

E-cigarette exposure triggers distal lung cell injury, persistent lung stress response, and antiviral immune suppression

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The mechanisms by which e-cigarette vaping (EV) affects lung health remain unclear. Clusters of EV-associated lung injury indicate that EV damages distal lung parenchyma and increases vulnerability to second-hit injury, including respiratory viral infections. Using human lung endothelial and epithelial cells and precision-cut lung slices, we investigated the mechanisms underlying distal lung cell injury and repair triggered by brief (24-hour) EV exposure. RNA-seq of lung tissue from golden Syrian hamsters evaluated the persistence of lung stress responses (10 days after 5 days of EV exposure) and the effect of EV on host defense against influenza A virus and SARS-CoV-2. EV disrupted the barrier function of human distal lung cells through JNK stress response signaling, triggered autophagy with impaired flux, suppressed mTOR signaling and cell proliferation, and culminated in apoptosis. Transcriptional responses in EV-exposed hamster lungs revealed persistent activation of JNK signaling, autophagy, barrier dysfunction, tissue remodeling, and impaired Th1 immunity. EV increased SARS-CoV-2 viral burden, downregulated antiviral genes (*Ifit1*, *Isg15*, *Nfkbia*), and amplified oxidative stress and IL-12 signaling. Short-term EV exposure triggered stress-induced distal lung cell injury with persistent changes in antiviral immunity and molecular pathways associated with tissue remodeling. That may increase susceptibility to viral infections and contribute to lung disease.

Introduction

Electronic cigarette (e-cigarette) vaping (EV) has emerged as a popular alternative to tobacco smoking (1, 2). EV poses important risks to lung health (3, 4), most dramatically reflected in a cluster of cases of acute respiratory failure from EV product use-associated lung injury (EVALI) (5), which was attributed to concurrent EV use with vitamin E or tetrahydrocannabinol or infection with respiratory viruses (5, 6). This suggests that EV predisposes to a second-hit injury of the distal lung, which may include weakening of the epithelial and endothelial lung barriers, thereby inducing acute lung inflammation and edema. Additionally, subtle repetitive lung barrier dysfunction has been implicated in chronic obstructive pulmonary disease (COPD) (7, 8) and pulmonary fibrosis (9, 10). We have shown that both nicotine-containing and nicotine-free EV weakens the lung endothelial cell barrier (11) through unknown mechanisms. EV exposure of primary human small airway epithelial cell (hSAEC) cultures and human precision-cut lung slices (PCLSs) triggers an exaggerated inflammatory response to viruses, weakening antiviral signaling (12, 13). EV has been shown to increase angiotensin-converting enzyme 2 (ACE2) levels, particularly in male mice (14), which could increase the susceptibility to coronavirus infection. These reports indicate that EV has deleterious effects on the host response to subsequent hits such as respiratory viral infections.

Stress-induced signaling pathways, including c-Jun N-terminal kinase (JNK), are critical regulators of cellular responses to environmental insults like reactive oxygen species or aldehydes that are present in EV (11). JNK signaling has been linked to increased endothelial and epithelial permeability, impaired repair, cell death, autophagy (15, 16), and inflammation. Although autophagy is a protective mechanism during stress, dysregulated autophagy may cause cell death and barrier dysfunction (17). Prior studies implicating JNK in autophagy in cigarette smoke-exposed cells suggest a role in EV-induced lung injury (17).

Authorship note: TCR and KSS are co-first authors.

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To better understand the acute and chronic effects of EV, most investigations relied on laboratory mice, revealing increased oxidative stress (11, 18), emphysema-like lung remodeling (19, 20), and extrapulmonary fibrosis (21). Further, most studies of EV in human specimens have focused on proximal airways (21). Additional animal modeling, complemented with studies in human distal lung specimens, is essential for bridging knowledge gaps. We study here golden Syrian hamsters (*Mesocricetus auratus*) because of their human-resembling small airway architecture (22) and inflammatory responses to respiratory viruses, including severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (23). Hamsters respond to acute EV exposure by upregulating airway epithelial inflammatory gene and oxidative stress responses (24), rendering it optimal to study EV effects on lung barriers and responses to influenza A virus (IAV) and SARS-CoV-2.

We show that EV triggers a JNK-mediated stress response that impairs distal lung barrier function and autophagy, with effects that persist even after cessation of EV exposure, manifesting as alterations in immune and tissue remodeling pathways that change host responses to acute respiratory viral infections.

Results

Barrier dysfunction of endothelial and epithelial monolayers following EV exposure. Trans-endothelial/epithelial electrical resistance (TEER, or TER) measured by electric cell-substrate impedance sensing (ECIS) and transcellular dextran permeability measured by fluorimetry were recorded over time following EV addition. In primary human lung microvascular endothelial cells (HLMVECs), exposure to EV resulted in a dose-dependent reduction in barrier function (Figure 1A). When normalized to before exposure, TER decreased immediately following EV concentrations (5 mM, 7.5 mM, and 10 mM) that simulate casual vaping in humans, with the 10 mM concentration showing the most profound and sustained effect (Figure 1B). EV impaired hSAEC-KT (25) barrier function in a dose-dependent manner (Figure 1C), with concentrations of 7.5 mM and 10 mM showing the greatest effect. In primary hSAECs differentiated into a pseudostratified layer at the air-liquid interface for 3 weeks, EVs reduced barrier function, as assessed by FITC-labeled dextran transmigration (Figure 1D). The slower kinetics compared with those measured by ECIS in the submerged cells likely reflect differences in assay methodology and sensitivity.

Autophagy in lung endothelial and epithelial cells following EV exposure. Next, we determined the effect of EVs on repair via stress-induced autophagy. In HLMVECs, EV exposure increased levels of microtubule-associated protein-1 light chain 3 β (LC3B-II), with a punctate appearance on immunofluorescence imaging, indicating autophagosome formation (Figure 2, A and B, and Supplemental Figure 1A; supplemental material available online with this article; <https://doi.org/10.1172/jci.insight.198757DS1>), and p62/sequestosome 1 (SQSTM1) (Figure 2, A and C, and Supplemental Figure 1B), consistent with autophagy without full autophagolysosomal degradation. Phospho-ribosomal protein S6 (phospho-S6) was reduced by EV (Figure 2D), indicating suppressed mechanistic target of rapamycin (mTOR), consistent with induction of autophagy. EV reduced cell proliferation, as indicated by decreased EdU staining (Figure 2E). EV increased ceramide levels in plasma membranes and perinuclear regions and increased the cleaved caspase-3 translocation to the nucleus (Figure 2, F and G), consistent with apoptotic signaling.

In hSAEC-KT, EV increased LC3B-II and p62/SQSTM1 levels (Figure 3, A–E) and markedly decreased cell proliferation, measured by EdU (Figure 3F). EV increased apoptosis, as indicated by increased ceramide staining at the plasma membrane (Figure 3G) and increased cleaved PARP1, cell rounding, and nuclear condensation (Figure 3H). Similar responses were observed in cells exposed to EV juice condensate (Figure 3I) and in human PCLSs exposed to EV (Figure 3, J and K).

JNK inhibition improves barrier function and autophagy completion in EV-exposed HLMVECs. To determine the mechanism by which EV triggers incomplete autophagy and barrier dysfunction, we next interrogated the MAPK pathways. In HLMVECs, under conditions of decreased barrier integrity induced by EV (7.5 mM), pretreatment with a pan-JNK inhibitor (SP600125, 50 μ M) significantly attenuated this effect (Figure 4, A and B), indicating that JNK contributes to barrier disruption. Western blotting showed that JNK inhibition significantly reduced EV-induced LC3B and p62/SQSTM1 levels (Figure 4, C and D). Complementary immunofluorescence microscopy showed that transformed HLMVECs pretreated with a JNK inhibitor, compared with vehicle, had reduced accumulation of p62/SQSTM1 (Figure 4, E and F). Co-immunostaining for p62/SQSTM1 and the lysosomal marker LAMP1 (Figure 4F) revealed that in EV-exposed cells, most p62-stained puncta did not colocalize with lysosomes (indicating reduced autophagolysosomal fusion). While JNK inhibition reduced the abundance of p62-stained puncta, it had no effect on LAMP1-stained lysosome abundance, suggesting that JNK does not impact lysosomal biogenesis.

Human Lung Microvascular Endothelial Cell Barrier Function

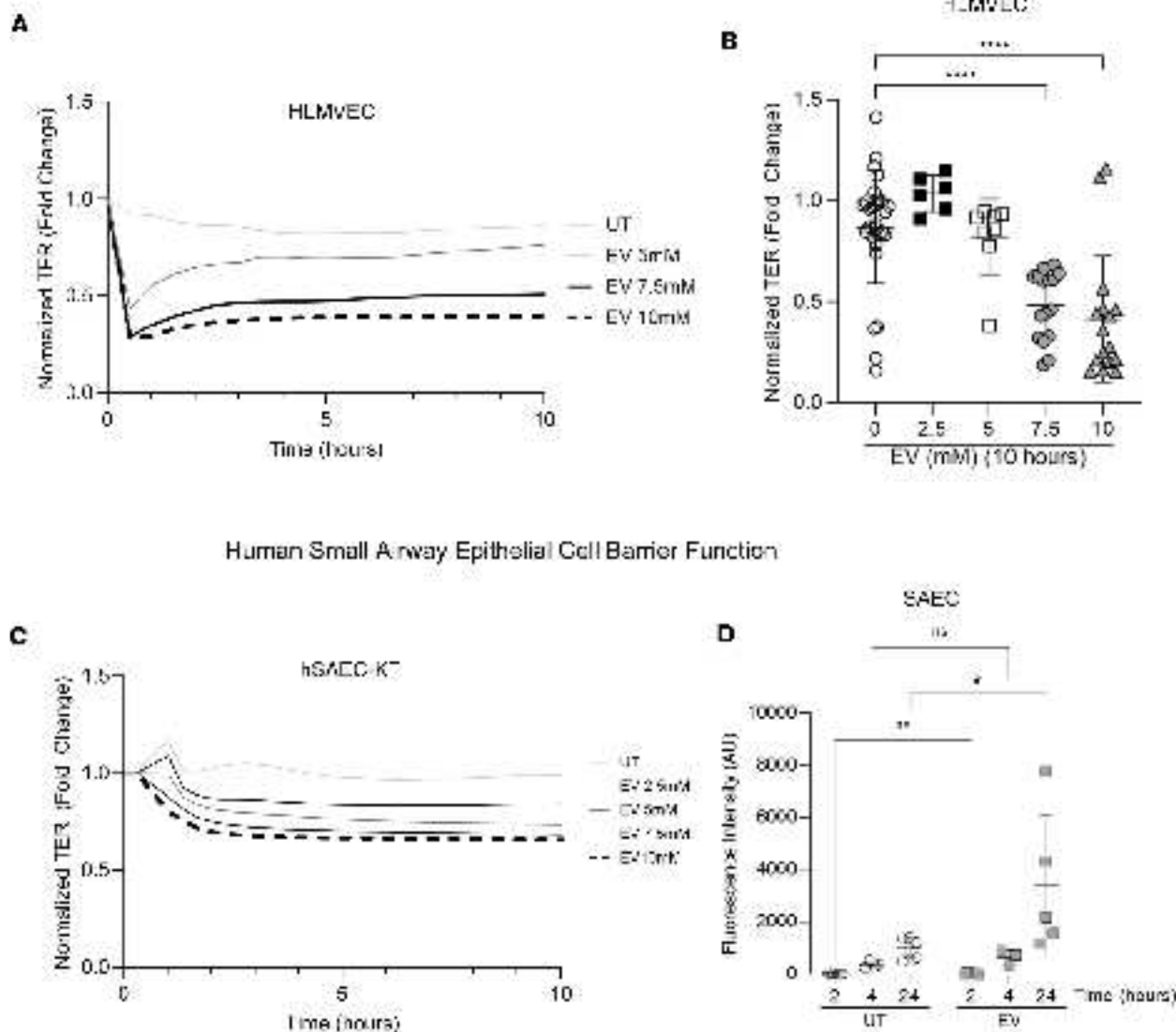


Figure 1. Barrier function of endothelial and epithelial mono layers following EV exposure. (A and C) Representative tracings of kinetics of trans-endothelial/epithelial electrical resistance (TER), normalized and expressed as fold change versus initial (prior to exposure), of primary human lung microvascular endothelial cells (HLMVECs; A) and transformed human small airway epithelial cells (hSAEC1-K7; C) exposed to EV at the indicated concentrations. (B) Scatterplot of normalized TER measured in HLMVECs exposed to EV for 10 hours. Mean $\pm$ SEM; symbols are individual biological replicates; 1-way ANOVA with Tukey's test ($n = 6-28$, **** $P < 0.0001$). (D) Scatterplot of fluorescence intensity, measured in arbitrary units (AU) in the basolateral chamber of FITC-labeled dextran (4 kDa) apically applied to a primary hSAEC pseudotransfected layer grown on a Transwell to air-liquid interface for 21 days and exposed to EV (7.5 mM) for the indicated times. Mean $\pm$ SEM; symbols are individual biological replicates; 1-way ANOVA ($n = 6-29$, * $P < 0.05$). UT, untreated.

