Cigarette smoke induced autophagy-impairment accelerates lung aging, COPD-emphysema exacerbations and pathogenesis

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At a Glance Summary:

Scientific Knowledge on the Subject: The CS exposure and aging are first and second leading causes of COPD-emphysema pathogenesis. Although various mechanisms related to the role of CS and aging in emphysema progression are independently described but there remains a gap in knowledge, if CS and aging involve a common process for disease pathogenesis and progression.

What This Study Adds to the Field: This study for the first time describes that both CS exposure and aging impair proteostasis/autophagy resulting in a unique alveolar pathology, aggresome-bodies, that mediates alveolar senescence and emphysema progression. In addition, the study describes a prognosis based intervention strategy where levels of aggresome-bodies can predict severity of disease that can be treated with an autophagy inducing antioxidant drug, cysteamine.
Abstract

Rationale: Cigarette-smoke (CS) exposure and aging are the leading causes of chronic obstructive pulmonary disease (COPD)-emphysema development, although the molecular mechanism that mediates disease pathogenesis remains poorly understood.

Objectives: To investigate the impact of CS-exposure and aging on autophagy, and pathophysiological changes associated with lung aging (senescence) and emphysema progression.

Methods: Beas2b cells, C57BL/6 mice and human (GOLD 0-IV) lung tissues were used to determine the central mechanism involved in CS/age-related COPD-emphysema pathogenesis.

Results: Beas2b cells and murine lungs exposed to CSE/CS showed a significant (p<0.05) accumulation of poly-ubiquitinated proteins and impaired-autophagy marker, p62, in aggresome-bodies. Moreover, treatment with autophagy-inducing antioxidant drug, cysteamine significantly (p<0.001) decreased CSE/CS-induced aggresome-bodies. We also found a significant (p<0.001) increase in levels of aggresome-bodies in the lungs of smokers and COPD-subjects in comparison to non-smoker controls. Furthermore, the presence and levels of aggresome-bodies statistically correlated with severity of emphysema and alveolar senescence. In addition to CS exposure, lungs from old mice also showed accumulation of aggresome-bodies, suggesting this as a common mechanism to initiate cellular senescence and emphysema. Additionally, Beas2b cells and murine lungs exposed to CSE/CS showed cellular apoptosis and senescence, which were both controlled by cysteamine treatment. In parallel, we evaluated the impact of CS on pulmonary exacerbation, using mice exposed to CS and/or infected with Pseudomonas aeruginosa (Pa), and confirmed cysteamine’s potential as an autophagy-inducing antibacterial drug, based on its ability to control CS-induced pulmonary exacerbation (Pa-bacterial counts) and resulting inflammation.

Conclusion: CS induced autophagy-impairment accelerates lung aging, and COPD-emphysema exacerbations and pathogenesis.
Introduction

The human airway is continuously exposed to environmental contaminants that make it predisposed to the risk of intermittent or stable pulmonary exacerbations. Moreover, repeated long-term environmental exposure to cigarette smoke (CS) activates reactive oxygen species (ROS), inflammatory-oxidative stress and apoptosis that leads to alveolar space enlargement and development of chronic obstructive pulmonary disease (COPD)-emphysema (2). COPD is the third major leading cause of death in the United States and the major risk factors for COPD-emphysema is cigarette smoke (CS) exposure and aging. CS exposure not only initiates COPD-emphysema but also compromises ability to the fight infection (2, 3, 36). Thus, COPD-emphysema is often associated with acute or repeated episodes of chronic infection that can lead to respiratory failure (43).

Although, it remains uncertain how some non-smokers develop emphysema. The causes of emphysema in non-smokers include alpha-1-antitrypsin deficiency (~5%) and premature aging-related conditions such as cutis laxa (46, 47). In non-smokers, emphysema presents morphologically similar features as in smokers. However, in non-smokers, disease develops very early, suggesting premature aging that may involve genetic and/or other pathological conditions. This implies that, in addition to CS-induced changes that are known to activate inflammatory-oxidative stresses and pathogenesis of emphysema, other genetic/age-related factors may also contribute to the structural changes in the alveoli (emphysema) that result in decreased lung function (21, 43).

Aging is defined as the progressive decline of homeostasis (including protein homeostasis or “proteostasis”) after the reproductive phase of life, leading to an
increasing risk of disease or death (21, 31, 45). With increasing age, pulmonary function progressively declines due to repeated infections and inflammation, followed by structural/morphological alveolar changes (21, 31, 45). Similarly, we anticipate that CS exposure or genetic changes can accelerate COPD-emphysema pathophysiology, suggesting the central role of aging-related mechanisms in emphysema progression. Similar to non-smokers, early age-related alveolar emphysema is seen in KL (Klotho) and SMP-30 (Senescence marker protein 30) gene knockout/mutant mice. CS exposure enhances emphysema severity in these mice (17, 21, 24) suggesting that CS can accelerate lung aging (46, 47). Thus, aging serves a critical role in emphysema pathogenesis, although the exact molecular mechanism remains unknown. Based on recent studies (8, 16, 44, 50), we hypothesized that CS- or age-related decline in proteostasis/autophagy could induce aggresome-bodies that activate chronic inflammatory-oxidative responses, and alveolar apoptosis and senescence. Thus, in the present study we first evaluated whether CS- or age-related proteostasis/autophagy-impairment could lead to pathophysiological changes associated with both lung aging and COPD-emphysema progression. Next, we assessed the therapeutic efficacy of prognosis based intervention strategy targeting aggresome-bodies in COPD-emphysema. The study not only demonstrate for first time the central mechanism involved in COPD-emphysema pathogenesis and progression but also explains the process for, emphysema development seen in small group of non-smokers or accelerated lung aging in smokers. Moreover, pre/clinical development of aggresome-dye based prognostic assay in this study for predicting emphysema, will allow early detection and intervention in COPD-emphysema subjects.
Methods

Human subjects and mice experiments

Paraffin embedded longitudinal sections were obtained from the NHLBI Lung Tissue Research Consortium (LTRC, NIH). COPD lung disease subjects were classified and graded based upon the emphysema stages defined by the Global Initiative for Chronic Obstructive Lung Disease (GOLD) and control (GOLD 0) and COPD (GOLD I-IV) stages (n=10-15 for each) were used (see Table 1,(7)). Aside from emphysema, no other underlying conditions affected non-COPD control or COPD subjects (GOLD I-IV), but one person in each group (GOLD I-IV) had first-degree blood relatives with chronic bronchitis. The study protocol was approved by the Institutional Review Board (IRB), Central Michigan University and Johns Hopkins University as exempt, and subject’s lung function data and other clinical parameters were obtained from each of the LTRC contributing centers without disclosing the subject’s name and information. These lung sections were analyzed via immunostaining and statistical analysis was performed as described below. For murine experiments, C57BL6/6 mice (ages 6-weeks to 19-months) were housed in a clean environment during the course of these studies in our satellite animal housing facility, following our CMU IACUC approved protocols. Mice were exposed to room-air, or chronic (28 weeks; 5 days/week) or sub-chronic (14 weeks; 5 days/week) cigarette smoke generated by burning 3RF4 research cigarettes (Tobacco Research Institute, University of Kentucky) using the TE-2 cigarette smoking machine (4, 5, 7, 33, 44). For chronic and sub-chronic experiments, air- or CS-exposed mice were also intraperitoneally treated with a daily dose of 0.01 M cysteamine (Sigma) for the last 10 days. In a parallel chronic CS experiment, air- or CS-exposed mice were
intronasally infected with 2x10^6 c.f.u *Pseudomonas aeruginosa* (PA01-GFP) for the final 5 days. In addition to sub-chronic-CS exposure groups, old mice (~12 months) were used as a positive control for age-related alveolar changes. In a separate experiment, aging-induced changes were verified by comparing lungs of young (6-months) and older (19-months) mice.

**In vitro experiments**

For *in vitro* studies, human bronchial epithelial cell line, Beas2B was cultured at 37°C with 5% CO₂ in DMEM/F12 (Dulbecco’s Modified Eagle Medium; Cellgro, Manassas, VA) supplemented with 10% FBS (Fetal Bovine Serum; Cellgro) and 1% PSA (Penicillin, Streptomycin, Amphotericin; Cellgro). For the smoke exposure experiments, cigarette smoke extract (CSE) was prepared as previously described (Tran et al., 2014). Beas2b cells were treated with cysteamine and/or CSE at various concentrations for 6 hrs or overnight.

