Discoveries

ORIGINAL RESEARCH COMMUNICATION

Master Autophagy Regulator Transcription Factor EB Regulates Cigarette Smoke-Induced Autophagy Impairment and Chronic Obstructive Pulmonary Disease–Emphysema Pathogenesis

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Abstract

Aims: Recent studies have shown that cigarette smoke (CS)-induced oxidative stress impairs autophagy, resulting in aggresome-formation that correlates with severity of chronic obstructive pulmonary disease (COPD)-emphysema, although the specific step in autophagy pathway that is impaired is unknown. Hence, in this study, we aimed to evaluate the role of master autophagy transcription factor EB (TFEB) in CS-induced COPD-emphysema pathogenesis.

Results: We first observed that TFEB accumulates in perinuclear spaces as aggresome-bodies in COPD lung tissues of tobacco smokers and severe emphysema subjects, compared with non-emphysema or nonsmoker controls. Next, Beas2b cells and C57BL/6 mice were exposed to either cigarette smoke extract (CSE) or subchronic-CS (sc-CS), followed by treatment with potent TFEB-inducing drug, gemfibrozil (GEM, or fisetin as an alternate), to experimentally verify the role of TFEB in COPD. Our *in vitro* results indicate that GEM/fisetin-mediated TFEB induction significantly ($p < 0.05$) decreases CSE-induced autophagy-impairment (Ub/LC3B reporter and autophagy flux assay) and resulting aggresome-formation (Ub/p62 coexpression/accumulation; immunoblotting and staining) by controlling reactive oxygen species (ROS) activity. Intriguingly, we observed that CS induces TFEB accumulation in the insoluble protein fractions of Beas2b cells, which shows a partial rescue with GEM treatment. Moreover, TFEB knockdown induces oxidative stress, autophagy-impairment, and senescence, which can all be mitigated by GEM-mediated TFEB induction. Finally, *in vivo* studies were used to verify that CS-induced autophagy-impairment (increased Ub, p62, and valosin-containing protein in the insoluble protein fractions of lung/cell lysates), inflammation (interleukin-6 [IL-6] levels in bronchoalveolar lavage fluid and iNOS expression in lung sections), apoptosis (caspase-3/7), and resulting emphysema (hematoxylin and eosin [H&E]) can be controlled by GEM-mediated TFEB induction ($p < 0.05$).

Innovation: CS exposure impairs autophagy in COPD-emphysema by inducing perinuclear localization of master autophagy regulator, TFEB, to aggresome-bodies.

Conclusion: TFEB-inducing drug(s) can control CS-induced TFEB/autophagy-impairment and COPD-emphysema pathogenesis. *Antioxid. Redox Signal.* 27, 150–167.

Keywords: TFEB, autophagy, COPD, emphysema, cigarette smoke, tobacco, ROS, oxidative stress

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Innovation

Cigarette smoke-induced autophagy-impairment mediates chronic obstructive pulmonary disease (COPD)–emphysema progression, but underlying mechanisms are unclear. Our study identifies cigarette smoke-induced accumulation of transcription factor EB (TFEB) in perinuclear aggresome-bodies as a novel mechanism for TFEB/autophagy-impairment. Moreover, our data demonstrate the potential of prognosis-based intervention strategy for COPD-emphysema.

Introduction

EXPOSURE TO FIRST- and/or secondhand tobacco smoke is the primary risk factor in the development and progression of chronic obstructive pulmonary disease (COPD) in the United States, which is a preventable, but life-threatening, lung condition characterized by the presence of chronic bronchitis, bronchiectasis, and/or emphysema (30, 42). The deleterious effects of cigarette smoke (CS) exposure are multifarious as it impacts several key cellular homeostatic processes, such as cell growth, protein processing, autophagy, and antibacterial immune defense mechanisms (19, 20, 37, 41, 55, 58). Extensive research into the underlying mechanism(s) of CS-induced lung damage has shown that oxidative stress (reactive oxygen species [ROS] activation) and inflammation are the prime causes of alveolar apoptosis and senescence resulting in emphysema pathogenesis (3, 4, 16, 40, 57). Specifically, ROS activation triggers autophagy-impairment leading to the accumulation of misfolded/damaged proteins as perinuclear aggresome-bodies (31, 37) that induce COPD-emphysema pathogenesis (58). Although we have recently shown that pharmacological autophagy induction can control lung damage in the murine models of CS-induced COPD-emphysema (51, 58), further studies were necessary to identify the specific defect in the autophagy pathway in COPD-emphysema subjects to design a potent and highly specific therapeutic strategy.

The search for transcription factors that govern the expression of key autophagy-related genes led to identification of transcription factor EB *(TFEB)*, a master regulator of important genes that govern lysosomal biogenesis and autophagy (35, 48, 49). The activation of TFEB is regulated by mammalian target of rapamycin protein kinase complex (mTORC1), which under normal cellular environment, phosphorylates TFEB at the lysosomal surface leading to binding of YWHA/14-3-3 proteins and cytosolic retention of TFEB (28, 33, 45, 50). Under conditions of starvation or alteration of lysosomal functions, TFEB translocates to the nucleus, where it enhances transcription of numerous genes involved in autophagy and lysosomal biogenesis (33, 39, 50). Several reports highlight the crucial role of TFEB in age-related neurodegenerative diseases, such as Parkinson's, Huntington's, and Alzheimer's, all of which involve autophagy-impairment and accumulation of disordered or aggregated proteins (34, 56, 60). Moreover, genetic or pharmacological induction of TFEB has been reported to benefit in several of these pathological conditions caused by autophagy-impairment, which includes lysosomal storage disorders, Alzheimer's disease (AD), and α1-antitrypsin (α1-AT) deficiency. (18, 26, 43, 44, 53).

Hence, based on our recent studies identifying tobacco smoke exposure and/or aging-mediated autophagy-impairment and resulting aggresome-formation as a prognostic indicator of COPD-emphysema severity (5, 37, 51, 55, 58), we wanted to evaluate the pharmacological effects of TFEB-mediated autophagy induction in CS-induced COPD-emphysema (using human/murine lung tissues) to develop a prognosis-based intervention strategy. We first tested the therapeutic potential of a known TFEB inducer, gemfibrozil (GEM), which is an FDA-approved fibrate drug used for lowering serum lipid levels (22, 46), in controlling CS-induced autophagy-impairment and the resulting alveolar tissue damage (emphysema). GEM treatment is known to not only induce TFEB protein levels and activity in brain cells (46) but also initiates anti-inflammatory and antioxidant responses (46). Hence, apart from its well-known application in controlling hyperlipidemia in coronary heart disease (2, 54), treatment with GEM has proven beneficial in several other pathological conditions, for example, rheumatoid arthritis (12, 46), diabetes (6, 46), and murine experimental autoimmune encephalomyelitis (13, 46). Moreover, it is important to note here that the prominent comorbidities of COPD are coronary heart disease and metabolic syndrome (7), for which GEM is already shown to be clinically beneficial (46, 54). We verified the GEM results with an alternate TFEB-inducing flavonoid drug, fisetin (FIS), which is available over the counter as a dietary antioxidant for brain health.

Thus, we evaluated the impact of GEM (or FIS) treatment on CS-induced oxidative stress, autophagy-impairment, and/or resulting alveolar damage (emphysema) to validate the role of TFEB in COPD-emphysema pathogenesis.

Results

Perinuclear accumulation of TFEB protein in human lungs correlates with severity of COPD-emphysema

