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Age-dependent Allergic Asthma Development and Cystathionine gamma-Lyase Deficiency

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Running Head: H_2S and age-dependent development of asthma

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Abstract

Aims: The pathogenic mechanisms for the higher prevalence of **a**llergic asthma in children than in adults have not been settled. The aim of the present study is to examine whether the age-dependent development of allergic asthma is caused by age-dependent expression of cystathionine gamma-lyase (CSE), a key enzyme that catalyzes the production of H_2S. ***Results:*** Allergic asthma was induced with ovalbumin in wild-type (WT) and CSE knock-out (KO) mice at young and old ages. CSE expression and H_2S production were lower in immune cells of young WT mice than those of old WT mice. Coincidently, more severe asthmatic symptoms with greater type-2 immunoreaction were found in young WT mice than old WT mice. H_2S supplementation reversed the asthmatic symptoms. Lower expression levels of CSE proteins were also found in human umbilical cord blood mononuclear cells in comparison with that of peripheral blood mononuclear cells from adult people. The age-dependent asthma propensity vanished in CSE-KO mice but these mice developed more severe asthma than WT mice. More splenocytes were differentiated to type-2 cytokine-generating cells in young WT mice and in CSE-KO mice at all ages. This differentiation was inhibited by H_2S donors. GATA3 translocation to the nucleus and type-2 immunoreaction of splenocytes were inhibited after GATA3 was *S*-sulfhydrated by H_2S. ***Innovation and conclusion:*** This study for the first time demonstrated that lower abundance of CSE expression and H_2S production enhances type-2 immunoreaction and renders a higher incidence of allergic asthma at a young age. As such, H_2S level may be a biomarker for asthma development and a H_2S-based strategy can be perceived for asthma prevention and treatment.

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Introduction

Over 50% of all asthma cases attribute to atopy (2). The first 1-3 years of human life are critical for the development of atopic (allergic) asthma (16,34). Immune maturation of atopic children lags behind non-atopic children, with a persistent type-2-biased immune response to allergens (32). On the other hand, asthma development was much less severe if he early childhood was spent on a farm (9). One theory speculates that this phenomenon is related to the maturation of the developing neonate immune system stimulated by early gut microbial colonization. For example, the development of allergic asthma was more prominent in germ-free animals than in animals with normal microbial flora (24). The linkage between microbiota colonization in the gut and asthma development, however, has been unknown.

Animals and humans at the neonatal stage have low density and phylogenetic diversity of their gut microbiota (8,17). Successive colonization of microbiota occurs in an age-dependent manner from weeks to years (13, 17). Gut microbiota can produce significant amount of H_2S (40). Thus, low H_2S production from microbiota in the gut during early childhood is expected. If this low level of exogenous H_2S cannot be compensated by H_2S produced by mammalian cells, the weakened anti-asthmatic effects of H_2S would result in early and more severe asthma attack. Butyrate and pyridoxal 5′-phosphate, produced by bacteria, up-regulate H_2S-generating enzymes, including cystathionine gamma-lyase (CSE), in mammalian cells (5, 19). CSE activities in brain, heart, aorta, liver, kidney, colon, cecum, and small intestine were higher in non-germ-free mice than in age-matched germ-free mice (33). In mouse liver tissues, the expression and activity of were very low after birth but at the age of 3 weeks the upregulation of CSE expression and activity reached the maximal levels (15). It appears that the age-dependent CSE/H_2S development bears a correlation with the age-dependent asthma development.

The activity of CSE/H_2S system has been implicated in asthma development. Adult asthma patients had lower plasma levels of H_2S but more severe asthma symptoms (43). Animal models of allergic asthma, rats or mice, also had lower H_2S levels in plasma and lung tissues than in age-matched healthy animals (7, 47). NaHS decreased the severity of ovalbumin (OVA)-induced asthma, including the elevated type-2 cytokines in bronchoalveolar lavage fluid. The preceding studies led us to hypothesize that endogenous activity of CSE/H_2S system in early life controls regulatess the type-2-biased immune response and that the low activities of CSE/H_2S system in childhood instigates the higher incidence of asthma.

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Results

Correlation of age and CSE levels with allergic asthma development

OVA was used to induce allergic asthma at the dosages normalized by the respective body weights of young (3-4 weeks) and old mice (7-8 months). OVA-treatments of young WT mice have significantly increased lung resistance and decreased lung dynamic compliance (Cdyn) in response to aerosolized methacholine (MCh) (Fig. 1*A*).Airway responsiveness was much weaker in OVA-treated old WT mice, only about 50% of lung resistance increase and Cdyn decrease of those in OVA-treated young WT mice (Fig. 1*A*). OVA-induced inflammatory cell influx in bronchoalveolar lavage fluid (BALF), especially eosinophils, from young WT mice was approximately 3 times of that in old WT mice (Fig. 1*B*). BALF from young WT mice also had significantly higher level of interleukin-4 (IL-4) than that from old WT mice (Fig. 1*C*). These results are consistent with a clinical observation that enhanced lymphocyte cytokine responses were associated with the early onset of atopy (37). More peribronchial and perivascular inflammatory infiltrates were found in lung tissues of young than in those of old WT mice (Fig. 1*D*).

Airway responsiveness to MCh challenge in non-asthmatic WT mice was also age-dependent. The airway resistance of young WT mice was more than three times of that of old WT mice. On the other hand, the airway resistance did not show difference between young and old CSE-KO mice although it was significantly greater than that of old WT mice (Fig. 1E). It should noticed that without OVA sensitization and challenges WT and KO mice at different ages did not develop asthma and airway inflammation (Fig. 1F).

In each of H&E-stained mouse lung slides, we counted 7 different bronchi to calculate total eosinophils. Each group of slides composed of 3-4 mice. There were 65±3 peribronchial

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eosinophils per bronchus in OVA-treated WT-young mice whereas only 10±5 in OVA-treated WT-old mice ($P<0.05$). OVA-treated young and old CSE-KO mice had 114±12 and 107±4 peribronchial eosinophils per bronchus, respectively ($P<0.05$ vs. OVA-treated young and old WT-mice).

MCh challenges of OVA-treated young CSE-KO mice caused similar airway resistance increase and Cdyn decrease as those of OVA-treated old CSE-KO mice (Fig. 1*A*). Inflammatory infiltrates in airways and type-2 cytokine levels (IL-4, IL-5 and IL-13) in BALF were comparable between OVA-treated young and old CSE-KO mice (Fig. 1*B*-*D*). CSE-KO mice exhibited greater airway hyper-responsiveness and more severe inflammation than age-matched WT mice (Fig. 1*A*-*D*).

In addition to immunologically challenging the mice with body-weight normalized dosages as described in Materials and Methods (Fig. 1A and B), we also treated young and old mice using fixed amounts of OVA (40 μg for sensitization and 100 μg for challenge). With this alternative OVA treatment regime, young WT mice also developed higher airway responsiveness and more inflammatory infiltrates than old WT mice (Fig. 2).

Correlation of CSE expression and type-2 cytokine production in allergic asthma development

Mouse mediastinal lymph cells and splenocytes were isolated 5 days after OVA-sensitization. These cells were re-stimulated with OVA for 3 days. In comparison with those from young WT mice, the cells from old WT mice produced lesser type-2 cytokines (IL-4, IL-5 and IL-13) after OVA re-stimulation (Fig. 3*A* and *B*). The levels of type-2 cytokines were similar between young and old CSE-KO mice (Fig. 3*A* and *B*). The cells from WT mice generated lesser type-2 cytokines than those from age-matched CSE-KO mice. CSE protein levels in splenocytes

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from young WT mice were lower than those in splenocytes from old WT mice (Fig. 3C). The protein levels of mercaptopyruvate sulfurtransferase (MST) in splenocytes were similar between young and old WT mice. Cystathionine β-synthase (CBS) proteins were undetectable in splenocytes of young and old WT mice (Fig. 3*D*). Thus, lower expression of CSE proteins in splenocytes from young WT mice resulted in lesser H_2S production in these cells than in the splenocytes from old WT mice (Fig. 3E).

CBS or MST proteins were not detectable, but CSE proteins were spotted, in human spleen tissue (Fig. 3*F*). Human umbilical cord blood mononuclear cells (CBMCs) expressed lesser CSE proteins than peripheral blood mononuclear cells (PBMCs) from adult humans (38-55 years old) (Fig. 3*G*). While CBS proteins were clearly detected in mouse liver tissues (positive control), they were not detectable in PBMCs or CBMCs. Low abundance of MST proteins was detected in PBMCs.

We also tested the therapeutic effect of NaHS at two doses, 14 and 39 μmole/kg (i.p.). NaHS at 39 μmole/kg (i.p., once a day) weakened airway hyper-responsiveness of OVA-sensitized CSE-KO mice to 55.2±14.5% (n=5, $p<0.05$) and limited airway inflammatory infiltrates. The relative total cell numbers in BALF from CSE-KO mice was reduced to 74.9±10.5%, taking the average numbers of total cells in BALF from OVA-sensitized CSE-KO mice without NaHS treatment as 100% (n=5). Instead of giving higher concentrations of NaHS, we decided in the present study to use NaHS at low concentration (14 μmole/kg, i.p.) but increased the frequency of NaHS administration to twice a day. This new treatment regime was composed of two days NaHS treatment before OVA sensitization, and continued NaHS treatment during and two days after sensitization. OVA re-stimulation-induced IL-4, IL-5 and IL-13 production was significantly decreased by NaHS treatments, with IL-4 being decreased

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most dramatically, in lymph cells and splenocytes from both young WT mice and CSE-KO mice (Fig. 3*H* and *I*). NaHS slightly decreased IL-4 level in splenocytes, but not in lymph cells, from old WT mice. NaHS increased IL-5 but decreased IL-13 production in splenocytes, and itdecreased both IL-5 and IL-13 production in lymph cells from old WT mice (Fig. 3*H* and *I*). These results indicate that the effect of CSE deficiency on type-2 immunitywas mediated by H_2S, and that the inhibitory effect of NaHS, as a H_2S donor, on type-2 immune response was greater in the absence of endogenous H_2S.

Furthermore, OVA-induced airway hyper-responsiveness to MCh as alleviated significantly by NaHS treatments by about 2 times in young/old CSE-KO mice and 1.6 times in young WT mice in comparison with saline treatments (Fig. 4*A*). OVA-challenged young WT mice and young/old CSE-KO mice also had lesser airway inflammatory cell infiltration, fewer peribronchial and perivascular inflammatory infiltrates in the lung, and lower levels of IL-4 and IL-5 in BALF after NaHS treatments in comparison with saline treatment control (Fig. 4*B*-*D*). NaHS treatment of old WT mice did not significantly change their airway responsiveness and inflammatory infiltration (Fig. 4*A*-*D*). These data suggest that NaHS treatment during sensitization phase protects against ensuing allergic asthma development when the expression of CSE and endogenous H_2S level are suppressed.

Differentiation of naïve immune cells and its inhibition by H_2S *in vitro*

Cultured splenocytes were differentiated into type-2 cytokine-generating cells under specific culture conditions. Splenocytes from old WT mice secreted much lesser type-2 cytokines than those from young WT mice (Fig. 5*A*). In consistence with *in vivo* OVA-sensitization results (Fig. 3*A* and *B*), CSE-KO mouse splenocytes did not show such an age-

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related difference in their type-2 cytokine-generating abilities when polarized *in vitro* (Fig. 5*A*). Splenocytes from CSE-KO mice generated more IL-4, IL-5 and IL-13 than those from WT mouse (Fig. 5*A*). The inhibitory effect of NaHS treatments on type-2 cytokine production in splenocytes from CSE-KO mice was concentration-dependent (Fig. 5*B*).