**Western blotting**

After room-air or CS exposures, cells or lung tissues were harvested and protein lysates were prepared using RIPA buffer (Pierce) containing 1x protease inhibitor cocktail (Pierce). Briefly, lung tissues were homogenized and sonicated for 2-3 minutes on ice with 10-15s pulses. While cultured cells were washed with ice-cold PBS and directly lysed with ice-cold protein lysis buffer on plates by gentle scraping followed by 5-10 minutes constant shaking at room temperature. Soluble- and insoluble- protein fractions were separated by centrifugation at 13,000 rpm for 15 minutes at 4°C. Total protein lysate was used for protein quantification using Bradford assay and soluble- (50µg) and/or insoluble- protein fractions (pellet, isolated from equal amount of protein
~500µg, for each sample) were separated on 10% SDS-PAGE and transferred to 0.45µm nitrocellulose membranes (BioRad) for immunoblotting. Non-specific binding sites on membranes were blocked with 5% non-fat milk at room temperature for an hour on a shaker. Membranes were incubated overnight at 4°C with mouse monoclonal ubiquitin (SantaCruz, 1:1000), rabbit polyclonal p62 (SantaCruz, 1:500), rabbit polyclonal VCP (SantaCruz, 1:500), rabbit polyclonal Sirt1 (SantaCruz, 1:500) and rabbit polyclonal p53 (SantaCruz, 1:500) as primary antibodies. Later the membranes were washed several times (3-5X, ~ 30 mins) with PBS-tween buffer (0.5% tween-20 in 1xPBS) and incubated with 1:5000 goat horseradish peroxidase-conjugated anti-mouse (BioRad) or anti-rabbit (BioRad) antibodies for an hour at room temperature followed 3-5X (~30 mins) washing as described above. The Clarity™ Western ECL substrate (BioRad) was used for chemiluminescence detection of immunoblots using a LI-COR C-Digit™ Blot Scanner. Images were captured with Image Studio Lite 5.0 and analyzed using this or Image J software.

**Caspase-3/7, DNA Fragmentation and Senescence assays**

Apoptotic cell death was quantified in total-protein lysates of lungs harvested from young (6-weeks) and older mice (19-months) by using commercially available Caspase-Glo® 3/7 kit (Promega Corp). Briefly, lung total-protein lysates were plated onto 96 well plates. Freshly prepared caspase-3/7 reagent was added, followed by 1 hr incubation at room temperature. Luminescence was quantified as described previously (48). For DNA fragmentation assay we isolated DNA using TAE Lysis Buffer and followed the protocol as previous described(25). For senescence assay, histochemical staining kit (Sigma-Aldrich) was used according to manufacturer’s instruction. Briefly, 0.5 million Beas2b
cells were plated on a 6-well plates overnight at 37°C with 5% CO₂. Following Beas2b cell treatments as described above, cells were fixed with 10% fixation buffer for 7 minutes at room temperature followed by 2X wash with 1x PBS and incubation with X-gal staining solution at 37°C overnight. SA-β-galactosidase positive (blue) cells were counted under a Nikon ECLIPSE TS100 microscope equipped with Infinity-2 camera (Lumenera Corporation) and Infinity Analyze 6.4.1 software. Data are expressed as percentage change in number of senescent cells in comparison to the control group. Standard sudan B Black (SBB; M P Biomedicals) staining was used to quantify levels of senescent cells in lung sections obtained from mice (Air/CS-exposed or old mice) and human (COPD, GOLD 0-IV) lungs. Briefly, staining solution was prepared by dissolving 0.7 g of SBB in 100 ml of 70% ethanol, followed by overnight stirring at room temperature. The stain solution was then filtered and stored in the dark in an airtight container. The lung sections were then stained with SBB for 1 minute and washed with PBS (2X). Images were taken on a Nikon ECLIPSE TS100 microscope equipped with the Infinity2 camera (Lumenera Corporation) by using the Infinity Analyze 6.4.1 software.

Aggresome-, H&E- and immuno- staining and TEM

The longitudinal sections of mouse lungs were deparaffinized by passing through series of xylene, graded alcohol (100, 95 and 70%) and distilled water. These sections were processed for antigen retrieval by treatment with hot Tris-EDTA for 5-10 mins, and cooled at room temperature (RT). For aggresome staining, sections were incubated with ProteoStat® Aggresome Detection reagent (Enzo Life Sciences) and Hoechst 33342 nuclear stain for 30 minutes followed by 2X washes with 1x PBS before mounting.
coverslips for microscopy. For H&E staining, Hematoxylin-Mayers (Lillie’s Modification) reagent was added and sections were incubated for 5 mins at RT. The sections were rinsed 2x with distilled water and bluing reagent was added to cover the sections and incubated for ~15 seconds followed by 2x rinse with distilled water. Next, Eosin-Y solution was added and sections were incubated for 3 mins at RT. Sections were rinsed with absolute alcohol and further dehydrated in 3 changes of absolute alcohol before mounting coverslips with 30% glycerol. Images were captured using a Nikon ECLIPSE TS100 microscope equipped with Infinity 2 camera (Lumenera Corporation) and Infinity Analyze 6.4.1 software as described recently (4, 5). For immunofluorescence studies, sections were blocked with 5% normal goat (or other antibody specific) serum for 1 hr at room temperature followed by incubation with 1 µg primary antibody (mouse monoclonal Ubiquitin, rabbit polyclonal p62 or rabbit polyclonal Sirt1, Santa Cruz) prepared in 5% goat or donkey serum (Jackson Immunological), 0.4% BSA (Sigma) and 0.2% Triton X-100 (Sigma) solution for 1 hr at room temperature. Detection of primary antibodies was performed using 1 µg of goat anti-rabbit Alexa 488 (Santa Cruz) and/or donkey anti-mouse Texas Red (Santa Cruz) secondary antibodies. Nucleus was counterstained with Hoechst (10µg/ml) for 5 minutes and washed with 1xPBS. The coverslips were mounted upon slides with 30% glycerol and the images were captured using the Zoe™ Fluorescent Cell Imager (Bio-Rad), as recently described. The Transmission electron microscopy (TEM) was performed as we recently described. Briefly, Beas2b cells were grown on 6-well plates and were exposed to room-air or indicated concentration of CSE for 6 hrs. Cells were washed with 1x PBS and harvested
by low speed centrifugation (1250 rpm, 10 mins) and processed for TEM as described previously(41).

**Flow Cytometry Analysis**

The bronchioalveolar lavage fluid (BALF) cells were re-suspended in cell staining buffer (Biolegend) containing Fc receptor (TruStain fcX; Biolegend) blocking solution for 10 minutes at 4°C. The cells were incubated with 1 µg/ml of CD4-Phycoerythrin(secondary)-conjugated rat monoclonal (Santa Cruz), rat monoclonal Mac-1 (BD Biosciences), mouse monoclonal-ubiquitin (Santa Cruz) or rabbit polyclonal-p62 (Santa Cruz) for 20 minutes at 4°C. Cells were washed twice with ice-cold cell staining buffer (Biolegend), and Ub, p62 and Mac-1 immunostained cells were detected by incubating samples with Alexa Texas red-anti-mouse (1µg; Santa cruz), Alexa 488-antirabbit (1µg; Santa-cruz) or Phycoerythrin-anti-rat secondary antibodies. The stained cells were further washed with ice-cold cell staining buffer (Biolegend) and re-suspended in 1% paraformaldehyde. Results were acquired and analyzed using BD FACS Aria flow cytometer and BD FACS Diva 8.0 analysis software as recently described(41).

**ELISA, MPO activity and Bacterial Survival Assays**

Supernatants from bronchoalveolar lavage fluid (BALF) were quantified for IL-6 and IL-1β using ELISA kits (eBiosciences) and following manufacturer’s instructions. Quantification of myeloperoxidase (MPO) levels in mouse BALF was similarly performed using MPO ELISA kit (Hycult Biotechnology), following manufacturer’s instructions. For bacterial assay, the right or left lung from mice infected with PA01-GFP were homogenized in 1X PBS containing 0.05% Tween-20. Lung tissue homogenates
were serially diluted in 1 ml volume of PBS and spread upon LB agar plates supplemented with 1% cabenecillin (Sigma) because our Pa bacterium is transformed with antibiotic resistant GFP plasmid for fluorescence visualization. The plates were incubated overnight at 37°C and colony counts were used to determine the counts of colony forming units (CFU) to quantify bacterial survival in the lungs.