We have recently observed that CS or aging induces autophagy-impairment and resulting aggresome-formation that correlates with the severity of COPD-emphysema lung disease, which can serve as a prognostic biomarker (55, 58). Thus, we found that underlying autophagy-impairment is a common mechanism for CS/age-related chronic obstructive lung disease initiation and progression. To identify autophagy mechanism impaired by CS exposure, we wanted to verify the role of the master autophagy regulator, TFEB, in the pathogenesis of COPD-emphysema in murine and human lungs. Hence, we first analyzed changes in expression and localization of TFEB protein in longitudinal lung tissue sections of COPD-emphysema subjects (GOLD 0 to GOLD I–IV) and found that nuclear localization of TFEB (TFEB activation) decreases with severity of emphysema, while its perinuclear localization (in aggresome-bodies) increases with emphysema severity (from GOLD 0 to GOLD IV; Fig. 1A, D, $p < 0.05$, $r = -0.95$; white arrows: nuclear TFEB localization and red arrows: perinuclear TFEB accumulation). We observed a significant increase in perinuclear TFEB localization in severe (GOLD III) and very severe (GOLD IV) emphysema subjects compared with non-emphysema (GOLD 0) controls as well as mild or moderate emphysema (GOLD I–II). Furthermore, we demonstrate that smokers (GOLD I–IV) show a significant increase in perinuclear localization of TFEB compared with nonsmokers (Fig. 1A–C,
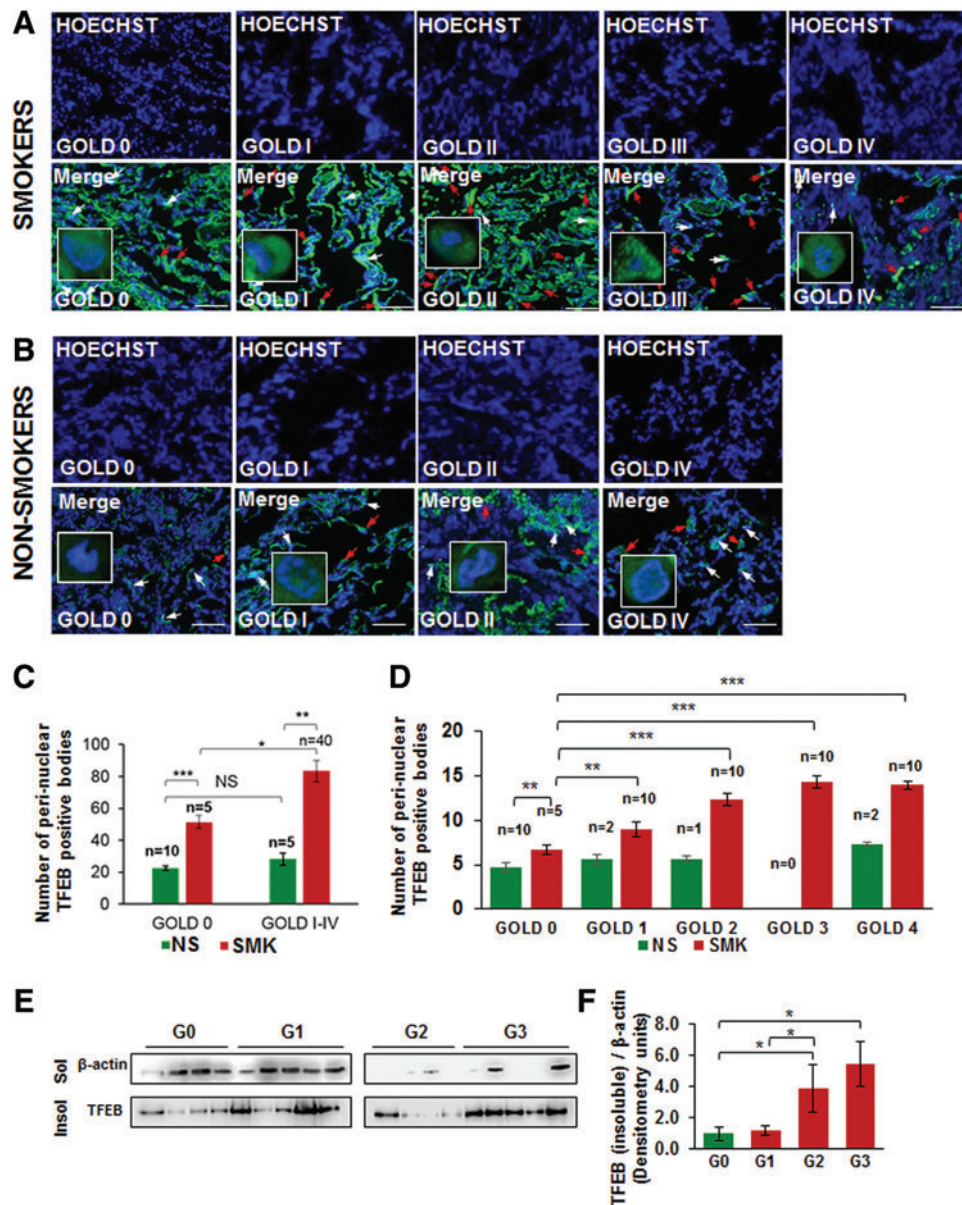
FIG. 1. **TFEB accumulates in perinuclear spaces in smokers and/or COPD subjects with emphysema. (A, B)** The fluorescent or confocal imaging (*insets*) of immunostained human lung tissue sections collected from non-emphysema (GOLD 0) and COPD subjects (GOLD I–IV) with emphysema shows that the nuclear localization of TFEB (TFEB activation) decreases with severity of emphysema (*white arrows*), while its perinuclear localization increases with disease/emphysema severity (from GOLD I–IV; *red arrows*). As anticipated, the increase in perinuclear localization in GOLD IV emphysema subjects is limited by substantial tissue destruction. Nuclei are stained with Hoechst (*blue*) dye for both protein localization and observing cellular changes due to tissue destruction. High-resolution confocal image *insets* show the representative positive or negative staining marked by *red* or *white arrows*. **(C, D)** The data compiled from high-resolution fluorescent and confocal images (*insets*) indicate that smokers (SMK, GOLD I–IV) show a significant increase in perinuclear localization of TFEB compared with nonsmokers (NS, GOLD 0 or GOLD I–IV). Moreover, smokers with greater disease severity (from GOLD I–IV) demonstrate a higher increase in accumulation of TFEB in perinuclear spaces (*red arrows*) compared with GOLD 0 subjects that show nuclear localization of TFEB (*white arrows*). Our data suggest the role of CS-induced TFEB-autophagy-impairment in COPD-emphysema pathogenesis. The correlation analysis for perinuclear TFEB-positive aggresome-bodies and FEV1-% predicted (lung function) showed significant increase in number of TFEB+ perinuclear staining with lung function decline, $r = -0.95$. Scale bar, 100 μM, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $n = 5–10$. **(E, F)** Western blots showing a significant increase in TFEB protein accumulation in the insoluble protein fractions (aggresomes) isolated from moderate (GOLD II) and severe (GOLD III) COPD-emphysema subjects compared with GOLD 0 non-emphysema controls ($*p < 0.05$; $n = 4–5$). The data supplement our above findings and confirm that TFEB protein accumulates in perinuclear spaces (potentially as aggresome-bodies) in the lungs of smokers and COPD-emphysema subjects. CS, cigarette smoke; COPD, chronic obstructive pulmonary disease; TFEB, transcription factor EB. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

GOLD 0 or GOLD I–IV, $p < 0.05$). In addition, smokers with greater disease severity (GOLD I–IV) show a comparatively higher increase in TFEB accumulation in aggresome-bodies compared with GOLD 0 subjects (Fig. 1C). Moreover, we verified that TFEB protein accumulates in the insoluble protein fractions (aggresomes) isolated from the lung tissues of moderate and severe COPD-emphysema subjects (GOLD II and III) compared with non-emphysema controls (GOLD 0) (Fig. 1E, F, and Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/ars). Thus, our data suggest the mechanistic role of master autophagy regulator, TFEB, in autophagy-impairment in smokers and COPD-emphysema subjects, which we verified using the *in vitro* and murine experimental models of CS-induced COPD-emphysema.

Cigarette smoke-induced TFEB accumulation in aggresome-bodies impairs autophagy in COPD-emphysema

Next, we verified our observation that TFEB protein accumulates in the perinuclear spaces as aggresome-bodies using an aggresome-specific dye. Thus, we analyzed the changes in colocalization of TFEB protein with an aggresome dye (PROTEOSTAT® Aggresome detection kit) in longitudinal lung tissue sections of COPD-emphysema subjects (GOLD I–IV) and observed that TFEB-aggresome colocalization (yellow) increases with emphysema severity in COPD subjects (GOLD I–IV) compared with non-emphysema (GOLD 0) controls (Fig. 2A, C; red arrows and insets, $r = -0.88$). We also found that smokers (GOLD 0 to GOLD IV) have higher TFEB-aggresome colocalization compared with nonsmoker COPD-emphysema or control subjects (Fig. 2B, $p < 0.05$). The Hoechst (Blue) dye was used to stain the nuclei to visualize the tissue area used for immunostaining (Supplementary Fig. S2). This suggests that CS induces accumulation of TFEB into aggresome-bodies that results in TFEB/autophagy-impairment in COPD-emphysema subjects (4, 55). We also verified this hypothesis by autophagy flux assay (colocalization of green fluorescent protein [GFP] and red fluorescent protein [RFP], LC3B-yellow puncta bodies, red arrows) and observed that CSE-impaired autophagy can be controlled by GEM or FIS-mediated TFEB induction (Fig. 2D, E, $p < 0.05$). The data suggest the significant role for TFEB in regulating CS-induced autophagy-impairment that required further analysis of TFEB induction strategies in controlling CS-induced oxidative stress, autophagy-impairment, and resulting emphysema.

Pharmacological TFEB inducers restore CSE-mediated autophagy-impairment and inhibit aggresome-formation

Several studies have identified the therapeutic benefit of TFEB induction in age-related pathological conditions that involve aggregation of damaged/misfolded proteins (11, 14, 15). We have earlier shown that CS and aging also induce aggregation of misfolded proteins (as aggresome-bodies) due to proteostasis/autophagy-impairment in murine/human lung (58), thus we evaluated here the pharmacological potential of TFEB induction in rescuing CS-induced aggresome-formation and resulting emphysema. First, we compared GEM, a known TFEB inducer (18), with previously tested autophagy inducer drugs (5, 51, 55, 58) and found that GEM treatment significantly ($p < 0.01$) elevates TFEB protein expression levels in Beas2b cells, compared with cysteamine (CYS) and carbamazepine (CBZ) (Fig. 3A, B, and Supplementary Fig. S3). Next, we found that GEM/FIS-mediated TFEB induction ameliorates CSE-induced autophagy-impairment (Ub-RFP and LC3B-GFP colocalization; aggresome-formation) in human bronchial epithelial cells (Beas2b) (Fig. 3C–F, $p < 0.05$). Thus, GEM/FIS-mediated TFEB induction is capable of controlling CSE-induced autophagy-impairment and resulting aggresome-formation and/or emphysema progression.

We demonstrate here that CSE-induced ubiquitin (Ub) and/or p62/sequestosome 1 accumulation in the insoluble protein fractions as aggresome-bodies can be controlled by using GEM-mediated TFEB induction (Fig. 4A, B). Furthermore, we demonstrate that the CSE-induced accumulation of ubiquitinated proteins in the insoluble protein fractions of Beas2b cells is significantly (GEM, $p < 0.05$) reduced by

FIG. 2. TFEB accumulates in aggresome-bodies in smokers and COPD subjects with increasing severity of emphysema that results in autophagy flux impairment. (A–C) Immunostaining of human lung tissue sections collected from non-emphysema (GOLD 0) and COPD-emphysema subjects (GOLD I–IV) shows the colocalization of TFEB (*green, white arrows*) in perinuclear aggresome-bodies (*red*; aggresome-specific dye, *insets*). This TFEB-aggresome colocalization (*yellow, insets*) statistically correlates with the severity of emphysema (from GOLD I–IV; *red arrows*) and lung function (FEV1-% predicted) decline, $r = -0.88$. We also observed that the increased tissue destruction in GOLD IV emphysema subjects may be influencing further increase in TFEB-positive aggresome-bodies compared with severe (GOLD III) COPD-emphysema subjects. The Hoechst (*blue*) dye was used to stain the nuclei to visualize the tissue area used for immunostaining (Supplementary Fig. S2). Moreover, smokers (GOLD 0 to GOLD IV) with incrementing emphysema severity show a higher TFEB-aggresome colocalization, verifying TFEB-autophagy-impairment in smokers and COPD-emphysema subjects. Scale bar, 25 μM, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; $n = 5$–10 per group. **(D)** Beas2b cells were incubated with BacMam reagent (with Premo™ Autophagy Tandem Sensor RFP-GFP-LC3B) for 16 h, followed by treatment with CSE (5%), GEM (10 μM), and/or FIS (25 μM) for 12 h. Fluorescence microscopy was used to quantify the number of GFP/RFP-positive autophagosomes that suggests an impaired autophagy flux (Scale bars, 100 μm). The *red arrows* indicate *yellow* puncta bodies, while *white arrows* show GFP-positive cells. **(E)** The data analysis of GFP-RFP-positive puncta bodies is shown as mean ± SEM of $n = 6$ replicates (**$p < 0.01$, ***$p < 0.001$). Results indicate that CSE induces autophagy flux impairment, while autophagy-inducing drugs (GEM/FIS) can restore autophagy flux in the presence of CSE. Chloroquine (endosomal acidification and autophagy inhibitor) is used as a positive control to identify RFP-GFP-positive (*yellow*) autophagosomes indicative of autophagy flux inhibition. The *insets* **(A, D)** show the representative magnified image for positive or negative staining, marked by *red* or *white arrows*. FIS, fisetin; GEM, gemfibrozil. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

GEM/FIS treatment (Fig. 4C, D, and Supplementary Figs. S4, S5). Moreover, CSE treatment also induced the accumulation of valosin-containing protein (VCP), p62, and/or the master autophagy regulator, TFEB, in the insoluble protein fractions, which can be controlled by GEM/FIS-mediated autophagy induction (Fig. 4C, D, and Supplementary Figs. S4, S5). This suggests the critical role of TFEB in CSE-induced autophagy-impairment as GEM/FIS-mediated TFEB-autophagy induction can control CSE-induced aggresome-formation. The data also verify our findings in COPD subjects (Figs. 1 and 2) and suggest a novel mechanistic role of TFEB in CS-induced COPD-emphysema pathogenesis.

