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# Redox regulation of metabolism in asthma

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# Redox regulation of metabolism in asthma

## *New insights into the roles of Glutathione-S-transferase P*

Cheryl van de Wetering

The University of Vermont

@ Cheryl van de Wetering, 2021

Redox regulation of metabolism in asthma
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# Redox regulation of metabolism in asthma

## *New insights into the roles of Glutathione-S-transferase P*

**Dissertation**

To obtain the degree of Doctor at Maastricht University,

on the authority of the Rector Magnificus, Prof. Dr. Rianne M. Letschert,

in accordance with the decision of the Board of Deans,

to be defended in public on Monday May 10, 2021 at 16:00 hours

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# 1

# General introduction and Thesis Outline

## Asthma

Asthma is a complex pulmonary disorder that affects approximately 25 million people in the U.S. and 300 million people worldwide (1). This disease is characterized by mucus metaplasia, airway remodeling, inflammation, and airway hyperresponsiveness (AHR) (2). Asthma can occur at any age and typical asthma symptoms include shortness of breath, wheezing, coughing, as well as airway obstruction (3), although the severity and frequency of the symptoms between patients varies significantly (4). In addition, asthma can be induced by a variety of direct and/or indirect triggers with different responses driven by distinct pathways. It is therefore hard to define and characterize patients with asthma. However, several subtypes of asthma exist, and these clinical manifestations include allergic asthma, severe and steroid-resistant asthma, occupational asthma, as well as exercise or cold air-induced asthma. The fundamental causes of asthma are still not fully understood, but are likely to be a combination of genetics and external factors which predisposes individuals to hypersensitive reactions (5).

*Asthma pathophysiology*

Chronic exposure to environmental factors such as air(borne) pollutants, particulate matter, pathogens as well as allergens and irritants, can induce oxidative stress and pro-inflammatory responses in the lung in susceptible individuals. Persistent, low-grade (chronic) inflammation and oxidative stress are major drivers of tissue damage and abnormal repair that characterize the different pathological features. The pathophysiology of asthma is recognized by structural changes in the airways, including epithelial cell mucus metaplasia, smooth muscle hypertrophy and hyperplasia, subepithelial fibrosis, changes in submucosal gland cells, and increased blood vessel formation (**Figure 1**). These structural changes may ultimately lead to irreversible epithelial damage and airway remodeling, which is thought to induce the aforementioned symptoms of the disease (2,6).

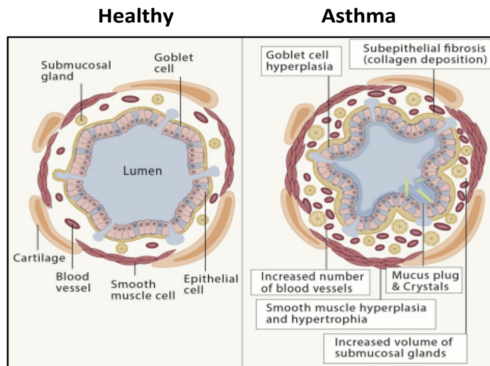


**Figure 1:** Schematic representation of histological characteristics of the airways in a lung of a healthy control subject (left) and the structural changes in a patient with severe asthma (right) (6, 7).

*Type 2 immune response*

The first line of defense against the daily occurrence of allergens is the airway epithelium, which acts as a protective barrier between the external environment and the submucosa of the lungs. Subsequently, the epithelial cells activate the immune system to neutralize toxins, maintain metabolic homeostasis, and regulate tissue regeneration pathways (8). However, for sensitive people with allergies, the immune system mistakenly identifies these allergens as foreign, harmful substances, causing a disruption to normal body functions by the release of substances that can affect the lungs and subsequently cause an asthma exacerbation or symptoms of asthma. Dysregulation of the immune response, resulting in chronic activation and/or augmentation thereof, may therefore drive asthma pathogenesis.

Sensitization against e.g. the aeroallergen house dust mite (HDM) activates both innate and adaptive immune responses, which includes type 2 immune responses (9). The initiation of the type 2 immune response is believed to be triggered by lung epithelial cells upon contact with environmental stimuli including allergens (2). Once activated, airway epithelial cells induce the production of immune regulators Interleukin (IL)-33, IL-25, and Thymic Stromal Lymphopoietin (TSLP) (8), which results in recruitment/chemotaxis of various innate immune cells (**Figure 2**). This response is thought to lead to the development of asthma in susceptible individuals because they have pre-existing atopy, specific genetic risk factors in regulators of type 2 inflammation or other vulnerabilities (2). These inflammatory mediators, including dendritic cells, subsequently stimulate local type 2 CD4+ lymphocytes

(Th2 cells) and type 2 innate lymphoid cells (ILC2) to produce various other interleukins, such as IL-4, IL-5, IL-9, and IL-13 (10), which are responsible for the production of immunoglobulin E (IgE), activation and recruitment of eosinophils and mast cells, and the induction of goblet cell hyperplasia, mucus hypersecretion and airway hyperresponsiveness (6, 8) (**Figure 2**). Eosinophils can also produce various type 2 cytokines (IL-4, 5, 13, 25) and chemokines, and attract leukocytes to sites of inflammation. Additionally, airway eosinophils are known to produce GM-CSF and IL-8, and airway epithelial cells produce IL-6 and IL-8 in response to allergens, which both result to an influx of airway neutrophils.

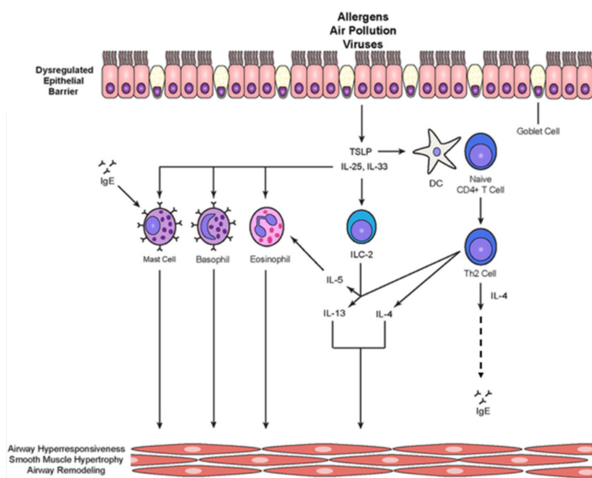


**Figure 2:** Schematic overview of the mechanism of type 2 inflammation in (severe) asthma, adapted from (11).

*Asthma phenotypes and endotypes*

Asthma is a heterogeneous disease and because of its complexity, it is classified as a syndrome rather than a disease (5, 12). Current treatments for asthmatic patients include bronchodilators and/or anti-inflammatory agents that mostly target specific manifestations including eosinophilic inflammation. However, there remains a significant patient population for whom these treatments are ineffective, as some asthmatics indicate severe and persistent poorly controlled disease with frequent symptoms, and exacerbations as well as airway obstruction. Based on genetic susceptibility, environmental risk factors, age, gender, atopy, prognosis, BMI, and response to therapies, severe asthma is characterized into type 2 and non-type 2 inflammatory endotypes (2, 4, 11). Type 2 inflammation is characterized by allergic sensitization, eosinophilic inflammation and exacerbations, including atopic asthma, a predisposition towards developing certain allergic hypersensitivity reactions, and can be further divided into the phenotypes early onset

asthma, late onset asthma, and eosinophilic asthma. Patients classified with the non-type 2 inflammation endotype are often non-allergic and non-atopic, are often steroid resistant, and are often obese, which includes increased oxidative stress, and present with neutrophilic inflammation (2, 13). However, it is hard to define patients into endotypes and phenotypes as overlap exists (11). For example, the largest group of patients can be defined in the early onset group, which are characterized by an allergic component, but not all early onset patients are associated with type 2 inflammation. The symptoms of older and obese asthmatics are often more severe, especially once these patients develop steroid resistance, and lack asthma control. Furthermore, obese asthmatics can also be characterized in an early onset atopic asthma phenotype based on a Th2-high profile in which allergic asthma is complicated by the presence of obesity, or into late onset non-atopic asthma in which patients display a Th2-low profile, where the development of asthma is a consequence of obesity (14). While endotypes remain relatively underdefined, they represent distinct molecular mechanisms that underlie characteristics of the (severe) asthma phenotypes. Therefore, characterization of specific biomarkers as pharmacological target for each phenotype would lead to a better approach and more personalized treatment, which could ultimately result in a decrease of exacerbation rates and symptoms and an improved lung function and quality of life.

*Mouse models of allergic airways disease*

The use of *in vivo* models of asthma has dramatically increased our understanding of the pathogenesis of allergic asthma. In humans, asthma is induced by inhalation of allergens. It is estimated that approximately 85% of patients with asthma are allergic to the most common aeroallergen HDM (9, 15, 16), and HDM allergens represent as one of the leading triggers for persistent respiratory allergies as well as asthma. Therefore, at present, one common mouse model of allergic airways disease uses repeated intranasal instillations of HDM (17, 18). In this model, the allergic inflammatory responses are largely dependent on recognition by epithelial cells (9), and the production of pro-inflammatory mediators including IL-33, IL-25, and TSLP, which is an important feature given that the epithelium plays a critical role in driving innate immune responses in the airways. Previous studies utilizing HDM to induce allergic airways disease in mice moreover demonstrated an

immune response with both eosinophilic and neutrophilic flux, production of IgE, and steroid resistance (17, 18). Moreover, pathophysiological features of asthma including airway inflammation, mucus metaplasia, fibrotic remodeling and methacholine-induced airway hyperresponsiveness were also observed (17-19), which together makes this a representative model to study underlying mechanisms in (Th2-high severe) allergic asthma. Another widely used model of allergic airways disease is the conventional Ovalbumin (OVA) mouse model (20). However, controversy exists regarding its clinical relevance as exposure to inhaled OVA is associated with the development of immunological tolerance (20, 21). Therefore, to elicit allergic sensitization, OVA is often intraperitoneally administered with an adjuvant, which avoids sensitization via the airways and thereby omits recognition of inhaled substances by the airway epithelium, which is believed to be a crucial response in asthmatic patients. Moreover, the immune response observed in the OVA model is primarily Th2-driven, with eosinophilic influx but little to no neutrophilic influx and no steroid resistance (20, 22).

## Asthma and metabolism

Cellular metabolism consists of catabolic and anabolic pathways to maintain the energy balance of a cell. Cells use glucose, fatty acids and amino acids as substrates to generate intermediates that drive glycolysis, the tricarboxylic acid (TCA) cycle, and mitochondrial oxidative phosphorylation to generate adenosine triphosphate (ATP) (**Figure 3**) (23). On the other hand, these metabolic pathways also regulate the maintenance of redox balance and the production of mediators that alter the epigenetic landscape and signal transduction of cells (23, 24).

Cellular metabolism plays a key role in innate immunity, and alterations of these metabolic processes may contribute to impaired innate immune effector function and pathology (25). The precise alterations in cellular metabolism that are induced in lung epithelial cells during asthma pathogenesis are still not known. However, cells that are exposed to proinflammatory cytokines and growth factors display upregulation of the glycolysis pathway to synthesize macromolecules and produce oxidants, rather than energy, to meet the demand of cell proliferation, proinflammatory mediator release or anti-microbial

effects. Indeed, increases in the glycolysis pathway (reprogramming) have been shown to facilitate the polarization of pro-inflammatory macrophages, which are important in direct host-defense against pathogens, and activation of immune cells that are known to be important in asthma pathology including dendritic cells, mast cells and neutrophils (23).

*Glycolysis and metabolic reprogramming*

The glycolysis pathway is the uptake of glucose by cells through glucose transporters via a 10 step cascade to form pyruvate from phosphoenolpyruvate (PEP), which is catalyzed by Pyruvate Kinase M (PKM) in the lung (**Figure 3**) (25). Different isoenzymes of PK are expressed, depending upon the different metabolic functions of the tissue. PKM1 is expressed in many differentiated tissues such as the muscle and brain (26), whereas PKM2 is highly expressed during embryonic development as well as in proliferating cells (27), and can adopt multiple structural forms that dictate its intracellular function (see Chapter 4, 5) (27, 28).

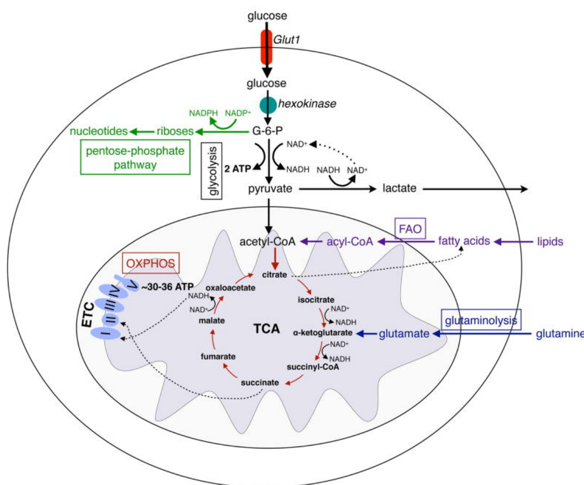


**Figure 3:** Schematic overview of major metabolic pathways in immune cells (25).

Pyruvate can enter the mitochondria to undergo oxidative phosphorylation, in which cells use enzymes to oxidize nutrients, thereby producing ATP, but alternatively, in the presence of low oxygen levels in the cells, under hypoxic conditions (anaerobic glycolysis), pyruvate can also be metabolized to lactate, via lactate dehydrogenase (LDH) (**Figure 3**). In the mid 1900's, Otto Warburg and his group described the phenomenon of aerobic glycolysis, the metabolism of glucose to form lactate in the presence of oxygen, a feature seen in tumor cells and other metabolically active cells, to support cell growth (29). This 'Warburg-effect' in cells is associated with increased glucose uptake and lactate over-production. Interestingly, aerobic glycolysis also generates nicotinamide adenine dinucleotide

phosphate (NADPH) that is important in protection against oxidative stress, and preserves the carbon backbone of glucose to fuel the synthesis of macromolecules, which is called the pentose phosphate pathway (30, 31).

