CYSTATHIONINE γ-LYASE DEFICIENCY ENHANCES AIRWAY REACTIVITY AND VIRAL-INDUCED DISEASE IN MICE EXPOSED TO SIDE-STREAM TOBACCO SMOKE

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Abstract

BACKGROUND: Environmental tobacco smoke (ETS) is a known risk factor for severe respiratory syncytial virus (RSV) infections, yet the mechanisms of ETS/RSV co-morbidity are largely unknown. Cystathionine γ-lyase regulates important physiological functions of the respiratory tract.

METHODS: We used mice genetically deficient in the cystathionine γ-lyase enzyme (CSE), the major H_2S-generating enzyme in the lung to determine the contribution of H_2S to airway disease in response to side-stream tobacco smoke (TS), and to TS /RSV co-exposure.

RESULTS: Following a two-week period of exposure to TS, CSE-deficient mice (KO) showed a dramatic increase in airway hyperresponsiveness (AHR) to methacholine challenge, and greater airway cellular inflammation, compared to wild type (WT) mice. TS-exposed CSE KO mice that were subsequently infected with RSV exhibited more severe clinical disease, airway obstruction and AHR, enhanced viral replication and lung inflammation, compared to TS-exposed RSV-infected WT mice. TS-exposed RSV-infected CSE KO mice had also a significant increase in the number of neutrophils in bronchoalveolar lavage (BAL) fluid and increased levels of inflammatory cytokines and chemokines.

CONCLUSION: This study demonstrates the critical contribution of the H_2S-generating pathway to airway reactivity and disease following exposure to ETS alone or in combination with RSV infection.

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INTRODUCTION

Second hand tobacco smoke, known also as environmental tobacco smoke (ETS), is a complex mixture of gases and particles that include smoke from the burning cigarette, cigar, or pipe tip (side-stream smoke, SS) and exhaled mainstream smoke (MS). It is involuntarily inhaled by nonsmokers, lingers in the air for hours after cigarettes have been extinguished, and can induce or exacerbate a wide range of diseases, from cancer to respiratory infections, chronic obstructive pulmonary disease (COPD) and asthma (24, 27). It is estimated that worldwide close to 50% of children under the age of 5 yr are exposed to ETS (28). Younger children represent also the most vulnerable population with regard to severe lung infections caused by respiratory syncytial virus (RSV), an enveloped, negative-sense single-stranded RNA virus of the *Pneumoviridae* family that is recognized as a leading cause of lower respiratory tract illness (LRTI) in young children. It is estimated that RSV produces over 64 million (M) cases of acute infections globally, and is responsible for acute emergency room visits for an estimated 2.1 M children under 5 years of age annually in the US (14). The clinical spectrum of RSV-caused disease in children ranges from a relatively mild "cold" to acute lower respiratory tract infections, which may be so severe to require mechanical ventilation (intensive care). Although infants with certain risk factors (prematurity, chronic lung disease, congenital heart disease or immunodeficiencies) have an increased risk for more severe RSV disease, the large majority of infants with RSV infections that require hospitalization are previously healthy (36). Therefore, the spectrum of disease in infants points to host determinants, viral-specific factors, and environmental insults that combined may lead to enhanced viral replication, inflammation of the airway mucosa, and lung injury, ultimately determining the severity of RSV infections (7, 9). With regard to environmental factors, different studies have reported that exposure to ETS may be associated with more severe manifestations of RSV infection (8). A study of more than 200 young children has revealed that maternal cigarette smoking, especially postnatal, is associated with the severity of RSV bronchiolitis infection in infants (2). Lanari et al. demonstrated that exposure to TS, in general, seems to worsen the severity of the bronchiolitis (19). Moreover, a study by Gurkan et al, in which cotinine levels were measured at index of hospitalization, showed that infants admitted to the hospital with severe RSV bronchiolitis were exposed more recently to TS than infants hospitalized for non-respiratory diseases (13). However, the mechanism(s) underlying such increased severity of RSV infection in subjects exposed to ETS are largely unknown.

Hydrogen sulfide (H_2S) is a cellular gaseous transmitter with known biological activities, including anti-inflammatory properties (32). Synthesis of H_2S involves two pyridoxal-5′-phosphate–dependent enzymes, cystathionine β-synthase [CBS] and cystathionine γ-lyase [CSE] that are involved in the metabolic pathway of L-cysteine. A third enzyme, 3-mercaptopyruvate sulfurtransferase (3-MST) is also involved in a cysteine aminotransferase (CAT) pathway (26). H_2S modulates ion channel and cellular function by a multitude of signaling pathways, including protein modification by S-sulfhydration and scavenging reactive oxygen species which affects protein function, cellular localization, stability, and resistance to oxidative damage [reviewed in (18, 35)]. In the respiratory tract, endogenous H_2S has been shown to participate in the regulation of important physiological functions

such as airway tone, pulmonary circulation, and cell proliferation and apoptosis, and to modulate lung fibrosis, oxidative stress, and inflammation (1). Based on this premise, the goal of our study was to investigate in an experimental mouse model the contribution of endogenous H_2S to airway responsiveness and inflammation in response to side-stream tobacco smoke (TS) as a surrogate of ETS exposure and its function in TS-enhanced RSV pathogenesis.

METHODS

RSV Preparation

RSV (Long strain) was grown in HEp-2 cells and purified by centrifugation on discontinuous sucrose gradients and viral pools were titered in plaque forming units (PFU)/mL using a methylcellulose plaque assay (see Supplementary Material) (25).

Ethics Statement

Animal care and use were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of the University of Texas Medical Branch at Galveston (Protocols: 9001002 and 1304014). The TS exposure was performed in the Inhalation Toxicology Core, UTMB. Mice were euthanized with high dose of anesthetic ketamine and xylazine, by an intraperitoneal injection.