Master autophagy regulator, TFEB, controls CSE-induced oxidative stress, aggresome-formation, cellular viability, and senescence

To further verify the role of TFEB in CS-induced COPD-emphysema pathogenesis, we modulated TFEB expression in Beas2b cells, followed by functional assays. Our data shows that CSE-induced significant increase in ROS activity is controlled by TFEB induction, using GEM/FIS (Fig. 5A, $p < 0.05$). Moreover, TFEB induction by GEM/FIS also rescues the CSE-mediated decrease in cell viability, as shown by the MTS assay (Fig. 5B, $p < 0.05$). We and others have
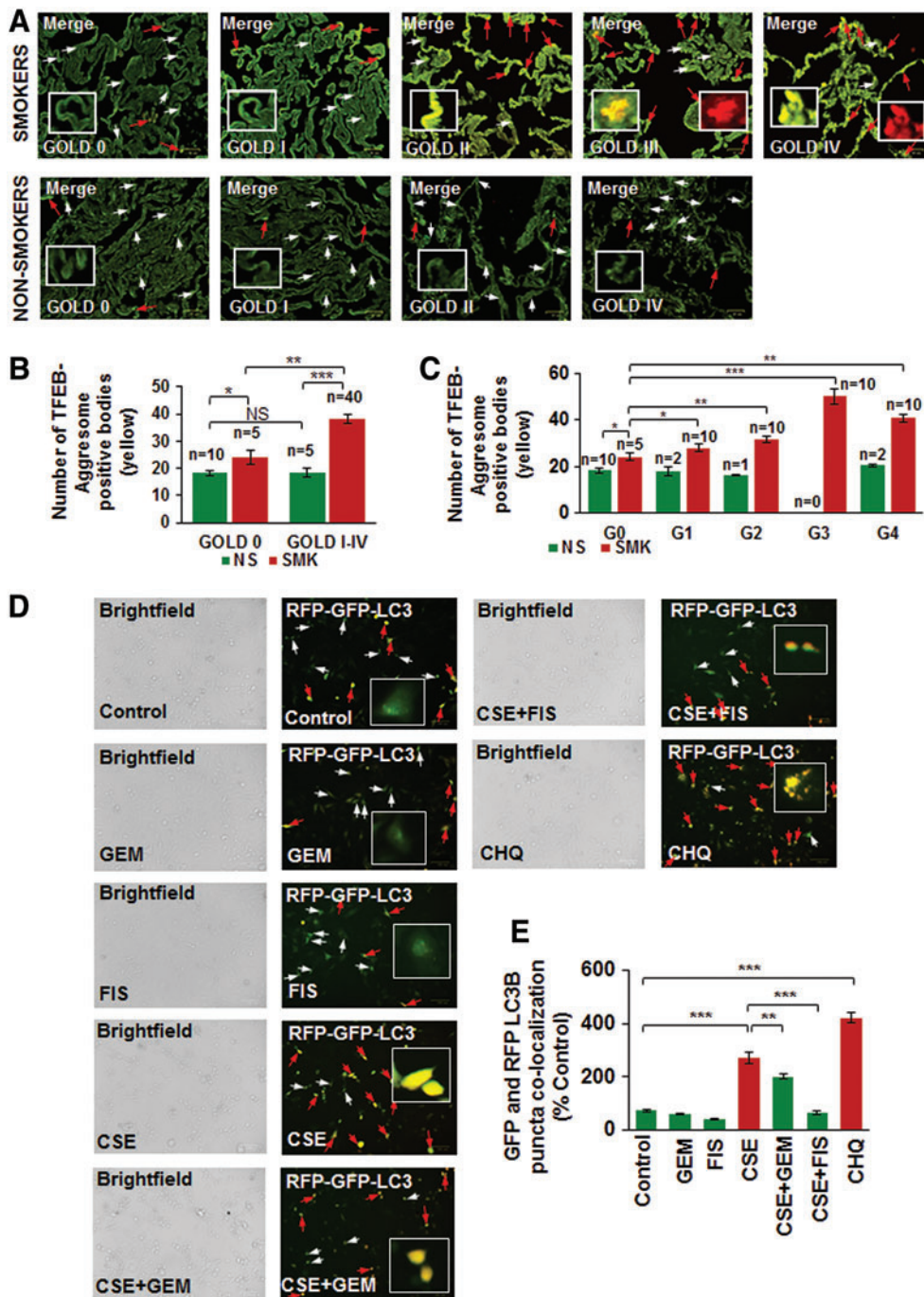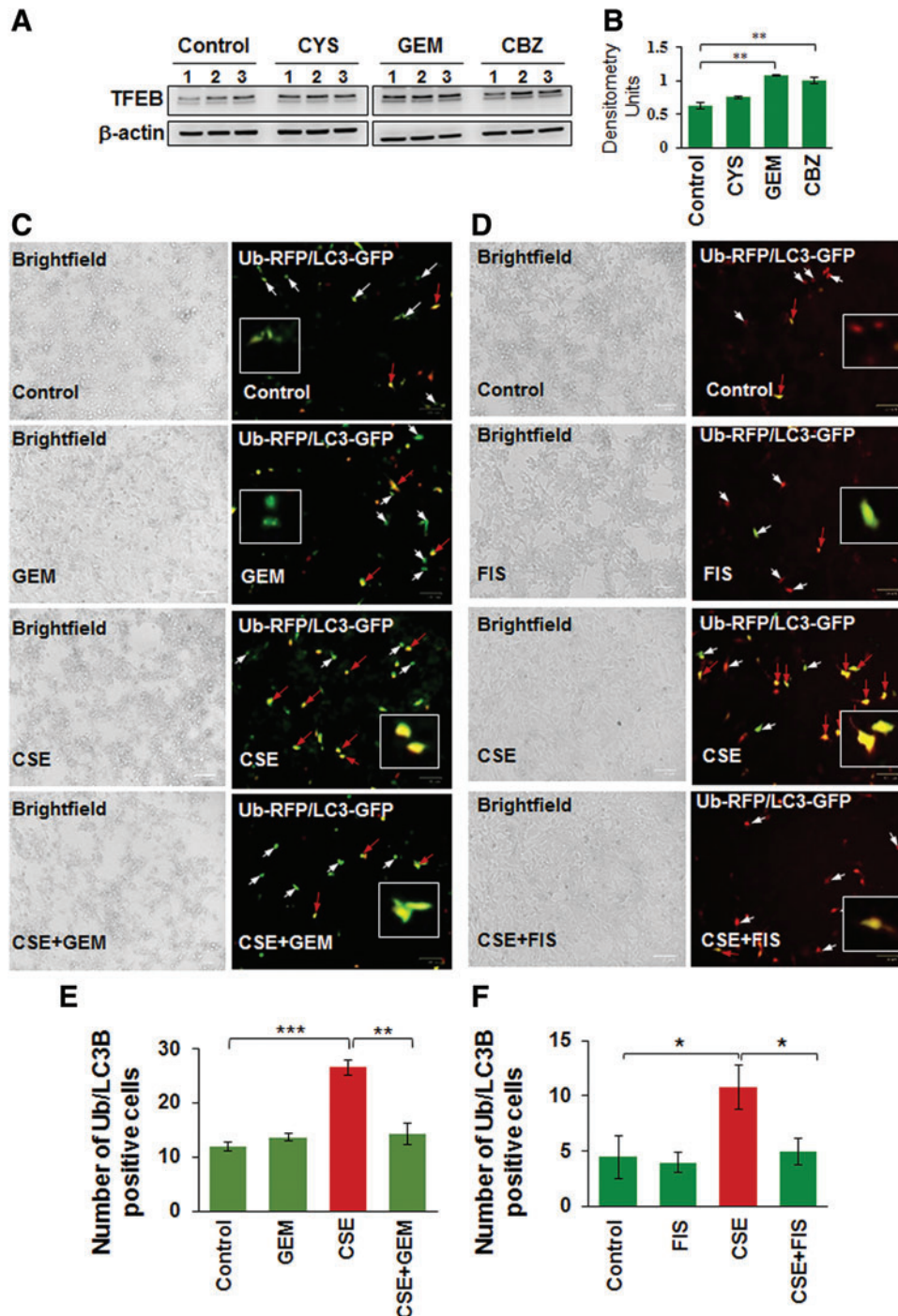
FIG. 3. TFEB-inducing drugs rescue CSE-induced autophagy-impairment. (A, B) Immunoblotting of total protein lysates isolated from Beas2b cells treated with different autophagy-inducing drugs (cysteamine, CYS, 250 μM; GEM, 10 μM; and carbamazepine, CBZ, 20 μM; 6 h) indicates that GEM and CBZ significantly induce TFEB expression in Beas2b cells ($n = 3$, **$p < 0.01$). **(C, D)** The Beas2b cells were cotransfected with RFP-(Ub) Ubiquitin and GFP-(LC3B), the autophagy protein light chain-3, plasmids. After 24 h post-transfection, cells were treated with 5% CSE, GEM (10 μM, *left panel*), and/or FIS (25 μM, *right panel*) for 12 h, followed by fluorescence microscopy (Scale bars, 100 μm). The fluorescent images were used to count the cells positive for colocalization (*yellow*, *red arrows*,) of ubiquitinated (Ub) proteins (*red*) and LC3B-puncta bodies (*green*), where *white arrows* show single staining. Data represent mean $\pm$ SEM, $n = 6$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and analysis is shown in **(E)** and **(F)**. The data indicate that CSE treatment induces Ub-LC3B coexpression as *yellow* puncta bodies (aggresomes), which is alleviated by TFEB-autophagy inducers, GEM or FIS. Thus, GEM/FIS-mediated TFEB activation has the potential to rescue CSE-induced autophagy-impairment. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars
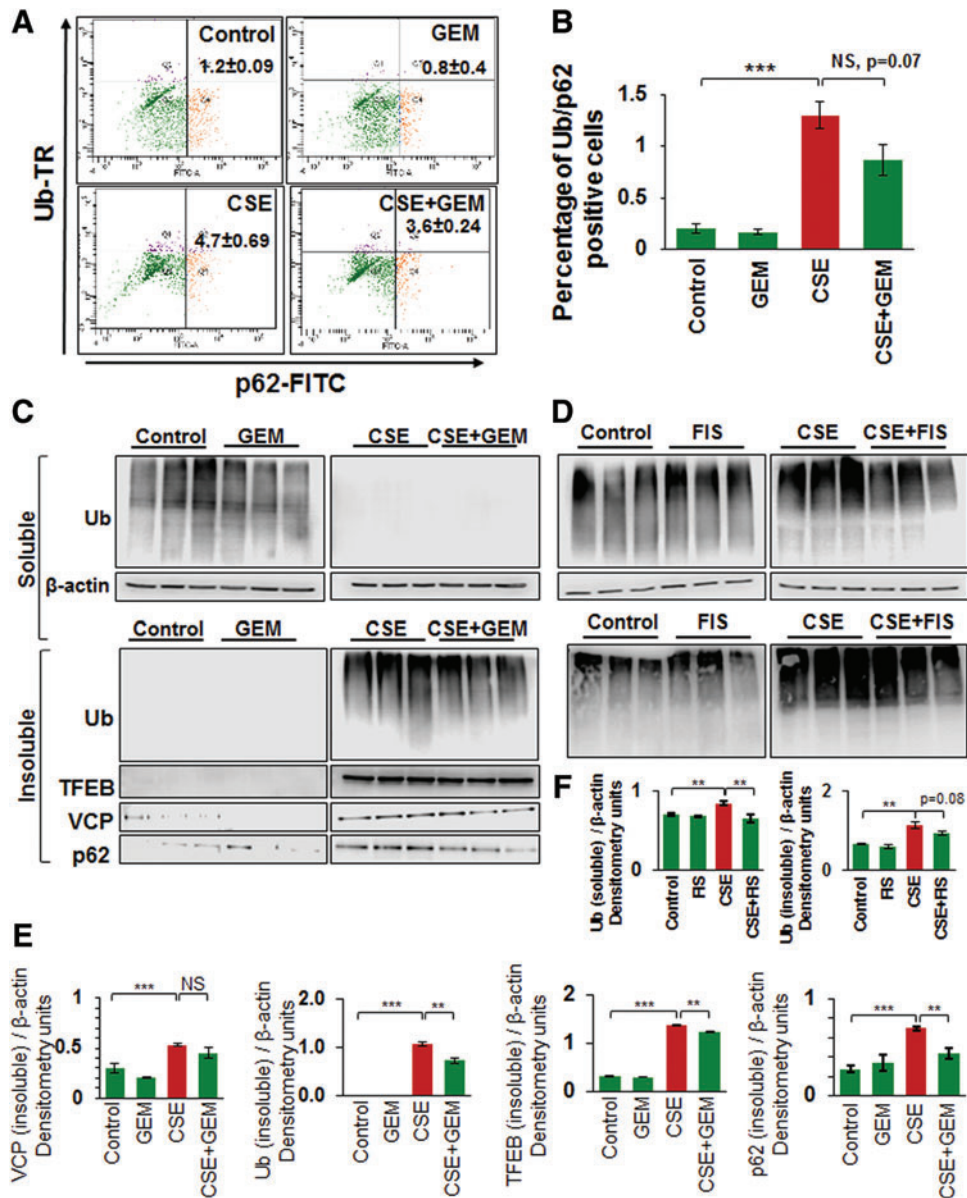
FIG. 4. TFEB induction controls CSE-induced autophagy-impairment. (A, B) Flow cytometry analysis verifying that CSE-induced coexpression of Ub and p62 (aggresome-bodies) is controlled by treatment with GEM (mean ± SEM, $n = 3$, $p = 0.07$). **(C, D)** Western blot analysis showing accumulation of ubiquitinated proteins (Ub), VCP, and TFEB in the soluble and insoluble protein fractions of Beas2b cells treated with 10% CSE and/or GEM (10 μM) for 6 h. β-Actin was used as the loading control. The data represent three replicates and show that GEM treatment significantly inhibits CSE-induced ubiquitinated protein and TFEB accumulation in the insoluble protein fractions (aggresome-bodies). **(D)** Similarly, FIS (25 μM)-mediated TFEB/autophagy induction also showed clearance of CSE-induced ubiquitinated proteins from soluble and insoluble protein fractions. Densitometry analysis of Western blots is shown in **(E** and **F)**. Data represent $n = 3$, mean ± SEM, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Data indicate that GEM or FIS-mediated TFEB induction controls CSE-induced autophagy-impairment. VCP, valosin-containing protein. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