During glycolytic reprogramming, proliferating cells reprogram/switch their intracellular metabolism from catabolic mitochondrial oxidative phosphorylation to aerobic glycolysis and other anabolic pathways. Glycolytic reprogramming occurs in metabolically active cells where there is a high demand for energy and the uptake of nutrients to engage cell activation and proliferation and is accompanied with increases in lactate production and accumulation at sites of for example chronic inflammation and tumor environments. Similar to tumor cells, immune cells also modify their metabolic requirements to acquire appropriate immune function in response to infections or stress to proliferate and activate their defense mechanisms. For example, Toll-like receptor-induced changes in glycolytic reprogramming regulates dendritic cell activation, partly via the non-canonical kinases of the inhibitory kappa B kinase (IKK) family, TANK-binding kinase 1 (TBK1), and inhibitory kappa B kinase epsilon (IKBKE) (32, 33). Distinct glycolytic programs are essential for CD4+ T cell effector function (34), and interestingly, increases in lactate levels were reported in serum and proliferating CD4+ T cells of asthmatics compared to controls, which could be reduced by inhibition of aerobic glycolysis (35). Moreover, increases in the glycolysis pathway are associated with IL-33-mediated increases in cytokine production in mast cells, and in lipopolysaccharide (LPS)-induced airway smooth muscle cell proliferation (36, 37). In airway epithelial cells and platelets from obese asthmatics who are associated with severe disease, increases in glycolysis (basal and maximal respiration) were demonstrated compared to lean asthmatics and healthy subjects (38). However, the extent of increases in glycolysis in the epithelium of (allergic) asthmatics is still an unexplored area of research. Improved understanding of how (innate) cell metabolism is altered in asthma may allow for identification of new biomarkers and therapeutic targets.

## Asthma and redox stress

A maintained redox environment is a balance between the production of oxidants, also known as reactive oxygen species (ROS), and anti-oxidant activity. While oxidants were

classically thought to be harmful and damaging to cells through oxidative irreversible modifications of e.g. DNA, proteins and lipids, it is now appreciated that oxidants also regulate important processes that maintain cellular homeostasis (39). Indeed, ROS at physiological concentrations play a central role in redox signaling via multiple post-translational modifications (39). For example, hydrogen peroxide ($H_2O_2$) is a key agent in redox signaling through specific oxidation of protein targets which engage in metabolic regulation and stress response to support cellular adaptions to changing environment and stress. However, excessive concentrations of ROS leads to an imbalance between oxidant/antioxidant production and scavenging, which is called oxidative stress. Changes in the redox environment, and especially oxidative stress have been speculated to be a main risk factor for asthma development (40).

*Protein S-glutathionylation and Glutathione S-transferase P*

One known process of oxidative signaling is the reversible post-translational modification protein S-glutathionylation (PSSG), a process that regulates protein structure and function through the oxidation of glutathione (GSH) with reactive cysteine residues (41). The forward PSSG reaction can occur spontaneously, but can also be catalyzed enzymatically by Glutathione-S-transferases (GSTs), especially GSTP, whereas the reverse reaction, the deglutathionylation, is catalyzed by Glutaredoxins (GLRXs) (42, 43) (**Figure 4**). The process of PSSG involves intermediate sulfenylation, in which protein cysteines, containing a sulfhydryl side chain (-SH), reacts with an oxidant ($H_2O_2$) to form a sulfenic acid (-SOH) (**Figure 4**) (44). The protein cysteine of the sulfenic acid moiety can be S-glutathionylated by the addition of a GSH molecule, thereby partly protecting proteins from becoming irreversible overoxidized to sulfinic ($-SO_2H$) and sulfonic acids ($SO_3H$).

GSH is one of the most prevalent and important thiol buffers, with concentrations ranging from millimolar within cells, to high micromolar concentrations in airways. The ratio of GSH (reduced) and its disulfide, GSSG (oxidized), contributes to the redox potential of the cell and thereby contributes to redox homeostasis (45). GSTs protect cells from environmental and oxidative stress by conjugating GSH to different xenobiotics, and are therefore classified as a family of phase II detoxification enzymes (46). Of the cytosolic GST family,

GSTP is the highest expressed GST in the lung (epithelium) (47). Besides its role in antioxidant defenses, GSTP has been shown to form protein-protein interactions with several proteins thereby regulating cell signaling pathways. These ligand-binding partners include c-Jun N-terminal kinase (JNK), and tumor necrosis factor-receptor-associated factor 2 (TRAF2), which both play important roles in the regulation of survival and (programmed) cell death pathways (48, 49). As mentioned before, GSTP is a major regulator of the cellular redox environment and impacts protein cysteine oxidation by catalyzing the forward PSSG reaction (**Figure 4**) (43).

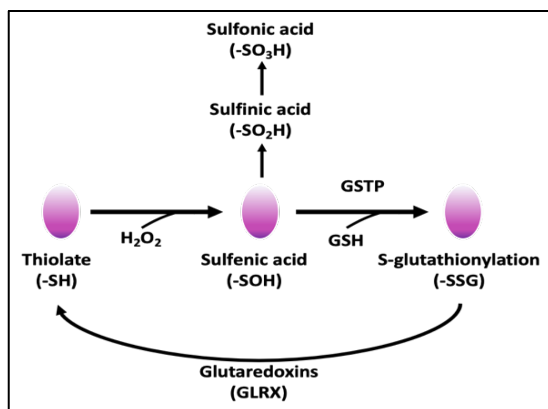


**Figure 4:** Schematic overview of the PSSG reaction, adapted from (50).

Dysregulation of oxidant production, and consequently, protein cysteine oxidation, due to PSSG reactions, may contribute to the pathogenesis of asthma by affecting cellular pathways that play an important role in the lung including cell metabolism and inflammation (51). Studies from our laboratory have shown that overall PSSG levels are increased in lungs of mice with allergic airways disease exposed to HDM compared to control lungs and contribute to disease (52). Although it is unclear if GSTP facilitates the forward reaction herein. Moreover, the expression of GSTP in the lung of different animal models of asthma has been studied with contrasting results (53-55). Given the important role of GSTP in PSSG chemistry, GSTP may play a prominent role in lung epithelial pathology during asthma. A more thorough description of the protein-S-glutathionylation process, the expression profile and functions of the different cytosolic GSTs, including GSTP, and their contribution to asthma (epithelial) pathology is described in chapter 2.

## Thesis outline

Allergic asthma is associated with distinct cellular and molecular mechanisms. Changes in cellular metabolism are recognized to promote inflammation, thereby contributing to the pathogenesis of lung diseases, including asthma. However, the precise metabolic alterations that are induced in immune and especially in lung structural cells during asthma pathogenesis, and mechanisms whereby changes in redox processes contribute to the progression of this disease, remain incompletely understood. Therefore, **the overall aim of this thesis was to examine the importance of an altered cellular metabolism during allergic airways disease and whether these metabolic changes are regulated by redox perturbations.** The main focus was to study the contribution of the (reprogrammed) glycolysis pathway in asthma pathogenesis, the signals that drive glycolysis as well as the role of Glutathione-S-transferase P (GSTP) herein, utilizing both *in vitro* and *in vivo* models of allergic airways disease.

An overview of the possible involvement of mammalian cytosolic GSTs in the pathogenesis of the chronic lung diseases asthma and COPD is described in **Chapter 2**. In this review, the different classes of GSTs, their expression profile in the lung as well as in epithelial cells in healthy subjects versus asthmatics are described. Moreover, the contribution of GSTs and their genetic variants to normal lung growth and development as well as their implication in the susceptibility to and progression of asthma and COPD are discussed.

Glycolysis is known to be important in the regulation of inflammatory responses. Therefore, in **Chapter 3** we examine whether glycolysis is altered and contributes to allergic asthma pathology using a mouse model of HDM-induced allergic airways disease, mouse tracheal epithelial cells as well as primary nasal epithelial cells and sputum samples derived from asthmatics or healthy subjects. Our results show that increased glycolysis is a critical feature of allergic airways disease by increasing inflammation, airway remodeling, and airway hyperresponsiveness. We moreover demonstrate that Interleukin-1(β) is the main signal that promotes glycolytic reprogramming during allergic airways disease.

The precise signals that elicit the glycolysis-associated pro-inflammatory responses in the lung (epithelium) remain unclear. As PKM2 catalyzes the final rate-limiting step in glycolytic reprogramming, **Chapter 4** investigates whether PKM2 promotes the pathogenesis of HDM-induced allergic airways disease. To this end, we examined the effect of activation of the glycolysis function of PKM2 with the small molecule activator TEPP46 in a HDM-induced mouse model of allergic airways disease as well as in Interleukin-1β treated mice and primary mouse tracheal epithelial cells. Glycolytic activation of PKM2 resulted in attenuation of the expression of pro-inflammatory mediators, and decreased mucus metaplasia, and subepithelial collagen during allergic airways disease.

In **Chapter 5** we examine whether the observed glycolytic reprogramming during allergic airways disease is due to redox perturbations by dysregulation of protein S-glutathionylation. As GSTP is the most abundantly expressed GST in the airway epithelium and catalyzes the forward glutathionylation reaction, we investigated if GSTP promotes HDM-induced allergic airways disease by utilizing wild-type and *Gstp*$^{-/-}$ mice. Interestingly, GSTP was found to promote HDM-induced allergic airways disease, in association with enhanced lactate levels. PKM2 was identified as a target of S-glutathionylation, which resulted in a decrease of its glycolytic activity. Moreover, GSTP interacts with PKM2, interferes with the PKM2 structure, and increases IL-1-induced pro-inflammatory signaling.

Finally, **Chapter 6** provides general discussion of this thesis and future directions as well as the implications and limitations of our findings.

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# 2

# Glutathione-S-transferases and their implications in the lung diseases asthma and Chronic Obstructive Pulmonary Disease: early life susceptibility?

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**ABSTRACT**

Our lungs are exposed daily to airborne pollutants, particulate matter, pathogens as well as lung allergens and irritants. Exposure to these substances can lead to inflammatory responses, and may induce endogenous oxidant production, which can cause chronic inflammation, tissue damage and remodeling. Notably, the development of asthma and Chronic Obstructive Pulmonary Disease (COPD) is linked to the aforementioned irritants. Some inhaled foreign chemical compounds are rapidly absorbed and processed by phase I, and phase II enzyme systems critical in the detoxification of xenobiotics including the glutathione-conjugating enzymes Glutathione-S-transferases (GSTs). GSTs, and in particular genetic variants of GSTs that alter their activities, have been implicated in the susceptibility to and progression of these lung diseases. Beyond their roles in phase II metabolism, evidence suggests that GSTs are also important mediators of normal lung growth. Therefore, the contribution of GSTs to the development of lung diseases in adults may already start in utero, and continues through infancy, childhood, and adult life. GSTs are also known to scavenge oxidants, and affect signaling pathways by protein-protein interaction. Moreover, GSTs regulate reversible oxidative post-translational modifications of proteins, known as protein S-glutathionylation. Therefore, GSTs display an array of functions that impact the pathogenesis in asthma and COPD.

In this review we will provide an overview of the specific functions of each class of mammalian cytosolic GSTs. This is followed by a comprehensive analysis of their expression profiles in the lung in healthy subjects, as well as alterations that have been described in (epithelial cells of) asthmatics and COPD patients. Particular emphasis is placed on the emerging evidence of the regulatory properties of GSTs beyond detoxification and their contribution to (un)healthy lungs throughout life. By providing a more thorough understanding, tailored therapeutic strategies can be designed to affect specific functions of particular GSTs.

**Key words:** Glutathione-S-transferases, protein S-glutathionylation, COPD, asthma, RNA-seq

**Abbreviations:**

GSH: Glutathione; GST: Glutathione-S-transferase; PSSG: Protein S-glutathionylation; COPD: Chronic Obstructive Pulmonary Disease; MAPK: Mitogen Activated Protein Kinase pathway; Cys: Cysteine; Prdx: Peroxiredoxin; Glrx: Glutaredoxin; MAPK: Mitogen activated protein kinase; JNK: c-Jun N-terminal kinase; TRAF2: Tumor necrosis factor receptor associated factor 2; $H_2O_2$: Hydrogen peroxide; SOH: Sulfenic acid; PM: Particulate matter; AHR: Airway hyperresponsiveness; BALF: Bronchoalveolar lavage fluid; NF-κB: Nuclear factor kappa B; Keap1: Kelch-like ECH-associated protein 1; Nrf2: Nuclear factor erythroid 2–related factor; STAT3: Signal transducer and activator of transcription 3; RNA-seq: RNA sequencing

## INTRODUCTION

Our lungs are constantly exposed to air and airborne pollutants (including $NO_2$, ozone), particulate matter, pathogens as well as respiratory allergens and irritants. Many of these agents are free radicals or initiate free radical reactions. Exposure to these exogenous pollutants or allergens gives rise to oxidative stress which can cause pro-inflammatory responses in the lung, and may subsequently induce endogenous oxidant production (1-5). The tripeptide glutathione (GSH, γ-L-glutamyl-cysteinyl-glycine) is the major anti-oxidant, non-protein thiol in cells, as well as in plasma and extracellular fluid of lungs. Glutathione-S-transferases (GSTs) protect cells from environmental exposures by their detoxification function to catalyze the conjugation of GSH (6, 7). Foreign chemical compounds inhaled into the lung are rapidly absorbed and processed for detoxification by phase I and II metabolizing enzymes. Notably, enzymes engaged in Phase I metabolism, including the cytochrome P450 system, will target these compounds by catalyzing different reactions including hydroxylation, oxidation and reduction to protect the cell (8, 9). Subsequently, during Phase II metabolism, Phase II enzymes, the GSTs and microsomal epoxide hydrolases, can catalyze the conjugation of Phase I modified xenobiotics to an endogenous water-soluble substrate, such as reduced GSH, or glycine. This will result in less toxic metabolites, which can now be more easily transported out of the cell by different transmembrane efflux pumps. Some compounds will not go through Phase I metabolism, but enter Phase II metabolism directly.