TS Exposure and Infection of Mice

Cystathionine γ-lyase (CSE) deficient mice (CSE KO) were used to examine the role of endogenous H_2S in the pathogenesis of TS exposure. 10–12 weeks male and female C57BL/6J mice (wild type, WT) used in this work were purchased from The Jackson Laboratory (Bar Harbor, Maine). CSE KO mice on C57BL/6J background were generously provided by Dr. Solomon Snyder, Johns Hopkins University, Baltimore, MD. Both male and female CSE KO and WT age-matched mice were used in the experiments. Groups of CSE –/ – (KO) and wild type control (WT) mice were subjected to whole body exposure of either cigarette generated TS (as side-stream, TS) or filtered air (5 h/day, 5 days/week, for two weeks). The Standard Reference Cigarettes 3R4F used in this study were obtained from the Tobacco and Health Institute at the University of Kentucky (http://www.ca.uky.edu/refcig). In some experiments, at the end of the two-week exposure period groups of mice were infected intranasally (i.n.) with 50 μl of RSV diluted in phosphate-buffered saline (PBS), at dose 10^6 PFU/mouse or were mock-inoculated using the same volume of PBS (sham infection). Daily determination of body weight, illness score, cytokine and chemokine concentration and cell differential counts in bronchoalveolar lavage (BAL) fluid, and airway function tests (see below) were performed exactly as we previously described (16). Mouse strains, side-stream cigarette smoke or air exposure, and infection with RSV or sham are identified in the paper as: WT or CSE KO, TS or Air, RSV or PBS. The time line of experimental protocol for air/smoke exposure and air/smoke exposure followed by either PBS or RSV infection is shown in Supplemental Figure S1a and Supplemental Figure S1b, respectively.

Statistical Analysis

All results are expressed as mean ± SEM for each experimental group. Data were analyzed using the GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA). Statistical significance was calculated between groups by one-way ANOVA followed by Tukey's *post hoc* test for samples with unequal variances. $p < 0.05$ value was selected to indicate significance. All experiments were repeated at least three times, data in figures are shown from a representative experiment. For full methods please refer to Supplementary Material online.

RESULTS

CSE Gene Deficiency Enhances TS -Induced AHR and Lung Inflammation

To establish the role of endogenous H_2S in a TS surrogate of ETS exposure we first measured airway function (AHR), cytokine and chemokine concentration and inflammatory cell recruitment in BAL fluid of mice lacking CSE a key enzyme in the biosynthesis pathway of H_2S (37). For pulmonary function, groups of CSE KO and WT mice were assessed for AHR in response to MCh by whole-body barometric plethysmograph (Buxco™) at approximately 24 h after the last exposure to TS (overall two week-long exposure). As shown in Figure 1a, AHR measured as Penh values was similar between air and TS-exposed WT mice. On the other hand, significant dose-dependent increase in AHR to MCh challenge was observed in CSE KO mice. Specifically, CSE KO mice had increased AHR when compared to WT mice even in conditions of air exposure ($p < 0.05$ at 25 and $p < 0.01$ at 50 mg/ml MCh doses). After exposure to TS, CSE KO mice showed a two-fold increase in Penh values at doses of 6 and 12.5 mg/ml, respectively and three-fold increase at doses of 25 and 50 mg/ml of MCh, compared to CSE KO Air mice. Compared to WT mice, CSE KO mice had even more dramatic increase in AHR, specifically a six -fold increase in Penh at 6 and 12.5 mg/ml, and ten-fold increase at 25 and 50 mg/ml MCh doses.

The total cell number in BAL fluid was not different between Air CSE KO and WT mice (Figure 1b). On the other hand, total number of BAL cells was significantly elevated both in WT and CSE KO mice after two weeks of TS exposure, compared to the respective Air control groups (Figure 1b), and in TS CSE KO compared to TS WT mice. In particular, macrophage cell counts in BAL increased with TS exposure compared to respective Air controls, but significantly more so in CSE KO mice than in WT (Figure 1c, $p < 0.05$), and in TS CSE KO mice compared to TS WT mice. Exposure to TS resulted also in a significant increase in the number of neutrophils ($p < 0.01$) and lymphocytes ($p < 0.05$) in CSE KO mice compared to Air CSE KO mice, and also relative to TS WT mice (Figures 1d and 1e). In general, we did not observe significant differences BAL neutrophils and lymphocytes numbers between Air and TS WT animals.

To determine the profile of major inflammatory molecules that could explain the increased influx of cells into the lungs of CSE KO mice, we measured a panel of inflammatory cytokines in samples of BAL fluids by a bio-plex array, 24h after the two week period of TS or control filtered air exposure. Most of the cytokines/chemokines included in the 18-cytokine array were undetectable in BAL of WT or CSE KO mice, with the exception of

those presented in Figure 1f (IL-6, CXCL1, CCL2, and CCL4). Baseline levels (i.e. Air mice) of IL-6 and CXCL1 were significantly increased in the BAL fluid of CSE KO mice compared to WT mice (p<0.05 for IL-6, and p<0.001 for CXCL1). No significant differences were observed for any of these four cytokines between smoke- and Air WT mice. On the other hand increased levels of CCL2 and CCL4 were observed in TS compared to Air CSE KO mice (p<0.05). In addition, CSE KO mice exposed to TS showed significantly increased BAL concentration of IL-6 (p<0.05), CXCL1, (p<0.001), CCL2 (p<0.05) and CCL4 (p<0.05), compared to WT mice.

Next, we determined whether cigarette smoke exposure alters the expression of the H_2S-producing enzyme CSE in WT mice. Lungs samples were harvested at day 1 post-infection to assess mRNA levels of CSE. As shown in Figure 1g, mRNA levels of the H_2S-producing enzyme CSE are significantly increased in TS exposed WT mice when compared with Air WT mice ($p < 0.05$).

