstructive Pulmonary Disease in epithelial cells, while autophagic flux is impaired in isolated alveolar macrophages following cigarette smoke exposure (8, 9). On the other hand, autophagy is reportedly decreased in bleomycin-induced fibrosis *in vivo*, and in human Idiopathic Pulmonary Fibrosis and Cystic Fibrosis (10, 11). The role and state of autophagy in silicosis has not been determined, but may differ from these other lung diseases due to alternative mechanisms of action.

Chronic NLRP3 inflammasome activity and cell death is reported to drive development of pathology following silica exposure (12). Previous studies have shown that innate immune function is sufficient for the development of fibrosis in a mouse model of silicosis, suggesting a critical role for macrophages in the chronic inflammatory response (13). A primary mechanism by which silica induces NLRP3 inflammasome activation and cell death in macrophages is through permeabilization of phagolysosomes (14). The autophagic pathway also shares lysosomes as a common endpoint for degradation of sequestered material. Correlative findings suggest that loss of lysosomes is associated with increased autophagosomes in lung macrophages as the severity of silicosis increases, and may indicate impaired autophagic degradation (15). However, it is also possible that increased autophagosome formation is a direct result of cellular injury rather than impaired autophagic

flux. Therefore, the consequences of silica exposure on autophagic flux through the lysosome have not been fully elucidated.

In the current study, we investigated impacts of silica exposure on autophagic flux as well as the contribution of autophagy in lung macrophages to silica-induced inflammation and disease. For this study we used Atg5^{fl/fl}LysM-Cre⁺ mice, which lack essential machinery necessary for the formation of autophagosomes in cells that express high levels of lysozyme, which include macrophages and other myeloid cell populations (16). Therefore, we expected that there would be greater inflammation and disease following silica exposure in mice in which autophagic activity has been impaired.

Materials and Methods

Particle preparation

Acid washed crystalline silica (Min-U-Sil-5, mean particle diameter 1.5-2 μ m) was obtained from Pennsylvania Glass Sand Corp (Pittsburgh, PA, USA). Silica was determined to have insignificant levels of endotoxin (LPS) by the Limulus amoebocyte lysate assay (Cambrex, Walkersville, MD, USA) as previously described (13, 17). Prior to instillation into mice or addition to cell cultures, silica particles were suspended in PBS and sonicated for >1 min (550 watts @ 20 kHz) by a cup-horn sonicator in a circulating water bath (Misonix, Inc. Farmingdale, NY, USA).

Mice

Transgenic GFP-LC3 mice, which express green fluorescent protein (GFP) fused to microtubule-associated protein 1A/1B-light chain 3 (LC3), were generously donated by Dr. Aruni Bhatnagar (University of Louisville, KY, USA) and have been previously described (18). Homozygous Atg5^{fl/fl} mice were obtained from the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan, courtesy of Dr. Noboru Mizushima (19). LysM-Cre⁺ mice were obtained from Jackson Laboratories. Crossing of homozygous Atg5^{fl/fl} mice with LysM-Cre⁺ to generate the Atg5^{fl/fl}LysM-Cre⁺ mice was tracked and confirmed by PCR as previously described (19, 20). Autophagy impairment in Atg5^{fl/fl}LysM-Cre⁺ mice was confirmed by Western Blot analysis of depleted Atg5 (data not shown) and blocked lipidation of LC3-I to LC3-II in isolated Bone Marrow derived Macrophages (BMdM) and Alveolar Macrophages (AM). Both male and female C57Bl/6 (Jackson Laboratories, Bar Harbor, ME, USA), GFP-LC3 and Atg5^{fl/fl}LysM-Cre⁺ mice were used in equal numbers for all studies. All transgenic mouse strains used were on a C57Bl/6 background. Mice were housed in micro-isolators in a specific pathogen-free facility under a 12:12-hr light-dark cycle. Food, bedding, and cages were sterilized by autoclaving. Mice were used between 8-16 wk of age. The University of Montana Institutional Animal Care and Use Committee (Missoula, MT, USA) approved all procedures performed on the animals.

