H_2S AS A NOVEL BIOMARKER AND THERAPEUTIC TARGET FOR ASTHMA

By

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ABSTRACT OF DISSERTATION

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Asthma is a chronic inflammatory disease with hyper-responsive bronchoconstriction and airway remodeling, leading to extensive airway narrowing. The pathophysiology of asthma remains unclear. The literature shows that the cystathionine gamma lysase (CSE)/ hydrogen sulfide (H_2S) system participates in the regulation of airway contractility and immune response. In this PhD thesis study we found that CSE was a major enzyme responsible for endogenous H_2S synthesis in the lung and spleen. CSE gene knock-out (CSE-KO) dramatically decreased H_2S production rates in these two organs. In an asthma model established via ovalbumin (OVA)-sensitization and challenge, airway resistance of CSE-KO mice (12-16 weeks old), in response to aerosolized methacholine (MCh) at 12.5 mg/ml, was two times higher than that of wild type (WT) mice (12-16 weeks old). CSE-KO mice also developed more peribronchial inflammation and had higher levels of type-2 helper T cell (T_H2) cytokines in bronchoalveolar lavage fluid (BALF). As allergic asthma is more prevalent among children than in adults, we next used young (3-4 weeks old) and old (7-8 months old) mice to observe whether the CSE/H_2S system was involved in early onset asthma. With the same intensity and duration of OVA-treatments, young WT mice developed much more severe asthma with greater lung resistance, higher levels of eosinophils and T_H2 cytokines in BALF, and more peribronchial inflammation than did old WT mice. This age-dependent propensity for immunoreaction and asthma development resulted from lower levels of CSE expression and H_2S production in splenocytes from young mice, which was reversed by H_2S supplementation. Human umbilical cord blood mononuclear cells also had lower level of CSE proteins than peripheral blood mononuclear cell from human adults. CSE-KO mice had more severe asthma than WT mice but without age-dependent variation.

Lower endogenous levels of CSE/H_2S promoted the differentiation of splenocytes into type-2 cytokine-generating cells in young WT mice and in CSE-KO mice at all ages. This effect was suppressed byH_2S supplementation. CSE/H_2S-induced inhibition of type-2 immunity was not mediated by STAT-6 activation. Instead, H_2S caused S-sulfhydration of GATA3 in splenocytes and decreased GATA3 nuclear translocation, leading to the inhibition of type-2 immunity. We also found that CSE expression in the airways of WT mice increased in an age-dependent manner. In the absence of allergen exposure, lower abundance of CSE in young WT mice or absence of CSE in young/old CSE-KO mice aggravated airway responsiveness to MCh challenge by more than twice compared to old WT mice. In conclusion, CSE/H_2S in peripheral lymph tissues and the lung suppresses allergen-induced type-2 immunity, airway responsiveness and the consequential asthma. Lower activity of the CSE/H_2S pathway renders a higher incidence of allergic asthma in childhood.

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Abbreviations

AHR: airway hyper-responsiveness

ALI: acute lung injury

ANOVA: analysis of variation

Atf3: activating transcription factor 3

BALF: bronchoalveolar lavage fluid

Bm: basement membrane

BSA: bovine serum albumin

Bv: blood vessel

CBMC: cord blood mononuclear cells

CBS: cystathionine beta-synthase

Cdyn: dynamic compliance

CGRE: conserved GATA3 response element

CHILD: Canadian Healthy Infant Longitudinal Development

CLIC: chloride intracellular channel

CNS: non-coding sequences

CSE: cystathionine gamma-lyase

CSE/H_2S: cystathionine gamma-lyase/hydrogen sulfide

CSE-KO: cystathionine gamma-lyase gene knock out

DC: dendritic cell

ECL: enhanced chemiluminescence

Ep: epithelium

FEV1: forced expiratory volume in 1 second

GINA: Global Initiative for Asthma

GPX2: glutathione peroxidase 2

GSTA4: glutathione S-transferase alpha 4

HDM: house dust mite

H_2S: hydrogen sulfide

HSV: hypersensitive site V

HTV: high tidal volume

IE: intronic enhancer

IFNγ: interferon gamma

IL: interleukin

InsP3: inositol-1, 4, 5-trisphosphate

ip: intraperitoneal injection

K_{ATP} channel: ATP-sensitive potassium channel

KO: knock out

LCA: latent class analysis

LPS: lipopolysaccharide

MHC: major histocompatibility complex

MST: mercaptopyruvate sulfurtransferase

MCh: methacholine

Na_2S: sodium sulfide

N, N-DPD: N, N-dimethyl-pphenylenediamine sulfate

NO: nitric oxide

NOS: nitric oxide synthase

NQO1: NAD(P)H dehydrogenase, quinine 1

NSL: nuclear localization sequence

OVA: ovalbumin

PAS: Periodic acid–Schiff

PASTURE: protection against Allergy—Study in Rural Environments

PBS: Phosphate-buffered saline

PBMC: peripheral blood mononuclear cell

PDVF: polyvinylidene difluoride membrane

PPG: d,l-propargylglycine

PRR: pattern recognition receptor

PTFE: polytetrafluoroethylene

RL: resistance of lung

ROS: Reactive oxygen species

R&C: resistance and compliance

SEM: standard error of the mean

Sm: smooth muscle

SMC: smooth muscle cells

SPF: specific pathogen-free

STAT6: signal transducer and activator of transcription factor-6

Tbet: T-box protein

T_H1: type 1 T helper cell

T_H2: type-2 T helper cell

TCR: T cell receptor

WT: wild type

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1. Chapter 1

INTRODUCTION

This chapter is an updated version of the original review article we published in Experimental Physiology, 2011 Sep; 96(9): 847-52. It is reproduced with permission from the publisher. Recent advances in asthma and hydrogen sulfide (H_2S) research have been added. Updated sections are denoted with italic subtitles.

Asthma is a chronic inflammatory disease, with hyper-responsive bronchoconstriction and airway remodelling, leading to extensive airway narrowing. The regulation of airway responsiveness and inflammation by endogenous H_2S during the pathogenic development of asthma has been suggested. Hydrogen sulfide can be produced in the lung and airway tissues via the actions of two H_2S-generating enzymes, cystathionine β-synthase (CBS) and/or cystathionine gamma-lyase (CSE). Abnormal metabolism and function of H_2S have been reported in experimental animals with asthma, especially in rat or mouse ovalbumin-induced asthma models. In patients with asthma, serum H_2S levels are significantly reduced. Supplementation with exogenous H_2S has been shown to mitigate the severity of asthma in experimental animals. It is hypothesized that decreased H_2S production in the lung and airway tissues may be used as an early-detection biomarker. H_2S-based therapy would represent a new treatment strategy for asthma. Major challenges for establishing the diagnostic and treatment values of H_2S include the differential expression of CSE and CBS in airways and their changes during asthma, the effects of H_2S on bronchoconstriction and airway remodelling, as well as the underlying mechanisms, and the detection of the changes in H_2S levels in airway tissues and in exhaled air.

1.1 Pathological Features and Phenotypes of Asthma

Asthma is a chronic inflammatory disorder of the airways, involving pathological changes in different types of cells and molecules. During asthma, activated mast cells,

increased eosinophils, T-helper 2 (T_H2) lymphocytes and neutrophils, epithelia cells, airway smooth muscle cells, fibroblast cells, pulmonary blood vessel smooth muscle cells, and endothelia cells release inflammatory factors to cause classic pathological changes such as subepithelial fibrosis, airway remodelling and airway hyper-responsiveness to allergens[1] (Fig.1-1). Inhaled corticosteroids and long-acting β_2-adrenoceptor agonists are now the mainstay of asthma treatment. These agents suppress airway inflammation and decrease bronchoconstriction, but they cannot cure asthma. In addition, approximately 5% of asthma patients are corticosteroid resistant[2]. The search is ongoing to develop novel or improved therapies for asthma, which include anti-IgE therapy, cytokine inhibitors, chemokine antagonists, phosphodiesterase-4 inhibitors and adhesion molecule blockers[2]. However, many of these experimental treatments are limited in their therapeutic capabilities[2,3]. For example, inhibition of interleukin-5 with mepolizumab results in the depletion of eosinophils from the circulation and sputum of patients with modest asthma, but has no effect on airway hyper-responsiveness and other symptoms or altered lung function in asthma[4]. In contrast, for the subgroup of asthmatic patients who have >3% eosinophils in their sputum, mepolizumab is effective in reducing asthma symptoms and preventing exacerbations[5,6]. These discrepancies highlight the clinical heterogeneity of asthma.

Clinically-based definitions of asthma distinguish phenotypes such as atopic and allergic asthma, adult-onset asthma, obesity-related asthma, smoking-related asthma, neutrophilic asthma, and exercise-induced asthma[7]. Recently, researchers adopted unbiased statistics methods such as latent class analysis (LCA)[8] and cluster analysis[9] to define asthma. Fig.1-2 shows 5 phenotypes of adult asthma established by Haldar P using cluster analysis. Results based on unbiased strategies overlap with those obtained using earlier clinical approaches. Age at disease onset has been found to be a key-differentiating factor of asthma phenotypes. Early-onset asthma is consistently related to atopic and allergic conditions, whereas later-onset asthma is generally less allergic, and more likely to be associated with obesity and eosinophilic or neutrophilic inflammation.

Early-onset asthma compared with later-onset asthma, is more likely to have higher amounts of T_H2 cytokines (type-2 cytokines), total and allergen-specific IgE, and be concomitant with other atopic diseases, such as allergic rhinitis and atopic dermatitis[9-11]. A large portion of persistent adult asthma originates in early childhood and has an atopic and allergic component. Results from the Third National Health and Nutrition Examination Survey[12] and a community-based birth cohort from Perth[13] revealed that over 50% of asthma cases could be attributed to atopy.

Childhood asthma generally manifest as wheezing. Wheezing is a common symptom in early childhood but many children who wheeze do not develop asthma by school age[14]. Multiple wheezing phenotypes and several asthma predictive phenotypes have been identified in large, longitudinal cohort studies of the general population, including Tucson Children's Respiratory Study[15], Avon Longitudinal Study of Parents And Children (ALSPAC) study[16], and a New Zealand longitudinal study[17]. According to ALSPAC, the most common phenotype is infrequent or transient infantile wheeze: approximately 75% of children in the general population were reported to have recurrent wheeze during the first 2–3 years of life but rarely afterwards. 9% of children have prolonged early wheeze with wheezing until 4.5 years of age and remitting by the age of 6. These children have increased airway responsiveness and lower school-aged lung function compared with the first phenotype. Intermediate-onset wheeze was seen in 3% children. In this phenotype, wheezing begins between 1.5 and 3.5 years of life and persists into later childhood. These children are characterized by allergic sensitization, higher levels of airway responsiveness, and lower lung function compared with the first phenotype. Late-onset wheeze (6%) defines children who have infrequent wheezing during their 0.5 to 3.5 years of life, but subsequently develop frequent wheezing. 7% of children already have persistent wheezing by the age of 6 months. They constitute the final group, "persistent wheeze" phenotype.

The intermediate-onset, late-onset, and persistent-wheeze groups may represent

asthma-predictive phenotypes meaning that children within these groups are most likely to experience recurrent asthma symptoms in later life[18]. Higher prevalence of T_H2 markers were seen in these three phenotypes compared to other wheezing phenotypes in the ALSPAC study and the Prevention and Incidence of Asthma and Mite Allergy (PIAMA) study[14,19].

1.2 Asthma Prevalence

A sharp increase has been seen in the global prevalence, morbidity, mortality, and economic burden associated with asthma over the last 40 years. After combining data from the Phase 1 International Study of Asthma and Allergies in Childhood (ISAAC, patients aged 6 to 7 years and 13 to 14 years) study[20,21] and the European Community Respiratory Health Survey (ECRHS, adults aged 20 to 44 years) study[22], the Global Initiative for Asthma (GINA) estimated 300 million people worldwide had asthma. They predict this number will increase to 400 million by 2025[23]. Findings from the cross-sectional World Health Survey show global prevalence of clinical asthma in adults is 4.5% and ranges from 1.0% in Vietnam to 21.5% in Australia (Fig. 1-3)[24]. Prevalence of asthma is higher (> 10%) in developed countries than in developing countries, but in recent years rates are increasing in developing regions as they become more westernized[23,24].

The greatest increase in asthma prevalence is seen in children (Fig.1-4)[25]. Asthma currently is the most common chronic disease among children and young adults. Acute asthma is the leading cause of hospital admission among children of all ages in Europe[25]. The 2001--2009 American National Health Interview Survey showed persistent age differences in asthma prevalence: prevalence among children (age <18 years) was 9.6% while prevalence among adults was 7.7%[26]. A study based on European Community Respiratory Health Survey (ECRHS) data, including 9091 men and women randomly selected from the general population at 29 centres from 14 countries, also showed that the incidence of allergic asthma decreased significantly with age[27]. Sequential phases of ISAAC studies showed that the percentage of

children with asthma symptoms increased significantly in Africa, Latin America and parts of Asia[28] over a 10-year period. Although overall prevalence of asthma is still lower in these lower income countries compared to more affluent countries, the prevalence of severe asthma has been higher[29]. This might be due to limited asthma care resources or lower awareness of wheeze as a marker of childhood asthma in those countries.

The prevalence of asthma among migrating populations was studied in phase three ISAAC by comparing data from Vancouver, Canada, with data from centres in China[30]. Prevalence of asthma was lowest in Chinese adolescents born and residing in mainland China, intermediate for Chinese adolescents who migrated during their childhood to Canada and highest for Chinese-Canadian adolescents who were born and raised in Canada.

1.3 Why Is Asthma Prevalence Increasing?

Geographic variation in asthma prevalence, the rise of asthma incidence seen in migrating populations[30,31]and the substantial increase in asthma prevalence within these few decades, support the hypothesis that environmental factors play a large role in the current asthma epidemic. Data on environmental exposures, collected in birth cohort studies, reveal that events and exposures in utero, in early infancy and during the preschool years have long term effects on asthma development. The risk of developing asthma is age-dependent, with the majority of asthma beginning in the preschool years (Fig.1-5)[32,33]. More than 130 birth cohorts focusing on asthma and allergy have been initiated within the last 30 years[34]. These cohort studies utilize multidisciplinary/multiparameter phenotypic assessments of subjects and track recruited populations throughout childhood and, where feasible, into adulthood, providing us with new insights into the development, persistence, remission and/or relapse of the disease.

These birth cohort studies identified two asthma risk factors: lower respiratory viral

infections and early life sensitization[32,35]. The asthma-predictive wheeze phenotypes (persistent, intermediate-onset, and late-onset wheezing phenotype) manifest higher prevalence of early sensitization to aeroallergens and associated T_H2 response[14,19]. Common aeroallergens associated with sensitization and asthma include house dust mites (HDM), furred pets, cockroaches, rodents, and mold[36].

Wheezing due to viral infections is very common during childhood, especially during the first years of life. For example, Tucson Children's Respiratory Study found 30% of children had at least one lower respiratory infection associated wheezing in the first 3 years of life[14]. Allergic sensitization preceded and increased the risk of wheezing in response to rhinovirus[37]. Symptoms in non-atopic children with virus-induced wheeze resolved by the early school years and normal lung function was retained[14,38]. In contrast, children who became sensitized to aeroallergens were much more likely to remain symptomatic into late childhood/adulthood and had lower lung function at school age[14,39-41].

Ulrik and Backer [42] assessed pulmonary function and atopy status of a group of children (7-17 years of age at enrollment) and reassessed them 6 years later. They found sensitization to house dust mites had a negative impact on the expected age-related rise in FEV1 (forced expiratory volume in 1 second). Children with either persistent or new atopy to house dust mites had lower lung function than those who remained unsensitized.

Longitudinal follow-up of birth cohorts in England[41]and Australia[40,43]showed that early sensitization was a risk factor for persistence of asthma. Western Australian Pregnancy Cohort Study confirmed atopy in early life increased risk of asthma independent of lower respiratory tract infections[13]. Furthermore, in many cases, exposure and sensitivity follow a dose–response relationship. The German Multicentre Allergy Study and a longitudinal birth cohort in Boston area found that exposure to high levels of perennial allergens early in life further aggravated this loss of lung function at school age[39] and conferred greater risk of asthma[44].

The asthma-promoting effect of early sensitization may result from its associated intense T_H2 polarized effector and long-term memory responses. In a longitudinal study[45], Turner *et al*. grouped 60 children into persistent infant-onset atopy (positive skin prick testing in infancy and at ages 6 and 11 years); early childhood-onset atopy (negative skin prick testing in infancy but positive at 6 and 11 years); and late childhood-onset atopy (negative skin prick testing in infancy and at 6 years but positive at 11 years of age). After testing peripheral blood mononuclear cell cytokine responses to HDM at 11 years of age, they found sensitization at an early age was associated with enhanced T_H2 cytokine responses and adverse asthma outcome. Mice immunized as neonates or at a young age (4 weeks or 8 weeks), but not those immunized at an older age (30-40 weeks), mounted T_H2-type-dominant memory responses and other asthma features when re-exposed to the same antigens [46].

T_H2 bias response is developed *in utero* to avoid maternal rejection[47]. T_H2 cytokine, IL-4, drives apoptosis of T_H1 cells and skews neonatal immunity toward T_H2 through IL-13Ralpha1 which is upregulated in neonatal T_H1 cells[48]. Murine neonatal $CD4^+$ lymph cell are deficient in mounting antigen-specific TH1 response in adoptive adult hosts[49].

After birth, the neonatal immune system undergoes extensive development and gradually limits T_H2 response and promotes T_H1 response. The kinetics of postnatal immune maturation processes vary in human populations. An increased or prolonged bias towards T_H2 response increases the risk of allergic asthma development[50]. For example, allergen-specific T_H2 response, measured by cytokine mRNA levels in cord blood mononuclear cells, is higher than in peripheral blood mononuclear cells from one-year old infants. This suppression of T_H2 response is not observed in atopic children[51], suggesting their immune maturation lags behind non-atopic children. Lower expressional levels of interferon gamma (IFNγ), the major T_H1 cytokine, at birth or in the first year of life are related to increased risk of wheeze and development of other atopic diseases[52,53]. T_H1 response is critical for repelling

intracellular pathogens; its deficiency may explain the high susceptibility to respiratory virus infections (such as rhinovirus and respiratory syncytial virus) in early childhood. In addition, these infections further alter immune responses and airway function and promote asthma development, especially in atopic children[54].

Clinical cohort studies have found that exposure to a microbially-rich environment *in utero* or early childhood was inversely related to incidence of childhood asthma. These exposures include, but are not limited to, growing up on a farm with livestock[55], ingestion of raw cow milk[56], and presence of dogs in the home. For example, PARSIFAL (Prevention of Allergy — Risk Factors for Sensitization Related to Farming and Anthroposophic Lifestyle) study, GABRIELA (Multidisciplinary Study to Identify the Genetic and Environmental Causes of Asthma in the European Community [GABRIEL] Advanced Study) and other studies, compared children who were raised on farms with those raised in the same rural communities but away from farms, or those raised in cities[57,58], and consistently found protective effects of living on a farm against asthma development (Fig. 1-6, Fig. 1-7). The protection was strongest when exposure occurred in utero, or within the first 2–3 years of life. The Protection against Allergy--Study in Rural Environments (PASTURE) birth cohort demonstrated that maternal farm-related exposures during pregnancy reduced prevalence of seasonal allergen-specific IgE responses in cord blood mononuclear cells and enhanced T_H1 response[59]. The Allergy and endotoxin (AlEX) study found that first year farm exposure suppressed grass-elicited T_H2-dependent immunoglobulin class-switch towards IgG1, IgG4 and IgE isotypes[60].

The protective effects of farm exposure are believed to be mediated by the increased amount and greater diversity of early gut microbial colonization, which subsequently triggers maturation of the developing neonatal immune system. Gut microbiota is of low density and phylogenetic diversity after birth[61,62]. Successive colonization of G*ram-positive cocci, Enterobacteria*, *Lactobacilli*, *Bifidobacteria etc*. happens in the first few weeks (in mice) or few years (in humans) of life. This process can be promoted by early life events such as breastfeeding, farm exposures and pet

ownership, or delayed by events such as caesarean section, and antibiotic usage. Normally, by the age of 3-5 weeks in mice or 3-4 years in humans, gut microbiota becomes comparatively stable with adult-like species diversity and population profile, although full development may take several more years in humans[61,63]. Delayed colonization, reduced diversity or disturbed balance of microbiota perturbs its immune regulatory functions. This notion is supported by an animal study in which germ-free animals exhibited persistent T_H2 biased response and were more likely to develop allergic asthma than non-germ-free animals[64]. Colonizing germ-free animals with *Bacteroides fragilis* induced maturation of the developing immune system and downregulated splenic T_H2 cytokine production[64].

The importance of microbiota in asthma development is illustrated in the evolved "hygiene hypothesis". The "hygiene hypothesis" was first proposed by Strachan in 1989 to elucidate the potential roles of improved hygiene in early life, reduction of family size and number of siblings (factors closely associated with the modern westernized lifestyle), as determinants of asthma susceptibility[65,66]. The hygiene hypothesis has been extended to include the interaction between microbiota and host immune balance. According to the updated theory, a modern/industrialized lifestyle leads to altered microbial diversity and loss of coevolved commensal microbes that have a strong effect on host immune responses[67]. The resulting dysbiosis fails to assist the host immune system in developing tolerance towards non-harmful antigen exposures and thus results in allergic disorders. The extended hygiene hypothesis is supported by studies performed in Eastern and Western European countries which showed significant differences in asthma prevalence and in the composition of gut flora between these two populations[68].

Taken together, environment factors have profound influences on asthma development. Early sensitization to aeroallergens and lower respiratory tract viral infections in early life collectively or independently skew immune response towards a T_H2 dominated phenotype and contribute to asthma inception[37]. On the other hand, exposures to microbial-rich environments promote balanced immune system

development and prevent both allergic sensitization and transient early wheezing[69]. It is critical to test the hygiene hypothesis in more clinical studies as this hypothesis implies that asthma incidence may be lowered simply by modifying lifestyle factors rather than requiring sophisticated medical interventions.

1.4 How is H_2S Related to Asthma Development?

1.4.1. Physiology of H_2S metabolism in the lung

Endogenous H_2S is produced in many tissues, primarily by two H_2S-generating enzymes, cystathionine β-synthase (CBS) and cystathionine gamma-lyase (CSE). Working together with cysteine aminotransferase (CAT), 3-mercaptopyruvate sulfur transferase (MST) may also produce H_2S in selective tissues[70]. Abundant levels of CSE and MST have been found in sea lion pulmonary arteries and bovine pulmonary arterial smooth muscle cells (SMCs)[71]. Human airway SMCs and human lung primary fibroblast MRC-5 cells both express CSE and CBS proteins[72,73]. In rat lungs, CSE is expressed in airway and pulmonary vessels[74]. In mouse lungs, we recently found that both CSE and CBS are mainly expressed in airway SMCs, pulmonary blood vessel SMCs and endothelial cells (Fig. 1-8). It appears that the expression patterns and extent of H_2S-producing enzymes in the lung and airway tissues are variable depending on the species and cell types.

1.4.2. Altered H_2S metabolism and the pathogenesis of asthma

Wu *et al.*[75] noted that the serum level of H_2S decreased from 75.2 ± 13.0 μM in healthy adult subjects to 55.8 ± 13.6 μM in patients with stable asthma and 31.3 ± 2.9 μM in patients with a severe acute exacerbation of asthma. In patients with acute asthma, serum H_2S levels correlated positively with forced expiratory volume and negatively with the amount of inflammatory cells in sputum, especially neutrophils. Whether the drop of serum H_2S in asthma patients is the cause or the consequence of asthma development has not been addressed. It is also not clear whether the pulmonary production of H_2S was altered in these patients. At any rate, these clinical observations were replicated in a later animal study. Chen *et al.*[74] found that

ovalbumin (OVA) treatments in rats led to 81% reduction of serum H_2S and 80% decrease in the rate of H_2S production in the lung. These decreases might be due to impaired CSE expression in lung tissues from these OVA-treated rats. Chen *et al.*[74] also found that peak expiratory flow was 55.4% lower in OVA-treated rats than in control rats, and the proportions of eosinophils, lymphocytes and neutrophils in bronchoalveolar lavage fluid were significantly increased. Administration of exogenous H_2S (NaHS) improved peak expiratory flow and alleviated airway inflammation and airway remodelling in this rat model.

1.4.3. H_2S *and immune response*

Asthma is characterized by an exaggerated T_H2 adaptive immune response. In asthma patients, aeroallergen exposures stimulate clonal expansion of T lymphocytes, particularly T_H2 cells, which secrete cytokines such as IL-4, IL-5, IL-9, and IL-13. These T_H2 cytokines orchestrate the allergic inflammatory cascade in asthma[76]. Various innate immune cells, including dendritic cells and type-2 innate lymphoid cells, have also been found to play an important role in promoting type-2 immune response[77] (Fig. 1-9).

Endogenous H_2S enzyme CSE is expressed in human primary peripheral blood mononuclear cell (PBMC) and human T cell lines such as HUT-78 and Jurkat cells[78,79]. Nanomolar concentrations of H_2S potentiate T lymphocyte activation *in vitro* as assessed by CD69 expression, IL-2 expression, and CD25 levels. Silencing H_2S-producing enzymes impairs T cell activation which can be rescued by the addition of Na_2S (sodium sulfide, 300 nM)[79]. H_2S treatment increased IFN-γ and IL-10 production in Jurkat cells[78]. Blocking CSE activity with PPG (dl-propargylglycine) delayed heart allograft rejection and abrogated type IV hypersensitivity by inhibiting T_H1 type factors T-bet, IL-12, and IFN-γ. H_2S also modulates innate immune responses. For example, H_2S up-regulates CD11b and G protein-coupled receptor kinase 2 in neutrophils and improves their migration and survival in sepsis induced by cecal ligation and puncture[80].

1.4.4. H_2S and pulmonary inflammation

Both pro-inflammatory and anti-inflammatory effects of H_2S have been reported in different lung disease models.

1.4.4.1 Anti- inflammatory effects

In a rat intra-tongue vein lipopolysaccharide (LPS) injection-induced acute lung injury (ALI) model, NaHS treatments increased mitochondrial activity in a dose-dependent manner, lessened mitochondrial lipid peroxidation and mitochondrial swelling, and limited lung damage[81]. A bolus injection of Na_2S (0.5 mg/kg) combined with continuous infusion at a rate of 0.2 mg/kg/h for 24 hours reduced burn- and smoke-induced fluid accumulation in the lung, improved pulmonary gas exchange and decreased mortality during the 96 h experimental period[82]. H_2S administrated after burn and smoke inhalation also increased anti-inflammatory cytokine IL-10, decreased pro-inflammatory cytokine IL-1β, and significantly decreased mortality in mice[83].

In rats challenged with aerosolized live *streptococcus pneumoniae,* NaHS infusion at 36 μM/kg/h reduced heart rate and body temperature and preserved mitochondrial function[84]. Using a ventilator-induced lung injury model, Aslami *et al*. found continuous infusion of NaHS reduced pulmonary inflammation and improved oxygenation in a dose-dependent manner. Although this NaHS treatment also reversibly lowered body temperature, the author believed its anti-inflammatory effect was mediated via other mechanisms because mere induction of deep hypothermia did not protect against inflammatory damage in the lung[85]. Microarray analysis revealed that H_2S administration activated genes related to inhibition of inflammation and apoptosis such as Atf3 (activating transcription factor 3), an anti-inflammatory and anti-apoptotic regulator[86].

1.4.4.2 Pro- inflammatory effects

CSE expression in the lung and serum H_2S levels increase significantly in rats with acute pancreatitis-associated lung injury. Blocking CSE activity with PPG or

knocking out the CSE gene significantly alleviated lung damage[87,88], which suggests that endogenous H_2S generated by CSE may play a key proinflammatory role in acute pancreatitis-associated lung injury.