The EV-induced increase in LC3B-II was significantly reduced by inhibitors of JNK, p38 MAPK, or ERK MAPK pathways (Figure 4C), whereas only JNK inhibition reduced EV-induced p62/SQSTM1 accumulation (Figure 4D). Therefore, JNK signaling is a critical mediator of both barrier dysfunction and autophagy dysregulation, whereas multiple MAPK pathways are involved in EV-triggered autophagosome formation.

JNK inhibition attenuates barrier dysfunction and autophagy in EV-exposed hSAECs. EV-induced barrier disruption in hSAEC1-K7 was attenuated by a pan-JNK inhibitor (Figure 5A). Confirming that EV induced JNK activation, immunoblotting and immunofluorescence demonstrated increased phospho-JNK levels (Figure 5, B and C, and Supplemental Figure 1C). The EV-triggered accumulation of LC3B-II and p62/SQSTM1 was reduced by JNK inhibition (Figure 5, B, D, and E), indicating improved autophagy completion. EV reduced phospho-S6, indicative of mTOR inhibition regardless of JNK inhibition (Figure 5, F and G), implying that JNK acts downstream or independently of mTOR in regulating the completion of autophagy.

Human Lung Microvascular Endothelial Cells

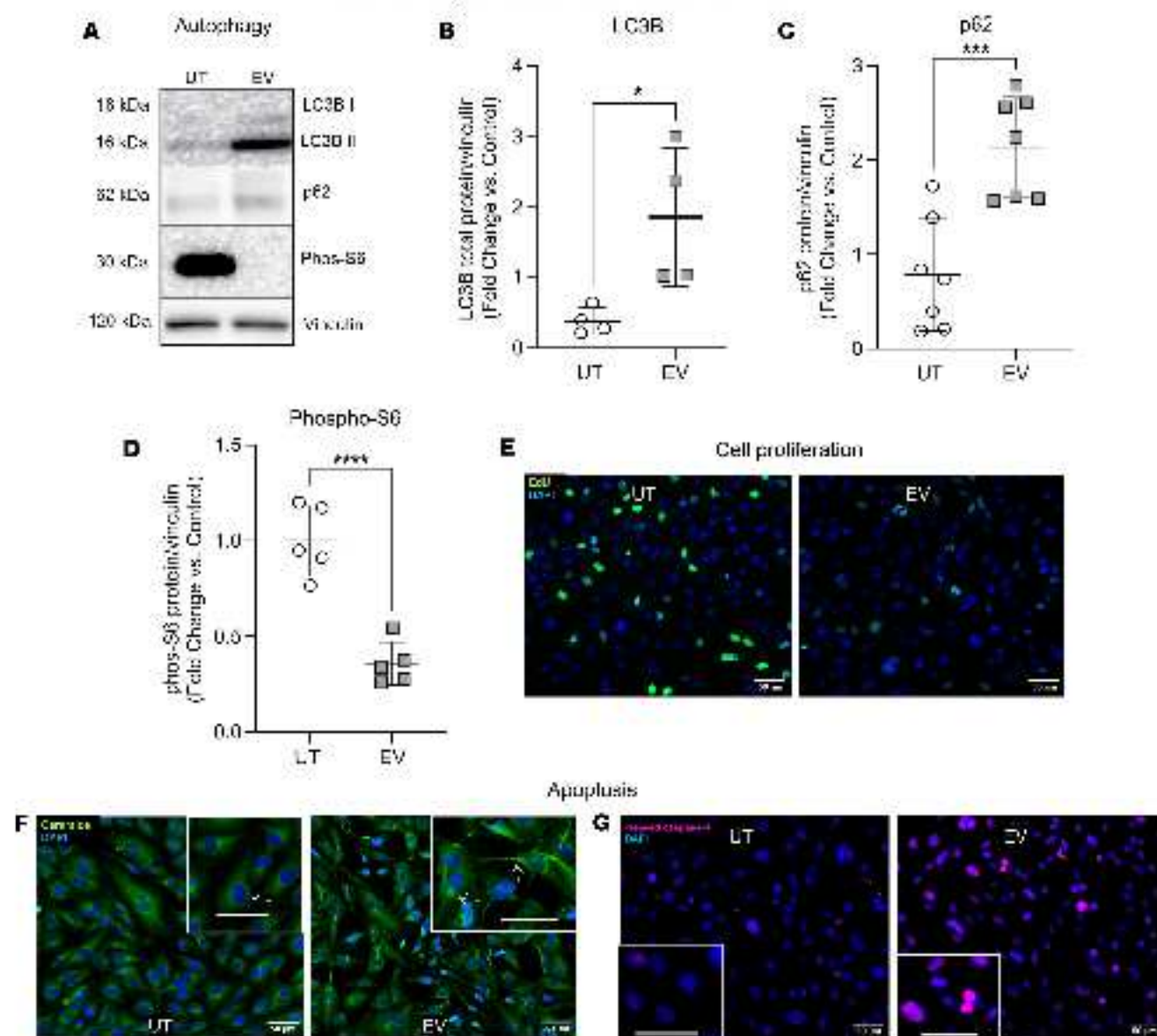


Figure 2. Effect of EV exposure on HLMVEC autophagy. (A) Representative Western blots of LC3B-II, p62/SQSTM1, and phosphorylated S6 in HLMVECs exposed to EV (7.5 mM, 24 hours). (B–D) Scatterplots of respective protein abundance measured by densitometry and expressed relative to vinculin loading control. Mean $\pm$ SEM; symbols are in individual biological replicates; unpaired *t* test (B, $n = 4$, $^*P = 0.03$; C, $n = 7$, $^{***}P = 0.002$; D, $n = 5$, $^{****}P < 0.0001$). (E–G) Representative immunofluorescence image of cells exposed to EV (7.5 mM, 24 hours) and stained for nuclei with DAPI (blue) and for incorporated fluorescently labeled EdU (green; E), ceramide (green; F), or cleaved caspase-3 (magenta; G). UT, untreated. Note that EV-exposed cells showed decreased proliferation; increased ceramide abundance predominantly localized perinuclearly (solid arrows in insets) and in plasma membrane (dashed arrow in inset); and increased cleaved caspase-3 abundance with almost exclusive nuclear localization (inset). Scale bars: 50 μ m.

Short-term EV exposure causes persistent cellular stress responses in hamster lungs. To gain further insights into repair processes and potential long-term consequences of a acute EV exposure, hamsters were exposed to EV aerosols at concentrations shown to simulate human exposure (26) via 5 consecutive daily nasal nebulizations. To evaluate persistent pulmonary effects of a relatively brief EV aerosol exposure, animals were allowed a 10-day recovery period, with lung tissue harvested on day 15 (Figure 6A). Expectedly, at the time of harvest, EV-exposed and recovered hamsters showed no clinical signs of illness, and no body weight changes (Supplemental Figure 2A), confirming that the model was nontoxic. Histopathology showed no signs of inflammation or fibrosis (Supplemental Figure 2, B and C). EV-exposed animals showed a 4-fold increase in p62 abundance in the distal lung parenchyma, an increase in p62 abundance

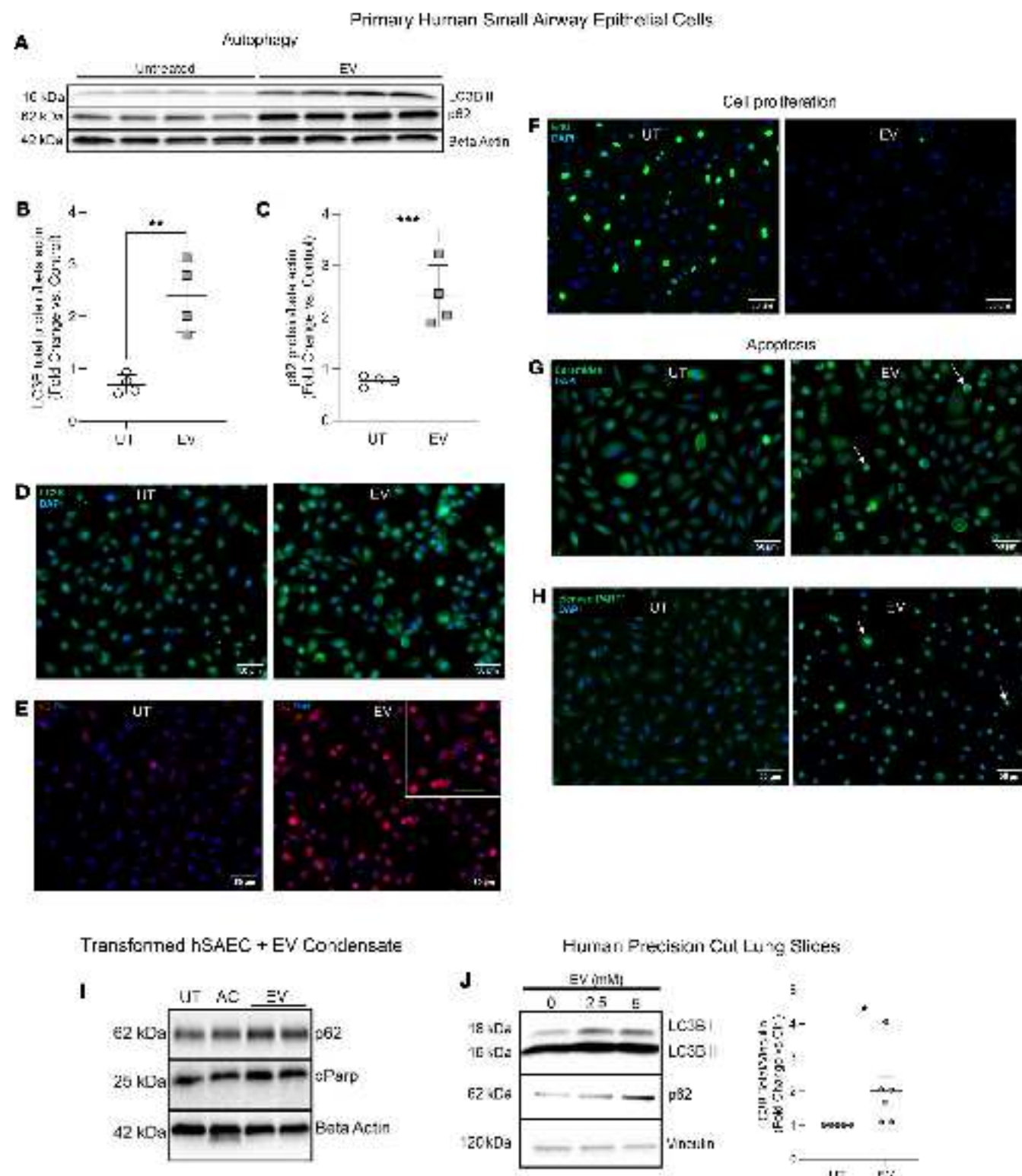


Figure 3. Effect of EV exposure on human lung small airway epithelial cell autophagy. (A) Representative Western blots of LC3B-II and p62 in hSAEC1-KT exposed to EV (7.5 mM, 24 hours). (B and C) Scatterplots of respective protein abundance measured by densitometry and expressed relative to β -actin loading control. Mean $\pm$ SEM; symbols are individual biological replicates; unpaired t test (B, $n = 4$, $**P = 0.003$; C, $n = 4$, $***P < 0.001$). (D–H) Representative immunofluorescence microscopy images of hSAEC1-KT exposed to EV (7.5 mM, 24 hours) and stained for nuclei with DAPI (blue) and for LC3B (green; D), p62 (red; E), incorporated fluorescently labeled EdU (green; F), ceramides (G), and cleaved PARP (green; H). (I) Representative Western blots of p62 and cleaved PARP (cPARP) with actin as loading control in hSAEC1 cells exposed to EV condensate or ambient air condensate (5%; 24 hours, $n = 3$). UT, untreated. (J) Representative Western blots of LC3B-I/II and p62 with vinculin as loading control in human precision-cut lung slices exposed to EV at the indicated concentrations ($n = 5$) with relative changes measured by densitometry shown as fold change vs. UT ($*P < 0.05$). Scale bars: 50 μ m.

colocalizing with CD31-positive endothelial cells lining small pulmonary arteries, and a significant increase in p62 in epithelial cells lining small airways (Figure 6, B and C), consistent with impaired autophagic flux. We noted cleaved caspase-3 presence in EV-exposed lungs, even at 10 days after exposure (Figure 6D). Costaining for CD31 and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) revealed that endothelial cells had DNA strand breaks at 10 days after EV exposure (Figure 6E). These results suggest modest but persistent apoptosis in distal lungs following EV exposure.