**Data analysis**

Data were analyzed using GraphPad Prism 4.0/6.0 software (GraphPad Prism, San Diego, CA). Statistical analysis was performed using one-way ANOVA followed by Tukey’s post-hoc test or two tailed t-tests. Statistical values of p<0.05 were considered to be significantly different.
Results

Aggresome levels correlate with smoking history, alveolar senescence and severity of emphysema in COPD subjects

To identify the role of aggresome-formation in CS-induced COPD-emphysema, we obtained human lung tissue sections from non-emphysema controls (GOLD 0) and COPD-emphysema (GOLD I-IV) subjects that included both smokers (current/ever) and non-smokers (never). We analyzed these samples to determine the levels of aggresome-bodies in relation to severity of emphysema in COPD subjects in comparison to non-emphysema subjects. Human lung tissue sections (GOLD 0 to GOLD IV) were stained with a ProteoStat® dye to detect and quantify levels of aggresome-bodies. We found significant increase in levels of aggresome-bodies with severity of emphysema (GOLD 0-III, p<0.05, Fig 1A), alveolar senescence (Fig 1B) and decline in lung function (FEV1%-predicted). However, GOLD IV COPD-emphysema subjects showed fewer aggresome-bodies (Fig 1A) in comparison to non-emphysema (GOLD 0) group due to significant tissue destruction and alveolar senescence in GOLD IV subjects (Fig 1B). Moreover, smokers (current/ever, Fig 1A) showed significantly higher number of aggresome-bodies in comparison to non-smokers (never, Fig 1C), suggesting the role of smoke exposure in aggresome-formation (Fig 1A vs 1C), and resulting senescence (Fig 1B vs 1D) and lung function decline. Based on this study and our previous findings, we believe aggresome-bodies can be developed as a prognostic biomarker for cigarette smoke induced COPD-emphysema progression.

CSE exposure induces ultrastructural changes, autophagy-impairment, aggresome-formation and cellular senescence
We used Transmission Electron Microscopy (TEM) to analyze ultrastructural changes that characterize the effect of cigarette smoke extracts (CSE) in modulating in vitro morphological changes in Beas2b cells. Compared to air-exposed control cells, 10% CSE induced increased accumulation of peri-nuclear aggresome-bodies. In addition, we also found that CSE exposure leads to membrane damage and disintegration (Fig 2A) indicative of apoptosis-mediated cell death. To confirm our previous findings (6, 8, 33, 44) that CS induces aggresome-formation and to determine whether treatment with an antioxidant may alleviate CS-induced aggresome-formation, Beas2b cells were treated with freshly prepared CSE (10%) and cysteamine (250 µM) for 6 hrs. Soluble and insoluble protein fractions prepared from Beas2b cells exposed to room-air, 10%-CSE and/or cysteamine were used for immunoblot analysis (Fig 2B). Soluble protein fractions from air-exposed cells showed basal levels of ubiquitinated proteins. Similarly, Beas2b cells treated with cysteamine displayed no increase in amount of total ubiquitinated protein in soluble fractions. However, significant increase in accumulation of total poly-ubiquitinated proteins was observed in insoluble protein fractions prepared from Beas2b cells exposed to CSE (10%, 6hr); the translocation of ubiquitinated-proteins from the soluble to insoluble protein fraction is indicative of aggresome-formation (Fig 2B). Treatment with cysteamine (250 µM) significantly decreased the aggresome-formation in Beas2b cells exposed to CSE (10%). To determine whether CSE induced aggresome-formation would result in cellular senescence, we quantified changes in protein levels of p53 and Sirtuin1 by immunoblotting (Fig 2B). p53 belongs to the family of transcription factors important in regulating cell viability and cell death (51). We observed a decrease in p53 in soluble protein fractions of 10%-CSE treated cells that is
indicative of a reduction in DNA damage recognition and repair. Sirtuin1 (Sirt1) is implicated in control of cellular senescence (54). Abrogation of Sirt1 was observed in the soluble protein fractions prepared from Beas2b cells upon exposure to 10% CSE in comparison to air-exposed cells; the loss of Sirt1 is indicative of cell cycle arrest and cellular senescence. We have recently demonstrated cysteamine’s antioxidant potential to modulate electronic-cigarette-vapor (eCV) impaired autophagy and aggresome-formation (41). In this study, cysteamine inhibited eCV induced ROS activity in vitro and nitrotyrosine adduct in vivo. Hence, we anticipated that cysteamine would impact CSE exposed cells in a similar fashion. Beas2b cells treated with cysteamine (250 µM) for 6 hrs alleviated CSE-induced accumulation of ubiquitinated proteins in the insoluble protein fraction and rescued protein levels of p53 and Sirt1 (Fig 2B). This data supports our previous findings, suggesting cysteamine’s potential as an antioxidant and autophagy inducer. Furthermore, we performed histochemical analysis of senescence-associated-β-galactosidase (SA-β-gal) activity and found that Beas2b cells exposed to 10%-CSE exhibited significant (p<0.001) increase in SA-β-gal-positive (blue) cells in comparison to air-exposed or cysteamine-treated cells. Pre-incubation with cysteamine (250 µM, 6 hrs) decreased (p<0.0001) expression of CSE (10%) induced SA-β-gal-positive (blue) cells (Fig 2C). These results indicate that CSE exposure results in cellular senescence, the onset of which is delayed by the action of the autophagy inducing antioxidant, cysteamine. Further, studies are needed to identify the presence of any necrosis. Although, both human subject and in vitro data validate our hypothesis that CS impaired autophagy mediated aggresome-formation accelerates senescent pathophysiology (aging) and emphysema progression.
Cysteamine reduces CSE impaired autophagy and aggresome-formation

To further substantiate that CS impairs autophagy and induces aggresome-formation, we performed an autophagy flux assay using the Premo™ autophagy tandem sensor RFP-GFP-LC3B kit. Beas2b cells were transduced with 30 particles per cell of BacMam reagent containing LC3B tagged to acid-sensitive GFP (green fluorescence protein) and RFP (red fluorescence protein). The formation of autophagosomes (yellow or green fluorescence) and autolysosomes (red fluorescence) was quantified via flow cytometry. We observed significant increase in RFP (p<0.0001) and GFP (p<0.0001) fluorescence and their colocalization (yellow; p<0.0001) in Beas2b cells upon 6 hrs of CSE (10%) exposure in comparison to air-exposed cells. Pretreatment of CSE exposed Beas2b cells with cysteamine for 6 hrs showed significantly reduced expression of RFP (p<0.001), GFP (p<0.01) and their co-localization (yellow; p<0.0001) (Fig 3A) in comparison to CSE treatment. To further validate our findings, we performed fluorescence microscopic analysis of Beas2b cells transduced with tandem LC3B-RFP-GFP construct. In comparison to air-exposure, we observed significant increase in bright RFP and GFP fluorescence as puncta-bodies and their co-localization (p<0.0001) in Beas2b cells treated with CSE (10%) for 6 hrs (Fig 3B). Pretreatment of Beas2b cells with cysteamine showed significant decrease in CSE induced co-localization of RFP and GFP fluorescence puncta bodies (p<0.0001) in comparison to CSE exposure (Fig 3B and 3D). The autophagy or endosome-lysosome fusion inhibitor, chloroquine (90 µM, 12 hrs) used as a positive control, showed a significant (p<0.0001) increase in co-localization of RFP and GFP fluorescence puncta-bodies (Fig 3B). In order to verify our western blot results demonstrating the formation of aggresome-bodies upon exposure.
to CSE, we performed microscopic visualization of aggresome-bodies using the ProteoStat® Aggresome Detection dye. Beas2b cells were pre-incubated with cysteamine (250 µM) and/or CSE (10%) for 6 hrs. Significant increase (p<0.001) in number of aggresome-bodies (red fluorescence) was observed in CSE treated cells in comparison to air-exposed control group (Fig 3C). However, cysteamine treatment significantly reduced (p<0.001) CSE induced aggresome-bodies (Fig 3C and 3E). As a positive control, Beas2b cells were treated with proteasome inhibitor, MG132 (5 µM, 12 hrs), which exhibited significant increase in number of aggresome-bodies. These results indicate cysteamine’s potential as an antioxidant and autophagy inducer to alleviate CSE impaired autophagy and aggresome-formation.