It is still unclear whether H_2S is pro- or anti-inflammatory in lung diseases. Variations in doses of H_2S supplementation, administration routes, H_2S releasing rate and timing of treatments may all contribute to observed controversies. For example, Francis *et al.*[89] found inhalation of 1 or 5 ppm H_2S during high tidal volume (HTV) ventilation had no effect on lung injury. Inhalation of 60 ppm H_2S, however, upregulated pulmonary expression of inflammatory mediators such as chemoattractant CXCL-2, leukocyte adhesion molecules CD11b and L-selectin, and worsened lung injury. Notably, pre-treating the animals with a bolus infusion of Na_2S (0.55 mg/kg) reduced pulmonary CXCL-2 and CD11b expression, elevated Nrf2-dependent antioxidant genes (NQO1, GPX2, and GST-A4) and attenuated pulmonary edema[89].

1.4.5. H_2S and oxidative stress

Mitochondrial dysfunction and oxidative stress are associated with the onset and progression of asthma[90]. Activated inflammatory cells generate more reactive oxygen species (ROS). In contrast, antioxidants reduce mitochondrial dysfunction and oxidative stress in asthmatic mice[91]. Antioxidants have been used to prevent and treat mitochondrial abnormalities in asthma patients[92]. Macromolecule antioxidants such as vitamins E and C cannot enter mitochondria to scavenge reactive oxygen species. In contrast, gasotransmitter H_2S can freely cross both plasma and mitochondrial membranes. Once inside mitochondria, H_2S acts as a reducing agent, decreasing oxidative stress and enhancing endogenous antioxidant defenses, leading to the preservation of both mitochondrial structure and function[93]. Hu *et al.*[94] reported that H_2S prevented rotenone-induced mitochondrial membrane depolarization, cytochrome *c* release and decrease in Bcl-2/Bax in a human-derived dopaminergic neuroblastoma cell line. These protective effects of H_2S may be mediated by mitochondrial ATP-sensitive potassium (K_{ATP}) channels. To date, it is unclear whether H_2S can attenuate airway inflammation and hyper-responsiveness in asthma

by affecting the mitochondrial/oxidative stress pathway.

1.4.6. H_2S and airway smooth muscle cells

Higher airway responsiveness has been observed in both young humans and young animals compared with older people or animals[46,95,96]. Airway smooth muscle cells (ASMCs) are the final controllers of airway contraction. Hyper-responsiveness of ASMCs in early life increases risk of asthma in childhood[97,98]. Lung and airway smooth muscle maturation have been suggested as contributors to this age-dependent discrepancy in airway responsiveness at various levels, i.e. alterations of lung volume, elastic recoil, structure of the airway wall, interaction of the airway wall with surrounding lung parenchyma, and properties of airway smooth muscle cells, such as M2 and M3 muscarinic receptors levels in airway smooth muscle[96,99].

H_2S induces smooth muscle relaxation via its actions on two different types of ion channels. One is the K_{ATP} channel located on vascular SMCs and the other is the small to medium conductance K_{Ca} channel located on vascular endothelial cells. The opening of these two channels by H_2S leads to membrane hyperpolarization and smooth muscle relaxation. Therefore, H_2S can be characterized as an endothelium-derived relaxing factor[100].

In contrast to the abundant studies on various functions of H_2S in the cardiovascular system, little is known about the physiological effects of H_2S on the respiratory system. Perry *et al.*[73] investigated the regulatory role of H_2S on the proliferation of human airway SMCs. H_2S supplementations, both the fast-releasing 'donor' NaHS and the slow-releasing 'donor' GYY4137, suppressed airway SMC proliferation and interleukin-8 release due to the inhibited phosphorylation of ERK-1/2 and extracellular regulated kinase1/2 p38 mitogen-activated protein kinase. These effects of H_2S donors were not altered by the inhibitor of CSE or by the manipulation of K_{ATP} channel opening or nitric oxide (NO) levels. In other studies, administration of NaHS or the CSE blocker PPG alleviated or aggravated, respectively, airway hyper-responsiveness in both the rat cigarette smoke exposure model and the OVA-induced

asthma model[74,101]. NaHS relaxed rat tracheal rings that had been precontracted with acetylcholine or potassium chloride in a concentration-dependent manner. However, this NaHS-induced relaxation was not blocked by inhibitors of nitric oxide synthase (NOS) or by denudation of epithelium[101]. A reduction in intracellular calcium level induced by H_2S (due to reduced calcium influx) has been shown in airway smooth muscle cells, which may underlie the H_2S-induced relaxation of airway smooth muscle[102]. Airway SMCs contribute not only to airway narrowing in asthma but also to bronchial inflammation through the secretion of inflammatory factors and recruitment and activation of inflammatory cells[103]. Given the high expression levels of H_2S-generating enzymes in airway SMCs and the demonstrated muscle-relaxing effect of H_2S, it is hypothesized that reduced endogenous H_2S levels due to the down-regulation of H_2S-producing enzymes may constitute a significant factor in the pathogenesis of asthma.

1.4.7. H_2S and microbiota

Gut microbiota can up-regulate CSE activity/expression and endogenous H_2S production *via* metabolic products in the colon[104]. For example, gut microbiota synthesize pyridoxal 5′-phosphate, the cofactor for CSE activation. CSE expression can also be upregulated by butyrate[105], an end-product of microbial fermentation of plant polysaccharides which cannot be digested by humans[104]. Products generated from the interaction between microbiota and the intestinal immune system, such as IL-10, also promote colonic CSE expression and H_2S synthesis[106]. CSE activity, assessed by cystathionine consumption in the cecum, colon, small intestine, kidney, liver, aorta, heart, and brain, was lower in 11-12 weeks old germ-free mice compared to age-matched mice raised in a conventional specific pathogen-free (SPF) environment[107].

1.5 H_2S as a Biomarker of Asthma

Detecting the presence of or changes in asthma biomarkers in nasal air is an attractive approach for early detection of asthma symptoms, monitoring treatment effect and

evaluating asthma prognosis. An ideal biomarker of clinical relevance should be reliable and repeatable, sensitive and specific for intervention effects, and easy to sample and detect[108]. Biomarkers in nasal air can be detected non-invasively and repeatedly. A large number of inflammatory factors (including adenosine, ammonia, hydrogen peroxide, isoprostanes, leukotrienes, prostanoids, NO, peptides, and cytokines) have been studied in exhaled breath condensate. Some of these factors correlate with eosinophilic airway inflammation and the treatment schedule of corticosteroid therapy[109]. However, these biomarkers do not always reflect asthma severity or predict therapeutic outcomes[110]. There is a need for new biomarkers with high specificity and sensitivity for asthma; H_2S represents a promising biomarker for asthma.

As mentioned earlier, serum H_2S correlates with airway inflammation and with the severity of different respiratory diseases. It appears that serum H_2S may be used as a marker for airway inflammation and lower respiratory tract infections[74,75]. However, since many non-respiratory diseases can affect serum H_2S levels, the measurement of blood H_2S is non-specific as well as invasive. As a gasotransmitter, H_2S shares many attributes with NO and also exists in exhaled breath so it too can be sampled non-invasively. As one of the most important breath markers of lung diseases, exhaled NO has been studied extensively in multiple conditions including asthma, chronic obstructive pulmonary disease and cystic fibrosis[111]. Because it is affected by pathophysiological conditions of oral and dental health, H_2S in exhaled oral air does not accurately reflect H_2S metabolism in the respiratory system. This oral contamination can be avoided by measuring nasal H_2S. Although the specific variations in exhaled H_2S during different phases of asthma are still uncertain, exhaled nasal H_2S has great potential as a marker of the health or specific disease status of lung and airway tissues. It would be desirable to develop new technologies to detect H_2S in nasal air as a biomarker for asthma as well as other respiratory diseases.

1.6 Animal Asthma Models

One of the most frequently used animal asthma models uses ovalbumin challenges. The animals are sensitized using ovalbumin with an adjuvant, typically aluminum hydroxide, and then challenged with ovalbumin by nebulization or intranasal administration. Ovalbumin challenge induces antigen-specific T_H2 cell responses. Activated T_H2 cells produce T_H2-associated cytokines such as IL-4, IL-5 and IL-13. The ovalbumin model replicates features of human allergic asthma including airway hyper-responsiveness, airway inflammation and airway remodelling. It should be noted, however, that asthma is heterogeneous in origin and that distinct phenotypes and different pathogenic mechanisms are probably involved. Some types of asthma are dependent on T_H2 cell activation while others are not[112]. Ozone-induced asthma models, for example, are characterized by airway neutrophilia, natural killer T cell and macrophages but not eosinophilic or T_H2 inflammation in the lung[113]. Nevertheless, ozone exposure in animals induces severe airway hyper-reactivity, a cardinal feature of asthma[113].

1.7 Technological Challenge of Detecting H_2S in Nasal Air

Measurement of H_2S in exhaled breath, especially nasal air, is attractive because it best represents physiological conditions (because of minimal moisture loss and reduced contamination from oral sources). Breath samples contain very low concentrations of H_2S and are characterized by high moisture levels; both these factors challenge the detection power and ability of analytical technologies. Moreover, H_2S presents difficult chemical and physical properties for the detection technology.

Technical challenges related to sampling are nasal air collection, choice of storage media and maintenance of sample integrity. Nasal air sampling in experimental animals requires devices that suit the animal physiology to ensure representativeness. Measurement of H_2S in biological materials is difficult owing to its volatility, tendency to undergo oxidation, adsorption onto containers and the sample introduction system, and loss during processing. H_2S is corrosive; acceptable results

can be expected only if inert materials (such as polytetrafluoroethylene (PTFE), polychlorotrifluoroethylene, vinylidene polyfluoride, polyamide, ethylene-propylene or high-quality stainless-steel) are used in sample collection, storage and sample introduction systems.

Challenges in nasal air analysis include moisture tolerance and detection power. A limited number of analytical techniques have been used for measuring H_2S in breath, biological tissues and fluids (Table 1-1). These include gas chromatography, electrochemical detectors, spectrophotometry, high-performance liquid chromatography and an optical fibre chemical sensor, although the elevated H_2S values reported with the latter cast significant doubt on the accuracy of the technique in light of the low occurrence of H_2S in human breath[114,115]. Improved technologies (such as selected ion flow tube mass spectrometry and proton transfer reaction mass spectrometry) show promise for real-time analysis of H_2S levels in human breath[114]. Most techniques either fail to accommodate the high moisture in nasal air or lack sufficient sensitivity. Nevertheless, gas chromatography is the most widely used technique, perhaps due to its capability to separate and detect traces of volatile compounds and the high sensitivity possible using a combination of thermal desorption and pulsed flame photometric detection.

Figure 1-1

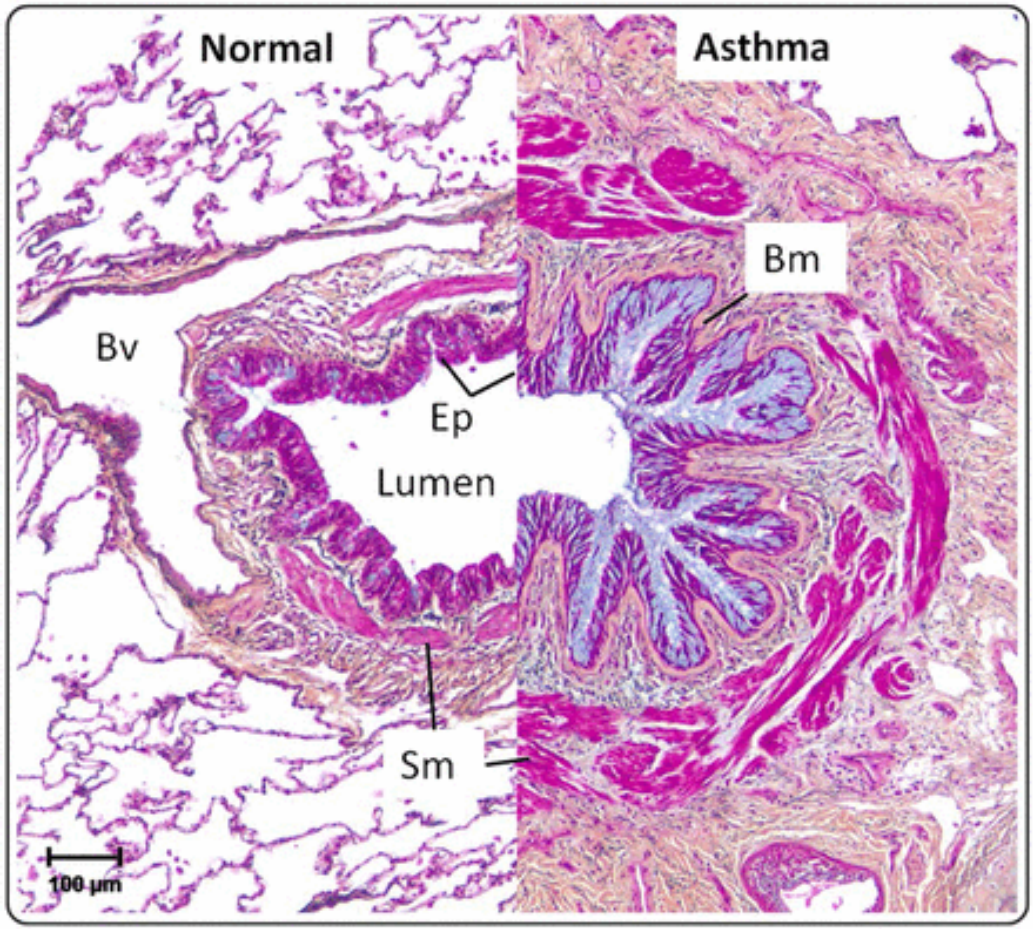


Fig. 1-1 Airways in asthma undergo substantial structural remodeling. Histological sections of a medium sized airway from a person without asthma and a patient with severe asthma stained with Movat's pentachrome stain. In asthma, the epithelium (Ep) shows mucus hyperplasia and hyper-secretion (blue), and thickening of the basement membrane (Bm). Smooth muscle (Sm) volume is also increased in asthma. Bv=blood vessel. Reproduced from Ref. 1 with permission.

Figure 1-2

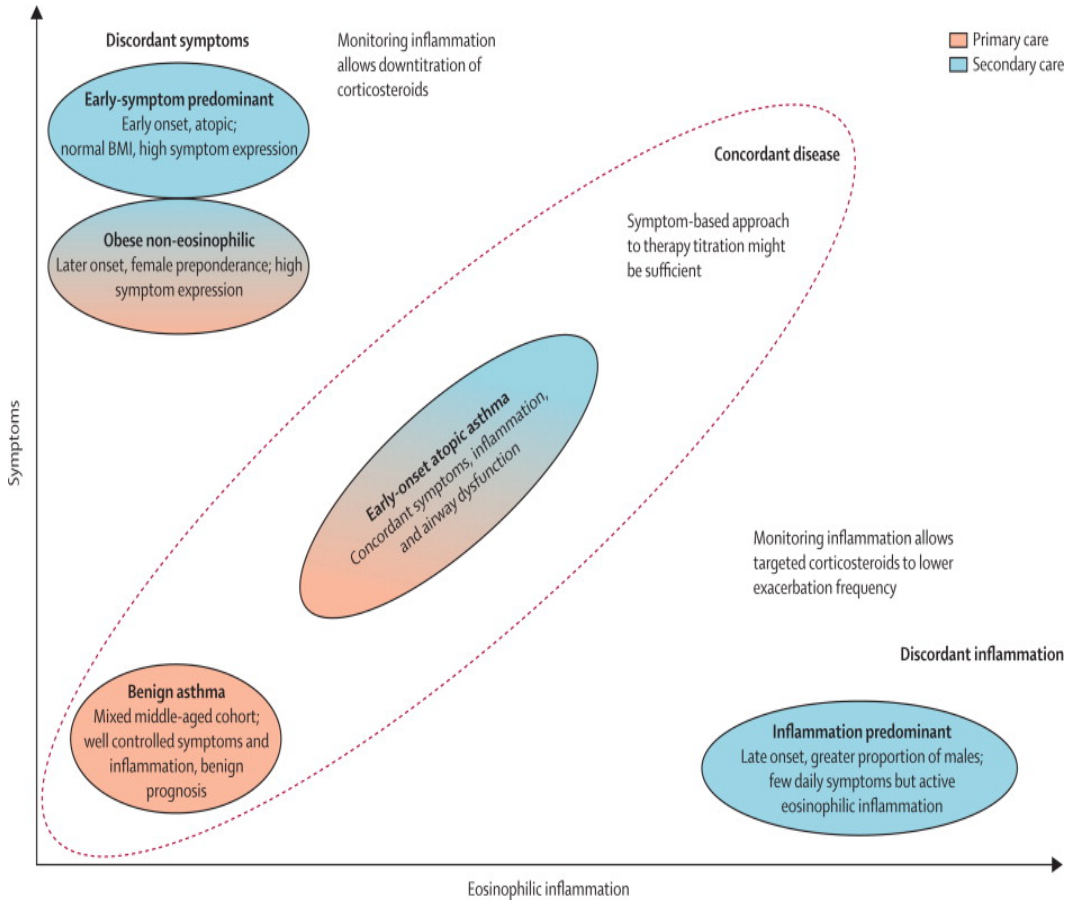


Fig. 1-2 Clinical phenotypes of adult asthma, identified by cluster analysis. Clusters of patients are plotted according to their relative level of symptoms and degree of bronchial eosinophilic inflammation. The graph highlights that patients with greater discordance between symptoms and inflammation are more difficult to treat and may be best followed up in specialized asthma centres. Reproduced from Ref. 116 with permission.

Figure 1-3

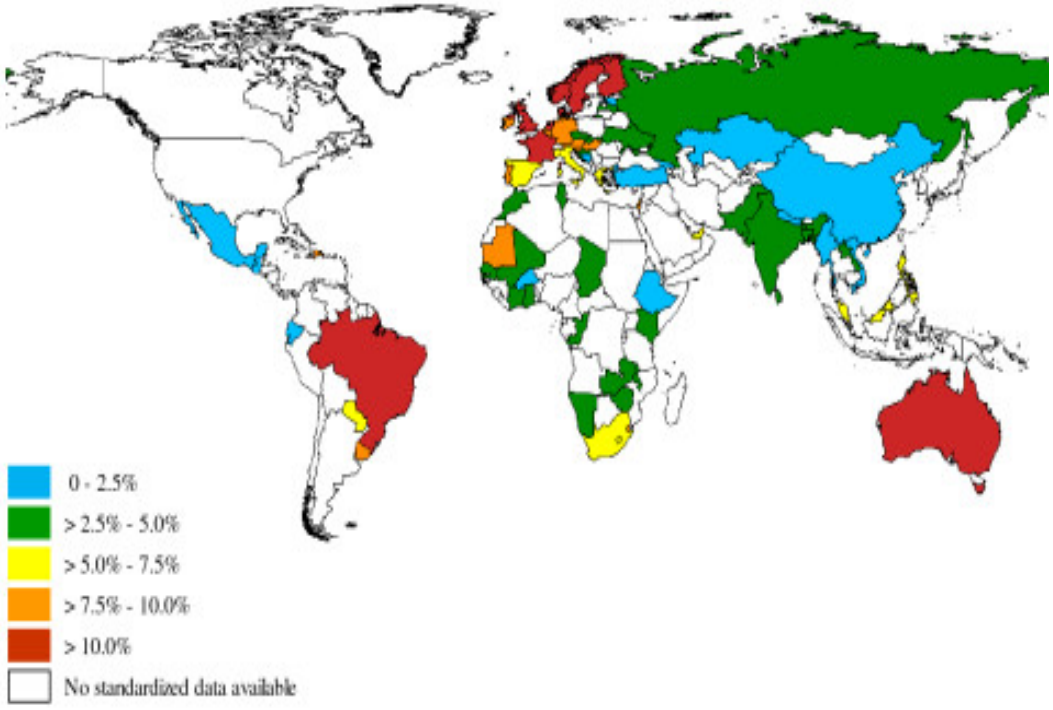


Fig. 1-3 Worldwide prevalence of clinical asthma. Reproduced from Ref. 24 with permission. The graph was generated with data from the World Health Survey (2002-2003). 70 of the 192 WHO member countries conducted this standardized cross-sectional survey. 178,215 individuals aged 18 to 45 years self-reported on questions relating to physician-diagnosed asthma, clinical/treated asthma, and wheezing in the last 12 months.

Figure 1-4

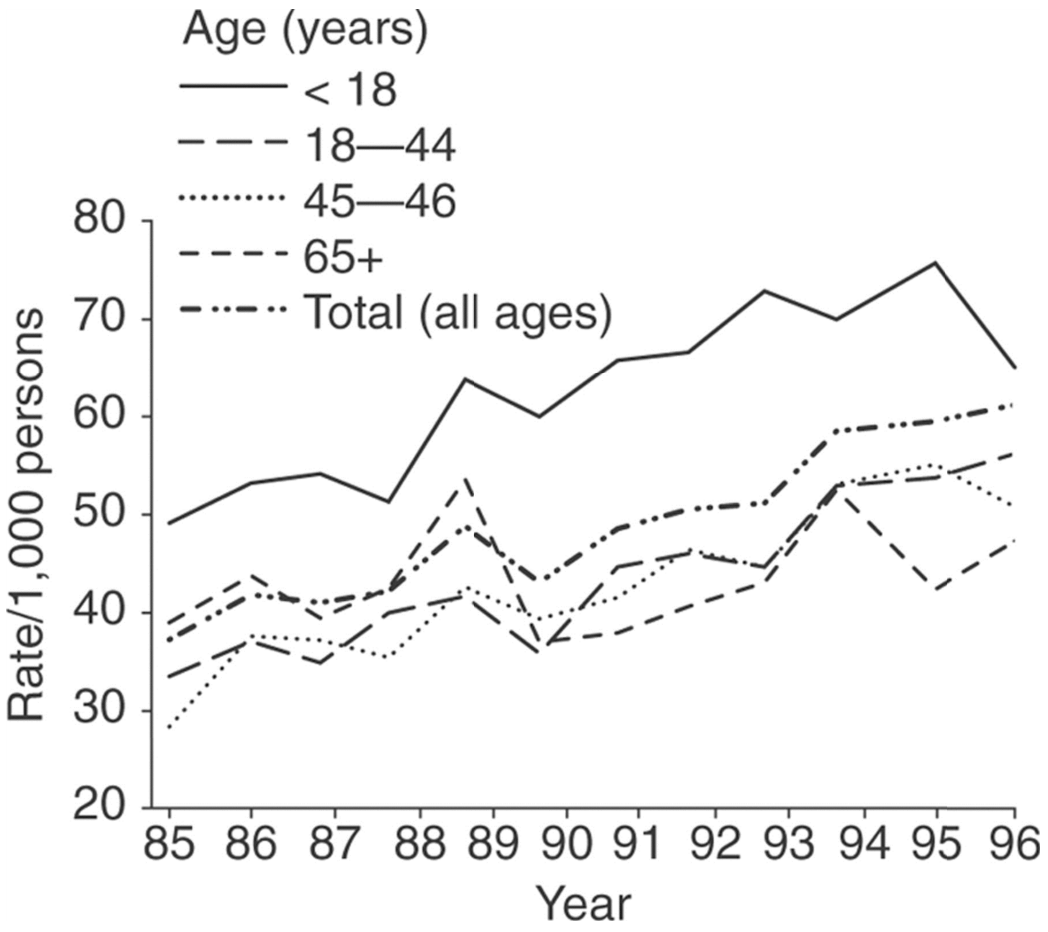


Fig. 1-4 Trends in the prevalence of asthma, by age, in the United States (from 1985 to 1996). The greatest increases are seen in children. Reproduced from Ref. 25 with permission.

Figure 1-5

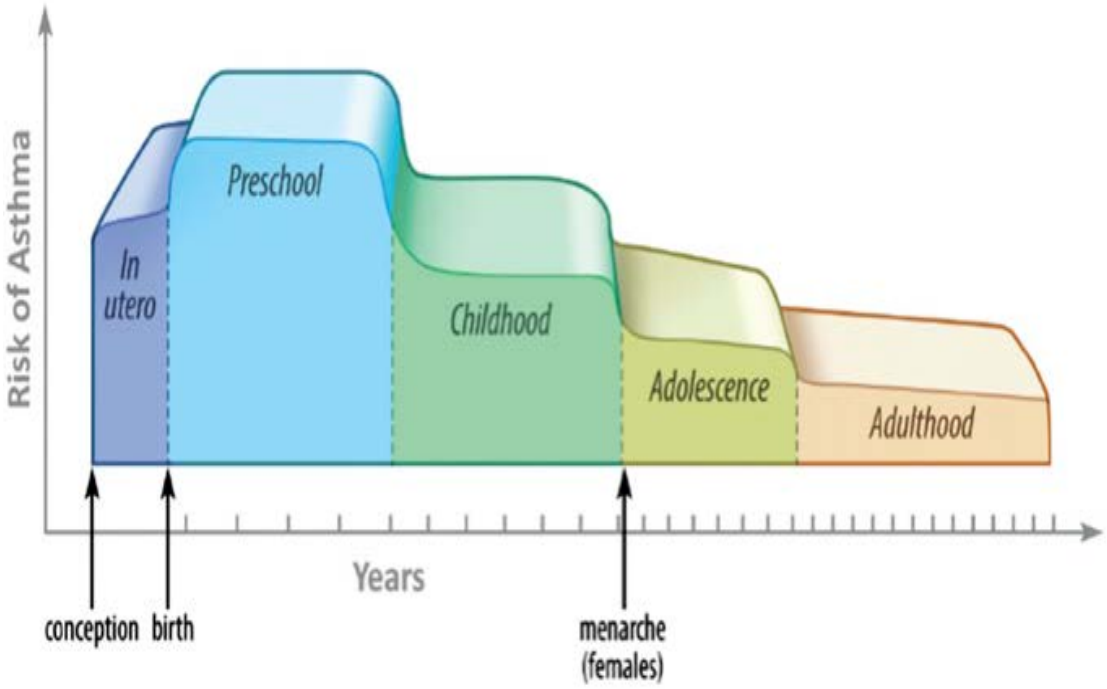


Fig.1-5 The risk for developing asthma is age dependent, with most asthma beginning in the preschool years. Box sizes represent estimates of the relative importance of each developmental period in childhood. Reprinted with permission of the American Thoracic Society. Copyright © 2015 American Thoracic Society. Jackson DJ, *et al.* 2014, Asthma: NHLBI Workshop on the Primary Prevention of Chronic Lung Diseases. Ann Am Thorac Soc. Suppl 3:S139-45.

Figure 1-6

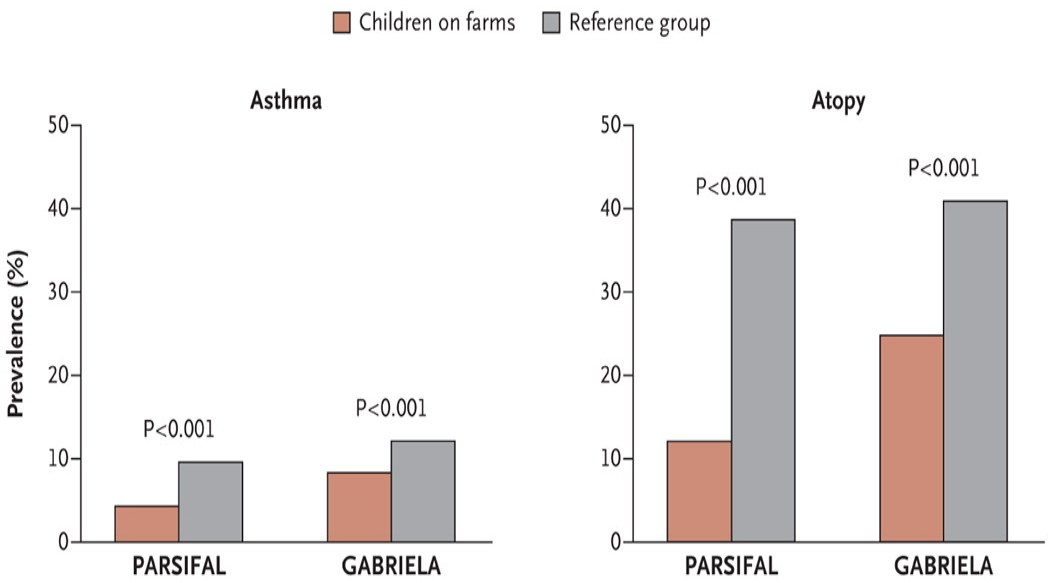


Fig. 1-6 Prevalence of asthma and atopy among children living on farms as compared with reference groups. The PARSIFAL study population included 6843 school-age children 6 to 13 years of age, and the GABRIELA study population included 9668 children between 6 and 12 years of age. Calculations of prevalence in GABRIELA were weighted on the basis of the total number of children who were eligible for inclusion in the study (34,491 children). This study was published in the New England Journal of Medicine in Feb 2011. Reproduced with permission from New England Journal of Medicine Ref. 57, Copyright Massachusetts Medical Society.