recently observed that tobacco smoke/e-cigarette vapor (eCV) exposure leads to cellular senescence (1, 51) *via* ROS activation that accelerates lung aging and COPD-emphysema pathogenesis (36). We demonstrate here that CSE-mediated increase in number of senescent cells is significantly diminished by treatment with TFEB-inducing drugs, GEM/FIS (Fig. 5C, D, $p < 0.05$). The present data confirm the importance of TFEB-mediated functional autophagy in regulating CS-induced cellular senescence.

Next, we utilized TFEB knockdown (using TFEB shRNA) in Beas2b cells and observed that even partial inhibition of TFEB protein expression induces significant accumulation of ubiquitinated proteins in the insoluble protein fraction (aggresomes, similar to CSE treatment) that could be restored by treatment with TFEB-inducing drug, GEM (Fig. 5E, F, and Supplementary Fig. S6, $p < 0.05$). We also observed that TFEB inhibition leads to increased ROS activity and senescence in Beas2b cells,

which can be controlled by GEM-mediated TFEB induction (Fig. 5G–I). Thus, our data provide substantial proof-of-concept evidence supporting the crucial role of TFEB in CS-mediated oxidative stress, autophagy-impairment, and aggresome-formation involved in COPD-emphysema pathogenesis.

GEM alleviates CS-mediated autophagy-impairment in murine lungs by rescuing TFEB from aggresome-bodies

Recent studies from our group demonstrate that tobacco smoke/nicotine vapor exposure leads to autophagy-impairment and resulting aggresome-formation in the murine and/or human lungs (5, 55, 58), although the exact mechanism of autophagy-impairment is unknown. Hence, we utilized a similar *in vivo* approach as previously described (58) to evaluate the effect of GEM-mediated master autophagy regulator induction on CS-induced aggresome-formation. Our data shows that subchronic-CS (sc-CS)-induced accumulation of ubiquitinated proteins (Ub, aggresomes), p62 (impaired autophagy marker), and valosin-containing protein (VCP, a component of the retrograde translocation complex) in the insoluble protein fractions diminished by GEM treatment (Fig. 6A, B, and Supplementary Figs. S7, S8). Moreover, we report a novel observation that sc-CS exposure leads to increased accumulation of TFEB (master autophagy regulator) protein in the insoluble protein fractions as aggresome-bodies. This aggregation may plausibly render TFEB protein unavailable for performing its normal cellular functions (transcriptional regulation of crucial autophagy genes), thus leading to autophagy-impairment.

Apart from the impairment of autophagy, CS-induced oxidative stress triggers the unfolded protein response (UPR), which regulates the transcriptional program toward either cell survival or apoptosis, depending on the strength and duration of the oxidative insult (23). Sustained accumulation of misfolded or damaged proteins in the lungs due to perturbed autophagic clearance mechanisms leads to unabated activation of UPR in COPD subjects (23), promoting COPD progression (23). GRP94 is an ER-resident molecular chaperone (ER stress protein) that plays a central role in induction of the UPR (10, 59). Upon sc-CS exposure, we observed a modest induction of GRP94 protein levels (Fig. 6A, soluble fraction) compared with aggresome/impaired autophagy markers (Fig. 6B, Ub, p62, VCP, insoluble protein fractions), suggesting that long-term CS exposure induces significant accumulation of proteins in aggresome-bodies instead of ER. Overall, our data identify CS-induced accumulation of TFEB in aggresome-bodies as a novel mechanism for autophagy-impairment in murine lungs that is rescued by GEM treatment.

GEM-mediated TFEB activation rescues sc-CS-induced inflammation and autophagy-impairment

The coexpression/localization of ubiquitinated proteins (Ub) and p62 (impaired autophagy marker) is a marker of autophagy-impairment (5, 51, 58) that can be used to quantify changes in number of aggresome-bodies. Thus, to further ascertain the efficacy of GEM-mediated TFEB activation in controlling sc-CS-induced aggresome-formation, we performed flow cytometry analysis of bronchoalveolar lavage fluid (BALF) cells and show that GEM-mediated TFEB induction significantly ($p < 0.05$) rescues sc-CS-induced Ub and p62 (impaired autophagy marker) coexpression, indicating restoration of autophagy (Fig. 7A, B). TFEB induction by GEM also has the potential to alter CS-induced inflammatory cell phenotypes (Th1 to Th2 switching) by initiating anti-inflammatory mechanisms such as reduction of IL-6 and other inflammatory cytokines (46). Similarly, we demonstrate here that GEM-mediated TFEB induction significantly ($p < 0.05$) reduces sc-CS-induced IL-6 levels (Fig. 7C). Since elevated IL-6 levels are associated with CS-induced emphysema (4), reduction in sc-CS-induced IL-6 levels supports our notion that GEM-mediated TFEB induction is a potent therapeutic strategy for controlling COPD-emphysema pathogenesis.

FIG. 5. TFEB controls CSE-induced oxidative stress, aggresome-formation, cellular viability, and senescence. (A) Beas2b cells were treated with CSE (5%), GEM (10 μM), and/or FIS (25 μM) for 12 h and cell proliferation was assessed by MTS assay. The data show that CSE inhibits Beas2b cell survival, while TFEB-inducing drugs (GEM/FIS) can partially rescue CSE-impaired cell proliferation. **(B)** CM-DCFDA-based ROS assay shows that CSE-induced ROS activity can be alleviated by treatment with TFEB-inducing antioxidants, GEM or FIS (mean $\pm$ SEM, *$p < 0.05$, ***$p < 0.001$). **(C, D)** Beas2b cells were treated with CSE (5%), GEM (10 μM), and/or FIS (25 μM) for 12 h and the number of senescent cells were counted (*blue, black arrows*). The data summarized for $n = 6$ replicates (mean $\pm$ SEM, ***$p < 0.001$) indicate that TFEB-inducing drugs (GEM/FIS) can control CSE-induced senescence. **(E)** Beas2b cells were transfected with TFEB-shRNA for 24 h and/or treated with GEM (10 μM) for the final 12 h of transfection. Western blots show that TFEB-shRNA significantly inhibits the protein expression of TFEB that was restored by GEM treatment (TFEB/autophagy inducer, *upper panel*, soluble fraction). Knockdown of TFEB also shows significant accumulation of ubiquitinated proteins in the insoluble protein fraction as anticipated (similar to CSE exposure), which could be rescued by GEM treatment (*lower panel*). **(F)** β-Actin was used to normalize the expression of each protein and densitometry analysis of the blots is shown ($n = 3$, mean $\pm$ SEM, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). **(G)** TFEB inhibition by shRNA induces ROS activity, which could be inhibited by a parallel GEM treatment ($n = 3$, mean $\pm$ SEM, **$p < 0.01$, ***$p < 0.001$). **(H)** Beas2b cells were transfected with TFEB-shRNA for 24 h and/or treated with GEM (10 μM) for the final 12 h of transfection. The number of senescent cells (*blue, black arrows*) was quantified under the light microscope to quantify changes in senescence on TFEB inhibition and/or induction. **(I)** The analysis of the senescence data shows that TFEB knockdown increases the number of senescent cells and parallel treatment with TFEB/autophagy-inducing drug, GEM, controls this increase in senescence. The data confirm the functional role of TFEB-mediated autophagy in regulating CS-induced oxidative stress and resulting autophagy-impairment and cellular senescence. CM-DCFDA, chloromethyl derivative of 2′,7′-dichlorodihydrofluorescein diacetate; ROS, reactive oxygen species. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