Oxidants, also known as reactive oxygen species (ROS) are produced by living organisms as a result of normal cellular metabolism. At physiological concentrations, they function as signaling molecules to regulate (patho)physiological processes. However, in excessive concentrations, oxidants that overwhelm the antioxidant defense system induce adverse modifications to cell components such as lipids, proteins, and DNA (10). This shift in balance between oxidants and antioxidants in favor of oxidants is termed oxidative stress (11). Aside from protecting cells from environmental toxins, GSTs also protect cells from oxidative stress (6, 7). Oxidative stress contributes to the development of asthma and Chronic Obstructive Pulmonary Disease (COPD) (12). Ambient outdoor air pollution from

the use of biomass fuels, ozone, and tobacco smoking have been associated with decreased lung volume and growth (3-5). Epidemiological studies demonstrate that exposure to ambient air pollutants induces episodes of acute respiratory exacerbations and bronchitis, which increases the progress of asthma and COPD (13-16).

Genetic variations, including polymorphisms in cytosolic human GSTs, can change a person's ability to cope with inhaled environmental stressors and toxins, and can thereby contribute to the risk to develop inflammatory lung diseases associated with these exposures, as well as aggravate the course of these conditions (17-19). Examples include the increased risk for asthma and COPD in subjects carrying null variants of the Mu and Theta class of GST, which lead to a loss of protein expression and therefore detoxification activity (20-25). The Glutathione-S-transferase P (GSTP) class Ile105Val polymorphism, which also results in decreased GST activity towards chloro-2,4-dinitrobenzene (CDNB) (26) has been linked to asthma and COPD in some studies as well (27, 28). In this review we will briefly describe the characteristics and main functions of the different classes of human cytosolic GSTs, and focus on their impact on the redox state. We will provide an overview of their implications in lung development and the diseases asthma and COPD.

**Historical overview of Glutathione-S-transferases**

Despite their most well-known function as a family of Phase II detoxification enzymes, GSTs were first introduced as ligandins in the 1970's, as GSTs were known to bind toxins and function as transport proteins (29, 30). GSTs are present in different subcellular compartments including the cytosol, endoplasmic reticulum (ER), mitochondria, nucleus and plasma membranes. Human GSTs can be divided into distinct families, namely cytosolic, mitochondrial, also known as the kappa class, and membrane bound microsomal GSTs, also known as MAPEG (membrane-associated proteins involved in eicosanoid and glutathione metabolism) (30, 31). Cytosolic GSTs are further classified based on sequence similarities, and physical and structural properties. In humans, the cytosolic GSTs can be further divided into 7 functional classes: Alpha, Mu, Omega, Pi, Theta, Sigma and Zeta (31). Mitochondrial GSTs are soluble enzymes and have structural similarities with cytosolic GSTs, whereas microsomal GSTs are integral membrane proteins, which are evolutionarily unrelated to the

other classes of GSTs. For the scope of this review we will only discuss the human cytosolic GSTs. GST Sigma remains uncharacterized and will not be discussed.

**Human Cytosolic Glutathione-S-transferases families**

Mammalian cytosolic GSTs are the best-characterized family group of GST proteins. The multiple members in each class share a common fold, and the GST isoenzymes within a class typically share more than 40% sequence homology, while between classes less than 25% sequence homology occurs (32, 33). GSTs are dimeric enzymes with, in most cases, both subunits being from the same class of GST (and forming homodimers) (**Figure 1A-B**) (34). The monomers are approximately 25-26 kilo Dalton (kDA) in size (30). The GST monomer consists of two distinct domains, the N-terminal thioredoxin-like domain, which comprises about 1/3 of the protein, and a C-terminal alpha-helical domain (35). The N-terminal thioredoxin-like domain is highly conserved in all GST isozymes and contains a redox active CXXC motif (32). This structure is common to several proteins from a thioredoxin fold superfamily, including glutaredoxins (GLRXs), glutathione peroxidases, and peroxiredoxins, which bind cysteine or GSH with high affinity (36-39). The highly specific GSH binding site, the G-site, is located within the N-terminal domain (32). The C-terminal domain and a loop from the N-terminal domain together function to shape the co-substrate binding site, the H-site (xenobiotic binding site), which binds various hydrophobic and electrophilic substrates (32, 40), and is proposed to be adjacent to the G-site (41). Dimeric GSTs enhance protein stability, and GST catalysis occurs by binding the substrate at the H-site, thereby attacking xenobiotics, and binding GSH at the G-site which together form the well-conserved active site of the enzyme. Near the N-terminus, a specific amino acid residue activates the thiol cysteinyl side chain by attack of the sulfur atom of GSH and lowers its pKa through hydrogen-bonding. In the GST Alpha, Mu, and Pi isoenzymes, the activating residue constitutes a conserved tyrosine (Y-GSTs), while in the Theta, and Zeta classes this is a serine, and in the Omega class a cysteine (S/C-GSTs) (42-26). As such, unlike the other GSTs, the active site cysteine of GST Omega (GSTO) is able to form a disulfide bond with GSH. These residues have been shown to be essential for catalytic activity, as replacement of specific conserved residues by site-directed mutagenesis of for example

tyrosine 7, lowered GSTP activity toward CDNB, and ethacrynic acid (47). CDNB is the most common GST substrate used in classical GST activity assays, however, not all GSTs can use CDNB as a substrate, and thus the activity of certain GSTs may be underestimated or even undetected when using this substrate. The activity of GSTs is moreover dependent upon GSH supply from γ-glutamylcysteine synthetase and glutathione synthetase, and on transporters to remove GSH conjugates from cells. Compounds that induce GSTs (often GST substrates) or that are recognized as substrates share a common chemical signal, namely a carbon-carbon double bond, adjacent to an electron-withdrawing group. The variability in co-substrates between the different GSTs is mainly reflected by different amino acids residues in the H-site (35). In addition to the variations in co-substrates, the different classes of human cytosolic GSTs are expressed in different organs.

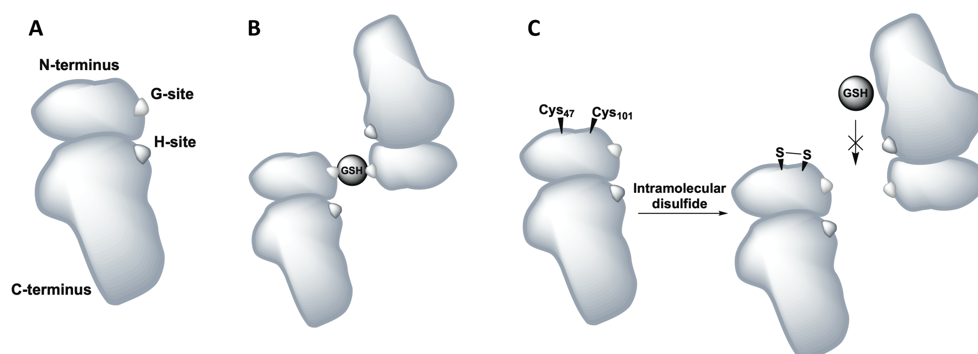


**Figure 1: Schematic representation of the structure of a Glutathione-S-transferase P molecule.** ***A,*** Schematic of a GSTP monomer with the N- and C-terminal domains including the glutathione binding site, the G-Site, and the xenobiotic binding site, the H-Site. ***B,*** Schematic of a GSTP dimer (forming homodimers) binding a GSH molecule on the G-sites in each monomer. ***C,*** GSTP contains cysteines residues regulating its catalytic activity. Intra-subunit disulfide bond formation between Cys47 and Cys101 residues results in steric hindrance for GSH binding. All figures are schematic representations and the actual position of the binding sites and cysteine residues may deviate from the original 3D crystal structure.

In addition to their roles in xenobiotic metabolism through glutathione conjugation, GSTs also have other catalytic activities (**Table I**). For example, GSTM, GSTP, and GSTO have been shown to control the activity of members of the mitogen activated protein kinase (MAPK) pathway (48). Some GSTs play an important role in biosynthesis of hormones (steroid hormone isomerase activity), are able to degrade tyrosine, display glutathione peroxidase activity, and may catalyze the reduction of organic hydroperoxides

to their corresponding alcohols (31, 49). GSTs also have biological functions independently of their catalytic activity (**Table I**). GSTs are capable of binding non-substrate ligands by forming protein-protein interactions with critical proteins involved in controlling stress responses, apoptosis, and proliferation (48). The ligand binding moreover facilitates the intracellular transport of numerous hydrophobic and amphipathic compounds such as bilirubin, heme, steroids and bile salt. This binding often results in the inhibition of GST's activity. Furthermore, GSTs have been shown to regulate protein structure and function by their capability of conjugating GSH to proteins in a process called protein S-glutathionylation (PSSG) (50). Based upon the aforementioned versatility of GSTs, the potential roles of GSTs in the susceptibility to and progression of various chronic lung diseases, thus likely extends beyond the classic xenobiotic metabolism, which we will highlight in a later section. First, we will briefly describe the main characteristics, isotypes, functions as well as expression profile per class of human cytosolic GST.

*Glutathione-S-transferase Alpha*

GST Alpha (GSTA) is expressed in a wide variety of tissues with high expression in the liver, kidneys, adrenal glands, intestine, and in the testis, and at medium-low levels in a wide range of other tissues including the lung (51). The GST Alpha class genes are located in a cluster mapped to chromosome 6 and contains five different isoforms: GSTA1, GSTA2, GSTA3 (expression is rare), GSTA4, and GSTA5, although GSTA5 has never been confirmed as a functional gene (51). Of the GSTA isoforms, the GSTA4 protein is identical in length and shares 54% sequence identity to human GSTA1 and GSTA2.

Aside from being a Phase II detoxification enzyme, the human Alpha class genes are known to metabolize bilirubin, and heme in the liver. The GSTA enzymes furthermore display glutathione peroxidase activity which helps to protect cells and tissues through detoxification of oxidants and lipid peroxidation products. The GSTA enzymes are moreover known to possess glutathione-dependent steroid isomerase activity (especially GSTA3) (49, 51) and have activity towards polycyclic aromatic hydrocarbons, epoxides and alkenyl products of lipid peroxidation (4-hydroxynonenal), especially GSTA4 (51, 52).

**Table I**: GSTs main known functions

| GST class | Functions |
|---|---|
| GSTA | Detoxification<br>Steroid isomerase activity (49, 51)<br>Glutathione peroxidase activity (51, 52)<br>Lipid peroxide reductase (4-HNE) (51, 52) |
| GSTM | Detoxification<br>Lipid peroxidation<br>Suppression of ASK1: inhibits cJUN and p38 – inhibition of cytokine and stress-induced apoptosis (56)<br>Catalyzes forward PSSG reaction (57) |
| GSTO | Detoxification<br>Modulates ryanodine receptors in ER (68)<br>Dehydroascorbate and Monomethylarsonate reductase activity (66, 70, 71)<br>Affects cell survival/apoptosis pathways- (de)phosphorylation of ERK1/2, AKT and JNK (73)<br>Diarylsulfonylurea binding protein: inhibits ATP-induced processing of IL-1β (75)<br>Forward and reverse PSSG (69) |
| GSTP | Detoxification<br>JNK: inhibits apoptosis (81, 82)<br>TRAF2: inhibits activation of JNK and p38; reduces cell death and stress responses (83)<br>Catalyzes forward PSSG reaction (50) |
| GSTT | Detoxification<br>Lipid peroxide reductase (91)<br>Sulfatase activity (289) |
| GSTZ | Detoxification<br>Catabolism of phenylalanine and tyrosine (degradation) (290)<br>Maleylacetoacetate isomerase (98)<br>Conversion of dichloroacetic acid to glyoxylic acid (β-oxidation) (99, 100, 290)<br>Metabolism of α-haloacids (290) |

Table describes the main functions of the different human cytosolic GST isoenzymes 4-HNE: 4-hydroxy-2-nonenal; ASK1: apoptosis signal-regulating kinase 1; PSSG: Protein S-glutathionylation; JNK: c-Jun N-terminal kinase; ERK: Extracellular signal-regulated kinases; Akt: Protein Kinase B; ATP: Adenosine Triphosphate; IL-1β: Interleukin-1beta; TRAF2: tumor necrosis factor receptor associated factor 2

Functional, allelic, single nucleotide polymorphisms (SNP) occur in an SP1-binding element of GSTA1 and in the coding regions of GSTA2 and GSTA3, leading to either low expression or reduced activity towards among others fatty acid hydroperoxides (51). These properties suggest Alpha class GSTs to be involved in susceptibility to diseases with an environmental component (such as cancer, asthma, and cardiovascular disease).

*Glutathione-S-transferase Mu*

The GST Mu (GSTM) class exists of 5 isoforms, GSTM1 to GSTM5, which are located on chromosome 1 (53, 54) and which are expressed in different organs. GSTM is highly expressed in most tissues including the liver, lungs, muscle, kidney, and brain. GSTM uses CDNB, and participates in the detoxification of carcinogenic compounds such as polycyclic aromatic hydrocarbons, aromatic amines and other organic compounds, including benzone(a)pyrene, styrene-7,8-oxide, and trans-stilbene oxide (55). GSTM1 has been shown to bind (non-substrate dependent) and suppress apoptosis signal-regulating kinase 1 (ASK1) activity (56), a MAPK kinase kinase (MAPKKK) that activates c-Jun N-terminal kinase (JNK), a protein important in stress responses and pro-apoptotic signaling, and p38 pathways, that regulate cytokine and stress-induced apoptosis. Moreover, GSTM has been shown to catalyze the forward PSSG reaction (57), although not much is known about its specific PSSG targets.

The genes encoding the Mu class of enzymes are known to be polymorphic, especially GSTM1 exists as genetic variants. GSTM1 Lys173Asn (rs1065411) encodes proteins that form active mono- and heterodimeric enzymes. dGSTM1*1x2 is a unique variant in which the GSTM1 gene is duplicated (58). The GSTM1 null variant on the other hand contains a homologous deletion of a 16 kb segment, which leads to a loss of protein expression and detoxification activity in homozygotes (59, 60). The prevalence of the null genotype is very high in the general population (up to 66%; in Caucasians ~ 50%), but varies across ethnicities (61, 62). In context of the lung, the GSTM null phenotype is associated with an increased risk of lung cancer, especially in Asians (20, 63) and has also been linked with an increased risk of inflammatory lung diseases (24, 61, 64, 65).