Persistent transcriptomic responses in hamster lungs following brief EV exposures. To comprehensively profile biological processes, signaling, and disease associations driven by EV exposure, we performed RNA-seq of hamster lungs and analyzed pathway enrichment. RNA-seq, filtered for log₂ fold change greater than 1 or less than -1 and *P* less than 0.05, revealed about 200 upregulated and about 300 downregulated lung genes. In this analysis, while many genes achieved *P* value significance, far fewer met the more stringent FDR (*q*) threshold, likely because of brief, nontoxic exposure followed by recovery, the inherent variability in transcriptomic data, and the relatively low number of animals per group. However, the observed nominal significance of differentially expressed genes may provide biologically meaningful information that will require further corroboration.

To validate the results obtained in human cells, we first interrogated the JNK signaling pathway. Although this was not the top differentially regulated pathway during the recovery from acute EV exposure, interactive pathway analysis predicted that EV activates the JNK signaling pathway as a result of (nominally) significant increase in expression of the upstream JNK activators *Spag9* (JNK-interacting protein-4; 4-fold; *P* = 0.03) and *Aida* (axin-interacting protein-1; 9-fold; *P* = 0.02) (Figure 7, A–C), as well as the downstream JNK mediator *JunD*, which was also increased 5-fold (*P* = 0.003). In addition to *JunD*, a central signaling pathway hub linking cellular stress to barrier function and autophagy, another notable effector change was the decrease in *Dach1* (Dachshund homolog 1), predicted to impair autophagy completion (27) and trigger lung epithelial cell apoptosis, promoting fibrosis (28). EV decreased *Dach1* by 50% (*P* = 0.0007) while upregulating *Ager* (advanced glycation end product receptor), *Eng* (endoglin), *IL1β*, *Igfb4* (integrin β₄), and *Tlr4*, which may link EV-induced JNK activation to barrier dysfunction and autophagy. These results demonstrate cross-validation between the human cell experiments and the animal model, reinforcing the biological significance of the identified pathways.

We determined which transcripts are most differentially modified by remote EV exposure in hamster lungs. The top 20 transcripts that were most increased by prior EV exposure in hamster lungs and that reached nominal statistical significance (Table 1) included genes involved in protein degradation (such as ~40-fold increase in ubiquitin-conjugating enzyme E2 O), innate immune defense (~14-fold increase in BPI fold containing family B member 1), coagulation and inflammation (such as ~13-fold increase in tissue factor), cell adhesion and proliferation (such as tumor-associated calcium signal transducer 2 and LY6/PLAUR domain containing 2), and stress response (Sumo1/sentrin peptidase 1, Nudix hydrolase 13, and *JunD*). The 20 transcripts most decreased by prior EV exposure in hamster lungs and that reached nominal statistical significance (Table 2) were also genes involved primarily in proteostasis and matrix remodeling, such as ubiquitin-conjugating enzyme E2 D1, protease, serine 35, and osteopontin, with *UB5* (ubiquitin-like protein 5) registering a statistically significant decrease of more than 600-fold (*P* = 4.51×10^{-5} , *q* = 0.04). Also downregulated were genes involved in mitochondrial function and the immune response, such as galectin-9, *Cxcr2*, and *Nod2*. These findings suggest that EV contributes to a persistent stress response and signaling that impact immune function and tissue remodeling.

When examined by enrichment scores, the top 5 functional categories of gene pathways impacted by EV (Figure 7D) included cellular movement, followed by the cellular assembly and organization pathway, suggesting cytoskeletal remodeling; cell death and survival; cellular development; and cellular function and maintenance. These pathways are consistent with EV-triggered activation of autophagy, stress-adaptive survival, and cellular repair mechanisms.

When analyzed using patterns related to biological responses or disease associations, pathway enrichment analysis revealed significant activation of actin cytoskeleton, hepatic fibrosis, and neutrophil extracellular trap signaling (Supplemental Figure 3A) as well as cancer, inflammatory disease, and cell-mediated immune response (Supplemental Figure 3B). Interestingly, gene expression was predominantly inhibited in NET, RHOGDI, and Th1 pathways, suggesting immune suppression or impaired inflammatory resolution. Other activated pathways were linked to metabolism and endocrine regulation, with notable enrichment of the P2Y purinergic receptor signaling pathway, given its key role in airway inflammation, mucus hypersecretion, vascular reactivity, and pulmonary surfactant homeostasis.

Human Lung Microvascular Endothelial Cells

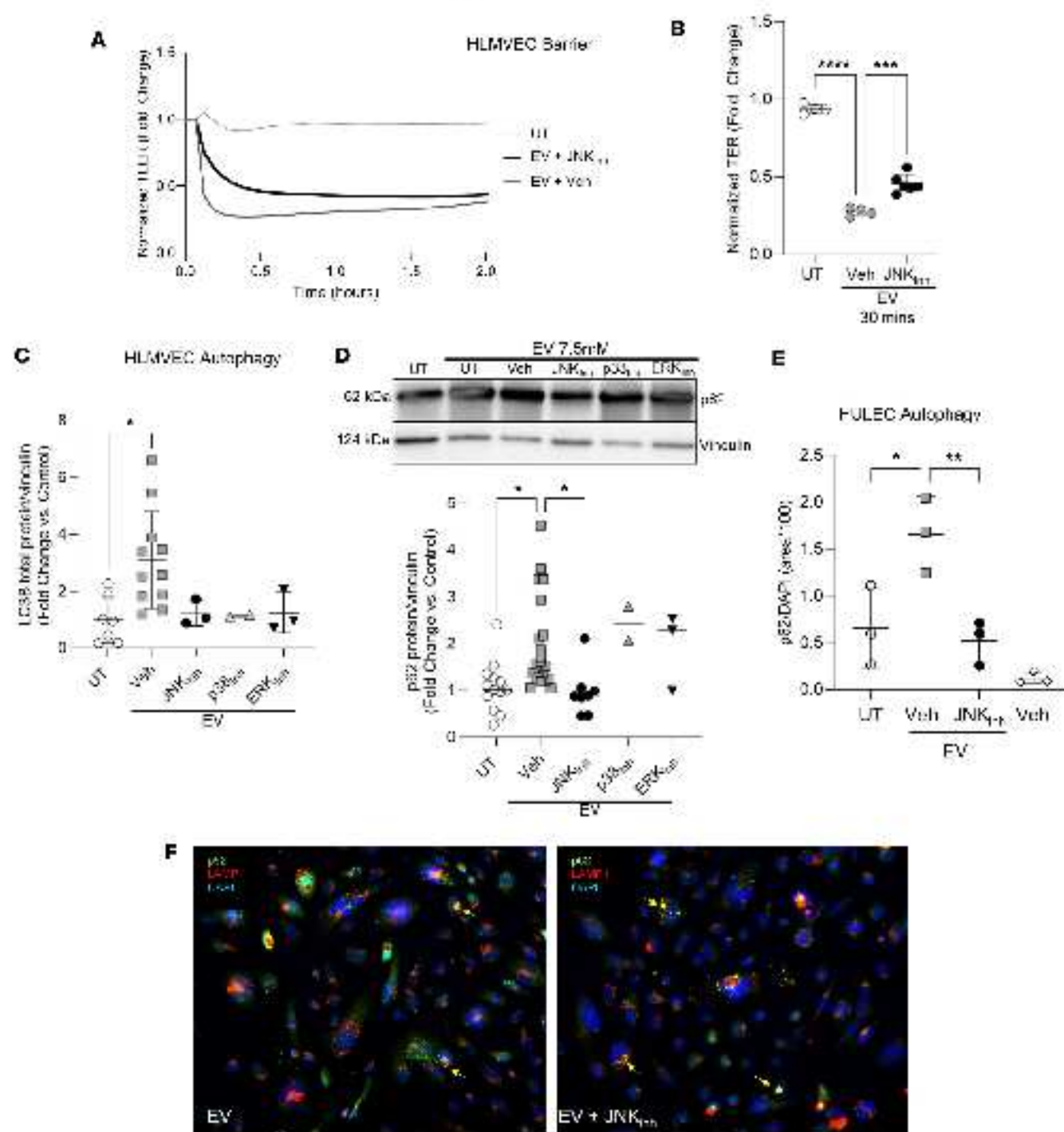


Figure 4. Role of JNK in EV-induced human lung microvascular endothelial autophagy. (A) Representative tracings of kinetics of normalized TER expressed as fold change versus initial measurement in HLMVECs exposed to EV (7.5 mM) in the presence of JNK inhibitor (JNK_{inh}, SP600125, 50 μ M) or vehicle (Veh; DMSO, 1%). (B) Scatterplot of normalized TER measured in HLMVECs exposed to EV (7.5 mM, 30 minutes) in the presence of JNK_{inh} or Veh. Mean $\pm$ SEM; symbols are individual biological replicates; 1-way ANOVA, Tukey's test ($n = 4-6$, *** $P < 0.001$, **** $P < 0.0001$). (C and D) Scatterplot of densitometry of LC3B-II (C) and p62 (D) relative to vinculin loading control in HLMVECs exposed to EV (7.5 mM, 24 hours) in the presence of JNK_{inh}, p38 MAPK inhibitor (p38_{inh}, SB202190, 30 μ M), ERK inhibitor (ERK_{inh}, PD98059, 50 μ M), or Veh. Mean $\pm$ SEM; 1-way ANOVA, Tukey's test (C, $n = 2-11$, * $P = 0.03$, D, $n = 2-22$, * $P = 0.01$). Representative Western blot of p62 and vinculin is shown in D. (E) Scatterplot of relative abundance of p62 relative to DAPI detected by immunofluorescence (IF) microscopy and measured by blinded automatic image analysis, in HULECs exposed to EV (7.5 mM, 24 hours) in the presence of Veh or JNK_{inh}. Mean $\pm$ SEM; 2-way ANOVA, Tukey's test ($n = 3$, * $P < 0.05$, ** $P < 0.01$). (F) Representative IF images of HULECs exposed to EV in the presence of vehicle (left) or JNK_{inh} (right), immunostained for p62/SQ5 TM1 (green), lysosomes (LAMP1, red), and nuclei (DAPI, blue). Yellow arrows indicate superimposed p62 and LAMP1 staining (yellow) suggesting autophagolysosome fusion events. Original magnification $\times 40$. UT, untreated.

Short-term EV exposure alters host lung responses to acute respiratory viral infections in hamster lungs. The transcriptional changes in immune response pathways indicate that EV may modulate host responses to respiratory viral infections. We designed the experiments to address the following two questions: does EV pre-exposure modify the lung injury response to SARS-CoV-2; and does EV pre-exposure modify the effect of co-viral infection (with IAV) on the lung injury induced by SARS-CoV-2? The goal was not to evaluate (the effect of EV on) IAV-induced acute lung injury; rather, IAV served solely as a coinfection with SARS-CoV-2, reflecting a common clinical scenario. Given our focus on SARS-CoV-2-mediated injury, we selected a literature-supported time point corresponding to peak SARS-CoV-2 lung injury, and we exposed hamsters to daily EV for 5 days, followed by SARS-CoV-2 (3 days later); or, sequentially, to both IAV (the following day) and SARS-CoV-2 (3 days later) (Figure 8A). Lungs were harvested on day 7 after SARS-CoV-2 infection (during the injury phase). This experimental design also enabled us to test the effect of IAV pre-infection on host responses to SARS-CoV-2, with or without EV exposure.

To test the effect of EV on upper airway virus infectivity, we measured oropharyngeal SARS-CoV-2 viral titers during the first 3 days following infection (Figure 8B). A 3-way ANOVA factoring time, EV exposure, and IAV infection showed that time ($P = 0.004$) played a significant role, and that EV significantly increased viral titers of SARS-CoV-2 ($P = 0.04$) (Figure 8B). Although IAV infection reduced SARS-CoV-2 titers, this did not reach statistical significance in this analysis ($P = 0.13$). As expected, at 7 days after SARS-CoV-2, animals had significant weight loss that was more pronounced in male animals (Supplemental Figure 2B). EV pre-exposure significantly ($P = 0.04$) worsened weight loss in female animals infected with SARS-CoV-2 (Figure 8C), while males pre-exposed to EV had less weight loss ($P < 0.0001$) (Figure 8C). A 3-way ANOVA factoring EV exposure, IAV coinfection, and sex showed that IAV and sex had a significant impact on SARS-CoV-2-induced weight loss ($P = 0.004$), with IAV attenuating and male sex accentuating the SARS-CoV-2-induced weight loss, and that EV significantly interacted with these 2 variables ($P = 0.0006$), modifying their effect on weight loss (Supplemental Figure 2B).