GEM-mediated TFEB/autophagy activation rescues sc-CS-induced alveolar apoptosis and emphysema

In this study, we verified that sc-CS-mediated colocalization of ubiquitinated proteins (Ub) and p62 (impaired autophagy marker) in the murine lung sections (Fig. 8A, merge panel, white arrows) is alleviated by GEM treatment (Fig. 8B). The high-resolution confocal images from these lung sections show a clear increase in perinuclear accumulation of Ub-p62 (yellow) in sc-CS-exposed mice that is reduced by GEM treatment (Fig. 8C). Moreover, we also validate our human lung tissue (COPD subjects) and cell line (Beas2b) data and report that CS exposure induces a significant increase in perinuclear localization of TFEB in aggresome-bodies (Fig. 8D, F, yellow arrows), which was rescued by treatment with its therapeutic inducer, GEM. Finally, GEM-mediated TFEB activation significantly decreases the CS-induced alveolar airspace enlargement (mean alveolar diameter, Lm, Fig. 8E, G) and caspase-3/7 activity (lung cell apoptosis; Fig. 8H), validating that our strategy of TFEB induction has a potential to therapeutically control the progression of COPD-emphysema. Overall, our data suggest that CS induced perinuclear accumulation of TFEB as a novel mechanism for CS-mediated autophagy-impairment and
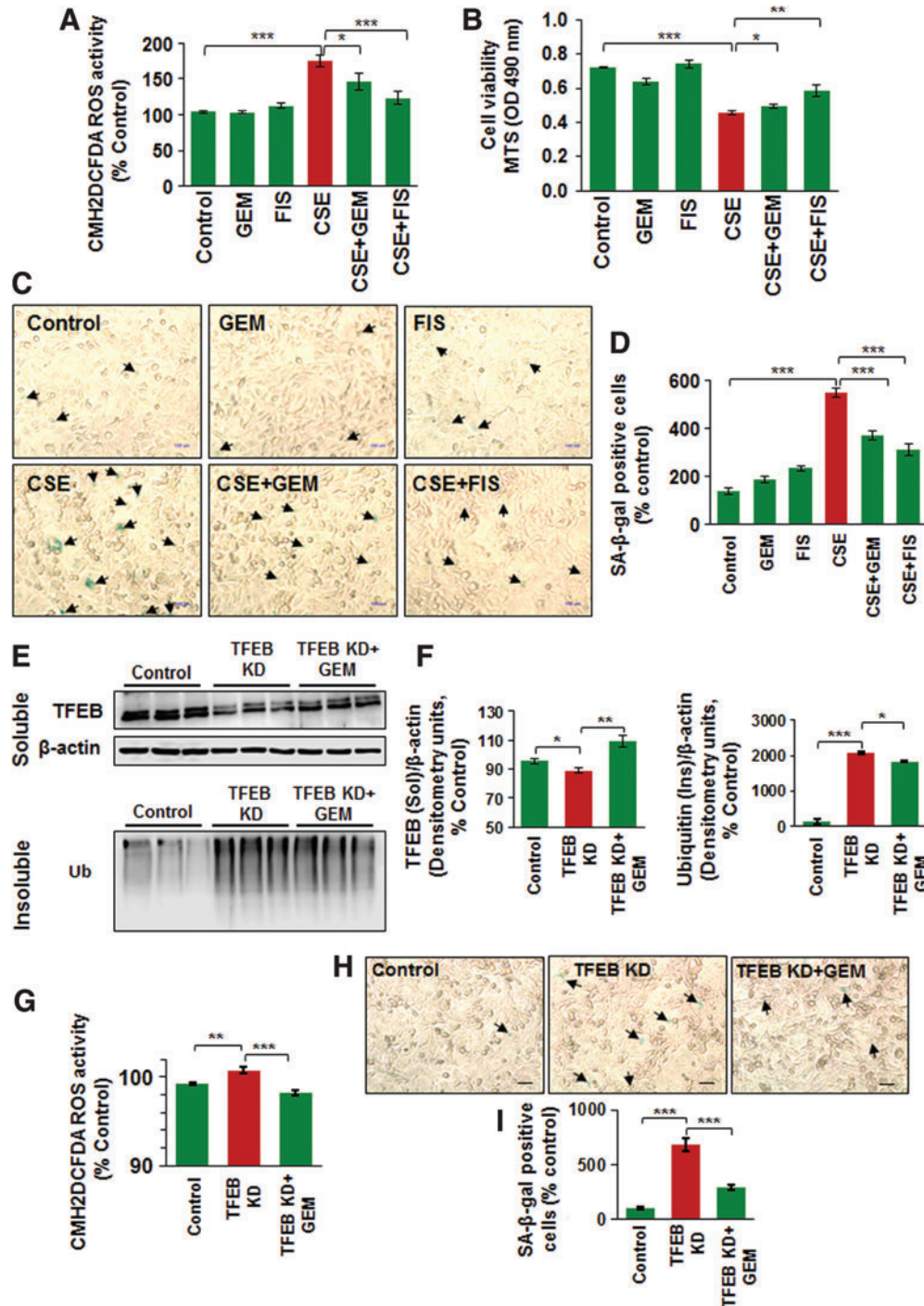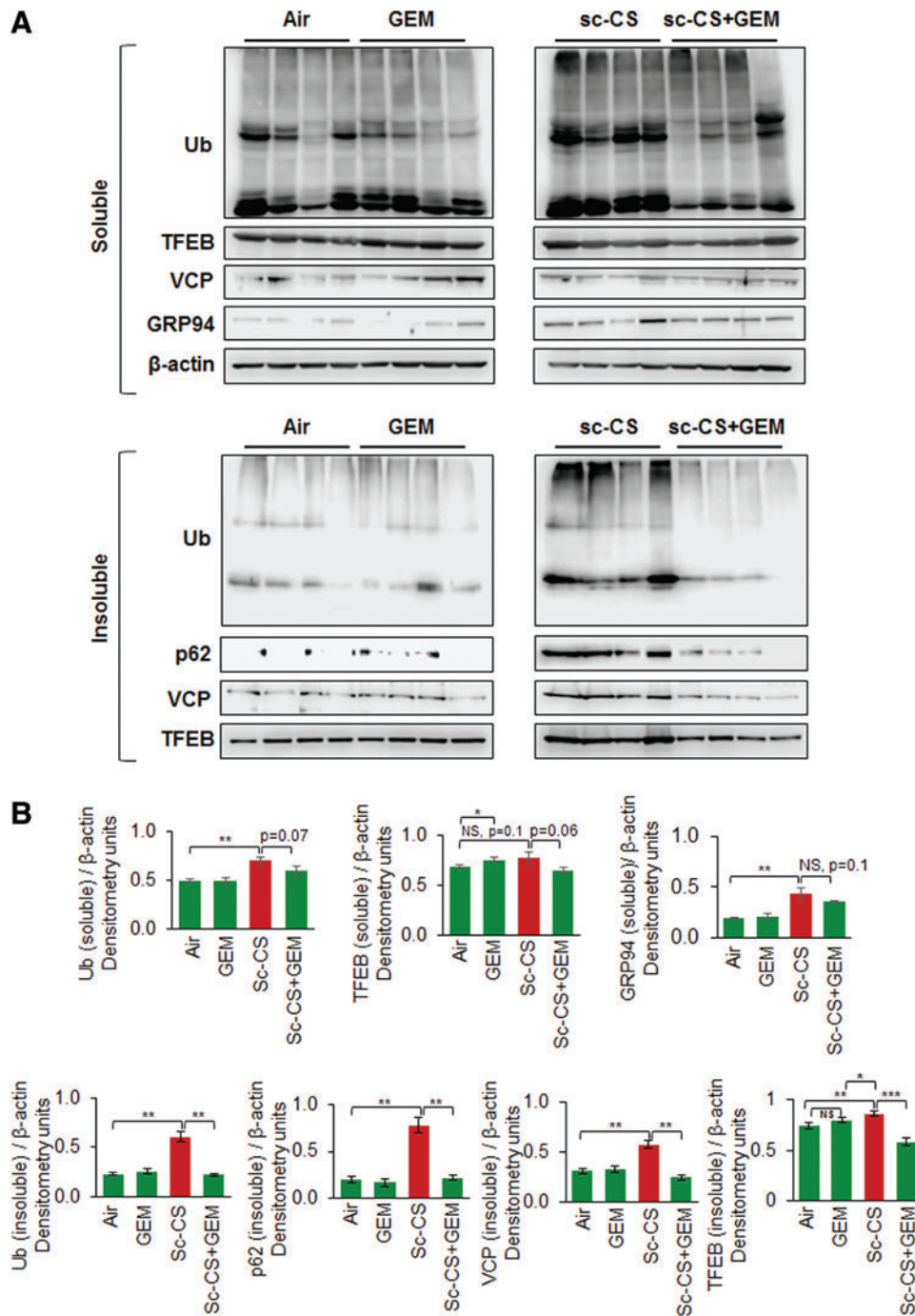
FIG. 6. GEM rescues TFEB protein from aggresome-bodies as a mechanism to control sc-CS-induced autophagy-impairment. (A) Western blot analysis illustrating the levels of ubiquitinated proteins (Ub), p62 (impaired autophagy marker), TFEB (master autophagy regulator), and GRP94 (ER stress marker) in the soluble and insoluble protein fractions of murine lung protein lysates collected from C57BL/6 mice exposed to room air or subchronic cigarette smoke (sc-CS, 2 months, 5 h/day, 5 days/week) and/or treated with GEM [i.p., 40 mg/kg bw, one dose alternate days for 10 days]. Expression of each protein was normalized to β-actin loading control. **(B)** The data (mean $\pm$ SEM, $n = 4$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$) confirm our findings that GEM treatment significantly diminishes CS-induced autophagy-impairment as evident by rescue of Ub, p62, VCP, and TFEB proteins from the insoluble protein fractions (aggresome-bodies). As anticipated, we only see a mild induction of ER stress marker, GRP94, levels (Fig. 4B, soluble fraction) compared with impaired-autophagy markers (Fig. 4B, Ub, p62, VCP, insoluble protein fractions), suggesting that misfolded proteins are accumulating mainly in aggresomes over ER. Our data suggest that sc-CS-induced autophagy-impairment is mediated by TFEB accumulation in aggresomes that can be rescued by GEM treatment. i.p., intraperitoneal; sc-CS, subchronic cigarette smoke. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars
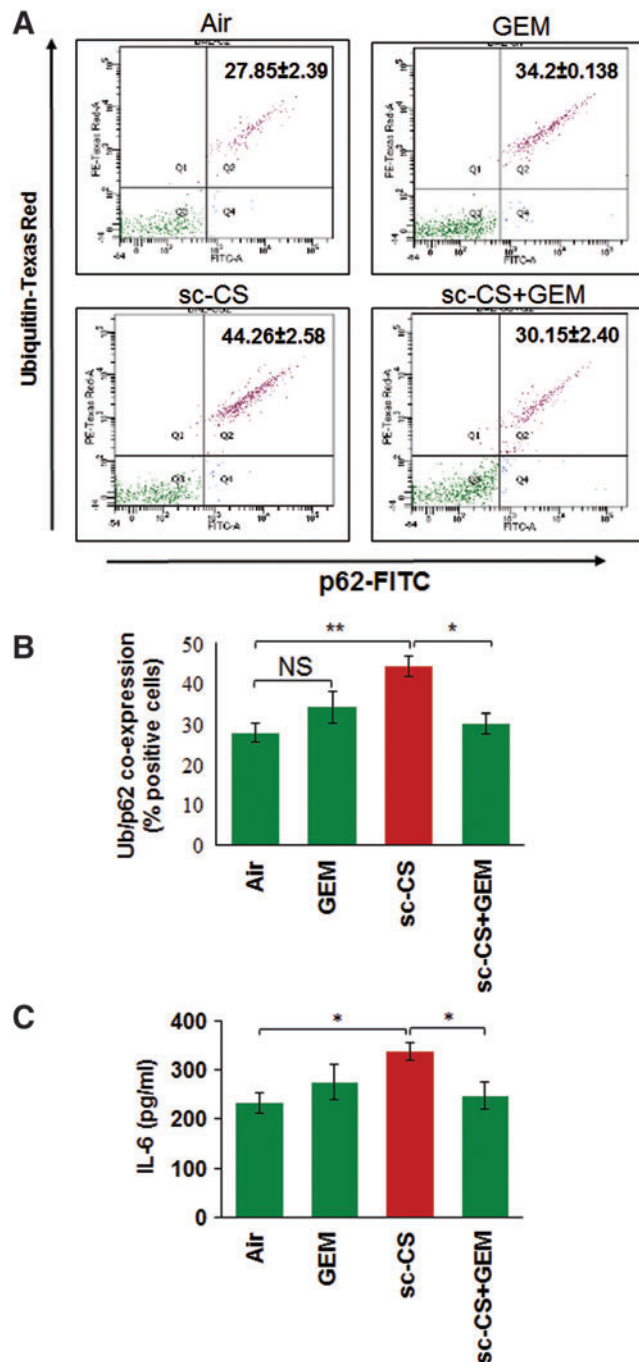
pharmacological induction of TFEB by GEM as a strategy to compensate for nonfunctional aggresome-trapped TFEB protein by inducing TFEB/autophagy for controlling inflammatory-oxidative stress, apoptosis, and resulting COPD-emphysema.

