Sulforaphane prevents and reverses allergic airways disease in mice via anti-inflammatory, antioxidant, and epigenetic mechanisms

Simon G. Royce1,2,3,4 · Paul V. Licciardi4,5 · Raymond C. Beh1,2 · Jane E. Bourke3 · Chantal Donovan6,7,8 · Andrew Hung9 · Ishant Khurana10 · Julia J. Liang1,9 · Scott Maxwell10 · Nadia Mazarakis1,4,5,11 · Eleni Pitsillou1,9 · Ya Yun Siow1 · Kenneth J. Snibson11 · Mark J. Tobin12 · Katherine Ververis1,2 · Jitaporn Vongsivivut12 · Mark Ziemann10,13 · Christan S. Samuel14 · Mimi L. K. Tang5,15,16 · Assam El-Osta10 · Tom C. Karagiannis1,2

Received: 1 July 2022 / Revised: 13 October 2022 / Accepted: 21 October 2022 / Published online: 1 November 2022 © Crown 2022

Abstract
Sulforaphane has been investigated in human pathologies and preclinical models of airway diseases. To provide further mechanistic insights, we explored L-sulforaphane (LSF) in the ovalbumin (OVA)-induced chronic allergic airways murine model, with key hallmarks of asthma. Histological analysis indicated that LSF prevented or reversed OVA-induced epithelial thickening, collagen deposition, goblet cell metaplasia, and inflammation. Well-known antioxidant and anti-inflammatory mechanisms contribute to the beneficial effects of LSF. Fourier transform infrared microspectroscopy revealed altered composition of macromolecules, following OVA sensitization, which were restored by LSF. RNA sequencing in human peripheral blood mononuclear cells highlighted the anti-inflammatory signature of LSF. Findings indicated that LSF may alter gene expression via an epigenetic mechanism which involves regulation of protein acetylation status. LSF resulted in histone and α-tubulin hyperacetylation in vivo, and cellular and enzymatic assays indicated decreased expression and modest histone deacetylase (HDAC) inhibition activity, in comparison with the well-known pan-HDAC inhibitor suberoylanilide hydroxamic acid (SAHA). Molecular modeling confirmed interaction of LSF and LSF metabolites with the catalytic domain of metal-dependent HDAC enzymes. More generally, this study confirmed known mechanisms and identified potential epigenetic pathways accounting for the protective effects and provide support for the potential clinical utility of LSF in allergic airways disease.

Keywords Sulforaphane · Allergic airways disease · Airway hypersensitivity · Antioxidant · Anti-inflammatory · Epigenetic regulatory mechanisms

Introduction
Sulforaphane (SFN) is a dietary isothiocyanate released from the precursor glucoraphanin by the action of plant myrosinase or gut microbiota β-thioglucosidases [1–3]. In addition to the production of isothiocyanates, the hydrolysis of glucoraphanin results in the formation of nitrile compounds [4, 5]. Sulforaphane is metabolized through the mercapturic acid pathway [6]. The first step involves glutathione conjugation (SFN-GSH) mediated by glutathione S-transferase (GST) enzymes [7, 8]. Glutathione conjugates are cleaved further to generate the cysteinylglycine conjugate (SFN-Cys-Gly), cysteine conjugate (SFN-Cys), and N-acetylcysteine conjugate (SFN-NAC), catalyzed by γ-glutamyltranspeptidase, cysteinylglycinase, and N-acetyltransferase, respectively [7, 8].

Sulforaphane has been widely investigated in numerous human models of disease including cancer chemoprevention, metabolic disorders, and neurological conditions [9–11]. Further, SFN has been studied in numerous preclinical and clinical models of lung damage and airway diseases [12–17]. The lack of definitive findings from clinical studies to date most likely reflects issues with extract preparations and dosage regimes [18–20]. A notable exception to this is the beneficial effects of SFN in airborne pollution studies [21, 22]. Mercapturic acids are detoxification products formed from GST-catalyzed reactions that are being used in clinical trials as biomarkers of exposure to environmental and industrial
Sulfuraphane has been shown to enhance the detoxification of toxic and carcinogenic airborne pollutants by increasing the elimination of mercapturic acids [21, 22].

A multitude of mechanisms of action, predominantly involving activation of cellular antioxidant and anti-inflammatory pathways, has been associated with the beneficial effects of SFN in models of disease. It is well known that SFN activates nuclear factor erythroid 2-related factor 2 (Nrf2), and in response to cellular stress, this transcription factor translocates into the nucleus and binds to the antioxidant response element (ARE) [20, 24, 25]. The interaction of Nrf2 and coactivator proteins with ARE results in the induction of phase II detoxification and antioxidant enzymes, such as heme oxygenase-1 (HO-1), NAD[P]H:quinone oxidoreductase-1 (NQO1), superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH) [24]. Under basal conditions, Nrf2 is sequestered in the cytosol by Kelch-like ECH-associated protein 1 (Keap1) [24]. The differential biological effects of (R)- and (S)-enantiomers of SFN have also been
Sulforaphane prevents and reverses allergic airways disease in mice via anti-inflammatory,…

Fig. 1 L-sulforaphane protects from bronchial mucosal damage in a murine chronic model of allergic airways disease. Chemical structures of antioxidant isothiocyanate, L-sulforaphane (LSF), and broad-spectrum histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA) (A), used in this study. The ovalbumin (OVA)-induced chronic murine model of allergic airways disease (AAD) was investigated (B). Hematoxylin and eosin (H&E) stained sections of formalin-fixed paraffin-embedded mouse lung tissue derived from the prevention model show LSF attenuates epithelial thickening induced by OVA. Little or no peribronchial inflammatory infiltrate was observed for the saline and OVA-LSF mice, while severe to moderate peribronchial inflammatory infiltrate was present in the OVA-vehicle (OVA-VEH) and OVA-SAHA mice respectively (C). Immunofluorescence staining of Annexin V (red), a membrane apoptotic marker and histone deacetylase enzyme (HDAC) 8 (green) (D). Strong staining of Annexin V was found in the bronchial epithelium and peribronchial inflammatory cells in mice treated with OVA-VEH and OVA-SAHA. Weak Annexin V staining was observed in the OVA-LSF mice and no staining was found in the epithelium of the control saline group. Merged image: nucleus stained with 4′,6-diamidino-2-phenylindole (DAPI) (blue), Annexin V (red), HDAC8 (green). Morphometric analysis of epithelial thickness (E) and subepithelial collagen (F) from H&E stained MBC lung sections shows both OVA-VEH and OVA-SAHA mice significantly elevated epithelial and subepithelial thickness compared to saline and OVA-LSF mice. Image J analysis of the mean fluorescence intensity of Annexin V expression showed significantly elevated expression in OVA-VEH and OVA-SAHA mice compared to saline and OVA-LSF mice. Data shown as the mean ± SEM; **p<0.01 and ***p<0.001

explored; however, there is conflicting evidence from studies [26–28]. Recently, it has been shown that L-sulforaphane (LSF), which is also known as (R)-sulforaphane, causes a reduction of pro-inflammatory cytokine and chemokine production and alleviates inflammation by targeting antigen-presenting cells [29].

Here, we investigated the effects of administration of LSF in a murine model of ovalbumin (OVA)-induced chronic allergic airways disease (AAD). This model of chronic AAD recapitulates several features of human asthma including airway inflammation, airway remodeling and airway hyperresponsiveness (AHR) [30–33]. Apart from conventional histological, and immunohistological analyses, we performed genome-wide mRNA-Seq to confirm the anti-inflammatory signature of LSF in human peripheral blood mononuclear cells (PBMC). Furthermore, we examined epigenetic mechanisms associated with LSF by investigating histone deacetylase enzyme (HDAC) expression and histone acetylation status following LSF administration both in vitro and in the animal model.

Materials and methods

Chemicals. In animal studies, LSF (Santa Cruz Biotechnology, Dallas, Texas, USA) was used at a dose of 5 mg/kg dissolved in 1% (v/v) dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA) in 0.9% (v/v) normal saline solution (Baxter Health Care, NSW, Australia) and suberoylanilide hydroxamic acid (SAHA) (Sigma) was used at 100 mg/kg in 1% (v/v) DMSO in 0.9% (v/v) saline. The selected doses are known to be biologically active and have been used in experimental murine models of disease without significant toxicity [34, 35]. Chemical structures of LSF and SAHA are shown in Fig. 1A. For in vitro studies, LSF, SAHA, and methacholine (MCh) were all purchased from Sigma. LSF and SAHA were dissolved in 100% (v/v) DMSO and stored as stock solutions of 20 mM (−80 °C), 10 mM (−20 °C), and 1 M (−80 °C), respectively.

Animals. Given the relative ease of care, female mice were chosen for our experiments. On the basis of previous observations using sulforaphane in animal models of lung pathologies, it is anticipated that LSF will behave in an analogous manner in male and female mice [25, 36]. Six-week-old female BALB/c mice were obtained from Walter and Eliza Hall Institute Bioservices (prevention model; Parkville, VIC, Australia) and Monash Animal Services (reversal model; Clayton, VIC, Australia). Mice were housed under specific pathogen-free conditions, maintained at 21 °C, 12-h light/dark cycle, fed standard laboratory chow (Barestoc Stockfeeds, Pakenham, VIC, Australia), and water ad libitum. The BALB/c mice strain was chosen for their strong Th2 responses in OVA-induced AAD models [37]. Due to their increased sensitivity to OVA and AAD models, female mice were chosen for this study [37, 38]. The prevention model experimental protocol was approved by the Murdoch Children’s Research Institute Animal Ethics Committee (approval no. A597). The reversal model experimental protocol was approved by the Monash University Animal Ethics Committee (MARP/2012/085). All mice were provided an acclimatization period of 4–5 days before any experimentation. All experimental procedures followed the Australian guidelines for the care and use of laboratory animals for scientific purposes.

Mouse model of chronic allergic airways disease. To assess the effects of LSF in chronic AAD, two models were used to determine protective effects and ability to reverse AAD in an established model of OVA-induced chronic AAD. This model was chosen for its pathological similarities to human asthma, including increased allergic responses indicated by increased immunoglobulin E against OVA (OVA-specific IgE), AHR and remodeling changes such as epithelial remodeling, goblet cell metaplasia, and subepithelial collagen deposition (fibrosis). Treatment groups were divided into the prevention and reversal models according to the timelines shown in Figs. 1B and 2A. Detailed protocols of the prevention model [39] and reversal model [40] have been described previously.