Figure 1-7

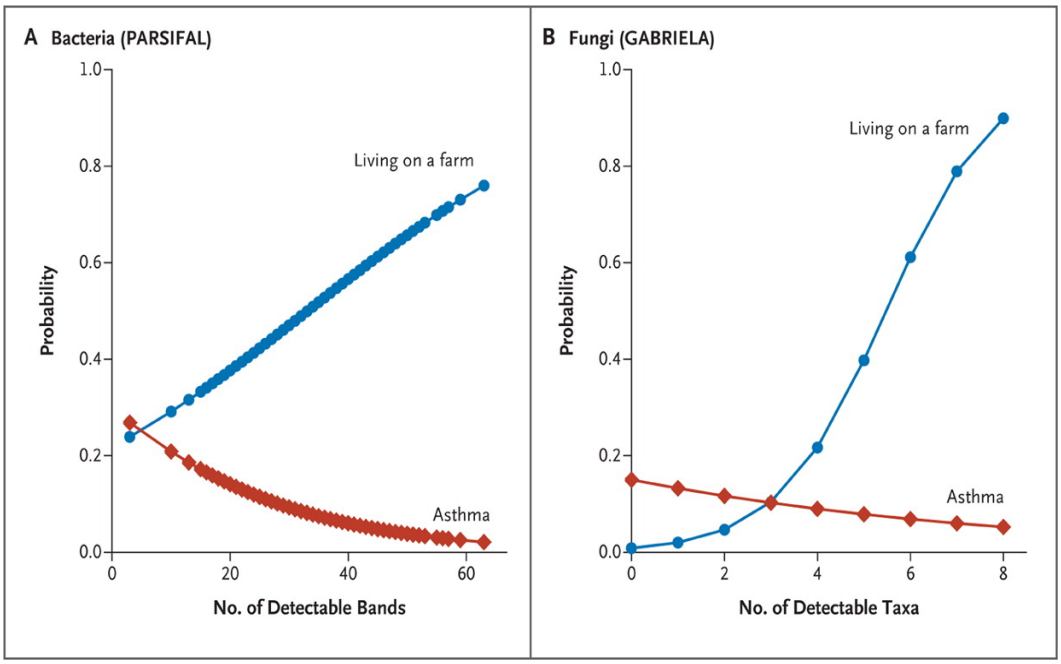


Fig. 1-7 Relationship between microbial exposure and the probability of asthma. In both the PARSIFAL study and GABRIELA, the range of microbial exposure was inversely associated with the probability of asthma. The PARSIFAL study population included 6843 school-age children 6 to 13 years of age, and the GABRIELA study population included 9668 children between 6 and 12 years of age. This study was published in the New England Journal of Medicine in Feb 2011. Reproduced with permission from New England Journal of Medicine Ref. 57, Copyright Massachusetts Medical Society.

Figure 1-8

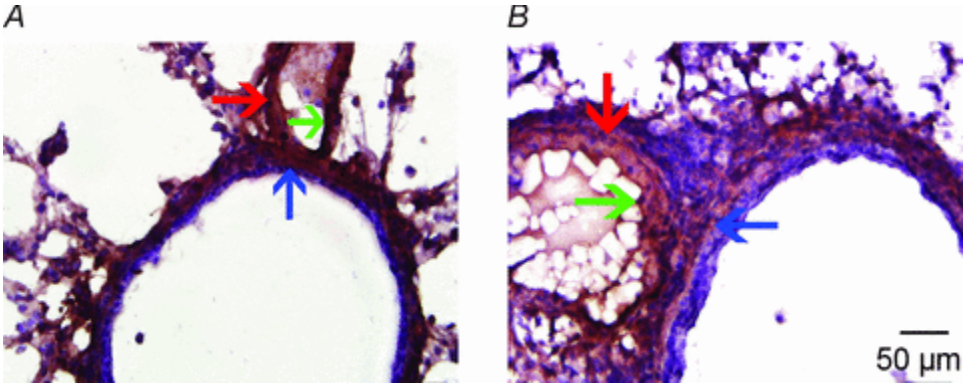


Fig.1-8 Distribution of cystathionine gamma-lyase (CSE) and cystathionine β-synthase (CBS) in the lung tissues of mice. Immunohistochemical staining shows that the expressions of CSE (A) and CBS (B) are mainly located in airway smooth muscle cells (blue arrow), vascular smooth muscle cells (red arrow) and vascular endothelial cells (green arrow). Published in Experimental Physiology, 2011 Sep; 96(9): 847-52. Reproduced with permission.

Figure 1-9

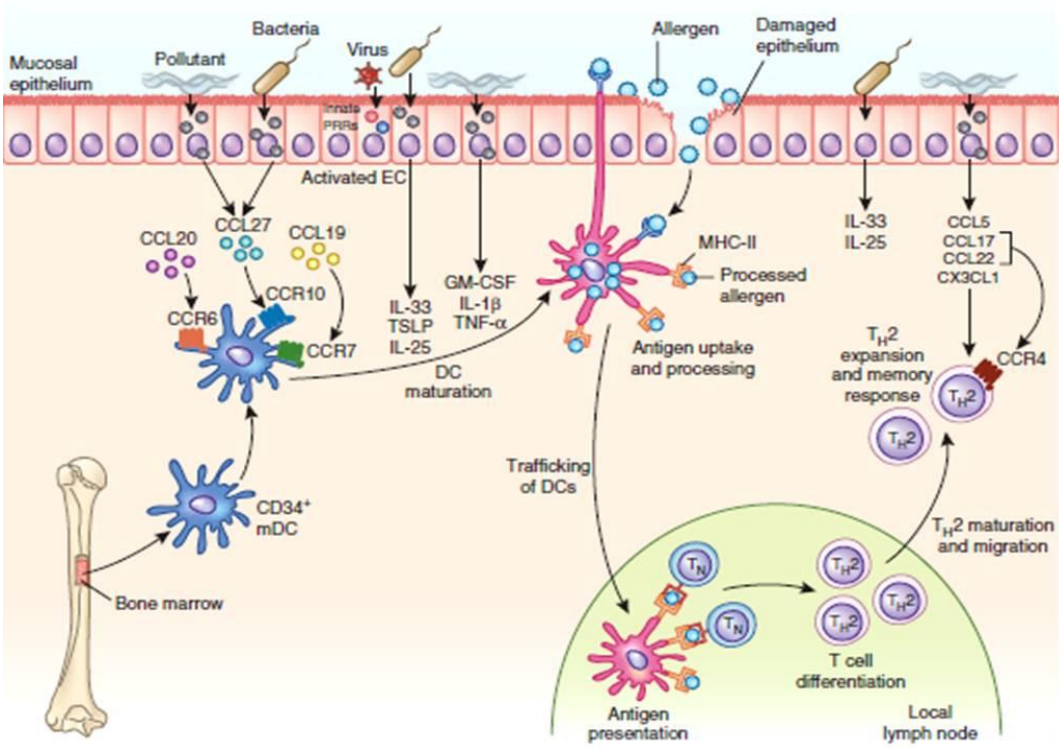


Fig. 1-9 Primary sensitization of the airways in the induction of allergic-type asthma. Perturbation of airway epithelium with infection and pollutants provides the initial danger signal and activates innate signaling receptors. This leads to chemokine secretion from airway epithelial cells and trafficking of immature dendritic cells (DCs) to the mucosal epithelium. DCs respond to danger signals via pattern recognition receptors (PRRs), triggering their maturation into competent antigen-presenting myeloid-type DCs. Once activated, mature DCs process allergens detected through processes extended into the airways, or by capturing allergens that have breached the epithelium. The allergen-loaded DCs then migrate to local lymph nodes where they interact with naive T cells via TCR, MHC class II and co-stimulatory molecules to drive differentiation of T cells. Additional epithelial-derived cytokines and chemokines such as IL-33, IL-25, CCL17, and CCL22 influence DC activation and T_H2 maturation and migration into the mucosa. Reproduced from Ref. 76 with permission.

Table 1. Analytical techniques used for the detection of H_2S in biological samples

Method	Sample type	Sample container and processing	LOQ/LOD	Drawbacks	Reference
Electrochemical	Rat breath	Real time	50 p.p.b.	Low sensitivity	Insko *et al.* (2009)
Gas chromatography	Mouse tissue and alveolar air	Polypropylene syringe/homogenization	1 p.p.b.	Invasive	Furne *et al.* (2008)
Portable gas chromatography	Human oral and nasal air	Polyethylene-coated balloon/cryotrapping	4 p.p.b.	Poor repeatability	Tangerman & Winkel (2008)
Selected ion flow tube mass spectrometry	Human breath	Real time	300 p.p.t.v.	Low sensitivity	Ross (2008)
Spectrophotometry	Trout plasma	Chemical treatment	—	Invasive; prone to error	Dombkowski *et al.* 2004
Optical fibre sensor	Mouth air	Catalytic reaction; sample held in polyvinyl fluoride bags and PTFE cell	10 p.p.b.	Prone to sulfur-oxidation and noise; lengthy	Rodríguez-Fernández *et al.* 2002

Abbreviations: LOQ/LOD, limit of quantification/limit of detection; p.p.b., parts per billion; p.p.t.v., parts per trillion by volume; PTFE, polytetrafluoroethylene.

Table 1-1 Analytical techniques used for detection of H_2S in biological samples. Published in Experimental Physiology, 2011 Sep; 96(9): 847-52. Reproduced with permission.

Hypothesis and Objectives

Asthma is a chronic inflammatory disease with hyper-responsive bronchoconstriction and airway remodelling, leading to extensive airway narrowing. Patients with allergic asthma usually develop their symptoms within the first 1-3 years of life. High incidence of allergic asthma in children closely relates to a T_H2 biased immune response after birth[47]. Multiple cohort studies consistently found that living on a farm during early life was protective against childhood asthma[57]. This protection is believed due to increased early gut microbial colonization, which subsequently triggers maturation of the developing neonate immune system.

Gut microbiota not only produces significant amount of H_2S[117] but also up-regulates CSE in the hosting mammalian cells via its metabolic products such as pyridoxal 5′-phosphate and butyrate[104,105]. CSE activity in cecum, colon, small intestine, kidney, liver, aorta, heart, and brain was lower in 11-12-week germ-free mice than in age-matched non-germ-free mice[107]. Gut microbiota is of low density and limited phylogenetic diversity after birth[61,62]. Successive colonization with *Gram-positive cocci, enterobacteria, lactobacilli* and *bifidobacteria* happens in the first few weeks of life in mice and first few years of life in humans[61,63]. Consistent with the observations of germ-free animals and lower microbiota colonization in early life, CSE activity and expression levels in mouse liver are very low after birth and increase gradually until reaching their peaks at 3 weeks of age[118]. Considering the stimulatory effect of microbiota on CSE expression in host organs and the time frame of microbiota colonization after birth, there appears to be a direct correlation between the age-dependent colonization of microbiota and CSE expression in the host. It is reasoned that early-life microbial exposure may suppress systemic T_H2 biased response via up-regulating CSE/H_2S in mammalian host cells.

Involvement of CSE/H_2S in asthma development has been suggested in human and animal studies. Plasma levels of H_2S were lower in patients with stable asthma and correlated negatively with the severity of asthma[75]. In animal allergic asthma models

(8-week-old rats or 12-week-old mice), H_2S levels in plasma and lung tissues were lower than in non-asthmatic control animals[74]. Exogenous H_2S supplementation reduced OVA challenge-induced airway hyper-responsiveness, eosinophil infiltration and T_H2 cytokine levels in bronchoalveolar lavage fluid (BALF).

Hypothesis of this study: endogenous level of CSE/H_2S is a determining factor for asthma development via suppression of biased T_H2 response and that age-dependent changes in CSE/H_2S levels explain the higher incidence of asthma in childhood.

The objectives of this study include:

1. To explore the protective role of endogenous H_2S against allergen-induced allergic asthma using both wild type (WT) and CSE-KO mice;
2. To investigate whether CSE/H_2S plays a key role in the initiation of childhood asthma;
3. To examine the mechanisms underlying the asthma-modulating effects of H_2S;
4. To search for effective H_2S-based treatment regimes.

2. Chapter 2

MATERIALS AND METHODS

2.1 Chemical and Antibodies

Anti-mouse CD3ε, CD28, IL-4, and IFN-γ antibodies were purchased from eBioscience (USA). Anti-STAT6, phos-STAT6, STAT-4, phos-STAT4, GATA3 and Tbet antibodies were purchased from Santa Cruz Biotechnology (USA). Anti-CBS antibody was purchased from Abnova (Taiwan). Anti-CSE antibody was purchased from Proteintech (USA). Anti-3MST was purchased from Sigma-Aldrich (USA), Lambin B1 was purchased from Abcam (USA). Mouse IL-2, IL-4 and IL-12 were purchased from Peprotech (Canada). NaHS, ovalbumin (OVA), protein inhibitor cocktails, penicillin-streptomycin, β-mercaptoethanol, RPMI Medium 1640, methacholine (Mch), red blood cell lysis buffer and bovine serum albumin were purchased from Sigma-Aldrich (USA). PMA and ionomycin were purchased from eBioscience (USA).

2.2 Animals

For in vivo experiments, homozygous CSE knockout (KO) and age-matched wild-type littermates (WT) on C57BL/6J × 129SvEv background were used[119]. All animals were under diurnal lighting conditions. All experimental procedures were approved by Lakehead University Animal Care Committee (ACC) and performed in accordance with the guidelines set by the Canadian Council on Animal Care (CCAC). All efforts were made to minimize suffering and the number of animals used.

2.3 Allergic Asthma Model

In chapter 3, 12-16 week old WT and CSE-KO mice were used to establish allergic asthma models. Animals were sensitized to OVA with two intraperitoneal injections (ip) of 40 μg OVA/1 mg Alumina/mouse (grade V; Sigma-Aldrich, St. Louis, MO)

emulsified in 2.25 mg of alum hydroxide (AlumImject; Pierce, Rockford, IL) in a total volume of 200 µl on day 0 and day 7. On day 21 to day 23, mice were challenged with 100 µg OVA in 40 µl PBS by intratracheal inhalation after being anesthetized with isofluorane (Baxter, Canada). Control groups were treated identically except treatment solutions did not contain OVA.

In chapter 4, to investigate age-dependent development of allergic asthma, allergic asthma was induced in young and old mice (3-4 weeks old and 7-8 months old, respectively) (Fig. 4-1). 2 µg OVA per gram body weight and 5 µg OVA per gram body weight were used to sensitize and challenge old mice, respectively. Some animals were treated with NaHS (14 µM/ kg ip twice daily) starting two days prior to sensitization and continuing until two days after sensitization (day minus-2 through day 2 and day 5 through day 9) (Fig. 4-1).

2.4 Measurement of Lung Function

A resistance and compliance (R&C) system (Fig.2-1, Buxco, USA) was used to test airway resistance in response to increasing concentrations of aerosolized methacholine (1.56, 3.13, 6.25, 12.5 mg/ml) following procedures as listed below: 1) Mice underwent acute non-recovery operation for trachea cannulation. The mice were anesthetized with Ketamine (100 mg/kg, ip) + Xylazine (8.5 mg/kg, ip). Then a 1.5-2 cm longitudinal incision on the ventral side of the neck was made to expose the trachea. A silk suture (6-8 cm) was placed underneath the trachea with curved forceps before a small T-shaped incision was made on the trachea. A cannula was inserted 5-8 mm into the trachea and secured with the suture. 2) The cannulated mice were mechanically ventilated with a Buxco FinePointe R&C system (Buxco, USA). Body temperature of the mice was maintained between 36.0 °C and 37.5 °C using a heating pad. 3) Mice were ventilated (tidal volume: 0.2 ml, frequency: 150 breaths/min), and exposed to methacholine (MCh) aerosol for 20 s, then dynamic resistance was measured for 3 min. The average dynamic resistance response was recorded for each concentration of MCh. Data were collected and processed using FinePointe Software

(Buxco, USA).

2.5 Bronchoalveolar Lavage Fluid Collection

Mouse lungs were flushed three times with 0.8 ml PBS/1% BSA to obtain BALF. Aliquots of the pooled, non-centrifuged BALF were used to count total live and dead cells using trypan blue exclusion. The remaining BALF was centrifuged and the supernatant was stored at -80°C for determining IL-4, IL-5, IL-13, and eotaxin levels with ELISA (R&D). Cyto-centrifuged samples were re-suspended with PBS to reach a final concentration at $8x10^5$ cells /ml. 80 µl of the cell suspension was used for cytospin preparations. The obtained slides were stained with Wright–Giemsa stain (Sigma-Aldrich, USA) for white cell counting. At least 200 cells per slide were counted using a light microscope under 400 magnification. Percentages of eosinophils, neutrophils, macrophages and lymphocytes were calculated.

2.6 Lung Histology

Left lobes of lungs were fixed in 5 ml of 10% buffered formalin and embedded in paraffin. Lung samples were sectioned at 4 µm and stained with H&E reagents (Sigma-Aldrich, USA) following standard procedures. Briefly, slides were deparaffinized with Xylene (Sigma-Aldrich, USA) for 3 x 10 minute periods, then hydrated by sequential passage through 100% ethanol (2 x 3 min), 95% ethanol (3 min), 70 % ethanol (3 min) and distilled water. The sections were stained with hematoxylin for 5 min and rinsed under running tap water for 15 min before differentiation in a 0.1% acid alcohol solution (1-3 s). After being well rinsed in ddH_2O, the slides were counter stained with Eosin (Sigma) for 20 seconds. Slides were then dehydrated by sequential passage through 90% ethanol (2 x 3 min), 100% ethanol (2 x 3 min), and Xylene (2 x 5 min). Once dry, the slides were mounted with resinous mounting medium (Fisher, USA). Pathological changes were examined under a light microscope equipped with a camera (Olympus, Canada).

To quantitatively analyze the pulmonary inflammatory response (in Chapter 4), H&E

stained lung sections were examined using light microscopy with x40 objective. Eosinophils surrounding seven bronchi were counted in each lung slide and the average number of eosinophils per bronchus was calculated.

2.7 Immunohistochemistry

The right lobes of the lungs were fixed in 5 ml of 10% buffered formalin for 6-10 hours then transferred to a 10% sucrose Solution (130 mM Na2HPO4, 30 mM KH_2P0_4, 10% (w/v) sucrose, 0.01% sodium azide, pH 7.2) for 24 h. The fixed lungs were put into vinyl molds, and mounted in frozen tissue matrix OCT. The tissues in OCT were frozen slowly and gradually on top of liquid nitrogen and then stored at -80 °C until sectioning.

The frozen lung tissues were cut at 5 µm and used to detect CSE and CBS following standard immunohistochemistry protocol: 1) The slides were thawed at room temperature for 10-20 min before being fixed in methanol for 10 min; 2) The fixed slides were then rinsed in PBS (3 x 5 min) and incubated in a 0.3% H_2O_2 solution in PBS for 10 min to block endogenous peroxidase activity; 3) The slides were rinsed in PBS (3 x 5 min). Non-specific bindings were then blocked with a blocking buffer (1x PBS/0.3% Triton-X 100/5% normal goat serum) for 60 min at room temperature in a humidified chamber; 4) Blocking solution was removed and the slides were incubated with 400 µl primary antibody (CBS and CSE were diluted at 1:200 in blocking solution) overnight at 4 °C in a humidified chamber; 5) Primary antibodies were removed by rinsing the slides in PBS (3 x 5 min). The slides were then incubated with 400 µl secondary antibody (diluted at 1:400 in blocking solutions) at room temperature for 1 h in a humidified chamber; 6) Secondary antibodies were removed by rinsing the slides in PBS (3 x 5 min). The slides were incubated with 400 µl DAB (Sigma, USA) for 5-10 min until the desired color intensity was reached. DAB substrate solution was prepared by mixing 1 ml DAB chromagen with 1ml DAB buffer; 7) The slides were rinsed in PBS (3 x 5 min) and stained with Hematoxylin following the steps indicated in 2.6; 8) Finally the slides were dehydrated and

mounted with coverslips using resinous mounting medium.

2.8 Western Blotting

Tissues or cells were homogenized in ice-cold RIPA buffer (Thermo Scientific, USA; 25 mM Tris-HCl, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS). Protein inhibitor cocktails (Sigma, USA) were added to RIPA buffer right before use. Lysates were incubated on ice for 10 min and then centrifuged at 15,000 g at 4 °C for 15 min. Protein concentrations of the supernatants were determined using Biorad protein assay (Bio-Rad, USA) following manufacturer's instructions. The supernatants with equal amounts of protein were then mixed with sample buffer (4% SDS, 0.125 M Tris-HCl, 20% glycerol, 10% 2-mercaptoethanol, 0.015% bromophenol blue) and denatured at 100 °C for 10 min. 80 μg tissue protein samples or 60 μg cell protein samples were loaded per lane and electrophoresis was performed in 10% SDS–polyacrylamide gels. The gel was subsequently run in running buffer (Tris-base 25 mM, Glycine 192 mM, SDS 0.1% w/v) at 50 V for stacking gel and 100 V for separating gel using the Mini-PROTEAN® Electrophoresis System - Bio-Rad (Bio-Rad Laboratories, Inc, USA). Proteins separated by the gel were then transferred to polyvinylidene difluoride membrane (PDVF, 0.45 μm pore size) using the mini-trans blot Electrophoretic Transfer Cell (Bio-Rad Laboratories, Inc, USA). The PDVF membrane was blocked in non-fat milk (5%, w/v) in PBST (0.1% v/v Tween-20 in PBS) for 1 h at room temperature. The membrane was then incubated with primary antibody overnight at 4 °C followed by 1 h incubation with HRP linked secondary antibody (Sigma, USA) at room temperature. The bands were visualized using an ECL detection reagents kit (Biosciences, Canada) according to the manufacturer's instructions. The blots were subsequently quantified using ImageJ software.

2.9 Measurement of H_2S Production in the Lung

H_2S production in the lung was measured following procedures described previously[120]. Briefly, mouse lung tissues were homogenized in ice-cold 50 mM potassium phosphate buffer (pH 6.8). For each reaction, 300 μl lung homogenate

were added to a mixture of 100 µl L-cysteine (100 mM), 100 µl pyridoxal 5' phosphate (PDP, 2 mM), and 250 µl potassium phosphate buffer (0.4 M, pH 7.4). 300 µl potassium phosphate buffers (50 mM), instead of lung homogenate, were added in blanks. Cryovial test tubes (2 mL) with a piece of filter paper (2×2.5 cm^2) were used as the central wells, each containing 500 µl trapping solution (1% zinc acetate, 2% sodium EDTA, pH 12.7) to trap H_2S. The flasks were flushed with N_2 for 1 min and then sealed with Parafilm®. Reactions were initiated by transferring the flasks from ice to a 37ºC shaking water bath. After incubation for 90 min, 0.5 ml of 50% (w/v) TCA was injected into the flasks (central wells had to be avoided). The flasks were incubated in the shaking water bath at 37 ºC for another 60 min to trap H_2S completely. Upon finishing incubation, all contents in the central wells were carefully transferred to a 15 ml testing tube with 3.5 ml ddH_2O. 500 µl of 20 mM N, N-dimethyl-pphenylenediamine sulfate (N, N-DPD in 7.2 M HCl) and 500 µl of 30 mM FeCl3 in 1.2 M HCl were added to the testing tubes in the stated sequences. After another 20 min of incubation at room temperature, the absorbance of the resulting solution was measured with a spectrophotometer at 670 mm.

2.10 H_2S Concentration Measurement

H_2S generated by splenocytes was measured as described by Kartha *et al*[121] with modifications. In brief, splenocytes were cultured in 50 ml cell culture flasks, the tops of which were pre-coated with 5 ml zinc-agar. The composition of the agar layer was 800 µl zinc acetate (45 mM, Sigma-Aldrich, USA), 180 µl NaOH (3 M, Sigma-Aldrich, USA) and 4.02 ml sterilized agar (1% w/v, Sigma-Aldrich, USA). After being cultured for 72 h, the splenocytes and media were removed carefully without disturbing the agar layers. The culture flasks were then re-orientated with the agar layers down. 2 ml of N, N-dimethyl-p-phenylenediamine chloride (N, N-dpd, 40 mM in 7.2 M HCl; Sigma-Aldrich, USA) was added to each flask to incubate the agar layers for 10 min at room temperature. Iron chloride reagent of 400 µl ($FeCl_3$, 30 mM in 1.2 M HCl; Sigma-Aldrich, USA) was then added and mixed with the N, N-dpd. After 20 min incubation, 200 µl of the contents in the flask were transferred to a 96-

well microtiter plate. Absorbance was measured at 670 nm using a Synergy 2 Multi-Mode Reader (BioTek, Winooski, USA). H_2S concentration was calculated against a standard curve that was generated with NaHS (concentrations range from 5 to 250 μM) using setups identical to the cell culture flasks.

2.11 In vitro Re-stimulation Assay

Spleens and mediastinal lymph nodes were collected 5 days after OVA sensitization from 6 mice per group (Supple. 4.1). Splenocytes and lymph node cells were prepared by mashing pooled mediastinal lymph nodes and spleens through a 40 μm cell strainer (BD Biosciences, USA). Erythrocytes from spleens were removed using Red Cell Lysis Buffer (Sigma-Aldrich, USA). Cells were seeded at a final concentration of 1×10^6/ml in 24-well plates and re-stimulated with OVA (100 μg/ml) for 72 h. IL-4, IL-5 and IL-13 levels in the supernatant were tested with ELISA (R&D, USA) following the manufacturer's instructions.

2.12 T_H2 Cell Differentiating Culture

Spleens were collected from normal mice without any sensitization or treatment. Splenocytes were isolated following the method described in section 2.11. Splenocytes (200 μl 1 million/ml) were seeded in 96-well plates. Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 500 U penicillin-streptomycin (Sigma-Aldrich, USA) and 50 μM β-mercaptoethanol (Sigma-Aldrich, USA), anti-mouse CD3ε (5 μg/ml, plate-bound, 145-2C11; eBioscience, USA), anti-mouse CD28 (2 μg/ml, clone 37.51; eBioscience, USA), mouse IL-4 (20 ng/ml, Peprotech, Canada), mouse IL-2 (20 ng/ml, Peprotech, Canada) and anti-mouse IFN-γ (5 μg/mL, R4-6A2; eBioscience, Canada). These conditions promote T_H2 cell differentiation. In some experiments, NaHS (0.3, 1, 3, 10, and 30 μM, Sigma-Aldrich, USA) was added to the culture medium. On day 3, the cells were passaged and cultured for an additional 3 days on 24-well plates containing IL-2 (20 ng/ml) andIL-4 (20 ng/ml) with or without the addition of NaHS (0.3, 1, 3, 10, and 30 μM). After the differentiation culture, splenocytes were re-

stimulated with PMA/ionomycin (1:1000; Cat No. 00-4970, eBioscience) for 24 h. Culture medium was harvested and cytokine levels in the medium were measured with ELISA (R&D, USA).