Bone Marrow derived Macrophages

BMdM were generated as described previously (17). Briefly, BMdM were obtained by flushing the femur and tibia following sacrifice of C57Bl/6, GFP-LC3, and Atg5^{fl/fl}LysM-Cre⁺ and/or Littermate control (Atg5^{+/+}LysM-Cre⁺ or Atg5^{fl/fl}LysM-Cre⁻, no difference was

observed in responses between littermate controls used) mice. Bone marrow isolate was then cultured in T75 culture flasks with 20 mL of RPMI (10 % FCS) overnight for stromal cell elimination. After 24 hr, the non-adherent fraction which included progenitor stem cells was removed and seeded in new T75 flasks (15×10^6 cells/flask) in 20 mL RPMI (10 % FCS) and 40 μ L M-CSF (10 ng/mL stock, R&D Systems, Minneapolis, MN, USA) added to each flask. Cultures were maintained for 7-10 days with re-feeding every 3-4 days.

In vitro studies

BMdM from $Atg5^{fl/fl}$ LysM-Cre⁺ mice and littermate control were plated at 1×10^5 cells/well in a 96 well plate and co-exposed to silica (100 μ g/mL) and LPS (20 ng/mL) to prime the NLRP3 inflammasome. A dose of 100 μ g/mL of silica was used for these studies based on prior reports by our laboratory in which this dose was shown to maximize inflammasome activity while minimizing cell death (27). After 24 hr, cell supernatants were collected and assessed for markers of inflammation and cell death. For analysis of GFP-LC3 in BMdM, cells were plated in a 96 well culture dish with a glass bottom coverslip (MatTek Corp. Ashland, MA, USA) at 1×10^5 cells/well. Macrophages were then exposed to silica (100 μ g/mL) with or without Bafilomycin A1 (100 nM, EnzoLife Sciences, Farmingdale, NY, USA) to inhibit autophagic flux. BMdM were also treated with 3-methyladenine (3-MA, 5 μ M, Sigma-Aldrich, Saint Louis, MO, USA) to inhibit autophagosome formation. After 24 hr, GFP-LC3 was assessed by laser scanning cytometry.

In vivo studies

$Atg5^{fl/fl}$ LysM-Cre⁺ mice and littermate control mice were instilled with silica at 40 mg/kg (~1 mg/mouse), which has previously been shown to induce sufficient acute inflammation *in vivo* (13, 17). Mice were sacrificed 24 hr after silica exposure for analysis of the whole lung lavage fluid and cell differentials. Mice were lavaged by instilling, aspirating, and re-instilling 1 mL of cold PBS (3X) to concentrate lavage cytokines. An additional two more rounds of 1 mL PBS instillation/aspiration were performed to maximize cell retrieval. Isolated whole lung lavage cells were counted using a Coulter Z2 particle counter (Beckman Coulter, Brea, CA, USA). Cells were then stained for differential analysis using Wright's Geimsa and a Hematek 2000 autostainer (Miles-Bayer-Siemens Diagnostics, Deerfield, IL, USA). Macrophage area on differential slides was assessed via ImageJ Analysis Software. Using similar methods, whole lung lavage cells were isolated from C57Bl/6 or GFP-LC3 mice 7 d following silica exposure for analysis of autophagy by confocal microscopy or Western Blot. For chronic studies, $Atg5^{fl/fl}$ LysM-Cre⁺ mice and littermate control mice were instilled with silica (40 mg/kg) once a week for 4 consecutive weeks as described previously (13, 17). Twenty-eight days after the final instillation (day 56), the mice were sacrificed and their lungs inflated with 4% paraformaldehyde for histology. Fixed lungs were embedded in paraffin and sectioned (7 μ m), and mounted on Superfrost+ VWR slides (VWR, Radnor, PA, USA). Sections were stained with Gomori's Trichrome (EMD Chemicals, Gibbstown, NJ, USA) or hematoxylin-eosin (RAS Harris Hematoxylin and Shandon Alcohol Eosin) using a Leica ST5010 Autostainer (Buffalo Grove, IL, USA).