*Glutathione-S-transferase Omega*

GST Omega (GSTO) is mainly expressed in the liver, notably in macrophages, glial, and endocrine cells, as well as in other tissues including for example the kidney, lung, and gallbladder (66). The GSTO class has two isoforms, GSTO1, and GSTO2, located on chromosome 10 (45), and a third sequence appears to be a reverse-transcribed

pseudogene, GSTO3p, identified on chromosome 3 (66). GSTO1 and GSTO2 are two homodimeric proteins that display 64% sequence homology (67). GSTO2 is strongly expressed in the testis, liver, kidney, and skeletal muscle, whereas GSTO1 is more abundantly expressed in the heart, in gastrointestinal tissues, and moderately in the lung (66). The Omega class is structurally and functionally distinct from other eukaryotic GSTs, as it comprises a unique 19 residue N-terminus, which is similar to the tertiary structure of GLRXs, the main deglutathionylation enzymes (described in a later section).

Interestingly, GSTO enzymes have poor activity towards common GST substrates, such as CDNB, but exhibit alternate functions in cellular redox homeostasis as well as enzymatically modulating ryanodine receptors, calcium channels in the ER (68). GSTO1 plays an important role in the S-glutathionylation cycle as it functions both as a catalyst of the forward reaction or as a deglutathionylation enzyme depending on the specific conditions (69). Moreover, GSTO2 displays high glutathione-dependent thioltransferase and dehydroascorbate reductase activities, which are activities more characteristic of the glutaredoxins. Another novel activity of GSTO1 is the reduction of monomethylarsonic acid to monomethylarsonous acid (monomethylarsonate reductase activity), the rate-limiting step in the biotransformation of inorganic arsenic (66, 70, 71). GSTO1 was originally identified as the ubiquitin ligase, p28, and is associated with anti-cancer drug resistance (72). Additionally, it has been suggested that GSTO1 affects cell survival by activating survival and inhibiting apoptotic signaling pathways, presumably due to binding to and (de)phosphorylation of ERK1/2, AKT and JNK (73). Moreover, GSTO1 is shown to translocate to the nucleus after heat shock and other stress conditions, but its function in the nucleus remains unclear (74). Lastly, the GSTO1 isoform has been identified as a diarylsulfonylurea binding protein and it is suggested that this interaction is responsible for inhibition of ATP-induced interleukin 1 beta (IL-1β) posttranslational processing (75).

Based on the use of overexpression constructs it is believed that the GSTO1 Ala140Asp polymorphic variant (rs4925) protein is expressed at a higher level compared to the wild-type protein (67). However, heat stability or activity towards CDNB or hydroxyethyl disulfide (measuring glutathione-dependent thioltransferase activity) is similar between

wild-type and GSTO1 Ala140Asp variants (66). Another group in contrast did find repressed thioltransferase activity of the Asp140 variant of GSTO1 (25% lower), but a difference in kinetics of its monomethyl arsenate reductase activity was not observed (76). When using more specific substrates, the kinetics of S-glutathionylation were found to be increased and deglutathionylation repressed for the Asp140 variant of GSTO1 (69). Protein expression of the GSTO2 Asn142Asp polymorphism (GSTO2*N142D; rs156697) was reduced to 76% compared to wild-type (67). Due to the insolubility of GSTO2, the activity of variants has not been assessed. In a genome wide-analysis, top ranked relations were found between the non-synonymous coding homozygous GSTO2 Asn142 and lung function in adults (77).

*Glutathione-S-transferase P*

Only one isoform of the GST P (GSTP) class is known to be expressed in humans, namely GSTP1, which is located on chromosome 11 (78, 79). GSTP1 is the GST most commonly expressed outside the liver, with main expression in the heart, lung, and brain. GSTP accounts for over 90% of the GST activity toward CDNB in the lung, with the remaining activity attributed to GSTM1 and GSTT1 (80). In addition, GSTP can bind to JNK by direct protein-protein interaction in a non-substrate dependent manner, thereby inhibiting the kinase activity and protecting cells against ($H_2O_2$-induced) cell death (81, 82). Another ligand binding partner of GSTP is tumor necrosis factor receptor associated factor 2 (TRAF2), a member of the TNF-α induced signaling, which in turn activates p38 and JNK (83), which is inhibited by GSTP binding. Moreover, GSTP is known to catalyze the forward PSSG reaction (50), which we will describe in more detail in the next section.

The GSTP gene is also known to be polymorphic (84). In the context of the lung, the GSTP1 Ile105Val variant (rs1695) is one of the most commonly studied polymorphisms linked to chronic lung diseases, and has a decreased GST activity towards CDNB (26, 85).

*Glutathione-S-transferase Theta*

GST Theta (GSTT) is predominantly found in the liver (hepatocytes), and is also expressed in the kidney (renal proximal tubule cells), gastrointestinal tracts, and lung (86-88). The Theta class includes the isoforms GSTT1 and GSTT2, which are both located on chromosome

22 (89, 90). GSTT1 and GSTT2 share 55% amino acid sequence identity. GSTT1 is important in phase II biotransformation of drugs and chemicals, and is involved in the detoxification of substrate intermediates which are produced during oxidative stress such as peroxidized lipids (91). GSTT also participates in the detoxification of smoke-derived small hydrocarbons such as ethylene oxide, as well as epoxy butanes and methyl bromide (22, 92, 93).

Similar to the Mu class of GSTs, a null allele of GSTT1 exists, which contains a deletion of approximately 54 kb, and results in loss of enzymatic activity (21-23). Individuals homozygous for this allele are at an increased risk for malignancies (head, neck, oral cavity) since the deletion results in decreased detoxification capacity of possible carcinogens (94). The prevalence of the null genotype varies across ethnic groups with the highest prevalence in Asians (Caucasians 13~26%; Asians 35~52%) (62, 95), and it has been linked to chronic lung diseases (24, 64, 65, 96).

*Glutathione-S-transferase Zeta*

GST Zeta (GSTZ) is found in a wide variety of endocrine tissues including the liver, stomach, and testis as well as in the pancreas, and is located in the cytosol as well as in the mitochondria. The gene spans approximately 10.9 kb, is composed of 9 exons, and is located on chromosome 14 (97).

Besides its role in detoxification, GSTZ also plays a role in the catabolism of tyrosine. In particular, GSTZ catalyzes the cis-trans isomerization of maleylacetoacetate to fumarylacetoacetate, therefore has GSTZ also been described as maleylacetoacetate isomerase (MAAI) (98). Interestingly, GSTZ1 shows the closest sequence similarity to GSTO1. However, GSTZ is the only enzyme in the GST family that plays a role in β-oxidation by catalyzing processes in the intermediary metabolism including the conversion of dichloroacetic acid to glyoxylate in a reaction that requires but does not consume GSH (99-100). GSTZ is moderately expressed in the lung, but research efforts on GSTZ are very limited and studies are needed to examine the involvement of GSTZ in the lung.

**Protein S-glutathionylation and deglutathionylation**

Changes in the redox environment have long been implicated in the pathophysiology of many pathological conditions, especially the imbalance between antioxidant/oxidant production and scavenging. Consequently, damage by oxidants includes irreversible oxidations of cysteines within proteins. The original thought of oxidants being 'bad actors' and damaging has shifted since we now know that low levels of oxidants such as hydrogen peroxide ($H_2O_2$) and nitric oxide (NO) regulate processes important in maintaining cellular homeostasis (101). Aside from being Phase II detoxification enzymes and the additional aforementioned functions, certain GSTs also play an important role in the process of PSSG by conjugating GSH to selective proteins. PSSG is a reversible post-translational modification (102), which regulates protein structure and function as it alters the shape, charge and size of the target protein. The process of PSSG can occur spontaneously/non-enzymatically, but can also be catalyzed enzymatically, by specifically the GSTs, GSTP (50), GSTO (69), and to a lesser extent GSTM (57). Non-enzymatic PSSG depends on the availability of GSH/GSSG and occurs via thiol-disulfide exchange reactions between GSSG and a protein cysteinyl residue or via reaction of GSH with an oxidized thiol derivative such as sulfenic acid (-SOH), thiyl radical (-S•) or S-nitrosyl (-SNO) (103). Depending on the protein, and targeted cysteine residue, PSSG can either activate or inhibit its function. GSTs have several substrates for glutathionylation which include transcription factors, kinases, structural proteins and enzymes involved in metabolism (104).

It is thought that both non-enzymatic, as well as enzymatically catalyzed formation of PSSG involves intermediate sulfenylation, the reaction of protein cysteines, containing a sulfhydryl side chain (SH), with an oxidant such as hydrogen peroxide ($H_2O_2$) to form a sulfenic acid (-SOH) intermediate. This SOH intermediate can be stabilized, or can give rise to sulfenamides (SN), or disulfide bonds (S-S) which can occur within a protein or between proteins (105). The SOH intermediate is believed to be recognized by GSH-bound GSTP (or other GSTs), which in turn catalyzes the S-glutathionylation of the protein cysteine. The S-glutathionylated protein can interact with another GSH moiety to release oxidized GSH (GSSG), which regenerates the reduced protein thiol. This latter reaction is catalyzed by

GLRXs under physiological conditions. The redox-dependent PSSG reaction is thought to protect proteins from further irreversible overoxidation to sulfinic ($SO_2H$) and sulfonic acids ($SO_3H$), but it has also been shown to play critical roles in the regulation of protein function. Worthy to mention is that only certain cysteine residues have been signified as 'reactive', meaning they can be readily oxidized, reduced and otherwise modified. Reactive cysteines are characterized as having a lower pKa (≤7) than that typical of cysteine, which ranges from 8 to 8.5. Detailed information on cysteine chemistry and modifications can be found elsewhere (103, 105-107).

The regulatory role of GSTP in the forward reactions of PSSG is based on the catalytic activity of the enzyme. GSTP contains 4 cysteines residues: Cys14, 47, 101, and 169. Intramolecular disulfide bonds have been shown to occur between Cys47 and Cys101, and disulfide formation also seemed to occur preferably between Cys14 and Cys169 when a disulfide bond between Cys47 and Cys101 had already been formed (108) (**Figure 1C**). Disulfide bond formation (intra-subunit) between Cys47 and Cys101 results in steric hindrance for GSH binding, which implies that these residues are located in an important region for GSH binding. Disulfide bonds can also be formed between Cys47 residues in different subunits, which can result in conformational change and inactivation of GSTP (108). GSTP itself can be auto-S-glutathionylated on Cys47 and Cys101, changing its secondary structure with a resultant impact on the structures within the monomer of GSTP (41). This can reduce the catalytic activity of GSTP (50) and affect the interaction of GTSP with ligand-binding proteins. For example, Cys47 and Cys101 in GSTP have been shown to be critical for the interaction and regulation of JNK. S-glutathionylation on both cysteines thus acts as an oligomer switch and can cause GSTP multimerization and inactivation of the enzyme (50).

Whereas the forward PSSG reaction can be catalyzed by GSTs, in mammalian, GLRXs, or thioltransferases are the main deglutathionylating enzymes (109, 110). GLRXs are part of the family of thioltransferases (110), and there are four known GLRXs in mammalian cells; GLRX1 (cytosolic), GLRX2 (mitochondrial/nuclear), GLRX3/PICOT (cytosolic) and GLRX5 (mitochondrial), of which GLRX1 is most efficient at deglutathionylating proteins. For

the current knowledge on GLRXs, their impact on lung diseases and PSSG targets relevant to these lung diseases, we direct the reader to another review that we recently published (37). Other enzymes that have been implicated in catalyzing the reverse reaction under certain conditions, include sulfiredoxins (111), as well as GSTO1 (69). The GSH binding of GSTO1 distinguishes this GST from typical GSTs as the active site cysteine residue, Cys32, in GSTO1 creates a mixed disulfide with GSH. Mutating Cys32 ablated the deglutathionylation activity of GSTO1 (69). ß-actin is a protein that is specifically deglutathionylated by GSTO1 (69).

**Glutathione-S-transferases and their implication during early life in lung diseases**

*GST presence in the lungs*

Prenatal as well as postnatal environmental and lifestyle exposures may affect lung development, in part due to increased oxidant production. Especially at birth, newborns are highly susceptible to increases in oxidative stress levels, as for example the partial oxygen pressure increases from 20 to 25 mm Hg in utero to 100 mm Hg in the extra uterine environment, because of the increased metabolic activity to maintain breathing, and body temperature. This abrupt change exposes newborns to high oxidative stress levels (112). Pre-term birth is associated with increased oxidative stress as higher levels of oxidative stress markers have been reported in pre-term compared to full-term newborns (113). Moreover, pre-term infants often require assistance to breathe which includes the need for supplemental oxygen and ventilatory support. The amount of supplemental oxygen is crucial as too much oxygen has been shown to increase oxidative stress, and ‘pure’ (100%) oxygen may cause inflammation, and emphysema in the lungs of newborn mice (114). Additionally, increased oxidative stress is even thought to cause pre-term birth (113, 115), although the contribution of oxidative stress itself is hard to interpret since other factors may contribute as well. Antioxidant systems in the lungs are therefore crucial in maintaining perinatal redox balance, and it is surprising that very few studies have investigated the expression profile of antioxidants including the cytosolic GSTs pre-, during and post-birth. In mouse lungs, GSTs are expressed at low levels before birth (with the exception of GSTP which showed high levels during gestation), highly increased within one week after birth,

and decreased again two to three weeks after birth (116). Overall GST activity in the distal airways increased over this time window, which may be an adaptation to the external environment. During human lung development, GSTP is the predominantly expressed GST isoenzyme up to 13 weeks of gestation (117). Another study showed that in a 14 week old fetus, GSTP is present in all differentiated epithelial cells (118). As development proceeds with the differentiation of progenitor cells into type I and type II pneumocytes, the appearance of GSTP negative cells coincided. Two independent studies found that GSTA and GSTM expression was continuous throughout development in the lung, albeit at respectively moderate to low levels (117, 119).