2.13 T_H1 Cell Differentiating Culture

Mouse splenocytes were isolated as described in section 2.11. Splenocytes (200 µl, 1 million/ml) were seeded in 96-well plates. Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 500 U penicillin-streptomycin (Sigma-Aldrich) and 50 µM β-mercaptoethanol (Sigma-Aldrich), anti-mouse CD3ε (5 µg/ml, plate-bound, 145-2C11; eBioscience), anti-mouse CD28 (2 µg/ml, clone 37.51; eBioscience), mouse IL-12 (20 ng/ml, Peprotech) , mouse IL-2 (20 ng/ml, R&D) and anti-mouse IL-4 (5 µg/ml, 11B11, eBioscience). These conditions promote T_H1 cell differentiation. In some experiments, NaHS (0.3, 1, 3, 10, and 30 µM) was added to the culture medium. On day 3, the cells were passaged and cultured for an additional 3 days on 24-well plates containing IL-2 (20 ng/ml) and IL-12 (20 ng/ml) with or without NaHS (0.3, 1, 3, 10, and 30 µM). After the differentiation culture, splenocytes were re-stimulated with PMA/ionomycin (1:1000; Cat No. 00-4970, eBioscience) for 72 h. Culture medium was harvested and IFN-γ levels in the medium were measured with ELISA (eBioscience, USA).

2.14 ELISA

IL-4, IL-5, IL-13, and eotaxin levels in BALF and cell culture medium were tested using a commercial ELISA kit (R&D, USA); IFN-γ levels in the medium were measured with mouse IFN gamma ELISA Ready-SET-Go (eBioscience, USA). ELISAs were performed according to the manufacturer's protocols.

2.15 Nuclear and Cytoplasmic Protein Extraction

Splenocytes were cultured under T_H1 or T_H2 differential conditions for 4 days. Cytoplasmic and nuclear proteins were extracted using NE-PER Nuclear and

Cytoplasmic Extraction Reagent (Pierce, Thermo Scientific, USA) following the manufacturer's instructions. Briefly, cells were incubated with Cytoplasmic Extraction Reagent (CER I) supplemented with protease inhibitors for 15 min and then CER II was added. After vortexing, cells were spun to pellet at 8000 rpm for 5 min at 4 °C. The supernatant (cytoplasmic extract) was removed and stored at -80 °C until use. The nuclear pellets were resuspended in ice-cold Nuclear Extraction Reagent (NER) supplemented with protein inhibitors and vortexed on the highest setting for 15 s (every 10 min, for a total of 40 min). The lysed nuclei were centrifuged at 16,000 g) for 10 min. For nuclear translocation assays, nuclear and cytosolic proteins were immunoblotted with mouse anti-GATA3 antibody (1:200, Santa Cruz Biotechnology, USA).

2.16 Reactive Oxygen Species Detection

Freshly isolated splenocytes were cultured for 24 h in plates with or without pre-coated anti-CD3ε antibody. Then ROS activities in splenocytes were measured using 2′,7′-dichlorofluorescein diacetate (DCFDA) with the ROS assay kit (Abcam, USA). Briefly, approximately 1×10^6 splenocytes were incubated in 20 μM DCFDA for 45 min at 37 °C according to the manufacturer′s instructions. Incubated cells were then washed with PBS and re-suspended in 1ml PBS. Fluorescence intensity of a single splenocyte was measured using flow cytometry (λ_{ex} = 485 nm, λ_{em} = 535 nm).

2.17 Modified Biotin Switch Assay for *S*-sulfhydration Detection

The *S*-sulfhydration assay was performed as described previously[122]. Briefly, 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] supplemented with 100 μM deferoxamine. Lysates were centrifuged at 13,000 g for 30 min at 4 °C. Supernatant was added to blocking buffer [HEN buffer with 2.5% SDS and 20 mM methyl methanethiosulfonate (MMTS)] at 50 °C for 20 min with frequent vortexing. The MMTS was then removed with pre-chilled acetone and the proteins were precipitated at -20°C for 20 min. After acetone removal, the proteins were re-suspended in HENS

buffer (HEN buffer with 1% SDS). Biotin-HPDP (N- [6-(biotinamido) hexyl] - 3'-(2'-pyridyldithio) propionamide) in dimethyl sulfoxide was added without ascorbic acid. After incubation for 3 h at 25 °C, biotinylated proteins were precipitated by streptavidin-agarose beads. Beads were washed five times with HENS buffer. The biotinylated proteins were eluted by SDS-PAGE sample buffer and subjected to Western blotting analysis using anti-GATA3 antibody (1:200, Santa Cruz Biotechnology, USA).

2.18 Site-directed Mutagenesis of GATA3

pFAG-GATA3 was purchased from Addgene (MA, USA). Single mutations 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) [123]. All primer sequences used in this study were listed in Table 4-1. The correct mutant was confirmed by DNA sequencing at the MOBIX lab of McMaster University, ON, Canada.

2.19 Statistical Analyses

Data analysis and graph generation were performed using GraphPad Prism Software version 5.0. Statistical differences were calculated using two-way ANOVA (for comparisons among WT-young, WT-old, KO-young and KO-old), and unpaired *t* tests (for comparisons between WT and CSE-KO or control and NaHS treatment groups). Mann Whitney test was used when variance was different between groups or data were not normally distributed. One-way ANOVA was used for comparing data among groups with various NaHS treatments. Results were considered significant at $P \leq 0.05$. All data are expressed as mean ± standard error of the mean (SEM).

Figure 2-1

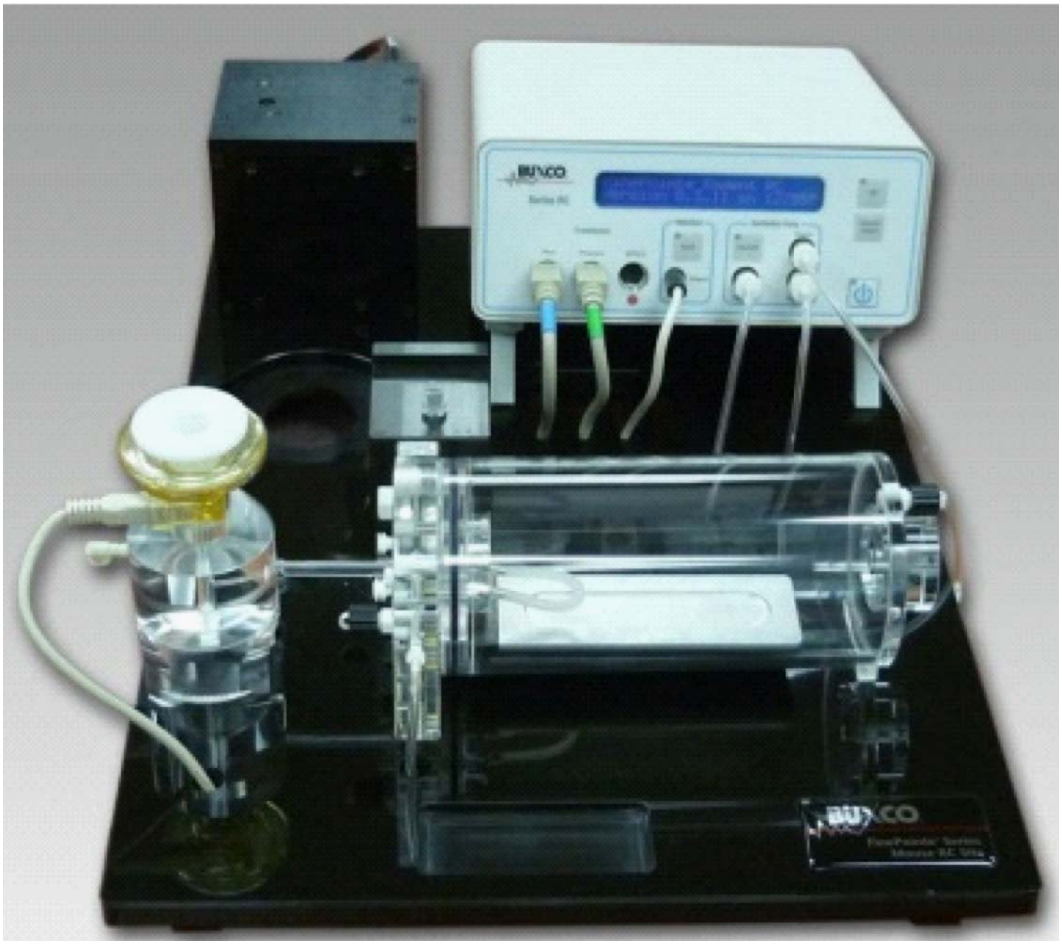


Fig. 2-1 R&C (resistance and compliance) system (Buxco, USA)

3. Chapter 3

The Inhibitory Role of H_2S in Airway Hyper-responsiveness and Inflammation in a Mouse Model of Asthma

[Data included in Chapter 3 were published in American Journal of Pathology, 2013 Apr; 182(4): 1188-95. They are reproduced with permission from the publisher. The experiments conducted by other co-authors are referred to as Appendix 1 and Appendix 2. I assisted in these experiments by performing lung function tests and histological staining.]

3.1 Abstract

Cystathionine gamma-lyase (CSE) is one of the major enzymes producing H_2S in lungs, participating in the regulation of respiratory functions. In allergic diseases, the role of CSE-derived H_2S in eosinophil-dominant inflammation has been unclear. The objective of this study was to explore the protective role of H_2S against allergen-induced airway hyper-responsiveness (AHR) and inflammation. CSE expression and H_2S production rates were assessed in lung tissues from mice with ovalbumin (OVA)-induced acute asthma. AHR, airway inflammation, and T_H2 response in wild-type (WT) mice were compared with those in *CSE* gene knockout (CSE-KO) mice. CSE expression was absent and H_2S production rate was significantly lower in the lungs of CSE-KO mice when compared with WT littermates. OVA-challenge decreased lung CSE expression and H_2S production in WT mice. CSE deficiency resulted in aggravated AHR, increased airway inflammation, and elevated levels of T_H2 cytokines such as IL-5, IL-13 and eotaxin-1 in bronchoalveolar lavage fluid after OVA-challenge. The aforementioned alterations were reversed by exogenous H_2S treatment. More importantly, NaHS supplementation rescued CSE-KO mice from the aggravated pathological process of asthma. In conclusion, the CSE/H_2S system appears to play a critical protective role in asthma development and may present new options for asthma prevention and treatment.

3.2 Introduction

H_2S, known for centuries as a noxious and toxic gas, is now recognized as a third gasotransmitter (after nitric oxide and carbon monoxide) with an important regulatory role in chronic respiratory diseases, and cardiovascular and metabolic disorders[119,124-127]. The role of H_2S in inflammation, however, is still controversial[125]. Some studies have showed that exogenous H_2S has a pro-inflammatory effect in various inflammatory models including lipopolysaccharide-induced endotoxemia, acute pancreatitis, and cecal ligation and puncture-induced sepsis[128-130]. Other researchers have reported anti-inflammatory effects of H_2S[131-133]. The concentration and the release rate of exogenous H_2S donors are the main factors in determining whether H_2S is anti-inflammatory or pro-inflammatory. High concentration and fast-releasing rate of H_2S results in a pro-inflammatory effect while low concentration and slow-releasing rate of H_2S is anti-inflammatory[133,134].

Asthma is one of the most common chronic inflammatory diseases. Its prevalence has markedly increased throughout the past 2 decades[135]. Eosinophilia and T_H2 cytokine production are two hallmarks of allergic asthma[136]. Increased T_H2 cytokines (such as IL-4, IL-5, and IL-13) cause immunoglobulin isotype switching of B cells to produce IgE, promote the growth, maturation, and activation of T_H2 cells and eosinophils, and result in airway hyper-responsiveness (AHR), mucus production, and airway remodeling[136]. Previous studies have shown the existence of CSE and/or cystathionine β-synthase (CBS) in peripheral lung tissues of mice and rats[74,137]. Decreased CSE expression and serum H_2S levels were observed in the rat asthma model and in asthmatic patients[74,75,138]. Serum H_2S level is positively correlated with lung function parameters such as forced expiratory volume in 1 second (FEV1) in both adult and pediatric asthma[75,138], and negatively correlated with sputum counts of total cells and the percentage of sputum neutrophils[75]. These results suggest a possible role of endogenous H_2S in the development of asthma. All of these previous studies, however, were based on neutrophil-dominant airway inflammation[74,75] which

is different from the eosinophil-dominant airway inflammation in allergic asthma[136]. Whether endogenous H_2S plays a role in the development of eosinophil-dominant inflammation, as occurs in allergic asthma and atopic dermatitis, remains unknown.

Our previous studies have found that CSE is the main H_2S-producing enzyme in peripheral tissues such as liver, pancreas, and aorta[119,126]. Compared to WT mice, endogenous H_2S levels of CSE-KO mice were decreased by 50% in serum, and by 80% in vascular tissues and pancreases[119,126]. The lower level of endogenous H_2S in CSE-KO mice causes hypertension[119], delays the onset of streptozotocin-induced diabetes[126], promotes proliferation of smooth muscle cells[139], and inhibits vascular endothelial growth factor-induced microvessel formation and wound healing[140]. To date, the exact role of endogenous H_2S in systemic or local inflammation has not been studied by knocking out the *CSE* gene. Given the critical regulatory role of the CSE/H_2S system in various physiological and pathological conditions, we hypothesized that the endogenous CSE/H_2S pathway may play an important role in the pathogenesis of allergic asthma.

In the present study, we induced asthma in CSE-KO mice and WT mice via OVA-treatments. With these models, we examined the alterations of CSE expression and H_2S production in the lung, as well as changes in AHR, airway inflammation, and T_H2 response. In addition, the effects of exogenous H_2S treatments on asthma development were observed in both WT and CSE-KO mice.

3.3 Results

OVA-Challenge Reduces CSE Expression and H_2S Production Rate in the Lung

Age-matched WT and CSE-KO mice (12-16 weeks old) were used to establish allergic asthma models following procedures listed in section 2.3. In CSE-KO mice, CSE proteins were not detectable in the lung (Fig.3 -1 A). Sensitized WT mice displayed a slight, but significant decrease in CSE expression in the lung after OVA-challenge (0.70 ± 0.05 versus 0.52 ± 0.03; $P < 0.05$) (Fig.3 -1 A). The levels of CBS

expression, another H_2S-producing enzyme, were similar in the lungs of WT and CSE-KO control mice. Even after OVA-challenge, CBS expression was not changed in either WT or CSE-KO mice (Fig.3 -1 A). Rate of pulmonary H_2S production in CSE-KO mice was four times less than that of WT mice (0.27 ± 0.04 versus 1.38 ± 0.06 nmol/g per minute; $P < 0.05$) (Fig.3 -1 B). H_2S production rate was reduced by 40% in lungs of WT-OVA mice in comparison with WT-CON mice (0.83 ± 0.10 versus 1.38 ± 0.06 nmol/g per minute; $P < 0.05$) (Fig.3 -1 B). In CSE-KO mice, OVA-challenge had no effect on lung H_2S production (Fig.3 -1 B).

CSE Deficiency Aggravates OVA-Induced Airway Inflammation, and Elevates Cytokine Levels in BALF

No difference was detected in AHR to increased doses of MCh between WT and CSE-KO control mice (Fig.3 -2 A). OVA-challenge induced a significant increase in AHR in both WT and CSE-KO mice with the latter being more severe (Fig.3 -2 A). The resistance of lung (RL) in response to MCh at 12.5 mg/mL and 25 mg/mL was 2- and 1.62- fold higher, respectively, in CSE-KO-OVA mice in comparison to WT-OVA mice (13.13 ± 1.58 versus 6.52 ± 0.56 and 26.25 ± 3.47 versus 16.18 ± 0.98 cmH_2O/mL per second, respectively, both $P < 0.05$) (Fig.3 -2 A).

The number of leukocytes in BALF was not different between WT and CSE-KO control mice. However, OVA-challenge resulted in significantly greater counts of total cells and eosinophils in BALF from CSE-KO mice compared with WT mice (Fig.3 -2 B). The number of eosinophils in CSE-KO-OVA mice was almost threefold higher than that in WT-OVA mice (148.70 ± 16.63 versus 50.66 ± 11.35 × 10^4/mL; $P < 0.05$) (Fig.3 -2 B). Histological examination revealed widespread patchy inflammatory infiltrates around peribronchial and perivascular areas in lungs of WT-OVA and CSE-KO-OVA mice with more serious infiltrates in the latter (Fig.3 -2 C). IL-4, IL-5, IL-13 and eotaxin-1 in BALF were hard to detect or found at very low levels in control mice, and increased significantly after OVA-challenge (Fig.3 -2 D-G). Compared to WT-OVA mice, IL-5 and IL-13 were 3.49-fold and 1.75-fold higher, respectively in CSE-KO-OVA mice (Fig.3 -2 D, E). However, there was no

significant difference in IL-4 levels between the WT-OVA and CSE-KO-OVA groups (Fig.3 -2 F). Among OVA-challenged mice, eotaxin-1 level in BALF was higher in CSE-KO mice than in WT mice (90.25 ± 4.23 versus 41.13 ± 3.45 pg/mL; $P < 0.05$) (Fig.3 -2 G).

NaHS Treatment Alleviates OVA-Induced AHR and Airway Inflammation and Lowers Cytokine Levels in WT Mice

NaHS treatments of WT mice attenuated OVA-induced AHR in response to MCh at 12.5 and 25 mg/mL compared with vehicle treatments (both $P < 0.05$) (Appendix 1A). Total and differential cell counts including eosinophils, macrophages, and lymphocytes were significantly decreased in BALF by NaHS treatments (Appendix 1B). Of note, the number of eosinophils was reduced almost by 50% after NaHS treatments compared to vehicle treatments (70.23 ± 7.41 versus 36.32 ± 8.71 × 10^4/mL; $P < 0.05$) (Appendix 1B). Histological sections of the lung also showed less inflammatory cell infiltration in OVA-challenged WT mice after NaHS treatments (Appendix 1C). In assessment of cytokines in BALF, NaHS treatments significantly attenuated the OVA-induced IL-5, IL-13, and eotaxin-1 compared with vehicle treatments, whereas they had no significant effect on IL-4 (Appendix 1 D-G). As shown in Appendix 1, pretreatment with glybenclamide did not reverse the inhibitory effect of NaHS on the OVA-induced AHR, airway inflammation or cytokine levels in BALF.

NaHS Supplementation Reduces Asthmatic Severity in CSE-KO-OVA Mice

AHR to MCh at 12.5 and 25 mg/mL in KO-OVA mice was decreased (by 41.68% and 32.40% respectively) after NaHS treatment (10.94 ± 1.42 versus 6.38 ± 0.91 and 22.93 ± 2.57 versus 15.16 ± 1.77 cmH_2O/mL per second, respectively; both $P < 0.05$) (Appendix 2A). NaHS applications significantly reduced total and differential cell counts in BALF in comparison with vehicle treatments (Appendix 2B). Significantly, eosinophil counts in CSE-KO-OVA mice were reduced by 51% after NaHS treatment (105.2 ± 9.7 to 51.5 ± 6.7 × 10^4/mL; $P < 0.05$). Decreased inflammatory cell infiltrates in lung tissues were also observed after NaHS treatments in CSE-KO-OVA

mice (Appendix 2C). NaHS treatments significantly decreased the levels of IL-5, IL-13 and eotaxin-1 in BALF compared to vehicle treatments whereas it had no effect on IL-4 (Appendix 2D). Moreover, NaHS treatment decreased IL-13 in CSE-KO-OVA mice to the basal level (Appendix 2D).

3.4 Discussion

CSE is a major H_2S-production enzyme in mouse lungs, although at least one other pulmonary H_2S-producing enzyme (CBS) exists[127,137]. Lung CSE expression in WT mice was significantly reduced after OVA-challenge, accompanied by a significant decrease in H_2S production (Fig. 3 -1 B). The OVA-challenge, however, had no effect on lung CBS expression, even when CSE was deficient (Fig.3 -1 A). Based on these results, it is suggested that the decrease in endogenous H_2S production in WT mice is due to reduced CSE expression after OVA-challenge. T_H2 cytokines, such as IL-4 and IL-13, can down-regulate CSE expression and activity in human airway smooth muscle cells. It is likely that elevated T_H2 cytokines, produced by inflammatory cells which influx to the lung after OVA-challenge, suppressed CSE expression and consequent H_2S production in the lung.

Accumulating evidence has shown altered endogenous H_2S metabolism and a potentially protective role of exogenous H_2S in many conditions of neutrophil-dominant inflammation[131-133]. Herein, we investigated the role of the endogenous CSE/H_2S system in eosinophil-dominant airway inflammation. In CSE-KO mice, CSE deficiency and lower H_2S production in the lung caused profound airway inflammation, elevated levels of T_H2 cytokines, and aggravated AHR after OVA-challenge (Fig.3 -2). Exogenous H_2S supplements to WT-OVA mice or KO-OVA mice, on the other hand, decreased or reversed OVA-induced AHR, reduced inflammatory infiltration and alleviated airway remodeling (Appendix 1, Appendix 2).

T_H2 cytokines orchestrate inflammatory cascades in asthma. For example, IL-4

facilitates naïve T cell differentiation into T_H2 cells; IL-4 and IL-13 induce isotype switching to IgE production in B cells; IL-5 promotes eosinophil maturation and survival[141]. These mature eosinophils were then recruited into the lung by eotaxin[136,142,143]. Deficiency in CSE boosted OVA-challenge-induced IL-5, IL-13 and eotaxin-1 production, which led to increased eosinophil infiltration in the lungs of CSE-KO mice. Exogenous H_2S supplementation significantly decreased cytokine levels of IL-5, IL-13 and eotaxin-1, and reduced, even rescued, OVA-induced eosinophilic airway inflammation in WT-OVA mice and in KO-OVA mice. These results suggest that endogenous and exogenous H_2S protects against the development of OVA-induced allergic airway inflammation by suppressing T_H2-associated cytokine expression and reducing eosinophil recruitment.

The exact molecular mechanism underlying the inhibitory role of H_2S in inflammation is still not well understood. Studies have shown that H_2S is involved in regulation of anti-inflammation through inhibition of NF-κB activation[133,144]. Recently, a direct effect of endogenous H_2S on NF-κB activation has been reported[145]. CSE-generated H_2S directly sulfhydrates the p65 subunit of NF-κB at cysteine-38, and then antagonizes tumor necrosis factor-α–induced cell death[145]. NF-κB also plays an important role in allergic inflammation[146,147]. Thus, it would be interesting to investigate whether H_2S plays its anti-asthmatic role via sulfhydration of NF-κB.

Development and maintenance of AHR in asthma depends on high eosinophil infiltration and T_H2 cytokine production, particularly IL-13 and to a lesser extent IL-4[148-150]. Thus enhanced T_H2 cytokine production might also contribute to the observed higher AHR in KO-OVA mice. Our study and others have shown that H_2S directly relaxes vascular smooth muscle by increasing K_{ATP} channel currents and hyperpolarizing membranes[120,151]. H_2S can also relax nonvascular smooth muscle in a K_{ATP} channel-independent manner[101,102]. Chen *et al*[101] have shown that exogenous NaHS directly relaxed airway smooth muscle pre-contracted with acetylcholine and potassium chloride, an effect that cannot be reversed by glybenclamide. In our study,

glybenclamide did not affect the inhibitory effect of exogenous H_2S on OVA-induced AHR. Taken together, these data imply that H_2S might reduce AHR in asthma models via two different avenues, i.e. relaxing airway smooth muscle in a K_{ATP} channel-independent manner, and inhibiting T_H2 cytokine production.

Our animal asthma model in the current study is suitable for the study of acute inflammatory events in human asthma[152], although it cannot mimic the chronic features of human asthma such as epithelial detachment, increased smooth muscle mass and peribronchial collagen deposit[153]. It would be intriguing to observe the effect of endogenous H_2S on the development of airway remodeling during asthma with CSE-KO mice using an OVA-mouse model recently reported[154].

In summary, CSE is the main H_2S-production enzyme in the mouse lung. CSE-derived endogenous H_2S is a protective factor against the development of asthma via inhibition of T_H2 cytokines and eotaxin-1 secretion. Exogenous H_2S supplementation suppresses asthmatic pathogenesis. These results establish, for the first time, a critical role of the endogenous CSE/H_2S system in the development of allergic asthma, and suggest the potential of CSE/H_2S based novel asthma therapies.

Figure 3-1

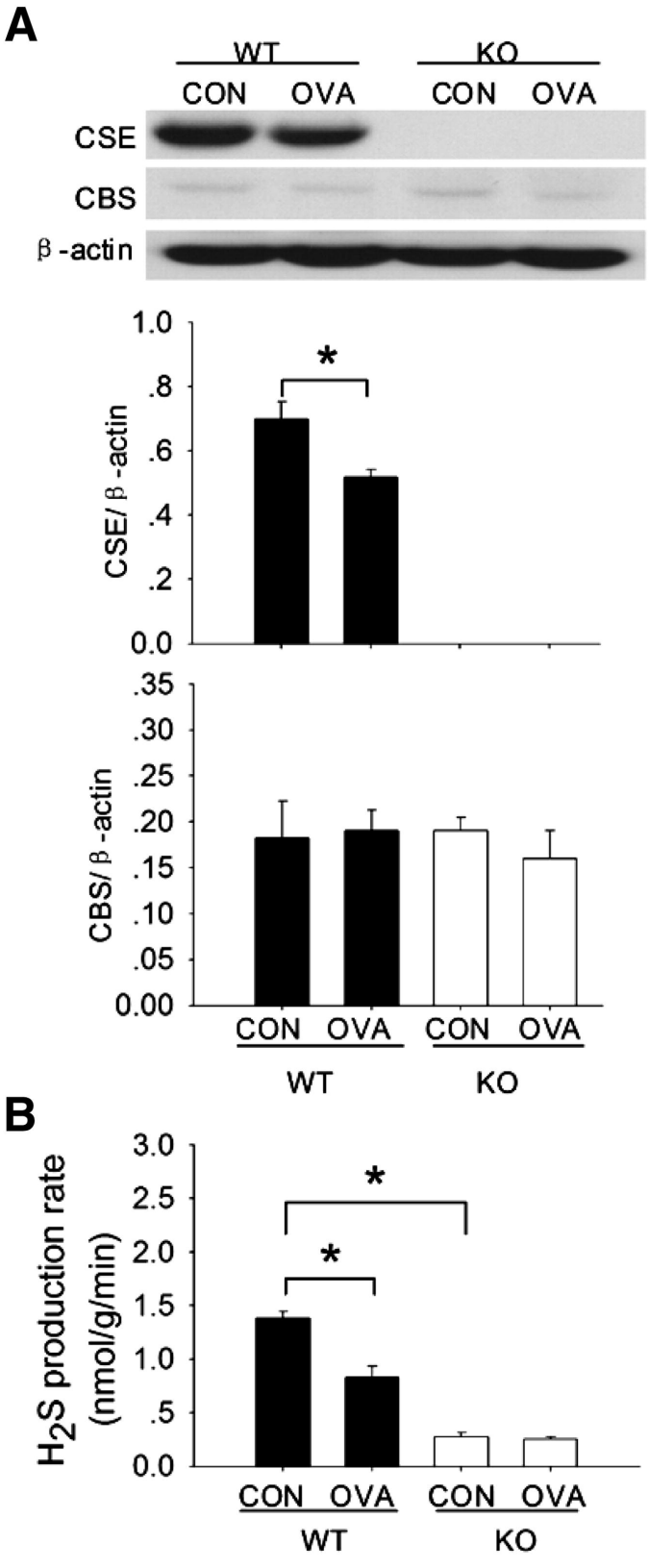

A
WT
KO
CON
OVA
CON
OVA
CSE
CBS
β-actin
CSE/β-actin
1.0
.8
.6
.4
.2
0.0
*
CBS/β-actin
.35
.30
.25
.20
.15
.10
.05
0.00
CON
OVA
CON
OVA
WT
KO
B
H2S production rate
(nmol/g/min)
3.0
2.5
2.0
1.5
1.0
.5
0.0
*
*
CON
OVA
CON
OVA
WT
KO

Fig.3-1 OVA-challenge reduced CSE expression and H_2S production in mouse lungs. OVA-sensitized WT and CSE-KO mice were challenged with OVA (WT-OVA and KO-OVA). Sensitized mice challenged with saline were used as controls (WT-CON and KO-CON). Twenty-four hours after the last OVA-challenge, lung tissues were dissected for Western blot analysis (**A**) and H_2S production detection (**B**). Six to ten mice were used for each group. $*P < 0.05$.