Cysteamine reduces CS- and age- related aggresome-formation in murine lungs

To confirm our preliminary in vitro results, chronic-CS-exposed C57BL/6 mice were intraperitoneally (i.p.) administered with a daily dose of 0.01 M cysteamine (in 100 µl volume) for 10 days and/or infected intranasally with *Pseudomonas aeruginosa* (*Pa*, 2x10^6) for 5 days (7, 49). Soluble and insoluble protein fractions separated from whole-lung protein lysates were subjected to immunoblot analysis for changes in levels of ubiquitinated proteins, p62 (aberrant-autophagy marker) and VCP/p97 (valosin containing protein). In comparison to air-exposed controls, chronic-CS-exposed or *Pa*-infected mice showed significant increase in accumulation of ubiquitinated proteins in insoluble protein fractions, suggesting translocation of ubiquitinated proteins from the soluble to insoluble fraction. In addition, significant increases in the protein levels of the aberrant-autophagy marker, p62, and VCP were observed in insoluble protein fractions prepared from lung tissue collected from C57BL/6 mice exposed to chronic-CS and/or
infected with *Pa*, suggesting impaired autophagy/proteostasis and aggresome-formation. Moreover, cysteamine treatment substantially attenuated accumulation of ubiquitinated-proteins, p62 and VCP in the insoluble protein fraction (Fig 4A).

Lungs harvested from sub-chronic-CS-exposed and old (~12 months) mice similarly displayed significant increase in levels of ubiquitinated proteins (p<0.001), p62 (p<0.01) and VCP (p<0.05) in the insoluble protein fractions. Furthermore, immunoblotting analysis displayed significant reduction (p<0.01) in the levels of Sirt1 in soluble protein fractions prepared from lung tissues of C57BL/6 mice exposed to sub-chronic-CS or old C57BL/6 mice in comparison to air-exposed mice. Cysteamine treatment reduced the CS/age-related increase in levels of ubiquitinated-proteins, p62 and VCP in insoluble protein fractions and recovered Sirt1 levels in soluble protein fractions in comparison to sub-chronic-CS exposures (Fig 4B). The results of these experiments indicate that sub-chronic-CS exposure results in autophagy-impairment, aggresome-formation and accelerated lung aging while autophagy induction by cysteamine is capable of alleviating these changes. We further verified these results by microscopically analyzing CS- and age- related changes in protein- localization and expression as discussed below.

**Cysteamine rescues CS-induced aggresome-formation, senescence and alveolar space enlargement**

To verify our western blot and immunofluorescence results, we stained longitudinal lung sections collected from mice exposed to chronic-CS, treated with cysteamine and/or infected with *Pa*, with ProteoStat® Aggresome Detection dye to visualize aggresome-bodies via fluorescence microscopy. Significant increase in aggresome-formation (red-
fluorescence) was observed in lung sections collected from mice exposed to chronic-CS (p<0.0001) and/or infected with Pa (p<0.0001) in comparison to air-exposure. Moreover, treatment of chronic-CS-exposed mice with cysteamine significantly decreased the (p<0.0001) number of aggresome-bodies in comparison to chronic-CS-exposed mice (Fig 5A and 5B). Significant increase in aggresome staining was also observed in sub-chronic-CS-exposed (p<0.0001) and old (p<0.0001) positive control groups in comparison to air-exposed mice (Fig 5D). Moreover, treatment of sub-chronic-CS-exposed mice with cysteamine reduced the number of aggresome-bodies in comparison to sub-chronic-CS exposure (Fig 5D and 5F). Next, we evaluated whether exposure to CS and/or infection with Pa could lead to alveolar space enlargement and senescence in lungs via morphometric analysis of Hematoxylin and Eosin (H&E) and Sudan B Black (SBB) stained lung sections. Significant increase in alveolar space and senescence was observed in lungs harvested from mice exposed to chronic-CS (p<0.0001) and/or infected with Pa (p<0.0001) in comparison to air-exposed mice. However, even greater increase in (p<0.0001) alveolar space diameter was observed in mice exposed to chronic-CS and infected with Pa. Treatment with cysteamine significantly decreased senescence and alveolar space enlargement in chronic-CS-exposed mice (p<0.0001) in addition to chronic-CS-exposed mice (p<0.0001) infected with Pa (Fig 5A and 5C). We also observed significant increase in alveolar space and senescence in lungs harvested from mice exposed to sub-chronic-CS (p<0.0001) and old mice (p<0.0001) in comparison to air-exposed mice (Fig 5D and 5E). The old mice group served as a positive control, and displayed greater (p<0.0001) senescence and alveolar space enlargement than the increase exhibited by sub-chronic-CS-exposed mice (p<0.0001) in
comparison to air-exposure. Treatment with cysteamine significantly decreased (p<0.0001) senescence and alveolar space enlargement in sub-chronic-CS-exposed mice (Fig 5D and 5E). In comparing the impact of CS-exposures upon alveolar space enlargement and senescence, chronic-CS-exposure displayed significantly (p<0.05) more alveolar space enlargement and senescence in comparison to sub-chronic-exposure (Fig 5A/C vs 5D/E), as anticipated. The results of these experiments demonstrate that accumulation of aggresome-bodies accompanies senescent changes in lung tissue morphology dependent upon levels of CS exposure, Pa infection and/or aging. Moreover, we demonstrate cysteamine’s potential to control CS-induced aggresome-formation, senescence and emphysema pathogenesis.

Cysteamine rescues CS-induced autophagy-impairment in BALF inflammatory cells

To determine whether chronic-CS-exposure can activate inflammatory cells via autophagy impairment, we performed flow cytometry analysis of bronchoalveolar lavage fluid (BALF) harvested from mice exposed to chronic-CS and/or treated with cysteamine. Infection of mice with Pa served as a positive control for the analysis of inflammatory cells. In comparison to air-exposed BALF, substantial increase in the number of CD4+ T-cells (cluster of differentiation 4, T-cell marker) and Mac-1+ macrophages (macrophage 1 antigen, macrophage marker) was observed in chronic-CS-exposed and/or Pa infected BALF. Cysteamine treatment reduced levels of CD4+ T-cells and Mac-1+ macrophages (Fig 6A), indicating cysteamine’s potential to control CS-induced inflammatory responses. Similarly, we analyzed the levels of ubiquitin and p62 in BALF inflammatory cell population and found substantial increase in the number
of cells showing co-expression of ubiquitinated-proteins and the aberrant-autophagy marker p62, in chronic-CS-exposed and/or *Pa* infected mice in comparison to air-exposed group. Cysteamine treatment attenuates the co-expression of ubiquitinated-proteins and p62 in chronic-CS-exposed and/or *Pa* infected mice (Fig 6B). We also analyzed BALF harvested from mice exposed to sub-chronic-CS and/or treated with cysteamine. Increase in levels of CD4+ T-cells and Mac-1+ macrophages were observed in sub-chronic-CS-exposed mice in comparison to air-exposed mice. No increase in activated CD4+ T-cells was observed in the old mice group in comparison to air-exposed mice, indicating that old mice possess a weakened immune response due to aging. Cysteamine treatment reduced levels of sub-chronic-CS induced CD4+ T-cells and Mac-1+ macrophages in comparison to sub-chronic-CS-exposed mice (Fig 6C).

Moreover, changes in pro-inflammatory cytokines, IL-6 and IL-1β were analyzed in these experimental groups to verify these findings (Fig 8).