CSE Deficiency and TS Exposure Enhance RSV Pathogenicity

To investigate the role of endogenous H_2S in an experimental model of smoke exposure and RSV infection, we exposed CSE KO and WT mice to TS or Air as described in the previous section, followed 24h later by infection with RSV. Using daily body weight as an indicator of clinical disease, we found that early body weight loss after RSV inoculation was similar between air and TS exposed WT mice (which peaked at day 1, Figure 2a). In control condition of air exposure, RSV-infected CSE KO mice exhibited enhanced body weight loss at the peak of the clinical disease when compared with infected WT mice (p<0.05). Following exposure to TS, RSV-infected CSE KO mice showed significant greater body weight loss beginning at day 1, peaking at day 2 and delayed recovery to baseline weight, compared with WT mice and also compared to Air RSV-infected CSE KO mice (p<0.05, Figure 2a). No differences in body weight loss were observed among groups of WT and CSE KO mice either Air- or TS-exposed which were PBS inoculated (data not shown). Next, we investigated the role of CSE deficiency and smoke exposure on RSV-mediated airway obstruction and AHR. As shown in Figure 2b, RSV-infected CSE KO mice that were exposed to TS had significantly increased baseline airway obstruction compared to both Air RSV-infected CSE KO infected mice or Air RSV-infected WT mice [(at day 1 after infection, returning to baseline at day 5 post-infection (p<0.05)]. TS RSV-infected CSE KO mice also exhibited significantly greater AHR in response to aerosolized MCh (day 5 post-infection), compared to Air RSV-infected CSE KO as well as to air or TS RSV-infected WT mice (Figure 2c).

To determine whether CSE deficiency and exposure to smoke would alter viral replication, groups of mice were sacrificed at day 5 after RSV infection and lung tissue collected to determine virus titer by a plaque assay. As shown in Figure 2d, no significant difference was observed in peak viral titer between air and TS exposed RSV-infected WT mice. On the other hand Air RSV-infected CSE KO mice had significantly higher peak titers in the lung compared with Air infected WT mice (p<0.001). Overall, the highest peak titers were found in the lung of CSE KO mice exposed to TS and subsequently infected with RSV, which had

significantly higher peak virus titers compared to both Air RSV infected CSE KO mice (p<0.05) and to smoke exposed RSV-infected WT mice (p<0.01).

For histopathology studies, lungs from mock- and RSV-infected CSE KO and WT mice, TS or Air, were collected at days 5 after infection, formalin-fixed, H&E stained, and qualitatively and quantitatively analyzed using an established grading score (from 0, absent to 4, severe). Mock-infected (PBS) animals from TS or Air had no inflammatory infiltrates in the lung. For RSV-infected mice, quantification of pulmonary inflammation showed no significance differences between TS or Air WT mice (Figure 2e). On the other hand, lungs from TS exposed and RSV-infected CSE KO mice showed diffuse inflammation with perivasculitis, peribronchiolitis, alveolitis, and vasculitis when compared with all other groups. Overall, quantification of the pulmonary inflammation by alveolitis and bronchiolitis scores indicated significantly greater airway pathology at day 5 in TS exposed RSV-infected CSE KO mice compared to WT mice ($p < 0.05$, Figure 2e). Representative images for each group at day 5 after infection are shown in Figure 3 (a,b).

CSE Deficiency and TS Increase Airway Inflammatory Cells Following Infection

Overall, the numbers of total inflammatory cells, neutrophils and lymphocytes subpopulations were greater in BAL fluids of TS /CSE KO mice. As shown in Figure 4a, a significant greater number of total BAL cells was observed in TS /CSE KO/PBS mice, compared with all other PBS groups (day 1, p<0.05). The difference in the total number of cells persisted at day 5 (p<0.05). Differential H&E staining revealed a slight increase (non-significant) of the number of macrophages in BAL at day 1 post-infection for smoke exposed CSE KO/PBS mice (Figure 4b), but significantly more neutrophils (Figure 4c) and lymphocytes (Figure 4d) at both day 1 and 5 in these animals compared to all other PBS groups. Exposure to air or TS followed by RSV infection was characterized by increase in the total number of cells, macrophages, neutrophils and lymphocytes in BAL of CSE KO and WT mice compared with Air/PBS mice (Figures 4a–d). There were no statistically significant differences between smoke and Air/WT RSV mice for total cells or numbers of macrophages and neutrophils at day 1, respectively day 5 post-infection. At day 5 post-infection, we observed a significant increase in the number of lymphocytes in the BAL for smoke exposed vs Air RSV-infected WT mice (p<0.05, Figure 4d).

However, the total cells in BAL were significantly increased at day 1 compared with day 5 post-infection for air and TS exposed WT and CSE KO mice after RSV infection (p<0.001). The most robust cell inflammation was observed as early as day 1 and persisting at day 5 following RSV infection in CSE KO mice that had been exposed to TS, in regard to total cells (p<0.001, Figure 4a), number of neutrophils (p<0.001, Figure 4c) and lymphocytes (p<0.01, Figure 4d), compared to other RSV-infected groups (Figures 4 c–d). The percentage of BAL macrophages, neutrophils and lymphocytes are presented in Supplemental Figure S2.