Signal transducer and activator of transcription factor-6 (STAT6) regulates naïve T cell differentiation and type-2 cytokine production (28). It can be activated by IL-4 and IL-13 (12). We found that IL-4 induced phosphorylation of STAT6 to the same degree in WT and CSE-KO mice (Fig. 5*C*). In IL-4-treated splenocytes from young CSE-KO mice, NaHS treatments did not alter STAT6 phosphorylation (Fig. 5*D*).

GATA-3 is a transcription factor that binds to the DNA sequence "GATA". It participates in the regulation of innate and adaptive type-2 immune response (14). GATA3 distribution between nuclear and cytosolic fractions was investigated. The successful separation of nuclear from cytosolic fractions was confirmed by the lack of β-actin and presence of lambin b1 in the nuclear fraction, and the presence of β-actin and lack of lambin b1 in the cytosolic fraction (Fig. 5*E* and *F*; Fig. S1). In differentiated splenocytes from young WT mice, nuclear GATA3 protein levels were much higher than in those from old WT mice. In contrast, the levels of nuclear GATA3 proteins were similar in differentiated splenocytes from young and old CSE-KO mice (Fig. 5*E*). NaHS treatments during differential culture decreased concentration-dependently the expression of nuclear GATA3 in splenocytes from young CSE-KO mice (Fig. 5*F*). In the experiments described in Fig. 5*B, D,* and *F,* only CSE-KO splenocytes were used in order to study NaHS effect in the absence of endogenous H_2S.

GATA3 *S*-sulfhydration induced by H_2S

S-sulfhydration is a post-translational modification mechanism in which cysteine-SH groups are converted to –SSH (27). The levels of *S*-sulfhydrated GATA3 proteins were significantly higher in old WT mouse spleens than in those from young WT mice, and these levels in WT mouse spleens were much higher than in the spleens from young/old CSE-KO mice (Fig. 6*A*). GATA3 *S*-sulfhydration in HEK-293 cells, in which CSE was heterologously overexpressed, was substantially increased by NaHS and decreased by dl-propargylglycine (PPG) (Fig. 6*B*). *S*-sulfhydration of GATA3 proteins was suppressed by double mutation of cyteine-84/182 (GATA3-C84/182) or cysteine-84/248 (GATA3-C84/248) (Fig. 6*C*). Decreased GATA3 nuclear translocation was suggested by the higher ratio of nucleus/cytosol GATA3 with mutated GATA3 than with wild-type GATA3 (Fig. 6*D*).

Deficiency in type-1 immune response also contributes to atopy and asthma development (21). The effect of CSE expression on type-1 immune response was examined *in vitro* using cultured splenocytes. Under the culture conditions favoring T cell differentiation into type-1 cytokine-generating cells, splenocytes from old WT mice generated more interferon gamma (IFN-γ), a type-1 cytokine, than those from young WT mice (Fig. 7*A*). Splenocytes from CSE-KO mice produced lesser IFN-γ than those from WT mice (Fig. 7*A*). NaHS did not have effect on IFN-γ production except at the high concentration of 30 μM (Fig. 7B). Both STAT4 and T-box protein (T-bet) are essential for the initiation and maintenance of type-1 immune response (36). The IL-12-induced STAT4 phosphorylation was not affected by H_2S (Fig. 7*C* and *D*). Lower nuclear level of T-bet was found in differentiated splenocytes from young/old CSE-KO mice as well as young WT mice (Fig. 7*E*). NaHS treatment (30 μM), on the other hand, increased nuclear T-bet level (Fig. 7*F*). It was reported previously that the inhibition of CSEwith PPG decreased the levels of T-bet, IL-12 and IFN-γ, delaying heart allograft rejection (39).

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Age-dependent defects in CSE/H_2S signaling in the lung and airway tissues

CSE protein was detected in both human and mouse lungs (Fig. 8*A*, *B*). Following the same patterns as seen in mouse splenocytes and human blood mononuclear cells (Fig. 3), CSE expression in lungs was low in 4-week WT mice, increased at the age of 8-week, and reached the highest level at 16 weeks of age (Fig. 8*B*). Thereafter, CSE protein levels in WT mouse lungs remained at the same age as in 16-week mice. In mouse airway tissues, CSE protein levels followed the same age-dependent patterns as seen in lungs from age-matched WT mice (Fig. 8*C*). CBS and MST proteins were also detected in airway tissues from WT mice. However, their expression levels did not alter as the mice became aged (Fig. 8*C*). Furthermore, H_2S production was lowest in WT mouse lung and bronchial tissues at the age of 4 weeks. At the age of 8 and 16 weeks, H_2S production reached the highest levels in WT mouse bronchi and lungs, respectively (Fig. S2).

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Discussion

Early childhood is accompanied by immune immaturity, which favors type-2 dominant reactivity and renders children higher susceptibility of developing allergic asthma (1, 3). Low expression of CSE may explain this prevalence of childhood asthma attack. Our study found that young WT mice have lower expression CSE in their immune cells than in their counterparts of old mice. OVA-sensitization of young WT mice resulted in more type-2 immune responses and more severe allergic asthma symptoms than OVA-sensitized old WT mice. On the other hand, *cse* gene knock-out enhanced type-2 immune response and worsened asthma development independent of animal ages. The aforementioned outcomes of *cse* gene knock-out can be ascribed to the decreased endogenous H_2S level. H_2S at nanomolar concentrations potentiated T lymphocyte activation *in vitro* (25). H_2S also up-regulated G protein-coupled receptor kinase 2 and CD11b in neutrophils (35). Our current study showed that NaHS alleviated OVA-sensitization-induced type-2 response in peripheral lymph tissues *in vivo*. NaHS limited the differentiation of splenocytes, *in vitro*, into type-2 cytokine-producing cells. The frequency and concentrations of *in vivo* NaHS administrations in the current study were selected according to literature and our previous studies. H_2S concentrations in mammalian tissues range from 30 to 100 μmole/kg (22). NaHS (14 μmole/kg, i.p.) was used in the present study due to its effectiveness and physiological relevance. Numerous previous studies have used this NaHS concentration for *in vivo* animal treatment (7, 47, 48).

Type-2 cytokine genes are clustered on mouse chromosome 11 or human chromosome 5 (20). Our study shows that CSE/H_2S-induced inhibition of type-2 immune response is not mediated by STAT6 (10). Instead, H_2S inhibited asthma development by *S*-sulfhydrating GATA3 to decrease its nuclear translocation (Fig. 9). GATA3 is considered a master regulator in

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type-2 response. It directly binds to *Il-5* and *Il-13* promoters. By acting on type-2 cytokine locus, GATA 3 regulates chromatin modification. GATA3-deficient $CD4^+$ T cells cannot differentiate into T helper 2 (T_H2) cells (49).

Mouse and human GATA3 contain 12 conserved cysteine residues. Eight of them are located in the zinc finger region, limited their likelihood to be modulated. Among the remaining 4 cysteines, H_2S *S*-sulfhydrates C84, C182, and C248. C248 locates at the very beginning of nuclear localization sequence (NLS) of GATA3. *S*-sulfhydration of C84, C182 and C248 may alter the structure of GATA3 and decrease its binding to nuclear import protein importin-a (23,46). Once C-84/182 or C-84/248 were double mutated, GATA3 *S*-sulfhydration level was decreased its nuclear expression increased. The proposed mechanisms for CSE/H_2S mediated age-dependent asthma development are summarized in Fig. 9.

The immature immune system in childhood is also marked with weak protective type-1 immune response (21). Previous studies showed that IFN-γ levels and atopy were inversely associated (31). Type-1 cytokines and T-bet suppress the expressions of type-2 cytokine and GATA3 genes. We found that higher CSE expression and endogenous H_2S levels in old WT mice facilitated the differentiation of type-1 cytokine-generating cells by increasing nuclear T-bet expression. Exogenous NaHS also facilitated IFN-γ production albeit not as strong as its inhibitory effect on type-2 cytokine generation.

Airway is the end target of asthma attack. Airway hyper-responsiveness in childhood increases the prevalence of childhood asthma (4, 38). Airway contraction and ventilation are determined by the contractility and proliferation of airway smooth muscle cells (ASMCs) as well as an array of inflammatory mediators secreted from these cells (50). In the present study, we confirmed the age-dependent expression pattern of CSE in WT mouse lungs as well as in WT

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mouse airways. While CSE and CBS are both involved in the reverse-transsulfuration pathways, the former cleaves cystathionine and the latter generates cystathionine, and both generate H_2S from L-cysteine cleavage. On the other hand, the production of H_2S by MST requires the formation of the immediate substrate 3-mercaptopyruvate from L-cysteine (42). Neither CBS nor MST proteins showed an age-dependent expression profile in the involved organs in our study, suggesting that the age-dependent asthma development in our study does not bear a causative relationship with the CBS or MST. We showed previously the expression of CSE in vascular smooth muscle cells of mouse lung and ASMCs (7, 41). We show here that MCh-induced airway responsiveness was much higher in young WT mice and young/old CSE-KO mice than that of old WT mice (Fig. 1*E*). The mechanisms underlying H_2S-induced relaxation of ASMCs including the opening of K_{ATP} channel (11) or inhibition of Ca^{2+} release through inositol-1,4,5-trisphosphate receptors (6). Low abundance of CSE in young mice results in decreased endogenous H_2S level, leading to the development of airway hyper-responsiveness. H_2S may also directly inhibit the release of pro-inflammatory mediators, such as IL-8, from ASMCs (29). In old WT mice, CSE/H_2S system in ASMCs is matured and fully developed, which can offer protection against local inflammation and ASMC constriction during allergen exposures. We have previously reported the therapeutic effects of NaHS treatments during the OVA-challenge phase (7, 47), consistent to the local effects of H_2S in the lung observed in the present study.

In summary, endogenous H_2S production in peripheral lymph tissues and the lung and exogenous H_2S supplementation inhibit allergic asthma development by suppressing allergen-induced type-2 immune response and airway hyper-responsiveness. Lack of endogenous H_2S production due to lower CSE expression in the early stage of development may underlie higher

incidence of childhood allergic asthma. Our findings may help the development of H_2S-based, in addition to NO-based or anti-oxidant based (26, 30), prevention and treatment strategies for pediatric asthma.

Innovation

The development of allergic asthma is more often and more severe in childhood. The mechanisms for this higher prevalence of childhood asthma than in adulthood remain elusive. The present study for the first time demonstrates that deficiency of CSE/H_2S pathway is responsible for the higher prevalence of allergic asthma development at young ages due to the lack of H_2S-induced S-sulfhydration of GATA3 protein and the suppression of type-2 immune response. H_2S supplementation at young ages may be considered as a novel protective tactic against allergic asthma.

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Materials and Methods

H_2S production measurements

H_2S generated by mouse lung tissues was measured as described previously (44). Briefly, the isolated mouse lung tissues were homogenized with 50 mM ice-cold potassium phosphate buffer (pH 6.8). The tissue homogenates were incubated with L-cysteine (10 mM, Sigma, St. Louis, MO) for 90 min at 37°C, and then trichloroacetic acid (Sigma) was added to stop the reaction. The level of methylene blue generated by the interaction of H_2S with N,N-dimethyl-p-phenylenediamine sulfate (Sigma) was determined with a FLUOstar OPTIMA microplate spectrophotometer (BMG LABTECH, Germany) at 670 nm.