Prevention model. Briefly, mice were sensitized with two intraperitoneal (i.p) injections of 10 μg of grade V chicken egg OVA (Sigma) and 1 mg of aluminum...
potassium sulfate adjuvant (alum) (Sigma) in 0.5 mL of 0.9% (v/v) saline, which was subsequently administered via i.p on days 0 and 14. Mice were then challenged by whole body inhalation exposure (nebulization) to aerosolized 2.5% (w/v) OVA in saline for 30 min, three days per week for six weeks using an ultrasonic nebulizer (NE-U07, Omron Corporation, Tokyo, Japan), between days 21 and 63 to establish AAD. Following exposure to nebulized OVA, mice were treated with 5 mg/kg LSF (OVA-LSF) or 100 mg/kg SAHA (OVA-SAHA, n = 6) or vehicle control (OVA-VEH, n = 15) by i.p injection, three days per week for six weeks, which represent doses known to be effective and nontoxic [41, 42]. A fourth group of mice were sensitized with 1 mg alum in 0.5 ml saline on days 0 and 14 and were challenged with saline aerosols three days per week for six weeks (n = 15). Mice were euthanized with an
L-sulforaphane reverses bronchial remodeling in a murine model of chronic allergic airways disease. In the reversal model, L-sulforaphane (LSF) was administered following ovalbumin (OVA) sensitization (A). Representative photomicrographs of histological stained sections of formalin-fixed paraffin-embedded mouse lung tissue derived from the reversal model (B). Hematoxylin and eosin (H&E) staining shows LSF reduces peribronchial inflammatory cell infiltration induced by OVA (Bi). Masson’s trichrome staining shows a thin epithelium (arrow) and little subepithelial collagen (arrowhead) in the saline and OVA-LSF mice (Bii). Airways in OVA-vehicle (OVA-VEH) mice display epithelial thickening (arrow) and severe subepithelial collagen deposition (arrowhead). Silver impregnation staining highlights that LSF attenuates the increased reticular fibers (R) and collagen fibers (C) found in the OVA-VEH mice (Biii). Gomori’s aldehyde-fuschin staining shows an increase of elastic fibers (royal blue) of the smooth muscle in the OVA-VEH mice that is not present in the saline and OVA-LSF mice (Biv). Blood vessels (BV) stained positive. Sirius red staining imaged under polarized light differentiates between collagen type I (red/yellow) and type III (green) in lung sections (Bv). Morphometric analysis of Masson’s trichrome stained sections shows OVA-VEH had significantly elevated epithelial thickness (C) and subepithelial collagen thickness (D) when compared to both saline and OVA-LSF mice. Analysis of type I and III collagen from Sirius red stained sections indicates that OVA-VEH significantly elevates type I collagen when compared to all other groups (E). Airway hyperresponsiveness to methacholine was recorded using plethysmography 48 h following the final treatment and nebulization (F). Increasing doses of methacholine was administered and maximal resistance values (cmH2O/mL/sec) after 5 min were recorded. OVA-VEH mice had elevated airway resistance at the highest methacholine dose as compared to saline mice. OVA-LSF mice had significantly lower airway resistance at the highest methacholine dose compared to those of OVA-VEH mice. All data represented as the mean ± SEM; *p < 0.05 **p < 0.01 and ***p < 0.001.

Reversal model. Saline control mice (n = 6) and vehicle control mice (OVA-VEH, n = 6) were sensitized and nebulized to saline or OVA as described in the prevention model. Mice were treated with saline or 5 mg/kg LSF (OVA-LSF, n = 8) by i.p injection 24 h after the last OVA nebulization five times every 72 h between days 64 and 76. Mice were euthanized by i.p injection of ketamine and xylazine (200 µg/g: 10 µg/g) 48 h after the last treatment on day 78.

Measurement of airway reactivity in vivo. AHR was assessed by plethysmography, 14 h after the final treatment with LSF or vehicle using a mouse invasive plethysmograph (Buxco Electronics, Troy, NY). Following anesthetization using an i.p injection of ketamine and xylazine (200 µg/g: 10 µg/g), mice were tracheostomized and cannulated via the jugular vein. Mice were ventilated with a small animal respirator (Harvard Apparatus, Holliston, MA) delivering 0.01 ml/g bodyweight at a rate of 120 strokes/min. A baseline airway resistance was recorded (Biosystem XA; Buxco Electronics), then every three minutes after increasing intravenous doses of MCh (3.1, 6.3, 12.5, 25, 50 and 100 mg/ml) in phosphate-buffered saline. The change in airway resistance from baseline level per dose was recorded in Finepoint (Buxco Electronics).

Measurement of airway reactivity ex vivo. Preparation of precision cut lung slices and measurement of responses to MCh were performed in OVA-VEH-sensitized mice as previously described [43, 44]. Briefly, lungs were inflated with warm 2% (w/v) ultra-pure low melting point agarose (Invitrogen, Carlsbad, CA, USA) in Hanks’ balanced salt solution supplemented with 40 mM HEPES (HBSS, Sigma), via a tracheal cannula. After solidification of the agarose at 4 °C (20 min), a single lobe was mounted in cold HBSS to a vibratome (VT 1000S, Leica Microsystems, Wetzler, Germany) in preparation for serial sectioning (150 µm). Measurements of airway responses to MCh were performed using a gravity-fed system where lung slices were perfused at a constant rate (5 min) for each condition, 300 nM MCh, increasing doses of LSF (3, 10, 15 and 30 µM) and HBSS in resting periods.

Lung histopathology. Lung tissues were dissected, and right lung lobes were fixed with 10% (v/v) neutral buffered formalin (Sigma) for 24 h, routinely processed and embedded in paraffin. Lung Sects. (3 µm) were assessed by standard histological staining procedures; hematoxylin and eosin (H&E) for inflammatory cell infiltration, Masson’s trichrome for epithelial and subepithelial collagen deposition, silver impregnation for detection of reticular and collagen fibers, Gomori’s aldehyde-fuschin for elastic fibers and sirius red staining for differentiation of collagen type I and type III.

Airway tissue inflammation cell score. Airway inflammatory cell infiltration was determined from H&E stained sections. A minimum of ten airways per mouse were captured using an Olympus FSX100 microscope mounted with a digital camera (Olympus, Tokyo, Japan). The degree of airway inflammatory cell infiltration around the bronchi was scored by two independent blinded investigators using the following score scale: 0 = no inflammatory cells; 1 = 1–3 layers of inflammatory cells; 2 = 4–6 layers; 3 = 7–10 layers and congestion; or 4 = > 10 layers of inflammatory cells and severe congestion. For each mouse, a minimum of ten airways were observed and the average scores were taken.

Morphometric analysis of structural changes. Epithelial thickness and subepithelial collagen thickness were assessed from Masson’s trichrome stained sections. A minimum of ten airways per mouse were captured using an Olympus FSX100 microscope mounted with a digital camera (Olympus). Bronchi measuring between 150 and 350 µm luminal diameter were analyzed using Image ProPlus software (v6.0, Media Cybernetics, MD, USA) after calibration with a reference micrometer slide. The thickness of the bronchial epithelial layer was measured by tracing around the basement membrane and the luminal surface of epithelial, and calculating the mean distance between the two. Subepithelial collagen thickness was also measured by
tracing around the outer extent of the total collagen layer in the submucosal region, around the basement membrane, and the mean distance between these lines calculated. Differentiation of collagen type I and type III was assessed from Sirius red F3B (Sigma) stained slides. Sirius red staining was captured under polarized light using an Olympus BX61 microscope (Olympus) automated with a FVII digital camera to differentiate between the types of collagen. Type I (red and yellow under polarized light) and type III (green under polarized light) collagen was quantified using ImageJ analysis software (FIJI v1.48a, NIH, Bethesda, MD).

**Immunohistochemistry.** Formalin-fixed paraffin-embedded sections were used to evaluate the protein expression of α-smooth muscle actin (α-SMA) to identify airway smooth muscle, caveolin-1, superoxide dismutase 2 (SOD2), and phosphorylated Smad2. Primary antibodies: mouse monoclonal anti-αSMA (1:150, Dako, Glostrup, Denmark); rabbit polyclonal anti-caveolin-1 (1:1000, Santa Cruz Biotechnology); rabbit monoclonal (EPR2560Y) anti-SOD2 (1:250, Abcam, Cambridge, UK); and rabbit polyclonal anti-phospho-Smad2 (ser465/467) (1:500, Cell Signaling Technology, Danvers, MA, USA), were bound, biotinylated, and detected using streptavidin horseradish peroxidase (Dako). The chromogen 3,3-diaminobenzidine (DAB, Thermo Fisher Scientific, Waltham, MA, USA) and anti-HDAC8 (1:250, Sigma) all diluted in 1% (v/v) bovine serum albumin (BSA, Sigma). Primary antibodies were conjugated with secondary antibodies, goat anti-mouse Alexa 488 (Molecular Probes Invitrogen) and goat anti-rabbit 546 (Molecular Probes) diluted in 1% (v/v) BSA (1:500) and mounted in Prolong Gold Antifade with 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen). Slides were incubated overnight at 4 °C before imaging. Annexin V images were acquired using an Olympus BX61 motorized upright fluorescence microscope automated with FVII Camera (Olympus) using a 10x/0.3 and 20x/0.5 U PLAN FL objectives and fluorescence filter cubes used included: DAPI (λex: 350/50, λem: 460/50 nm), FITC (λex: 470/40, λem: 525/50 nm) and TRITC (λex: 545/30, λem: 620/60 nm). All other antibodies were acquired using the Nikon A1R-si resonant scanning confocal system with upright Nikon D-Eclipse microscope fitted with an automated digital camera (Nikon Ti, Tokyo, Japan) in association with Nikon NIS-Elements AR 3.2 64-bit software (NIS Elements Advanced Research, Tokyo, Japan). Samples were captured using a 60x/1.0 W DIC N2 ɔe/0 oil 2.8 Nikon Japan NIR Apo immersion objective (NA = 1.4). Images were acquired with sequential excitation: 405 nm (λem: 450/50), 488 nm (λem: 490/525) and 568 nm (λem: 562/95) laser lines and collected in 512 × 512 pixel format (mono 16-bit) with a 0.5 µm step size and 2.2 pixel/dwell. A minimum of five airways per mouse were acquired, and images were processed and analyzed for mean total fluorescence (FL) intensity using ImageJ (FIJI). Nrf2 foci and line scan analysis was assessed using Image J processing tools (FIJI).