**Cysteamine treatment resuces CS-induced inflammatory cytokine levels**

After quantifying CS-exposure-induced activation of immune cells, we analyzed whether CS exposure can increase levels of pro-inflammatory cytokines, including IL-6 and IL-1β. We performed Enzyme-Linked Immunosorbant Assay (ELISA) using supernatants collected from BALF harvested from mice exposed to chronic-CS and/or treated with cysteamine. Infection of mice with *Pa* served as a positive control for the analysis of inflammatory cytokines. Significant increase in levels of IL-6 (p<0.0001) was observe in chronic-CS-exposed mice in comparison to air-exposed mice. Treatment with cysteamine alleviated (p<0.0001) IL-6 accumulation in comparison to chronic-CS-exposed mice (Fig 8A).
To determine the effect of cysteamine upon *Pa* infection, BALF collected from mice exposed to air, treated with cysteamine and/or infected with *Pa* were analyzed for levels of IL-6. Analysis showed an extremely significant (*p*<0.0001) increase in IL-6 levels in comparison to air-exposed mice. Cysteamine treatment of chronic-CS-exposed mice significantly (*p*<0.0001) alleviated levels of IL-6 in comparison to *Pa*-infected mice. Air-exposed or cysteamine-treated mice displayed no significant change in IL-6 levels (Fig 8B), as anticipated. Chronic-CS-exposed mice infected with *Pa* exhibited a significant increase (*p*<0.001) in IL-6 levels in comparison to air-exposed mice that was significantly (*p*<0.001) alleviated upon treatment with cysteamine (Fig 8C). ELISA analysis was also used to quantify changes in IL-1β levels in BALF supernatants collected from chronic-CS-exposed mice. Significant (*p*<0.0001) increase in inflammatory IL-1β levels was observed in chronic-CS-exposed mice in comparison to the air-exposed group. Treatment of chronic-CS-exposed mice with cysteamine significantly (*p*<0.0001) alleviated levels of IL-1β in comparison to chronic-CS-exposed mice (Fig 8D).

Murine infection (i.t.) with *Pa* alone exhibited significantly (*p*<0.001) increased IL-1β levels in comparison in un-infected mice. *Pa*-infected mice treated with cysteamine displayed significant (*p*<0.001) reduction in IL-1β levels that resembled un-infected mice (Fig 8E). However, chronic-CS-exposed mice infected with *Pa* displayed significant increase (*p*<0.0001) in IL-1β levels in comparison to air-exposed mice. Mice exposed to chronic-CS, infected with *Pa* and treated with cysteamine displayed reduction (*p*<0.0001) in IL-1β levels in comparison to mice exposed to chronic-CS and infected with *Pa* (Fig 8F). We also analyzed the supernatants from BALF harvested sub-
chronic-CS-exposed mice and old mice (positive control) to determine IL-1β levels. As shown in Fig 8D, mice exposed to sub-chronic-CS exhibited significant increase (p<0.01) in IL-1β levels in comparison to air-exposed mice; the increased levels of IL-1β were reduced (p<0.01) upon treatment with cysteamine. Moreover, old mice displayed increase (p<0.01) in IL-1β levels (Fig 8G) that suggests IL-1β’s potential contribution in age-related immune modulation. In summary, the results of these experiments verify the therapeutic potential of cysteamine in reducing levels of IL-6 and IL-1β, key mediators of chronic- and sub-chronic-CS and/or age-related lung inflammation.

Next, we performed a bacterial survival assay to test the viability of *Pa* in infected lungs of mice exposed to air or chronic-CS. Significant increase in bacterial load (colony forming units, CFU) was observed in *Pa*-infected (p<0.0001) and/or chronic-CS-exposed mice (p<0.0001) in comparison to air-exposed mice. Treatment with cysteamine attenuated bacterial load in *Pa*-infected (p<0.001) and/or chronic-CS-exposed mice (p<0.0001; Fig 8H) in comparison to *Pa*-infected and/or chronic-CS-exposed group, confirming the antibacterial property of cysteamine that may be attributed to autophagy induction. The activation of Mac1+macrophages, CD4+ T-cells and cytokines led us to inquire about age-related neutrophil activity in elderly lungs. Activity of neutrophil granules was measured via Myeloperoxidase (MPO) activity assay in plasma samples collected from 6-weeks-young and 19-months-older C57BL/6 mice. We found significant increase in MPO activity in old mice in comparison to the young mice (p<0.05), as anticipated, verifying age-related changes in immune function (Fig 8I). To determine age-related apoptotic changes, we measured caspase-3/7 activity in the old and younger mice groups and found that old mice exhibited significant (p=0.01)
increase in caspase-3/7 activity in comparison to young mice (Fig 8J), suggesting age-
dependent changes in cell survival.

**Cysteamine attenuates cigarette smoke (CS)- and age-related aggresome formation.**

To confirm our western blot results demonstrating autophagy impairment and
aggresome-formation in chronic-CS-exposed mouse-lung lysates (Fig 9A), we used
ubiquitin and p62 antibodies to immunostain lung-tissue sections. We observed
significant (p<0.01) increase in levels of ubiquitinated proteins, p62 and VCP within peri-
nuclear aggresome-bodies upon exposure to chronic-CS in comparison to air-exposed
or cysteamine-treated lung sections. In comparison to chronic-CS-exposed mice, we
observed substantial attenuation of ubiquitinated poteins, p62 and VCP staining upon
treatment of CS-exposed mice with cysteamine (Fig 9A, p<0.05). We also
immunostained lung sections obtained from mice exposed to sub-chronic-CS and the
older mice group for the detection of ubiquitinated proteins and p62. We found
significant (p<0.01) increase in ubiquitin and p62 co-staining in sub-chronic-CS exposed
and old (~12-months) mice lungs in comparison to air exposure. Cysteamine treatment
(i.p.) of mice exposed to sub-chronic CS decreased levels of ubiquitinated-proteins and
p62 in comparison to mice exposed to sub-chronic-CS (Fig 9B, p<0.05). To test the
potential of CS to induce cellular senescence, lung tissues were immunostained for
Sirt1. We found that cysteamine treatment in mice exposed to sub-chronic-CS results in
substantial (p<0.05) recovery of Sirt1 protein to levels seen in control and cysteamine
treatment groups. No Sirt1 staining was observed in sub-chronic-CS-exposed or old
mice groups (Fig 9B), further supporting the potential of cysteamine to alleviate both
aggresome-formation and cellular senescence. To determine whether aggresome formation increases with age, lung sections from young (6-months) and older/aged (19-months) C57BL/6 mice were immunostained for ubiquitin and VCP. Aged/older mice displayed substantial (p<0.05) increase in perinuclear accumulation of ubiquitin and VCP in aggresome-bodies (Fig 9C), indicating increased aggresome-formation in aged mice. These data suggest that aggresome-bodies play a critical role in initiating cellular senescence and can explain CS-induced respiratory failure and the resulting mortality seen in elderly subjects.

Discussion

The first- and second- leading cause of COPD-emphysema pathogenesis is cigarette smoke (CS) exposure and aging. Albeit several decades of research on CS-induced COPD-emphysema, the third leading cause of death in the USA, it is not apparent whether CS exposure and aging involve a common mechanism for lung disease pathogenesis. To address this gap in knowledge, we designed this study to identify the central mechanism modulated by both CS and aging that results in COPD-emphysema pathogenesis. Based on recent studies from our group and others, we predicted that CS exposure and aging could impair proteostasis. These recent studies suggest that CS exposure induces accumulation of ubiquitinated proteins in COPD-emphysema lungs, indicating proteostasis impairment (8, 16, 44, 50). Expanding upon this observation, we investigated whether CS-induced proteasomal/autophagy dysfunction leads to perinuclear accumulation of ubiquitinated proteins in aggresome-bodies. We found that
levels of these bodies decreased upon treatment with an autophagy inducing drug, carbamezapine (44) suggesting that CS exposure may impair both proteasomal and autophagy activities, leading to unique alveolar pathology, aggresomes that may persuade emphysema pathogenesis. In the current study, we not only aimed to verify if CS induced aggresome-formation mediates emphysema pathogenesis but also if age-related proteostasis/autophagy decline mediated aggresome-formation is a central mechanism for accelerated lung aging and emphysema pathogenesis (Fig 7).