Analysis of bronchoalveolar lavage fluid inflammatory cells revealed that SARS-CoV-2 significantly decreased the abundance of macrophages and increased that of lymphocytes, neutrophils, and eosinophils (Figure 8, D–I). EV pre-exposure decreased macrophage ($P = 0.01$) and increased lymphocyte ($P = 0.04$) abundance only in female animals coinfecting with IAV and SARS-CoV-2 (Figure 8, E and G). A 3-way ANOVA factoring EV exposure, sex, and IAV coinfection showed a significant effect of IAV on (increasing) lymphocyte predominance and (decreasing) neutrophil predominance in response to SARS-CoV-2 ($P < 0.05$ for each) (Supplemental Figure 2, D and E), consistent with interference, and confirmed significant interaction of EV with sex and with sex and IAV on macrophage abundance ($P < 0.05$ for each) (Supplemental Figure 2C).

Histological analysis was consistent with increased inflammatory injury induced by SARS-CoV-2 in comparison with uninfected lungs, regardless of EV exposure (Figure 8, J and K). A 3-way ANOVA factoring EV exposure, sex, and IAV coinfection showed only a trend of impact of EV ($P = 0.09$) and sex ($P = 0.07$) on lung injury induced by SARS-CoV-2 (Supplemental Figure 2G).

Overall, these results show that remote short-term exposure to EV is sufficient to alter the susceptibility to respiratory infection with SARS-CoV-2, as evidenced by increased viral titers and (sex-dimorphic) weight loss. However, these changes were insufficient to alter lung inflammation and injury in response to SARS-CoV-2. Our data also indicate that 3 days of pre-infection with IAV was sufficient to interfere with SARS-CoV-2 infection, reducing its infectivity and its detrimental effect on weight loss, preventing the decrease in, and even increasing, macrophage abundance, increasing lymphocytes, and decreasing eosinophils. EV was synergistic with IAV in its effects on body weight and macrophage and lymphocyte inflammation in females, suggesting it may share mechanistic features of viral interference.

EV exposure changes transcriptome responses to respiratory viral infections. To gain insight into potential mechanisms underlying antiviral host responses triggered by EV, the transcriptomic profiles of lungs pre-exposed to EV and then infected with SARS-CoV-2 were compared with those of animals infected but unexposed to EV. Several significant effects were noted (Table 3), including EV increases in *Mmp13* (3-fold; $P = 9.0 \times 10^{-7}$, $q = 0.001$), *Mmp12* (3-fold; $P = 0.0001$, $q = 0.035$), and *osteopontin* (*Spp1*; 2-fold; $P = 0.0002$, $q = 0.05$) that, coupled with downregulation of *Timp1* and *Fmod*, suggested that EV enhances lung tissue remodeling following virus infection. Downregulation of *Scl31a1* is essential for cytochrome c oxidase (by 30%, $P = 4.2 \times 10^{-4}$, $q = 3.87 \times 10^{-5}$) and for critical components of the electron transport chain, indicating significant effects on mitochondrial responses to viral infection. EV-pre-exposed lungs

Human Small Airway Epithelial Cells

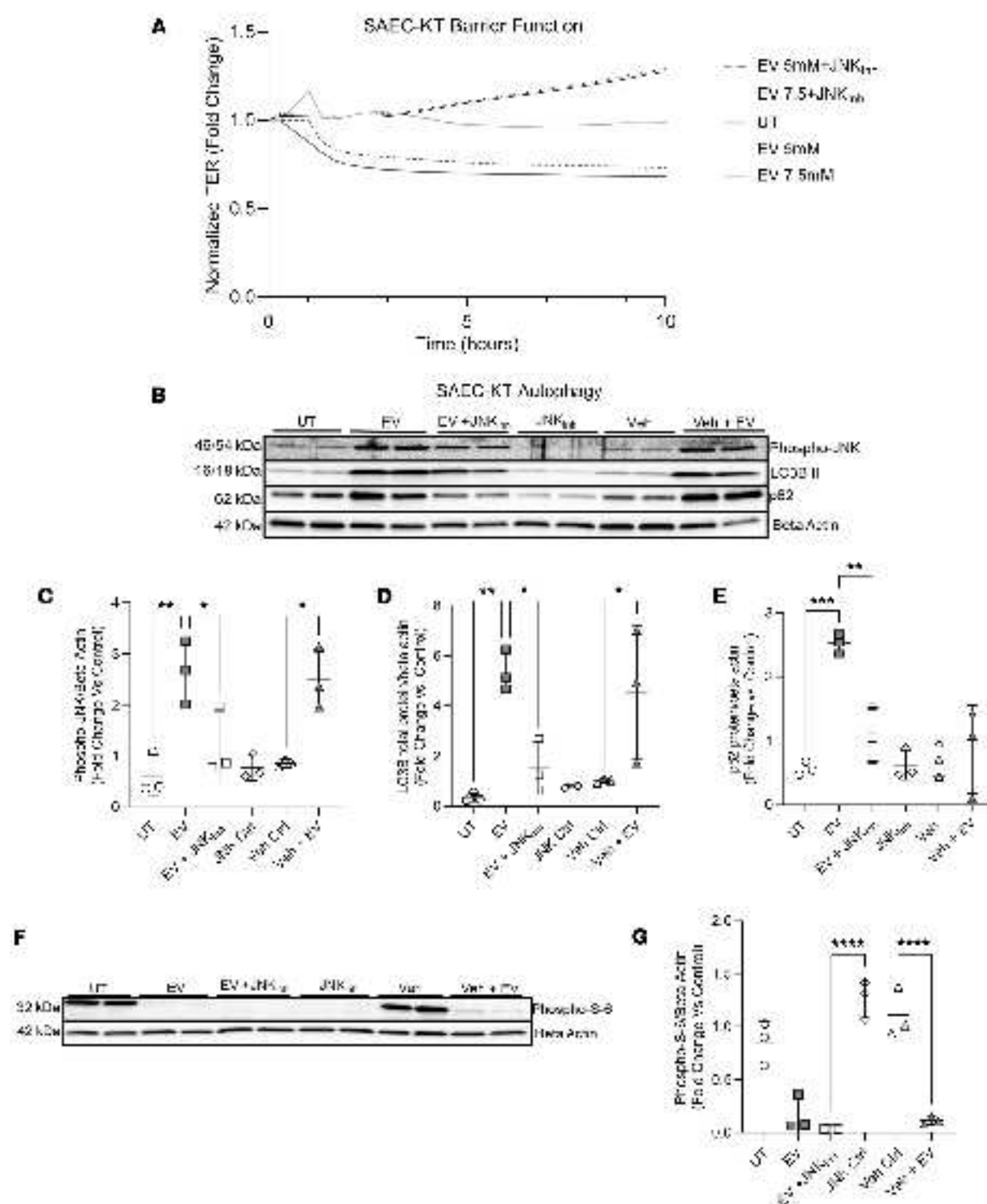


Figure 5. Role of JNK in EV-induced human lung small airway epithelial autophagy. (A) Representative tracings of kinetics of TER normalized and expressed as fold change versus initial measurement in hSAEC1-KT exposed to EV (5 or 7.5 mM) in the presence of JNK_{inh} (SP60125, 50 μ M) or vehicle (DM50). (B–E) Representative Western blots (B) and respective densitometries of phospho-JNK (C), LC3B-II (D), and p62 (E) relative to β -actin (as loading control) in hSAEC1-KT exposed to EV (5 or 7.5 mM, 24 hours) in the presence of JNK_{inh} (SP60125, 50 μ M) or vehicle. (F and G) Representative Western blots (F) and respective densitometries of phospho-S6 (G) relative to β -actin. (C–E and G) Mean $\pm$ SEM; symbols are individual biological replicates ($n = 3$ in each, 1-way ANOVA, Tukey's test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$). UT, untreated.

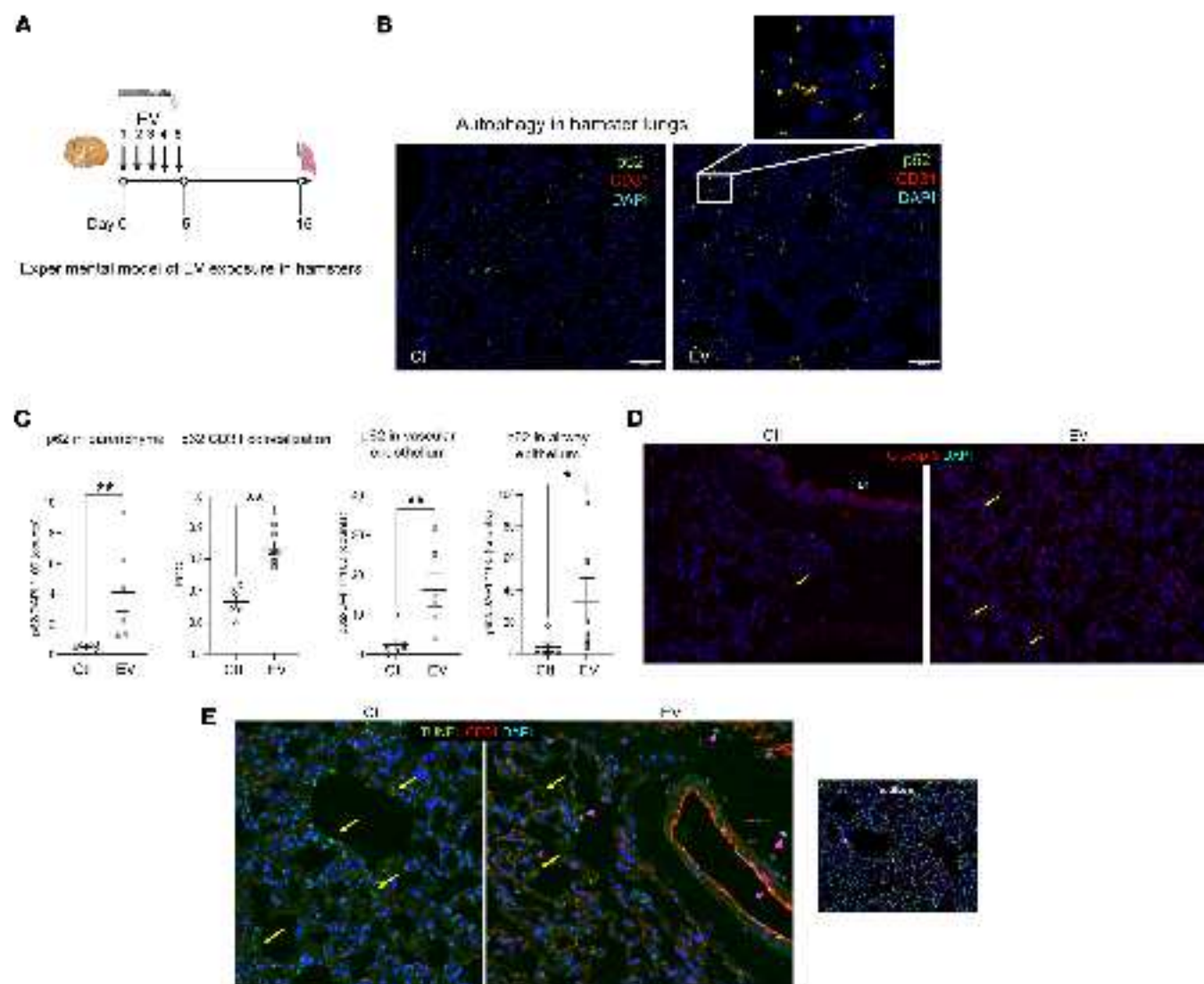


Figure 6. Persistent effects of brief EV exposure on distal lung autophagy in golden Syrian hamsters. (A) Schematic of experimental design depicting daily EV exposure via inhalation for 5 consecutive days, followed by removal from exposure for 10 days and harvest at day 15. (B) Representative immunofluorescence images of EV-exposed hamster lung parenchyma stained for nuclei (DAPI, blue), autophagy marker p62/SQSTM1 (green), and endothelial cell marker CD31 (red) (scale bar size: 10.0 µm; inset: 50x magnification). (C) Respective scatterplots of abundance of p62 in the parenchyma, including endothelial p62-CD31 cell colocalization (PCC) scores; in the vascular endothelium; and in the airway epithelium. * $P < 0.05$, ** $P < 0.01$. (D and E) Representative immunofluorescence images of EV-exposed hamster lung parenchyma stained for nuclei (DAPI, blue) and cleaved caspase-3 (red; yellow arrows), ×10 magnification. (D) or DNA strand break marker TUNEL (green; yellow arrows) and endothelial cell marker CD31 (red), ×20 magnification (E). Note the colocalization of TUNEL/CD31 in EV-exposed lung parenchyma (magenta arrowheads). As a positive control, lung slides were treated with DNase.

responded to SARS-CoV-2 with significantly increased neuritin (*NRN1*; 4-fold; $P = 9.26 \times 10^{-5}$, $q = 0.033$), a pro-angiogenic molecule with neuroendocrine signaling effects, and decreased TMF-regulated nuclear protein 1 (*TRNP1*; by 80%; $P = 2.7 \times 10^{-10}$), an inhibitor of cell proliferation. Pathway analysis showed that lungs pre-exposed to EV exhibited decreased phagosome formation, decreased immune cell death, and enhanced NETosis, IL-12 signaling, muscle cell death, and vascular lesion formation in response to SARS-CoV-2 infection (Supplemental Figure 4A). When EV preceded both IAV and SARS-CoV-2, similar patterns of gene expression were observed, with EV-exposed lungs showing upregulation of genes critical for extracellular matrix integrity and downregulation of genes involved in energy production and protein synthesis (Table 4).