**Focal plane array-Fourier transform infrared (FPA-FTIR) microspectroscopy.** Lung samples were sectioned (4 µm) using a Leica RM 2135 microtome (Leica Biosystems) onto calcium fluoride (CaF2) windows (Crystran, Dorset, UK). The sections were subsequently deparaffinized by two consecutive 5 min washes with xylene (Sigma-Aldrich) and stored in desiccator prior to FPA-FTIR microspectroscopy. The acquisition of FPA-FTIR chemical images was performed using an offline FPA-FTIR microspectroscopic system (Bruker Optik GmbH, Ettlingen, Germany) at the Australian Synchrotron Infrared Microspectroscopy (IRM) beamline (Clayton, VIC, Australia) as described previously [41]. Briefly, the FPA-FTIR images were acquired in transmission mode using a Bruker Hyperion 3000 FTIR microscope, with a liquid nitrogen cooled 64×64 element FPA detector and a matching 15× objective and condenser (NA = 0.40), coupled to a Bruker Vertex 70 FTIR spectrometer with an internal thermal (Globar™) IR source. Each FPA-FTIR image (4×4 grid) was acquired within the 4,000–800 cm⁻¹ spectral region with a sampling area of 180×180 µm². The spectral images were collected with 8 cm⁻¹ resolution, 64 co-added scans, Blackman-Harris 3-term apodization, Power-Spectrum phase correction, and
a zero-filling factor of two, which were set as default acquisition parameters using the OPUS 7.2 imaging software suite (Bruker). Background spectra were measured using the same acquisition parameters, by focusing on a clean surface area of the CaF2 window without any tissue. FPA-FTIR images were pre-processed for an atmospheric compensation, baseline correction (concave rubberband algorithm) and vector normalization. The spatial distribution of lipids were generated based on integrated areas under the lipid region (3,000–2,800 cm⁻¹). Spectra localized to the bronchial epithelium (104 spectra for saline, 156 spectra for OVA-VEH and 114 spectra for OVA-LSF) were extracted from the FPA-FTIR images. The extracted spectra were then aged and converted into its corresponding second derivative using a 25 smoothing point Savitzky-Golay algorithm in OPUS 7.2 software (Bruker). The derivatization process, specifically second derivatization, has been widely used in spectroscopic analysis because this mathematical approach not only eliminates the baseline effect, but also allows accurate detection and positive identification of band components that are hidden in the presence of broad overlapping spectral features.

**HDAC8 activity profile.** To determine the in vitro HDAC inhibitor capacity of LSF against the metal-dependent HDAC1-11 enzymes, direct fluorescence-based enzymatic inhibition assays were performed by Reaction Biology Corp. (Malvern, PA, USA) using the fluorogenic peptides for HDAC1-11 enzymes, direct fluorescence-based enzymatic inhibitor capacity of LSF against the metal-dependent HDAC8 activity profile. 

**Chemokine and cytokine multiplex bead assay.** Chemokines and cytokine production from mononuclear cells was measured by multiplex array. PBMCs were incubated with LSF (15 µM and 30 µM), and SAHA (10 µM) or left untreated for 24 h at 37 °C, 5% (v/v) CO₂. Supernatants were harvested following centrifugation (800 g; 10 min) and measured for the presence of IL-1β, IL-6, IL-8, IP-10, MIP-1β, and TNF-α using the multiplex bead assay (Bio-Rad Laboratories Inc. Hercules, CA, USA) according to the manufacturers’ procedures. All supernatants were assayed undiluted, in triplicate. Briefly, a standard curve was prepared using fourfold serial dilutions of the cytokine standards provided using RPMI-FCS. Assay controls were prepared by adding known concentrations of the cytokine standard using a 1:2 and 1:4 dilution in RPMI-FCS. Culture medium was used as a negative control. Anti-cytokine beads and matched anti-cytokine biotinylated reporters were measured on a Luminex 200 Bio-analyzer, and data were analyzed in LuminexIS v2.3 software (Luminex Corporation, Austin, TX, USA), and concentrations reported in pg/ml.

**RNA-Seq.** PBMCs were cultured with or without 15 µM LSF for 24 h prior to RNA extraction, which comprised of TRIzol (Invitrogen) cell lysis followed by RNeasy (Qiagen, Hilden, Germany) column purification with on-column DNase treatment. Equal amounts of purified RNA (5 mg per sample) were used in the construction of sequencing libraries (RNA-sequencing library preparation kit, Illumina, San Diego, CA, USA). Sequencing of RNA-Seq libraries was performed on a Genome Analyzer IIX with a 36-cycle (v4) sequencing kit (Illumina). Sequence reads were extracted using Pipeline (v1.6) software (Illumina) before alignment (GRCh38 cDNA reference, release-101, Ensembl) and quantification of transcript counts using Kallisto quant (v0.45.0) [45]. Transcript level counts were aggregated to genes, before sample normalization and differential expression analysis with Deseq2 (v1.28.1) [46]. Differential genes were ranked by significance and fold change as described previously [47], prior to gene set enrichment analysis (GSEA) of gene ontology (GO) gene sets (v7.2) [48], using ‘mitch’ (v1.0.8) [49] with Mitch settings: priority = ‘effect.’ Heat maps and differential expression plots for RNA data were generated using ggplot2 (v3.3.2) [50].

**Western blotting.** To determine the effects of LSF on the expression of class I and II HDAC enzymes, protein was assessed in A549 cells using immunoblotting techniques as previously described [51]. Briefly, A549 cells were treated with 15 µM LSF and 10 µM SAHA for 24 h prior to cell lysis using mammalian protein extraction reagent (MPER, Thermo Fisher Scientific). Protein was measured using the
exhaustiveness of 2048. The top 10 predicted LSF binding potential binding sites. The searches were conducted with an ing the entire protein surface for an exhaustive search of dimensions x-size: 77, y-size: 60, and z-size: 60, encompass-
ligand search grid, identical for all HDACs modeled, with
assumed to be rigid. AutoDockTools was used to define the
were processed using AutoDockTools (v1.5.6) [62] to cre-
formed using Autodock Vina. All HDAC and LSF PDB files
[61] using default settings base [60] and energy minimized with Open Babel (v2.2.3)
structures were obtained from the PubChem data -
energy gradient convergence criterion of 0.01 kcal/mol/Å. All HDAC structures were
loops were filled in using Modeller (v10.2) to generate five
structures from the RCSB Protein Data Bank (PDB) exemplifying three distinct conformations of HDAC8 co-crystallized with different inhibitors (PDB ID: 1T67, 1T64, 1VKG) were used [53]. Docking to HDAC1, HDAC2 and HDAC3 were performed on PDB ID: 4BKX, 4LY1 and 4A69, respectively [54–56]. Due to its role in the regulation of α-tubulin acetylation, HDAC6 was also studied. Docking was performed on the catalytic domain 2 of HDAC6 using PDB ID: 5EDU [57]. For each HDAC structure, the first protein chain was isolated, water molecules and other ligands were removed, and the zinc atom was retained for subsequent docking calculations. For two of the HDAC8 structures (PDB ID: 1T67 and 1VKG), missing loops were filled in using Modeller (v10.2) to generate five models, selecting the structure with the lowest zDOPE score [58]. All HDAC structures were energy-minimized using GROMACS (v2018.4) [59] with the GROMOS forcefield and 53A6 parameter set. Each HDAC structure was energy minimized using the steepest descent algorithm, and an energy gradient convergence criterion of 0.01 kcal/mol/Å. Ligand structures were obtained from the PubChem database [60] and energy minimized with Open Babel (v2.2.3) [61] using default settings. Docking calculations were performed using Autodock Vina. All HDAC and LSF PDB files were processed using AutoDockTools (v1.5.6) [62] to create their corresponding PDBQT files. All rotatable torsions for the ligands were activated, while HDAC structures were assumed to be rigid. AutoDockTools was used to define the ligand search grid, identical for all HDACs modeled, with dimensions x-size: 77, y-size: 60, and z-size: 60, encompassing the entire protein surface for an exhaustive search of potential binding sites. The searches were conducted with an exhaustiveness of 2048. The top 10 predicted LSF binding poses for each HDAC model were visualized and analyzed using Visual Molecular Dynamics (v1.9.2) (VMD, Beckman Institute, Urbana, IL, USA) and Maestro (v2020-1) (Maes-
Statistics. All data are expressed as mean ± SEM, where each n represents a single mouse (> five airways per mouse unless otherwise specified) or a single blood donor (n= 4). Groups were compared using a one-way analysis of variance (ANOVA) with a 95% confidence interval and Tukey post hoc test where appropriate using GraphPad Prism, v7.01 (GraphPad).

Results

Protective effects of L-sulforaphane in the ovalbumin-induced model of allergic airways disease

We used the well-established OVA-induced chronic model of AAD to investigate protection and reversal of pathological features associated with OVA sensitization by LSF (Figs. 1A, B and 2A). As a positive HDAC inhibitor control, we also explored the protective effects of the FDA approved HDAC inhibitor SAHA (Fig. 1A) [63]. Using H&E staining, we observed epithelial thickening, increased goblet cell metaplasia and inflammatory cells peripheral to the bronchial epithelium and basement membrane in sensitized and challenged mice (OVA-vehicle; OVA-VEH) (Fig. 1C). The membrane apoptotic marker, Annexin V, was used to assess for apoptosis in the bronchial epithelium (Fig. 1D), and morphometric analysis was used to examine epithelial (Fig. 1E) and subepithelial thickness (Fig. 1F). These findings highlighted the effects of OVA sensitization and challenge in the OVA-VEH group compared to the saline control mice. LSF attenuated the pathological features associated with OVA-VEH sensitization, whereas SAHA was not effective in this model (Fig. 1G).

L-sulforaphane reverses ovalbumin-induced allergic airways disease

Histological staining was performed to assess for structural changes including epithelial thickness, goblet cell metaplasia and inflammatory infiltrate (Fig. 2B). Similar to the prevention model, we observed goblet cell metaplasia and inflammatory cell infiltrate in the OVA-VEH-sensitized and challenged mice compared to saline control mice and mice treated with LSF. An array of histological stains was performed to assess collagen and reticular fibers in the reversal model (Fig. 2B).

Masson’s trichrome stain was used to highlight the overall change in collagen deposition and changes to epithelial
and subepithelial thickness highlighting the beneficial effects of LSF (Fig. 2Bii). Silver impregnation of collagen and reticular fibers showed weak staining in the saline- and LSF-treated mice in comparison to the OVA-VEH group (Fig. 2Biii). Gomori’s aldehyde-fuchsin was used to highlight elastic fibers as seen in the fibers surrounding the blood vessels (Fig. 2Biv). We observed an increase in elastic fibers surrounding the smooth muscle in the OVA-VEH-sensitized mice that was not present in the saline control or mice treated with LSF. Picrosirius red staining was used to show the differential changes in type I and type III collagen deposition to determine the efficacy of LSF treatment in reducing the deposition of type I collagen observed in sensitized airways (Fig. 2Bv). Morphometric analysis of Masson’s trichrome-stained airways showed LSF significantly attenuated the increases in epithelial thickness (Fig. 2C) and subepithelial thickness (Fig. 2D). Analysis of sirius red-stained airways showed collagen type III deposition was not affected in the OVA-VEH sensitized mice (Fig. 2E). In contrast, collagen type I was significantly increased following OVA-VEH sensitization compared to saline control mice. Treatment of sensitized mice with LSF significantly reduced the augmentation of collagen type I. To assess AHR, mice were challenged with nebulized MCh, and the change in resistance from baseline was measured by invasive plethysmography (Fig. 2F). MCh-induced AHR was significantly elevated in OVA-VEH mice at doses above 12.5 mg/ml MCh compared to saline mice. These effects were significantly attenuated by LSF mice at doses above 25 mg/ml MCh compared to the OVA-VEH-sensitized mice.