Laser Scanning Cytometry

GFP-LC3 was assessed using a CompuCyte iCys Laser Scanning Cytometer (LSC; Westwood, MA, USA). Non-adherent BMdM were removed prior to analysis by gentle washing of each well once with PBS. GFP-LC3 positive cells were counter-stained with Molecular Probes HCS NuclearMask Blue (Life Technologies, Carlsbad, CA, USA) according to manufacture's instructions. GFP-LC3 was detected using a 488 nm laser as the excitation source and a PMT detector with a 530/30 nm bandpass filter. The nuclear staining was excited with a 405 nm laser and detected with a 440/30 nm bandpass filter/PMT set. The iCys was programmed to make 0.15 μm x-steps (setting pixel size/resolution) on an automated stage using a 60x inverted objective to interrogate a field size of 150 μm $\times$ 125.6 μm each step. Individual passes of the 488 nm and 405 nm lasers were used to avoid any spectral overlap of the “blue” and “green” fluorescent signals. A threshold of “blue” fluorescence was set such that the software draws a contour around the nucleus of the cell. The contour is then expanded by 25 pixels to include the cytoplasm of the cell. Each cell (as defined by nuclear staining) is plotted on a histogram showing green Median Fluorescent Intensity (MFI). Additionally, GFP puncta were counted per each cell using standard integrated filters and threshold within the CompuCyte software. Regions were defined within each well of the 96-well plate to include approximately 1500 cells to achieve sufficient sample representation. Isolated lung lavage cells from GFP-LC3 mice were seeded at 1×10^5 cells/well in RPMI in an 4-well glass bottom dish (Greiner Bio-One, Monroe, NC, USA), counterstained with Molecular Probes HCS NuclearMask Blue (Life Technologies), and examined immediately using an Olympus FV 1000 IX inverted laser scanning confocal microscope.

Cytokine and Cytotoxicity Assays

BMdM cell supernatants and whole lung lavage fluid were assayed for LDH activity (Promega, Madison, WI, USA). Cytokines IL-1 β , CXCL1, and IL-1 α were measured by ELISA (R&D systems, Minneapolis, MN, USA). HMGB1 was assessed by an in-house ELISA as previously described (21). IL-18 was also assessed by an in-house ELISA (22). Extracellular cathepsin activity was assessed as previously described by our laboratory (23). Briefly, 2 μg Z-LR-AMC (specific to cathepsin B, cathepsin L and cathepsin V; R&D systems) in PBS was added to 50 μL of whole lung lavage fluid in a total reaction volume of 150 μL . The assays were incubated at 37°C for 1 h then fluorescence was measured using a plate reader at 380 nm excitation and 460 nm emission.

Western Blot

BMdM or cells isolated from lung lavage fluid were lysed directly in RIPA buffer containing HALT™ protease inhibitors (Life Technologies). For *in vivo* assessment of autophagic markers, whole lung lavage cells from 4 mice were pooled for each *N*. Lysates were run on 4-12% Bis-Tris SDS-PAGE gels. Anti-LC3, p62, ubiquitin, and GAPDH antibodies were obtained from Cell Signaling (Danvers, MA, USA). GAPDH was used as the loading control. Protein bands were quantified with Image J software (NIH, Bethesda, MD, USA).

Microscopy and pathology scoring

Gomoris Trichrome and H&E stained mouse lung tissue sections were imaged using a Zeiss Axioskop attached to a Zeiss digital camera. Two expert observers scored the lung disease using a 5-point scale (0, 1, 2, 3, and 4) with 0 being no effect and 4 being maximum lung pathology evident. Scores were derived from observing multiple whole lung sections to prevent regional bias. The scorers were blinded to mouse treatments. Cronbach's- α was used to assess the reliability between the scorers. Inter-scorer reliability was significant at 0.86.

Statistical analysis

Statistical analyses involved comparison of means using a one- or two-way ANOVA followed by a Bonferroni's test or Holm-Šidák test to compensate for increased type I error. Unpaired *t* test was utilized for analysis of Western Blots or other data sets that included simple comparisons between two groups. Statistical power was > 0.8 to determine sample size. Statistical significance was defined as a probability of type I error occurring at $< 5\%$ ($P < 0.05$). The minimum number of experimental replications was 3. Graphics and analyses were performed on PRISM 5.0 and 6.0.