CSE Deficiency and TS Enhance BAL Cytokines and Chemokines Following RSV Infection

RSV infection is a potent inducer of cytokine and chemokine production, and increased chemokine release has been shown to play an important role in RSV-induced lung

inflammation and to correlate with disease severity. We have recently shown that in the absence of CSE, RSV infection induced significantly higher levels of the cytokines IL-6, IL-12 (p40) and TNF-α, and chemokines CCL2, CCL3, CCL4 and CCL5 compared to CSE competent infected mice (15). To investigate whether a two week period of exposure to TS along with CSE deficiency affected RSV-induced cytokine response, BAL samples were collected at days 1 and 5 after infection from CSE KO and WT mice and were assessed for cytokines and chemokine levels by a multiplex array. TS exposure and RSV infection in WT mice was associated with significant increase levels for IL-12 (p40), G-CSF ($p<0.05$), CCL4 ($p<0.05$), and CCL5 ($p<0.01$) when compared with Air/WT RSV-infected group at day 1 after infection. BAL concentrations of IL-6, IL-12(p40), and TNF-α in TS /CSE KO RSV-infected mice were significantly higher at day 1 compared to Air/CSE KO RSV-infected mice ($p<0.05$), and to TS /WT RSV-infected mice ($p<0.01$) (Figure 5a). Concentrations of IL-6 and IL-12(p40) remained higher also at day 5 in TS / CSE KO RSV-infected mice compared with TS /WT RSV-infected mice. In addition, exposure to TS followed by RSV infection in CSE KO mice lead to an enhanced production of the chemokines CCL2 ($p<0.001$), CCL3 ($p<0.001$), CCL4 ($p<0.01$), and CCL5 ($p<0.001$), compared to RSV-infected WT mice at day 1 post-infection (Figure 5b). Similar findings were observed with the chemokines CCL3 ($p<0.01$), CCL4 ($p<0.01$), and CCL5 ($p<0.05$) when compared to Air/CSE KO RSV-infected mice.

DISCUSSION

This study shows that in absence of CSE mice that have been exposed to TS for a short period of two weeks had a dramatic increase in airway reactivity to MCh challenge (24h after last smoke exposure)(Figure 1). Even in mice exposed solely to filtered air, airway reactivity was significantly more pronounced than in WT mice with intact H_2S-generating capacity. CSE is a key enzyme that controls production of H_2S in the lung (1, 6) and CSE KO mice exhibit a profound depletion of H_2S in several peripheral tissues (37). In other rodent studies levels of endogenous H_2S were found to be decreased in the lung of rats challenged with ovalbumin. Serum levels of H_2S correlated positively with H_2S levels in lung tissues and peak expiratory flow, and negatively with allergic inflammation and airway smooth muscle hyperplasia (4). In a mouse model of asthma, lack of CSE expression resulted in increased AHR after MCh challenge and some evidence of lung Th2-driven inflammation (38). In our study, although TS exposure was associated with an increased in BAL neutrophils and lymphocytes, levels of Th1 or Th2 cytokines (i.e. IFN-γ and IL-4) in BAL samples of TS - or Air mice, either WT or CSE KO, were at the lowest level of detection or were not significant different among different groups when mice, including when subsequently infected with RSV (data not shown). One report examining the effect of TS alone on airway function/reactivity in mice has shown that side stream TS exposure for a short period (days) resulted in increased AHR to MCh challenge, however mouse strain (ICR) as well as the source of TS (Marlboro) were different from those in our study (12).

We also show for the first time that compared to control WT animals, a relatively short period of TS exposure enhances RSV pathogenicity in mice that are CSE deficient, including: 1) peak of clinical disease as measured by body weight loss, as well as delayed recovery; 2) increased airway obstruction, measured at day 1 after RSV inoculation; 3)

enhanced AHR to MCh challenge at day 5 post-infection; 4) greater viral replication in the lung, and 5) more severe lung inflammation, characterized by peribronchiolitis and alveolitis. These pathogenic responses in TS -exposed/RSV-infected CSE KO mice were associated with an increase in concentration of inflammatory cytokines IL-6, IL-12(p40), and TNF-α and chemokines CCL2, CCL3, CCL4, and CCL5. Other studies in C57BL mice have demonstrated the effect of co-exposure to TS (direct exposure) and RSV on parameters of lung fibrosis, chronic inflammation, protease and cytokine expression but using longer protocols of exposure to smoke (23) and repeated viral infections, as models of COPD (11). Airway function was not examined in those studies. One report examining the effect of TS alone on airway function/reactivity in mice has shown that side stream TS exposure for a short period (days) resulted in increased AHR to MCh challenge, however mouse strain (ICR) as well as the source of TS (Marlboro) were different from those in our study (12). Previous work by others in a BALB/c mouse model of double infection with RSV (neonatal/ adult challenge) has shown that exposure to side-stream smoke from birth to 35 days was associated with increase in eosinophils and reduced levels of Th1 cytokines in BAL following RSV challenge (28). In our study, levels of IFN-γ and IL-4 and Th1/Th2 ratio in BAL samples at day 5 post-infection by a high sensitive ELISA were no significantly different in any of the treatment groups, including those expose to TS and CSE deficient (data not shown).

Endogenous H_2S is involved in critical physiological functions of the respiratory tract and its dysregulation appears to be involved in disease such as COPD and asthma (1). For example, smokers have lower levels of H_2S, when compared with nonsmokers with acute exacerbation of COPD or healthy controls (5). Moreover, in patients with stable COPD, serum H_2S levels were found to be significantly lower in those with more severe obstruction, correlated positively with the percentage of predicted forced expiratory volume in 1 second (FEV_1) values, and negatively with neutrophils in sputum (5). In other studies, patients with COPD have been shown to have lower levels of expression of CSE protein and CBS mRNA (31). Patients with COPD that had lower levels of exhaled H_2S had greater number of eosinophils in sputum, worse lung function, and more frequent exacerbations (40). In asthma, several studies have shown that both children and adult patients have lower levels of serum and exhaled H_2S [reviewed in (1)], with lower H_2S levels being correlated with more altered pulmonary function tests and severity of their disease (33, 39). Elegant studies in mice have also shown that H_2S contributes to lung development, expression of CSE and CBS enzymes in airway epithelium and lung vessels, and demonstrated their critical function in the process of alveolarization and pulmonary vascular development (21). The same group has shown that systemic administration of the H_2S slow-releasing donor GYY4137 partially restored arrested alveolarization in an experimental model of bronchopulmonary dysplasia (22).