Pathway analyses indicated activation of fibrosis and actin cytoskeletal reorganization (Supplementary Figure 4A) and downregulation of antiviral genes and innate immune responses, as well as those involved in immune recruitment and resolution (Supplemental Figure 4B). Signaling by IL-12, a central Th1 immune

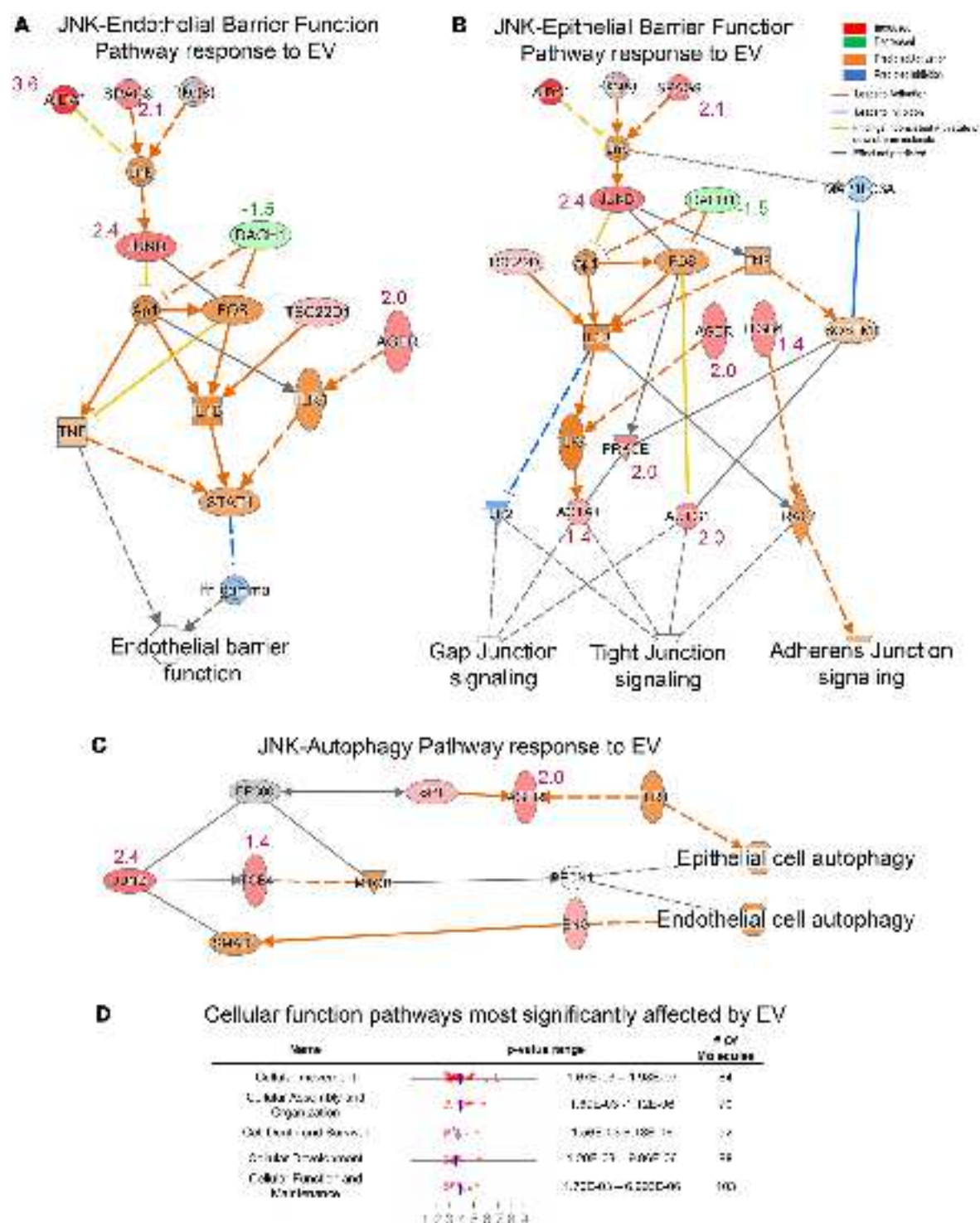


Figure 7. Interactivity map of JNK signaling induced by brief distant EV exposure in hamster lungs. (A–C) Persistent EV effects on JNK-anchored signaling pathways linked to endothelial barrier function (A), epithelial barrier function (B), and cell autophagy (C). Molecules that are predicted or measured as activated are shown in orange or red, respectively (with indicated log₂ fold changes), and those that are predicted or measured to be inhibited are shown in blue or green, respectively. Note predicted JNK activation by upstream regulators (e.g., SLC41A3, TRAF1, ALC1), and downstream activation of JunB and Ap1 and of proinflammatory mediators such as IL1B. Jnk is predicted to drive autophagy in epithelial and endothelial cells through pathways involving TLR4 and ENG, respectively. JunB activation (red), which promotes autophagy via transcriptional activation of ITGB4 and AGC1, should lead to mTOR upregulation and subsequent autophagy regulation through BECN1. However, downstream mTOR targets are not increased, suggesting its activity is suppressed. AGC1/TLR4 signaling drives epithelial autophagy, whereas ENG signaling mediates endothelial autophagy. (D) Dot plot graph and summary table showing the P value distribution for pathways involved in cellular and molecular function and the corresponding number of molecules involved.

Table 1. Top 20 upregulated genes in hamster lung following EV exposure

Gene	Log ₂ fold change	P value	Full name and function
Ube2o	4.363	0.00463	Ubiquitin-conjugating enzyme E2 O; involved in protein ubiquitination and degradation
Tacstd2	4.22	0.0009	Tumor-associated calcium signal transducer 2; involved in cell adhesion and cancer progression
Bpifb1	3.856	0.0107	BPI fold containing family B member 1; lipid-binding protein involved in innate immunity
F3	3.711	0.012	Coagulation factor III (tissue factor); initiates blood coagulation and promotes inflammation
Sft2d2	3.629	0.0397	SFT2 domain-containing protein 2; functions in vesicle trafficking and membrane fusion
Senp1	3.452	0.0211	SUMO1/sentrin peptidase 1; regulates de-SUMOylation and protein stress response
Nudt13	3.304	0.036	Nudix hydrolase 13; involved in nucleotide metabolism and cellular stress responses
Iqck	3.036	0.0103	IQ motif containing K; putative regulator of calcium signaling pathways
Mcm9	2.898	0.0205	Minichromosome maintenance complex component 9; essential for DNA replication and genome stability
Gas2l1	2.828	0.0159	Growth arrest specific 2 like 1; cytoskeletal protein involved in actin stabilization
Clkm	2.814	0.0224	Creatine kinase, muscle; key enzyme in energy metabolism and ATP regeneration
Enpp1	2.778	0.0128	Extracellular nucleotide pyrophosphatase/phosphodiesterase 1; regulates extracellular nucleotide levels and mineralization
Znf365	2.603	0.0103	Zinc finger protein 365; involved in DNA repair and genomic stability
Ube2s	2.581	0.0169	Ubiquitin-conjugating enzyme E2 S; targets proteins for degradation during cell cycle progression
Cacnb4	2.541	0.047	Calcium channel voltage-dependent β_4 subunit; regulates calcium influx in signaling pathways
Cand2	2.511	0.0291	Cullin-associated NEDD8-dissociated protein 2; implicated in muscle contraction and signaling pathways
Fam189a2	2.442	0.0217	Family with sequence similarity 189 member A2; membrane protein linked to signal transduction
Jund	2.41	0.00033	Jund proto-oncogene; component of the AP-1 transcription factor; regulates stress and inflammation
Lypd2	2.389	0.0108	LY6/PLAUR domain containing 2; involved in cellular adhesion and signaling
Chst2	2.383	0.026	Carbohydrate sulfotransferase 2; regulates glycosylation and ECM remodeling

cytokine that stimulates IFN production and IL-23 and suppresses Th17 cell responses, was increased by EV in doubly infected lungs (Supplemental Figure 4, C and D). These changes suggest that EV exposures are favorable for viral persistence and may promote chronic inflammation in response to viral infections.

IAV pre-infection altered transcriptional responses to SARS-CoV-2 infection, enhancing pulmonary and hepatic fibrosis pathways (Supplemental Figure 5A), increasing estrogen receptor signaling (Supplemental Figure 5, A and D), and suppressing interferon signaling (Supplemental Figure 5, B and C) and cell proliferation while increasing DNA damage and inhibiting coronavirus pathogenesis pathways (Supplemental Figure 5B).

Discussion

These results indicate that even short-term EV exposure has detrimental effects on lung health, with EVs potentially directly injuring distal lung cells, triggering a stress response that decreases the barrier function of small airway epithelial and lung microvascular endothelial cells, and leading to incomplete cell repair via stress-autophagy that may culminate in apoptosis. This stress response persists for at least 10 days after short-term EV exposures, with transcriptomic changes that predict decreased immune defenses against respiratory viruses, and notable alterations in proteostasis and tissue remodeling that might herald the development of chronic lung diseases following habitual EV use.

The pulmonary consequences of vaping remain an important public health concern marked by important knowledge gaps and the misconception that EV is a safer alternative to cigarette smoking. By focusing on the distal lung, our study addresses unmet needs (29), complementing knowledge of the harmful effects of EV on the proximal airways (30–32) or on specific cell populations (33). By using a popular commercial EV product and integrating studies of human distal lung structural cells with those in laboratory animals that share distal lung anatomy and immune responses to respiratory virus infection with humans, our study has clinical relevance. However, we did not dissect potentially distinct (11, 12, 34–36) nicotine-dependent versus nicotine-independent effects, or those of various flavorings, and, owing to BSL-3 regulations at the time of the study, we relied on aerosolizing EV from condensate, which lacks short-lived volatile compounds.

Acute EV exposure of human lung endothelial and epithelial cells disrupted the distal lung structural cell barrier function, thereby weakening lung defenses and promoting inflammation, a process pertinent to the development of several chronic lung diseases, including COPD and lung fibrosis, which are also

Table 2. Top 20 downregulated genes in hamster lung following EV exposure

Gene	Log ₂ fold change	P value	Full name and function
Ube2d1	-1.626	0.00089	Ubiquitin-conjugating enzyme E2 D1; involved in protein degradation and ubiquitin signaling
C15orf48	-1.629	0.00225	Chromosome 15 open reading frame 48; involved in immune response and inflammation
Bms1l	-1.934	0.038	Breast cancer metastasis suppressor 1-like; suppresses metastasis and regulates gene expression
Nod2	-1.977	0.00088	Nucleotide-binding oligomerization domain 2; pattern recognition receptor in immune response
Rab5b	-2.02	0.0103	RAB5B, member RAS oncogene family; regulates endocytosis and intracellular trafficking
Nrn1	-2.059	0.0195	Neuritin 1; promotes neuronal survival, plasticity, and repair
Cd274	-2.087	0.00916	CD274 (PD-L1); immune checkpoint protein that inhibits T cell activation
Aoah	-2.132	0.0193	Acylcoacyl hydrolase; detoxifies bacterial lipopolysaccharides to modulate inflammation
Cxcr2	-2.397	0.0108	C-X-C chemokine receptor type 2; receptor involved in neutrophil chemotaxis and inflammation
Ms4a7	-2.633	0.0364	Membrane-spanning 4-domains A7; linked to immune cell function
Tbx21	-2.827	4.64×10^{-4}	T-box transcription factor 21; controls differentiation of T-helper cells
Spp1	-2.861	0.0103	Secreted phosphoprotein 1; osteopontin, regulates immune responses and tissue remodeling
Dlx5	-3.163	0.0189	Distal-less homeobox 5; regulates bone and craniofacial development
Mars2	-3.226	0.0177	Mitochondrial methionyl-tRNA synthetase 2; catalyzes aminoacylation of mitochondrial tRNA
Lgals9	-3.459	0.0176	Galactin-9; modulates immune responses, apoptosis, and inflammation
Pss35	-3.505	0.0305	Protease, serine 35; involved in ECM remodeling and proteolytic activity
Mps10	-3.666	0.00181	Mitochondrial ribosomal protein S10; essential for mitochondrial protein synthesis
Slc13a4	-6.006	0.0207	Solute carrier family 13 member 4; sodium-dependent phosphate transporter
Dmp1	-7.569	0.0152	Dentin matrix protein 1; involved in bone mineralization and phosphate regulation
Ubl5	-9.245	4.51×10^{-4}	Ubiquitin-like protein 5; regulates mitochondrial stress response and RNA splicing

characterized by decreased lung barrier function. Building on previous findings that EV decreases lung microvascular endothelial cell barrier function (11), current results add mechanistic insight and demonstrate a similar permeability increase in small airway epithelial cells when exposed to EV experimental formulation as vapor, juice, or condensate.