Results

Silica particle exposure increases autophagy in macrophages

Crystalline silica is reported to cause lysosome membrane permeabilization, NLRP3 inflammasome activity, and the activation of cell death pathways (14). Silica's ability to compromise lysosomal integrity and cause intracellular damage likely has broad impacts on other cell pathways, including autophagy, however this relationship has not been investigated sufficiently. Therefore, to assess the effects of silica exposure on autophagy, we exposed BMdM from GFP-LC3 mice to silica and measured changes in GFP-LC3 expression and puncta formation after 24 hr. BMdM were used for these studies due to their consistency in response with Alveolar Macrophages (AM) in prior studies by our laboratory (17, 24). Bafilomycin A1 and 3-MA were added to BMdM in order to assess whether silica exposure causes autophagic induction, increased autophagic flux, and/or blocked autophagic flux (25, 26). Silica exposure caused a marginal increase in median fluorescent intensity (MFI) of GFP-LC3 per cell and a significant increased number of GFP-LC3 puncta/cell (Figure 1A, 1B, and 1C). 3-MA effectively blocked autophagy in silica-exposed BMdM, indicating silica exposure results in autophagic induction. As expected, Bafilomycin A1 treatment of control cells resulted in an accumulation of GFP-LC3. When Bafilomycin A1 was added to silica-exposed cells, there was an increase in GFP-LC3 when compared to Bafilomycin A1 only treated cells, which further supports the notion that silica exposure is increasing autophagosome formation but not necessarily blocking autophagic flux through the lysosome.

To further confirm that silica exposure was increasing autophagy rather than blocking autophagic flux, we assessed LC3-I and LC3-II, the autophagy associated ubiquitin-specific chaperone p62, and total ubiquitinated protein levels by Western Blot (25, 28). Both intracellular p62 and total ubiquitinated protein levels were elevated in BMdM 24 hr following silica exposure (Figure 1D). Silica exposure increased LC3-II, consistent with

increased autophagosome formation and with the observed increase in number of GFP-Puncta/Cell shown in Figure 1B. When silica-exposed cells were treated with Bafilomycin A1, we saw further increases in LC3-II, p62, and total ubiquitinated protein when compared to Bafilomycin A1-treated control. Together, our findings that silica with Bafilomycin A1 caused further accumulation of autophagic protein compared to Bafilomycin A1 alone supports the notion that silica exposure increases induction and/or the amount of carrier material moving through the autophagic pathway in macrophages, but does not necessarily result in impaired autophagic flux through the lysosome.

Silica exposure enhances autophagy in AM *in vivo*

To determine if silica exposure increases autophagy in AM *in vivo*, we instilled C57Bl/6 mice with crystalline silica and assessed autophagy markers in isolated lung lavage cells lysates after 7 d. Lung lavage cells at this time are primarily composed of AM with some neutrophil influx (Figure 2A). Isolated AM from silica-exposed mice exhibited increased LC3-I and LC3-II levels, indicating increased autophagy (Figure 2B). P62 levels were also significantly elevated (Figure 2B), as well as were total ubiquitinated protein (Supplemental Figure 1). Isolated lung lavage cells from GFP-LC3 mice at 7 d had increased GFP-LC3 fluorescence and puncta (Figure 2D), further supporting our findings by Western Blot. Together, this data provides consistency between silica exposures' ability to increase autophagy in BMdM *in vitro* with similar outcomes in AM *in vivo*.

Autophagy deficiency in macrophages enhances cell death and HMGB1 release

Autophagy has been reported to be a primary mechanism for the degradation of assembled inflammasomes (5). Consequently, autophagy can potentially regulate the secretion of inflammasome-associated cytokines. The role of autophagy in regulating NLRP3 inflammasome activity following exposure to silica has not been previously described. Additionally, autophagy is generally regarded as a cell survival mechanism, and may act to prevent silica-induced cytotoxicity. In order to test the role of autophagy in silica-induced NLRP3 inflammasome activity and cell death, we generated BMdM from Atg5^{fl/fl}LysM-Cre⁺ mice, which are deficient in autophagosome formation. Though Atg5^{fl/fl}LysM-Cre⁺ mice have been utilized by multiple other groups (16, 20, 29, 30), we confirmed BMdM generated from these mice exhibited impaired autophagy to establish reproducibility of the model. This included determining Atg5 depletion (data not shown) and defective lipidation of LC3-I to LC3-II (Supplemental Figure 2A). This was consistent with isolated AM (Supplemental Figure 2B). We then exposed BMdM generated from littermate control and Atg5^{fl/fl}LysM-Cre⁺ mice to silica and LPS, which was added simultaneously with the particle in order to prime the NLRP3 inflammasome and cause cytokine production over 24 hr. Silica exposure did not significantly increase extracellular IL-1 β levels in BMdM cultures from Atg5^{fl/fl}LysM-Cre⁺ mice when compared to littermate controls (Figure 3A). However, there was spontaneous (background) release of IL-1 β at low levels from non silica-exposed autophagy deficient BMdM. This suggested some basal level of NLRP3 inflammasome activity in macrophages in which autophagy is impaired. Atg5^{fl/fl}LysM-Cre⁺ macrophages were more susceptible to cell death than littermate controls, as indicated by increased LDH in cell supernatants following silica exposure (Figure 3B). HMGB1 release has been associated with NLRP3 inflammasome activation and cell death with bacterial