Figure 3-2

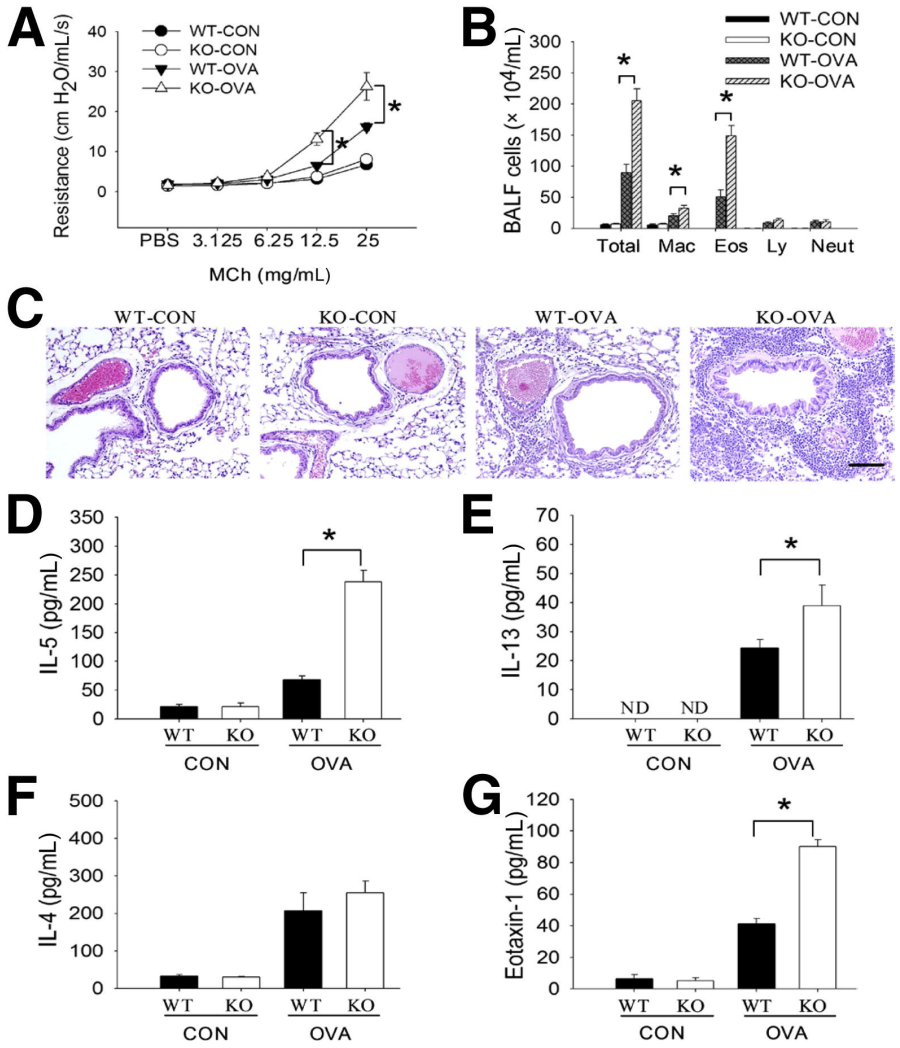
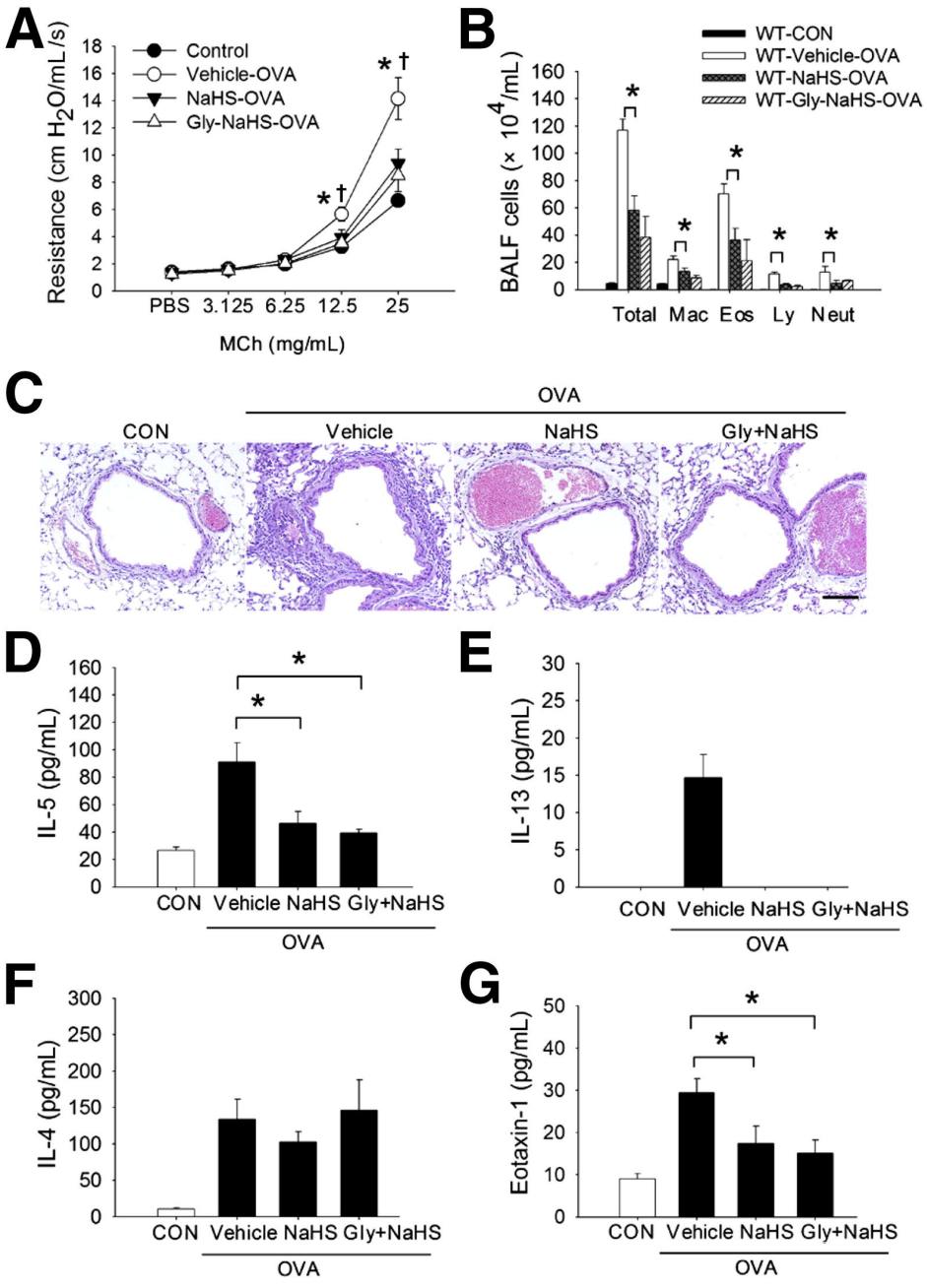
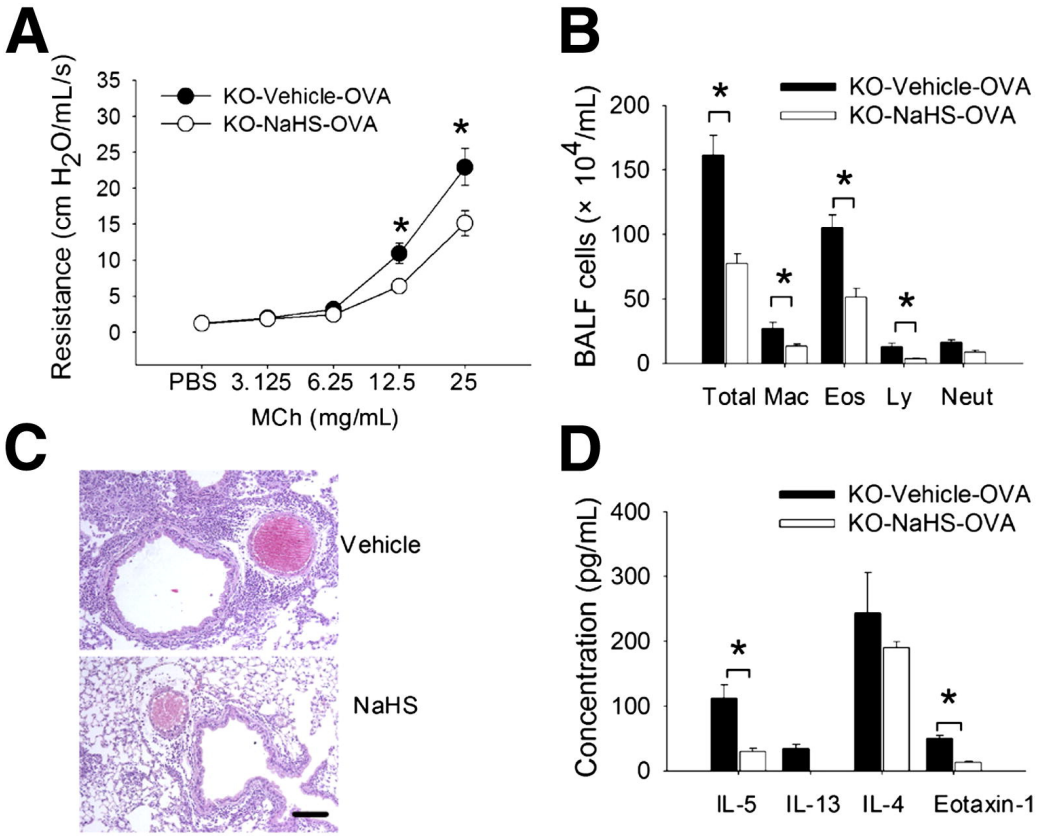


Fig.3-2 CSE gene deficiency and asthmatic damage. A Increased AHR in KO-OVA mice. Twenty-four hours after last OVA-challenge, AHR to inhaled methacholine (MCh) was measured by a resistance and compliance system. **B** Total and differential cell counts in BALF. BALF was recovered and total cell counts were determined after AHR measurement. Cytospins of leukocytes were stained with Wright-Giemsa and differential cell counts were performed for 200 cells. Eos, eosinophils; Ly, lymphocytes; Mac, macrophages; Neut, neutrophils. **C** Representative H&E staining of lung tissues. Scale bar: 100 μm. **D–G** Levels of IL-5, IL-13, IL-4, and eotaxin-1 in BALF were assessed by ELISA. For each group, 8 to 10 mice were used. *$P < 0.05$. ND, not detected.

Appendix 1


A
Resistance (cm H2O/mL/s)
Control
Vehicle-OVA
NaHS-OVA
Gly-NaHS-OVA
PBS 3.125 6.25 12.5 25
MCh (mg/mL)
B
BALF cells (× 10^4/mL)
WT-CON
WT-Vehicle-OVA
WT-NaHS-OVA
WT-Gly-NaHS-OVA
Total Mac Eos Ly Neut
C
OVA
CON
Vehicle
NaHS
Gly+NaHS
D
IL-5 (pg/mL)
CON Vehicle NaHS Gly+NaHS
OVA
E
IL-13 (pg/mL)
CON Vehicle NaHS Gly+NaHS
OVA
F
IL-4 (pg/mL)
CON Vehicle NaHS Gly+NaHS
OVA
G
Eotaxin-1 (pg/mL)
CON Vehicle NaHS Gly+NaHS
OVA

Appendix 1 NaHS treatments reversed OVA-induced AHR, airway inflammation, and cytokine levels in BALF in WT mice. OVA-sensitized WT mice were injected with 40 μmol/kg glibenclamide (Gly) i.p, plus 14 μmol/kg NaHS or vehicle (dimethyl sulfoxide) 30 minutes before and 8 hours after each OVA-challenge. Sensitized mice were challenged with saline as the control. **A** Decrease in OVA-induced AHR after NaHS treatment was not reversed by pretreatment with Gly. Twenty-four hours after the last OVA-challenge, AHR to inhaled methacholine (MCh) was measured by a resistance and compliance system. *$P < 0.05$ compared to WT-NaHS-OVA group; $^{\dagger}P < 0.05$ compared to WT-Gly-NaHS-OVA group. **B** Total and differential cell counts in BALF. After AHR measurement, BALF was recovered and total cell counts were determined. Cytospins of leukocytes were stained with Wright-Giemsa and differential cell counts were performed for 200 cells. Eos, eosinophils; Ly, lymphocytes; Mac, macrophages; Neut, neutrophils. **C** Representative H&E staining of lung tissues from the control group, and OVA-challenged groups treated with vehicle, NaHS alone and Gly plus NaHS. Scale bar = 100 μm. **D–G** Levels of IL-5, IL-13, IL-4, and eotaxin-1 in BALF were assessed by ELISA. For each group, 8 to 10 mice were used. *$P < 0.05$.



Appendix 2 Reversal of asthmatic severity by NaHS supplementation in CSE-KO mice. OVA-sensitized CSE KO mice were given 14 μmol/kg NaHS (ip) or vehicle (ip) 30 minutes before and 8 hours after each OVA-challenge. **A** Decrease in OVA-induced AHR in CSE KO mice after NaHS treatment. Twenty-four hours after last OVA-challenge, AHR to inhaled MCh was measured with a resistance and compliance system. **B** Total and differential cell counts in BALF. After AHR measurement, BALF was recovered and total cell counts were determined. Cytospins of leukocytes were stained with Wright-Giemsa and differential cell counts were performed for 200 cells. Eos, eosinophils; Ly, lymphocytes; Mac, macrophages; Neut, neutrophils. **C** Representative H&E staining of lung tissues. Scale bar = 100 μm. **D** Levels of IL-5, IL-13, IL-4, and eotaxin-1 in BALF. For each group, 8 to 10 mice were used. $*P < 0.05$.

4. Chapter 4

Early Onset of Allergic Asthma Due to Age-dependent Expressional Deficiency of Cystathionine gamma-lyase in Young Mice

4.1 Abstract

Allergic asthma is more prevalent in children than adults, an observation for which the pathogenic mechanisms remain unclear. We found that human umbilical cord blood mononuclear cells had lower levels of cystathionine gamma-lyase (CSE) proteins, the major endogenous H_2S generating enzyme, than peripheral blood mononuclear cell from adult people. In animal models, we observed more severe allergic asthma with higher type-2 immunity in wild-type (WT) young mice than in old mice. This age-dependent propensity of immune reaction and asthma development resulted from lower levels of CSE expression and H_2S production in young mice, and could be reversed by H_2S supplementation. CSE gene knock-out (KO) mice had more severe asthma than WT mice but without the age-dependent asthma propensity. Lower endogenous CSE/H_2S in young WT mice and in CSE-KO mice at all ages promoted the differentiation of splenocytes into type-2 helper T cytokines-generating cells, an effect that could be suppressed by H_2S supplementation. H_2S caused *S*-sulfhydration of GATA3 in spleen cells and decreased GATA3 nuclear translocation, leading to the inhibition of type-2 immunity. In conclusion, H_2S suppresses allergen-induced type-2 immunity and the consequential asthma development. Lower activity of the CSE/H_2S pathway in early life renders a higher incidence of allergic asthma in childhood.

4.2 Introduction

Asthma is characterized by chronic airway inflammation and airway hyper-responsiveness[26,155]. Although asthma can manifest at any age, the initiation of allergic sensitization and first asthmatic symptoms most often occur during the first 1-3 years of life[116,156, 32]. Results from the Third National Health and Nutrition Examination Survey[12] and a community-based birth cohort from Perth[13] revealed that over 50% of asthma cases can be attributed to atopy. High incidence of atopic (allergic) asthma in children is closely associated with a type-2 helper T cell (T_H2) biased immune response after birth[8]. T_H2 response is essential for eliminating parasitic infections and for the development of atopic diseases, including asthma. T_H2 bias is evolved *in utero* to avoid maternal rejection[47]. After birth the neonatal immune system undergoes extensive development and gradually limits T_H2 response. The kinetics of postnatal immune maturation process vary in human populations and an increased or prolonged bias towards T_H2 response increases the risk of allergic asthma development[50]. For example, allergen-specific T_H2 response, measured by cytokine mRNA levels in cord blood mononuclear cells, is greater than in peripheral blood mononuclear cells from one-year old infants. This age-related suppression of T_H2 response is not observed in atopic children[51], suggesting that their immune maturation lags behind non-atopic children.

Early post-natal factors are critical for immune maturation and asthma development. Multiple cohort studies, including the current Canadian Healthy Infant Longitudinal Development (CHILD)[157,158] and the Wayne County Health, Environment, Allergy, and Asthma Longitudinal Study, have showed various early life events (such as breastfeeding, growing up on farms or having pets) could protect against asthma development [57,159]. The observed protective effects of these exposures are believed to be mediated through increasing early gut microbial colonization, which subsequently triggers the maturation of the developing neonatal immune system. This notion is supported by studies carried out on germ-free animals. These animals exhibited persistent T_H2 biased response and were more likely to develop allergic asthma than

animals exposed to normal microbial flora[64]. Colonizing germ-free animals with *Bacteroides fragilis* induced maturation of their developing immune system and down-regulated splenic T_H2 cytokine production[64]. Recent studies[160], using novel high-throughput techniques, highlight the effects of gut bacterial colonization on systemic immune responses. Little is known, however, about how microbiota colonization in the gut suppresses systemic T_H2 biased response in early childhood.

CSE is one of the enzymes that produce H_2S in mammalian tissues. CSE activity, assessed by cystathionine consumption, in cecum, colon, small intestine, kidney, liver, aorta, heart, and brain was lower in 11-12-week germ-free mice than in age-matched non-germ-free mice[106]. Gut microbiota can up-regulate CSE activity/expression and endogenous H_2S production *via* metabolic products in the colon[104]. For example, gut microbiota synthesize pyridoxal 5′-phosphate, the cofactor for CSE activation. CSE expression can also be upregulated by butyrate[105], an end-product of microbial fermentation of plant polysaccharides which cannot be digested by humans[104]. Products, such as IL-10, generated from the interaction between microbiota and the intestinal immune system, also promote CSE expression and H_2S synthesis[106].

Gut microbiota is of low density and phylogenetic diversity after birth[61,62]. Successive colonization with *Gram-positive cocci*, *Enterobacteria*, *Lactobacilli* and *Bifidobacteria* happens in the first few weeks of life in mice and first few years of life in humans. This process can be accelerated by early life events such as breastfeeding, exposure to farms and pet ownership or delayed by other events such as caesarean section and antibiotic usage. Normally, by the age of 3-5 weeks in mice or 3-4 years in humans, gut microbiota becomes comparatively stable with adult-like diversity and population profile, although full development may take several more years in humans[61,63]. Consistent with the observations of germ-free animals and of lower microbiota colonization in human/animal infants, CSE activity and expression in mouse liver are very low after birth and increase gradually until reaching their peaks at 3 weeks of age[118]. CSE activity, reflected by cysteine formation rate, in the brain of

1 day old rats was 3 nmol/h/mg protein, but rose to 13 nmol/h/mg protein (near adult levels) by the second week[161]. In the preliminary study, we found that CSE expression in infant mouse lungs also increased gradually and reached its adult level by 12-16 weeks. Considering the stimulatory effect of microbiota on CSE expression in host organs and the time frame of microbiota colonization after birth, there appears to be a direct correlation between age-related colonization of microbiota and CSE expression in the host.

The involvement of CSE/H_2S in asthma development has been suggested in both human and animal studies. Plasma levels of H_2S were lower in adult asthmatics and inversely related to the severity of asthma[75]. In allergic-asthma animal models (8-week-old rats or 12-week-old mice), H_2S levels in plasma and lung tissues were lower than in non-asthmatic control animals[74]. Exogenous H_2S supplementation reduced asthma features induced by ovalbumin (OVA) challenges, including airway hyper-responsiveness, eosinophil infiltration and elevated T_H2 cytokine levels in bronchoalveolar lavage fluid (BALF). We hypothesize that the endogenous level of CSE/H_2S is key in suppressing the biased T_H2 response in early life and that age-dependent changes in CSE/H_2S levels underlie the high incidence of asthma at a young age.

4.3 Results

Age- and CSE-dependent allergic asthma development

Allergic asthma was induced with OVA in young (3-4 weeks old) and old mice (7-8 months old) (Fig. 4-1). OVA-sensitizations and challenges of young wild-type (WT) mice increased lung resistance by 351.1±85.2% and decreased lung dynamic compliance (Cdyn) by 58.2±6.4% in response to aerosolized methacholine (MCh) at 12.5 mg/ml (Fig. 4-2 A). OVA-induced airway responsiveness was much weaker in old WT mice than in young WT mice with only a 170.4±50.6% increase in lung resistance and 39.0±5.9% decrease in Cdyn, respectively (Fig. 4-2 A). OVA-challenge-induced BALF inflammatory cell influx, especially by eosinophils, was

approximately 2 times more in young WT mice than in old WT mice (Fig. 4-2 B). The IL-4 level in BALF from young WT mice was also higher than in BALF from old WT mice (Fig. 4-3 A). Similar patterns of elevation were seen with IL-5 and eotaxin in young WT mice, although not as pronounced as IL-4 (Fig. 4-3 A). Histological analysis of lung tissues showed more peribronchial and perivascular inflammatory infiltrates in young WT mice than in old WT mice (Fig. 4-3 B and Fig. 4-4). Thus, allergen exposure at a young age induces more severe allergic asthma symptoms than the same exposure at an older age.

OVA-treated young and old CSE-KO mice responded similarly to MCh challenges with airway resistance increasing by 712.6±85.9% and 661.8±92.6% and Cdyn dropping by 74.9±3.5% and 80.3±2.1%, respectively (Fig. 4-2 A). After OVA-challenges young and old CSE-KO mice had comparable inflammatory cell infiltration in their airways and T_H2 cell cytokine levels (IL-4, IL-5 and IL-13) in BALF (Fig. 4-2 B and Fig. 4-3 A). Extensive inflammation was seen in lung tissues from young and old CSE-KO mice (Fig. 4-3 B). Airway hyper-responsiveness and inflammation were more severe in CSE-KO mice than in WT mice of the same age. It is clear that the elimination of CSE expression abolished age-related asthma susceptibility and worsened allergic asthma at all ages.

CSE expression levels and immunoreactions in OVA-sensitized mice

To correlate expression levels of H_2S-generating enzymes with T_H2 cytokine production, we isolated mouse mediastinal lymph cells and splenocytes 5 days after OVA-sensitization and then re-stimulated these cells *in vitro* with OVA for 3 days. A previous study showed that an OVA-specific T cell response appeared in mediastinal lymph nodes and spleens 4 days after systemic OVA immunization (ip)[162]. Lymph cells and splenocytes from old WT mice produced less IL-4, IL-5 and IL-13 after OVA-re-stimulation than those from young WT mice (Fig. 4-5 A & B). No such difference was observed between young and old CSE-KO mice (Fig. 4-5 A & B). The cells from CSE-KO mice generated more T_H2 cytokines than the cells from WT mice of the same age. Splenocytes from young WT mice had much lower abundance of

CSE proteins than did splenocytes from old WT mice (Fig. 4-6 A). Expression of the other two H_2S-generating enzymes, 3-mercaptopyruvate sulfurtransferase (MST) and cystathionine β-synthase (CBS), was also examined. MST protein levels were comparable in splenocytes from young and old WT mice (Fig. 4-6 C). CBS proteins were undetectable in both groups (Fig. 4-6 C). Splenocytes from young WT mice generated less H_2S than did splenocytes from old WT mice (Fig. 4-6 B). The same pattern was observed in human samples. CSE proteins, but not CBS or MST proteins, were detected in human spleen tissue (Fig. 4-6 D). Peripheral blood mononuclear cells (PBMC) from human adults (38-55 years old) had more CSE proteins than umbilical cord blood mononuclear cells (CBMC) (Fig. 4-6 D). Neither PBMC nor CBMC expressed CBS proteins. Low abundance of MST proteins was detected in PBMC (Fig. 4-6 D).

To test whether the observed effects of CSE deficiency were due to endogenous H_2S deprivation, we administered NaHS (14 μmol/kg ip twice daily) to mice beginning two days prior to OVA-sensitization and continuing until two days after sensitization (Fig. 4-1). At the end of this NaHS treatments regime, the mice were sacrificed and mediastinal lymph cells and splenocytes were isolated. NaHS treatment significantly reduced OVA-re-stimulation-induced IL-4, IL-5 and IL-13 production in lymph cells and splenocytes from both young WT mice and CSE-KO mice at all ages, with IL-4 being decreased most dramatically (Fig. 4-7 A & B). In old WT mice, NaHS only slightly, though significantly, decreased IL-4 level in splenocytes but failed to do the same in lymph cells. In the same groups of cells from old WT mice, NaHS increased IL-5 and decreased IL-13 production in splenocytes. However, it decreased both IL-5 and IL-13 production in lymph cells. It is reasoned, therefore, that the type-2 immunity modulating effect of CSE was mediated by H_2S, and that exogenous H_2S treatment was more efficient in suppressing type-2 immunity when endogenous H_2S level was minimal.

In another group of mice, the animals were not sacrificed after NaHS treatments regime as described above. Instead, they were kept for another two weeks during

which time they were challenged with OVA on day 21 to 23 to establish asthma models (Fig. 4-1). NaHS treatments significantly alleviated OVA-challenge-induced airway resistance to MCh by 1.6 times in young WT mice and about 2 times in young and old CSE-KO mice compared with saline-treatments (Fig. 4-8 A). NaHS-treated young WT mice and young/old CSE-KO mice exhibited less airway inflammatory cell infiltration after OVA-challenges compared with saline-treated mice, mainly due to fewer eosinophils and neutrophils in the lung (Fig. 4-8 B). T_H2 cytokines IL-4 and IL-5 in BALF also decreased in NaHS-treated young WT mice and young/old CSE-KO mice (Fig. 4-9 A). Consistent with BALF cell counting, fewer peribronchial and perivascular inflammatory cells were present in lung tissues of NaHS-treated young WT mice and young/old CSE-KO mice (Fig. 4-9 B). Negligible changes in airway responsiveness and inflammatory infiltration were seen in NaHS-treated old WT mice in comparison with saline-treated mice (Fig. 4-8 and Fig. 4-9). These data suggest that when the functionality of CSE/H_2S system is compromised, NaHS treatments during sensitization phase have a protective effect against the development of ensuing allergic asthma.

H_2S-induced inhibition of naïve immune cell differentiation *in vitro*

To explore the mechanisms by which H_2S modulates immune response, splenocytes were cultured *in vitro* under the conditions for T cell differentiation into T_H2 cytokine-generating cells (see section 2.12). The differentiated cells were stimulated for 24 hours with PMA/ionomycin to generate cytokines. Old WT mouse splenocytes produced less IL-4, IL-5 and IL-13 than young WT mouse splenocytes (Fig. 4-10 A). Old CSE-KO mouse splenocytes generated IL-4 and IL-5 in amounts similar to young CSE-KO mouse splenocytes (Fig. 4-10 A). The amounts of IL-4, IL-5 and IL-13 generated by CSE-KO mouse splenocytes were greater than those generated by WT mouse splenocytes (Fig. 4-10 A). NaHS treatments during the differentiation culture suppressed T_H2 cytokine generation in CSE-KO mouse splenocytes, in a concentration-dependent manner (Fig. 4-10 B).

Since some of the cellular protection effects of H_2S are mediated by its anti-oxidant

effect[163,164,165], H_2S might suppress type-2 immunity by inhibiting production of reactive oxygen species (ROS). However, anti-CD3 stimulation-induced ROS production in splenocytes did not vary with endogenous H_2S level (Fig. 4-11).