For measuring H_2S generated by splenocytes (18), splenocytes were cultured in 50 ml cell culture flasks, the top of which were pre-coated with 5 ml zinc-agar. After 72 h culture, the agar layers were dissolved in 2 ml of N, N-dimethyl-p-phenylenediamine chloride (40 mM N, N-dpd in 7.2 M HCl). Iron chloride reagent (400 μl, 30 mM $FeCl_3$ in 1.2 M HCl) was then added. Absorbance was measured at 670 nm using Synergy 2 Multi-Mode Reader (BioTek, USA). H_2S concentration was calculated against a standard curve that was generated with NaHS at concentrations from 5 to 250 μM.

H_2S production from mouse bronchus tissues was measured and semi-quantitated using a modified lead sulfur method. Mouse bronchial tissues of 50 mg were homogenized in PBS buffer supplemented with 10 mM cysteine and 2 mM pyridoxal 5'-phosphate (PLP). A lead acetate H_2S detection strips (Sigma, Aldrich, USA) was placed above the liquid phase in a closed container (covered 96-well plate) and incubated about 3 hours at 37˚C until lead sulfide darkening of the paper occurred. Then the reacted H_2S detection strips were scanned with a CanoScan LiDE 700F scanner. The optical densities in arbitrary units of the reacted H_2S strips from the scanned images

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were analyzed and quantitated using imageJ software (1.47V, NIH, USA) and the data were plotted for different age groups.

Allergic asthma model

CSE gene knockout mice were generated in our lab on C57BL/6J×129SvEv background as previously described (44). Young (3-4 weeks old) and old (7-8 months old) WT and age-matched CSE-KO mice were sensitized with OVA (2 μg per gram body weight i.p., Grade V, Sigma-Aldrich) in 100 μl aluminum hydroxide gel solution on days 0 and 7. Isoflurane-anesthitzed mice were challenged with OVA (5 μg/gram body weight) in 30-40 μl phosphate buffered saline (PBS) by intratracheal inhalation on day 21 to day 23. NaHS treatments of selected groups of mice were conducted (14 μmole/kg i.p. twice a day) two days prior to sensitization until two days after sensitization (day minus-2 through day 2 and day 5 through day 9). All animal experiments were conducted in compliance with guidelines of Canadian Council on Animal Care and approved by the Animal Care Committee of Lakehead University, Ontario, Canada.

Measurement of lung function

To test airway resistance in response to increasing concentrations of aerosolized methacholine (1.5, 3.1, 6.2, 12.5 mg/ml), an R&C (resistance and compliance) system (Buxco, USA) was used. Data collection and process were accomplished with a FinePointe Software (Buxco, USA).

Collection of bronchoalveolar lavage fluid (BALF) and examination of lung histology

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To obtain BALF, mouse lungs were flushed three times with 0.8 ml PBS/1% BSA. Total cell numbers were counted using a hemocytometer. BALF cells were cytospined and stained with Wright–Giemsa staining solution for cell differential counting. The supernatants of BALF were then stored in -80°C for determining cytokine levels with ELISA (R&D, USA).

The fixation of the left lobes of lungs was taken in 5 ml of 10% buffered formalin and the fixed tissues embedded in paraffin. The lung sections at 4 μm were stained with H&E reagents.

Splenocytes and lymph cells isolation

Mediastinal lymph nodes and spleens were mashed through a 40 μm cell strainer to obtain splenocytes and lymph node cells. Red Cell Lysis Buffer was used to remove erythrocytes from spleens. Cells were washed with PBS and RPMI 1640 medium before use.

Type-2 cytokine-generating cell differentiation culture

Splenocytes (200 μl, 1 million/ml) were seeded in 96-well plates and cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 500 U penicillin-streptomycin and 50 μM β-mercaptoethanol. Mouse IL-4 (20 ng/ml, Peprotech, Canada), mouse IL-2 (20 ng/ml, Peprotech, Canada), anti-mouse IFN-γ (5 μg/mL, R4-6A2; eBioscience, USA), anti-mouse CD3ε (5 μg/ml, plate-bound, 145-2C11; eBioscience), anti-mouse CD28 (2 μg/ml, clone 37.51; eBioscience) were included in the culture medium,. In the experiments described in Fig. 5*B, D* and *F*, the culture medium contained NaHS at different concentrations. On day 3, the cells were passaged and cultured for an additional 3 days in 24-well plates containing IL-2 (20 ng/ml), IL-4 (20 ng/ml) with or without the addition of NaHS at different concentrations. Following the differentiation culture, splenocytes were re-stimulated with PMA/ionomycin

(1:1000, eBioscience) for 24 hours. ytokine levels in the culture medium were assayed with ELISA (R&D).

Western blot

Whole cell lysates of mouse splenocytes, human peripheral blood mononuclear cells (PBMC), and human cord blood mononuclear cells (CBMC) were prepared using RIPA lysis buffer (Thermo Scientific, USA) supplemented with Protease Inhibitor Cocktail (Sigma-Aldrich, USA). Human spleen and lung samples were purchased from Cure line (USA). PBMCs and CBMCs were purchase from Precision Bioservices (USA) and PromoCell (Germany). Immunoblotting was performed using mouse anti-CBS (1:1000, Abnova, Taiwan), rabbit anti-CSE (1:1000, Proteintech, USA), rabbit anti-MST (1:500, Sigma-Aldrich, USA), rabbit anti-phospho-STAT6, rabbit anti-STAT6 and mouse anti-GATA3 antibody (1:200, Santa Cruz Biotechnology, USA).

To detect nucleus GATA3 expression, nuclear extracts were prepared with NE-PER Nuclear and Cytoplasmic Extraction Reagent (Pierce, Thermo Scientific, USA) following the manufacturer's instructions.

S-sulfhydration detection

Modified biotin switch assay was performed as described previously (27). HEK 293 cells and spleen tissues were sonicated in HEN buffer (250 mM Hepes-NaOH (pH 7.7), 1 mM EDTA, and 0.1 mM neocuproine) containing 100 μM deferoxamine. After centrifuge, supernatant was blocked with HEN buffer containing 2.5% SDS and 20 mM methyl methanethiosulfonate (MMTS). The proteins were precipitated at -20°C and re-suspended in HEN buffer containing 1%

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SDS. The samples were further incubated for 3 hours at 25^{o}C with biotin-HPDP (N-[6-(biotinamido)hexyl]- 3'-(2'-pyridyldithio) propionamide) in dimethyl sulfoxide added. Biotinylated proteins were precipitated by streptavidin-agarose beads and eluted by SDS-PAGE sample buffer. Thereafter, Western blotting analysis was conducted using anti-GATA3 antibody (1:200, Santa Cruz Biotechnology).

Eosinophils counting

H&E stained lung sections were assessed under light microscope with x640 magnification. Seven bronchi were chosen randomly in each lung slide. Eosinophils surrounding each bronchus were counted and the average number of eosinophils per bronchus was calculated.

Type-1 cytokine generating-cell differentiation culture

Splenocytes (200 μl, 1 million/ml) were cultured in 96-well plates with RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 500U penicillin-streptomycin and 50 μM β-mercaptoethanol. Mouse IL-12 (20 ng/ml, Peprotech), mouse IL-2 (20 ng/ml, R&D), anti-mouse IL-4 (5 μg/ml, 11B11, eBioscience), anti-mouse CD3ε (5 μg/ml, plate-bound, 145-2C11; eBioscience), and anti-mouse CD28 (2 μg/ml, clone 37.51; eBioscience), were included in the culture medium. NaHS at different concentrations was added to the culture medium in selective groups of experiments. On day 3, the cells were passaged and cultured for additional 3 days in 24-well plates containing IL-2 (20 ng/ml), IL-12 (20 ng/ml) with or without NaHS at different concentrations. Following the type-1 immune cell differentiation culture, splenocytes were re-stimulated with PMA/ionomycin (1:1000; eBioscience) for 72 hours. IFN-γ level in the culture medium was assayed with ELISA (eBioscience).

OVA re-stimulation *in vitro*

Splenocytes and mediastinal lymph nodes were collected 5 days after OVA sensitization from 6 mice for each group. These were re-stimulated with OVA (100 μg/ml) for 72 h. The levels of IL-4, IL-5, and IL-13 in the supernatant were assayed with ELISA.

Mutagenesis of GATA3

Single mutation at cyteine-84 (GATA3-C84), cysteine-182 (GATA3-C182), cyteine-248 (GATA3-C248), cysteine-375 (GATA3-C375) and double mutation at cyteine-84/182 (GATA3-C84/182) or cysteine-84/248 (GATA3-C84/248) were conducted using the Quick Change Site-Directed Mutagenesis kit (Stratagene, USA) (45). DNA sequencing was performed to confirm the correctness of the mutants at the MOBIX lab of McMaster University, ON, Canada. pFAG-GATA3 was purchased from Addgene (MA, USA).

Statistical analyses

The comparisons among WT-young, WT-old, KO-young, and KO-old mice were made using two-way ANOVA. The comparisons between control and NaHS treatment groups were made using unpaired *t* tests. One-way ANOVA followed by Dunnett post test were used for comparisons among different NaHS treatment conditions. Mann Whitney test was used when variance was different among groups or data were not normally distributed. $P < 0.05$ is considered statistically significant. All data are expressed as mean ± SEM. Data analysis and graph preparation were performed using GraphPad Prism Software version 5.0.

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Author Disclosure Statement

The authors have declared no conflict of interest.

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List of Abbreviations

ASMCs = airway smooth muscle cells

BALF = bronchoalveolar lavage fluid

CBMCs = cord blood mononuclear cells

CBS = cystathionine β-synthase

CLIC4 = chloride intracellular channel protein 4

CSE = cystathionine γ-lyase

Cdyn = lung dynamic compliance

H_2S = hydrogen sulfide

IFN-γ = interferon gamma

Interleukin = IL

KO = knock out

MCh = methacholine

MMTS = methyl methanethiosulfonate

MST = mercaptopyruvate sulfurtransferase

NaHS = sodium hydrosulfide

NLS = nuclear localization sequence

OVA = ovalbumin

PBMCs = Peripheral blood mononuclear cells

PBS = phosphate buffered saline

PPG = dl-propargylglycine

SSH = S-sulfhydration

STAT6= Signal transducer and activator of transcription factor-6

T-bet = T-box protein

T helper 2 cells = T_H2 cells

WT = wild type

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Figure Legend.