Both cell types exposed to EV underwent autophagy with incomplete autophagolysosomal degradation and inefficient repair, as indicated by increased apoptosis. There is ample evidence that stress-induced autophagy is coupled with decreased cell proliferation rates to enable cell survival and repair (37). However, autophagy can directly precede and trigger apoptosis if stress levels are excessive and persistent, or if autophagy is impaired and/or decoupled from other cell repair processes (38). Acute EV exposures induced enrichment of ceramide in the plasma membrane and nuclear translocation of caspase-3, events that signify apoptosis (39–44). Although the levels of EV exposure studied did not induce massive apoptosis, and apoptotic markers were recorded many hours after the loss of barrier function, it is possible that apoptosis could weaken the barrier of the microvascular endothelium and the small airway epithelium. Apoptosis was also modest during recovery from EV exposure in hamster lungs and was primarily found in the distal lungs and lung endothelial cells.

Our study implicated JNK as a key signaling event in both EV-induced barrier dysfunction and dysfunctional autophagy. Other MAPKs activated by stress, such as p38 MAPK (45), have been implicated in the effects of EV on neutrophils (46), but not on rat lung microvascular endothelial barrier dysfunction (11). In previous studies, we identified both p38 and JNK as mediators of cigarette smoke-induced cytoskeletal changes in lung endothelial cells, but not barrier dysfunction (47). Interestingly, JNK inhibition did not affect S6 phosphorylation, suggesting the presence of additional mechanisms of mTOR signaling during EV exposure.

As expected (31, 48), short-term EV exposures in hamsters, as in mice, and especially when followed by a recovery window, are insufficient to trigger appreciable systemic changes. Our data build on findings published by Hinds et al. (24) on 2-day whole-body nicotine solution exposure in hamsters, with immediate histological and targeted gene transcription assessments showing increases in the inflammation-related genes IL-1 β , ELANE (NETosis gene), and tissue factor and in profibrotic genes. Our study complements these results by capturing previously unreported persistent effects of acute EV exposures and by providing a much broader transcriptomic assessment, which shows that IL-6 elevations (2.2-fold; $P = 0.01$) and IL-1 β reductions (by 50%; $P = 0.01$) persist even after EV cessation. In addition, we show that EV induces persistent changes in cytoskeletal dynamics and cell death and survival pathways, suggesting that it amplifies inflammation, promotes tissue remodeling, and impairs immune function. Repeated activation of

EV exposure followed by respiratory viral infection in hamsters

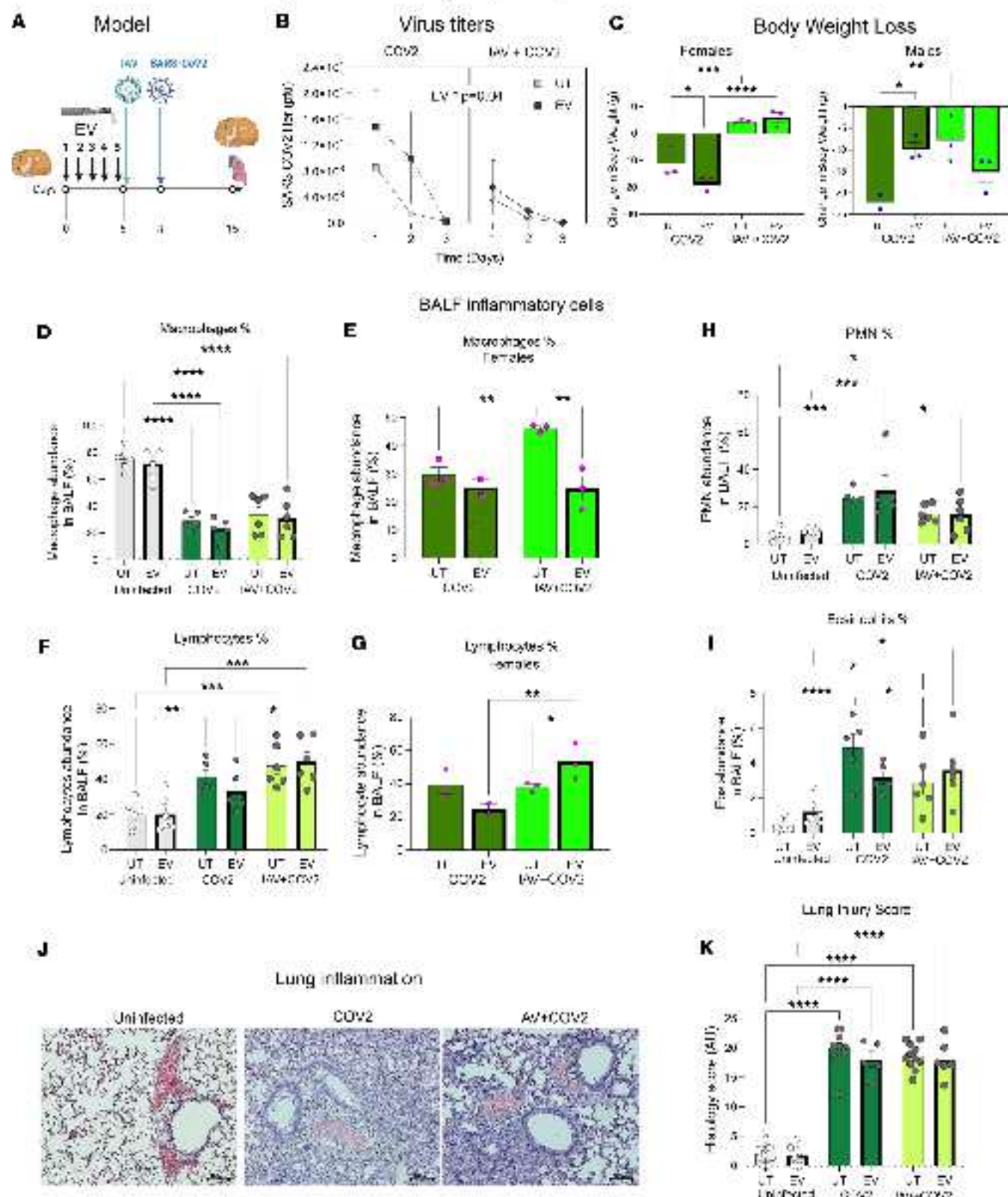


Figure 8. Effects of brief prior EV exposure on inflammatory responses to respiratory viral infections in hamsters. (A) Experimental timeline: Exposure to EV for 5 days prior to intranasal administration of influenza A virus (IAV), followed 3 days later by intranasal administration of SARS-CoV-2 (COV2). Viral titers were measured at days 1, 2, and 3 after infection with SARS-CoV-2; lung tissues and bronchoalveolar lavage fluid (BALF) were collected at 7 days after infection with SARS-CoV-2 (10 days after cessation of EV exposure). (B) Upper respiratory viral titers obtained by nasal swabs at the indicated times (days).

after SARS-CoV-2 infection. Mean $\pm$ SEM; 3-way ANOVA with a *P* value for the EV of 0.04. (C) Change in body weight in female and male hamsters from pre-infection weight. Mean $\pm$ SEM; 2-way ANOVA with Fisher's least significant difference (LSD). (D–I) Quantification of indicated inflammatory cell population abundance in cytosplins of BALF. Mean $\pm$ SEM; 2-way ANOVA with Fisher's LSD. (J) Representative H&E-stained lung tissue sections from uninfected, SARS-CoV-2-infected, and coinfecting (IAV+CoV-2) hamsters show exuberant inflammatory infiltrate in SARS-CoV-2-infected lungs. Scale bars: 100 μ m. (K) Unbiased histological scoring of inflammatory lung injury (based on epithelial thickening and inflammatory cell infiltration). Mean $\pm$ SEM; 2-way ANOVA with Fisher's LSD. For all graphs, **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001; dots are individual animals. UT, untreated.

these pathways could lead to persistent airway and vascular remodeling observed in chronic lung diseases. Pathway analysis following sequential EV, IAV, and/or SARS-CoV-2 exposures suggests that EV has a detrimental effect on the lung's ability to respond to SARS-CoV-2 infection, driven by a complex interplay between antiviral immune suppression, augmented tissue remodeling, and mitochondrial dysfunction signaling. Although not a study focus, nonlethal IAV pre-infection had an overall protective, antiinflammatory priming effect on subsequent SARS-CoV-2 infection, also known as interference (49). In contrast, EV exerted a detrimental priming effect on respiratory viral infections, with exaggerated inflammatory and tissue remodeling responses and impaired injury resolution. We noted a significant interaction between EV effects and animal sex. Given the small number of animals in each sex subgroup, these findings will need to be validated in future studies. Another limitation is that we did not investigate the effect of EVs on IAV-induced lung injury or recovery, as this topic has recently been explored. Instead, we used IAV to model a coinfection with SARS-CoV-2, a common clinical scenario (50, 51).

In conclusion, this study finds that even short-term EV exposure activates stress responses, induces cell injury, and reduces repair in human lung parenchyma cells, changes that persist after EV exposure. Even brief EV exposures decrease antiviral immunity, increasing susceptibility to SARS-CoV-2 and influenza virus infections while triggering molecular pathways involved in lung tissue remodeling. Identifying JNK signaling as a modifiable pathway may guide future research on therapeutic strategies to mitigate EV-induced lung injury. These findings provide mechanistic evidence linking EV exposure to impaired lung defense and distal lung remodeling, such as lung fibrosis, informing risk assessments of EV safety and prevention measures against respiratory virus exposures in habitual EV users.

Methods

Sex as a biological variable. Both sexes were included in human-cell-based and animal studies. When available, both sexes were used equally in the study design and interpretation.

Reagents. All reagents were purchased from Sigma-Aldrich/EMD Millipore unless otherwise stated.

Cell cultures. All cell cultures were maintained in sterile conditions at 37°C and 5% CO₂. Primary human lung microvascular endothelial cells (HLMVECs) were obtained from Lonza (CC-2527) and maintained in their appropriate media with standard growth factor supplements (Lonza). Immortalized HLMVECs (HULECs, ATCC, CRL-3244) were grown in MCDB131 (Corning, 45001-112) medium and used in submerged culture. h-TERT-immortalized human small airway epithelial cells (hSAEC1-KT, ATCC, CRL 4050) were grown in PneumaCult-Ex Plus Medium (StemCell Technologies, 05040), characterized for full differentiation at air-liquid interface (Supplemental Figure 6), and used in experiments at earlier stages of differentiation, while submerged in medium. Primary hSAECs were isolated from lungs of deidentified organ donors that had not been used for transplantation and that were donated for medical research. The lungs were obtained from the National Jewish Health Human Lung Tissue Core, which obtains deidentified lungs through the National Disease Research Interchange (Philadelphia, Pennsylvania, USA), the International Institute for the Advancement of Medicine (Edison, New Jersey, USA), or the Donor Alliance of Colorado (Denver, Colorado, USA). The Committee for the Protection of Human Subjects at National Jewish Health, Denver, Colorado, deemed this research as exempt from IRB human research. Donors were without a history of lung disease, were lifelong non-smokers, and had no lung injury as indicated by a PaO₂/FiO₂ greater than 300, a chest radiograph that indicated no acute process, and a time on the ventilator of less than 5 days. Small airway ($\leq$ 2 mm diameter) epithelial cells were collected from the distal lung using a 2 mm bronchoscopy brush (Conmed) and placed in sterile PBS. Cells were isolated by centrifugation, resuspended in PBS, counted, and plated onto an irradiated NIH 3T3 fibroblast feeder layer in F-medium. Once visible colonies had formed (7–10 days), they were removed with 0.25% trypsin (Corning, 25-053-CI) and plated on double-collagen-coated 12-well Transwells (Advanced BioMatrix, 5005, and Corning, 3460). Cells were maintained in PneumaCult-ALI-S medium (StemCell Technologies, 05001) at 5% CO₂ and 37°C for 21 days.