Previous studies suggested that chronic cigarette smoke contributes to cellular senescence and accelerated lung aging (6, 16, 21, 44). Therefore, we first investigated whether cigarette smoke (CS) induced proteostasis- and autophagy- impairment leads to cellular senescence and accelerated lung aging. Our group and others have recently found that CS exposure leads to proteostasis dysfunction resulting in accumulation of ubiquitinated-proteins and impaired autophagy marker, p62, in aggresome-bodies as a potential mechanism to initiate COPD-emphysema pathogenesis (4, 33, 44, 53). To verify these findings, we first exposed human bronchial epithelial cells (Beas2b) to cigarette smoke extract (CSE) and found accumulation of ubiquitinated-proteins and p62 in the cellular insoluble-protein fractions. The protein expression of p53 was either decreased or abrogated, potentially leading to oxidative stress and initiating cell cycle arrest and apoptosis upon CS exposure (35, 38). Next, we evaluated the impact of CSE upon the aging marker, sirtuin1 (Sirt1) (18). We found that Sirt1 protein levels were decreased upon CS exposure, further strengthening earlier observations (20, 37, 54) and suggesting that CS-mediated oxidative stress can down regulate Sirt1 levels as a mechanism to accelerate lung aging. This study for the first time verified that presence
and levels of aggresome pathology in COPD subjects is dependent upon cigarette
smoke exposure and emphysema severity that statistically correlates with alveolar
senescence.

Next, we used transmission electron microscopy (TEM) (44) to verify our findings and
observed that CS induced accumulation of aggresome-bodies around the perinuclear
region. Moreover, the loss of cellular and nuclear membrane is apparent, the common
features of apoptotic cell death. To validate our in vitro experimental data, we utilized an
in vivo experimental setup where C57BL6/7 mice were exposed to chronic- or sub-
chronic- CS. As anticipated, chronic/sub-chronic-CS exposed or aged control group
mice showed increased accumulation of ubiquitinated-proteins, p62 and VCP in
insoluble fractions, suggesting the role of autophagy impairment in CS and age-related
aggresome-formation. The number of aggresome-bodies was strictly dependent upon
levels of CS exposure. Moreover, aged mice showed lower levels of Sirt1 in soluble
fractions in comparison to air-exposed mice, suggesting that CS- and age- related
proteostasis/autophagy impairment not only results in aggresome-formation but also
affects cellular responses such as senescence (6, 22, 23, 27, 33, 42, 44, 50, 52).

Bacterial infections are common and known to cause intermittent or stable
exacerbations of COPD leading to progressive decline of lung function. Despite the
availability of antibiotic treatments they can only prevent 40% of pulmonary
exacerbations and lead to drug resistance, resulting in failure to recover or revert
disease from chronic stages (34, 39, 40). The chronic infections of P. aeruginosa in
COPD, resemble patterns observed in cystic fibrosis (32). The mechanism for recurrent
pulmonary exacerbations in these diseases warrants further investigation although we
anticipate the role of CS mediated autophagy impairment in this process. We observed that *P. aeruginosa* infection augments accumulation of ubiquitinated proteins, suggesting that even *P. aeruginosa* infection can induce autophagy-impairment. Nonetheless, in COPD subjects CS or aging impairs autophagy that is then anticipated to induce pulmonary infections due to lack of autophagy clearance of bacteria and/or viruses. Thus, having identified the role of CS and age-related autophagy impairment in alveolar senescence, emphysema and pulmonary exacerbations, we aimed to validate our observations using an autophagy-inducing drug.

The US Food and Drug administration (FDA) recently approved cysteamine drugs such as Procysbi and Linovex for treatment of other clinical conditions. These cysteamine-based drugs possess anti-bacterial-mucoactive and antioxidant properties that inhibit oxidative stress and restore autophagy, suggesting their potential in treating COPD-emphysema. The autophagy-inducing effect of cysteamine/cystamine is known to involve its ability to not only control ROS-activity but also restoring BECN1 (beclin-1) and p62 (sequestosome-1) levels (11, 12, 29, 30). Hence, although classical antioxidants can be helpful in restoring autophagy but may not be sufficient in chronic stages where inhibiting Ub-p62 accumulation is required. Moreover, cysteamine possess mucolytic properties that can facilitate both its delivery and therapeutic effect in chronic obstructive lung diseases. Hence, in this study, we questioned whether cysteamine would rescue CS-impaired proteostasis/autophagy, the resulting cellular senescence/apoptosis and emphysema progression. We found that cysteamine-induced autophagy can reduce aggresome-formation, CS-induced alveolar senescence/death and emphysema progression indicating its potential for treating
COPD subjects. We anticipate that cysteamine’s anti-oxidant, autophagy inducing and anti-bactericidal properties may be contributing to the reversal of the disease, which warrants further investigation, as autophagy induction by carbamezapine in our prior study did not completely reverse this disease (44). In fact, cysteamine can clear CS-induced *P. aeruginosa* infection by inducing autophagy. Hence, we anticipate that autophagy induction by cysteamine or other drugs could be viable as a therapeutic strategy for treating cigarette-smoke or age-related changes leading to recurrent pulmonary exacerbations and lung function decline.

When gaseous and particulate CS come into contact with the mucosal surface, the immune system induces secretion of inflammatory mediators that promote recruitment of immune cells and inflammation (28). Therefore, we next evaluated whether chronic or sub-chronic CS exposure and aging impacts host immunity by analyzing inflammatory cells and secretion of cytokines by these cells. Flow cytometry analysis of bronchoalveolar lavage (BALF) for T-lymphocytes and macrophages showed that CS induced influx of macrophages (Mac-1) and activation of CD4+ T-cells along with increased co-localization of ubiquitin and p62 in cells isolated from CS-exposed mice (Fig 5B). In addition, an increase in number of Mac1+macrophages and CD4+ T-cells in BALF cells was observed upon CS exposure, and the administration of cysteamine reduced this increase. Furthermore, ELISA analysis showed an increase in IL-6 and IL-1β levels upon CS exposure, as anticipated (1, 9, 10, 13, 15, 19, 26), and cysteamine treatment attenuated CS-induced IL-6 and IL-1β levels. The old control group showed increased inflammatory responses similar to CS exposed mice. We observed age-related increase in neutrophil myeloperoxidase activity and caspase-3/7 related
apoptotic cell death that is anticipated to further augment the effect of smoke exposure.

Our data indicates that CS-impaired autophagy serves as a mechanism for severe emphysema progression by inducing aggresome-formation that impact innate and adaptive immune responses leading to chronic inflammation. CS exposure is known to augment \textit{P. aeruginosa} biofilm formation, suggesting that smoking suppresses the activity of immune cells and delays clearance of microbes from lungs (14).

In summary, we observed that chronic and sub-chronic CS exposure induced autophagy-impairment and the resulting aggresome-formation initiates both inflammatory signaling and age-related changes such as senescence that can lead to the decline in lung function seen in severe COPD-emphysema subjects. We anticipate that the severity of COPD-emphysema can be predicted based upon quantitative levels of novel prognostic biomarker, aggresomes. Moreover, our data suggests an application of novel prognosis-based intervention strategy where autophagy-inducing drug such as cysteamine can be used to clear aggresome-bodies and resulting emphysema. We aim to further validate these findings in BALF samples obtained from COPD-emphysema subjects and anticipate that flow cytometry/microscopy based aggresome quantification-assay developed in this study can help with development of proposed prognosis based intervention strategy.

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Figure Legends

**Fig 1. Tobacco/cigarette smoking induces aggresome-formation and emphysema in COPD subjects.** (A, C) ProteoStat® aggresome-body dye based staining (red) of human lung-tissue sections collected from non-emphysema (GOLD 0) and COPD subjects (GOLD I-IV) with emphysema. Nuclei are stained with Hoechst (Blue). The data shows an increase in number of aggresome-bodies that correlates with the severity of emphysema (GOLD I-III) in COPD subjects in comparison to non-emphysema control (GOLD 0) lung sections. However, lung sections collected from severe emphysema-COPD (GOLD IV) subjects exhibit fewer aggresome/autophagy-bodies in comparison to less severe emphysema (GOLD III) but it was still higher than control smoker or non-smoker subjects and was similar to GOLD II. (B, D) Sudan B Black staining of human lung-tissue sections collected from smoker and non-smoker emphysema (GOLD I-IV, B) and non-emphysema (GOLD 0, D) show that smoking induces senescence in both control and emphysema subjects. Moreover, significant increase is seen in levels of senescent alveolar cells depending upon the severity of emphysema (GOLD I-IV). Significant tissue destruction is apparent in GOLD IV subjects that is anticipated to be a consequence of irreversible senescence induced by prolonged smoke exposure. (E, F) Moreover, the aggresome-formation process is clearly smoking-exposure-dependent, as non-smoker samples from GOLD-0 or GOLD-I-IV did not show significantly elevated levels of aggresome-bodies in comparison to COPD lungs (GOLD I-IV) of smokers. Scale bar 100 µm.