Activation of signal transducer and activator of transcription factor-6 (STAT6) is critical for antigen-induced T_H2 cytokine production and naïve T cell differentiation[166]. STAT6 can be activated by two key T_H2 type cytokines, IL-4 and IL-13, through the shared subunit IL-4Rα of their cognate receptors[167]. Mouse splenocytes with different endogenous H_2S levels were treated with IL-4 (50 ng/ml) for 1 hour. Splenocytes from WT and CSE-KO (young and old) mice had comparable levels of total STAT6 proteins. IL-4 induced phosphorylation of STAT6 was also comparable among these four groups of mice (Fig. 4-12 A). NaHS treatments did not alter STAT6 phosphorylation in IL-4-treated young CSE-KO mouse splenocytes (Fig. 4-12 B). Therefore, the CSE/H_2S-induced inhibition of type-2 immune response is not mediated by STAT6. Instead, GATA3 surfaced as the main molecular target of H_2S. GATA3 is the final mediator of many signal pathways in regulating innate and adaptive type-2 immunity[168,169]. It selectively induces T_H2 cytokine production and inhibits T_H1 differentiation[170]. We assessed nuclear GATA3 expression in splenocytes cultured under the conditions favoring T cell differentiation into T_H2 cytokine-generating cells. Nuclear GATA3 protein levels were much higher in differentiated splenocytes from young WT mice than in those from old WT mice. Differentiated splenocytes from young and old CSE-KO mice expressed similar levels of nuclear GATA3 proteins, levels higher than those from WT mouse splenocytes (Fig. 4-12 D). NaHS treatments during differentiating culture decreased nuclear GATA3 expression in young CSE-KO mouse splenocytes in a concentration-dependent manner (Fig. 4-12 E). To minimize potential interference of endogenous H_2S on NaHS effects, only CSE-KO splenocytes were used in the experiments described in Fig. 4-10 B, Fig. 4-12 B and Fig. 4-12 D.

H_2S-induced GATA3 *S*-sulfhydration

One of the molecular mechanisms for H_2S-induced post-translational modification of

proteins is *S*-sulfhydration where cysteine-SH groups are converted to –SSH[122]. *S*-sulfhydrated GATA3 protein levels were significantly greater in spleens from old WT mice than in those from young WT mice, whereas the sulfhydrated GATA3 levels in young/old CSE-KO mouse spleens were much lower than in WT spleens (Fig. 4-13 A). In HEK-293 cells with heterologous overexpression of CSE, GATA3 *S*-sulfhydration was remarkably decreased by CSE inhibitor dl-propargylglycine (PPG, 1 mM) and increased by NaHS (30 μM) treatments (Fig. 4-13 B). Site-directed mutagenesis revealed that GATA3 was *S*-sulfhydrated at cysteine 84, 182, and 248 (Fig. 4-13 C). Double mutation of cyteine-84/182 (GATA3-C84/182) or cysteine-84/248 (GATA3-C84/248) decreased endogenous H_2S as well as NaHS induced *S*-sulfhydration of GATA3 proteins (Fig. 4-14 A). The ratio of nucleus/cytosol GATA3 level was higher with mutated GATA3 than with wild-type GATA3 (Fig. 4-14 B), suggesting that *S*-sulfhydration of GATA3 decreases its nuclear translocation.

We also tested the effect of CSE expression on T_H1 cell differentiation. Splenocytes were cultured under T_H1 differentiation condition (plate-bound anti-mouse CD3ε, anti-mouse CD28, mouse IL-12, mouse IL-2, and anti-mouse IL-4) and then re-stimulated with PMA/ionomycin for 72 hours to generate IFN-γ. Splenocytes from old WT mice generated more IFN-γ than those from young WT mice (Fig. 4-15 A). CSE-KO mouse splenocytes produced less IFN-γ than WT mouse splenocytes (Fig. 4-15 A). On the other hand, NaHS increased IFN-γ production only at the highest concentration used in this study (30 μM) (Fig. 4-15 B).

T_H1 cytokine IL-12 and T-box protein (T-bet) are essential for the initiation and maintenance of the T_H1 response. By binding to IL-12R, IL-12 activates T_H1-specific transcription factor STAT4, leading to more IFN-γ production and T-bet expression. T-bet controls T_H1 differentiation by directly inducing IFN-γ and IL-12Rβ2 chain gene transcription as well as repressing T_H2 lineage commitment[171]. CSE deficiency led to lower nuclear levels of T-bet in differentiated splenocytes from young WT and young/old CSE-KO mice (Fig. 4-15 E). NaHS (30 μM) treatment, again, increased nuclear T-bet levels (Fig. 4-15 F). IL-12-induced STAT4 phosphorylation[172], which

initiates the differentiation of naïve T cells into T_H1 cells, was not affected by H_2S (Fig. 4-15 C & D). Our observation is consistent with an earlier report[173], in which blocking CSE activity by dl-propargylglycine delayed heart allograft rejection and abrogated type IV hypersensitivity by inhibiting T_H1 type factors T-bet, IL-12 and IFN-γ.

4.4 Discussion

Immune immaturity in early childhood favors type-2 dominant effector and memory responses to harmless antigens. This renders children more susceptible to developing allergic asthma[174,175]. Our study found that CSE expression in lymph cells played a key role in suppressing T_H2 response; its deficiency contributed to greater type-2 immunity and consequent asthma development in early life. CSE expression levels in splenocytes from young mice and neonatal human CBMC are significantly lower than those in splenocytes from old mice and adult human PBMC. Antigen immunization primes immune cells with low CSE for preferential T_H2 cytokines expression: OVA-sensitization of young WT mice resulted in more T_H2 cytokine-generating cells in peripheral lymph tissues, including mediastinal lymph nodes and spleens, than in old WT mice. This result is consistent with clinical observations that link early life sensitization with augmented T_H2 cytokine responses and adverse asthma outcome[45]. Such age-dependent differences were not observed in CSE-KO mice in which CSE expression is eliminated. OVA-sensitization induced comparable T_H2 responses in young and old CSE-KO mice. The elevated T_H2 response in peripheral lymph tissues evoked more dramatic inflammation and asthmatic symptoms in OVA-immunized WT young mice when they were re-exposed to aerosol OVA-challenges. Asthma development in young and old CSE-KO mice, on the other hand, was comparable. These data suggest that the age-related changes in CSE expression in lymph systems during immune sensitization controlled the OVA-induced immune response and consequent asthma development.

The immune-modulating effect of CSE is mediated mainly through H_2S, an important

gasotransmitter. H_2S can up-regulate CD11b and G protein-coupled receptor kinase 2 in neutrophils and improve their migration and survival in sepsis induced by cecal ligation and puncture[80]. Nanomolar concentrations of H_2S potentiate T lymphocyte activation *in vitro*. Our current study showed that H_2S was also responsible for the observed protective effect of CSE against asthma development[79]. Administering NaHS during the sensitization phase significantly alleviated OVA-induced T_H2 priming in peripheral lymph tissues and prevented subsequent asthma development.

T_H2 cytokines play critical roles in the development of allergic asthma [176]. IL-4, and to a lesser extent IL-13, promote IgE production, which subsequently binds to its high-affinity receptors on basophils and mast cells. These effector cells release cytokines, chemokines, histamine, heparin, serotonin and proteases, resulting in airway smooth muscle constriction, inflammatory cell infiltration, etc. IL-5 is critical for the maturation, differentiation and migration of eosinophils. IL-13 stimulates mucus production by goblet cells and airway hyper-responsiveness. After T_H2 polarizing culture, splenocytes from old WT mice secreted much lower levels of T_H2 cytokines than splenocytes from young WT mice. Consistent with our results of *in vivo* OVA-sensitization (Fig. 4-4 A&B), CSE-KO splenocytes showed no age-dependent differences in their T_H2 cytokine-generating abilities when polarized *in vitro*. NaHS treatments, in a concentration-dependent manner, suppressed T_H2 cytokine generation in young CSE-KO mouse splenocytes cultured under T_H2 polarization conditions. Clearly, the levels of CSE proteins and H_2S production in splenocytes decisively affect their differentiation toward T_H2-generating cells.

The genes of T_H2 cytokines are clustered on human chromosome 5 and mouse chromosome 11[177]. Multiple signal pathways modulate their expression. IL-4-induced STAT6 phosphorylation is a critical early signal in inducing T_H2 cytokine expressions[178]. By binding to type-I IL-4R, IL-4 activates janus kinase 1 and 3, and subsequently phosphorylates STAT6. The phosphorylated STAT6 translocates into nucleus and binds to its targeted DNA sequences such as the promoter of T_H2 master regulator - GATA3. CSE/H_2S-induced inhibition of type-2 immunity is not mediated

by STAT6 activation. Instead, our data showed that H_2S inhibited asthma development by *S*-sulfhydrating GATA3 so that its nuclear translocations were lessened (Fig. 4-14 C). GATA3 is expressed in naïve $CD4^+$ T cells and is upregulated dramatically during T_H2 differentiation *via* both IL-4-STAT6-dependent and -independent signaling pathways[179]. GATA3 regulates chromatin modification at the T_H2 cytokine locus by binding to regulatory elements such as conserved non-coding sequence (CNS)-1, hypersensitive site V (HSV)/CNS2, intronic enhancer (IE)/HSII and conserved GATA3 response element (CGRE). GATA3 also binds directly to *Il-5* and *Il-13* promoters. GATA3-deficient $CD4^+$ T cells fail to differentiate into T_H2 cells even when the cells are cultured under T_H2-skewing conditions[180]. Overexpression of GATA3 in developing T_H1 cells, on the other hand, can lead to T_H2 cytokine gene expressions.

There are 12 cysteine residues in human and mouse GATA3 (Fig. 4-16). They are conserved between humans and mice. Eight out of 12 cysteine residues locate in the zinc finger region which cannot normally be modulated (Fig. 4-16, green frames). Among the remaining 4 cysteine residues (Fig. 4-16, red frames), H_2S can *S*-sulfhydrate three cysteine residues (C84, C182 and C248) (Fig. 4-13 C).

GATA3 contains nuclear localization sequence (NLS) which facilitates its nuclear translocation by binding to nuclear import protein importin-a[181]. Malik *et al.* found that *S*-Nitrosylation of chloride intracellular channel protein (CLIC4) enhanced its association with importin-a[182]. CD spectra analysis and trypsinolysis of the modified protein suggested this enhancement was likely due to structure change of CLIC4 protein caused by *S*-nitrosylation[182]. C248 of GATA3 locates at the very beginning of NLS (Fig. 4-16, yellow frames). Thus, it is very possible that *S*-sulfhydration of C84, C182 and C248 alters the structure of GATA3 and the affinity of the importin-a–NLS interaction. Double mutation of cyteine-84/182 or cysteine-84/248 decreased GATA3 *S*-sulfhydration level and increased its nuclear expression. Considering the important role of GATA3 in the initiation and maintenance of type-2 response, our results

suggest a novel and critical mechanism for the protective effect of H_2S against asthma.

Another feature of the immature immune system in childhood is the absence of strong protective T_H1 polarizing signals. T_H1 cells produce IFNγ and are essential to repel intracellular pathogens. Defective T_H1 response is associated with more severe virus infections and atopy [183]. T_H1 cells from young mice and infants have limited IL-12 and IFN-γ-releasing capacity[184]. The Protection against Allergy—Study in Rural Environments (PASTURE) showed high allergen-specific IgE concentrations in cord blood related to decreased IFN-γ levels[53]. Other epidemiologic studies also suggest an inverse association between IFN-γ levels and atopy[185]; a positive correlation of total IgE levels with IL-4, IL-5 and IL-13 levels[186]. T_H1 cytokines and T-bet inhibit T_H2 responses by suppressing the expressions of GATA3 and T_H2 cytokine genes. Thus, upregulating T_H1 responses in early life can restore the T_H1/T_H2 balance and promote tolerance to aeroallergens. Our study found that upregulated CSE/H_2S in old WT mice favors T_H1 differentiation by promoting nuclear T-bet expression. Although less robust than its effects on suppressing T_H2 cytokines production, exogenous H_2S facilitated IFN-γ production. Limited intestine microbiota diversity is associated with weak T_H1 response during the first 2 years of life [61], an effect that might be mediated by the low expression of CSE/H_2S at a young age.

Susceptibility to asthma is also associated with defects in the airways. Airway smooth muscle cells (ASMCs) are the final effecters in controlling airway contraction, and their hyper-responsiveness in early life increase the risk of asthma in childhood[97,98]. In addition to its contractile properties, ASMCs secrete a wide variety of inflammatory mediators during asthma progression. These mediators activate and recruit T cells, mast cells, neutrophils, and eosinophils[187]. Both the number and size of ASMCs are increased in children with asthma[188]. We confirmed CSE expression in human lung samples (Fig. 4-17 A) and found that CSE expression in WT mouse lung increased with age (Fig. 4-17 B). Our previous studies showed that CSE was

expressed in mouse ASMCs and vascular smooth muscle in mouse lungs[74,137]. In the current study we found that airway CSE expression increased in an age-dependent manner, an observation not apparent with CBS or MST (Fig. 4-18). In the absence of allergen exposure, airway responsiveness to MCh challenge (12.5 mg/ml) of young WT mice and young/old CSE-KO mice, was more than two times higher than that of old WT mice (Fig. 4-19). H_2S is known to relax ASMCs by opening the K_{ATP} channel[189] or inhibiting Ca^{2+} release through inositol-1,4,5-trisphosphate (InsP3) receptors[102]. Therefore, low abundance of CSE in young mice might alter intrinsic properties of ASMCs and lead to airway hyper-responsiveness because of decreased endogenous H_2S production. H_2S also affects the immunomodulatory function of ASMCs by inhibiting their release of pro-inflammatory mediators such as IL-8[73] or upregulating anti-inflammatory mediators such as prostaglandin E[190]. Sufficient CSE/H_2S in ASMCs likely allows old WT mice to exert a 'braking' effect on local inflammation and ASMC constriction during allergen exposures. These local effects of H_2S in the lung explain the therapeutic effect of NaHS treatment during the OVA-challenge phase as shown in previous studies[74,191]. NaHS treatment in the OVA-challenge phase decreased IL-5 and IL-13 levels in BALF of both WT and CSE-KO mice[191]. This effect is more likely due to inhibited migration of leukocytes to the lung rather than to inhibited T_H2 cytokine generation in effector lymphocytes; NaHS treatment suppressed T_H2 cytokines generation from established effector cells only at 50 μM or higher concentrations[68].

During OVA-sensitization, primary T_H2 response is initiated; naïve T cells differentiate into T_H2 cells. In the OVA-challenge phase, a secondary T_H2 response occurs; differentiated memory T_H2 cells proliferate and migrate to the lung and airways where asthmatic reactions develop. Thus H_2S acts mainly on lymphocyte differentiation in the OVA-sensitization phase whereas in the OVA-challenge phase, altered H_2S metabolism targets the airways. At these different phases, independently and synergistically, an altered CSE/H_2S pathway contributes to allergic asthma development.

In conclusion, endogenous H_2S production in the lung and peripheral lymph tissues suppresses allergen-induced type-2 immunity and consequent asthma development. Lack of endogenous H_2S due to lower CSE abundance in early development may explain the higher incidence of allergic asthma in children. This discovery sheds light on potential H_2S-based prevention and treatment strategies for asthma.

Figure 4-1

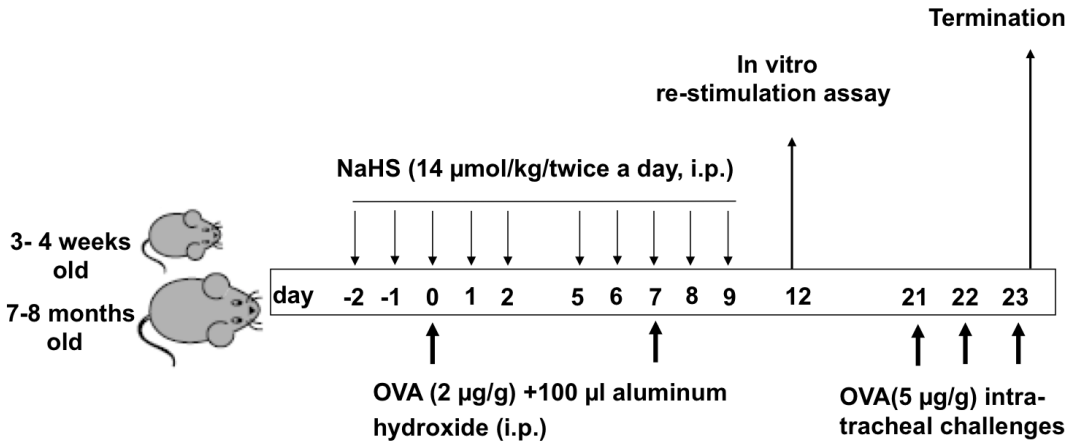


Fig. 4-1 Experimental protocol for establishing OVA-induced mouse allergic asthma. AHR, airway hyper-responsiveness; ip, intra-peritoneal injection. μg/g, μg per gram body weight.

Figure 4-2

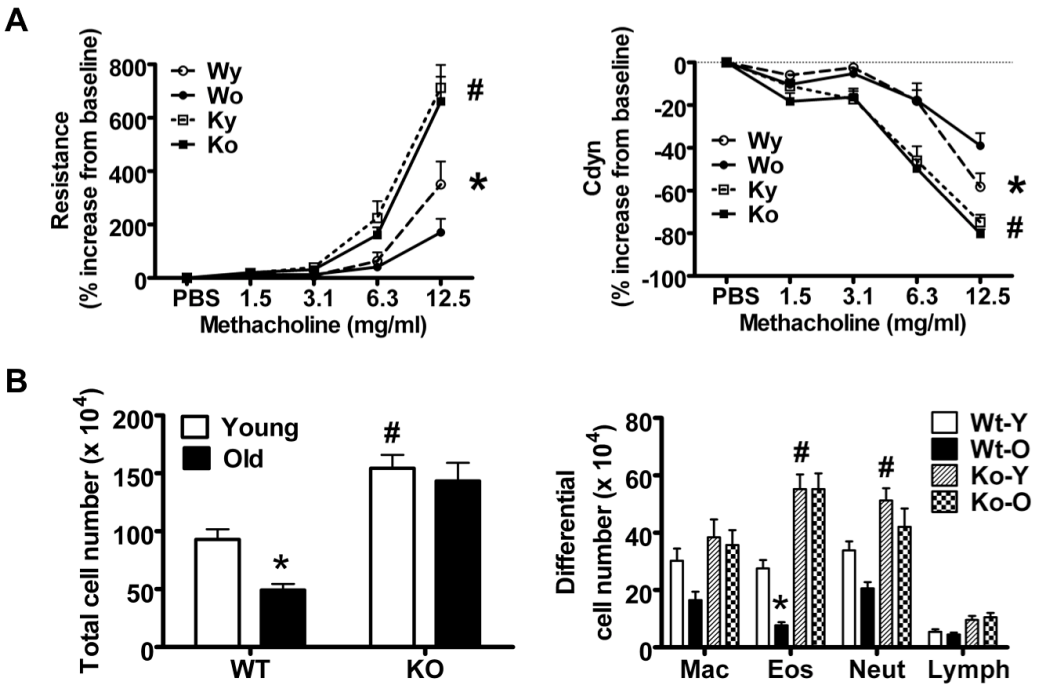


Fig. 4-2 CSE deficiency increased airway hyper-responsiveness and airway inflammatory infiltrates in OVA-induced mouse allergic asthma model. A Changes in mouse airway responsiveness, measured as airway resistance and compliance (Cdyn) with increasing doses of methacholine. WT-y: wide-type young mice; WT-o: wide-type old mice; KO-y: CSE-knockout (KO) young group; KO-o: CSE-KO old group. **B** Changes in the total cell numbers, macrophages (Mac), eosinophils (Eos), neutrophils (Neut) and lymphocytes (Lymph) in bronchoalveolar lavage fluid (BALF). The results in WT-y, WT-o, KO-y and KO-o were generated from 12, 10, 15 and 12 animals, respectively. Statistical significance was determined with two-way ANOVA. * $P< 0.05$ for WT-young compared with WT-old mice; # $P <0.05$ for CSE-KO mice compared with WT mice.

Figure 4-3

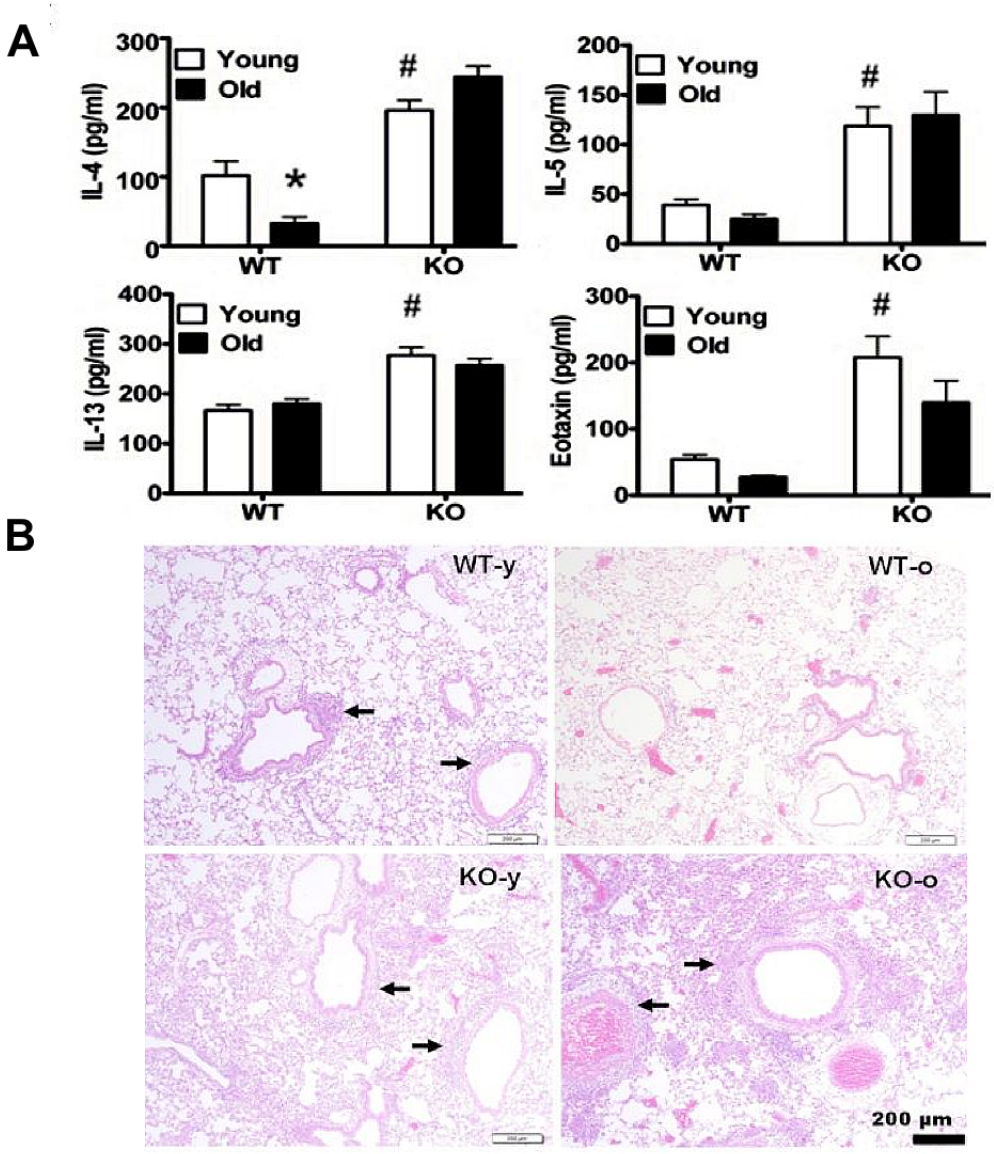


Fig. 4-3 CSE deficiency increased type-2 cytokines in BALF and severity of asthma in OVA-induced mouse allergic asthma model. A Changes in different cytokine levels in BALF. **B** Representative H&E-stained mouse lung tissues. Scale bars, 200 μm. The results in WT-y, WT-o, KO-y and KO-o were generated from 12, 10, 15 and 12 animals, respectively. Statistical significance was determined with two-way ANOVA. * $P< 0.05$ for WT-young compared with WT-old mice; # $P <0.05$ for CSE-KO mice compared with WT mice.

Figure 4-4

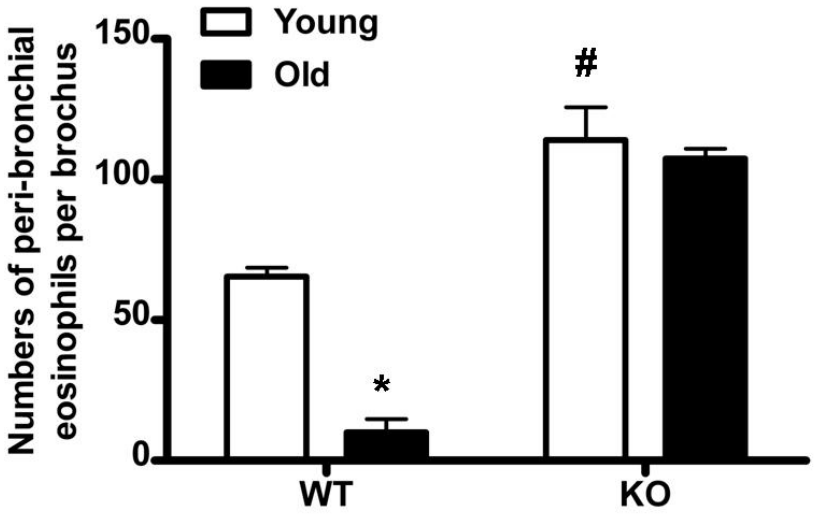


Fig. 4-4 Increased numbers of peribronchial eosinophils in OVA-treated mice. Total eosinophils from 7 different bronchi in each H&E-stained mouse lung slide were counted. Slides from 3-4 different mice were counted for each group. * $P<0.05$ for WT-young mice compared with WT-old mice; # $P<0.05$ for CSE-KO mice compared with WT mice.

Figure 4-5

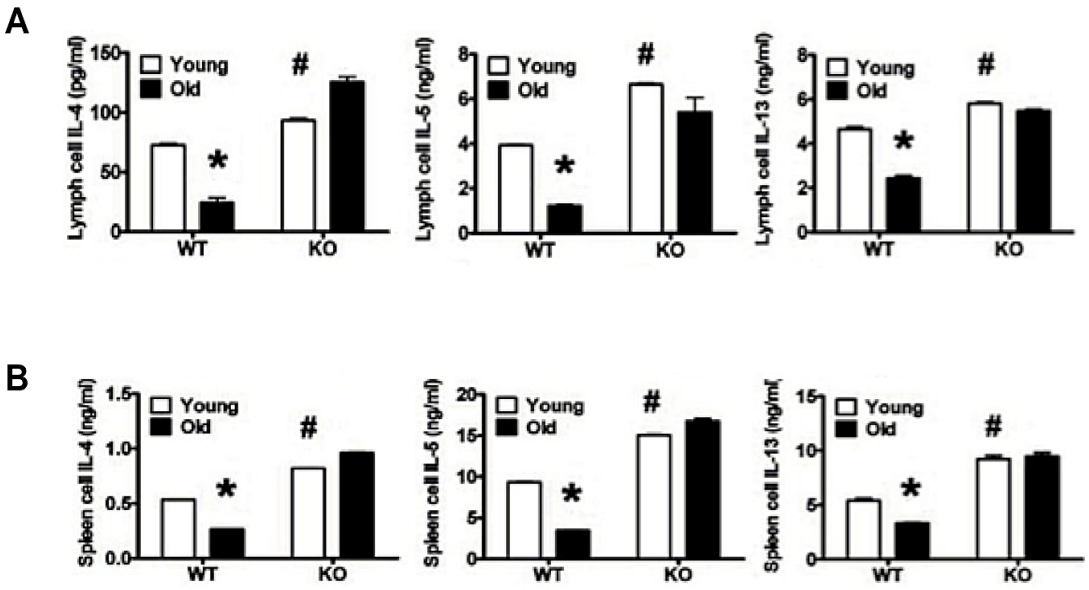


Fig. 4-5 CSE/H_2S suppressed OVA-sensitization-induced differentiation of mediastinal lymph cells and splenocytes. **A-B** Changes in cytokine levels in the culture media of mediastinal lymph cells and splenocytes (ELISA). Mediastinal lymph nodes and spleens were collected 5 days after OVA-sensitization from 6 mice per group. Isolated mediastinal lymph cells (**A**) and splenocytes (**B**) were then re-stimulated in vitro with OVA for 3 days. Differences among WT-young, WT-old, KO-young and KO-old groups were determined by two-way ANOVA test. *$P < 0.05$ for WT-young mice compared with WT-old mice; #P <0.05 for CSE-KO mice compared with WT mice.