Table 3. Differentially expressed genes in EV-exposed hamsters infected with SARS-CoV-2

Gene name	Log ₂ fold change	P value	P _{adj}	Full name and function
Nrnt1	2.13	9.26×10^{-4}	0.033	Neurtin 1; promotes neuronal growth and survival; involved in nervous system development
Pop7	2.10	0.0001536	0.045	Ribonuclease P protein subunit; involved in tRNA processing
Aqp3	1.97	3.36×10^{-4}	0.001	Aquaporin 3; water and glycerol channel important for cellular water homeostasis
Mmp13	1.68	9.01×10^{-7}	0.001	Matrix metalloproteinase 13; collagenase involved in ECM remodeling
Mmp12	1.60	0.000104	0.036	Matrix metalloproteinase 12; regulates elastin degradation in tissue repair and inflammation
Dexi	1.52	6.98×10^{-4}	0.031	Dexamethasone-induced protein; possibly involved in stress responses
Pcbp1	1.48	8.90×10^{-5}	0.001	Poly(rC)-binding protein 1; regulates mRNA stability and translation
Rps15a	1.45	7.32×10^{-5}	0.000	Ribosomal protein S15a; essential for ribosome assembly and protein synthesis
Egl-3	1.33	6.58×10^{-4}	0.031	Egl-9 family hypoxia-inducible factor 3; regulates cellular oxygen sensing and hypoxia response
Irx5	1.29	0.0002307	0.052	Iroquois homeobox 5; involved in developmental processes and cell differentiation
Spp1	1.11	0.0002497	0.052	Secreted phosphoprotein 1; key player in immune regulation and bone remodeling
Egfl6	1.09	2.02×10^{-4}	0.015	EGF-like domain multiple 6; involved in cell adhesion and angiogenesis
Krcc1	1.07	3.28×10^{-4}	0.001	Krüppel-related zinc finger protein; regulates transcription and chromatin remodeling
Ndufb6	1.06	6.05×10^{-6}	0.005	NADH:ubiquinone oxidoreductase subunit B6; component of mitochondrial complex I
Golph3	0.97	4.12×10^{-4}	0.023	Golgi phosphoprotein 3; regulates Golgi trafficking and cell growth
Kctd5	0.85	7.86×10^{-4}	0.033	Potassium channel tetramerization domain 5; involved in protein-protein interactions
Zfp36h	0.71	0.000181	0.048	Zinc finger protein 36 like 1; regulates mRNA decay and inflammatory responses
Smc4	0.66	0.0002219	0.052	Structural maintenance of chromosomes 4; involved in chromosome condensation and cohesion
Pdap1	0.62	0.0001864	0.048	PDGFA-associated protein 1; regulates cell growth and proliferation
Nsf1lc	-0.85	0.000126	0.041	NSF1 cofactor p47; involved in membrane trafficking and protein complex disassembly
Sfpr3	-0.89	0.0001868	0.048	Sphingosine-1-phosphate receptor 3; regulates vascular development, immunity, and inflammation
Ndufa3	-1.07	0.0001725	0.048	NADH:ubiquinone oxidoreductase complex assembly factor 3; required for mitochondrial complex I assembly
Fam20c	-1.31	2.45×10^{-5}	0.016	FAM20C Golgi kinase; regulates biomineralization and ECM homeostasis
Serpinc1	-1.44	6.03×10^{-4}	0.030	Serpin family C member 1; regulates complement cascade and inflammation
Slc31a1	-1.64	4.30×10^{-6}	0.000	Solute carrier family 31 member 1; copper transporter important for cellular metabolism
Gbp5	-1.77	9.22×10^{-4}	0.033	Guanylate binding protein 5; key player in innate immune response and inflammation
Pex1lg	-1.77	7.98×10^{-4}	0.001	Peroxisomal biogenesis factor 1lg; involved in peroxisome proliferation and metabolism
Tmp1	-2.49	2.77×10^{-10}	0.000	TM1 domain-containing protein 1; regulates neural stem cell proliferation and differentiation

Human PCLS. Right upper lung lobes from deidentified non-smoking donors with no lung disease or infection were obtained from the International Institute for the Advancement of Medicine or the Donor Alliance of Colorado. Lungs were inflated with 1.5% low-melting agarose (42°C) and sliced into consecutive sections of 450 μ m thickness using a Compressome VF-300 vibratome (Precisionary Instruments). The slices were transferred to 24-well plates containing Dulbecco's modified Eagle medium (Thermo Fisher Scientific) with antifungal agents and antibiotics and incubated in a humidified incubator at 37°C, 5% CO₂.

EV exposure of cells. To generate EV, we used JUUL pods with 5% (59 mg/mL) nicotine, commercialized as Virginia Tobacco (JUUL), with a ratio of 30:70 propylene glycol/vegetable glycerin. We tested several forms of EV, as juice or aerosol, in submerged and air-liquid interface exposure models, respectively. For submerged cultures, to determine whether the vaporized juice has similar effects to the non-vaporized juice on the outcomes tested, we generated EV condensate.

JUUL juice was prepared by dilution of JUUL liquid in serum-free medium to a 10 $\times$ stock solution that was then added to submerged cell cultures or PCLSs to achieve a final concentration of 2.5–10 mM nicotine. A VITROCELL VC 1 apparatus (VITROCELL Systems GmbH) was used to generate EV aerosol, which was used to either obtain EV condensate used in submerged cultures, or directly expose to EV SAECs at the air-liquid interface. The VITROCELL protocol consisted of a modified Cooperation COR-ESTA-recommended method 81, with puff volume, 55 mL; puff duration, 3 seconds; puff frequency, 30 seconds; puff hold time, 0 seconds; puff exhaust time, 8 seconds; and puff number, 75 puffs. The EV condensate collected was considered 100% and used at a 5% final concentration in submerged cultures, with ambient air used to generate a control condensate at similar concentrations. For experiments using

Table 4. Genes significantly regulated by EV in hamsters coinfecting with IAV and SARS-CoV-2

Gene name	Log ₂ fold change	P value	Full name and function
Upregulated			
Crisp4	4.11	5.07×10^{-4}	Cysteine-rich secretory protein 4; reproduction and immune response
Map2k1b	3.77	2.69×10^{-4}	Mitogen-activated protein kinase kinase kinase 19; stress response regulator
Myh3	3.62	4.97×10^{-4}	Myosin light chain 3; regulates cardiac muscle contraction
Sco1	3.45	4.75×10^{-4}	Synthesis of cytochrome c oxidase 1; mitochondrial respiration assembly
Cox8b	2.96	9.18×10^{-4}	Cytochrome c oxidase subunit 8B; part of electron transport chain
Hba-x	2.36	8.34×10^{-4}	Hemoglobin subunit alpha-X; oxygen transport
Cfap44	2.24	9.22×10^{-4}	Cilia- and flagella-associated protein 44; function in ciliary movement
Slc6a1	2.10	1.88×10^{-4}	Solute carrier family 6 member 1; bicarbonate transport
Marcks	2.00	1.37×10^{-4}	Myristoylated alanine-rich C-kinase substrate; regulates actin cytoskeleton
Neu2	1.98	8.88×10^{-4}	Neuraminidase 2; glycoprotein metabolism enzyme
Mfap4	1.89	5.36×10^{-4}	Microfibril-associated protein 4; connective tissue integrity
Pop7	1.79	2.06×10^{-4}	POP7 homolog; RNA and tRNA processing
Tnni3	1.72	2.89×10^{-4}	Troponin I3; regulates cardiac muscle contraction
Snca	1.65	1.13×10^{-4}	Synuclein α ; implicated in neurodegenerative disease
Pgl3	1.59	2.90×10^{-4}	6-Phosphogluconolactonase; involved in pentose phosphate pathway
Poli3d	1.57	1.60×10^{-4}	RNA polymerase III subunit D; transcribes small RNAs
Pnpla2	1.53	3.18×10^{-4}	Patatin-like phospholipase domain 2; lipid metabolism regulator
Kif23	1.51	2.92×10^{-4}	Kinesin family member 23; essential for cytokinesis
Klf9	1.49	6.71×10^{-4}	Kruppel-like factor 9; regulates cell differentiation
Alpl	1.47	1.14×10^{-4}	Alkaline phosphatase; bone mineralization enzyme
Washc1	1.29	6.10×10^{-4}	WASH complex subunit 1; actin cytoskeleton remodeling
Ahrgap35	1.21	1.50×10^{-4}	RhoGTPase-activating protein 35; regulates cell migration
Slc4a2	1.17	8.07×10^{-4}	Solute carrier family 4 member 2; bicarbonate transporter
Rps15a	1.16	3.31×10^{-4}	Ribosomal protein S15A; component of the ribosome
Ube2g	1.02	4.26×10^{-4}	Ubiquitin-conjugating enzyme E2 G2; mediates ubiquitination
Tmem63b	1.00	5.89×10^{-4}	Transmembrane protein 63B; calcium ion transport
RNF31	0.91	6.45×10^{-4}	Ring finger protein 31; ubiquitin ligase activity
Tm628	0.87	9.92×10^{-4}	Triplicate motif containing 28; chromatin remodeling and gene silencing
Aclr1	0.81	9.26×10^{-4}	Atypical chemokine receptor 1; chemokine scavenger receptor
Bcl2l1	0.77	5.66×10^{-4}	BCL2-like 1B; regulator of apoptosis
Arl16	0.77	4.85×10^{-4}	ADP ribosylation factor like 16; intracellular trafficking regulator
Tent5c	0.76	6.81×10^{-4}	Terminal nucleotidyltransferase 5C; adds nucleotides to RNA
Eif3b	0.73	1.01×10^{-4}	Eukaryotic translation initiation factor 3B; translation initiation
Ggh	0.72	1.96×10^{-4}	γ -Glutamyl hydrolase; folate metabolism
Col1a1	0.72	1.49×10^{-4}	Collagen β 1; ECM structural protein
Sptlc1	0.66	3.00×10^{-4}	Serine palmitoyltransferase long chain subunit 1; sphingolipid biosynthesis
Downregulated			
Cox8	-0.57	6.21×10^{-4}	Cytochrome c oxidase assembly factor 8; regulates mitochondrial respiratory function
Taf10	-0.85	5.27×10^{-4}	TATA box-binding protein-associated factor 10; part of transcription initiation factor complex
Saadh	-0.87	3.72×10^{-4}	Sarcosine dehydrogenase; catalyzes oxidative demethylation of sarcosine
Pxmp2	-0.90	8.12×10^{-4}	Peroxisomal membrane protein 2; involved in peroxisomal transport
Gabarapl	-0.91	9.38×10^{-4}	GABA type A receptor-associated protein like 1; autophagy-related protein
Npr2	-0.97	7.43×10^{-4}	Natriuretic peptide receptor 2; regulates cGMP signaling and vascular tone
Ranbp3	-1.07	3.30×10^{-4}	RAN-binding protein 3; mediates nuclear export processes
Ppp1r3c	-1.18	7.91×10^{-4}	Protein phosphatase 1 regulatory subunit 3C; regulates glycogen metabolism
Hb	-1.32	3.70×10^{-4}	Haptoglobin; binds free hemoglobin to prevent oxidative damage
Pigq	-1.37	9.05×10^{-4}	Phosphatidylinositol glycan anchor biosynthesis, class Q; essential for GPI-anchor synthesis
Rpl15	-1.61	3.18×10^{-4}	Ribosomal RNA processing 15 homolog; involved in rRNA maturation
Gtf3a	-1.71	1.63×10^{-4}	General transcription factor IIIA; zinc finger protein involved in 5S rRNA transcription
Fhod1	-1.71	4.17×10^{-4}	Formin homology 1 domain containing 1; regulates actin cytoskeleton assembly
Ctsa	-1.84	4.27×10^{-4}	Cathepsin A; lysosomal enzyme involved in protein degradation
Srsf25	-1.94	1.62×10^{-4}	Small nuclear ribonucleoprotein 25; part of spliceosome for mRNA splicing
Chrna1	-1.95	1.30×10^{-4}	Cholinergic receptor nicotinic α subunit; mediates synaptic transmission
2610318N02Rik	-2.13	3.41×10^{-4}	Uncharacterized protein; novel gene of unknown function
Rpl3	-2.36	3.63×10^{-4}	Ribosomal protein L3; component of the ribosome for protein synthesis
Brl1	-2.43	2.45×10^{-4}	RNA polymerase III transcription initiation factor 1; regulates RNA polymerase III activity
Zfp831	-4.16	8.63×10^{-4}	Zinc finger protein 831; putative transcriptional regulator

inhibitors, cells or PCLSs were exposed to EV once at the indicated final nicotine concentrations in the presence or absence of pan-JNK inhibitor (SP600125; 50 μ M, 10 mg; Selleckchem, S1460), ERK1/2 inhibitor (PD98059; 50 μ M; Fisher Scientific, NC0771630), p38 MAPK inhibitor (SB202190; 30 μ M, 5 mg; Sigma-Aldrich, S7067), or DMSO as vehicle control.