Molecular mechanisms associated with protection and reversal of ovalbumin-induced allergic airways disease by L-sulforaphane

Immunohistochemical staining of α-smooth muscle actin (α-SMA) was used to identify activation of the fibrotic pathway (Fig. 3Ai). The results showed that α-SMA stained myofibroblasts in the lamina propria was significantly elevated in the OVA-VEH-sensitized mice compared to saline control mice. Immunohistochemical images were quantitated to represent myofibroblasts per 100 μm of basement membrane length (Fig. 3Aii). The increases in the myofibroblasts observed in the OVA-VEH-sensitized mice were significantly reduced by treatment with LSF. Downregulation of the expression of Caveolin-1 in OVA-VEH-sensitized mice was evident by immunohistochemistry (Fig. 3Bi). Analysis of the total area of Caveolin-1 expression highlights the significant decrease in OVA-VEH-sensitized mice compared to the saline controls and OVA-LSF treated mice (Fig. 3Bii).

We further assessed airway contraction to MCh in precision cut lung slices from OVA-challenged mice exposed to LSF ex-vivo. Representative traces measuring the percentage of the airway lumen from the initial area in lung slices from the OVA-VEH mice showed bronchial contraction following perfusion of MCh at a dose of 300 nM (Fig. 3C). Directly following a five-minute perfusion of MCh to induce airway narrowing, LSF was perfused for the same period. Airway dilation was achieved after approximately one minute of perfusion, in a concentration-dependent manner up to 15 μM LSF (Fig. 3Ci) and 30 μM LSF (Fig. 3Cii).

Focal plane array-Fourier transform infrared (FPA-FTIR) microspectroscopy was used to investigate changes in spatial distribution of the lipid compositions. As shown in the chemical images (Fig. 3Di), lung sections of the OVA-sensitized mouse presented higher intensities of lipids in the bronchial epithelium, compared to those observed for the saline and OVA-LSF mice. The average absorbance and second derivative spectra were calculated from spectra taken from the bronchial epithelium (Fig. 3Dii and Diii). In this study, absorbance and second derivative spectra were used together for comparing specific lipid bands in the high-wavenumber region. The main spectral features in the high-wavenumber region include the broad ν(O–H) stretching band centred at ~3285 cm⁻¹, and ν(CH₂/CH₃) stretching modes from methylene/methyl groups of lipids, which are presented as triplet bands within 3000–2800 cm⁻¹ spectral range. By a comparison, the ν(O–H) stretching band was shifted from 3290 cm⁻¹ in the saline-treated control mouse, to 3292 and 3284 cm⁻¹ in the OVA-VEH and OVA-LSF mice groups respectively, suggesting that OVA treatment caused an alteration of intra- and intermolecular H-bonding in the lung tissue. There was no significant peak shift observed for the ν(CH₂/CH₃) stretching modes. However, the FPA-FTIR images of lipids clearly reveal changes in the intensity of these ν(CH₂/CH₃) absorption peaks, showing a decrease in lipid components in the OVA-LSF mice, and an increase of the lipid components in the OVA-VEH mice when compared with the saline group (Fig. 3Dii).

L-sulforaphane modulates key antioxidant defense pathways

To confirm LSF as an inducer of phase II detoxifying enzymes, immunofluorescence was used to detect the presence of Nrf2 in the prevention and reversal models (Fig. 4A). Strong staining of Nrf2 was observed in both models in OVA-LSF treated mice. Analysis of the total fluorescence (FL) and foci formation count highlighted significant increases on OVA-LSF treated mice in the prevention (Fig. 4Bi and Bii) and reversal model (Fig. 4Biii and Biv). To investigate the localization of Nrf2 foci, line scan analysis of DAPI and Nrf2 staining patterns was performed (Fig. 4C). Nrf2 was found in areas of strong DAPI staining associated to heterochromatin in saline and OVA-VEH-sensitized mice and elevated staining...
Fig. 3 Molecular effects of L-sulforaphane in the chronic allergic airways disease model. Immunohistochemistry was performed on formalin-fixed paraffin-embedded mouse lung tissue derived from the reversal model. Representative photomicrographs of α-smooth muscle actin (α-SMA) expression (Ai) and immunohistochemical morphometric analysis (Aii) show staining for α-SMA in the myofibroblasts in the lamina propria was significantly elevated in ovalbumin-vehicle (OVA-VEH) mice compared to saline and ovalbumin-L-sulforaphane (OVA-LSF) mice. LSF significantly attenuated the reduced expression of Caveolin-1 following OVA sensitization in OVA-VEH mice as observed by immunohistochemical images of Caveolin-1 (Bi) and morphometric analysis in bronchial epithelium (Bii). Data shown as the mean ± SEM; *p < 0.05 and ***p < 0.001.

The comparison of small airway responses to methacholine (MCh) in precision cut lung slices from OVA-VEH mice (C). Representative traces show bronchial constriction as a percent of the initial lumen area in response to 300 nM MCh and subsequent bronchial dilation following a step-wise increase in concentrations of LSF to a maximum of 15 µM (Ci) and 30 µM (Cii). Focal plane array-Fourier-transform infrared spectroscopy (FPA-FTIR) chemical images of lipid distributions of lung sections derived from the reversal model (Di). Average absorbance spectra (obtained from > 100 individual spectra) from the bronchial epithelium (Di) and their corresponding second derivative spectra (Diii) highlight key changes in spectral patterns in lipid regions.

 Springer
sometimes outside of heterochromatic regions was seen in OVA-LSF treated mice. The expression of the antioxidant SOD2 was assessed using immunohistochemistry in the reversal model and OVA-LSF reversed OVA-induced reduction in the expression levels (Fig. 4D). Expression of HO-1 was investigated via immunofluorescence in both models (Fig. 4Ei). These findings indicated that LSF treatment of OVA-sensitized mice could reverse the significant elevation seen in OVA-VEH mice in both the prevention (Fig. 4Eii) and reversal models (Fig. 4Eiii).

**L-sulforaphane suppresses cytokine and chemokine secretion and downregulates pro-inflammatory genes**

Histologically (H&E)-stained lung sections were analyzed for inflammatory cell infiltrate. OVA sensitization resulted in a significant increase in inflammation compared to saline control mice. In both models, LSF-treated mice had significantly lower inflammation scores compared with OVA-VEH mice (Fig. 5A).

We used immunohistochemistry to determine if LSF would affect the expression of pro-fibrotic genes via the TGF-β/smad2 pathway. We showed LSF significantly increased Smad2 expression compared to saline and OVA-VEH mice (Fig. 5B).

We investigated cytokine and chemokine production from human PBMCs treated with or without LSF (15 µM, 30 µM) and 10 µM SAHA for 24 h using a multiplex bead assay (Fig. 5C). We showed LSF at both concentrations (15 µM, 30 µM) significantly attenuated the production of all cytokines and chemokines tested, while SAHA only significantly decreased the secretion of IL-1β (Fig. 5C).

To investigate whether these findings were reflected in the transcriptome, RNA-sequencing libraries were constructed using total RNA obtained from PBMCs from healthy donors (n = 4) following ex vivo treatment with or without 15 µM LSF for 24 h (Fig. 5D). Differentially expressed (DE) genes were determined using Deseq2 (n = 4, LSF: no LSF) and subjected to gene set enrichment analysis (GSEA) of GO gene sets, which were ranked by effect. Of the top 50 ranked gene sets shown (Fig. 5Di), only six were upregulated, while 44 sets were downregulated. We additionally filtered differential genes (FDR < 0.05) from the top 100 gene sets for key terms including chemokine, cytokine, immune and inflammation, all of which were predominately downregulated and most notable for CCL and CXCL chemokines (Fig. 5Dii). Genes encoding the proteins investigated in Fig. 5B and C are also highlighted as selected genes, which include: TNF (TNFα), SMAD2, IL6, IL1B (IL-1β), CXCL8 (IL-8) and CCL4 (MIP-1β).

**L-sulforaphane reduces the expression of histone deacetylase (HDAC) enzymes and inhibits HDAC activity, mediating hyperacetylation of histone and non-histone proteins**

To evaluate the epigenetic effects of LSF in airways, immunofluorescence was used to assess the acetylation status of histones (H2B, H3 & H4) and non-histone protein α-tubulin (Fig. 6A, B, and C). The findings indicated that LSF administration resulted in hyperacetylated histone H2B in both the reversal and prevention models (Fig. 6B) and hyperacetylated H3, H4 histones and α-tubulin in the reversal model (Fig. 6C). Furthermore, we explored the expression of HDAC8 using immunofluorescence in the prevention model and the results show that HDAC8 expression was significantly elevated in OVA-VEH-sensitized mice compared to saline mice and OVA-LSF treated mice (Fig. 6D). To further explore the effects of LSF on HDAC8, direct enzymatic inhibition assays indicated LSF inhibited HDAC8 activity, albeit, with a low potency compared to the positive control SAHA (EC50 = 92.07 ± 7.45 µM) (Fig. 6E). Immunoblot analysis of class I and class II HDAC enzyme expression in A549 lung cells treated with or without 15 µM LSF, validated that LSF decreased HDACs 6 and 8 expression, increasing acetylation of α-tubulin and histone H3 (Fig. 6F). The broad spectrum HDAC inhibitor, SAHA (10 µM) was used a positive control highlighting the comparatively modest HDAC inhibitory effects of LSF.

We used the molecular docking program, AutoDock Vina, to examine binding characteristics of LSF and its metabolites on the human class I HDACs: HDAC1, HDAC2, HDAC3 and HDAC8, and the class IIb HDAC6 [52]. We sought to investigate whether there are unique binding modes of LSF and its metabolites to HDAC8 that may explain the observed modulation of these enzymes by LSF compared to the other three members of the family. For docking to human HDAC8, we selected three structures that exemplified three distinct conformations due to the flexible loop B residues S30-K36, adjacent to the catalytic zinc (Fig. 6Gi) [53]. This flexibility occurs due to the HDAC8 sequence being two residues shorter than that of HDACs1-3, which have a more static binding pocket (Fig. 6Gi). In the partially and fully opened structures of HDAC8, a secondary pocket becomes accessible for ligand binding (Fig. 6Gii). Modulation of HDAC6 gene expression was also observed, thus further investigation of this protein was also warranted.