In humans, the expression of the different GSTs during lung development in early life remain poorly examined. However, a number of studies have examined the distribution of the different GSTs in easily obtained nasal epithelial cells and lung tissue in adults. The available RNA-sequencing (RNA-seq) data from nasal brushings (120) showed expression of *GSTA1, GSTA2, GSTA4, GSTM4, GSTO1*, *GSTP1, GSTT1,* and *GSTZ1* in healthy control subjects (**Figure 2A**). Microarray gene expression data from the Lung Genomics Research Consortium (LGRC) showed expression of most GST isoforms with the exception of *GSTA1*, *GSTA3*, and the *GSTT* class in non-COPD control subjects (**Figure 2B**). In lung tissue as well as in nasal epithelium, *GSTP1* mRNA levels were most abundant. GSTs are highly expressed in the airway epithelium, but it is unknown whether GSTs exert unique functions among the various epithelial cell subtypes. Recently described single cell RNA seq databases begin to shed light onto this question. We therefore examined the expression of GST isoforms using a recently published single cell RNA seq database that profiled single cells of bronchial biopsies as well as lung parenchyma (small respiratory airways and alveoli) upon lung resection surgery from healthy subjects (**Figure 4**) (121). The airway epithelium is in direct contact with inhaled air and consists of basal cells (progenitor cells that exhibit the capacity to self-renew and give rise to multiple types of differentiated airway epithelial cells), ciliated cells (move liquid over surface, to keep the airways clean of mucus and dirt), goblet cells (a mucus secreting cell), and club cells (previously known as Clara cells that protect the bronchiolar epithelium by secreting proteins including CCSP) (**Figure 3**) (122).

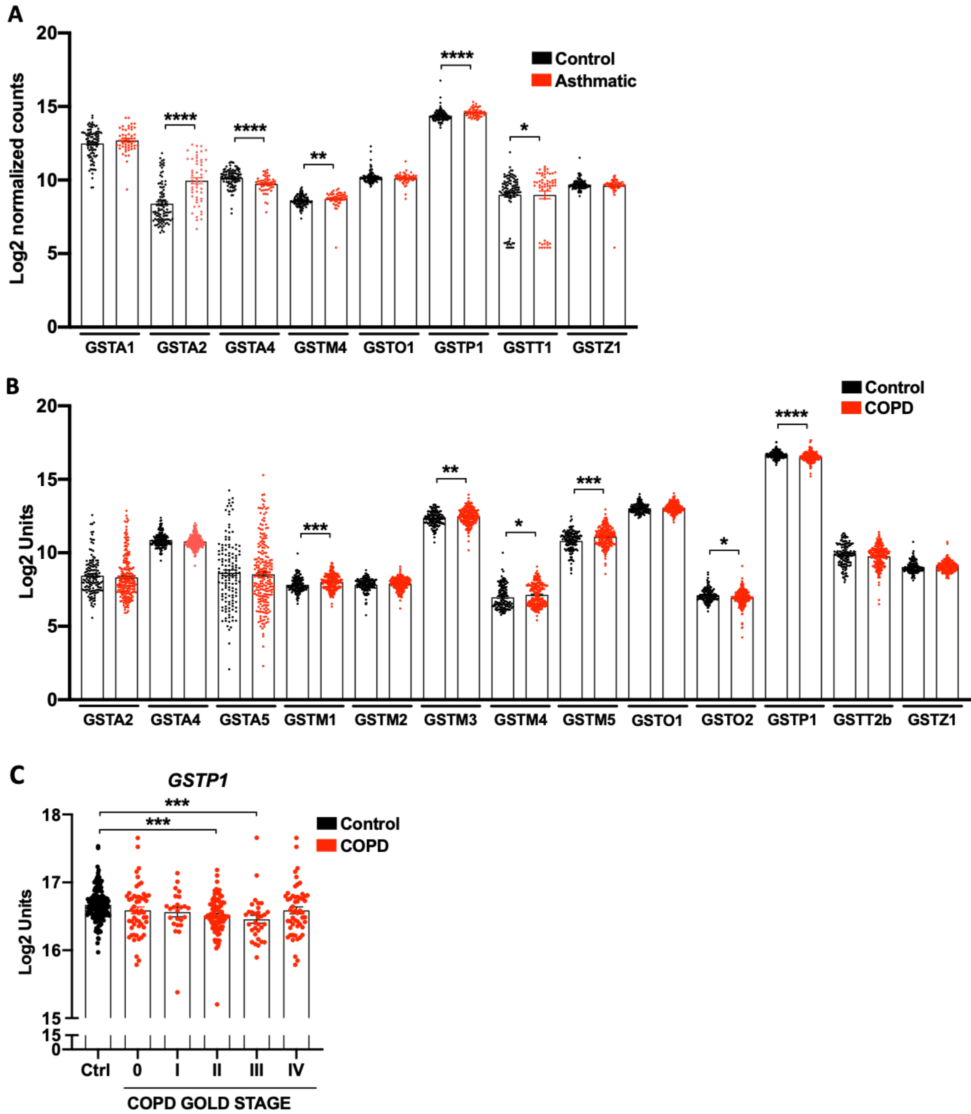


**Figure 2: Transcripts levels of cytosolic GSTs in patients with asthma and COPD.** ***A,*** **Control vs Asthma**: *GST* transcripts were assessed from publicly available RNA-sequence data from RNA isolated from nasal brushing of asthmatic patients (red; *n* = 53) compared with healthy subjects (black; *n* = 97). The full description of RNA isolation and processing from nasal brushings, and the process of the raw RNA-sequence data are published here (287). ***B,*** **Control vs COPD**. *GST* transcripts was obtained from publicly available microarray gene expression data from the Lung Genomics Research Consortium (LGRC) for COPD patients (red; *n* = 219), and non-diseased control tissues (black; *n* = 137) (data are available in the Gene Expression Omnibus (GEO) database accession no GSE47460). Results are shown as average ± SEM. Statistical significance was calculated using a Mann–Whitney U Test. *$p$-value <0.05; **$p$-value <0.01; ***$p$-value <0.001; ****$p$-value <0.0001. ***C,*** Differences in GST transcripts in between GOLD stages of COPD patients from the GLRC described in B*.* COPD: chronic obstructive pulmonary disease.

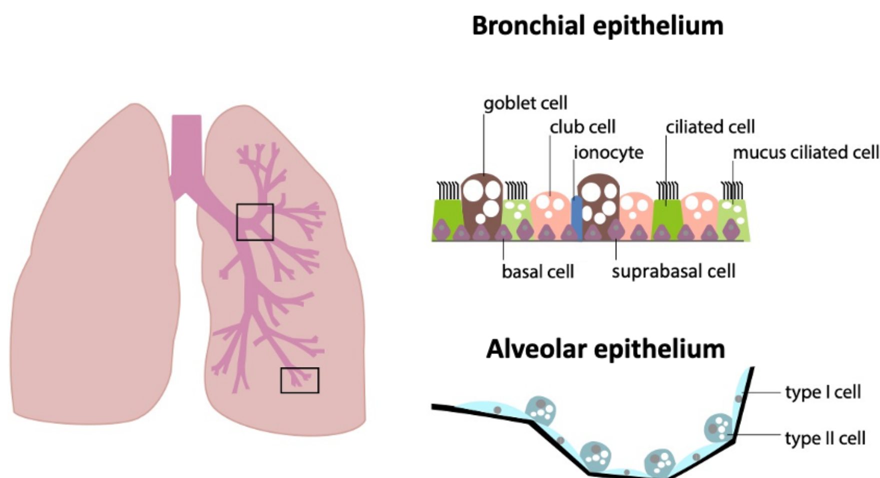


**Figure 3: Schematic representative overview of the different bronchial and alveolar epithelial cells of the lung.** Representative overview of the different epithelial cells in the bronchial epithelium (*top)*, including basal 1, basal 2, goblet, club, ciliated, and mucus ciliated cells, and ionocytes, as well as the Type I and Type II cells in the alveolar epithelium (*bottom)*.

Two discrete cell states were identified in basal, and ciliated epithelial cells. Basal cells were less-mature than suprabasal cells, which were more apically localized. Mucus ciliated cells are highly similar to ciliated cells, but co-express a number of mucus genes (121). **Figure 4A** shows that *GSTP1* is the most abundantly expressed GST in all respiratory epithelial cell types in controls, including type I and type II alveolar epithelial cells, and was even shown to be expressed in pulmonary ionocytes, a newly identified Foxi1$^{+}$ cell type which plays a role in clearing mucus from the airways (123, 124). *GSTO1* is also widely expressed in lung epithelial cell types, specifically in basal and suprabasal cells, as well as in ionocytes and type II alveolar cells in the parenchyma, while *GSTO2* is only moderately expressed in basal and suprabasal cells. *GSTA1* is highly expressed in ciliated, mucus ciliated, goblet and club cells and moderately expressed in basal and suprabasal cells. *GSTA2* is expressed in ciliated, and mucus ciliated cells, as well as in club cells, and to a lesser extent in goblet cells, while *GSTA4* was mainly expressed in basal and suprabasal cells, and also in type I and II epithelial cells in the parenchyma. Besides expression in epithelial cells, *GSTM3*, *GSTO1,* and *GSTP1* were highly expressed in fibroblasts and in smooth muscle cells in control subjects (**Figure 4B**). Similarly, *GSTA1,* and *GSTA4* were also expressed in smooth muscle cells and fibroblasts, as well as *GSTM5* in fibroblasts but to a lesser extent. *GSTO1*,

and *GSTP1* were also highly expressed in the endothelium. GSTs are also detectable in immune cells (**Figure 4B**). Especially *GSTP1,* and *GSTO1* were expressed in a variety of immune cells including mast cells, macrophages, monocytes, as well as B and T lymphocytes, and dendritic cells. *GSTA1* was moderate expressed in macrophages, as well as T and B lymphocytes.

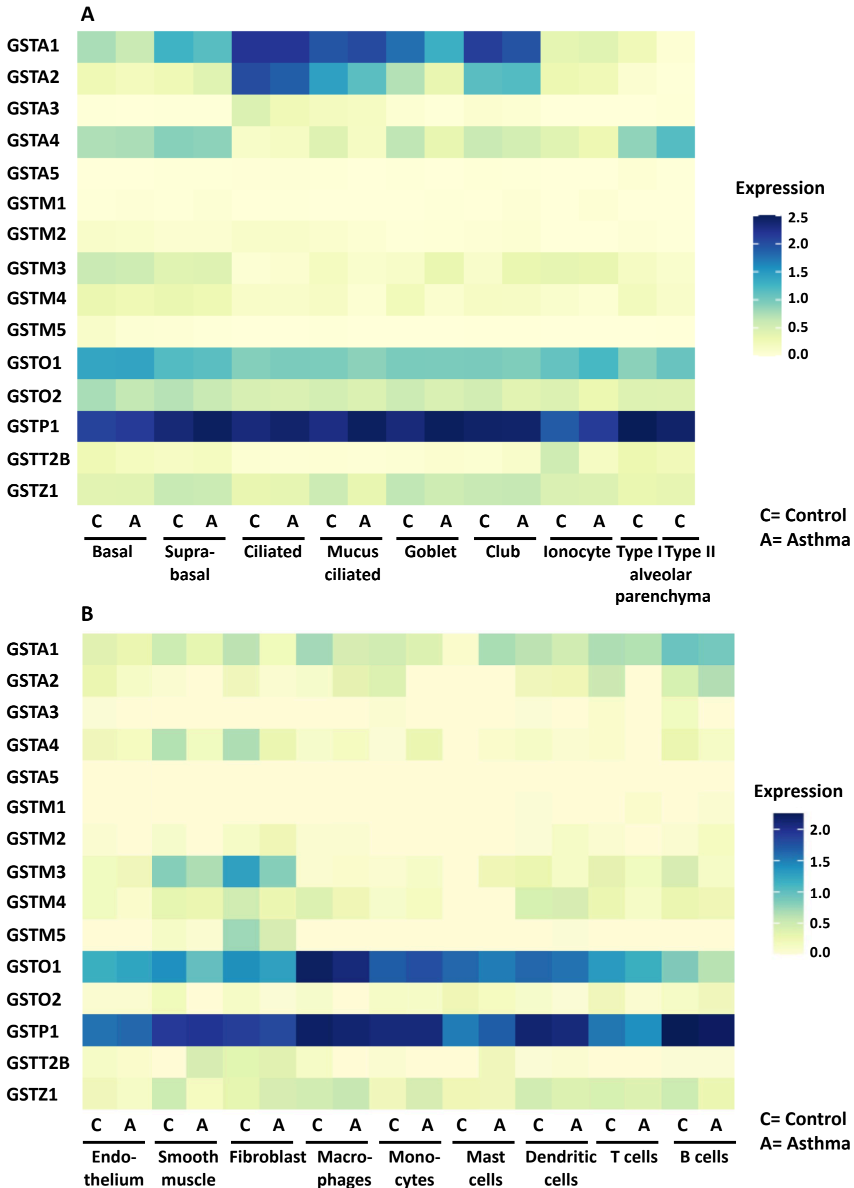

**Figure 4: Airway and lung epithelium transcripts expression of cytosolic Glutathione-S-transferases in patients with asthma and controls.** Heatmap of normalized gene expression of GST genes in single cell RNA seq data from expressing clusters of ***A,*** bronchial and alveolar epithelial cells, and ***B,*** other structural cells including immune cells, smooth muscle, fibroblasts, and endothelium in airway and lung epithelium split by asthma and control. The type I and type II alveolar cells in the parenchyma were only analyzed in controls (120). The single cell data consists of 22933 cells combined from bronchial biopsies from six healthy controls, six persistent asthma donors, and rejected lung donor material from six healthy controls. The data was processed with CellRanger version 2, ambient RNA corrected and normalized using SoupX (288) and analyzed using the R package Seurat, as described here (120). C= Control; A= Asthma.