Recent investigations in our laboratory have identified a novel antiviral role for H_2S (20). In airway epithelial cells, we showed that RSV inhibits the expression of CSE and H_2S and on the other hand increased its degradation. By studies using the CSE inhibitor propargylglycine (PAG), we demonstrated an increase in viral replication and cytokines production. On the other hand, when cells were treated with a slow-releasing H_2S chemical, GYY4137, RSV replication was significantly reduced. We have also shown that CSE KO have enhanced clinical disease AHR, viral replication and lung inflammation compared with

wild-type mice (15). Although the exact mechanism(s) leading to increased AHR in CSE KO mice exposed to TS alone and/or after RSV infection are not fully understood, our studies suggest that the H_2S pathway in the lung is critical in relaxing airway smooth muscle. Some studies have shown that H_2S relaxes vascular smooth muscle by increasing ATP-sensitive K^+ channel (K_{ATP}) channel currents and hyperpolarizing cell membrane (10), or by K_{ATP} channel–independent mechanisms, such as inhibition of Ca^{2+} release through the inositol-1,4,5-triphosphate receptor (3).

The association between parental or household tobacco smoke exposure and incidence of lower respiratory tract infections in infancy and the contribution of these factors to RSV disease is not fully clear (17) (30). However, when the severity of RSV bronchiolitis has been examined male sex and tobacco smoking by a household member were associated with need for both supplemental oxygen and mechanical ventilation (29). Studies in premature infants, newborns and children have shown that CSE expression increases with age (34), as levels of this enzyme would be developmentally delayed at the time of the most severe manifestations of RSV infection in vulnerable patients. We have previously shown that H_2S plays a critical role in experimental infection of mice by RSV, by reducing inflammation and viral replication in the lung as demonstrated by treatment with an H_2S donor that reduced lung neutrophilia and pathology (15). Based on the results of this study, we suggest that tobacco smoke, the most common and important indoor environmental pollutant may contribute to more severe manifestations of RSV infections in subjects who may be genetically predisposed due to a lower capacity to produce endogenous H_2S, i.e. young infants with immature H_2S-genarating enzymes or those who are more susceptible to the effect of RSV on reduction/degradation of H_2S (20).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

AKNOWLEDGMENTS

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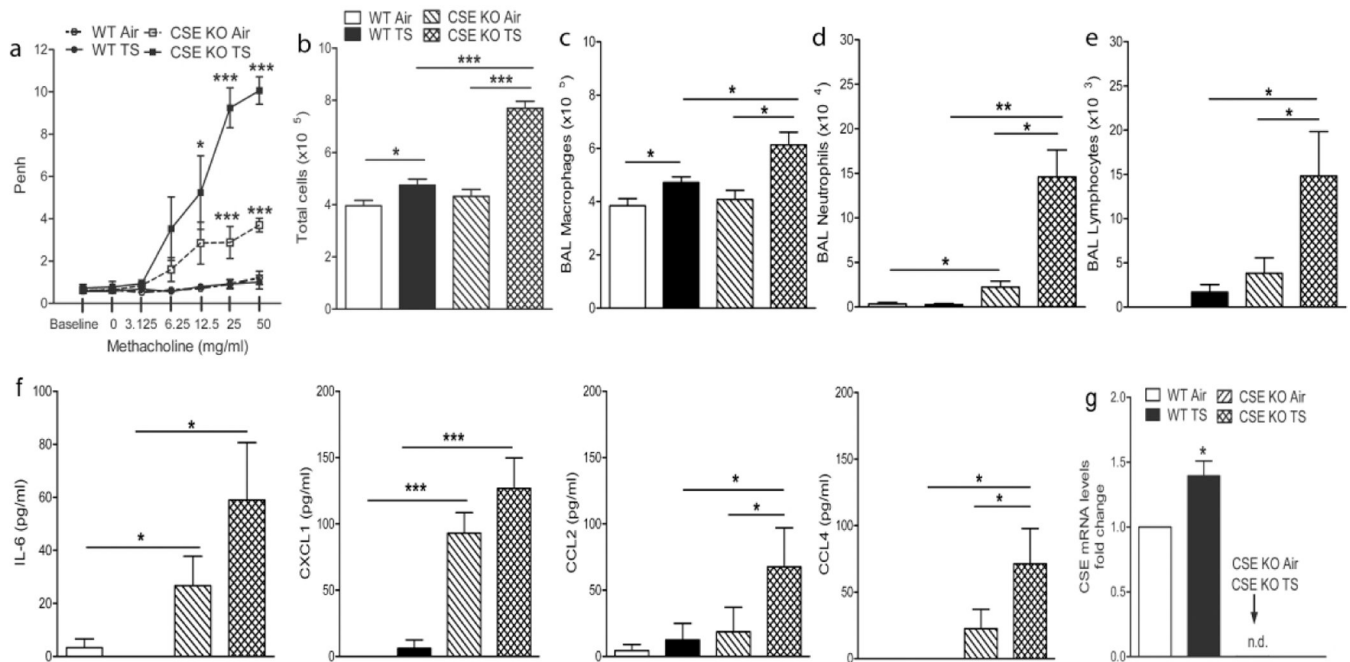
Figure 1.