infection, and exposure to carbon nanotubes (21, 31). We observed increased HMGB1 release from silica exposed Atg5^{fl/fl}LysM-Cre⁺ BMdM compared to silica-exposed littermate controls (Figure 3C). Autophagy deficiency did not decrease or enhance particle uptake (data not shown). Therefore, we suspect greater HMGB1 release was due to more cell death. This data is consistent with the notion that autophagy functions to prevent silica-induced cytotoxicity, and supports the proposal that autophagy deficiency results in some spontaneous NLRP3 inflammasome activity.

Autophagy deficiency enhances silica-induced acute inflammation in vivo

In order to determine if autophagy impairment in macrophages results in increased acute inflammation and cytotoxicity *in vivo*, we exposed Atg5^{fl/fl}LysM-Cre⁺ mice to silica and assessed parameters of inflammation in the whole lung lavage fluid after 24 hr. Silica exposure resulted in significant increases in total protein, HMGB1, and IL-18 in the lavage fluid of Atg5^{fl/fl}LysM-Cre⁺ mice compared to silica-exposed littermate controls (Figure 4A, 4B, 4C). IL-1 β was not significantly increased in Atg5^{fl/fl}LysM-Cre⁺ mouse lavage fluid compared to silica-exposed littermate controls, but was significantly increased over PBS-exposed animals (Figure 4D). We observed some spontaneous (background) IL-18 production in Atg5^{fl/fl}LysM-Cre⁺ mice (Figure 4C). Consistent with our findings in BMdM exposed to silica *in vitro*, LDH levels were greater in the lavage fluid of Atg5^{fl/fl}LysM-Cre⁺ mice compared to littermate controls (Figure 4E). Extracellular Cathepsin activity was also increased in autophagy deficient mice compared to littermate controls, though not significantly (Figure 4F). These data support the notion that depletion of autophagy in macrophages causes some basal NLRP3 inflammasome activity. Furthermore, the observation that inflammation and cytotoxicity (LDH) were exacerbated in Atg5^{fl/fl}LysM-Cre⁺ mice demonstrates a fundamental role for autophagic suppression of silica-induced lung injury.

Autophagy deficiency in macrophages causes a basal pro-inflammatory phenotype in vivo

In our assessment of the acute inflammatory response following silica exposure, some findings suggested a basal phenotype when autophagy was impaired, including spontaneous IL-18 production (Figure 4C). We also observed increased cell numbers, mainly due to increased neutrophils in the Atg5^{fl/fl}LysM-Cre⁺ mice (Figure 5A) suggesting background cellular inflammation. Neutrophil influx in PBS-exposed Atg5^{fl/fl}LysM-Cre⁺ mice correlated with elevated CXCL1 in the lavage fluid (Figure 5B). Other investigators have reported similar observations, as well as elevated levels of IL-1 α (16, 29). However, we did not observe increased IL-1 α in the whole lung lavage fluid of Atg5^{fl/fl}LysM-Cre⁺ mice, and there was no significant difference in IL-1 α after silica exposure between autophagy deficient and littermate control mice (Figure 5C). This discrepancy could be due to differences in amounts in the lung lavage fluid or more likely the interstitial/intracellular levels assayed with whole lung homogenization used in the other studies (16). During differential analysis, we noticed that AM from Atg5^{fl/fl}LysM-Cre⁺ mice were much larger than those observed in littermate controls (Figure 5D), which may indicate further differences in macrophage activity and phenotype. This data highlights the importance of macrophage-associated autophagy in maintaining homeostasis, and that knocking out autophagy in macrophages results in an

unusual basal phenotype that may contribute to greater inflammation following particle exposure.