The classical pan-HDAC inhibitor SAHA was used as a positive control. Docking results show interactions consistent with observed enzyme inhibition behavior, with SAHA generally in proximity to the catalytic zinc ion with binding affinities ranging from −6.5 to −7.5 kcal/mol across almost all HDAC structures examined (Fig. 6Gii).
and iii). For the partially opened HDAC8 structure, all ligands, including SAHA, bound to the secondary pocket (Fig. 6Gii). While this second pocket has a hypothesized
Role in inhibition, these results show that ligand binding to HDAC8 may vary depending on the protein conformation [53].

While SAHA consistently bound to the catalytic site of the HDAC enzymes, LSF and its metabolites were more varied in its binding locations. The top three modes of the LSF ligands did not bind to the catalytic site on HDACs 1-3. For HDAC8, binding of LSF ligands in proximity to catalytic residues was only observed for the fully open conformation, with weaker binding affinities compared to the positive control (Fig. 6Giv). The metabolites LSF-NAC and LSF-GSH demonstrated binding to the active site of catalytic domain 2 in HDAC6 (Fig. 6Gv). These findings suggest that LSF metabolites may be able to selectively modulate HDAC6 and HDAC8, albeit with a moderate propensity compared to SAHA.

Overall, our findings highlighted the protective effects of LSF in the OVA-induced model of chronic AAD. Mechanisms involving antioxidant and anti-inflammatory pathways account for the beneficial effects of LSF. LSF downregulated the expression of HDAC enzymes and displayed modest HDAC inhibition effects. Hyperacetylation of core histones and α-tubulin following administration in vivo represented key epigenetic mechanisms associated with the effects of LSF in the model of chronic AAD investigated (Fig. 7).

Discussion

We examined the effects of LSF in the OVA-induced murine model of chronic AAD, and LSF was found to prevent and reverse the pathological features associated with the model (Figs. 1 and 2). The OVA-induced murine model is well characterized and recapitulates several hallmarks of chronic asthma [30, 32, 41]. Our findings highlighted that the key features associated with the model, including epithelial thickening, goblet cell metaplasia, apoptosis in the bronchial epithelium, and inflammatory cell infiltration were prominent in OVA-VEH-sensitized mice. In the reversal model, LSF treatment significantly reduced the increase in collagen type I deposition and connective tissue fibers that were observed in OVA-VEH-sensitized mice (Fig. 2). Likewise, LSF significantly reduced the expression of α-SMA in myofibroblasts in the lamina propria, attenuated the expression of Caveolin-1 induced by OVA, and significantly increased the expression of phosphorylated Smad2 (Fig. 5). In accordance with previous studies, our findings indicated that LSF protected from MCh-induced increases in airway resistance both in vivo (Fig. 2), and ex vivo (Fig. 3) [12].

Caveolins are integral membrane proteins that play an important role in cellular processes including membrane trafficking, signal transduction, endocytosis, transcytosis, and intracellular cholesterol transport [64, 65]. The caveolin proteins are coded for by the Cav-1, Cav-2, and Cav-3 genes in vertebrates and their expression levels differ between the tissues [66]. Caveolin-1 is expressed in cells of the airways and lungs such as lung fibroblasts, alveolar epithelial cells, airway smooth muscle, alveolar and epithelial cells, and pulmonary vasculature [65]. There is also evidence to suggest that Caveolin-1 is expressed in macrophages, neutrophils, dendritic cells, and lymphocytes [65]. Reduced Caveolin-1 expression has been found to be associated with asthma and chronic inflammatory respiratory diseases [64]. Compared with controls, reduced Caveolin-1 mRNA and protein expression has been observed in murine models of AAD and in asthma patients [67, 68]. In response to ovalbumin, Caveolin-1-deficient mice have been shown to develop asthma-like responses such as increased collagen deposition and enhanced AHR [67, 69–71]. Moreover, Caveolin-1 is associated with the maintenance of airway epithelial barrier function [69]. The reduced membrane expression of Caveolin-1 in patients with asthma resulted in a reduction of junctional E-cadherin and β-catenin expression, epithelial barrier dysfunction, and increased levels of thymic stromal lymphopoietin (TSLP) [69].

The antioxidant effects of LSF involving activation of phase II detoxification pathways have been widely investigated [12]. Additionally, the molecular mechanisms
Sulforaphane prevents and reverses allergic airways disease in mice via anti-inflammatory,…

**Fig. 5** L-sulforaphane reduces inflammation via down-regulation of pro-inflammatory genes. Ovalbumin-L-sulforaphane (OVA-LSF) significantly attenuates inflammation observed in the ovalbumin-vehicle (OVA-VEH) mice in both the prevention and reversal model (A). Inflammatory cell infiltration of hematoxylin and eosin stained sections of mouse airways was scored by two independent blinded investigators using the scale: 0 = no inflammation; 1 = 3 layers of inflammatory cells; 2 = 6 layers; 3 = 10 layers and congestion; or 4 = > 10 layers of inflammatory cells and severe congestion. Representative immunohistochemical photomicrographs detecting phosphorylated Smad2 (Bi) performed on formalin-fixed paraffin-embedded mouse lung tissue derived from the reversal model. Morphometric analysis of stained sections shows OVA-LSF mice had a significant increase of phosphorylated Smad2 (Bi) expression compared to the saline and OVA-VEH mice. The anti-inflammatory effect of LSF was explored in peripheral blood mononuclear cells (PBMC) from healthy adults (n=4). PBMCs were treated with the indicated concentrations of LSF and 10 μM of suberylanilide hydroxamic acid (SAHA) (C), the chromatin modifying control compound. An array of chemokines and cytokines were measured by the multiplex bead array assay as indicated. All data are represented as the mean ± SEM; *p < 0.05 **p < 0.01 and ***p < 0.001. RNA-Seq was performed on adult PBMCs cultured with or without 15 μM LSF for 24 h. Differentially expressed (DE) genes were subjected to gene set enrichment analysis (GSEA) of gene ontology (GO) gene sets and examined for effect (D). Heat map of the top 50 gene sets according to effect demonstrates predominant downregulation of sets related to immune response (Di). The sets as shown are ordered by GSEA enrichment score according to analysis of combined DE genes n=4 (first column); individual sample data are also shown (columns 2 to 5). Heat map color gradient indicates GSEA enrichment score. Differential expression of genes from selected gene sets (Di). Genes from the top 100 gene sets ranked by effect were filtered for key terms including chemokine, cytokine, immune and inflammation. Individual genes (FDR < 0.05) are shown with log2 fold change alongside their gene set and corresponding GSEA enrichment score.

that mediate crosstalk between Nrf2 and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), which regulate oxidative stress and inflammation, have been explored [18, 19, 72]. Our findings demonstrated that LSF treatment resulted in the modulation of Nrf2 (Fig. 4). A significant increase in the overall expression of Nrf2 and Nrf2 foci was observed in OVA-LSF treated mice and DAPI staining showed that Nrf2 was predominantly localized in heterochromatic regions (Fig. 4). This is in line with recent findings, which associated Nrf2 with DNA damage response pathways highlighting the nuclear accumulation of Nrf2 and formation of foci at sites of DNA damage [73, 74].

An interesting finding was the significant overexpression of HO-1 in OVA-sensitized mice, which was diminished by administration of LSF in both the prevention and reversal models (Fig. 4). Expression of HO-1 is significantly upregulated in response to a wide variety of stimuli causing oxidative stress [75]. Investigation of the canonical pathway indicated that the expression of HO-1 was modulated by Nrf2. Our findings indicated that sensitization of mice with OVA resulted in the overexpression of HO-1, which was most likely independent of the Nrf2 pathway. A probable explanation was that OVA sensitization created oxidative stress releasing stimuli that may have inhibited the transcriptional repressor BACH1, resulting in increased expression of HO-1 (Fig. 4). It is understood that inhibition of BACH1 is sufficient for activation of HO-1 irrespective of the status of Nrf2 [75]. This OVA sensitization-mediated overexpression of HO-1 was consistent with previous findings in the context of asthma [76]. Therefore, we can assume that LSF ameliorates oxidative stress stimuli induced by OVA, which cause the overexpression of HO-1. Consistent with activation of the Nrf2 pathway, LSF elevated the expression of the major detoxification enzyme SOD2, which was significantly diminished by OVA sensitization compared to control mice (Fig. 4).

Our findings indicated reductions of inflammatory infiltrates in the in vivo model studied (Fig. 5). In human PBMC we showed significant reductions in pro-inflammatory cytokines and chemokines following LSF treatment. This included IL-1β, IL-6, TNF-α, IL-8, IP-10, and MIP-1β [25, 29]. We compared cytokine and chemokine release with the FDA approved pan-HDAC inhibitor SAHA in PBMC cells [77]. Apart from IL-1β, there was no significant modulation of other cytokine or chemokine secretion by SAHA at the concentration (10 μM) investigated (Fig. 5). Furthermore, in gene set enrichment analysis of RNA-Seq data in human PBMC, 44 gene sets largely related to the immune response were downregulated and six gene sets were upregulated following treatment with LSF. These findings were consistent in all four samples. Genes encoding for the expression of cytokines and chemokines such as IL-6, IL-1A, IL-1B, EB13, CXCL5, and CXCL1, were primarily downregulated, highlighting the anti-inflammatory signature of LSF [25, 29, 78].

A particularly interesting observation was the increase in lipid deposition in the bronchial epithelium of OVA-VEH-sensitized mice, as indicated by FPA-FTIR chemical images and spectral analysis (Fig. 3). Increased wall thickness has been shown to exacerbate airway resistance and more recently, fat-associated airway remodeling has been identified as a potential contributing factor [79, 80]. The relationship between adipose tissue within the airway wall and body mass index (BMI) in non-asthmatics versus asthmatics has been examined [79]. White adipose tissue was present in the outer airway wall in all subject groups and was predominantly found in large to medium sized airways [80]. Our FPA-FTIR data revealed that there was a significant modulation of lipid components following LSF administration with lipid compositions in airways resembling those of the control group, which was distinct from the OVA-VEH-sensitized mice (Fig. 3).

Epigenetic mechanisms, predominantly chromatin modifications involving the acetylation-deacetylation axis, have
Sulforaphane prevents and reverses allergic airways disease in mice via anti-inflammatory,...