Discussion

Tobacco smoke/nicotine vapor exposure induces inflammatory-oxidative stress *via* autophagy-impairment-mediated aggresome-formation (5, 51, 58) that is central to controlling the initiation and progression of tobacco smoke-induced obstructive lung pathology (55). Although we and

FIG. 7. **GEM-mediated TFEB induction controls sc-CS-induced autophagy-impairment and inflammation.** **(A, B)** Flow cytometry analysis of BALF cells isolated from C57BL/6 mice exposed to room air or subchronic cigarette smoke (sc-CS, 2 months, 5 h/day, 5 days/week) and/or treated with GEM [i.p., 40 mg/kg bw, one dose alternate days for 10 days]. Data shows that TFEB-inducing antioxidant, GEM, rescues sc-CS-induced Ub-p62 coexpression (impaired autophagy marker), suggesting restoration of autophagy. Data represent mean $\pm$ SEM, $n = 3$, $*p < 0.05$, $**p < 0.01$. **(C)** ELISA-based proinflammatory cytokine, IL-6, quantification in BALF samples isolated from each group (described for **A**) shows that GEM significantly inhibits sc-CS-induced IL-6 levels (mean $\pm$ SEM, $n = 3$, $*p < 0.05$). The data suggest that GEM-mediated TFEB induction rescues sc-CS-induced autophagy-impairment in the murine lungs. BALF, bronchoalveolar lavage fluid; IL-6, interleukin-6. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

others have successfully tested the efficacy of pharmacological autophagy induction in both *in vitro* and *in vivo* models of smoke-induced COPD-emphysema (55, 58), there is a gap in the understanding of the precise mechanism of tobacco smoke-induced autophagy-impairment. The activation of ROS by free radicals and pro-oxidative components of tobacco is obviously a major factor that may hamper cellular homeostasis by causing autophagy/proteostasis impairment (31), although we found that both eCV and nicotine can also induce ROS-mediated autophagy-impairment (5, 51) similar to tobacco smoke. Studies from other groups initially suggested that CS induces autophagy in COPD-emphysema (8, 9, 25, 27), although it was impaired in other lung diseases, which is discussed in detail in the recent review articles (32, 38). Subsequent studies have clarified that although acute CS exposure activates autophagy, repeated chronic exposures lead to autophagy flux impairment. The previous studies used LC3B as a marker to report CS-related autophagy changes, but subsequent studies utilized autophagy flux markers (p62 and ubiquitin accumulation in autophagosomes) and reporters (Premo™ Autophagy Tandem Sensor RFP-GFP-LC3B) to demonstrate that CS-induced autophagy-impairment accelerates COPD-emphysema progression (58). Thus, in this study, we wanted to further investigate the mechanism of CS-ROS-mediated autophagy-impairment.

We first evaluated the effect of CS exposure on transcription factor EB (TFEB; a key master regulator, which controls lysosomal biogenesis and expression of autophagy genes) expression and localization (33, 49) based on our observation in human COPD subject lungs. We initially found that TFEB protein localizes in the perinuclear region as aggresome-bodies in severe COPD-emphysema subjects (Fig. 1A), suggesting that aggresome sequestration of TFEB protein could be a potential mechanism of tobacco smoke-induced autophagy-impairment in COPD. In support of this finding, we demonstrate that significant increase in TFEB-aggresome colocalization statistically correlates with the severity of emphysema and smoking history in COPD-emphysema subjects (Fig. 2A, B). Thus, we used GEM (18) or FIS (26)-mediated TFEB/autophagy induction as an experimental tool with our well-established models of CS-induced COPD-emphysema progression to evaluate its functional role. Using both our *in vitro* (Beas2b cells) and

murine experimental models of COPD-emphysema (CS-exposed mice), we show that CS exposure leads to accumulation of TFEB protein in the perinuclear aggresome-bodies that can be rescued by treatment with GEM-mediated TFEB activation (Figs. 4, 6, and 8D). Moreover, we also demonstrate that TFEB induction by GEM/FIS treatment can control CS-induced inflammation (Fig. 7C), oxidative stress (ROS activation; Fig. 5B), autophagy-impairment, and resulting aggresome-bodies (Figs. 2–6), thus providing a proof-of-concept evidence that TFEB-mediated autophagy induction could rescue COPD-emphysema pathophysiology (Fig. 9).

Previous studies highlight the critical role of TFEB in the regulation and expression of autophagy genes and its mechanisms are well described (33, 39). Briefly, in normal physiological conditions, TFEB is retained in the cytoplasm by mammalian target of rapamycin (mTORC1)- or mitogen-activated protein kinase 1 (ERK2)- mediated phosphorylation, while in nutrient-deprived or lysosomal stress conditions, TFEB is dephosphorylated by the phosphatase calcineurin, which then promotes nuclear translocation of TFEB, where it regulates the transcription of numerous lysosomal biogenesis and autophagy genes. TFEB directly binds to the coordinated lysosomal expression and regulation (CLEAR) motif (lysosomal biogenesis) and also the promoter regions of various autophagy genes to induce target gene expression (39). The aberrant expression/activity of TFEB is evident in several pathological conditions, especially in age-related degenerative or lysosomal storage disorders, where TFEB impairment leads to accumulation of misfolded or damaged proteins in aggresome-bodies (34, 47). A recent study shows that genetic and chemical activation of TFEB clears the α-synuclein aggregates, which are associated with neurodegenerative diseases such as Parkinson's (24). These α-synuclein aggregates are formed by accumulation of misfolded α-synuclein due to impairment of protein degradation machinery that includes the ubiquitin–proteasome system and autophagy (24). Thus, TFEB-mediated autophagy induction is proposed as a strategy to clear α-synuclein protein aggregation (15, 24). Similarly, in AD, the neuronal accumulation of phosphorylated tau plays an important pathological role (26), and strategies to induce clearance of tau in neurons are developed as a therapeutic strategy to circumvent AD pathology (44, 52). Briefly, a recent report shows the potential of FIS, an organic flavo-noid compound, in enhancing clearance of aggregated tau protein *via* TFEB-mediated autophagy induction (26). In the context of lung diseases, pharmacological or genetic activation of TFEB, the master transcriptional activator of the autophagolysosomal system, is shown to be beneficial in rescuing the spontaneous pulmonary fibrosis developed in the PiZ murine model, wherein accumulation of misfolded α1-antitrypsin Z (ATZ) leads to lung proteinopathy (21). These promising findings led us to test whether pharmacological TFEB induction could rescue CS-induced autophagy-impairment and the ensuing aggresome pathologies initiating COPD-emphysema.

We first chose to use GEM as a pharmacological TFEB inducer based on previous reports (18) and its known anti-oxidant and anti-inflammatory properties, which may provide additional therapeutic advantage, as we have recently observed with autophagy-inducing antioxidant drug, CYS (5, 51, 58). Additionally, GEM is an FDA-approved lipid-lowering drug and is shown to be clinically beneficial in coronary heart disease and metabolic syndrome (46), two prominent comorbidities of COPD (7), thus warranting its evaluation in CS-induced chronic COPD-emphysema. Our present data demonstrate that GEM-mediated TFEB induction can alleviate CS-induced inflammatory-oxidative stress (IL-6 levels, ROS activation), autophagy-impairment, aggresome-formation (Ub-p62 coexpression/localization), apoptosis (caspase-3/7 activity), and resulting alveolar airspace enlargement (Lm, H&E staining). Mechanistically, our novel data shows that CS triggers accumulation of TFEB in perinuclear aggresome-bodies, thus rendering it unavailable to perform its normal function as a master autophagy activator. It is obvious that similar to other misfolded (ubiquitinated) proteins, CS modulates TFEB protein folding that promotes its accumulation into aggresome-bodies. Thus, we used GEM treatment to induce both the expression of TFEB protein and autophagy that will also allow rescue of additional TFEB protein from aggresome-bodies.