Autophagy deficiency in macrophages increases silica-induced lung pathology

Next, we examined whether autophagy deficiency in macrophages and other cells of myeloid origin results in greater chronic disease *in vivo* following silica exposure. Atg5^{fl/fl}LysM-Cre⁺ mice and littermate controls were exposed to silica (40 mg/kg) once a week for 4 consecutive weeks, then the mice were assessed 56 d following the first instillation for pathology and fibrosis (13). We observed no differences in weight between littermate controls and Atg5^{fl/fl}LysM-Cre⁺ over the course of this study (data not shown). Autophagy-deficient mice exposed to silica exhibited more significant pathology than littermate controls exposed to silica (Figure 6A). In parallel, we observed more pathology and fibrosis in the silica exposed Atg5^{fl/fl}LysM-Cre⁺ mice as determined by Trichrome staining (Figure 6B). PBS exposed Atg5^{fl/fl} LysM-Cre⁺ mice had slightly elevated pathology score compared to littermate controls, consistent with the altered phenotype and elevated IL-18 levels at baseline as previously discussed. These studies demonstrate autophagy in macrophages is important in mitigating silica-induced chronic disease.

Discussion

NLRP3 inflammasome activity in macrophages is a central driver of silica-induced chronic inflammation and lung disease (12) (13). An primary role for autophagy is to sequester and degrade inflammasomes and inflammasome cytokines such as pro-IL-1 β (5, 26, 32). Therefore, autophagic stimulation is being investigated as a potential therapeutic target for chronic inflammatory conditions. Ineffective autophagic suppression of NLRP3 inflammasome activity may, in part, be a mechanism contributing to chronic NLRP3 inflammasome activity with silica exposure. We initially hypothesized that silica-induced NLRP3 inflammasome activity would be due, in part, to impaired autophagic flux through the lysosome. This has been reported to be the case for rare-earth nanomaterials (33). There is some evidence of altered autophagic activity with silica exposure *in vivo*, including an accumulation of p62 within granulomas (34), though a very high dose of silica was used in those studies. Others reported that autophagy is impaired in isolated human lung AM from individuals with silicosis, including increased levels of LC3, p62, and Beclin-1 (15). In the same study, autophagy alteration by silica was suggested to contribute to AM sensitization to LPS stimulation, though exactly how this occurs was not clear. Our data showing increased LC3-II, p62, and total ubiquitination *in vitro* and *vivo* are consistent with reports in human AM (Figure 1 and 2). We were also able to show that 3-MA effectively inhibited silica-induced autophagy (Figure 1), indicating that silica-exposure is causing autophagic induction. Secondly, through inhibiting lysosomal activity with Bafilomycin A1, we demonstrated further accumulation of autophagic carrier proteins (Figure 1), which supports the hypothesis that the primary outcome of silica exposure on the autophagic pathway is induction and not necessarily impaired autophagic flux. Silica exposure is known to induce lysosome membrane permeabilization (LMP), which can result NLRP3 inflammasome activation and cytotoxicity (14). Increased autophagy following silica exposure may be a mechanism to mitigate the cytotoxic and pro-inflammatory outcomes of LMP.

Our studies are the first to utilize Atg5^{fl/fl}LysM-Cre⁺ mice to test the contribution of autophagy in macrophages to silica-induced NLRP3 inflammasome activity and chronic disease. Others have utilized this mouse model to test the role of myeloid cell-mediated autophagy in inflammation and disease burden with *Mycobacterium tuberculosis* (16). In that report, inflammation and disease were significantly enhanced in Atg5^{fl/fl}LysM-Cre⁺ mice compared to littermate controls. A second study by different investigators reported enhanced inflammation and disease with LPS and/or Bleomycin induced lung injury in Atg7^{fl/fl}LysM-Cre⁺ mice, which are also defective in autophagosome formation (29). We observed significantly greater levels of pro-inflammatory mediators IL-18 and HMGB1 (Figure 5), increased extracellular LDH, and chronic disease (Figure 6) in Atg5^{fl/fl}LysM-Cre⁺ mice with silica exposure. The studies strongly support an integral role for autophagy as an important mechanism in suppressing cell death with silica exposure as well as mitigating NLRP3 inflammasome activity.