**Fig 2. Cigarette Smoke (CS) exposure induced autophagy-impairment and aggresome-formation activates cellular apoptosis and senescence.** (A)
Transmission electron microscopy (TEM) images of Beas2b cells exposed to various concentrations of cigarette smoke extract (CSE) for 6 hrs. (I) Air-exposed control cells displaying the nucleus (N) and nuclear membrane (NM) (5598x magnification); (II) Beas2b cells exposed to 10% CSE show aggresome/autophagy bodies (AB) proximal to the nuclear membrane (NM) (10171x magnification). CSE treatment also shows some membrane disintegration along with the thinning of the nuclear membrane (NM) and peri-nuclear accumulation of aggresome/autophagy bodies (AB). III-IV represents higher magnification (46422x, 84190x) of I-II. The thin white arrow indicates the nuclear membrane (NM) and thick white arrows indicate the localization of aggresome bodies (AB). (B) Western blot analysis illustrating the levels of ubiquitinated proteins, sirtuin1 (SIRT1, senescence marker) and p53 (senescence/apoptosis mediator) in soluble protein fractions of control Beas2b cells and those treated with 250 µM cysteamine and/or 10% cigarette smoke extracts (CSE) for 6 hrs. Substantial accumulation of ubiquitinated protein was observed in the insoluble protein fraction of Beas2b cells treated with 10% CSE, suggesting that ubiquitinated proteins are translocated from soluble to insoluble protein fractions upon 6 hrs of 10% CSE exposure. Cysteamine attenuates the accumulation of ubiquitinated protein in insoluble fraction and partially restores normal SIRT1 and p53 levels in the soluble protein fraction. β-actin levels of the soluble protein fraction indicate equal loading of protein. (C) Senescence-associated beta-galactosidase (SA-β-gal) activity in Beas2b cells treated with cysteamine (6 hrs) and/or exposed to 10% CSE overnight was measured using a senescence cells histochemical staining kit. Senescent cells were visualized and identified via blue stain (black arrows), indicating positive SA-β-gal activity. Data are shown as mean ± SEM (n
3) of percentage change in SA-β-gal-positive blue senescent cells in comparison to air-exposed control (bottom panel). ***p<0.001 and ****p<0.0001.

**Fig 3. Cysteamine rescues CSE induced autophagy-impairment and aggresome-formation.** (A) Beas2b cells were transduced with Premo™ autophagy tandem sensor LC3B-RFP-GFP construct. After 24 hrs post-transfection, Beas2b cells were preincubated with 250 µM cysteamine and/or treated with 10% CSE for 6 hrs. Flow cytometry results showing significant increase in GFP fluorescence (autophagosomes), RFP fluorescence (autolysosomes) and their co-localization (autophagosomes) in comparison to air-exposed cells. Pre-incubation with cysteamine reduced GFP fluorescence, RFP fluorescence and their co-localization. The x-axis shows the log scale of LC3-GFP and y-axis shows the log scale of LC3-RFP fluorescence signals. The data represent the average (mean ± SEM) of three replicates (right panel). **p<0.01 and ***p<0.001 and ****p<0.0001. (B) Beas2b cells were pre/treated with chloroquine (CQ, 90 µM, 12 hrs) or cysteamine (Cys, 250 µM, 6 hrs) and/or treated with 10% CSE for 6 hrs. Fluorescence microscopy images showing autolysosomes (marked by the presence of red punctas, second column), autophagosomes (marked by the presence of green punctas, third column) and autophagosomes (marked by the co-localization of red and green puncta as yellow puncta bodies, fourth column). Scale bars, 56 µM. Fluorescence images were used to count the number of co-localized RFP-LC3B and GFP-LC3B punctas per image (yellow, fourth column). The data represent mean ± SEM of four replicates and analysis is shown in the bottom panel. Data suggest that CSE and CQ impair autophagy while cysteamine treatment restores CSE-impaired autophagy. *p<0.05, **p<0.01 and ***p<0.001. (C) Beas2b cells were treated with cysteamine (250
µM) and/or 10% CSE for 6 hrs. Cells were stained with ProteoStat® aggresome dye (red) and Hoechst nuclei dye (blue). MG132 (5 µM; 12 hrs) treatment was used as a positive control for this experiment. Images were captured by ZOE™ Fluorescent Cell Imager (Bio rad) and quantitative analysis of aggresome-bodies in each group is shown in bottom panel. Data suggest that CSE and MG132 induced aggresome/autophagy-bodies while cysteamine treatment restored CSE-impaired autophagy, as seen by decrease in the number of aggresome/autophagy bodies. Scale bars, 56 μM. (D, E) Statistical analysis of microscopy data in B and C as mean ± SEM (n = 3).

Fig 4. Chronic and sub-chronic CS exposure induces aggresome formation and age-related changes in murine lungs. (A) Western blot analysis illustrating the levels of ubiquitinated protein, the impaired autophagy marker p62, and VCP in soluble and insoluble protein fractions of mouse lung lysates collected from air- or chronic-CS-exposed C57BL/6 mice that were intraperitoneally (i.p.) injected with a daily dose of 0.01 M cysteamine for 10 days and/or infected intranasally (i.n.) with PA01-GFP (2x10^6) for 5 days. Expression of the above proteins was normalized to β-actin loading control. (B) Western blot analysis illustrating the levels of ubiquitinated protein, the impaired autophagy marker p62, and VCP and Sirt1 in soluble and insoluble protein fractions of mouse-lung lysates collected from C57BL/6 mice exposed to air or sub-chronic-CS and/or intraperitoneally (i.p.) injected with a daily dose of 0.01 M cysteamine for 10 days. Expression of the above proteins was normalized to β-actin loading control. The old mice (~12 months) were used as a positive control. Densitometry analysis (of B) is shown in right panel.
**Fig. 5 Cysteamine rescues CS-induced aggresome-formation, senescence and alveolar space enlargement.** (A, B, C) ProteoStat®-RED aggresome-dye was used for staining of lung sections from C57BL/6 mice exposed to air or chronic-CS, infected with PA01-GFP and/or treated with cysteamine. The lung sections from mice exposed to chronic-CS (or CS and *Pa*) display increased number of aggresome-bodies in comparison to air-exposed lungs. The chronic-CS-exposed mice (or CS and *Pa*) treated with 0.01 M cysteamine for 10 days exhibit reduction in CS-induced aggresome-bodies in comparison to chronic-CS-exposed mice (B). Nuclei were stained using Hoechst (blue) dye. Scale bars, 100 µM. Sudan B Black (SBB) and Hematoxylin and Eosin (H&E) staining of longitudinal lung sections show senescence and alveolar space enlargement in lung sections collected from chronic-CS-exposed mice in comparison with lung sections of room-air-exposed controls. The lung sections of mice treated with cysteamine display mitigation of chronic-CS-induced senescence and alveolar space enlargement. The lung sections from PA01-GFP (2x10^6) infected mice were used as a positive control for pulmonary exacerbation. The lung sections from chronic-CS-exposed mice infected with *Pa* display increased senescence and alveolar space diameter in comparison to samples collected from mice exposed to chronic-CS or *Pa* alone. Moreover, lung sections from chronic-CS-exposed mice infected with *Pa* and treated with 0.01 M cysteamine exhibit reduced senescence and alveolar space enlargement in comparison to samples collected from chronic-CS-exposed mice infected with *Pa*. (D, E, F) ProteoStat®-RED dye was used for aggresome staining of lung sections from C57BL/6 mice exposed to air or sub-chronic CS and/or treated with cysteamine. The old mice (~12-months) were used as a control group. The lung
sections from mice exposed to sub-chronic CS or old mice display an increase in number of aggresome-bodies in comparison to air-exposed mice. The lung sections of sub-chronic-CS-exposed mice treated with 0.01 M cysteamine for 10 days exhibit reduction in CS-induced aggresome-bodies. Sudan B Black (SBB) and Hematoxylin and Eosin (H&E) staining of longitudinal lung sections show senescence and alveolar space enlargement in lung sections collected from sub-chronic-CS-exposed and old mice in comparison with lung sections of room-air-exposed controls. The lung sections of mice treated with cysteamine display mitigation of sub-chronic-CS induced senescence and alveolar space enlargement. The lung sections from old mice were used as a positive control for senescence and alveolar space enlargement. The data analysis of microscopy figures in left panel (A, D) is shown in the right panel (B, C, E, and F). Nuclei are stained using Hoechst (blue) dye. Scale bars, 100 µM. **p<0.01 and ***p<0.001.