Figure 4-6

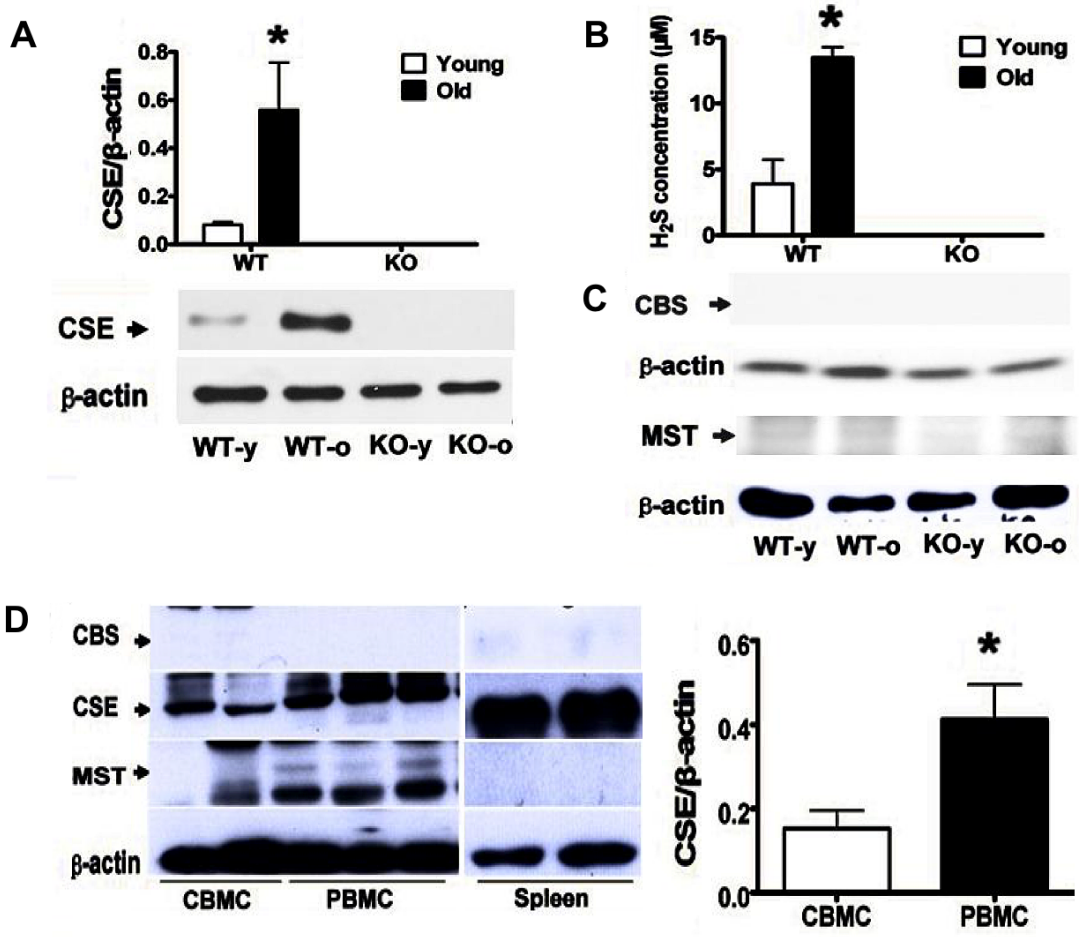


Fig. 4-6 The expression of H_2S generating enzymes in mouse and human samples. **A** CSE protein levels in splenocytes (*n*=4). **B** H_2S generated by cultured splenocytes (*n*=4). **C** CBS and MST protein levels in splenocytes. **D** CBS, CSE and MST protein levels in human cord blood mononuclear cells (CBMC, *n*=5), human peripheral blood mononuclear cells (PBMC, *n*=6) and a human spleen sample (*n*=1). **P* < 0.05.

Figure 4-7

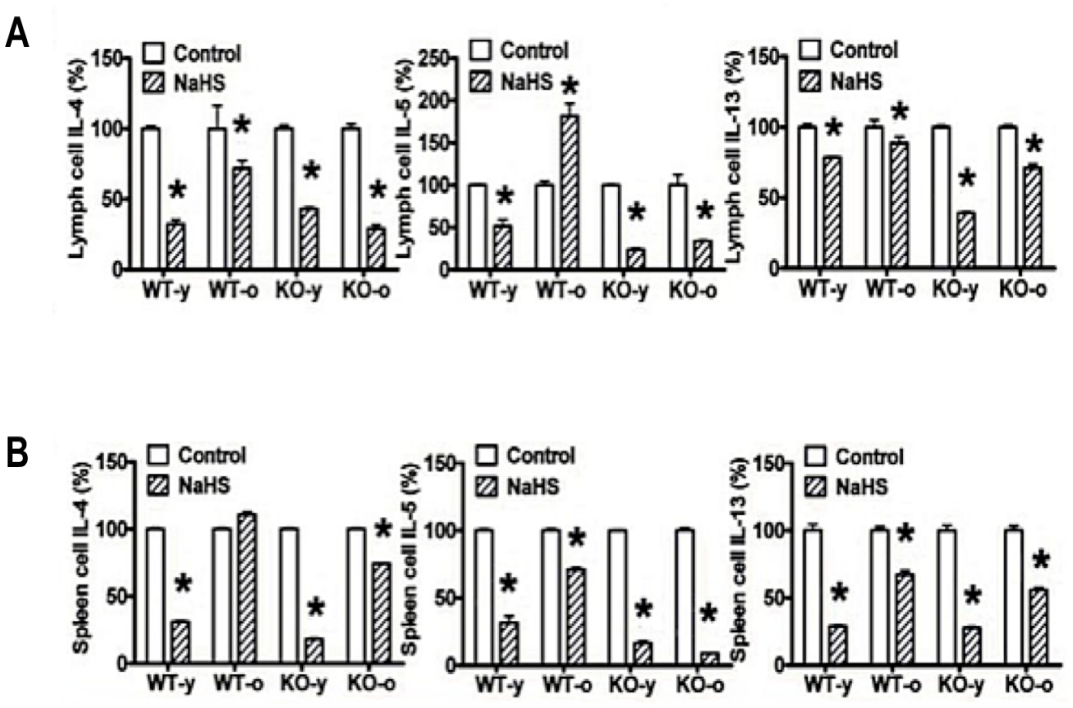


Fig. 4-7 H_2S supplementation suppressed OVA-sensitization-induced differentiation of mediastinal lymph cells and splenocytes. A-B Relative levels of cytokines in the culture medium of mouse mediastinal lymph cells **(A)** and mouse spleen cells **(B)**, measured with ELISA. Mean cytokine levels of control groups are set as 100%. Comparison between control group and NaHS-treatment group was made using unpaired *t* tests. **P* < 0.05.

Figure 4-8

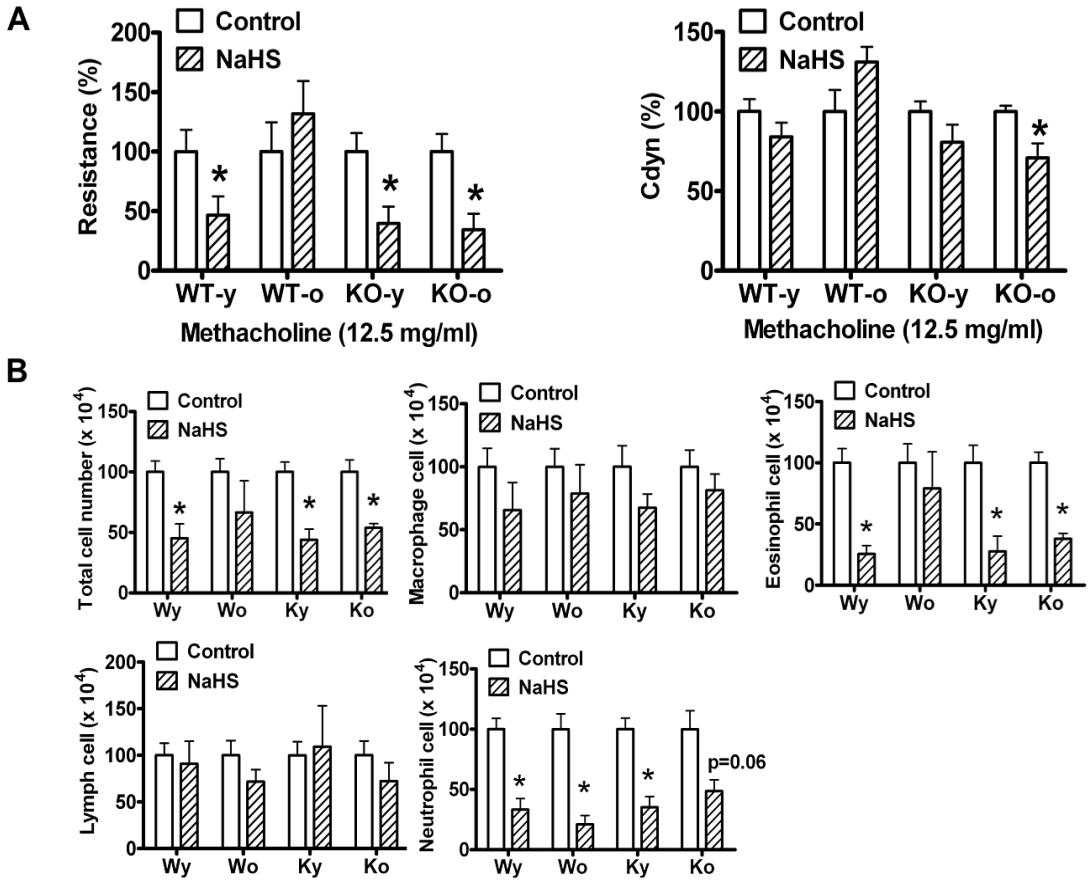


Fig. 4-8 NaHS treatment significantly reduced OVA-induced airway hyper-responsiveness and inflammatory infiltrates in CSE-KO mice and WT young mice. A Relative airway resistance and Cdyn of mice with or without NaHS treatment. Mean resistance and mean Cdyn values of respective control groups are set 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%. Comparison between control and NaHS-treatment group was made using unpaired t-test. *$P < 0.05$.

Figure 4-9

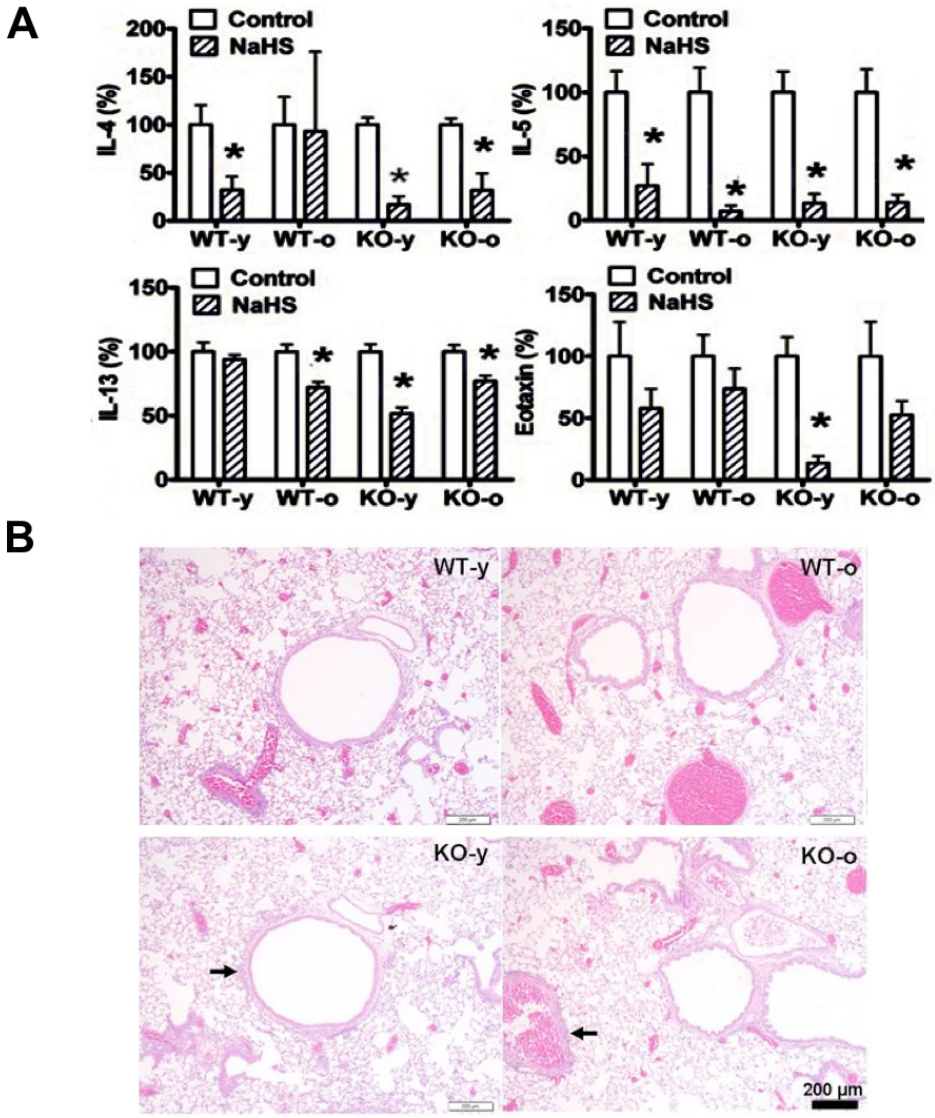


Fig. 4-9 NaHS treatment significantly reduced OVA-induced type-2 cytokine production and alleviated asthma severity in CSE-KO mice and WT young mice. Relative cytokine levels in BALF from the mice with or without NaHS treatment. Mean cytokine levels of respective control groups are set as 100%. **D** Representative H&E-stained lung tissues of NaHS-treated mice. Scale bars, 200 μm. The results in each group were collected from 6 animals. Comparison among control and NaHS-treatment group was made using unpaired t-test. **P* <0.05 for NaHS-treatment group compared with control group.

Figure 4-10

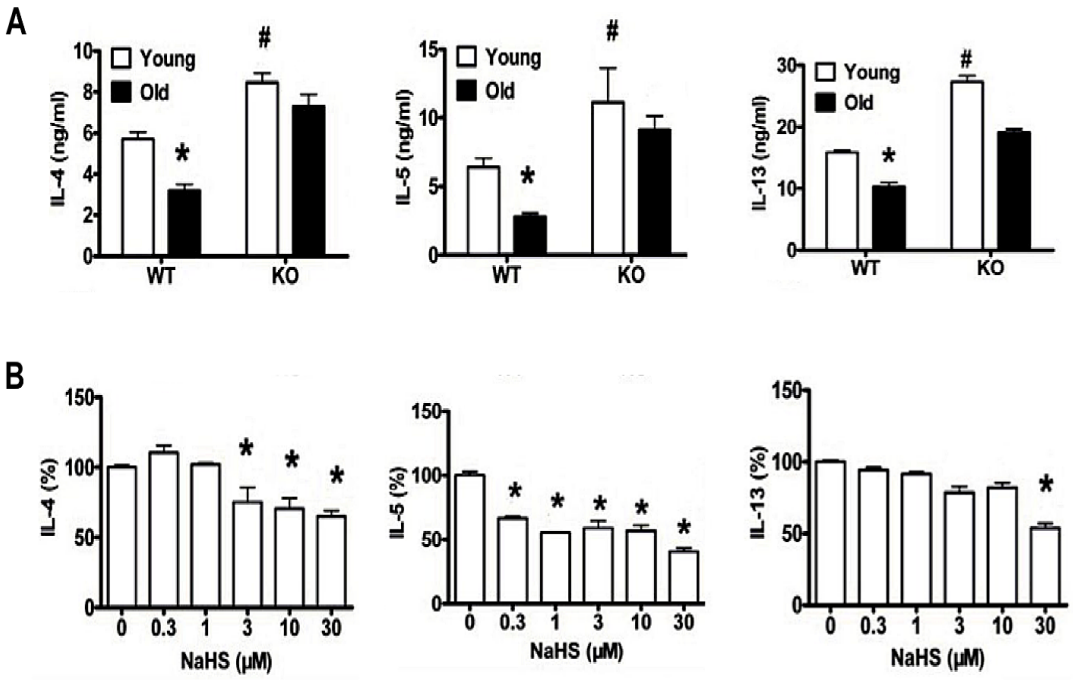


Fig. 4-10 Endogenous and exogenous H_2S reduced the differentiation of splenocytes into T_H2 cytokine-generating cells *in vitro*. A-B IL-4, IL-5 and IL-13 levels in the culture medium of splenocytes cultured in **(A)** the absence or **(B)** the presence of NaHS in various concentrations (see section 2.12). IL-4, IL-5 and IL-13 levels in the culture medium of splenocytes. Mean cytokine levels of NaHS-0 μM groups in (**B**) are set as 100%. Only CSE-KO-young splenocytes were used in (**B**) to minimize the potential interference of endogenous H_2S on NaHS effects. Differences among WT-young, WT-old, CSE-KO-young and CSE-KO-old groups were determined with two-way ANOVA in (**A**). Comparisons among different NaHS-treatment conditions in (**B**) were made using one-way ANOVA (followed by Dunnett post test). **P* < 0.05 for WT-young mice compared with WT-old mice and for NaHS-treatment group compared with NaHS-0 μM group; # *P* <0.05 for CSE-KO mice compared with WT mice.

Figure 4-11

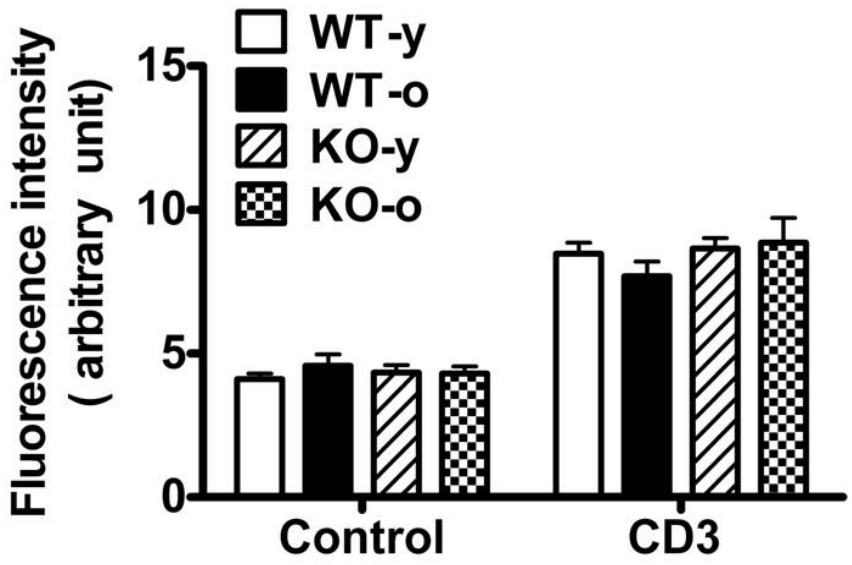


Fig. 4-11 Levels of reactive oxygen species (ROS) in spleen cells with or without anti-mouse CD3ε treatment. Splenocytes isolated from WT-young, WT-old, CSE-KO-young, and CSE-KO-old mice were cultured for 24 hours in the absence or presence of plate-bound anti-mouse CD3ε. ROS generated in splenocytes were measured using Abcam's DCFDA - Cellular Reactive Oxygen Species Detection Assay kit (n=4 for each group).

Figure 4-12

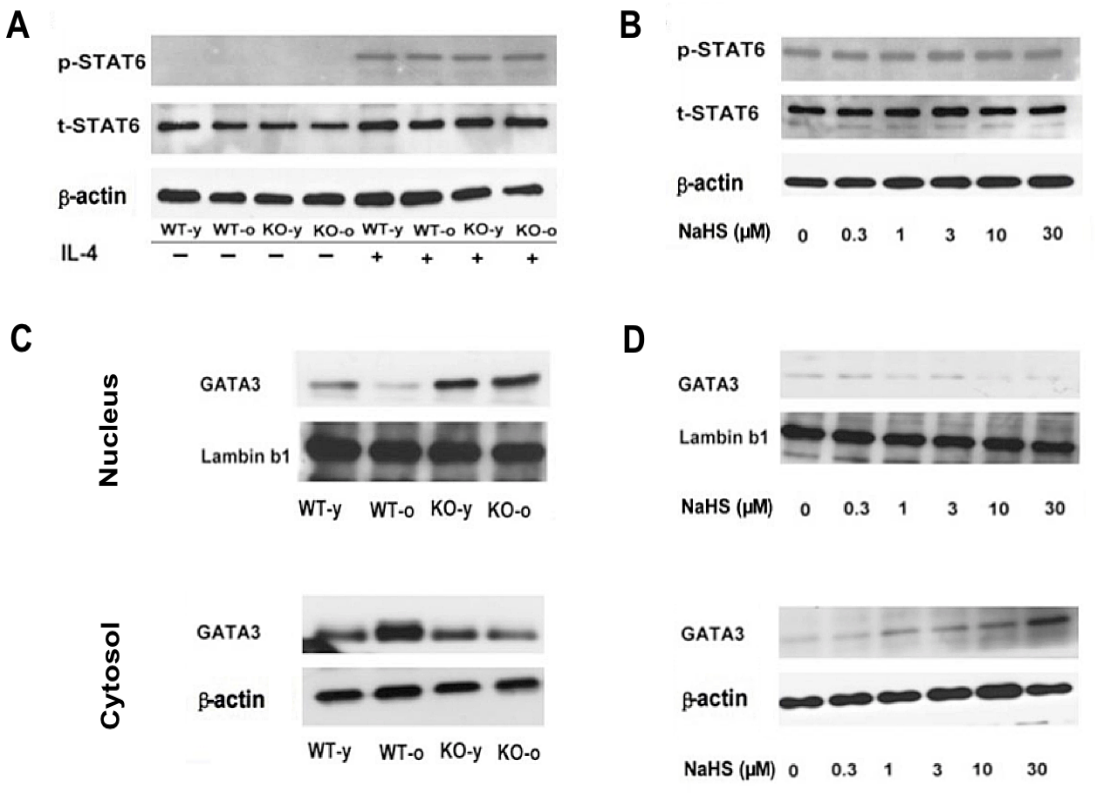


Fig. 4-12 The effect of CSE/H_2S system on STAT-6 and GATA3 pathways. A and **B** IL-4-treatment-induced STAT6 phosphorylation in splenocytes (n=3 for each group). **C** and **D** Nuclear and cytosolic GATA3 expression in differentiated splenocytes (n=4 for each group). Only CSE-KO-young splenocytes were used in (**B** and **D**) to minimize the potential interference of endogenous H_2S on NaHS effects.

Figure 4-13

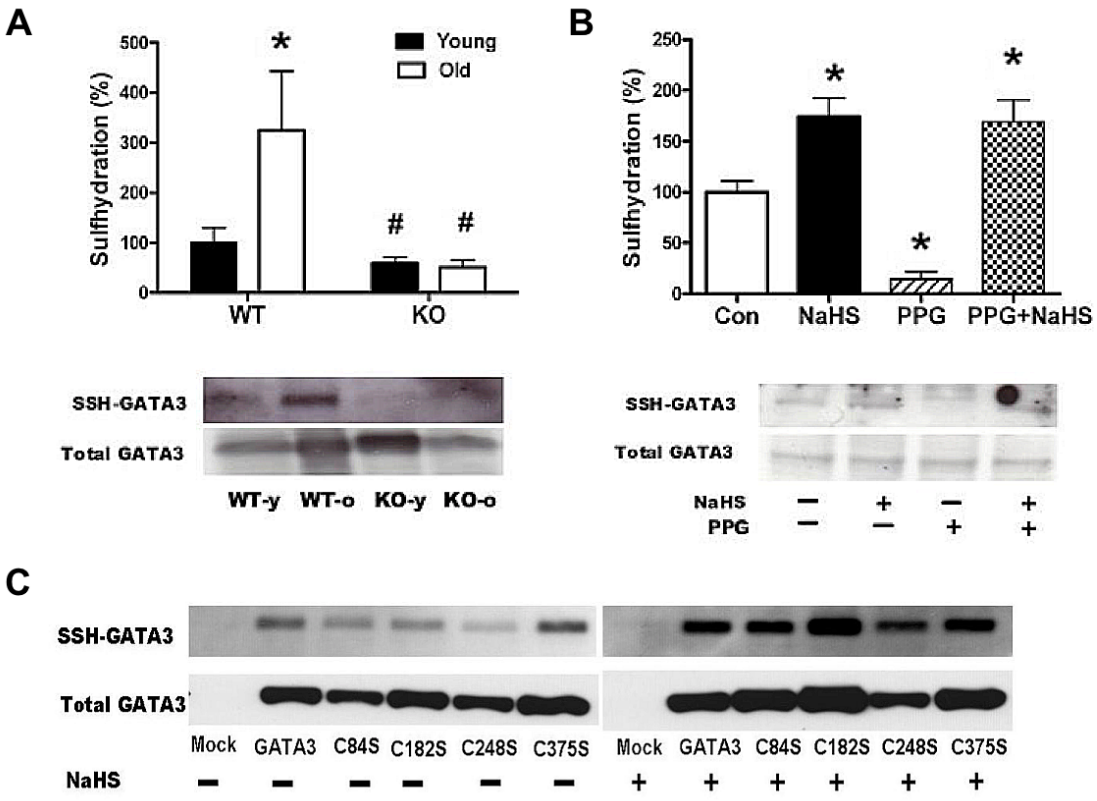


Fig.4-13 *S*-sulfhydration of GATA3. A Endogenous *S*-sulfhydration of GATA3 in mouse spleen tissues (*n*=4 for each group). **B** *S*-sulfhydration of GATA3 in HEK-293 cells with over-expressed CSE (*n*=3 for each group). PPG is dl-propargylglycine. **C** *S*-sulfhydration of GATA3 and its mutants heterologously expressed in HEK-293 cells. Four GATA3 cysteine residues (C84, C182, C248, and C375) were mutated into serines. *S*-sulfhydration of these mutants was tested with or without NaHS (10 μM) treatment. Differences among WT-young, WT-old, CSE-KO-young and CSE-KO-old groups in (**A**) were determined by two-way ANOVA. Comparisons among different treatment conditions in (**B**) were made using one-way ANOVA followed by Dunnett post test. **P* <0.05 when WT-young mice compared with WT-old mice, and for NaHS/PPG/PPG+NaHS compared with the control group in **(B)**, # *P* <0.05 for CSE-KO mice compared with WT mice in **(A)**.