As discussed above, TFEB-inducing strategies have been successfully used to rescue misfolded α-synuclein and tau proteins in neurological diseases (24, 26). In addition, GEM was recently shown to upregulate the expression of tripeptidyl peptidase (TPP1) in brain cells *via* the PPARα/RXRα pathway in late infantile neuronal lipofuscinosis, a disease


FIG. 8. TFEB/autophagy induction by GEM decreases sc-CS-induced alveolar airspace enlargement and aggresome-formation in the murine lungs. Immunostaining of longitudinal lung sections from C57BL/6 mice exposed to room air or subchronic cigarette smoke (sc-CS, 2 months, 5 h/day, 5 days/week) and/or treated with GEM [i.p., 40 mg/kg bw, one dose alternate days for 10 days] shows that GEM treatment significantly decreases **(A, B)** the CS-induced increase in the number of Ub-p62-positive aggresome-bodies (*yellow*, *red arrows*). Nuclei were stained using the Hoechst (*blue*) dye. Scale bars, 100 μM, *inset*: 10 μM. The *white arrows* are used to show the single staining in cells where aggresome-bodies were absent. **(C)** High-resolution confocal images **(A,** *third panel*) verify that sc-CS-induced perinuclear accumulation of Ub-p62 (*yellow*, $r = 0.58$) is diminished by GEM treatment. **(D)** Immunostaining of lung sections from the same groups of mice as in **(A)** shows that sc-CS exposure induces localization of TFEB into perinuclear aggresome-bodies (*red arrows*, *insets*) that is significantly rescued by GEM treatment **(F,** $r = -0.03$), implying that induction of master autophagy regulator, TFEB, inhibits its perinuclear accumulation potentially *via* restoration of autophagy. **(E)** H&E staining of longitudinal lung sections **(A,** *top panel*) shows a significant increase in alveolar airspace enlargement in lung sections from sc-CS-exposed mice compared with room air-exposed controls. **(G)** The lung sections (in **E**) of mice treated with GEM show significant inhibition of sc-CS-induced alveolar airspace enlargement. Data represent mean ± SD of $n = 4$ replicates (**$p < 0.01$, ***$p < 0.001$). Scale bars, 100 μM, *inset*: 10 μM. **(H)** The changes in alveolar apoptosis in each experimental group were quantified using caspase-3/7 assay. The data show that sc-CS-induced apoptosis can be controlled by treatment with TFEB/autophagy-inducing drug, GEM ($n = 4$, *$p < 0.05$, **$p < 0.01$). H&E, hematoxylin and eosin. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

caused by accumulation of mutant TPP1 protein (17). Thus, we concluded that GEM treatment similarly rescues misfolded TFEB (and other ubiquitinated proteins) from aggresomes by similar mechanisms, thereby restoring TFEB expression and transcriptional activity for normal functional autophagy response. To further validate the role of TFEB in COPD-emphysema pathogenesis, we utilized an alternative TFEB/autophagy inducer, FIS. In addition to TFEB-mediated autophagy induction (26), FIS is a potent antioxidant

activator of the Nrf2 pathway (26, 29) that can alleviate CS-ROS-mediated protein misfolding and aggregation as aggresome-bodies. As a proof of concept, fisetin-mediated TFEB/Nrf2 activation shows more significant inhibition of CSE-induced ROS activity and cellular senescence (Fig. 5B–D) compared with GEM treatment. Thus, FIS is a novel therapeutic agent to potentially rescue CS-mediated oxidative stress and protein aggregation by activating antioxidant response (via Nrf2) and autophagy (via TFEB). We also verified
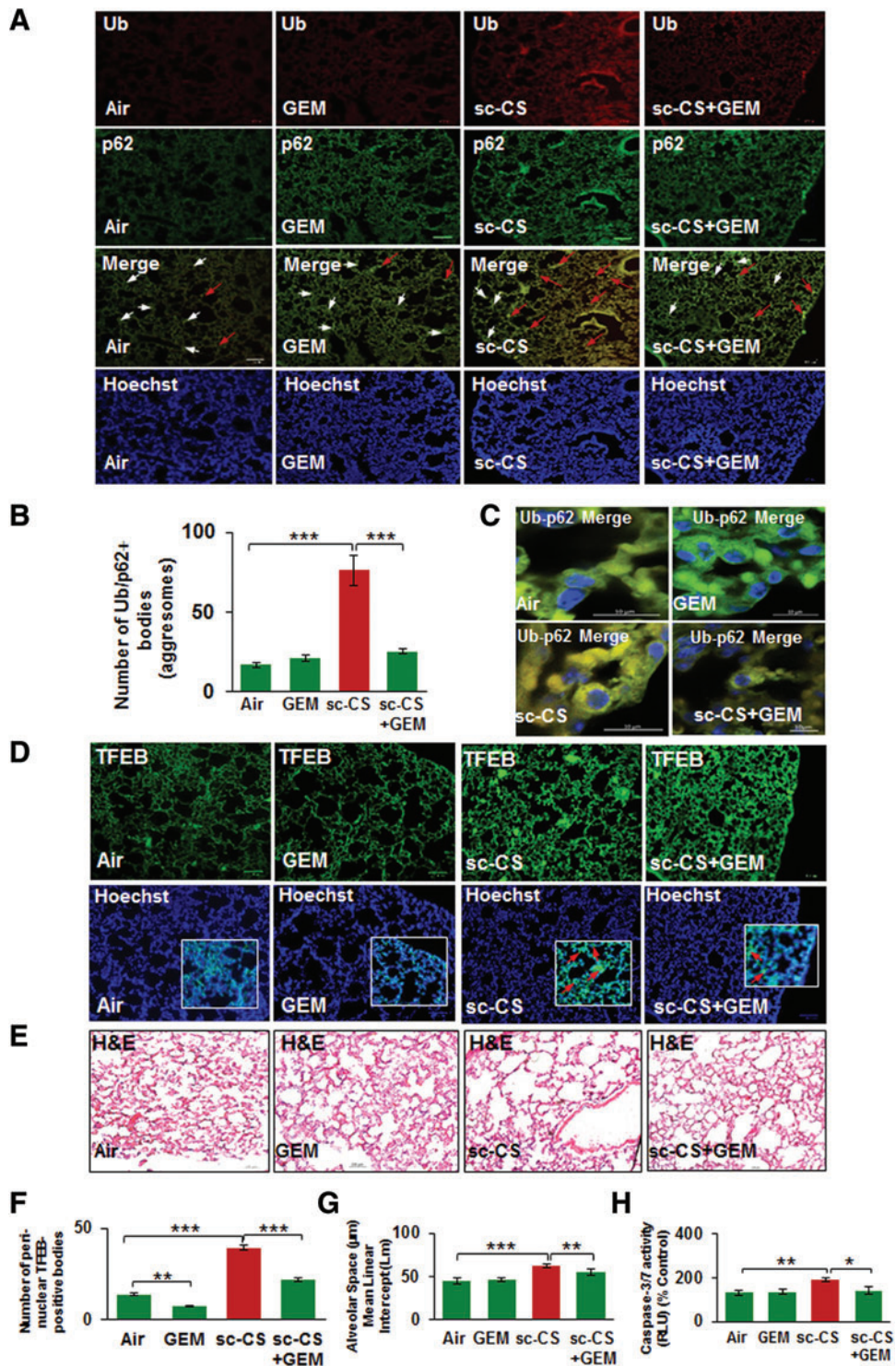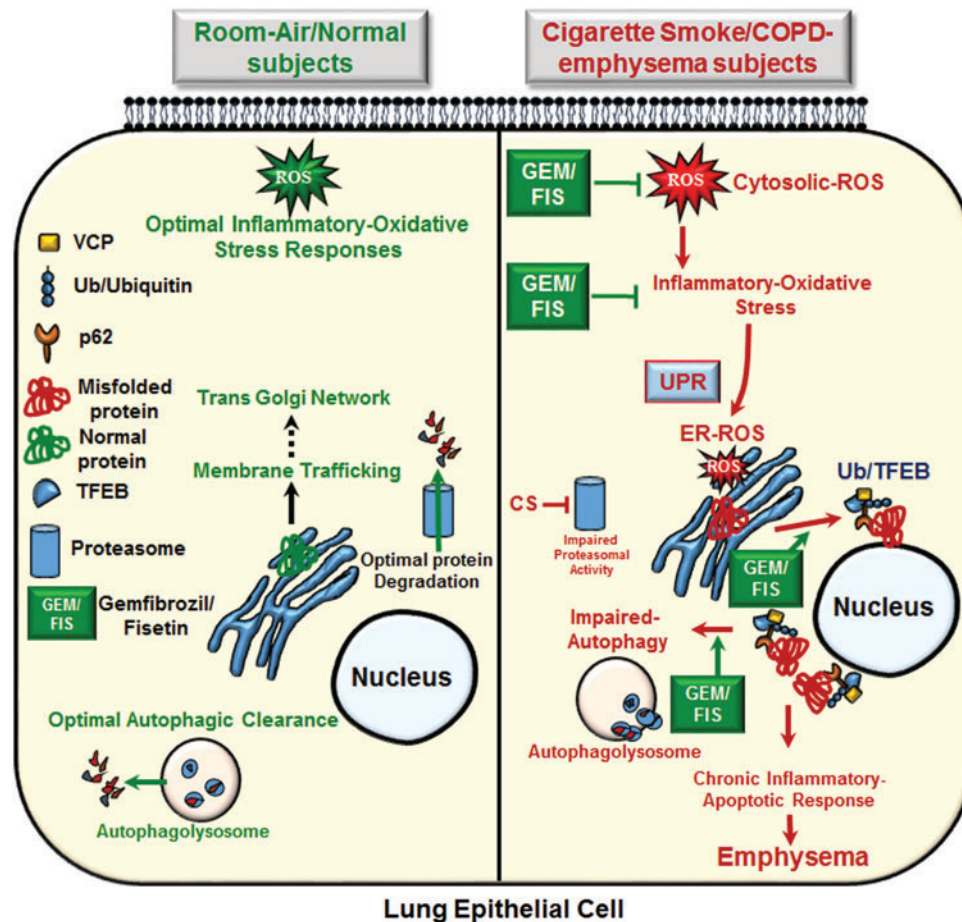
FIG. 9. Schematic showing cigarette smoke mediated TFEB/autophagy-impairment in COPD-emphysema. Under normal physiological conditions, the optimal inflammatory oxidative signaling restricts extracellular insults *via* the robust proteostasis/autophagy responses. Cigarette smoke exposure mediates ROS activation in lung epithelial cells that can induce inflammatory-oxidative stress, resulting in autophagy-impairment and formation of perinuclear aggresome-bodies that mediate COPD-emphysema pathogenesis. Moreover, CS-induced perinuclear accumulation of TFEB, the master autophagy regulator, in aggresome-bodies hampers autophagy responses in COPD-emphysema. TFEB inhibition induces ROS activity, resulting in aggresome-formation and cellular senescence/apoptosis, while treatment with TFEB-inducing antioxidant drugs (GEM or FIS) controls CS-induced inflammatory-oxidative stress, aggresome pathology, and alveolar apoptosis. Overall, smoke exposure impairs TFEB/autophagy activity, resulting in downstream functional changes associated with progression of COPD-emphysema pathophysiology. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

the role of TFEB in regulating CS-induced mechanisms involved in COPD-emphysema by using TFEB shRNA. Our data shows that TFEB inhibition induces ROS activity, ubiquitinated protein accumulation (autophagy-impairment), and cellular senescence (Fig. 5E–H) similar to CS/CSE exposure (Figs. 4C, 5B–D, and 6A). Moreover, these functional effects of TFEB knockdown were rescued by GEM-mediated TFEB induction.