CSE gene deficiency enhances TS -induced AHR and lung inflammation. Groups of CSE KO and WT mice were exposed to side-stream TS or Air (5 h/day, 5 days/week, for two weeks). TS /CSE KO mice have enhanced AHR after MCh challenge **(a)**, influx of total cells **(b)**, macrophages **(c)**, neutrophils **(d)** and lymphocyte **(e)** in BAL fluid, increased IL-6, CXCL1, CCL2, and CCL4 **(f)** compared to TS /WT mice and Air/CSE KO mice, and CSE mRNA levels in the lung **(g)**. Data are expressed as mean ± SEM (n = 4 mice (2 male and 2 female)/group and are representative of three independent experiments). *p<0.05.**p<0.01, ***p<0.001, CSE KO TS vs WT TS, CSE KO TS vs CSE KO Air, CSE KO Air vs WT Air, and WT TS vs WT Air.
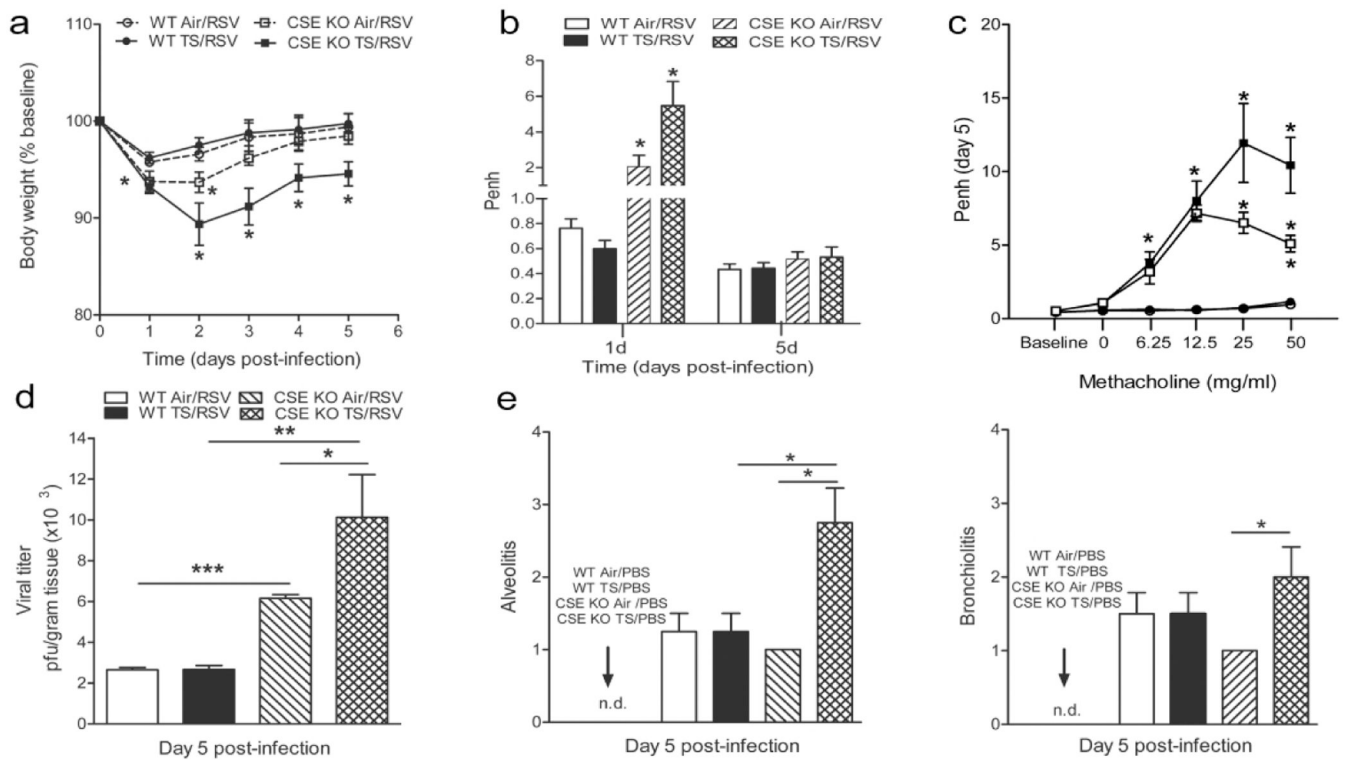
Figure 2.