Figure 6: L-sulforaphane alters the expression and activity of HDAC enzymes. Lung sections stained with anti-acetylated histone (red) and anti-acetylated α-tubulin (green) show strong staining in ovalbumin-L-sulforaphane (OVA-LSF) mice (A). Merged image: nucleus stained with DAPI (blue), histonemodified acetylated H3 and H2B (red), acetylated α-tubulin (green). H2Bac was analyzed in both models (B); H3ac and acetylated α-tubulin analyzed in the prevention model (C). Histone deacetylase (HDAC) 8 expression investigated in the prevention model of chronic allergic airways disease (AAD) (D). LSF reduces the enzymatic activity of HDAC8; EC50 = 92.07 ± 7.45 μM (SAHA EC50 = 306 nM in the same assay) (E). Data represented as the mean ± SEM; *p < 0.05, **p < 0.01 and ***p < 0.001. Western blot shows 15 µM LSF decreases HDAC6 and 8 expression and increases acetylated α-tubulin and H3ac in A549 lung cells (positive control = 10 µM SAHA) (F). Molecular docking of LSF to HDAC enzymes (G). Aligned structures of HDAC8, for PDB ID: 1T64 (closed, dark green), 1T67 (partially open, medium green), and 1VKG (closed, light green), with loop B residues highlighted. Silver sphere represents Zn (Gi). Sequence alignment between class I HDAC enzymes (Gi). Top three poses of positive control SAHA (dark blue) to HDAC8 structures (Gi), and HDACs1-3 and HDAC6 (6Giii), with binding affinities of the top pose stated in kcal/mol. The secondary binding pocket is highlighted in maroon. Binding to this site is indicated by values shown in maroon. Top three poses of LSF and its metabolites (LSF, blue; LSF-Cys, peach; LSF-NAC, orange; LSF-GSH, light red) to the open conformation of HDAC8 (Gi) and catalytic domain 2 of HDAC6 (Gi), with binding affinities of the top pose in the catalytic site indicated in kcal/mol. Values in maroon indicate binding to the secondary pocket for HDAC8, and distant to the active site for HDAC6. Predicted binding pose of LSF-NAC to the catalytic site of HDAC8 (Gi) and HDAC6 (Gi) is shown, with residues labelled. Hydrogen bond formation is indicated by dashed grey lines.

been implicated in numerous disease states including asthma and chronic obstructive pulmonary disease [42, 81–83]. One of the main mechanisms described is the downregulation of the HDAC2 enzyme resulting in aberrant acetylation of the glucocorticoid receptor and resistance to corticosteroid therapy [84]. This may call the potential clinical utility of HDAC inhibitors in AAD into question. However, HDAC inhibitors have well-known pleiotropic properties including modulating inflammatory and antioxidant pathways, and have shown beneficial effects in numerous models of disease [47, 85–89]. This includes studies with the prototypical hydroxamic acid Trichostatin A and short-chain fatty acid, valproic acid, in models of AAD [39, 90–92]. Here, unlike Trichostatin A, we show that the hydroxamic acid HDAC inhibitor SAHA was not effective in modulating airway responses in our model, further highlighting the complexities associated with the use of pleiotropic pan-HDAC inhibitors in airways disease (Fig. 1).

Our findings indicated that LSF modulated the acetylation of histones 2B and H3, and α-tubulin in the mouse model studied (Fig. 6). This is in accordance with previous work, which has indicated that SFN possesses HDAC inhibitor activity in vitro and in vivo [93–95]. Using in vitro enzymatic assays and in A549 human alveolar epithelial cells, we show that LSF preferentially inhibits HDAC enzymatic activity with very modest potency in comparison to the classical pan-HDAC inhibitor SAHA. Molecular modeling shows that LSF and its metabolites may modulate HDAC6 and HDAC8 enzymes by binding to the catalytic site (Fig. 6). While LSF metabolites are shown to bind to the active site, this is dependent on the conformation of HDAC8, suggesting that inhibitory effects may be modest. Inhibition of the expression and activity of HDAC8 by LSF is a particularly interesting observation, as it has been previously shown that a potent specific HDAC8 inhibitor (PCI-34501), attenuated airway responses in OVA-sensitized mice [96].

A limitation of our study is that the potential effects of key SFN metabolites were not explicitly investigated. We have recently shown that although not as potent as LSF, metabolites such as SFN-Cys and SFN-NAC, have important roles in anti-inflammatory pathways [29]. Here, we show acetylation of α-tubulin following in vivo administration of LSF, indicating modulation of HDAC6 (Fig. 6) [97]. According to previous observations, SFN-Cys is the metabolite that most likely possesses HDAC inhibition activity [98–100]. Therefore, it is worthy to systematically investigate the biological effects and bioactivity of SFN metabolites. However, these considerations do not detract from our findings which demonstrate accumulation of hyperacetylated histones and α-tubulin following administration of LSF in vivo (Fig. 6).

A further improvement of our study could be to examine the effects of LSF on the TGF-β/Smad signaling at different concentrations and timepoints. The role of Smad2 in fibrogenesis is complex. Our findings indicate that the expression of phosphorylated Smad2 was significantly increased compared to OVA-VEH and saline groups following the treatment with LSF (Fig. 5). In studies investigating renal and hepatic fibrosis, the overexpression of Smad2 was found to protect against TGF-β1-induced Smad3 phosphorylation and attenuated collagen matrix expression [101, 102]. Conversely, it has been reported that sulforaphane attenuates the increased phosphorylation of Smad2 upon treatment with TGF-β1 and is capable of reducing TGFβ-induced activation of myofibroblasts, as well as the production of extracellular matrix [103]. Sun et al. observed that sulforaphane inhibited the TGF-β1/Smad signaling pathway, ameliorated the phosphorylation of Smad2 and Smad3, and suppressed profibrogenic gene and protein expressions in an Nrf2-dependent manner [104]. As aforementioned, the conflicting findings regarding the effects of LSF on the regulation of Smad require further clarification in the context of chronic AAD.

Overall, our findings confirmed the efficacy of LSF in attenuating pathologies associated with AAD, involving activation of antioxidant and anti-inflammatory pathways (Fig. 7). Inhibition of HDAC enzymes by LSF and accumulation of acetylated core histones and α-tubulin in vivo following LSF administration represent an important epigenetic regulatory mechanism. Sulforaphane and high
GLUCORAPHANIN EXTRACTS HAVE HAD MIXED RESULTS IN HUMAN TRIALS AND CURRENTLY, THERE ARE NO THERAPEUTIC USES OF SFN OR EXTRACTS IN THE CLINIC TO DATE. HOWEVER, OUR FINDINGS ALONG WITH ACCUMULATED EVIDENCE, HIGHLIGHT THE CLINICAL POTENTIAL OF SFN AS EITHER A PROPHYLACTIC OR A THERAPEUTIC IN THE CONTEXT OF AAD. A PHARMACEUTICAL GRADE STABLE FORM OF LSF (SUCH AS SFX-01, EVGEN PHARMA [105, 106]), OR OPTIMIZED GLUCORAPHANIN-RICH EXTRACTS MAY ENABLE CLINICAL TRANSLATION.

Acknowledgements  We acknowledge the intellectual and financial support from McCord Research (Iowa, USA). The authors would like to acknowledge the use of the facilities provided by Monash University (Clayton, VIC, Australia) and the Murdoch Children’s Research Institute (Parkville, VIC, Australia) for their care and husbandry of the mice. The Gomori’s aldehyde-fuchsin and silver impregnation stains were performed by Ms Laura Leone at the Melbourne University Histology Platform (University of Melbourne, School of Biomedical Sciences, Parkville, VIC, Australia). The authors would like to acknowledge the use of the facilities provided by Monash Micro Imaging (MMI) at the Alfred Research Alliance (ARA, Melbourne, VIC, Australia) and, particularly, the expert assistance from Drs Stephen Cody and Iska Carmichael. FPA-FTIR imaging measurements were undertaken at the IRM beamline at the Australian Synchrotron, part of ANSTO (Clayton, VIC, Australia). Various figures in this manuscript were created with BioRender.com. We thank the National Computing Infrastructure (NCI), and the Pawsey Supercomputing Centre in Australia (funded by the Australian Government). Further, we thank the Spartan High Performance Computing service (University of Melbourne), and the Partnership for Advanced Computing in Europe (PRACE) for awarding the access to Piz Daint, hosted at the Swiss National Supercomputing Centre (CSCS), Switzerland. We acknowledge our use of the gene set enrichment analysis, GSEA software, and Molecular Signature Database (MSigDB) (Subramanian, Tamayo, et al. (2005), PNAS 102, 15545-15550, http://www.broad.mit.edu/gsea/).

Author contributions  SGR, JEB, AH, PVL, CSS, KJS, MLKT, AE, and TCK conceptualized the ideas and overarching aims. JEB, AH, PVL, CSS, KJS, MJT, JV, MLKT, AE, and TCK were involved in supervision. KV, CD, AH, IK, JLL, SM, NM, EP, MJT, JV, and MZ participated in the development and design of the methodology. KV.

Fig. 7 Mechanisms associated with the attenuation of ovalbumin-induced allergic airways disease by L-sulforaphane. Summary of the changes that occur to normal bronchioles in the ovalbumin (OVA)-induced chronic murine model of allergic airways disease (AAD) as observed in this study. The mechanisms associated with prevention and reversal of the pathobiology by L-sulforaphane (LSF) are depicted; antioxidant, anti-inflammatory and epigenetic mechanisms of action are shown. Methodologies used to detect changes are indicated in brackets; IF = immunofluorescence.
CD, IK, SM, NM, MIT, JV, and MZ conducted the research and investiga-
tion process. RCB, AH, JLL, EP, YYS, and MZ were responsible for
visualization and KV, EP, SGR, PVL, and TCK were involved in
production of the first draft of the manuscript.

Funding We would like to acknowledge intellectual and financial
support by McCord Research (Iowa, USA). AEO is supported by an
National Health and Medical Research Council (NHMRC) Senior
Research Fellowship (1154650). PVL is supported by an NHMRC
Career Development Fellowship (1146198). CD is supported by an
NHMRC Early Career Postdoctoral Fellowship (1120152). JLL is
supported by an Australian Government Research Training Program
Scholarship.

Data and materials availability RNA sequencing data are available
from GEO under the accession GSE160353. To review GEO access-
sion GSE160353 while it remains in private status, go the following
address and enter the access token: otmclcuoiwhjd. https://www.ncbi.
Data and materials availability

Declarations

Competing interests Epigenomic Medicine Program (TCK) was sup-
ported financially by McCord Research (Iowa, USA), which has a
financial interest in dietary compounds including sulforaphane. The
remaining co-authors declare that they have no direct financial relation
with the commercial identities mentioned in this manuscript that might
lead to a conflict of interest.

Ethics approval This study was performed in line with the principles of
the Declaration of Helsinki. Six-week-old female BALB/c mice were
obtained from Walter and Eliza Hall Institute Bioservices (preven-
tion model; Parkville, VIC, Australia) and Monash Animal Services (rever-
sal model; Clayton, VIC, Australia). The prevention model experi-
mental protocol was approved by the Murdoch Children’s Research
Institute Animal Ethics Committee (approval no. A597). The reversal
model experimental protocol was approved by the Monash Univer-
sity Animal Ethics Committee (MAP/R/2012/085). All experimental
procedures followed the Australian guidelines for the care and use of
laboratory animals for scientific purposes. Human peripheral blood
mononuclear cells (PBMC) were fractionated using Ficoll Paque (GE
Healthcare, Wauwatosa, Wisconsin, USA) from blood samples (healthy
participants; n=4) obtained from the Australian Red Cross Blood Bank
(Melbourne, VIC, Australia) under ethics project (#304/12) approved
by the Alfred Hospital Ethics Committee (Alfred Health, Melbourne,
VIC, Australia). Human epithelial lung A549 cells were purchased
from the American Type Culture Collection (ATCC, Manassas, VA,
USA).