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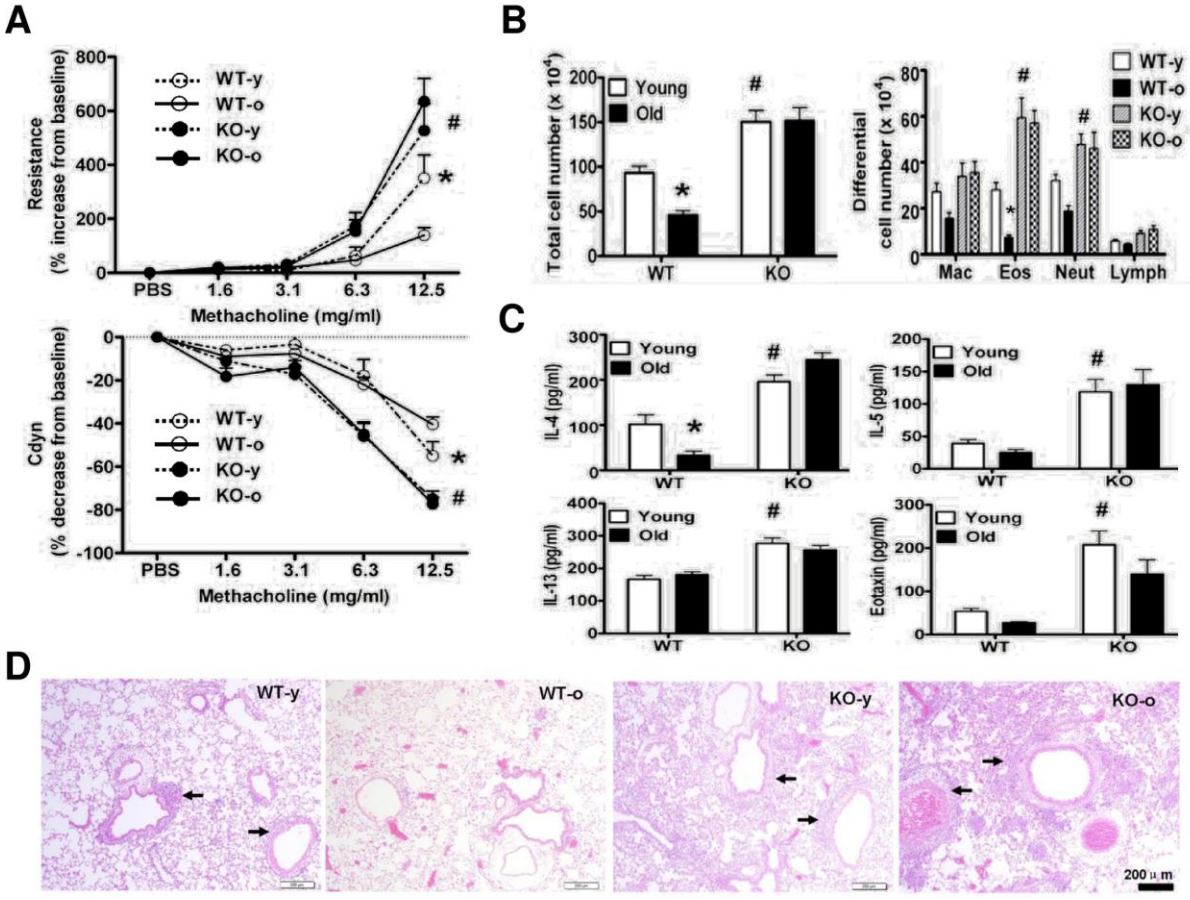
Fig. 1



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Supplement Figure 1

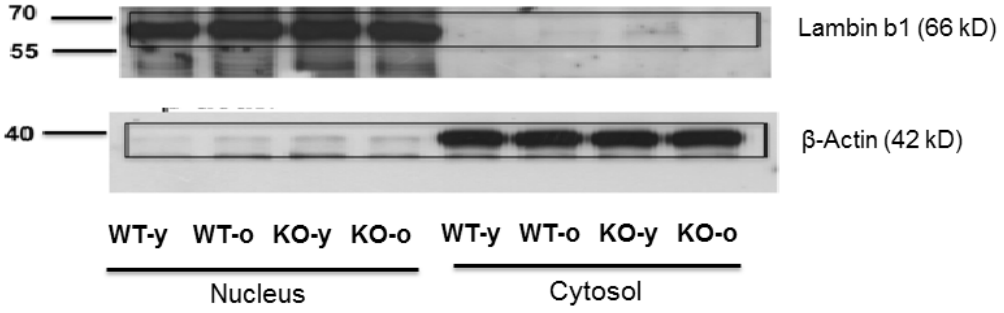

Fig. 1. CSE deficiency increased the severity of asthma in OVA-induced mouse allergic asthma model. (*A*) Mouse airway responsiveness measured as airway resistance and Cdyn. WT-y: WT young mice; WT-o: WT old mice; KO-y: CSE-KO young mice; KO-o: CSE-KO old mice. (*B*) Total cell numbers, macrophages (Mac), eosinophils (Eos), neutrophils (Neut) and lymphocytes (Lymph) in BALF. (*C*) Cytokine levels in BALF. (*D*) Representative H&E-stained mouse lung sections. Scale bars, 200 μm. Arrows point to peribronchial and perivascular inflammation. The results in each group were generated from 10-15 animals. Data are expressed as mean ± SEM. Statistical significance was determined with two-way analysis of variance (ANOVA). For A, B, & C, * P<0.05 for WT-young mice compared with WT-old mice; # P<0.05 for CSE-KO mice compared with young or old WT mice. (E) Airway responsiveness was measured as airway

resistance and Cydn. * P<0.05 when compared with WT old group. (F) Representative H&E-stained mouse lung sections. Scale bars, 200 μm. WT-y: WT young mice; WT-o: WT old mice; KO-y: CSE-KO young mice; KO-o: CSE-KO old mice. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars)

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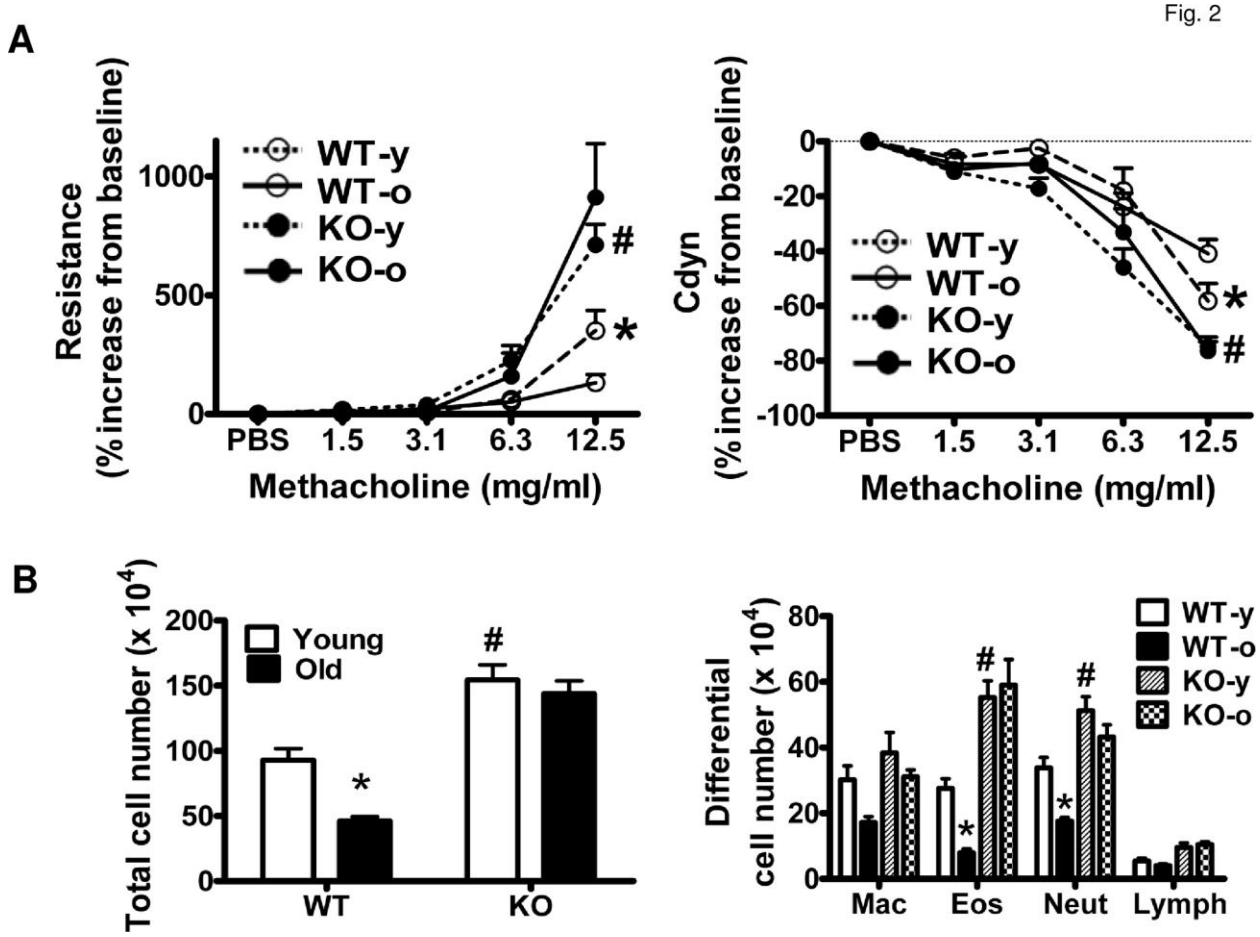
Fig. 2



Supplement Figure 2

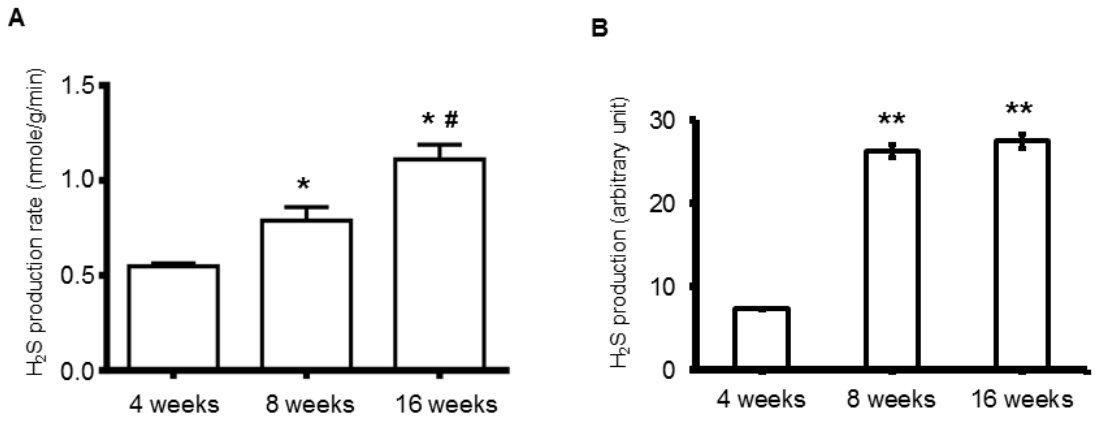


Fig. 2. CSE deficiency increased the severity of asthma in mouse allergic asthma model induced with equal amount of OVA treatments. (*A*) Mouse airway responsiveness measured as airway resistance and Cdyn. WT-y: WT young mice; WT-o: WT old mice; KO-y: CSE-KO young mice; KO-o: CSE-KO old mice. (*B*) Total cell numbers, macrophages (Mac), eosinophils (Eos), neutrophils (Neut) and lymphocytes (Lymph) in BALF. The results in each group were generated from 8-10 animals. Data are expressed as mean ± SEM. Statistical significance was determined with two-way ANOVA. * $P<0.05$ for WT-young mice compared with WT-old mice; # $P<0.05$ for CSE-KO mice compared with young and old WT mice.