Figure 4-14

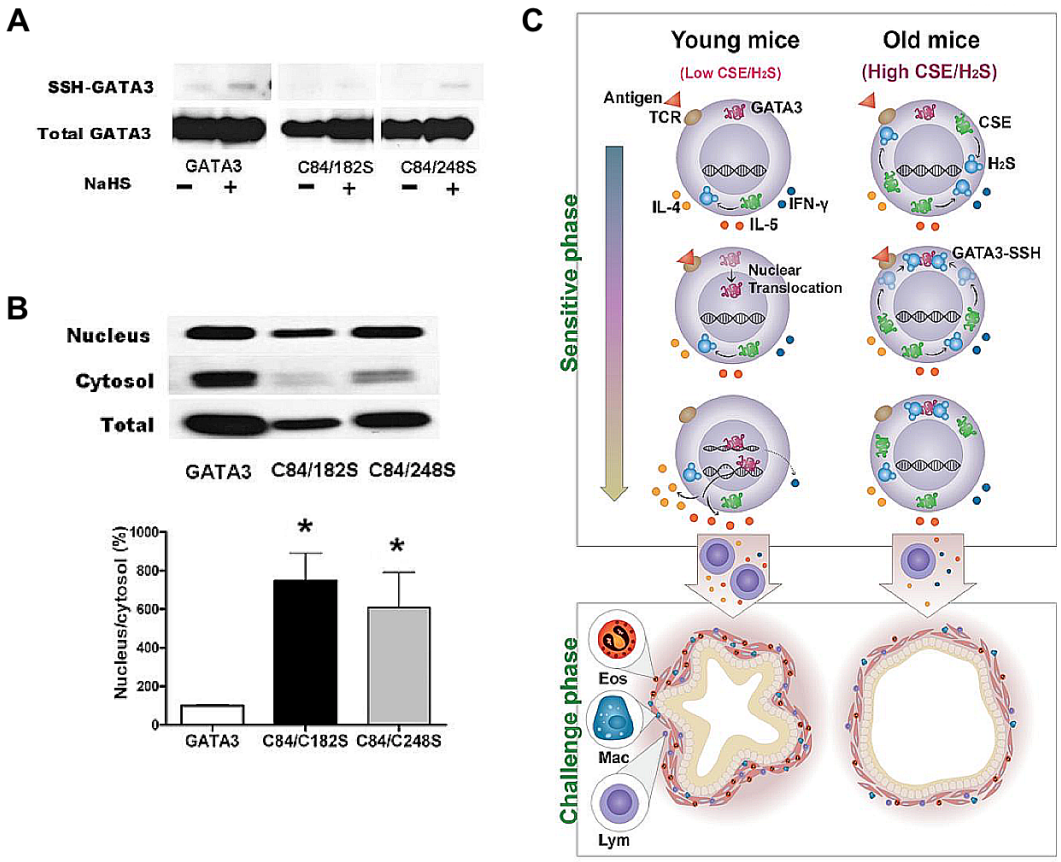


Fig.4-14 *S*-sulfhydration of GATA3 reduced its nuclear translocation. **A** *S*-sulfhydration of GATA3 with double mutations of C84/C182 or C84/C248 in HEK-293 cells. In these mutants, the targeted cysteines were replaced by serines. **B** Double mutation of C84/C182 or C84/C248 increased GATA3 nuclear expression in HEK-293 cells (n=4 for each group). **C** Proposed mechanisms for the CSE/H_2S pathway at different ages in the development of allergic asthma. Eos: eosinophils, Mac: macrophages, Lym: lymphocytes. Greater severity of asthma in young WT mice (compared to 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 cause decreased *S*-sulfhydration of GATA3, increasing its nuclear translocation. This promotes the differentiation of splenocytes into T_H2 cytokine-generating cells and the development of more severe asthma. *P <0.05.

Figure 4-15

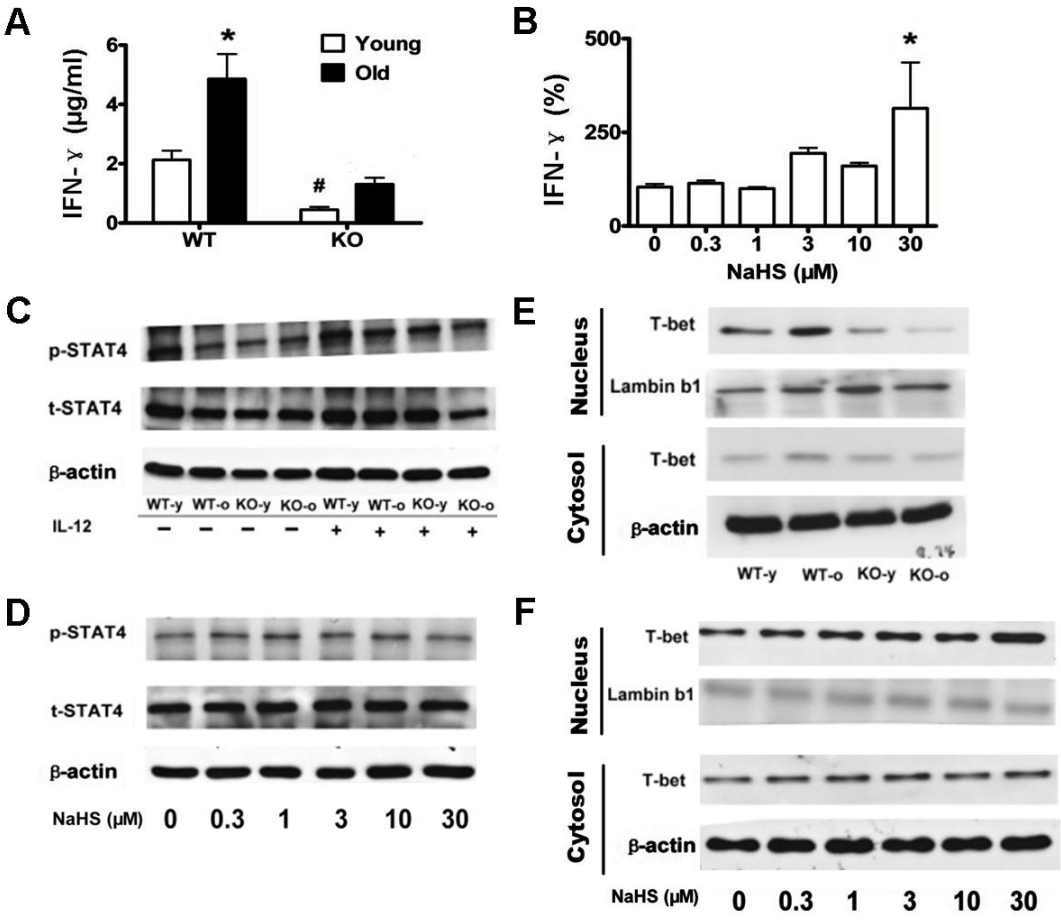


Fig. 4-15 Endogenous and exogenous H_2S enhanced the differentiation of splenocytes into IFN-γ-generating cells *in vitro*. A-B IFN-γ levels in the culture media of splenocytes. Mean IFN-γ level of NaHS-0 μM group in (**B**) is set as 100%. **C-D** IL-12-treatment induced STAT4 phosphorylation in splenocytes. **E-F** Nuclear and cytosolic T-bet expression in splenocytes. **P* < 0.05 for WT-young compared with WT-old mice and for NaHS-30 μM group compared with NaHS-0 μM group; # *P* <0.05 for CSE-KO mice compared with WT mice.

Figure 4-16

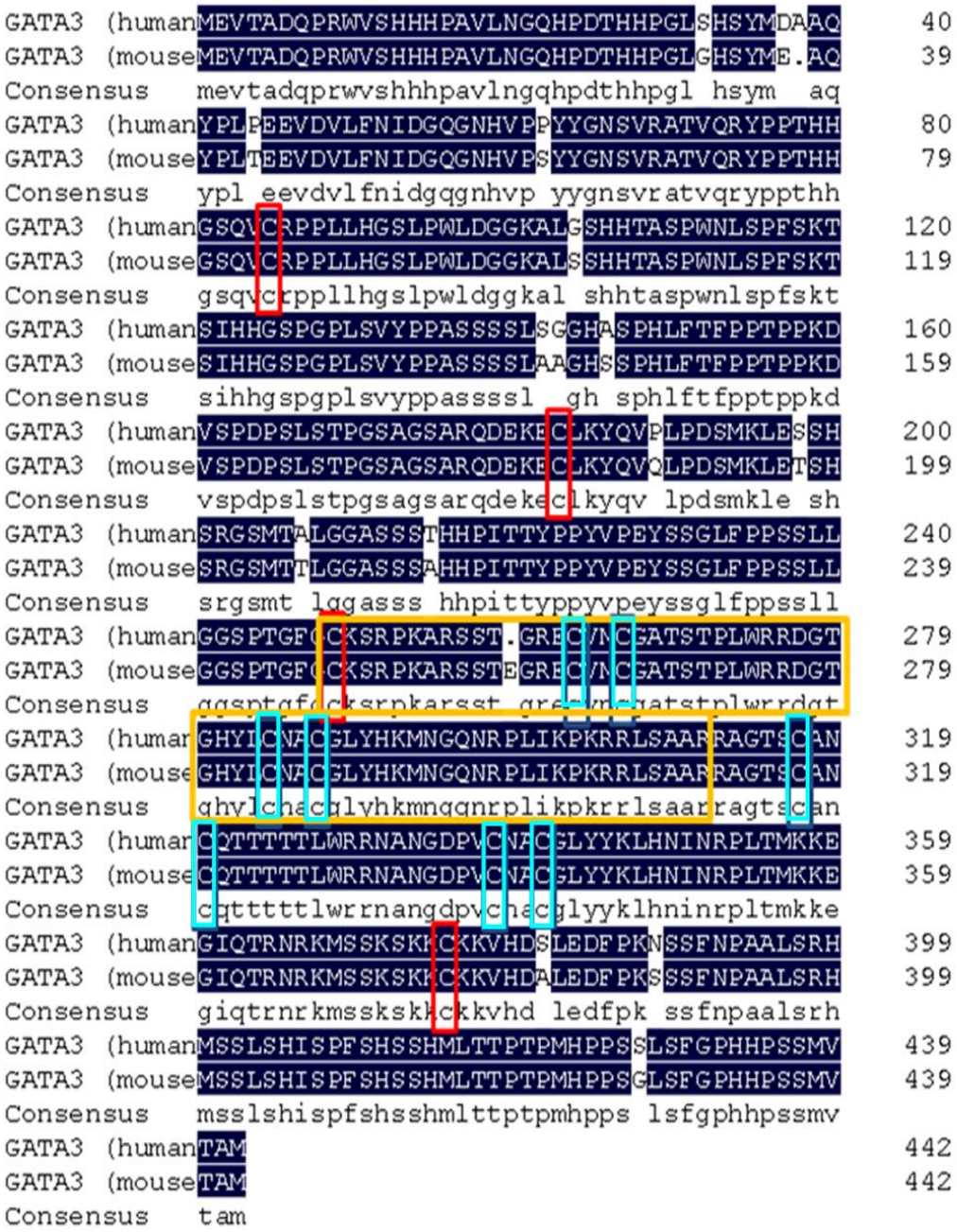


Fig. 4-16 Alignment of protein sequences of human and mouse GATA3. Eight of the 12 cysteine residues of GATA3 locate in the zinc figure region (green frames). The remaining 4 cysteine residues of GATA3 are shown with red frames. The nuclear localization sequence of GATA3 is indicated with yellow frames.

Figure 4-17

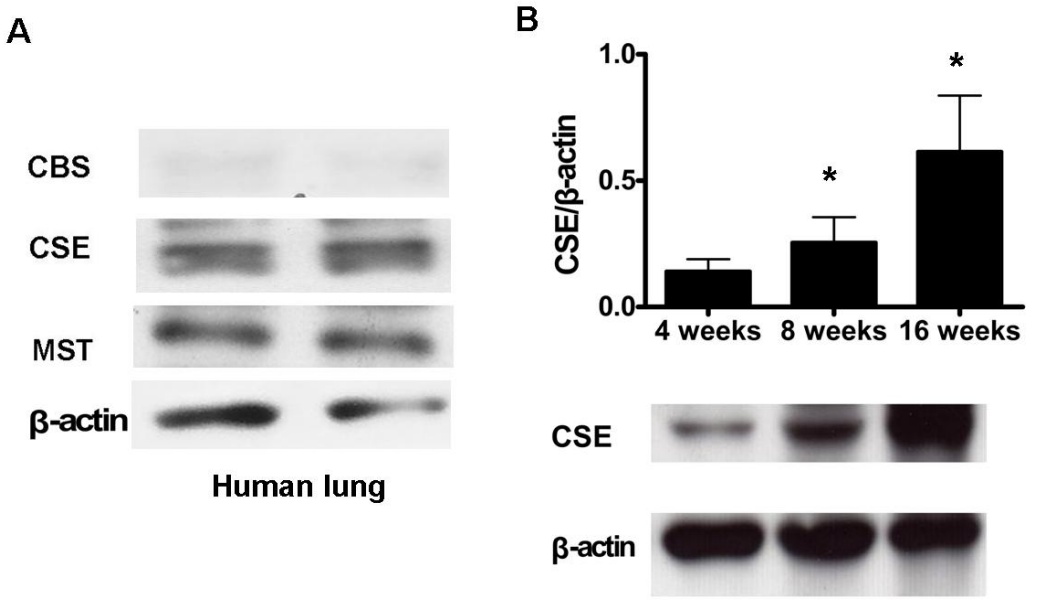


Fig. 4-17 CSE-expression levels in lung tissue from humans and WT mice. A CBS, CSE, and MST protein levels in human lung tissue. **B** CSE protein levels in lung tissues of WT mice at different ages (4-week, 8-week and 16-week-old) (n=4 for each group). *P<0.05 for both 8- and 16-week old WT mice compared with 4-week-old WT mice.

Figure 4-18

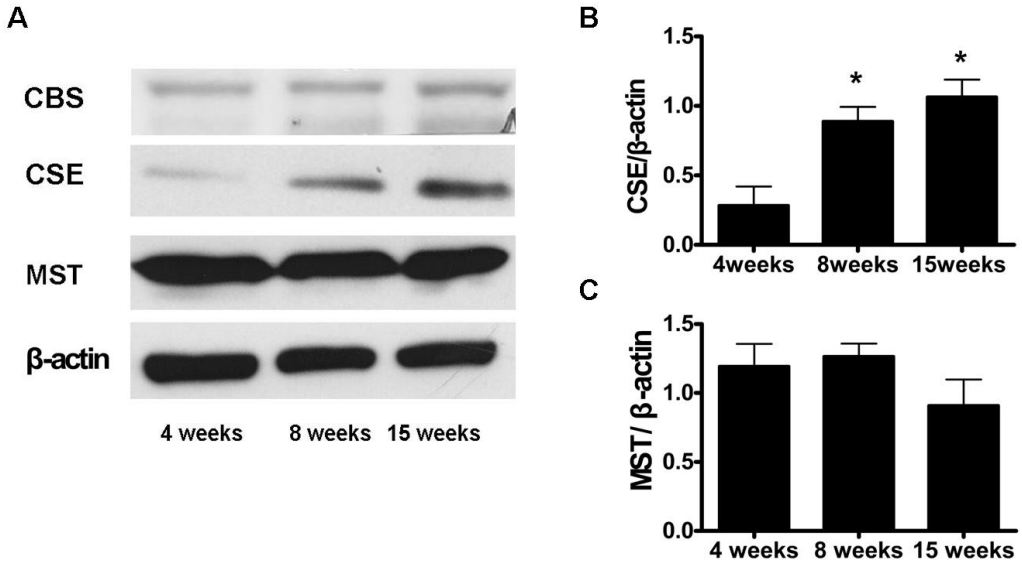


Fig. 4-18 The protein levels of H_2S-generating enzymes in WT mice airways. The tracheas and lung tissues of WT mice at different ages (4-week, 8-week and 15-week-old) were removed immediately after the animals were euthanized. Lung parenchyma was carefully removed until only the bronchial tree and trachea remained. CBS, CSE, and MST expression levels in the bronchial tree and trachea were tested by Western blot (n=4 for each group). * P<0.05 for both 8- and 15-week old WT mice compared with 4-week-old WT mice.

Fig. 4-19

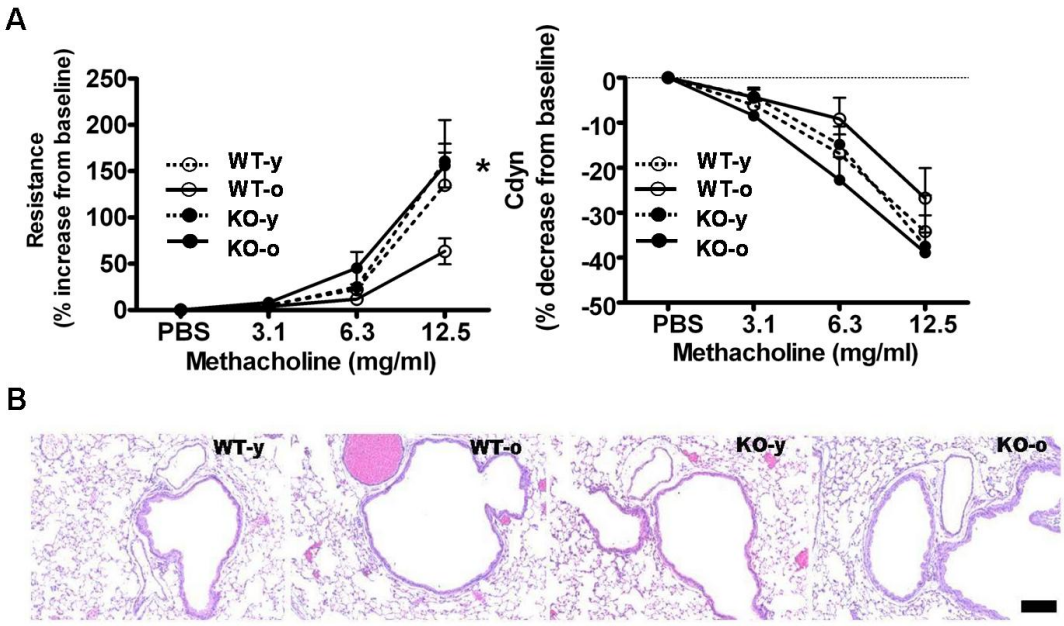


Fig. 4-19 Airway responsiveness and histological features of mice without asthma. A Airway responsiveness was measured as airway resistance and compliance (Cdyn) using increasing concentrations of methacholine. **B** Representative H&E-stained mouse lung tissues. Scale bars, 200 μm. WT-y: WT young mice; WT-o: WT old mice; KO-y: CSE-KO young mice; KO-o: CSE-KO old mice.

Table 4-1

Target site	Primer sequence	Purpose
C84	For 5' GAGCCAGGTATCACGCCCGCCTCTGCT 3'	mutation
	Rev 5' AGCAGAGGCGGGCGTGATACCTGGCTC 3'	
C182	For 5' CAAGATGAGAAAGAGAGCCTCAAGTATCAGG 3'	
	Rev 5' CCTGATACTTGAGGCTCTCTTTCTCATCTTG 3'	
C248	For 5' CTACCGGGTTCGGATCAAAGTCGAGGCCCAAG 3'	
	Rev 5' CTTGGGCCTCGACTTTGATCCGAACCCGGTAG 3'	
C375	For 5' GTCTAGCAAATCGAAAAAGAGCAAAAAGGTGCATGACGC 3'	
	Rev 5' GCGTCATGCACCTTTTTGCTCTTTTTCGATTTGCTAGAC 3'	
C84	For 5' ATGGAGGTGACTGCGGA 3'	sequencing
	Rev 5' GGAGGGTAAACGGACAGAG 3'	
C182	For 5' CCGCACCTCTTCACCTT 3'	
	Rev 5' GGAAGAGTCCGGAGCTGTA 3'	
C248	For 5' GAGTGCCTCAAGTACCAGG 3'	
	Rev 5' CGGTTCTGTCCGTTCATT 3'	
C375	For 5' CAGACCACCACAACCACACTC 3'	
	Rev 5' ATGTGGCTGGAGTGGCTGA 3'	

Table 4-1 Mutating and sequencing primers used in site-directed mutagenesis of GATA3.

5. Chapter 5

GENERAL DISCUSSION AND CONCLUSIONS

A sharp increase has been seen in the global prevalence, morbidity, mortality, and economic burden associated with asthma over the last 40 years. The greatest rise in asthma prevalence has been in children. Challenges in asthma management include understanding the cellular and molecular mechanisms for asthma, practical methods for early detection, monitoring symptoms and predicting prognosis, and the need for novel therapeutic approaches with improved efficacy and selectivity.

In previous studies we showed that H_2S at 1-3 mM relaxes tracheae in vitro. The studies of Fitzgerald, *et al.* and Castro-Piedras *et al.* suggest this H_2S effect might be mediated by opening K_{ATP} channel[189] or inhibiting Ca^{2+} release through InsP3 receptors[102]. Our current in vivo study further highlighted the importance of CSE/H_2S in modulating airway responsiveness (chapter 3). CSE deletion, which led to an 81% reduction of endogenous H_2S production in the lung, aggravated OVA-induced airway hyper-responsiveness in CSE-KO mice, while H_2S supplementation lowered OVA-induced airway hyper-responsiveness in both WT and CSE-KO mice. Besides relaxing vascular smooth muscle directly (by increasing K_{ATP} channel currents and hyperpolarizing membranes), H_2S may also reduce airway hyper-responsiveness by inhibiting T_H2 cytokine production. T_H2 cytokines, particularly IL-13 and L-4[148-150], augment stimulant-induced calcium influx and contractile responses in airway smooth muscle cells.

CSE deficiency aggravated OVA-induced T_H2 cytokines and eotaxin-1 production, leading to more eosinophil infiltration in the lungs of CSE-KO mice. Exogenous H_2S supplementation significantly decreased OVA-induced BALF cytokines as well as reducing eosinophilic airway inflammation in both WT and CSE-KO mice. These results suggest that both endogenous and exogenous H_2S protect against the development of OVA-induced airway hyper-responsiveness and allergic airway

inflammation.

It is known that the incidence of allergic asthma is age-dependent; most asthma begins during the preschool years. Structural and functional changes of asthma (including bronchial hyper-responsiveness, reticular basement membrane thickening and eosinophilic inflammation) start to develop between 1 and 3 years of age[192]. This places childhood asthma as the major target for primary prevention. Childhood asthma is usually studied separately because biomarkers and treatment strategies for adult asthmatics are not always applicable in children.

To investigate whether CSE/H_2S is involved in the development of childhood asthma, allergic asthma was induced with OVA in young (3-4 weeks old) and old (7-8 months old) mice. With this model, we demonstrate in chapter 4 that spleen and airway CSE expression, but not CBS or MST expression, increased with age. Lower levels of CSE expression and H_2S production in the spleens of young mice amplified the type 2 response towards antigens: OVA-sensitization of young WT mice resulted in more T_H2 cytokine-generating cells in peripheral lymph tissues with higher ensuing T_H2 cell cytokine levels (IL-4, IL-5 and IL-13) in BALF than OVA-sensitization caused in old WT mice. This result is consistent with other observations showing that sensitization at an early age is associated with enhanced T_H2 cytokine responses and adverse asthma outcomes[45]. H_2S supplementation, at the time of initial allergen sensitization, limited type 2 response, promoted maturity of the immune system and abridged asthma development, whereas CSE gene knock-out enhanced type 2 immune response and aggravated asthma symptoms. Inflammatory-cell infiltration in airways and T_H2 cell cytokine levels in BALF were comparable between young and old CSE-KO asthmatic mice. These pathological changes were more severe than in asthmatic WT mice of the same ages. It is clear that the elimination of CSE expression abolished age-dependent asthma development and worsened allergic asthma at all ages.

Using animals of different ages, we further confirmed the importance of CSE/ H_2S in

modulating airway responsiveness. CSE expression in the airways increased in an age-dependent manner. Airway responsiveness to MCh challenge of young WT mice, in the absence of allergen exposure, was more than twice that of old WT mice. Higher airway responsiveness at a young age has been observed in many other studies[46,95,193] and is related to increased risk of asthma in childhood[97,98]. MCh challenge induced comparable airway responsiveness in young and old CSE-KO mice, an effect of similar magnitude to that in young WT mice. Therefore, low abundance of CSE in young mice or deficiency of CSE in CSE-KO mice may alter intrinsic properties of airway smooth muscle and lead to airway hyper-responsiveness because of decreased endogenous H_2S production.

Taken together, our findings in chapter 3 demonstrate the important role of CSE/H_2S in regulating airway responsiveness and type-2 immunity. Results from chapter 4 not only confirm the findings in chapter 3 but also elucidate the different regulatory effects of the CSE/H_2S pathway in T_H2 and T_H1 responses and an essential role of the CSE/H_2S pathway in the inception of childhood asthma. These results speak to a H_2S-based therapeutic strategy for asthma.

Does this age-dependent expression CSE in mice resemble that of humans? CSE proteins, but not CBS or MST proteins, were detected in human spleen samples. Peripheral blood mononuclear cells (PBMC) from human adults (38-55 years old) had more CSE proteins than umbilical cord-blood mononuclear cells (CBMC). Neither PBMC nor CBMC expressed CBS proteins. Low abundance of MST protein was detected in PBMC. Direct comparison of CSE expression levels in splenocytes from young and old humans would be ideal to confirm the relevance of our animal study. There are no commercialized infant or children spleen tissues. (It is somewhat difficult to obtain samples by collaborating with pediatric surgeons because non-operative management of splenic trauma, with the potential of avoiding overwhelming post-splenectomy sepsis[194,195], is especially beneficial to children.) Spleen and peripheral blood are two major immune-system components. Cells from peripheral blood (PBMC or CBMC in newborns) share many characteristics of

splenocytes in terms of composition and function. Both are widely used to study type 2 immune response[196]. Comparing adult PBMC and infant CBMC should be a good substitute for the direct comparison of splenocytes from young and old humans. Therefore, to study whether CSE expression in immune cells varies with age, we chose commercially-available PBMC and CBMC.

It is currently unclear which specific cell type(s) express CSE in an age-dependent manner and which tuned the immune response in our study. We were restricted in exploring these questions due to lack of access to multi-channel flow cytometry. Based on our data and available literature[169], we hypothesize that dendritic cells express CSE in an age-dependent manner. Sufficient endogenous H_2S will modulate dendritic cells' response to antigen stimulation and subsequently refine the activating signals sent to naïve T cells by dendritic cells. Dendritic cells express high levels of major histocompatibility complex class I/II molecules, and costimulatory molecules CD80/CD86 and CD40. They are the most proficient antigen-presenting cells in terms of processing inhaled allergens, peptide antigens and soluble protein antigens. After taking up the antigen, dendritic cells migrate to the draining lymph nodes, where they activate and polarize naive T_H cells[197] .

To test this hypothesis, our future study will include: 1) using multichannel flow cytometry to determine whether dendritic cells are the major source of CSE in splenocytes; 2) isolating dendritic cells from WT mice at different ages to test whether CSE expression in dendritic cells is age-dependent; 3) co-culturing dendritic cells from CSE-KO mice with naïve T cells isolated from WT mice to test whether deficiency of endogenous H_2S in dendritic cells boosts their ability to induce T_H2 polarization; 4) in vitro experiments to observe which signal pathways in dendritic cells are changed by CSE/H_2S expression levels; 5) isolating dendritic cells from CBMC and PBMC to confirm whether the phenomenon observed in mice is parallel to humans.

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PUBLICATIONS AND CONFERENCE COMMUNICATIONS

Publications

1. Wang PP, Wu LY, Ju YJ, Wang R. 2015. Early onset of allergic asthma due to age-dependent expressional deficiency of cystathionine gamma-lyase in young mice. In the process of submission.
2. Zhang GS*, Wang PP*, Yang GD, Cao QH, Wang R. 2013. The inhibitory role of hydrogen sulfide in airway hyperresponsiveness and inflammation in a mouse model of asthma. Am J Pathol. 182(4):1188-95. doi: 10.1016/j.ajpath.2012.12.008. **(* equal contribution)**.
3. Wang PP, Zhang GS, Wondimu T, Ross B, Wang R. 2011. Hydrogen sulfide and asthma. Exp Physiol, 96(9):847-52.

Oral presentations

1. Wang PP, Zhang GS, Yang GD, Wang R. The role of cystathionine gamma lyase in asthma development. Thunder Bay COSC Canadian Oxidative Stress Consortium, 2012. 05.11.

2. Wang PP. H_2S as a novel biomarker and therapeutic target for asthma. Lakehead University Graduate Students Conference, 2011.02.17.

3. Wang PP, Yang G, Wang R. H_2S as a novel biomarker and therapeutic target for asthma. Sainte-Adèle, Canadian Physiological Society Winter Meeting, 2011.02.11.

Poster presentations

1. Wang PP, Wu LY, Ju YJ, Wang R. 2015. Gamma-lyase deficiency and early onset of allergic asthma in the mouse. American Thoracic Society International Conference, Denver, 2015,05.17.
2. Wang PP, Yang GD, Tang GH, Wang R. Insufficiency of cystathionine gamma lyase/H_2S in the lung of juvenile mouse contributes to airway hyper-responsiveness. Young Investigator Forum, Edmonton, 2014.05.28