Two additional important findings in this study include the observation that silica exposure increases IL-18 and HMGB1 (Figure 4), and release of these pro-inflammatory mediators is enhanced in autophagy deficient mice. HMGB1 is a nuclear architectural protein that is actively secreted from macrophages with active NLRP3 inflammasomes, and is passively released from dead or dying cells (30, 35, 36). Extracellular HMGB1 acts as a Danger Associated Molecular Pattern, and can contribute to sterile priming of the NLRP3 inflammasome (37). Exacerbated HMGB1 levels in the lavage fluid of silica-exposed Atg5^{fl/fl}LysM-Cre⁺ mice are consistent with increased cell injury (Figure 3B, 4E). The observation that Atg5^{fl/fl}LysM-Cre⁺ have increased neutrophil numbers at baseline (Figure 5), and high levels of HMGB1 after silica exposure could be due, in part, to more neutrophil cell death, though this was not investigated in these studies. Furthermore, HMGB1 secretion has been observed in models of oxidative stress, and oxidative stress is known to occur with silica exposure (38, 39). Consequently, increased extracellular HMGB1 in these studies may be directly associated with elevated oxidative stress. The presence of high levels of both IL-18 and HMGB1 in autophagy deficient mice, and greater chronic disease, indicate that these two inflammatory mediators could contribute more to particle-induced chronic lung disease than appreciated. IL-1R null mice exposed to Multi-walled Carbon Nanotubes (another particle that activates the NLRP3 inflammasome), while protected from excessive acute inflammation, develop more severe chronic disease (40). These studies indicate that IL-1R signaling may not be as important in chronic disease, but rather other signals including IL-18 and/or HMGB1 could be more important contributors to chronic pathology. This notion is further supported by the fact that we observed spontaneous IL-18 release and some lung pathology in PBS-exposed autophagy deficient mice (Figure 4C, Figure 6B). Under normal physiological conditions, a cytosolic pool of IL-18 is maintained (41). This may explain why IL-18 was the predominant cytokine released from Atg5^{fl/fl}LysM-Cre⁺ mice in our studies (41). Others have also reported increased neutrophils, IL-18, and spontaneous disease at baseline in Atg7^{fl/fl}LysM-Cre⁺ mice (29). IL-18 neutralizing antibodies but not Anakinra, the IL-1R antagonist, were effective at reducing spontaneous disease in Atg7^{fl/fl}LysM-Cre⁺ mice. This may likely be true for Atg5^{fl/fl}LysM-Cre⁺ mice, though this was not determined. Finally, IL-18 and HMGB1 have been implicated in autoimmune disorders such as SLE, which has greater prevalence among individuals with

silicosis (42, 43). Further studies will be needed to elucidate the role of HMGB1 and IL-18 in silica-induced lung inflammation and its subsequent contribution to systemic disease.

An additional novel finding of this work is that the macrophages in Atg5^{fl/fl}LysM-Cre⁺ mice were significantly larger than littermate controls. Defective autophagy has been reported to negatively impact lipid metabolism and cholesterol trafficking (44), as well as protein turnover. While the mechanism responsible for larger AM in the Atg5^{fl/fl}LysM-Cre⁺ was not investigated, these findings are consistent with accumulation of protein and lipids.

In conclusion, silica exposure increases autophagic activity in macrophages *in vitro* and *vivo* through a mechanism that is not necessarily due to impaired autophagic flux through the lysosome. Knocking out Atg5, which is required for autophagosome formation, causes enhanced acute inflammation (predominantly HMGB1 and IL-18 release), increased cell death, and exacerbated chronic disease. Furthermore, Atg5 depletion causes spontaneous NLRP3 inflammasome activity and disease, with IL-18 being the predominant NLRP3 inflammasome cytokine being released. These studies show the importance of autophagy in macrophages in suppressing silica-induced inflammation and cytotoxicity that drive chronic disease, and in maintaining lung homeostasis. The mechanisms described in these studies may also be critical for suppression of inflammation and disease following exposure to other NLRP3 inflammasome activating particles including asbestos, uric acid crystals, cholesterol crystals, and engineered nanoparticles, and supports future studies of the role of autophagy with exposure to these agents.

Supplementary Material

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Acknowledgements

The authors appreciate the technical support obtained through the CEHS Molecular Histology and Fluorescence Imaging, Inhalation and Pulmonary Physiology, and Fluorescence Cytometry Core facilities. We extend a special thanks to the technical staff of these cores including Britt Postma, Mary Buford, and Lou Herritt. We also acknowledge Dr. Noboru Mizushima for developing and providing the Atg5^{fl/fl} mice. We thank Dr. John Hoidal at University of Utah School of Medicine, for independently reviewing some of the data presented in this study. We also thank Dr. Elizabeth A. Putnam, Dr. Christopher T. Migliaccio, and Dr. Kevan Roberts at the University of Montana for independently reviewing and proof-reading the manuscript.