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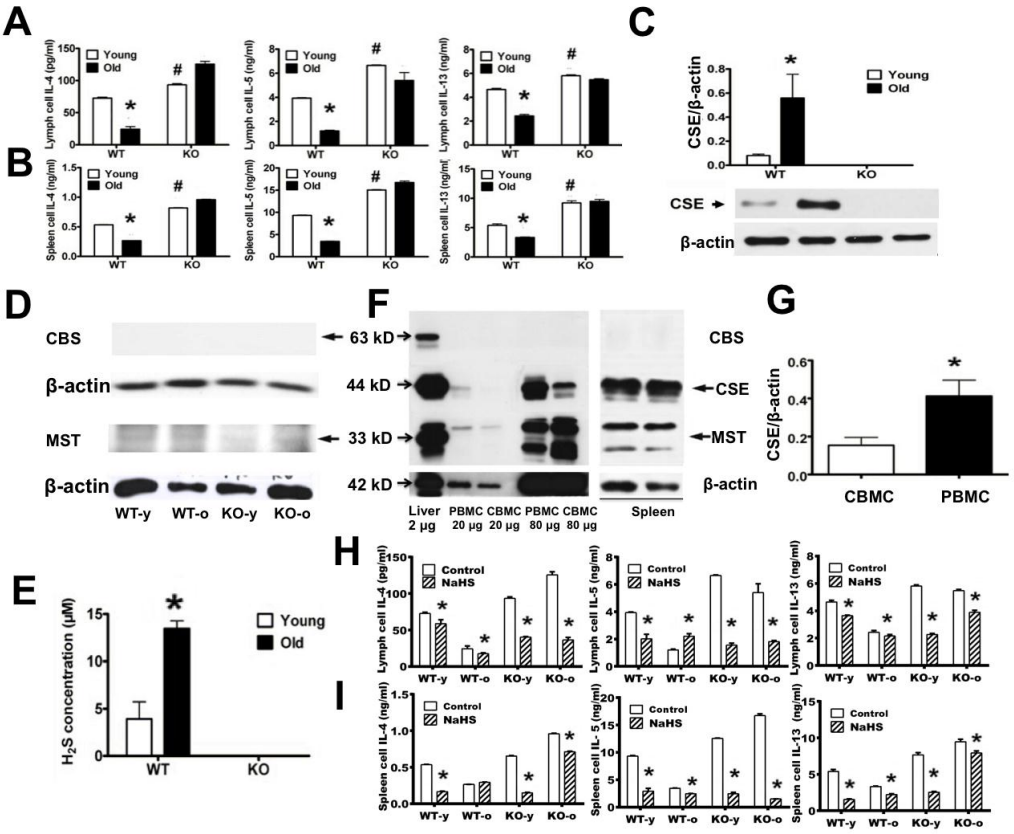
Fig. 3



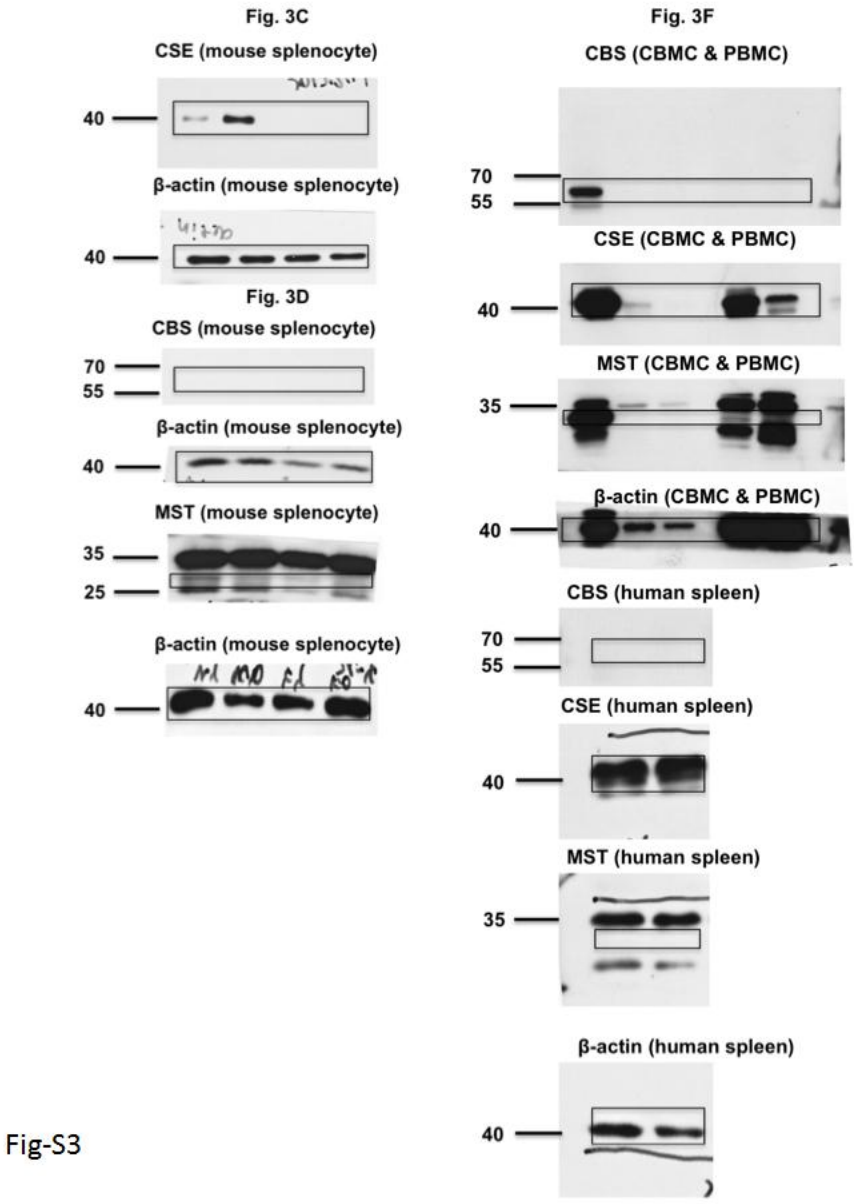


Fig-S3

Fig. 3. CSE/H_2S suppressed OVA sensitization-induced differentiation of mediastinal lymph cells and splenocytes. (*A*-*B*) Cytokine levels in the culture media of mediastinal lymph cells (*A*) and splenocytes (*B*), as assessed by ELISA (*n*=6). (*C*) CSE protein levels in splenocytes (*n*=4). (*D*) CBS and MST protein levels in splenocytes. (*E*) H_2S generated by cultured splenocytes (*n*=4). (*F*) CBS, CSE and MST protein levels in human cord blood mononuclear cells (CBMC),

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human peripheral blood mononuclear cells (PBMC), and human spleen sample. *(G)* Expression levels of CSE proteins in CBMC (n=5) and PBMC (n=6). (*H-I*) Relative cytokine levels in the culture media of mouse mediastinal lymph cells (*H*) and mouse splenocytes (*I*). Mean cytokine levels of respective control groups are set as 100% (*n*=6). Data are expressed as mean ± SEM. Statistical significance was determined with two-way ANOVA (comparisons among WT-young, WT-old, KO-young and KO-old groups) and unpaired *t* tests (comparisons between control and NaHS treatment groups). **P*<0.05 for WT-young mice compared with WT-old mice in (*A*, *B*, *C*, and *E*), PBMC compared with CBMC in (*G*), and NaHS treatment group compared with control group in (*H* and *I*); #*P*<0.05 for CSE-KO mice compared with WT mice at the same age.

The full unedited western blot images, corresponding to each and all gel images in this figure, can be found in supplemental Fig. 3S.