References

1. Zhang Y et al (1992) A major inducer of anticarcinogenic protec-
tive enzymes from broccoli: isolation and elucidation of struc-
ture. Proc Natl Acad Sci USA 89(6):2399–2403
2. Prochaska HJ, Santamaria AB, Talalay P (1992) Rapid detection
of inducers of enzymes that protect against carcinogens. Proc
Natl Acad Sci USA 89(6):2394–2398
3. Fahey JW et al (2015) Sulforaphane bioavailability from gluc-
oraphanin-rich broccoli: control by active endogenous myrosi-
nase. PLoS ONE 10(11):e0140963–e0140963
4. Angelino D et al (2015) Myrosinase-dependent and -indepen-
dent formation and control of isothiocyanate products of
glucosinolate hydrolysis. Front Plant Sci. https://doi.org/10.
3389/fpls.2015.00831
5. Matusheshi NV, Jeffery EH (2001) Comparison of the bioac-
tivity of two glucoraphanin hydrolysis products found in broc-
coli, sulforaphane and sulforaphane nitrile. J Agric Food Chem
49(12):5743–5749
6. Tortorella SM et al (2015) Dietary sulforaphane in cancer
chemoprevention: the role of epigenetic regulation and HDAC
inhibition. Antioxid Redox Signal 22(16):1382–1424
7. Vanduchova A, Anzenbacher P, Anzenbacherova E (2018) Iso-
thiocyanate from broccoli, sulforaphane, and its properties. J
Med Food 22(2):121–126
8. Amjad AI et al (2015) Broccoli-derived sulforaphane and
chemoprevention of prostate cancer: from bench to bedside.
Current Pharmacology Reports 1(6):382–390
9. Zimmerman AW et al (2021) Randomized controlled trial of
sulforaphane and metabolite discovery in children with Autism
Spectrum Disorder. Molecular Autism 12(1):38
10. Sun Y et al (2020) Protective effects of sulforaphane on type 2
diabetes-induced cardiomyopathy via AMPK-mediated activa-
tion of lipid metabolic pathways and NRR2 function. Metabol
Clin Experim 102:154002
11. Singh K et al (2014) Sulforaphane treatment of autism
spectrum disorder (ASD). Proc Natl Acad Sci USA 111(43):15550–15555
12. Brown RH et al (2015) Sulforaphane improves the bronchopro-
tective response in asthmatics through Nrf2-mediated gene path-
ways. Respir Res 16(1):106
13. Jiao Z et al (2017) Sulforaphane increases Nrf2 expression and
protects alveolar epithelial cells against injury caused by cigarette
smoke extract. Mol Med Rep 16(2):1241–1247
14. Cho H-Y et al (2019) Sulforaphane enriched transcriptome of
lung mitochondrial energy metabolism and provided pulmonary
injury protection via Nrf2 in mice. Toxicol Appl Pharmacol
364:29–44
15. Sudini K et al (2016) A randomized controlled trial of the effect
of broccoli sprouts on antioxidant gene expression and airway
inflammation in asthmatics. J Allergy Clin Immunol Practice. 4(5):932–940
16. An SS et al (2016) An inflammation-independent contrac-
tion mechanophenotype of airway smooth muscle in asthma. J
Allergy Clin Immunol 138(1):294–297.e4
17. Heber D et al (2014) Sulforaphane-rich broccoli sprout extract
attenuates nasal allergic response to diesel exhaust particles.
Food Funct 5(1):35–41
18. Mazarakis N et al (2020) The potential use of l-sulforaphane for
the treatment of chronic inflammatory diseases: A review of the
clinical evidence. Clin Nutr 39(3):664–675
19. Houghton CA (2019) Sulforaphane: its “coming of age” as a
clinically relevant nutraceutical in the prevention and treatment of
diabetes-induced cardiomyopathy via AMPK-mediated activa-
tion of glucose metabolism. J Diabetes Metab Drug Targets
19(2):83–91
20. Fahey JW, Kensler TW (2021) The challenges of designing and
implementing clinical trials with broccoli sprouts... and turning
evidence into public health action. Front Nutr 8:183
21. Kensler TW et al (2012) Modulation of the metabolism of air-
borne pollutants by broccoli sprout beverage: results of a rand-
omized clinical trial in China. Carcinogenesis 33(1):101–107
22. Egner PA et al (2014) Rapid and sustainable detoxication of air-
borne pollutants by broccoli sprout beverage: results of a rand-
omized clinical trial in China. Cancer Prevent Res (Philadelphia PA). 7(8):813–823
23. De Rooij M, Jan NM, Commandeur Nico PE (1998) Vermeulen
BEN Mercapturic acids as biomarkers of exposure to electro-
philic chemicals: applications to environmental and industrial
chemicals. Biomarkers. 3(4–5):239–303
24. Keum Y-S (2012) Regulation of Nrf2-mediated phase II detoxification and anti-oxidant genes. Biomol Therapeut 20(2):144–151
25. Al-Harbi NO et al (2019) Sulforaphane treatment reverses corticosteroid resistance in a mixed granulocytic mouse model of asthma by upregulation of antioxidants and attenuation of Th17 immune responses in the airways. Eur J Pharmacol 855:276–284
26. Abdull Razis AF, Iori R (2011) Ioannides C The natural chemopreventive phytochemical R-sulforaphane is a far more potent inducer of the carcinogen-detoxifying enzyme systems in rat liver and lung than the S-isomer. Int J Cancer 128(12):2775–2782
27. Abdull Razis AF et al (2011) Induction of epoxide hydrolase and glucuronosyl transferase by isothiocyanates and intact glucosinolates in precision-cut rat liver slices: importance of side-chain substituent and chirality. Arch Toxicol 85(8):919–927
28. Srovnałova A et al (2015) Effects of sulforaphane and its S- and R-enantiomers on the expression and activities of human drug-metabolizing cytochromes P450. J Funct Foods 14:487–501
29. Mazarakis N et al (2021) Examination of novel immunomodulatory effects of L-sulforaphane. Nutrients. 13(2):6002
30. Royce SG, Patel KP, Samuel CS (2014) Characterization of a novel model incorporating airway epithelial damage and related fibrosis to the pathogenesis of asthma. Lab Invest 94(12):1326–1339
31. Casaro M et al (2019) OVA-induced allergic airway inflammation mouse model, in Pre-Clinical Models: Techniques and Protocols. P.C. Guest, Editor. Springer New York: New York, NY. p. 297–301
32. Kim DI, Song M-K, Lee K (2019) Comparison of asthma phenotypes in OVA-induced mice challenged via inhaled and intranasal routes. BMC Pulm Med 19(1):241
33. Park JH et al (2012) Sulforaphane inhibits the Th2 immune response in ovalbumin-induced asthma. BBM Rep 45(5):311–316
34. Wu W et al (2019) Sulforaphane has a therapeutic effect in an atopic dermatitis murine model and activates the Nrf2/HO1 axis. Metabolites 15(6):4005–4014
35. Yan B et al (2017) Sulforaphane prevents bleomycin-induced pulmonary fibrosis in mice by inhibiting oxidative stress via nuclear factor erythroid 2-related factor-2 activation. Mol Med Rep 15(6):4005–4014
36. Kim H et al (2022) A metabolomics approach to sulforaphane efficacy in secondhand smoking-induced pulmonary damage in mice. Metabolites 12(6):518
37. Melgert BN et al (2005) Female mice are more susceptible to the development of allergic airway inflammation than male mice. Clin Exp Allergy 35(11):1496–1503
38. Hayashi T et al (2003) Less sensitivity for late airway inflammation in males than females in BALB/c mice. Scand J Immunol 57(6):562–567
39. Royce SG et al (2011) Protective effects of valproic acid against airway hyperresponsiveness and airway remodeling in a mouse model of allergic airways disease. Epigenetics 6(12):1463–1470
40. Royce SG et al (2009) Relaxin reverses airway remodeling and airway dysfunction in allergic airways disease. Endocrinology 150(6):2692–2699
41. Mazarakis N et al (2020) Investigation of molecular mechanisms of experimental compounds in murine models of chronic allergic airways disease using synchrotron Fourier-transform infrared microspectroscopy. Sci Rep 10(1):11713
42. Khurana I et al (2021) SAHA attenuates Takotsubo-like myocardial injury by targeting an epigenetic Ac/Dc axis. Signal Transduct Target Ther 6(1):159
43. Donovan C et al (2013) Differential effects of allergen challenge on large and small airway reactivity in mice. PLoS ONE 8(9):e74101
44. Donovan C et al (2015) Lipopolysaccharide does not alter small airway reactivity in mouse lung slices. PLoS ONE 10(3):e0122069
45. Bray NL et al (2016) Near-optimal probabilistic RNA-seq quantification. Nat Biotechnol 34(5):525–527
46. Love MI, Huber W, Anders S (2014) Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol 15(12):550
47. Rafiei H et al (2014) Vascular histone deacetylation by pharmacological HDAC inhibition. Genome Res 24(8):1271–1284
48. Mostha VK et al (2003) PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. Nat Genet 34(3):267–273
49. Kaspi A, Ziemann M (2020) mitch: multi-contrast pathway enrichment for multi-omics and single-cell profiling data. BMC Genom 21(1):447
50. Wickham HD (2016) ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag, New York
51. Ververis K, Karagiannis TC (2012) An atlas of histone deacetylase expression in breast cancer: fluorescence methodology for comparative semi-quantitative analysis. Am J Transl Res 4(1):24–43
52. Trott O, Olson AJ (2010) AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. J Comput Chem 31(2):455–461
53. Somoza JR et al (2004) Structural snapshots of human HDAC8 provide insights into the class I histone deacetylases. Structure 12(7):1325–1334
54. Watson PJ et al (2012) Structure of HDAC3 bound to co-repressor and inositol tetraphosphate. Nature 481(7381):335–340
55. Millard CJ et al (2013) Class I HDACs share a common mechanism of regulation by inositol phosphates. Mol Cell 51(1):57–67
56. Lauffer BE et al (2013) Histone deacetylase (HDAC) inhibitor kinetic rate constants correlate with cellular histone acetylation but not transcription and cell viability. J Biol Chem 288(37):26926–26943
57. Hai Y, Christianson DW (2016) Histone deacetylase 6 structure and molecular basis of catalysis and inhibition. Nat Chem Biol 12(9):741–747
58. Sali A, Blundell TL (1993) Comparative protein modelling by satisfaction of spatial restraints. J Mol Biol 234(3):779–815
59. Abraham MJ et al (2015) GROMACS: High performance molecular simulations through multi-level parallelism from laptops to supercomputers. SoftwareX 1–2:19–25
60. Kim S et al (2021) PubChem in 2021: new data content and improved web interfaces. Nucleic Acids Res 49(D1):D1388–D1395
61. O’Boyle NM et al (2011) Open Babel: An open chemical toolbox. J Cheminform 3:33
62. Morris GM et al (2009) AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility. J Comput Chem 30(16):2785–2791
63. Bolden JE, Peart MJ, Johnstone RW (2006) Anticancer activity of experimental compounds in murine models of chronic inflammatory respiratory diseases. Expert Rev Respir Med 8(3):339–347
64. Gosens R et al (2009) Caveslae and Cavelinos in the Respiratory System. Curr Mol Med 8(3):339–347
65. Williams TM, Lisanti MP (2004) The Caveolin genes: from cell biology to medicine. Ann Med 36(8):584–595
66. Bains SN et al (2012) Loss of caveolin-1 from bronchial epithelial cells and monocytes in human subjects with asthma. Allergy 67(12):1601–1604
Sulforaphane prevents and reverses allergic airways disease in mice via anti-inflammatory,…