Some of these new observations from the single-cell RNA seq study are a corroboration of much earlier studies examining the location of GSTs in lung tissue based upon immunohistochemistry. A study from the early 1990's indeed found that GSTP and GSTA were the most abundant GSTs in human lung tissue, and that these GSTs were primarily present in the bronchial epithelium (125, 126). It was furthermore shown that GSTP and GSTM were present in the distal airspaces, namely in alveolar type I and II cells and macrophages (126). GSTM1 was present at low levels in the lung, whereas GSTM2 was minimally present in the epithelium, and GSTM3 was located in the airway epithelium and smooth muscle of the lung (125). The GSTO1 protein was found primarily in apical parts of epithelial cells, in murine mouse lungs (127), while another study claimed, in humans, that GSTO1 was mainly expressed in alveolar macrophages, and to a lesser extent in airway and alveolar epithelium (128). GSTT was found to be poorly expressed in the lung, and the expression of GSTZ was moderate. Moreover, GSTs were also present in extracellular fluids as GSTA1, GSTM, GSTP, and GSTO1 were detectable in sputum supernatants of human subjects and GSTA, GSTO1, and GSTP1 were furthermore found in bronchoalveolar lavage fluid (BALF) and plasma (126, 128, 129). The extracellular presence and function of GSTs is of particular interest given the importance of GSH and regulatory enzymes in the protection of lung cells and tissues from the extracellular space. The unique expression profiles of GSTs in lung cells and within populations of airway epithelial cells point to specific functions of the diverse GSTs, which remain to be unraveled.

*Importance of GSTs in lung development and lung function during early life events*

Normal lung growth and development during early life and childhood are essential to reach maximal attainable adult lung function. Prenatal, postnatal, early life, and ongoing

childhood environmental exposures as well as genetic GST polymorphisms can affect lung development, lung maturation and adult lung injury and repair, events which are highly associated with each other (130, 131). Reduced lung growth results in a lower attained lung volume which increases adverse effects from exposure to respiratory toxins, the risks for acute symptoms from exacerbations of asthma or respiratory infections, and risks for chronic diseases such as COPD. Indeed, studies have shown that exposure to for example air pollution or tobacco smoke especially in association with GST polymorphisms affects pre- and postnatal lung development, increases the risk of asthma (symptoms) at younger age, and increases the risk for decreased lung function in adolescence which increased the susceptibility of developing COPD (4, 132-134).

Interestingly, there are some indications that GST polymorphisms are associated with the development of bronchopulmonary dysplasia (BPD), a severe pulmonary complication of premature birth. BPD is clinically defined as a dependence on oxygen past the 36$^{th}$ week of gestation and is characterized by an arrest in alveolarization and a reduced vascular network (135). The *GSTM1* null and combined *GSTM1*/*GSTT1* null genotypes (136), as well as the *GSTP1* Ile105 allele (137) have been associated with BPD, although some studies failed to confirm these associations (138-140). Importantly, these infants have a life-long risk to develop lung diseases, from asthma in childhood to adult lung diseases, such as asthma and COPD at more advanced age (132, 133).

Polymorphisms in GSTs of both the child and the mother during pregnancy are furthermore associated with lung function and the development of asthma in later life. It was shown that the presence of the *GSTT1* null allele in both the mother and the child was associated with a lower lung function and increased airways hyperresponsiveness (AHR) in the first year of life. Maternal smoking during pregnancy further exacerbated these effects (141). The detoxification capacity of both the mother and the child thus play a role in preventing adverse effects on early life lung development. Maternal exposure to particulate matter with a diameter of less than 2.5 micrometers ($PM_{2.5}$) at 35-40 weeks of gestation was furthermore found to be associated with decreased $FEV_1$ and FVC in early childhood (7 years of age), in particular in boys. In conjunction, this study demonstrated an increased

methylation of the GSTP1 promotor in nasal epithelial cells at age 7 when prenatal exposure to $PM_{2.5}$ was high in this same critical time-window (35-40 weeks) of pregnancy. A borderline significant association between GSTP1 methylation and decreased $FEV_1$ was furthermore established (142). In addition to the contribution of inherited genetic variants, this study demonstrates the role of epigenetic regulation of *GSTP1* expression by pollutants in lung function development.

In addition to direct effects of GST polymorphisms on the toxic effects of smoking of adults and the development of disease, a number of studies identified an interaction between GST variants, maternal smoking and lung function in children. Early-life wheezing was more prevalent in children with the wild-type *GSTP1* genotype whose mothers smoked (143). Similar results were obtained for the presence of the null allele of *GSTM1*, which in combination with maternal smoking led to a reduced lung function (144), and strongly increased the risk to develop asthma and asthma-associated symptoms (134, 145-147). *GSTM* and *GSTP1* Ile105Val variants have furthermore been associated with reduced lung function and growth until adolescence, independent of smoking (85, 148, 149). Moreover, *GSTT1* and *GSTM1* gene deletions are associated with chronic bronchitis in children (150).

Exposure to air pollution ($NO_2$ and $PM_{2.5}$) at birth and early life was also associated with increased asthma incidence through adolescence, showing the vulnerability of children in the perinatal period and the development of asthma at a later age (151). Moreover, 3 to 12 year old children with either the *GSTM1* null genotype or who were homozygous for the *GSTP1* Val105 allele, were more susceptible to asthma associated with environmental tobacco smoke exposure than those without these GST polymorphisms (152). The effect of environmental tobacco smoke was shown to be cumulative over time, as in 13 to 21-year olds with the *GSTM1* null allele, lung function declined with age as the peak expiratory flow rate was substantially reduced compared to 3 to 12 years old.

In 2007, the Framingham Heart study published results of their genome-wide analyses in relation to lung function in adults. Top ranked relations were found between the non-synonymous coding homozygous *GSTO2* Asn142 and $FEV_1$, as well as FVC (77). This association was later confirmed in a larger study, which additionally found an interaction

between lung function, *GSTO2* Asn142Asp and environmental tobacco smoke exposure (153). Surprisingly, the interaction of *GSTO2* Asp142 with in utero exposure to tobacco smoke associated with higher $FEV_1$ (153). Moreover, unique studies involving human experimental exposures have demonstrated that adult subjects with the null genotype of *GSTM1* show an increased susceptibility to the development of neutrophilic bronchitis in response to ozone, endotoxin, particulate matter and wood smoke (5, 154-156). Mechanistically, knock-down of *GSTM1* increased IL-8 release by bronchial epithelial cells in response to ozone as well as diesel particles, involving enhanced nuclear factor kappa B (NF-κB) activation and ROS production, and enhanced Erk/PI3K/Akt activation and ROS production respectively (6, 157). Importantly, a similar increase in IL-8 release was noted by epithelial cells derived from donors with the *GSTM1* null genotype (6). Thus, the latter studies suggest that GSTM1 appears to provide protection from a variety of insults and to protect from inflammation.

Numerous studies have shown that air pollution can cause acute and chronic morbidity, often related to lung damage (158, 159). Indeed, it has been estimated that ambient and household air pollution accounts indirectly for more than 6 million deaths per year, for which 50% is accountable due to COPD (160). Although it is unambiguous that PM and ambient air pollution are harmful for our lungs, a recent manuscript questioned the studies performed on the effects of air pollution on the development of chronic lung diseases (161), and suggested caution regarding the interpretation of claims that exacerbations and deaths were caused by particulate air pollution. More studies will be required to test the individual impact of each component (pollutant) to the susceptibility in developing disease and affecting mortality.

Alterations in lung development and maturation can increase the susceptibility to respiratory infections, acute exacerbations and even chronic lung disease. Moreover, children with GST polymorphisms are significantly more likely to have increased risk of acute respiratory illness if they are born to mothers who smoke during pregnancy or if they have been exposed to environmental tobacco smoke (134, 141, 144, 145). In the next

section we will review studies that have implicated GSTs in the pathogenesis of asthma and COPD.

**Asthma**

Asthma is a complex pulmonary disorder characterized by mucus metaplasia, airways hyperresponsiveness (AHR), airway remodeling, and inflammation, which affects approximately 300 million people worldwide (162). Asthma can be further divided into subgroups including exercise-induced asthma, occupational asthma, and allergy-induced asthma (163). Asthma also occurs in settings of obesity wherein the disease is notably severe (164, 165). Common symptoms include shortness of breath, wheezing and/or coughing, as well as chest tightness. Exposure to various irritants and allergens, e.g. airborne allergens (pollen, dust mites, mold spores), respiratory infections, physical activity, cold air, air pollutants such as smoke, and stress can trigger aforementioned symptoms of asthma. Oxidative stress has been speculated to be one of the main risk factors for asthma development (166).

Asthma is linked to chronic inflammation, and involves numerous cell types of the innate and adaptive immune system, including eosinophils, neutrophils, activated mast cells and type II immune cells, along others (167, 168). The pathophysiology of asthma is characterized by structural changes in the airways, such as subepithelial fibrosis, mucus metaplasia, smooth muscle hypertrophy and hyperplasia, increased blood vessel formation as well as dysregulation of epithelial barrier function (167). Asthma can develop at any age and due to the heterogeneity of the disease, it is hard to define and characterize patients with asthma (169). Current treatment for asthmatics include corticosteroids, and bronchodilators, which reduce symptoms, but do not cure disease. Moreover, these treatments are mostly effective in patients with typical asthma characteristics (atopic asthma) including eosinophilic inflammation, $T_H2$ immunity and acute exacerbations (167). Importantly, a subgroup of asthma patients with severe disease do not respond to these current treatments. The onset of asthma in these patients with severe disease is often independent of allergy and not always associated with eosinophilic inflammation. Conversely, these patients may have neutrophilic inflammation, are often steroid resistant

and their disease is associated with other factors including environmental and lifestyle factors (170).

**COPD**

COPD, one of the most common lung diseases in the world, is a chronic inflammatory lung disease characterized by airflow obstruction. Approximately 251 million people are affected by COPD globally which is about 8-10% of the adult population in developed countries (171). The World Health Organization has projected that COPD will become the 3rd leading cause of death globally by 2030 unless vital action is taken to reduce underlying risk factors (172). Main symptoms include difficulty breathing, wheezing and coughing, and excessive mucus production. Initially these symptoms are mild and often leave the disease undiagnosed. But as the disease progresses, they lead to major limitations in the execution of daily activities and negatively impact patients' quality of life. Current treatments alleviate some of the symptoms and slow disease progression, but the progression is not halted or reversed. The Global Initiative for Chronic Obstructive Lung Disease (GOLD) defines COPD as a common, preventable and treatable disease that is characterized by persistent airflow limitation that is due to airway and/or alveolar abnormalities caused by significant exposure to noxious particles or gasses (173). The severity of the disease is defined in GOLD stages (I->IV; A->D) from mild to more severe disease (173, 174).

The primary exposure linked to the development of COPD is cigarette smoke, followed by air pollution, chemical dusts and fumes. Despite the strong association with smoking, it is important to note that approximately 25% of COPD patients never smoked, and that the majority of smokers is spared from the disease (175). Genetic predisposition and gene-environment interactions thus play an important role in determining disease susceptibility. As COPD usually develops after the age of 40 and resembles features of normal lung aging, it is coined a disease of accelerated lung aging (176, 177). Because of the increase in the aging population, the still rising smoking prevalence in many countries, as well as the diminished air quality, the number of patients with COPD is likely to increase in the coming years.

The two most common conditions contributing to the loss of lung function in COPD are emphysema and chronic bronchitis (178). Chronic bronchitis represents an inflammatory state of the airways, which is associated with thickening of the bronchial wall and excess mucus production. In emphysema, damage to pneumocytes and proteolytic degradation of alveolar membranes leads to loss of alveolar septae. This reduces the surface area for gas exchange and leaves the small airways more prone to collapse. COPD is thus a complex clinical condition in which different components and mechanisms represent and contribute to the pathophysiology, as well as to its clinical presentation (173, 174, 179). Chronic inflammation and oxidative stress resulting from the exposure to noxious gasses and particles, and persisting after exposure ceases, are considered major drivers of the irreversible damage and aberrant repair that characterize the different pathological features.

The initial symptoms of COPD and asthma are very similar, and therefore sometimes difficult to distinguish during early stages of disease. One of the main differences between COPD and asthma is that COPD is mainly caused by irreversible damage elicited by smoke or other noxious inhaled particles and consequent chronic inflammation, and usually develops after the age of 40, whereas asthma is mainly caused by allergen-driven inflammatory reactions and can develop at any age (169, 177). The main inflammatory cells involved in COPD include neutrophils and macrophages, while smokers additionally have Th1 lymphocyte involvement, although some people with COPD also have eosinophil involvement, the predominant inflammatory cells in asthma. Similar to asthmatic patients with severe disease, current treatments for COPD patients reduce symptoms but do not recover the irreversible loss in functional lung tissue (173, 174). There is an urgent need to gain more insights into the underlying mechanisms of both chronic lung diseases to develop new treatment strategies to increase the quality of life and survival of patients with chronic lung diseases.

As mentioned in the previous section, GSTs display many important functions through with they may influence lung disease susceptibility. In the next section we will

review studies performed per class of GST, including the different GST isoenzymes and the known polymorphisms relevant to asthma and COPD.

**Glutathione-S-transferases in asthma**

*GSTA in asthma*

The studies that examined GSTA in asthma are very limited, which is surprising since the GSTA1/2 enzyme was found to be abundantly expressed in human lung tissue (125). One study reported the upregulation of *GSTA1* mRNA expression in induced sputum cells of asthmatics compared to healthy controls (**Table II**). This study also showed that GSTA was highly expressed in peribronchial inflammatory cells and in epithelial cells in ovalbumin-treated mice, although the mRNA expression in the whole lung was not different between ovalbumin-treated mice and their controls (180). RNA-seq data showed that *GSTA2* was upregulated, and *GSTA4* was downregulated in nasal brushings from asthmatics compared to controls (**Figure 2A**). Moreover, an earlier study highlighted *GSTA1* as a susceptibility locus for asthma, including the *GSTA1**-69C/T (rs3957357) polymorphism. Individuals with the −69T allele exhibited lower *GSTA1* expression (181). In two studies by the same group, asthmatics displayed a higher prevalence of the *GSTA1**-69C/T genotype compared to controls, and the *GSTA1**-69T polymorphism was associated with an increased risk of asthma and allergies in Italian adults (OR = 3.45; 95% CI: 1.80-6.62) (182, 183) (**Table III**).