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Fig. 4

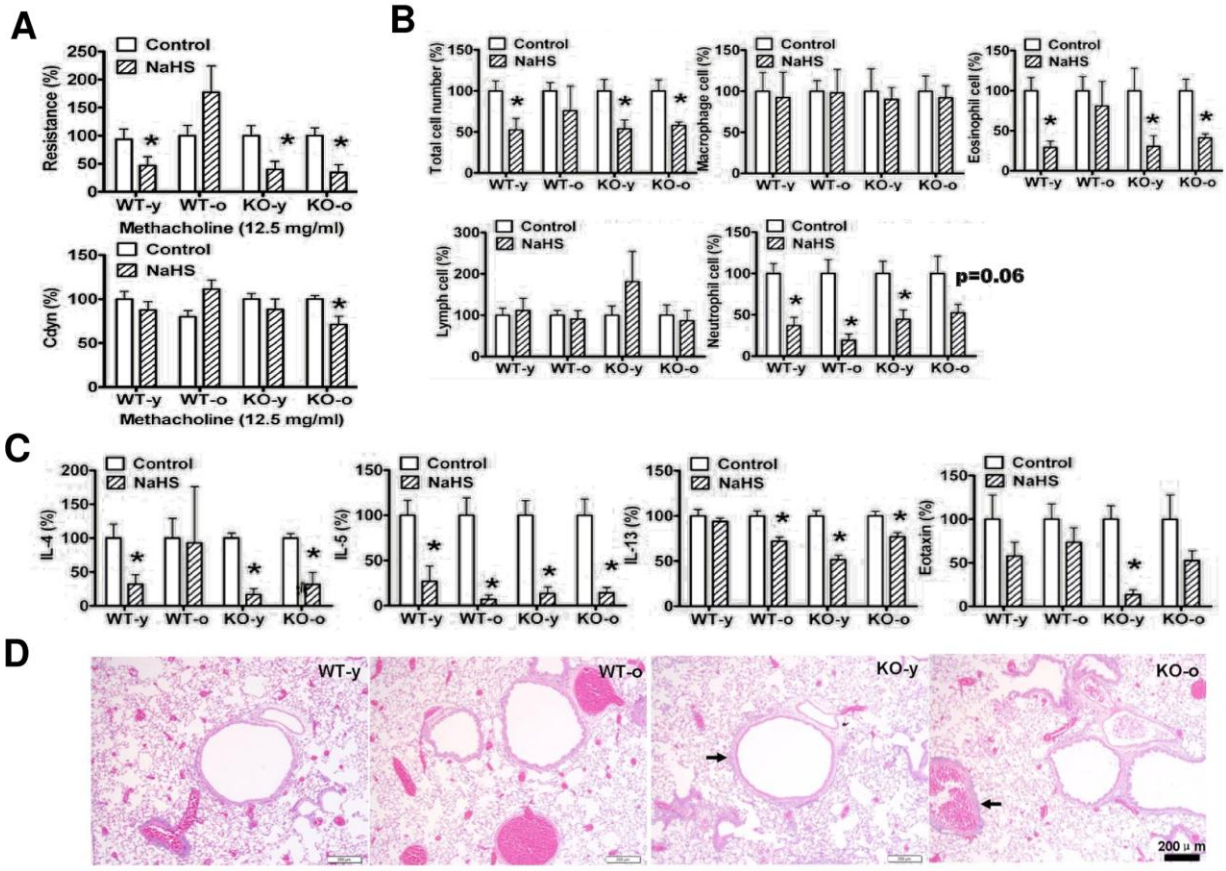

Fig-S4

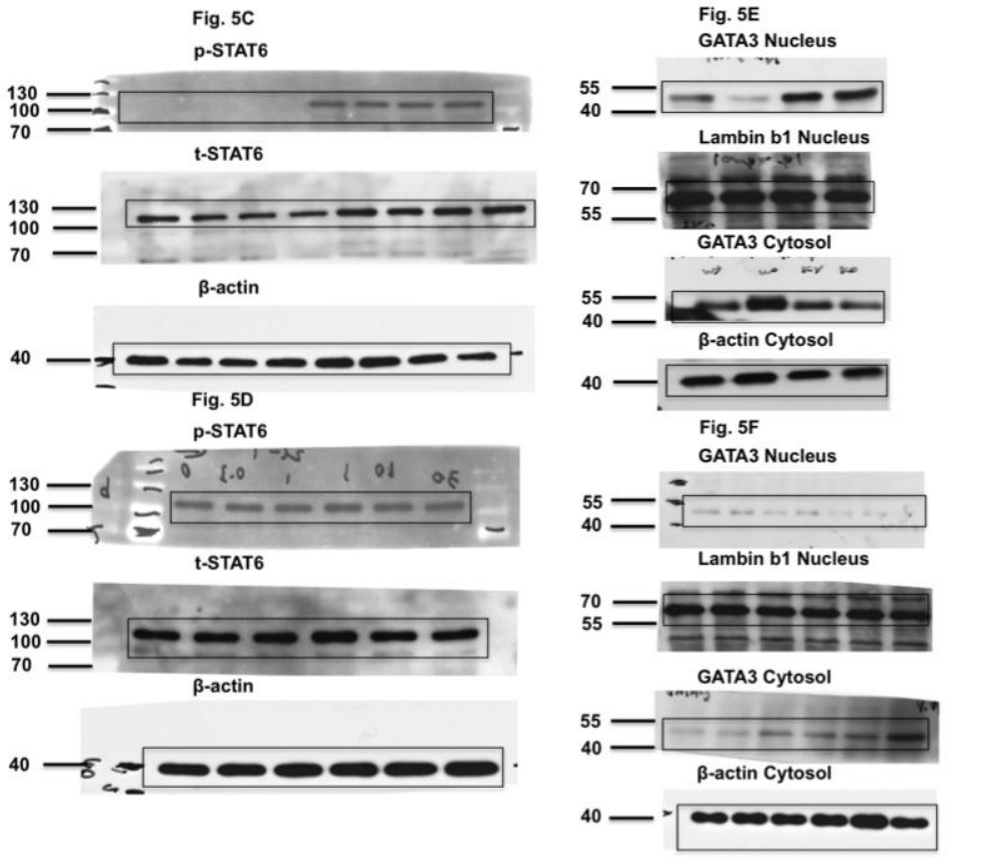

Fig. 4. NaHS treatment significantly improved OVA-induced allergic asthma symptoms in WT young mice and CSE-KO mice. (*A*) Relative airway resistance and Cdyn of mice with and without NaHS treatment. Mean resistance or mean Cdyn values of respective control groups are set as 100%. (*B*) Relative total cell numbers, macrophages (Mac), eosinophils (Eos), neutrophils (Neut), and lymphocytes (Lymph) in BALF. The average numbers of total cells, macrophages, eosionophils, neutrophils and lymphocytes in BALF from respective control groups are set as 100%. (*C*) Relative cytokine levels in BALF. Mean cytokine levels of respective control groups are set as 100%. (*D*) Representative H&E-stained lung sections of

NaHS-treated mice. Scale bars, 200 μm. The results in each group were collected from 6 animals. Data are expressed as mean ± SEM. Statistical significance was determined with unpaired t-test. *P <0.05 for NaHS treatment group compared with control group. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars)

Antioxidants & Redox Signaling
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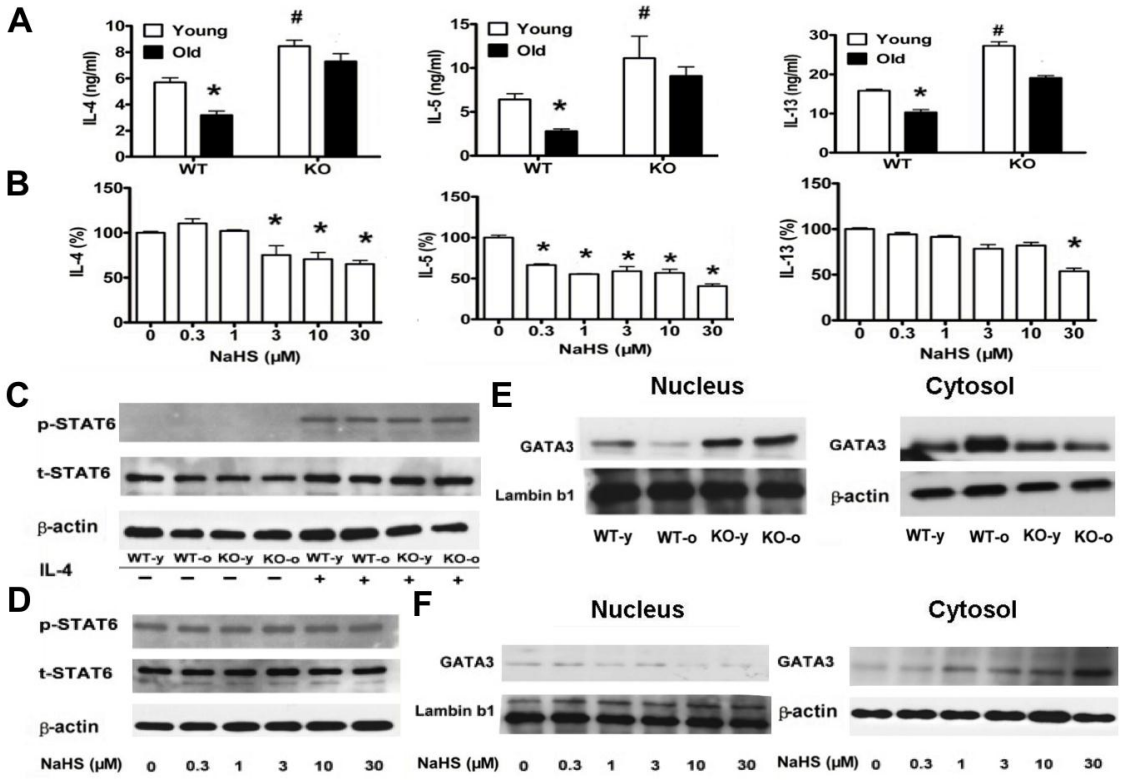
Fig. 5



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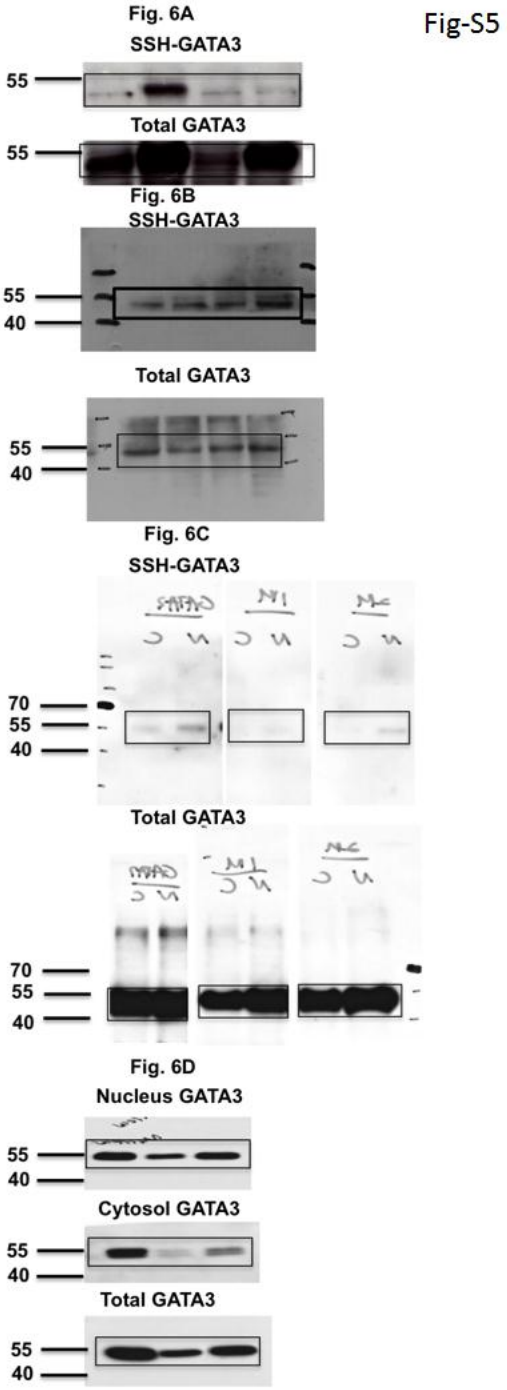

Fig. 5. Endogenous and exogenous H_2S reduced the differentiation of splenocytes into type-2 cytokine-generating cells *in vitro*. (*A*-*B*) IL-4, IL-5 and IL-13 levels in the culture medium of

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splenocytes with or without NaHS pre-treatment. Mean cytokine levels of NaHS-0 μM groups in (*B*) are set as 100%. (*C-D*) IL-4-treatment-induced STAT6 phosphorylation in splenocytes with or without NaHS pre-treatment. (*E-F*) Nuclear and cytosolic GATA3 expression in differentiated splenocytes with or without NaHS pre-treatment. Only CSE-KO-young splenocytes were used in (*B*, *D*, and *F*) to minimize the potential interference of endogenous H_2S on NaHS effects. *n*=4-6 in (*A and B*). Images in (*C-F*) represent 4 independent experiments. Statistical significance was determined with two-way ANOVA (comparisons among WT-young, WT-old, CSE-KO-young and CSE-KO-old groups) and one-way ANOVA followed by Dunnett post test (comparisons among different NaHS treatment conditions). *P<0.05 for WT-young group compared with WT-old group or NaHS treatment groups compared with NaHS-0 μM group; # P<0.05 for CSE-KO group compared with WT group at the same age. The molecular weights of STAT6 and GATA3 are 110 kD and 50 kD, respectively. The full unedited western blot images, corresponding to each and all gel images in this figure, can be found in supplemental Fig. 4S.