TS and CSE gene deficiency exacerbate clinical disease, viral replication, and AHR following RSV infection. Groups of CSE KO and WT mice were exposed to TS or Air as in Fig. 1, followed by infection with RSV as described in Material and Methods. **(a)** Effect of TS exposure on RSV induced disease. Change in body weight was measured over a period of 5 days after infection. **(b)** Airway obstruction represented by baseline Penh at day 1 and 5 after RSV infection. **(c)** AHR in response to MCh challenge at day 5 after RSV infection. **(d)** Increased RSV replication in the lung of TS /CSE KO mice, at day 5 after RSV infection lungs measured by plaque assay. **(e)** Lungs were harvested at day 5 after infection, fixed for slide preparation and H&E stained. Alveolitis and bronchiolitis inflammatory scores of prepared slides (scored as described in Materials and Methods). Data are expressed as mean ± SEM (n = 4 mice (2 male and 2 female)/group and are representative of three independent experiments). *$p<0.05$.**$p<0.01$, ***$p<0.001$ CSE KO TS /RSV vs WT TS / RSV, CSE KO TS / RSV vs CSE KO Air/ RSV, CSE KO Air/RSV vs WT Air/RSV, WT TS / RSV vs WT Air/RSV.
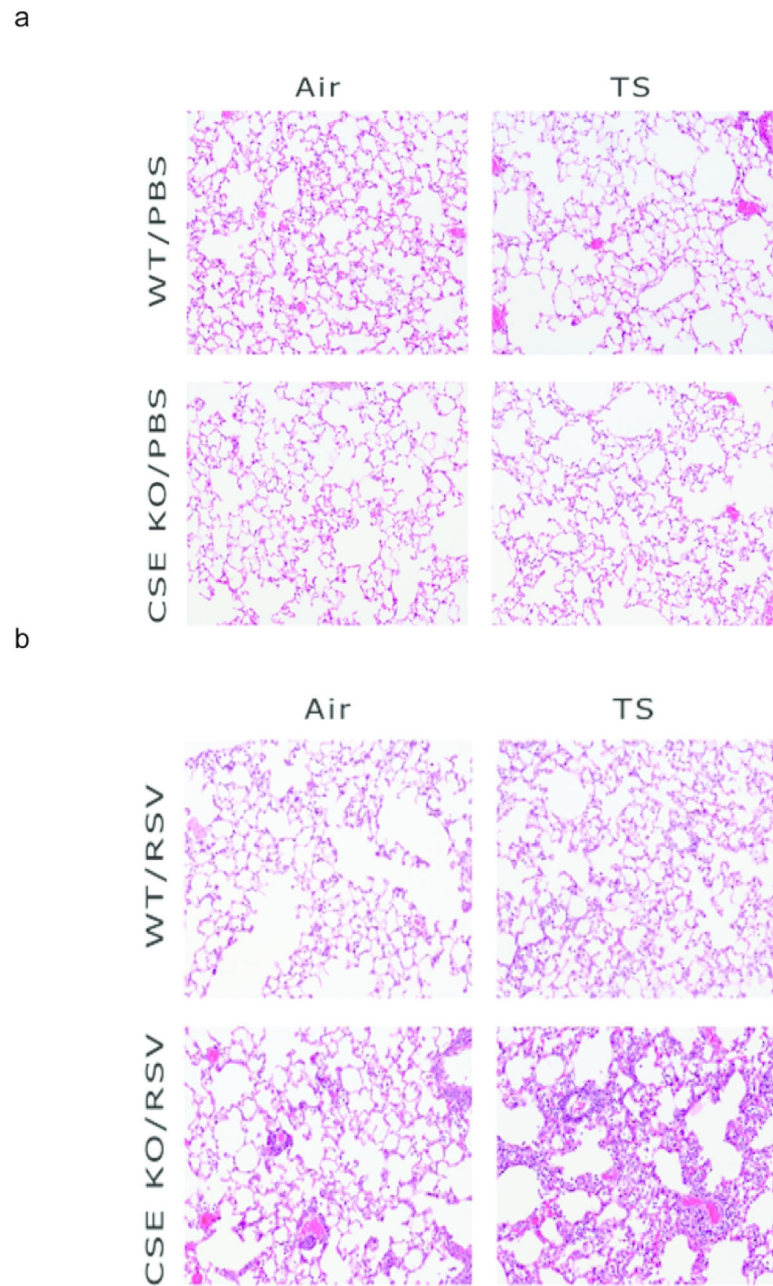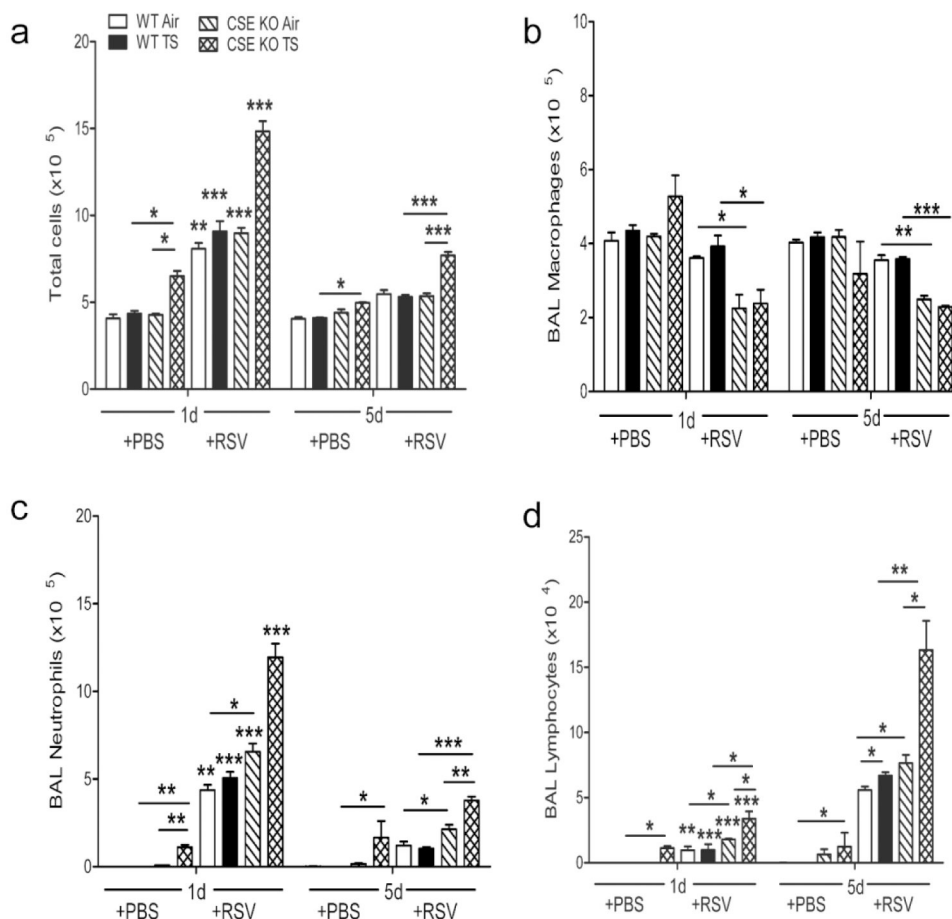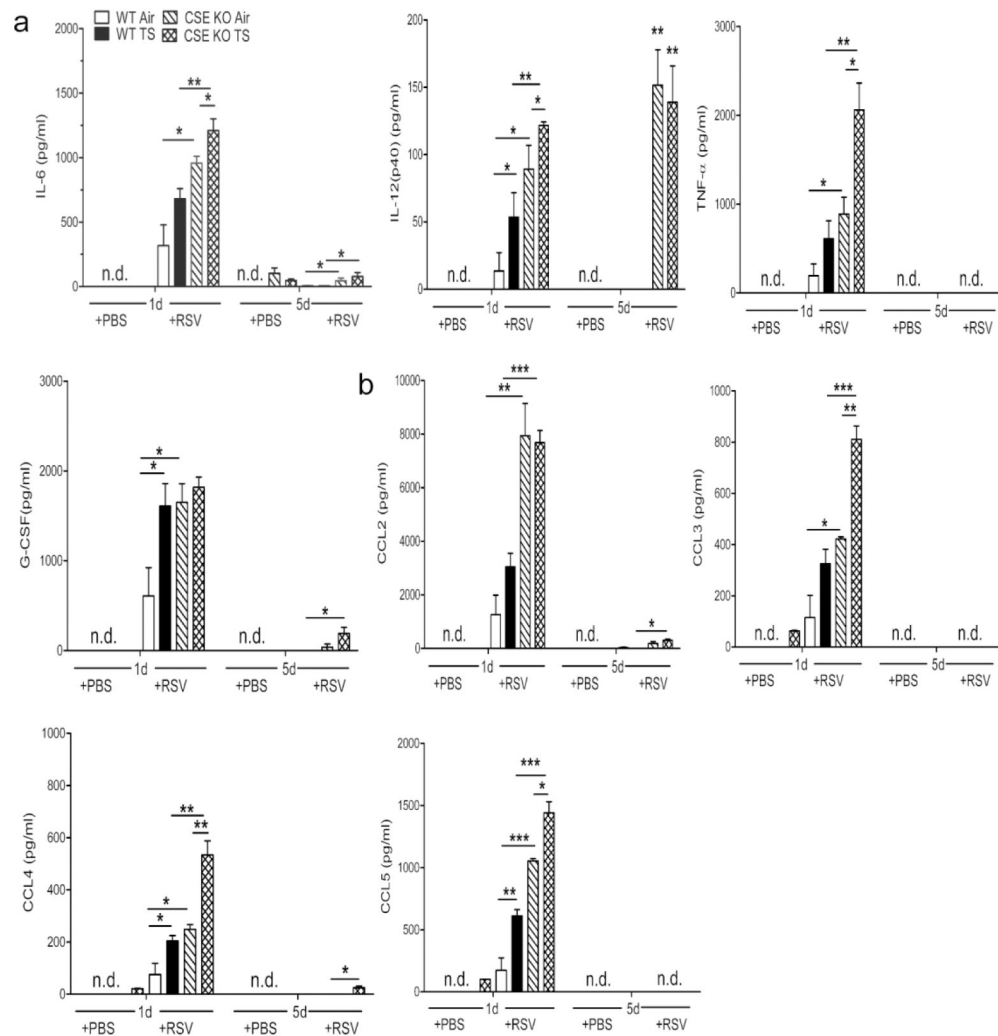
Figure 3.
TS and CSE gene deficiency exacerbate pulmonary inflammation. TS - or Air-exposed CSE KO and WT mice were infected with RSV or sham. Lungs were harvested at day 5 after infection, fixed for slide preparation and H&E stained. **(a)** Representative photomicrographs of lung sections from WT and CSE KO mice exposed to either Air or TS and then mock-infected with PBS. The lung sections are mostly unremarkable. **(b)** Representative photomicrographs of lung sections from WT and CSE KO mice exposed to either Air or TS and then infected with RSV. The sections from CSE KO TS/RSV mice show moderate to severe alveolitis, compared to minimal focal alveolitis observed in the other groups. Original magnification 20×.