68. Chen CM et al (2011) Downregulation of caveolin-1 in a murine model of acute allergic airway disease. Pediatr Neonatol 52(1):5–10
69. Hackett TL et al (2013) Caveolin-1 controls airway epithelial barrier function. Implications for asthma. Am J Respir Cell Mol Biol 49(4):662–71
70. Aravamudan B et al (2012) Caveolin-1 knockout mice exhibit airway hyperreactivity. Am J Physiol Lung Cell Mol Physiol 303(8):L669–L681
71. Gabehart KE et al (2013) Airway hyperresponsiveness is associated with airway remodeling but not inflammation in aging C57Bl/6 mice. Respir Res 14(1):110–110
72. Wardyn JD, Ponsford AH, Sanderson CM (2015) Dissecting molecular cross-talk between Nrf2 and NF-κB response pathways. Biochem Soc Trans 43(4):621–626
73. Sun X et al (2020) NRF2 preserves genomic integrity by facilitating ATR activation and G2 cell cycle arrest. Nucleic Acids Res 48:9109–9123
74. Peng D et al (2019) NRF2 antioxidant response protects against acidic bile salts-induced oxidative stress and DNA damage in esophageal cells. Cancer Lett 458:46–55
75. Gozzelino R, Jeney V, Soares MP (2010) Mechanisms of cell protection by heme oxygenase-1. Annu Rev Pharmacol Toxicol 50(1):323–354
76. Kitada O et al (2001) Heme oxygenase-1 (HO-1) protein expression and activity of histone deacetylases in human asthmatic airways. Am J Respir Crit Care Med 163(3):392–396
77. Starrett W, Blake DJ (2011) Sulforaphane inhibits de novo synthesis of IL-8 and MCP-1 in human epithelial cells generated by cigarette smoke extract. J Immunotoxicol 8(2):150–158
78. Elliott JG et al (2019) Fatty airways: implications for obstructive disease. Eur Respir J 54(6):1900857
79. Higami Y et al (2016) Increased epicardial adipose tissue is associated with the airway dominant phenotype of chronic obstructive pulmonary disease. PLoS ONE 11(2):e0148794
80. Adcock IM et al (2007) Epigenetic regulation of airway inflammation. Curr Opin Immunol 19(6):694–700
81. Royce SG, Karagiannis TC (2012) Histone deacetylases and their role in asthma. J Asthma 49(2):121–128
82. Ito K et al (2002) Expression and activity of histone deacetylases in human asthmatic airways. Am J Respir Crit Care Med 166(3):392–396
83. Barnes PJ (2010) Mechanisms and resistance in glucocorticoid control of inflammation. J Steroid Biochem Mol Biol 120(2–3):76–85
84. Adcock IM (2007) HDAC inhibitors as anti-inflammatory agents. Br J Pharmacol 150(7):829–831
85. Xu WS, Parmigiani RB, Marks PA (2007) Histone deacetylase inhibitors: molecular mechanisms of action. Oncogene 26(37):5541–5552
86. Karagiannis TC, El-Osta A (2007) Will broad-spectrum histone deacetylase inhibitors be superseded by more specific compounds? Leukemia 21(1):61–65
87. Lunke S et al (2021) Epigenetic evidence of an Ac/De axis by VPA and SAHA. Clin Epigenetics 13(1):38
88. Pirola L et al (2011) Genome-wide analysis distinguishes hyperglycemia regulated epigenetic signatures of primary vascular cells. Genome Res 21(10):1601–1615
89. Royce SG et al (2012) Effects of the histone deacetylase inhibitor, trichostatin A, in a chronic allergic airways disease model in mice. Arch Immunol Ther Exp (Warsz) 60(4):295–306
90. Choi JH et al (2005) Trichostatin A attenuates airway inflammation in mouse asthma model. Clin Exp Allergy 35(1):89–96
91. Ren Y et al (2016) Therapeutic effects of histone deacetylase inhibitors in a murine asthma model. Inflamm Res 65(12):995–1008
92. Ho E, Clarke JD, Dashwood RH (2009) Dietary sulforaphane, a histone deacetylase inhibitor for cancer prevention. J Nutr 139(12):2393–2396
93. Myzak MC et al (2007) Sulforaphane retards the growth of human PC-3 xenografts and inhibits HDAC activity in human subjects. Exp Biol Med (Maywood) 232(2):227–234
94. Myzak MC, Ho E, Dashwood RH (2006) Dietary agents as histone deacetylase inhibitors. Mol Carcinog 45(6):443–446
95. Liu ML et al (2020) HDAC8 inhibitor attenuates airway responses to antigen stimulus through synchronously suppressing galectin-3 expression and reducing macrophage-2 polarization. Respir Res 21(1):62
96. Hubbert C et al (2002) HDAC6 is a microtubule-associated deacetylase. Nature 417(6887):455–458
97. Nian H et al (2009) Modulation of histone deacetylase activity by dietary isothiocyanates and allyl sulfides: studies with sulforaphane and garlic organosulfur compounds. Environ Mol Mutagen 50(3):213–221
98. Dashwood RH, Myzak MC, Ho E (2006) Dietary HDAC inhibitors: time to rethink weak ligands in cancer chemoprevention? Carcinogenesis 27(2):344–349
99. Myzak MC, Dashwood RH (2006) Histone deacetylases as targets for dietary cancer preventive agents: lessons learned with butyrate, diallyl disulfide, and sulforaphane. Curr Drug Targets 7(4):443–452
100. Meng XM et al (2010) Smad2 protects against TGF-β/Smad3-mediated renal fibrosis. J Am Soc Nephrol 21(9):1477–1487
101. Zhang L et al (2015) Smad2 protects against TGF-β1/Smad3-mediated renal fibrosis. J Am Soc Nephrol 21(9):1477–1487
102. Fix C et al (2015) Smad2 protects against TGF-β1/Smad3-mediated collagen synthesis in human hepatic stellate cells during hepatic fibrosis. Mol Cell Biochem 400(1):17–28
103. Zhang L et al (2015) Smad2 protects against TGF-β1/Smad3-mediated collagen synthesis in human hepatic stellate cells during hepatic fibrosis. Mol Cell Biochem 400(1):17–28
104. Fix C et al (2019) Effects of the isothiocyanate sulforaphane on TGF-β1-induced rat cardiac fibroblast activation and extracellular matrix interactions. J Cell Physiol 234(8):13931–13941
105. Sun C, Li S, Li D (2016) Sulforaphane mitigates muscle fibrosis in mdx mice via Nrf2-mediated inhibition of TGF-β/Smad signaling. J Appl Physiol 120(4):377–390
106. Javaheri B et al (2017) Stable sulforaphane protects against gait anomalies and modifies bone microarchitecture in the spontaneous STR/Ort model of osteoarthritis. Bone 103:308–317
107. Simões BM et al (2015) Abstract 2319: Sulforaphane targets breast cancer stem-like cells in patient-derived cells and xenograft tumors. Cancer Res 75(15_Supplement): 2319–2319

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.
Authors and Affiliations

Simon G. Royce1,2,3,4 · Paul V. Licciardi4,5 · Raymond C. Beh1,2 · Jane E. Bourke3 · Chantal Donovan6,7,8 · Andrew Hung9 · Ishant Khurana10 · Julia J. Liang1,9 · Scott Maxwell10 · Nadia Mazarakis1,4,5,11 · Eleni Pitsillou1,9 · Ya Yun Siow1 · Kenneth J. Snibson11 · Mark J. Tobin12 · Katherine Ververis1,2 · Jitraporn Vongsivivut12 · Mark Ziemann10,13 · Chrishan S. Samuel14 · Mimi L. K. Tang5,15,16 · Assam El-Osta10 · Tom C. Karagiannis1,2

1 Epigenomic Medicine Laboratory, Department of Diabetes, Central Clinical School, Monash University, Alfred Centre, 99 Commercial Road, Melbourne, VIC 3004, Australia
2 Department of Clinical Pathology, University of Melbourne, Parkville, VIC 3010, Australia
3 Department of Pharmacology, Biomedicine Discovery Institute, Monash University, Clayton, VIC 3800, Australia
4 Infection and Immunity, Murdoch Children’s Research Institute, Melbourne, VIC 3052, Australia
5 Department of Paediatrics, The University of Melbourne, Melbourne, VIC 3010, Australia
6 Priority Research Centre for Healthy Lungs, Hunter Medical Research Institute, University of Newcastle, Newcastle, NSW 2305, Australia
7 Centre for Inflammation, Centenary Institute, Camperdown, NSW 2050, Australia
8 School of Life Sciences, Faculty of Science, University of Technology Sydney, Sydney, NSW 2007, Australia
9 School of Science, STEM College, RMIT University, VIC 3001, Australia
10 Epigenetics in Human Health and Disease Laboratory, Department of Diabetes, Central Clinical School, Monash University, Alfred Centre, 99 Commercial Road, Melbourne, VIC 3004, Australia
11 Faculty of Veterinary and Agricultural Sciences, University of Melbourne, Parkville, VIC 3010, Australia
12 ANSTO-Australian Synchrotron, Clayton, VIC 3168, Australia
13 School of Life and Environmental Sciences, Deakin University, Waurn Ponds, Warrnambool, VIC 3216, Australia
14 Cardiovascular Disease Program, Monash Biomedicine Discovery Institute and Department of Pharmacology, Monash University, Clayton, VIC 3800, Australia
15 Population Allergy Group, Murdoch Children’s Research Institute, Parkville, VIC 3052, Australia
16 Department of Allergy and Immunology, Royal Children’s Hospital, Parkville, VIC 3052, Australia