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Highlights

- Silica exposure increases autophagy in macrophages
- Autophagy deficient mice have enhanced inflammation and silicosis
- Autophagy deficiency in macrophages results in greater silica-induced cytotoxicity
- Autophagy deficiency in macrophages increases extracellular IL-18 and HMGB1

Figure 1. Autophagy is increased in vitro in BMdM 24 hr following silica exposure

(A) Representative images of GFP-LC3 expression in BMdM following exposure to silica (100 μ g/mL) with or without Bafilomycin A1 treatment (100 μ M) and 3-MA treatment (5 μ M). Images were captured using a 60X scan (B) Average GFP-LC3 puncta per cell for combined experiments ($N=3$). (C) Median Fluorescent Intensity (MFI) of combined experiments ($N=3$). (D) Representative western blot of three separate experiments showing LC3, p62, and total ubiquitination levels in BMdM exposed to silica with or without Bafilomycin A1 treatment (100 μ M). Graphs show median $\pm$ SEM. * $P < 0.05$ indicates significance with two-way ANOVA and a one-tailed post test.

Figure 2. Silica exposure increases autophagy in AM in vivo
 (A) Cell counts in the lung lavage fluid 7 d after silica exposure (40 mg/kg). (B) Representative Western Blot and densitometry graph of combined experiments showing LC3 and p62 levels in isolated AM 7 d following instillation of silica (40 mg/kg). (C) Representative images of AM isolated 7 d post silica exposure in GFP-LC3 mice (60X). $N=4$ for each treatment group. 2 mice were pooled for each N for sufficient protein for Western Blot analysis. Graphs show mean $\pm$ SEM. * $P < 0.05$, *** $P < 0.001$ indicates significance over PBS-exposed mice using a two-tailed t-test.

Figure 3. Autophagy deficient BMdM are more susceptible to cell death and have enhanced HMGB1 release

BMdM were exposed to silica (100 µg/mL) and LPS (20 ng/mL) and cell supernatants or lysates assessed after 24 hr. (A) IL-1β levels in cell supernatants 24 hr after particle exposure. (B) Percent extracellular LDH activity in cell supernatants (compared to activity from total cell lysates). (C) Extracellular HMGB1 24 hr after particle exposure. Graphs show mean ± SEM (*N*=6 for each group). **P* < 0.05, ****P* < 0.001 indicate significance with 2-way ANOVA.

Figure 4. Atg5 depletion in macrophages in vivo enhances acute inflammation

Autophagy deficient and littermate control mice were instilled with 40 mg/kg silica or PBS and assessed for indicators of inflammation in the lavage fluid after 24 hr including: (A) Total protein, (B) HMGB1, (C) IL-18, (D) IL-1 β , (E) LDH, and (F) cathepsin activity. Graphs show mean $\pm$ SEM ($N=8$ for all treatment groups). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to silica-exposed littermate control using a 2-way ANOVA.

Figure 5. Basal phenotype observed in Atg5^{fl/fl}LysM-Cre⁺ mice

Autophagy deficient and littermate control mice were instilled with 40 mg/kg silica or PBS and assessed sacrificed after 24 hr. (A) Total combined cell counts, and macrophage and neutrophil counts in the lavage fluid. (B) CXCL1 levels in the lavage fluid. (C) IL-1α levels in the lavage fluid. (D) Cytopins showing increased macrophage area on slide. Black Bar indicates 20 μm. Graphs show mean ± SEM (N=8 mice per treatment group). **P* < 0.05, ***P* < 0.01, ****P* < 0.001 when comparing to PBS-exposed littermate control.

Figure 6. Atg5 depletion in macrophages in vivo results in enhanced pathology and collagen deposition with silica exposure

Mice were exposed to silica (40 mg/kg) once a week for 4 consecutive weeks, and then sacrificed 28 days following the final instillation (day 56). (A) H&E stained lung sections showing increased pathology. (B) Trichrome stained sections showing increased collagen and pathology. Images were captured at 20X magnification. Black bars measure 50 μ m. Graphs show mean score $\pm$ SEM ($N=3$ for each treatment group) * $P < 0.05$, ** $P < 0.01$ indicates significance following rank transformation and post-hoc analysis using the Kruskal-Wallis test with Dunn's multiple-comparison post test.