In conclusion, we identify CS-induced accumulation of TFEB in aggresome-bodies as a specific novel mechanism for CS-mediated autophagy-impairment and resulting aggresome-formation and emphysema progression. Moreover, we propose that TFEB-positive aggresome-bodies can serve as prognostic biomarker for tobacco smoke-induced COPD-emphysema that can allow early detection and treatment of chronic obstructive lung diseases with proposed prognosis-based intervention strategy employing TFEB/autophagy-inducing drugs.

Materials and Methods

Human subject samples

Our study protocol was approved by the Institutional Review Board (IRB), Central Michigan University, and Johns Hopkins University as not a human subject research, under exemption no. 4, as subject's lung function data and clinical parameters were obtained from NHLBI Lung Tissue Research Consortium (LTRC, NIH) (4, 58) without disclosing any of the subject's identifiers or demographic information. The clinical severity, sample size, and classification of COPD subjects utilized GOLD stage classification as defined by Global Initiative for Chronic Obstructive Lung Diseases (GOLD). The sample size included lung samples from GOLD 0, $n = 15$ (smokers: 5 and nonsmokers: 10); GOLD I, $n = 12$ (smokers: 10, nonsmokers: 2); GOLD II, $n = 11$ (smokers: 10, nonsmokers: 1); GOLD III, $n = 10$ (smokers: 10, nonsmokers: 0), and GOLD IV, $n = 12$ (smokers: 10, nonsmokers: 2)

COPD-emphysema subjects. The smokers, who were mostly ex-smokers (as expected for COPD subjects), also included few current smokers; GOLD 0—0, GOLD I—1, GOLD II—2, GOLD III—1, and GOLD IV—0. We have previously reported the demographic information of the non-emphysema and COPD-emphysema subjects used in this study (4). Briefly, non-emphysema or COPD subjects had no other underlying condition (or α1-antitrypsin deficiency), other than emphysema for COPD subjects, although we had one patient in each COPD group (GOLD I–IV) who had his/her first-degree blood relative suffering from chronic bronchitis.

Murine experiments

All animal experiments were performed as per the guidelines of CMU Institutional Animal Care and Use Committee (IACUC). C57BL/6 mice (8 weeks, $n = 4$) were separated into four experimental groups: (1) room air, (2) GEM, (3) sc-CS, and (4) sc-CS+GEM. The room air or side-stream CS exposures were performed for 2 months, as per our previously described protocol (58). CS was generated by burning 3R4F (0.73 mg nicotine per cigarette) research-grade cigarettes (Tobacco Research Institute, University of Kentucky, Lexington, KY) and mice were exposed to direct whole-body smoke exposure (3 h/day, followed by another 2 h in the same chamber without burning more cigarettes) using TE2 smoking machine (Teague Enterprises). This methodology resulted in an average total particulate matter of 150 mg/m^3. The mice were either treated with GEM (40 mg/kg body weight; Sigma) or equal volume PEG vehicle control by intraperitoneal (i.p.) injection on alternate days for 10 days (5 doses) before the termination of experiment. Mice were sacrificed in accordance with our IACUC-approved protocols, and BALF and lung tissues were collected for further analysis by flow cytometry, immunoblotting, and immunostaining.

Cell culture, autophagy flux/reporter, and ROS assays

The human bronchial epithelial cell line, Beas2b, was used in the study as an *in vitro* model for cigarette smoke extract (CSE) exposure. Standard cell culture conditions were utilized as recently described (5). Briefly, cells were maintained at 37°C, 5% CO$_2$ atmosphere in DMEM/F12 media with 10% fetal growth serum (RMBIO) and 1% PSA (penicillin, streptomycin, and amphotericin; Invitrogen). The CSE was prepared by burning two to three 3R4F (0.73 mg nicotine per cigarette) research-grade cigarettes (Tobacco Research Institute, University of Kentucky, Lexington, KY) and bubbling the smoke directly into the serum-free DMEM/F12 media (20 ml). An OD (320 nm) of 0.74 was considered as 100% CSE and CSE final concentration was adjusted by using the cell culture media. For autophagy reporter assay, cells were transiently cotransfected with ubiquitin-RFP and LC3B-GFP plasmids using Lipofectamine® 2000 (24 h; Invitrogen) and treated with vehicle control (PEG), CSE (5%), and/or GEM (10 μM) for 12 h. The effect of CSE and the TFEB-inducing drugs on functional autophagy was quantified using the Premo Autophagy Tandem Sensor RFP-GFP-LC3B assay kit (Molecular Probes), as recently described (51). Briefly, Beas2b cells were incubated with BacMam reagent for 16 h, followed by treatment with CSE (5%), GEM (10 μM), and/or fisetin (FIS, 25 μM; Indofine Chemical Company) for 12 h. Images for both experiments were cap-

tured using the ZOE ™ Fluorescent Cell Imager (Bio-Rad) as we recently described (5, 58). The changes in ROS levels were quantified using the CM-DCFDA ROS indicator dye (Invitrogen) as per the protocol reported recently (5, 51). Briefly, cells were treated as above with CSE (5%), FIS (25 μM), and/or GEM (10 μM) for 12 h and dye was added for 30 min, followed by fluorescence measurement as described before (5). In parallel set of experiments, the effect of TFEB knockdown (using human TFEB-Mission™ shRNA; Sigma and Lipofectamine 2000 transfection reagent; Invitrogen) and/or GEM treatment on ROS activation was quantified using the chloromethyl derivative of 2′,7′-dichlorodihydrofluorescein diacetate (CM-DCFDA) ROS indicator dye.

Immunoblotting, flow cytometry, and ELISA

We used our previously described (5, 58) immunoblotting method to quantify changes in ubiquitinated proteins (Ub), p62 (aggresome marker), VCP (UPR/autophagy mediator), TFEB (master autophagy regulator), GRP94 (ER stress marker), and β-actin in soluble and/or insoluble protein fractions of murine lung or Beas2b cell protein lysates. All antibodies were procured from Santa Cruz Biotechnology (scbt), except β-actin, which was from Sigma. To verify immunoblotting data, flow cytometry analysis was used to quantify the percentage of Ub-p62+ cells (either in human Beas2b or murine BALF cells), using BD FACS Aria and FACS Diva software as we described recently (5, 58). Finally, downstream effects were quantified by measuring changes in levels of IL-6 cytokine in BALF samples using a mouse-IL-6 ELISA kit (eBiosciences), following the manufacturer's instructions as previously described (51, 58).

Immunofluorescence microscopy and lung morphometry

Paraffin-embedded longitudinal lung tissue sections (5 μM) were prepared from all four experimental murine groups (Control vehicle PEG, GEM, sc-CS, and sc-CS+GEM). The lung sections were deparaffinized and immunostained using our previously described protocol (58). Briefly, primary antibodies (1 μg/ml) against ubiquitin (mouse monoclonal, scbt), p62 (rabbit polyclonal, scbt), or TFEB (rabbit polyclonal, scbt), followed by secondary antibodies (1 μg/ml) anti-rabbit CFL488 and/or anti-mouse-Texas Red (Santa Cruz), were used to localize changes in protein expression and/or co/localization. The Hoechst dye was used to stain the nuclei for identifying nuclear/perinuclear localization and expression. Images were captured by either the ZOE Florescent Cell Imager (Bio-Rad) or the Nikon Eclipse TI confocal inverted laser scanning microscope and NIS Elements software using a 60×/1.42 numerical aperture (NA) oil immersion objective. The sections were also stained with H&E to assess the inflammatory state and morphometric changes. Briefly, changes in mean alveolar diameter, Lm, were quantified by bright-field microscopy as we recently described in detail (58).

We also used paraffin-embedded longitudinal lung sections (5 μM) from normal/non-emphysema and COPD-emphysema subjects for quantifying changes in TFEB expression and localization. Briefly, lung sections from normal control subjects (GOLD 0) and each COPD-emphysema GOLD stage (GOLD I–IV) were immunostained, using a

TFEB (1 µg/ml) primary antibody and anti-rabbit CFL-488 (1 µg/ml) secondary antibody. Nuclei were stained using the Hoechst dye, followed by microscopy analysis under ZOE Fluorescent Cell Imager and confocal microscopy, as described above. The PROTEOSTAT Aggresome Detection kit (Enzo Life Sciences) was used to perform the co-immunostaining with TFEB.

Cell proliferation, senescence, and caspase-3/7 assays

Beas2b cells were exposed to CSE (5%) and/or GEM/FIS at the indicated doses for 12 h, and cell proliferation was quantified using the standard MTT/MTS assay kit (Promega) following the manufacturer's protocol, as we recently described (5). The Senescence Cells Histochemical Staining Kit (Sigma) was used to quantify changes in number of senescent cells in the different treatment groups as previously described (5, 51). The caspase-3/7 activity was also quantified in murine lung tissue lysates using the Caspase Glo™ assay kit (Promega) following the manufacturer's protocol.

Statistical analysis

Data are shown as mean ± SEM (or SD, as indicated) of each experimental group. A two-tailed unpaired Student's t-test was performed to determine the significance between the data sets and a p-value of less than 0.05 was considered a significant change. In addition, Pearson's correlation analysis was used to determine the correlation between the two data sets and the correlation coefficient is shown as "r" value. For immunoblotting data, densitometry was used to quantify changes using the ImageJ software (NIH).

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Authors' Contributions

Conception and design: N.V.; analysis and interpretation: M.B. and N.V.; experimental contributions: M.B., N.V., N.P., D.S., and K.W.; and drafting of manuscript and editing: M.B. and N.V.

Author Disclosure Statement

No competing financial interests exist.

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Abbreviations Used

BALF = bronchoalveolar lavage fluid
CBZ = carbamazepine
CFTR = cystic fibrosis transmembrane conductance regulator protein
CM-DCFDA = chloromethyl derivative of 2′,7′-dichlorodihydrofluorescein diacetate
COPD = chronic obstructive pulmonary disease
CS = cigarette smoke
CYS = cysteamine
eCV = e-cigarette vapor
ER = endoplasmic reticulum
FIS = fisetin
GEM = gemfibrozil
GFP = green fluorescent protein
GOLD = Global Initiative for Chronic Obstructive Lung Disease
p62/SQSTM1 = sequestosome 1
RFP = red fluorescent protein
ROS = reactive oxygen species
TFEB = transcription factor EB
Ub = ubiquitin
VCP = valosin-containing protein