*GSTM in asthma*

*GSTM1* genes have been associated with an increased risk of asthma in children and adults (21). In the Children's Health Study in the 1990's, the *GSTM1* null genotype was associated with deficits in annual growth rates (FVC:-0.21%; 95% CI: -0.40, -0.03, and $FEV_1$:−0.27%; 95% CI, −0.50, −0.04) (85). Moreover, as mentioned before, the *GSTM1* null genotype has been linked to an increased risk of inflammatory lung diseases (61) including asthma, and atopy, a predisposition towards developing certain allergic hypersensitivity reactions (65, 184-186), especially in combination with environmental exposure (145). Asthma patients displayed a higher prevalence of the *GSTM1* null genotype compared to healthy individuals (63.4% vs. 40.8%; OR = 2.34; 95% CI, 1.31-4.20) (96), although this prevalence varies across

ethnic groups (187). However, there has been extreme between-study variability and publication bias, and the association between *GSTM1* and asthma sometimes disappeared when meta-analysis were restricted to the larger studies (184). In a recent 'updated' meta-analysis, including 26 case-control studies, a significant association was found for the *GSTM1* null polymorphism (OR = 1.452; 95% confidence interval (CI): 1.192-1.770) and the risk of asthma in both children (OR = 1.368; 95% CI: 1.051-1.781) and adults (OR = 1.859; 95% CI: 1.183-2.921) (24). Based on subgroup analysis by ethnicity, a significant association for GSTM1 and the risk of asthma was found in Europeans (OR = 1.303; 95% CI: 1.018-1.667), Africans (OR = 2.175; 95% CI: 1.560-3.031) and Latin-Americans (OR = 2.265; 95% CI: 1.375-3.729). One study so far also reported an association between the GSTM1 polymorphism rs412543 (G>C) and asthma (84) (**Table III**). *GSTM4* transcript levels were increased in nasal brushings from asthmatic patients compared to control subjects (**Figure 2A**), while *GSTM1* mRNA levels were shown not to be different in induced sputum samples from asthmatics compared to healthy controls (**Table II**) (180). These data on transcript levels are in contrast with the results of the meta-analysis that suggest that subjects with the *GSTM1* null polymorphism may display poorer lung function and may be of an increased risk of asthma.

*GSTO in asthma*

Although GSTO is not highly expressed in the lung, its expression can be induced in mouse lungs by allergens and arsenic, a component of cigarette smoke. The latter is of interest as GSTO1 also metabolizes arsenic by its monomethylarsonate reductase activity (71). *Gsto1* mRNA expression, but not protein, was indeed increased in an OVA- model of allergic airways disease (127). Recently, it has been shown in a mouse model of allergic airways disease (involving the house dust mite allergen), that *Gsto1* deficiency promoted a M2-macrophage phenotype (macrophages associated with wound healing and tissue repair), and increased nuclear HIF-1α levels in the lung, in association with increased eotaxin-induced eosinophilic airway inflammation (188). In humans, the distribution of the *GSTO1* genotypes, *GSTO1* Ala140Asp and *GSTO1**E155del, was nearly equal between control subjects and asthmatics (182) (**Table III**). Italian subjects with the *GSTO2* Asn142Asp

genotype were shown to have an increased risk of asthma (**Table III**) (182, 183). The *in vivo* mouse data suggests that *Gsto1* may be protective during allergic airway disease. However, future studies with a larger sample size of human subjects are required to determine the contributory role of GSTO1 and GSTO2 and their polymorphisms to asthma pathology.

*GSTP in asthma*

The mRNA expression of *Gstp* has been examined in different animal models of asthma with contrasting results. One study reported increases in total GST activity in BAL fluid and *Gstp* mRNA expression in lung tissue using an ovalbumin mouse model of allergic airways disease (180). In contrast, another study showed that *GSTP* transcript levels were down regulated and GST activity was decreased in lung tissues from wild-type mice challenged with house dust mite (189). These latter results in mice are in line with the downregulation of *GSTP* in nasal epithelial cells from asthmatic children (**Table II**) (189). In contrast, RNA-seq data show increased *GSTP* mRNA levels in nasal brushings of asthmatics (**Figure 2A**) while another other study reported no difference in *GSTP1* mRNA expression between induced sputum samples of asthmatics and healthy controls (180). The discrepancy in data from the mouse studies might be attributable due to differences in the allergen challenge regimen and differences in strains of mice used (180). *GSTP* knock-out mice also showed enhanced AHR, eosinophilia, airway remodeling, and goblet cell hyperplasia, although these results were also strain dependent, and only minor effects were observed in mice chronically stimulated with OVA (190). Interestingly, GSTP1 has been recently reported to increase the proteolytic activity of the protease Der p1 in the house dust mite allergen, suggesting that GSTP may promote house dust mite-induced immune responses (191).

Multiple studies have moreover examined the impact of *GSTP1* polymorphisms on the risk of asthma development and lung function. For example the noncoding polymorphisms rs1871042 (C>T) and rs947895 (C>A) were associated with lower odds of asthma (84). The haplotype corresponding to *GSTP1* rs6591256, rs17593068, rs1695, rs1871042, rs947895 was associated with a nearly five-fold increase in the odds of asthma (OR = 4.8, $p$ = 0.007) showing the complexity between GST variants and the relation with risk of disease. In particular, the *GSTP1* Ile105Val polymorphism (rs1695, A>G) has been

analyzed in a number of genetic association studies with conflicting outcomes (21, 27, 143, 184, 186, 192-194) (**Table III**). The *GSTP1* Ile105Val polymorphism modifies the substrate affinity of the GSTP1 enzyme, and decreases GST activity towards CDNB (26). Notable, individuals homozygous for the *GSTP1* 105Val genotype have an altered catalytic activity depending on substrates compared with individuals homozygous for the *GSTP1* 105Ile allele. Children (with asthma) who were homozygous for *GSTP1* Val105 allele showed slower lung function growth (85). Furthermore, the Val105 allele appears to play an important role in lung physiology in combination with environmental exposure. It was for instance associated with an increased susceptibility to breathing difficulties caused by ozone and an increased risk of sensitization to any allergen when exposed to elevated levels of traffic NO(x) (84, 195) and asthma when exposed to $PM_{2.5}$ or ozone (196). A study in German children could however not establish an association between the Ile105Val, as well as the Ala114Val (A114V; rs1138272) polymorphisms and bronchial asthma or AHR (**Table III**) (197). Surprisingly, both the wild-type and mutated allele have been associated with early-life symptoms an increased risk of asthma or atopy (27, 96, 143, 198, 199). These different outcomes might be accounted for by differences in ethnicities, age, and urbanization (194). However, when meta-analyses was performed on 28 different studies, including these independent genetic associations no significant association was found with asthma susceptibility for the *GSTP1* Ile105Val polymorphisms, although it is worth mentioning that high between-study heterogeneity was identified (200).

*GSTT in asthma*

Assessment of *GSTT1* mRNA level revealed that its expression was not different in induced sputum samples between asthmatics and healthy controls in one study (**Table II**) (180). The RNA-seq data revealed increases in *GSTT1* mRNA in nasal brushings of asthmatics compared to controls (**Figure 2A).** Just like *GSTM1*, atopic asthmatics displayed a higher prevalence of the *GSTT1* null genotype (96), and the *GSTT1* null genotypes have also been associated with (atopic) asthma and asthmatic symptoms (wheezing, cough, asthma attack) (21, 145, 185, 201). Meta-analyses however have shown contradicting results (65, 184). An 'updated' meta-analysis study including 26 case-control studies reported a significant association

between polymorphisms in *GSTT1* and the risk of asthma development in adults (OR = 2.312; 95%CI: 1.204-4.439) (24). Based on subgroup analysis by ethnicity, a significant increased risk was found only in Asians (OR = 2.105; 95% CI: 1.101-4.025) and Russians (OR = 2.747; 95% CI: 1.071-7.046). In the same year another meta-analysis study was published that included independent genetic studies using fixed, and random effects models, and also reported a significant association between asthma susceptibility and *GSTT1* null phenotype (pooled OR=1.33, 95% Cl = 1.10-1.60) (200) (**Table III**).

**Glutathione-S-transferases in COPD**

*GSTA in COPD*

Genetic variants in *GSTA* occur at a very low frequency and have not been examined in relation to COPD susceptibility. A microarray study on airway epithelium found an upregulation of *GSTA2* mRNA in smokers compared to non-smokers (202). These findings are in line with reports of enhanced *GSTA2* mRNA expression in bronchial biopsy material of smokers compared to non-smokers (203). Despite these consistent observations on *GSTA2* mRNA, lung GSTA protein levels were not different between control smokers and non-smokers. On the other hand, increased levels were observed in whole lung lysates of mild/moderate COPD patients compared to patients with very severe disease and controls (**Table II**). Importantly, the antibody used in this study showed two distinct bands on Western blot of whole lung lysates, which could represent GSTA1 and GSTA2. This was however not elucidated, nor were both bands quantified separately. In contrast, the number of GSTA positive central airway epithelial cells was decreased only in GOLD stage IV patients. Furthermore one GSTA immunoreactive band could be detected in induced sputum, and was shown to be increased in patients with chronic bronchitis and in patients with moderate to severe COPD compared to controls (128). A proteomics study on BALF in contrast found both GSTA1 and GSTA2 decreased in COPD patients compared to controls. Because of the smaller scale of the study and focus on identifying biomarkers that could discriminate between COPD and lung cancer, in which GSTA1 and GSTA2 were increased as well, no relations with disease severity were reported (204). In aggregate, these data on

GSTA(2) seem to fit the paradigm that this enzyme is elevated as part of the protective defense system against smoke components.

**Table II**: mRNA and protein expression differences between COPD or asthma vs control

| GST | Asthma vs control mRNA | Asthma vs control protein | COPD vs control mRNA | COPD vs control protein |
|---|---|---|---|---|
| GSTA | | | | Increased in lung lysates of mild-moderate COPD patients compared to very severe disease and controls; GSTA higher in induced sputum (127); GSTA+ airway epithelial cells decreased in GOLD stage IV patients. |
| GSTA1 | Increased in sputum (179) | | | Decreased in BALF (207) |
| GSTA2 | | | | Decreased in BALF (207) |
| GSTM1 | Not different in sputum (179) | | Lower in lung tissue (209); Increased in microarray from bronchial brushing (210); | |
| GSTM3 | | | Increased in microarray from bronchial brushing (210) | |
| GSTO1 | | | | Decreased in lung tissue and sputum compared to smokers and never smoker controls (127) |
| GSTP1 | Downregulation in nasal epithelial cells in children (192), no difference in induced sputum samples (179) | | Decreased in peripheral lung (209) | Increased in whole lung homogenates in patients with mild disease, number of GSTP+ cells did not differ (127); lower in BALF (207) |
| GSTT1 | Not different (179) | | | |

Table describes the differences in mRNA and protein expression of the different GST isoenzymes between COPD patients and controls or asthmatic patients and control subjects. COPD: Chronic Obstructive Pulmonary Disease; GST: Glutathione-S-transferase; BALF: Broncho alveolar lavage fluid

*GSTM in COPD*

Functional genetic variants of *GSTM1* have been found to be protective in COPD (205). Conversely, the *GSTM1* null genotype has been found to be associated with COPD susceptibility in various studies, with some exceptions (**Table III**). Reasons for discrepancies between studies may include the sample size, methodologies used and variants between

ethnic backgrounds. For this reason, meta-analyses have been performed which will be discussed below.

One study examined *GSTM1* gene expression in lung tissue of COPD patients compared to never-smoking and smoking controls as part of a candidate inflammatory and antioxidant gene study approach. They found significantly higher *GSTM1* mRNA expression in non-COPD smokers compared to both never smoking controls, as well as COPD patients (**Table II**). No significant correlations between *GSTM1* expression and lung function were observed (206). A microarray study on bronchial brushing also found increased *GSTM1* mRNA levels in healthy smokers compared to non-smokers, but also found levels to further increase in COPD patients (207). Another microarray study, focused specifically on antioxidant genes in airway epithelium, did not find such an upregulation of *GSTM1* (or *M3* or *M4*) mRNA in smokers compared to non-smokers (202). A similar lack of induction of *GSTM1* mRNA by smoking was reported in BAL cells and bronchial biopsies (203). We are unaware of studies that have examined GSTM1 protein or activities in lung tissue of COPD patients.

Protein level of GSTM3 in the lung was more abundant in current smokers than ex-smokers (125). Like *GSTM1*, *GSTM3* mRNA was also increased in healthy smokers compared to non-smokers, and further increased in COPD patients in a microarray study on bronchial brushing (207). Similarly, the microarray gene expression data available from the Lung Genomics Research Consortium (LGRC) also showed increases in *GSTM1*, *GSTM3*, *GSTM4*, and *GSTM5* in lung tissues from COPD patients (**Figure 2B**).

*GSTO in COPD*

Microarray data from lung tissue found a downregulation of *GSTO2* mRNA in COPD patients compared to controls (**Figure 2B**). The top-ranked association between lung function and *GSTO2* (rs156697) that was observed in the Framingham Heart study prompted us to examine *GSTO* polymorphisms in COPD (77). In a case-control study we did not demonstrate an association between lung function and *GSTO1* Asp140Ala or *GSTO2* Asn142Asp. However, an increased risk of COPD was found for the *GSTO2* 142Asp allele, as

well as the *GSTO1* 140Asp/GSTO2 142Asp haplotype (OR=1.39 95%CI 1.00-1.93, and OR=2.40, 95%CI 1.43-4.02, respectively) (208). Our unpublished observations in the COPD case-control study did show a significant relation between the *GSTO2* Asn142Asp genetic variant and plasma PSSG level, being the highest in the major allele carriers (data not shown). Although we do not know what the functional consequence of the *GSTO2* Asn142Asp variant is in the lung, an inefficient ascorbate regeneration could be a mechanism whereby GSTO contributes to the development of asthma and COPD due to a loss of detoxification (**Table I, Table III**).

Only protein expression of GSTO1 has been examined in COPD. In lung tissue homogenates of COPD patients, the GSTO1 protein expression was significantly lower compared to smoking and never smoking controls (**Table II**) (128). No significant relations to lung function parameters were found. Interestingly, GSTO1 could also be detected constitutively in cell culture supernatant of macrophage or bronchial epithelial cell lines and in induced sputum supernatant, suggesting that GSTO1 exerts functions extracellularly. The sputum of COPD patients also contained less GSTO1 compared to sputum of controls (128).