Figure 4.
TS and CSE deficiency increase airway inflammatory cells following infection. TS -or Air-exposed CSE KO and WT mice were infected with RSV or sham. BAL fluid was collected at different time points after infection and cell preparations were stained (Hema 3 stain, Fisher Scientific) and counted under the microscope (200 cells/slide). TS increased the influx of total cells **(a)**, macrophages **(b)**, neutrophils **(c)**, and lymphocyte **(d)** into the BAL of CSE KO PBS mice, which was further exacerbated in the RSV infected CSE KO mice, compared with WT animals. Data are expressed as mean ± SEM (n = 4 mice (2 male and 2 female)/group and are representative of three independent experiments). *p<0.05.**p<0.01, ***p<0.001 CSE KO TS/RSV vs WT TS / RSV, CSE KO TS / RSV vs CSE KO Air/ RSV, CSE KO Air/RSV vs WT Air/RSV, WT TS / RSV vs WT Air/RSV, CSE KO TS / PBS vs. WT TS / PBS, and CSE KO TS /PBS vs. CSE KO Air/PBS, WT Air/RSV day 1pi vs. WT Air/RSV day 5pi, WT TS/RSV day 1pi vs. WT TS/RSV day 5pi, CSE KO Air/RSV day 1pi vs. CSE KO Air/RSV day 5pi, CSE KO TS/RSV day 1pi vs. CSE KO TS/RSV day 5pi.

Figure 5.
TS and CSE deficiency enhance BAL cytokines and chemokines following RSV infection. TS - or Air-exposed CSE KO and WT mice were infected with RSV or sham. BAL fluid was collected at different time points after infection to measure cytokines and chemokines by a Bio-Plex array. TS /CSE KO RSV mice had significantly increased levels of cytokines IL-6, IL-12(p40), TNF-α, G-CSF **(a)** and chemokines CCL2, CCL3, CCL4, CCL5 **(b)** compared to TS/WT RSV and Air/CSE KO RSV mice. Data are expressed as mean ± SEM (n = 4 mice (2 male and 2 female)/group and are representative of three independent experiments). *p<0.05.**p<0.01, ***p<0.001 CSE KO TS /RSV vs WT TS / RSV, CSE KO TS / RSV vs CSE KO Air/ RSV, CSE KO Air/RSV vs WT Air/RSV, and WT TS / RSV vs WT Air/RSV.