**Fig. 6 Chrono**

**Chronic or sub-chronic CS exposure impairs autophagy/proteostasis and induces inflammation in bronchoalveolar lavage fluid (BALF) cells.** (A) Flow cytometry analysis of CD4 (Cluster of differentiation 4, T-cell marker) and Mac-1 (macrophage 1 antigen, macrophage marker) positive cells in BALF harvested from C57BL/6 mice exposed to chronic-CS and/or infected with PA01-GFP. Significant increase in CD4⁺ T-cells and Mac1⁺macrophages is displayed in BALF samples collected from mice exposed to chronic-CS and/or PA01-GFP infection. BALF cells from mice treated with 0.01 M cysteamine for 10 days exhibit reduction in the number of CD4⁺ T-cells and Mac1⁺macrophages in comparison to BALF collected from mice exposed to chronic-CS. (B) Flow cytometry analysis of BALF harvested from C57BL/6
mice exposed to room-air or chronic-CS and/or infected with Pa. BALF collected from mice exposed to chronic-CS or Pa displayed increased co-expression of ubiquitinated-protein (Ub) and the impaired-autophagy marker, p62. In addition, BALF collected from chronic-CS-exposed or Pa infected mice treated with 0.01 M cysteamine for 10 days attenuated induced co-expression of Ub and p62. (C) Flow cytometry analysis of BALF cells harvested from old (~12-months) and room-air or sub-chronic-CS-exposed adult (5-months) C57BL/6 mice. BALF collected from sub-chronic-CS exposed mice displays an increase in number of CD4⁺-T cells and Mac1⁺macrophages in comparison to room-air exposed control mice. BALF collected from sub-chronic-CS exposed C57BL/6 mice treated with cysteamine exhibits decreased number of sub-chronic-CS induced CD4⁺ T-cells and Mac1⁺macrophages in comparison to sub-chronic-CS exposed murine BALF cells. Moreover, BALF cells harvested from old mice show an increase in the number of Mac1⁺macrophages while no change was observed in CD4⁺T-cells in BALF cells collected from room-air exposed control.

**Fig 7. Schema describing tobacco-smoke-mediated proteostasis/autophagy decline as a mechanism for accelerated lung aging.** Tobacco/cigarette smoke (CS) exposure and aging can induce proteostasis and autophagy impairment, serving as a central mechanism to induce inflammatory-oxidative stress. Chronic-CS-exposure mediated steep decline in proteostasis/autophagy accelerates inflammatory-oxidative stress, apoptosis and senescence, serving as a mechanism for accelerated lung aging and emphysema progression.

**Fig 8. Autophagy induction by cysteamine reduces CS-induced inflammatory responses.** (A) Supernatants separated from BALF harvested from mice exposed to
chronic-CS and/or treated with cysteamine. Chronic CS-exposed mice showed
significant (****p<0.0001) increase in inflammatory cytokine, IL-6 levels. Treatment with
cysteamine restored (****p<0.0001) IL-6 levels as observed in air-exposed or
cysteamine treated mice. (B) Analysis of BALF samples from mice infected with PA01-
GFP showed extremely significant (****p<0.0001) increase in IL-6 levels and
cysteamine treatment significantly (****p<0.0001) alleviated IL-6 levels as compared to
PA01-GFP infected mice. No significant change in IL-6 levels was observed in air-
exposed or cysteamine treated mice. (C) The chronic CS-exposed mice infected with
PA-01-GFP induced increase (**p<0.001) in IL-6 levels which is significantly reduced
(**p<0.001) upon treatment with cysteamine. (D) In addition to IL-6 levels, significant
(****p<0.0001) increase in inflammatory IL-1β was observed in chronic CS-exposed
mice. Treatment with cysteamine reduced the CS induced IL-1β levels significantly
(****p<0.0001) in comparison to air-exposed control levels. (E) Infection with PA01-GFP
significantly (**p<0.001) increased IL-1β levels and that were restored (**p<0.001)
back to control levels with cysteamine treatment. (F) Cysteamine treatment reduced
(****p<0.0001) IL-1β levels in mice exposed to chronic-CS that showed significant
(****p<0.0001) increase in IL-1β. (G) Significant (**p<0.01) increase in inflammatory
cytokine, IL-1β was observed in mice exposed too sub-chronic CS. Levels of IL-1β were
reduced (**p<0.01) upon treatment with cysteamine. Old mice (~12-months) also
showed slight elevation (**p<0.01) in IL-1β levels. (H) Chronic CS-exposed mice were
infected with PA01-GFP (2x10^6) and/or treated with cysteamine followed by bacterial
survival assay using the lung homogenate. Significantly (~4 fold) higher bacterial load
(colony forming units, CFU) was observed in mice exposed to chronic-CS
(**p<0.0001) and/or infected with PA01-GFP mice (**p<0.0001) in comparison to the room-air controls (n=4). Treatment with cysteamine significantly (~ 2 fold) reduced the bacterial load in PA01-GFP (**p<0.001) and chronic CS exposed mice (**p<0.0001).

(I) Neutrophil activity was measured by Myeloperoxidase (MPO) assay in plasma samples obtained from 6-weeks young and 19-months older C57L/6 mice. We found significant (*p<0.028, n=3) increases in MPO activity in aged (19-months older) mice in comparison to adult (young) mice, indicating age-related basal changes in MPO activity.

(J) Moreover, we found significant constitutive increases (*p=0.01, n=3) in caspase-3/7 activity in aged (older) mice relative to adult (young) mice, thus indicating age-related susceptibility to mortality that can be exacerbated by chronic smoking and/or infection.

Fig 9. Cysteamine attenuates cigarette smoke (CS)- and age-related aggresome formation. (A) Fluorescence microscopic analysis for detection of ubiquitinated protein, p62 and VCP in lung sections from C57BL/6 mice exposed to air or chronic CS and/or treated with a daily dose of 0.01 M cysteamine for 10 days and intranasally infected with PA01-GFP (2x10^6) for 5 days as indicated. Samples collected from mice exposed to chronic CS and/or infected with PA01-GFP exhibit peri-nuclear localization of ubiquitinated protein (red), p62 (green) and VCP (green) within aggresome bodies. Nuclei (blue) are stained with Hoechst. Scale bar, 100 µm. (B) Fluorescence microscopic analysis for detection of ubiquitinated protein (red), p62 (green), and Sirt1 (red) in lung sections from C57BL/6 mice exposed to air or sub-chronic CS (2-month old + 3-months exposure) and/or treated with a daily dose of 0.01 M cysteamine for 10 days. Lung sections were also collected from old mice (~12-months) as a positive
control. The mice exposed to sub-chronic CS exhibit perinuclear co-localization of ubiquitinated protein (red) and p62 (green) within aggresome/autophagy bodies. The lung sections collected from old mice also display autophagy-bodies and substantial (p<0.05) decrease in Sirt1 expression similar to CS exposure. Nuclei (blue) are stained with Hoechst dye. Scale bar, 100 µm. (C) Fluorescence microscopic analysis for detection of ubiquitin (red) and VCP (green) in lung sections collected from adult (6-months, yng) and aged/older (19-months, old) C57BL/6 mice. In comparison to the adult mice, lungs collected from the aged/older mice display an increase in peri-nuclear ubiquitin accumulation and VCP co-localization; this is indicative of an age-related increase in aggresome-formation. Nuclei (blue) are stained with Hoechst. Scale bar, 100 µm.
A

Low magnification

Control

10% CSE

High magnification

Control

10% CSE

B

SOLUBLE

Ubiquitin

p53

Sirt1

β-actin

INSOLUBLE

Ubiquitin

C

SA-b-gal positive cells

Control

Cys

10% CSE

10% CSE+Cys

p<0.001

p<0.0001