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Fig. 6

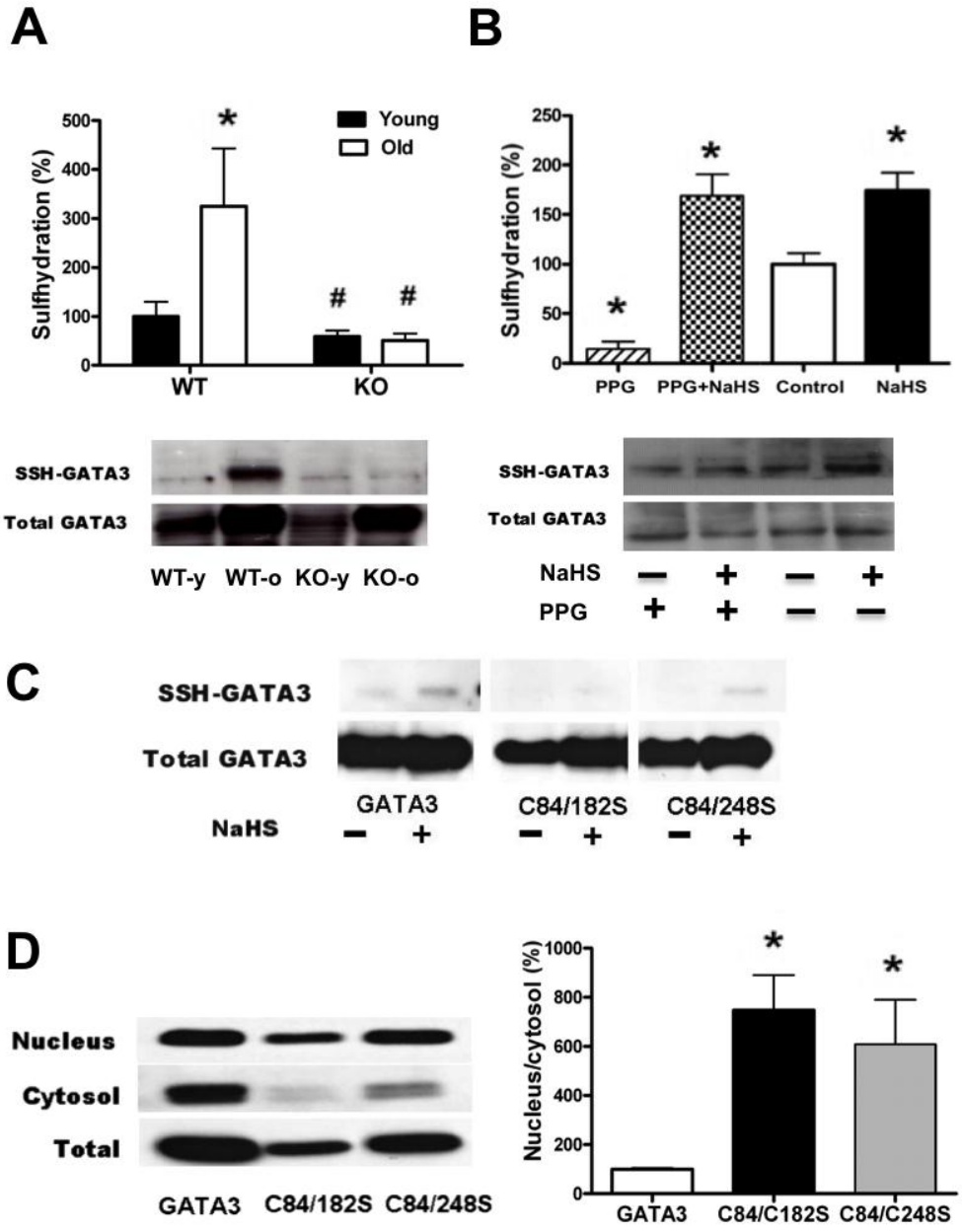

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Fig-S6

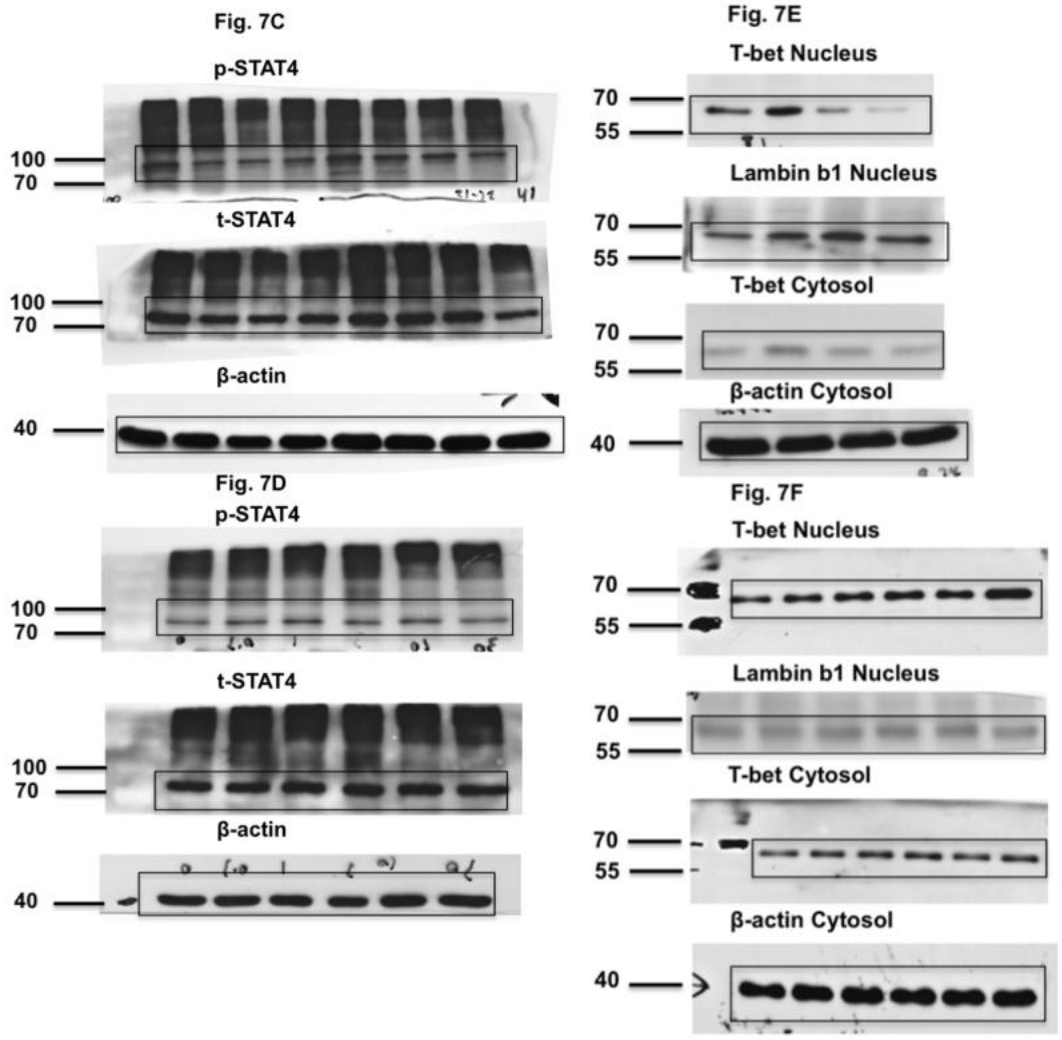

Fig. 6. *S*-sulfhydration of GATA3. (*A*) Endogenous *S*-sulfhydration of GATA3 in mouse spleens (*n*=4). (*B*) *S*-sulfhydration of GATA3 in HEK-293 cells (*n*=4). (*C*) *S*-sulfhydration of wild-type and mutated GATA3 in HEK-293 cells. In the mutants (C84/C182 or C84/C248), the targeted cysteines were replaced by serines. (*D*) Nuclear and cytosolic expressions of wild-type and mutated GATA3 in HEK-293 cells (*n*=4). Statistical significance was determined with two-way ANOVA (comparisons among WT-young, WT-old, CSE-KO-young and CSE-KO-old groups in A) and one-way ANOVA followed by Dunnett post test (comparisons among different groups in

B and D). *P<0.05 for WT-young mice compared with WT-old mice in (A), or NaHS (30 μM) and PPG (1 mM) compared with the control group in (B), and C84/182S and C84/248S mutants compared with wild-type GATA3 in (D). # P<0.05 for CSE-KO mice compared with WT mice at the same age in (A). The molecular weight of GATA3 is 50 kD. The full unedited western blot images, corresponding to each and all gel images in this figure, can be found in supplemental Fig. 5S.

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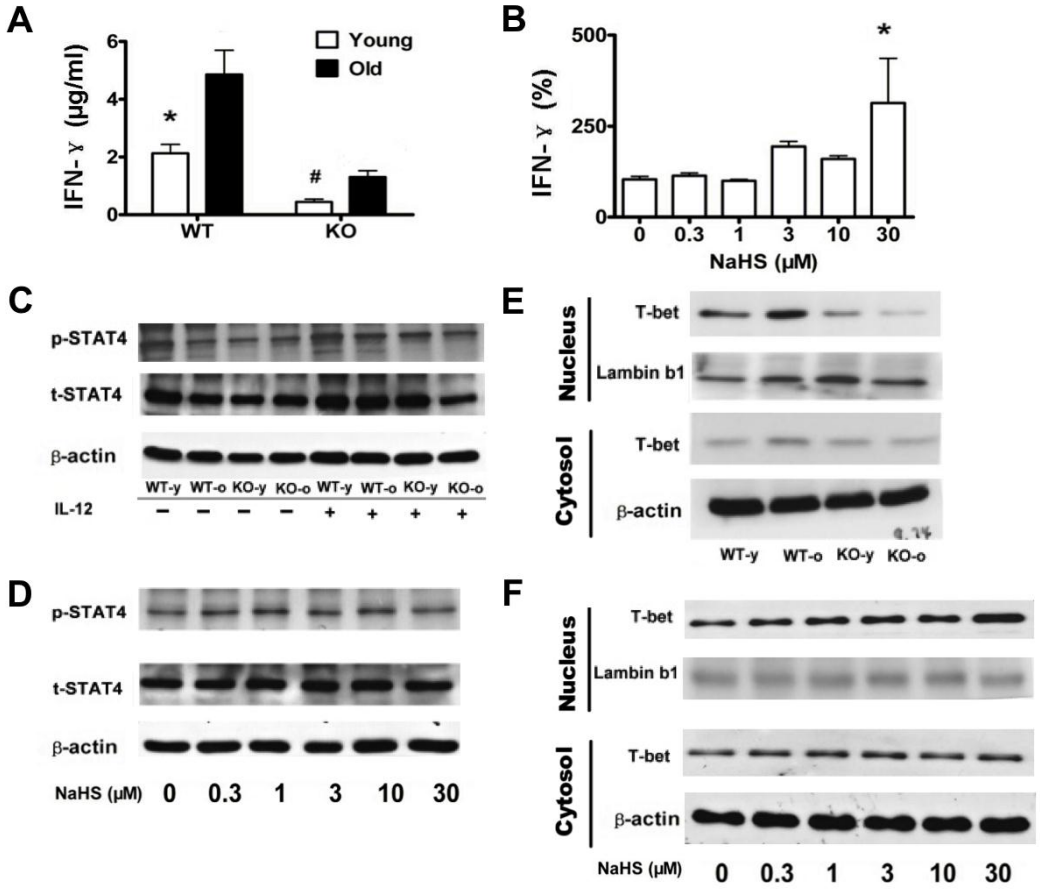
Fig. 7



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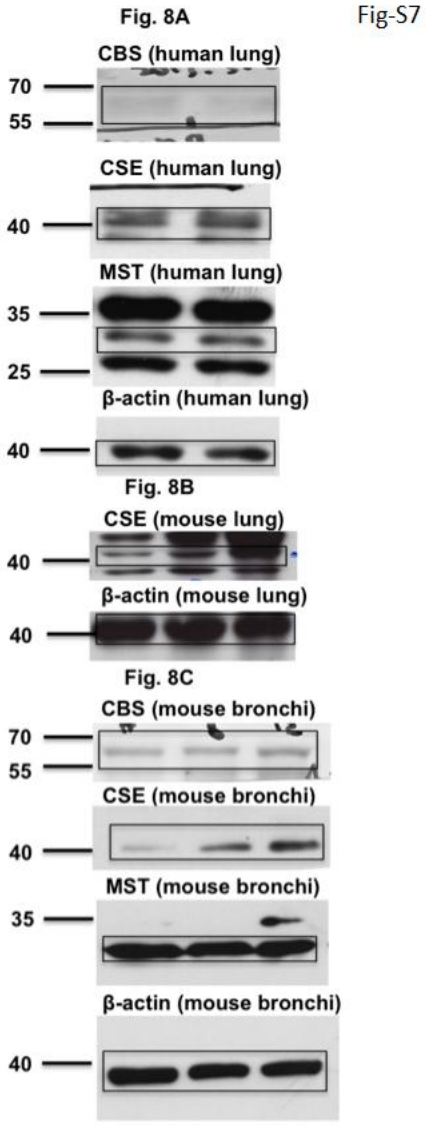

Fig. 7. Endogenous and exogenous H_2S enhanced the differentiation of splenocytes into IFN-γ-generating cells *in vitro*. (*A-B*) IFN-γ levels in the culture media of splenocytes. Mean IFN-γ level of NaHS-0 μM group in (*B*) is set as 100%. (*C-D*) IL-12-treatment induced STAT4 phosphorylation in splenocytes. (*E-F*) Nuclear and cytosolic T-bet expression in splenocytes. Images in (*C-F*) represent 4 independent experiments. Statistical significance was determined with two-way ANOVA (comparisons among WT-young, WT-old, CSE-KO-young and CSE-KO-old groups) and one-way ANOVA followed by Dunnett post test (comparisons among

different NaHS treatment conditions). *P<0.05 for WT-young compared with WT-old mice or NaHS-30 μM group compared with NaHS-0 μM group; # P<0.05 for CSE-KO mice compared with WT mice. The molecular weights of STAT4, Lambin b1, and T-bet are 89 kD, 66 kD, and 62 kD, respectively. The full unedited western blot images, corresponding to each and all gel images in this figure, can be found in supplemental Fig. 6S.