Cell monolayer barrier measurement was performed using ECIS (Applied Biophysics) with the Z-Theta 16-well array station. Cell monolayers were grown on 8W10E or 8W10E+ Cultureware to a capacitance of <1 nF or <10 nF, respectively, at 64 kHz. Measurements of electrical resistance (ohms) were collected at 4,000 Hz over time. A complementary assay to measure the barrier function of cells grown on inserts (0.4 μ m $\times$ 6.5 mm) used fluorescently labeled dextran (FITC or rhodamine B, 4K) applied to the apical side, followed by kinetic measurement of fluorescence in the basal compartment at excitation and emission wavelengths (FITC, excitation 485, emission 538; rhodamine B, excitation 544, emission 590).

Immunoblotting. Cells were resuspended in standard RIPA buffer (R0278, lot SLBL7395V) on ice for 30 minutes, collected by centrifugation, and quantified using standard BCA. Proteins were resolved by 4%–20% gradient PAGE and transferred using a semi-dry transfer apparatus (Bio-Rad) onto Immobilon-P PVDF membranes (MilliporeSigma, IPVH0010). Membranes were blocked in Pierce Protein-Free T20 (TBS) Blocking Buffer (Thermo Fisher Scientific, 37571) and washed in TBS with 0.1% Tween 20. Antibodies for immunoblotting were p62 (Abnova, H00008878M01), LC3B-II (Sigma-Aldrich, L7543), phospho-S6 (Cell Signaling Technologies, 2211S), anti-vinculin (Abcam, AB130007), and anti- β -actin (Fisher Scientific, A5441) and were incubated overnight at 4°C. Anti-mouse (NA931V, lot 9715064) and anti-rabbit (NA9340V, lot 10997954) HRP-conjugated secondary antibodies were from GE Healthcare.

Cell immunofluorescence microscopy. Human lung endothelial cells or hSAECs were plated on chamber slides (Falcon, culture slides, 354108) coated with either 12.5 μ g/mL fibronectin in PBS or 50 μ g/mL collagen type I in 0.02N glacial acetic acid, respectively. Experiments were conducted on cell monolayers at about 90% confluence. At the end of experiments, cells were fixed in 4% paraformaldehyde in PBS for 20 minutes at room temperature, then permeabilized in 0.5% of Triton X-100 in PBS.

For cell proliferation measurements, cells were incubated with the fluorescently labeled synthetic nucleotide EdU (5-ethynyl-2'-deoxyuridine) dissolved in DMSO (Invitrogen, C10637A), which was added 4 hours before experiment completion; briefly, 100 $\times$ EdU solution in PBS was added to each cell culture for a final EdU concentration of 10 μ M. The nuclear uptake of EdU was detected using the Invitrogen Click-iT Plus EdU Cell Proliferation Kit for Imaging, Alexa Fluor 488 dye (Thermo Fisher Scientific, C10637).

For immunofluorescence, after fixation and permeabilization, plated cells were blocked with 5% goat serum and 0.1% Triton X-100 in PBS for 1 hour at room temperature, then incubated overnight at 4°C with primary antibodies, followed by washing and incubation for 4 hours with respective secondary antibodies. This was followed by washing and nuclear staining with DAPI (400 ng/mL in 0.5% bovine serum albumin [BSA] and 0.01% Triton X-100 in PBS) for 20 minutes at room temperature, washing, and mounting using ProLong Diamond Antifade mounting solution (Invitrogen, P36970). Antibodies were diluted in an antibody buffer consisting of 1% BSA and 0.1% Triton X-100 in PBS. Primary antibodies were against p62 [1:100; mouse anti-SQSTM1-IgG2a(κ), Abnova, H00008878], LC3B (1:100; rabbit anti-LC3B, Sigma-Aldrich, L7543), PCNA [1:200; mouse anti-PCNA-IgG2a(κ), Novus, NB500-106], ceramides (1:200; mouse anti-ceramide-IgM [MID 15B4], Enzo, ALX-804196-T050), LAMP1 (1:100; rabbit anti-LAMP1, D2D11-XP, Cell Signaling Technology, 9091), and cleaved caspase-3 (1:200; rabbit anti-cleaved caspase-3, Abcam, ab2302). Primary antibodies were against mouse IgG2a(κ) (1:500; Alexa Fluor 594 goat anti-mouse IgG [H+L], Invitrogen, A11032), mouse IgM (1:500; Alexa Fluor 488 goat anti-mouse IgM [μ chain], Invitrogen, A21042), and rabbit IgG (1:500; Alexa Fluor Plus 488 goat anti-rabbit IgG [H+L], Invitrogen, A32731; or 1:500, Alexa Fluor 647 goat anti-mouse IgG [H+L], Invitrogen, A21245).

Animal studies. Golden Syrian hamsters (n = 6 per group, 3 months of age, male and female; Charles River) were exposed to EV (JUUL condensate; 70 puffs/100 μ L/dose in saline) or vehicle (saline control) via nebulization. Sex as a biological variable was considered in the design (both sexes were included equally) and data analysis and interpretation. EV condensate was collected from the aerosol of vaped JUUL in a side-arm Erlenmeyer flask and solubilized in saline as follows: 7 pods (each 700 μ L) were vaporized, and the condensate was brought up to 2 mL. Each animal was nebulized with EV or vehicle once per day for 5 consecutive days, and lungs were harvested 10 days after the last exposure (schematic in Figure 6A). The administration of nebulized EV was performed using a nebulizer unit (2.5–4.0 VMD, Aerogen) fitted with a cone that allowed nasal inhalation of the aerosol (5 minutes) by each animal at a time, as we previously

reported (11). To assess the effect of EV on respiratory virus infections, hamsters were infected at the end of EV exposure, on day 5, with influenza A virus (IAV; A/California/07/2009) by intranasal instillation of $6 \log_{10}$ PFU; and/or, on day 8, with SARS-CoV-2 (WA01) by intranasal instillation of $4 \log_{10}$ PFU (schematic in Figure 8A). As controls, uninfected animals were instilled intranasally with 100 μ L of PBS or 100 μ L of medium, respectively. Hamsters were sacrificed 7 days after SARS-CoV-2 infection. The left lobe was clamped, and the right lung was instilled with PBS (1 mL $\times$ 3) and lavaged, with each return collected separately as bronchoalveolar lavage fluid (BALF). The first return was used for cytopins. The right caudal lung lobe was excised and placed in Buffer RLT (RNA Lysis Tissue, QIAGEN) with freshly added β -mercaptoethanol. The left lung was inflated with ethanol (70%) and then immersed in formalin (10%), sectioned with a random uniform sampling method, and mounted on cassettes for paraffin embedding.

Long histochemistry and immunofluorescence. Cytospins obtained from the BALF were fixed and stained with a modified Diff-Quik stain using Giemsa Stain (Sigma-Aldrich, GS 1L-1L). Samples were fixed to slides using 100% methanol for 10 minutes at room temperature, and then Giemsa solution (in water) was added for 20 minutes at room temperature. After staining, rinsed slides were air-dried and mounted with Cytoseal 60 (Fisher Scientific, 23-244257). Images were taken at $\times 20$ magnification. Five hundred cells were assessed for each sample (blinded to the identity of the groups), differentiating among macrophages, lymphocytes, neutrophils, and eosinophils by morphological aspect and expressing their abundance as percentage of all cells counted.

For histological assessment, paraffin-embedded lung sections (4 µm thickness) were stained with hematoxylin and eosin (H&E) and then were assessed unbiasedly (blinded to the animal group identity) under light microscopy using a scoring system for inflammation as originally described (52) and previously applied for models of IAV lung infections (53) and hamster lung inflammation (54). The scoring was applied for each tissue section evaluated, using 5 criteria: (a) the number of bronchioles and bronchi with inflammatory cell infiltrate; (b) the severity of infiltrate of inflammatory cells in bronchioles and bronchi; (c) the severity of bronchiolar and bronchial luminal exudate; (d) the frequency of blood vessels with perivascular inflammatory cell infiltrate; and (e) the severity of pneumonia. H&E-stained slides were also assessed for lung fibrosis using the Ashcroft method, where each microscopic field of the biopsy was assigned an integer score from 0 (normal) to 8 (severe fibrosis).

For TUNEL staining, sections were deparaffinized in xylene, permeabilized with proteinase K, and post-fixed with 4% paraformaldehyde. They were then washed in PBS, incubated in TdT buffer, and then washed with 0.1% Triton X-100 in 3% PBS/BSA before being incubated in TUNEL reaction solution. Samples were washed with PBS before blocking in PBS/BSA-T. The primary antibody (diluted in PBS/BSA) was added and incubated overnight at 4°C. Samples were washed twice before addition of the secondary antibody for 4 hours at room temperature. The samples were washed twice with PBS before being incubated with DAPI and mounted. Images were obtained on an Echo Revolution Microscope (Discover Echo Inc.).

Bulk RNA-seq analyses. Total RNA was isolated using an All Prep DNA/RNA/Protein kit (QIAGEN, 80004). Bulk RNA (RNA-seq) was performed by Novogene using Illumina RNA-seq technology. The sequencing data were mapped through STAR software (<https://github.com/alexdobin/STAR/releases>), replicate multivariate analysis of transcript splicing (rMATS) (<https://rnaseq-mats.sourceforge.io/>), and clusterProfiler (<https://bioconductor.org/packages/release/bioc/html/clusterProfiler.html>). The list of differentially expressed genes was analyzed with QIAGEN's Ingenuity Pathway Analysis (IPA) using Core Analysis (datasets had a log₂ fold change cutoff of $\geq \pm 1$ and a *P* value cutoff of >0.05) to cross-reference against GO enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis results.

Statistics. Analyses of significance were performed using GraphPad Prism 10 software. A 2-tailed unpaired Student's *t* test was used for experiments in which 2 conditions were being compared. A *P* value less than 0.05 was considered statistically significant. Comparisons between groups used ANOVA followed by an intergroup comparison with Tukey's post hoc testing.

For RNA-seq analyses, a normalization method of DESeq2 was used, with the fold change being calculated with the equation $[\log_2(q_i) = \sum_j X_{ij}\beta_j]$. The P value was calculated using negative binomial distribution $[P(X = x) = \binom{n+x-1}{n-1}p^n(1-p)^x]$, and FDR was calculated using the Benjamini-Hochberg procedure:

$$[\text{FDR} = E(FDP(T_{(n)})) \leq \frac{\gamma}{k+2}]$$

Differential gene screening was performed with thresholds of $|\log_2(\text{fold change})| \geq 1$ and adjusted *P* value ≤ 0.05 . *Z* score was calculated through IPA software using $Z = \frac{\bar{X}}{s_y} = \frac{\sum_{i=1}^n X_i}{\sqrt{\frac{1}{n} \sum_{i=1}^n X_i^2}}$. Pathways with a *z* score $\geq \pm 2$ were considered significant.

Study approval. The studies were performed in line with the principles of the Declaration of Helsinki. This study used human biological samples from deidentified individuals and was deemed IRB exempt. All studies were conducted under approvals by the institutional IACUC (no. 1372) at Colorado State University, Fort Collins, Colorado, USA and National Jewish Health, Denver, Colorado, USA and the Institutional Biosafety Committee at National Jewish Health (20-029B) protocols.

Data availability. Bulk RNA-seq data generated in this study are available in the Zenodo repository at <https://doi.org/10.5281/zenodo.21676278>. Note that the original FASTQ and BAM sequencing files are unavailable for public deposition because the archived files were corrupted and could not be recovered. Supporting data values associated with the main and supplemental figures are provided in the Supporting Data Values file.

Author contributions

IP, HWC, and RB performed conceptualization. KSS, RB, EKV, JPB, SDG, GPD, and HWC developed methodology. TCR, KSS, IP, JPB, EKV, SDG, RAC, CM, CC, and JN performed investigation. NE performed visualization. IP and HWC acquired funding. IP and RB performed project administration. IP, RB, and HWC supervised the study. IP, TCR, and KSS wrote the original draft of the manuscript. IP reviewed and edited the manuscript. TCR assumed leadership of methodology and investigation after KSS relocated. TCR performed all pathway analyses and conducted experiments in SAECs; KSS oversaw the operationalization of the hamster experiment and coordinated with RB and the company performing RNA-seq, which conducted experiments in HLMVECs. They both contributed to writing. Therefore, they share first authorship.

Conflict of interest

IP is a scientific co-founder and consultant with Allinaire Therapeutics.

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