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Fig. 8

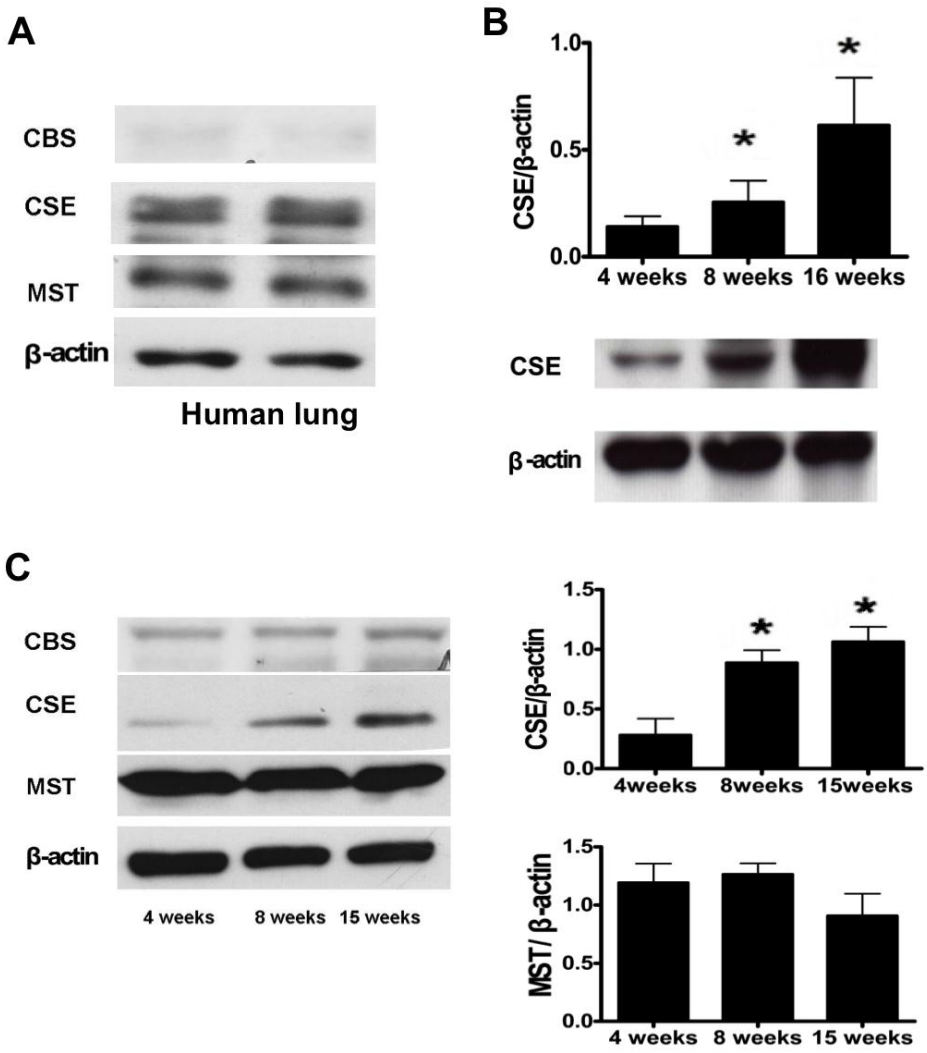

Fig. 8. Age-dependent expression of CSE proteins in human and mouse lung tissues. A) The expression of different H_2S-generating enzymes in human lung sample (purchased from Cure line, USA). B) CSE protein levels in the lungs from WT mice of different ages (n=4 for each group). *P<0.05 when compared with 4 weeks old WT mice. C) The protein levels of H_2S-generating enzymes in the airways of WT mice at different ages. CBS, CSE, and MST protein

levels in the bronchial tree and trachea (n=4 for each group). * P<0.05 when compared with 4 weeks old WT mice. The full unedited western blot images, corresponding to each and all gel images in this figure, can be found in supplemental Fig. 7S.

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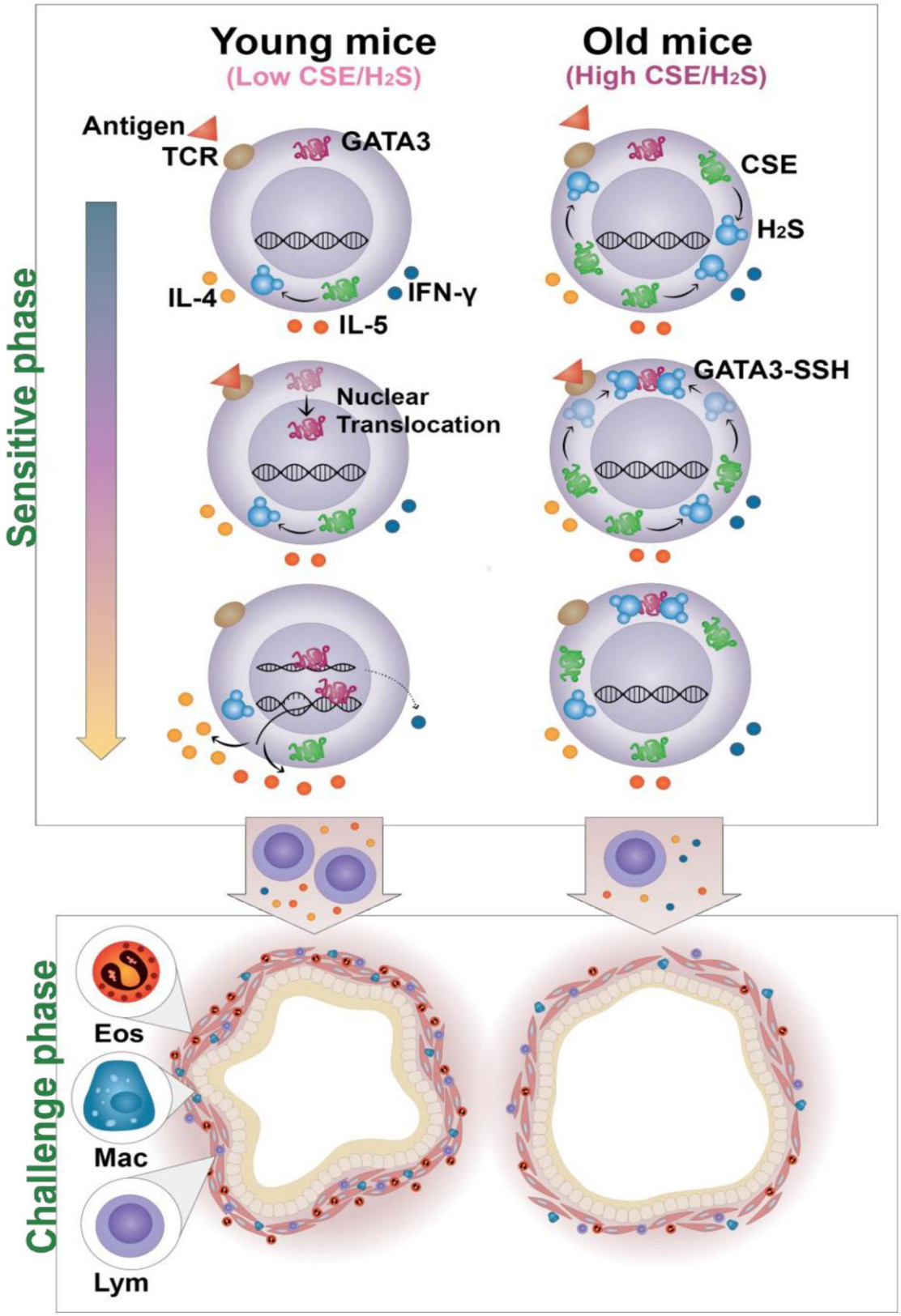

Fig. 9. Proposed mechanisms for the roles of CSE/H_2S pathway in the age-dependent

development of allergic asthma. Eos: eosinophils, Mac: macrophages, Lym: lymphocytes. The greater severity of asthma in young WT mice (compared with old WT mice) can be explained by the age-dependent CSE/H_2S expression pattern in splenocytes. Lower levels of CSE proteins and H_2S in splenocytes from the young WT mice result in decreased *S*-sulfhydration of GATA3, increasing its nuclear translocation. This promotes the differentiation of splenocytes into type-2 cytokine-generating cells and the development of more severe asthma. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars)

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Supplementary Information

Age-dependent Allergic Asthma Development and Cystathionine gamma-Lyase Deficiency

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Running Head: H_2S and age-dependent development of asthma

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Supplementary Figure Legends

Fig. S1. Confirmation of the separation of nuclear fraction from cytosolic fraction of mouse splenocytes. The β-actin and lambin B1 were applied as the cytosol and nuclear marker, respectively (2). Results are representative of three independent experiments with the same results. WT-y: WT young mice; WT-o: WT old mice; KO-y: CSE-KO young mice; KO-o: CSE-KO old mice.

Fig. S2. H_2S generated by mouse lungs and bronchi. (*A*) H_2S production by mouse lungs. n=3 measurements for each age group composed of 60-80 mice. (*B*) H_2S production by mouse bronchi. n=3 measurement for each age group composed of 17-20 mice. Data are expressed as mean ± SEM. Statistical significance was determined with one-way ANOVA. *P<0.05, **P<0.01 when compared with 4 weeks old mice, # P<0.05 when compared with 8 weeks old mice.

Fig. S3. The full unedited western blot images, corresponding to the gel images in Fig. 3.

Fig. S4. The full unedited western blot images, corresponding to the gel images in Fig. 5.

Fig. S5. The full unedited western blot images, corresponding to the gel images in Fig. 6.

Fig. S6. The full unedited western blot images, corresponding to the gel images in Fig. 7.

Fig. S7. The full unedited western blot images, corresponding to the gel images in Fig. 8.

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