*GSTP in COPD*

Microarray data from the LGRC also showed a downregulation of *GSTP* mRNA in COPD patients, particularly in GOLD Stage II, and III (**Figure 2B, C**). In line, mRNA levels of *GSTP* were reported to be downregulated in peripheral lung tissue of COPD patients compared to non-smoking controls and non-COPD smokers (**Table II**) (206). Furthermore, *GSTP* mRNA levels significantly correlated with parameters of pulmonary function and negatively correlated with cigarette smoking history. Contrasting the mRNA expression data in whole lung homogenates, the protein level of GSTP1 was increased in COPD patients with mild disease compared to both smoking and non-smoking controls, whereas the number of GSTP positive cells analyzed by IHC did not differ between these groups (**Table II**) (128). In line with the mRNA data, a proteomics study on BALF reported lower levels of GSTP1 in patients with COPD compared to controls (204). Overall, these results suggest that an impaired GSTP1-mediated xenobiotic-metabolizing activity may be correlated with

pathophysiological changes in COPD. In line with this possibility, GSTP has been reported to exert protective properties against cigarette smoke-induced cell death in fibroblasts (209).

As previously described, several genetic variations have been detected in *GSTP1*. In particular the frequencies of the *GSTP1* Ile105Val genotypes in COPD and risk of disease have been performed in a number of genetic association studies in different populations. However, these studies reported conflicting results, probably due to small sample sizes and different ethnic backgrounds (28, 210-214). Interestingly, the wild-type as well as the mutant allele have been associated with an increased risk for COPD, although some studies also failed to find any association at all (210, 213, 214). Also, meta-analyses result in divergent outcomes with respect to the associations between this *GSTP* polymorphism and the risk for COPD. One meta-analysis reported that the lle105Val polymorphism in *GSTP1* was not protective against COPD development in Asian populations (215, 216). Conversely, another meta-analysis study reported a significant correlation between the *GSTP1* Val/Val and COPD (217). The most recent and extensive analysis to date which included a total of 1892 cases and 2012 controls found no significant correlation between the *GSTP1* lle/Val polymorphism and COPD risk in general, nor in any ethnic subgroups (28). The relationship between *GSTP1* polymorphisms and COPD development is thus still not clear. Further studies involving larger populations and careful control with respect to age, sex, ethnicity and smoking behavior will be required to address the link between *GSTP1* polymorphisms and COPD risk.

*GSTT in COPD*

The *GSTT1* null genotype, has been associated with COPD susceptibility in general (25), and emphysema in particular (218). Similar to aforementioned conflicting data with other GSTs, not all studies could confirm the association between loss of *GSTT1* and enhanced COPD risk (64, 219). No studies to date have examined GSTT1 beyond the null polymorphism. The allele has not been identified as a risk factor for COPD or lung function decline in GWAS analyses, nor has the mRNA been reported in COPD. The protein and activity levels in lung tissue remain furthermore unexplored.

*Meta-analyses on genetic GSTT and GSTM polymorphisms and COPD*

Because of the inconsistencies between genetic studies, and the inconclusive outcomes on the association between GST polymorphism and COPD, a number of meta- and pooled analyses have been performed. A recent meta-analysis of the association of the null genotypes of *GSTM1* and *GSTT1* with COPD based on 37 case-control studies, including 4674 COPD patients and 5006 controls, showed that both null genotypes were more frequent in COPD patients compared to controls. A significant relation was furthermore found between these individual polymorphisms, as well as the combined presence of both null polymorphisms and COPD risk, with divergent effects in different ethnic groups (64, 95, 215, 219-221).

Combined *GSTM1* and *GSTT1* deficiency has furthermore been linked with accelerated age-related decline of lung function in males, irrespective of smoking status (222). Additional gene-gene interactions have been reported between *GSTM1* and *MMP1*, *9* and *12*, modifying not only the risk of COPD, but also the age of onset, as well as the severity of the disease (223). In combination with microsomal epoxide hydrolase genetic variants, the risk of COPD is further increased for *GSTM1* null and *GSTP1* Ile105 (224, 225). Furthermore, overall plasma GST activity decreased in COPD patients compared to control (226). In the same study, the GST polymorphisms that associated with COPD were *GSTM1* null and Val105Val *GSTP1*, although this association was only apparent when the GST genotypes were combined.

**Table III**: GST polymorphisms and their contribution to GST function, and the risk of the variant allele (compared to wild-type allele) on the chronic lung diseases asthma and COPD

| Genetic variant | Function | Asthma | COPD |
|---|---|---|---|
| GSTA1* -69C/T, rs3957357 | Promotor variant, reduced protein expression (51) | Increased risk in adults (181, 182); Associated with allergies (182) | No data |
| GSTM1 G>C rs412543<br><br>GSTM1 null | Promotor variant<br><br>16 kb deletion – loss of protein expression (59, 60) | Increased risk in children (84)<br><br>Increased risk to (atopic) children & adults (188, 189), especially in combination with exposure to tobacco smoke (144), meta-analysis (24, 65, 187); no association (190, 203, 204); increased risk but not when only restricted to larger studies (187) | <br><br>Increased risk, meta-analysis (64, 218, 222, 224); no association (25) |
| GSTO1 Ala140Asp (A140D), 419C>A, rs4925<br><br>GSTO1-E155del<br><br>GSTO2 Asn142Asp (N142D), 424A> G, rs156697 | Coding sequence variant with variable effects on activity (66, 67, 69, 76)<br><br><br><br>Coding sequence variant with reduced expression (67) | No association (181)<br><br>No association (181)<br><br>Increased risk in adults (181, 182) | Increased risk as haplotype with rs156697 (211)<br><br>Increased risk (211) |
| GSTP Ile105Val (L105V), 313A>G, rs1695<br><br>GSTP1 Ala114Val (A114V), 341C>T, rs1138272 | Coding sequence variant with decreased activity (26)<br><br>Coding sequence variant with not known activity | Decreased risk (188, 189, 197); no association (21, 200, 204); increased risk to (atopic) asthma in children & adults (27, 96); increased in combination with environmental exposure (198, 199); No significant association in meta-analysis (203)<br><br>Increased risk at young age (198) No association in children (200) | Decreased risk (216, 217); increased risk (213) – in particular in α1-antitrypsin deficiency (214) – meta-analysis (220). No association in meta-analysis (28, 219) |
| GSTT1 null | Whole or partial ~ 54 kb deletion - loss of protein expression (22, 23) | Increased risk to asthma attack in children (204), increased risk to atopic asthma (188), meta-analysis (24, 65, 203); no association (190); increased risk but not when only restricted to larger studies (187) | Increased risk (25), especially for emphysema (221), meta-analysis (64) |

Table describes different GST polymorphisms that have been associated with asthma or COPD in the literature.

COPD: Chronic Obstructive Pulmonary Disease; GST: Glutathione-S-transferase

**Differential expression of GSTs in epithelial cells in asthma and COPD**

Cytosolic GSTs are differentially and heterogeneously expressed in the lung; in specific bronchial- and alveolar epithelial cells, as well as in various immune and mesenchymal cells (**Table II / Figure 2-4**). We will focus here on their differential expression in epithelial cells as they display the most robust expression of GSTs and play important roles in lung pathologies. Upon activation, lung epithelial cells secrete pro-inflammatory mediators that recruit, activate, and/or promote differentiation and survival of immune cells, they release growth factors that control airway remodeling, and play an important role in lung repair through their (trans)differentiation capacities (227). The differential gene expression of GSTs could be associated with their respective physiological roles of basal, ciliated, club, and goblet cells in maintaining lung homeostasis, as well as with the versatile roles in which epithelial cells contribute to disease pathogenesis. For example, GSTs have been linked to epithelial plasticity, which is a key pathogenic feature in asthma as well as COPD. Indeed, epithelial plasticity often contributes to airway remodeling including mucus metaplasia in asthma, and squamous metaplasia in COPD (227). GSTA has been shown to promote epithelial-mesenchymal transition (EMT), the process whereby epithelial cells lose their cell polarity and cell-cell adhesion, and transform into mesenchymal cells, in lung cancer cells (228), and GSTO has been shown to inhibit membrane localization of E-cadherin (229). The regulatory properties of GSTs and their possible contribution to airway epithelial (dys)function in asthma and COPD will be described in the next sections.

Although the differential expression of GSTs in epithelial subtypes has not been investigated in COPD, multiple studies have examined the expression of cytosolic GSTs in (small) airway epithelial cells derived from healthy smokers compared to non-smokers. In small airway epithelium from healthy and smoking subjects combined, *GSTA1* was reported to be the most abundantly expressed isoenzyme of the GSTA class, followed by *GSTA2*, *GSTA3*, and *GSTA5*, data which are comparable to the RNA-seq data in **Figure 2.** Increased expression of *GSTA2*, *GSTA1*, as well as hypomethylation and upregulation of *GSTM1* and *GSTM5* were reported in small airway epithelium from smokers vs non-smokers (230-232). In smokers moreover, decreases in mRNA of *GSTO2* in airway basal cells were reported, in

association with decreased GSH levels and a decreased GSH/GSSG ratio (233). These data show that the small airway epithelium exhibit changes in GST gene expression in response to cigarette smoking, which could play a role in the development of smoking-associated lung disease (88). Interestingly, the RNA-seq database revealed, that the epithelial subtype expression of specific GSTs differs between healthy subjects and asthmatics (**Figure 4**). As mentioned before, *GSTA1*, *GSTO1,* and *GSTP1* are the main GSTs expressed in the different epithelial cell types. Interestingly, although the expression of *GSTP1* is already high in all subtypes or airway epithelial cells, the expression seems to elevate further in most epithelial subtypes in asthmatics (**Figure 4A**). In asthmatics, *GSTA1* expression was decreased in basal and suprabasal, goblet, and in club cells, while its expression was slightly increased in mucus ciliated cells compared to controls, which is in line with the RNA-seq data in nasal brushings (**Figure 2A**). *GSTA2* expression was decreased in ciliated, mucus ciliated and goblet cells in asthmatics compared to controls. The expression of *GSTO1* was found to be higher in ciliated cells and ionocytes of asthmatics, and lower in mucus ciliated cells, and club cells, while the *GSTO2* expression was decreased in basal and suprabasal cells in asthmatics compared to control. In smooth muscle, *GSTA4*, *GSTM3*, and *GSTO1* were higher expressed in controls than in asthmatics (**Figure 4B**). Interestingly, in asthmatics, the expression of *GSTA1*, *GSTA4*, *GSTM3*, *GSTM4*, *GSTM5*, *GSTO1* (and to a lesser extent *GSTP1*) all decreased in the fibroblasts, while *GSTO1*, and *GSTP* increased in the endothelium, compared to controls. As described before, GSTs are less ubiquitously expressed in immune cells (**Figure 4B**). The expression of *GSTA1*, and *GSTO1* seem to decrease in macrophages from asthmatics versus healthy controls. For monocytes, *GSTO1* expression increased in asthmatics compared to controls. Furthermore, mast cells display elevated expression levels of *GSTA1* and *GSTP1*, and lower expression of *GSTO1* in asthmatics versus controls. In asthmatics, dendritic cell expression of *GSTA1*, and *GSTO1* were decreased in comparison to healthy controls. Lastly, T- and B-lymphocytes isolated from asthmatics display reduced expression of *GSTO1*, versus healthy controls, and in asthmatic B-lymphocytes, elevated expression of *GSTA2* was observed, while *GSTP1* and *GSTA2* expression were decreased in T-lymphocytes. These differences in expression profile of each GST isoforms in epithelial

subtypes between asthmatics and controls should be further explored as they may give insights into the specific functions and contribution to disease.

**Biochemical reactions regulated by GSTs and their contributions to asthma and COPD**

Antioxidant systems are crucial to detoxify the constant exposure to toxic compounds. It is tempting to speculate that GSTs could be upregulated as part of an adaptive and protective response to detoxify these compounds and to scavenge the excessive levels of oxidant production/oxidative stress. Conversely, a failure of the lungs to respond proportionally to these challenges would result in damage, and disease development and progression. This failure can have a genetic or epigenetic origin (as previously reviewed), or can occur at the transcriptional or translational level. With respect to failure at the transcriptional level, a disturbance in (detoxification) protection against smoke components is often seen in more severe disease in COPD, and is related to Nuclear factor erythroid 2–related factor (Nrf2) repression. Nrf2 is a transcription factor that regulates numerous antioxidant and cytoprotective genes, including GSTs, and protect against oxidative damage triggered by injury and inflammation. The role of Nrf2 in controlling the expression of GSTs has been shown in studies using the homozygous *Nrf2*$^{-/-}$ mouse model, where it was found that the constitutive as well as inducible (by the antioxidants butylated hydroxy anisole, and ethoxyquin) expression of *Gsta1*, *Gsta2*, *Gstm1*, *Gstm2*, *Gstm3*, and *Gstm4* was impaired in the liver (234, 235). A recent comprehensive proteomic analysis moreover reported that GSTM1, GSTM2, GSTM3, GSTA3, GSTA4, and GSTP1 were all expressed at a constitutively lower level in the liver of *Nrf2*$^{-/-}$ mice (236). However, it remains to be determined whether *Nrf2* deficiency also alters GST expression in the lung. Previous studies have shown that a GSH deficiency and the exposure of cells to ROS or pro-oxidants enhances the induction of GSTs by activating Nrf2 (237). For example, it has been shown that increased concentrations of the lipid peroxidation product 4-HNE, leads to modification of cysteine residues in the Nrf2 inhibitor, Kelch-like ECH-associated protein 1 (Keap1), thereby stabilizing Nrf2 and allowing its nuclear accumulation. Increased GSTA4 transcription in association with enhanced GSH levels thereby result in an increased capacity to metabolize 4-HNE. Conversely, GSTs can negatively regulate Nrf2 activity by protecting Keap1 from cysteine

modifications that are required to stabilize and release the transcription factor. GSTs thus comprise a negative feedback system that indirectly controls the levels of other antioxidant and drug-metabolizing enzymes that are regulated through the Keap1/Nrf2 pathway. In the context of chronic lung diseases, NRF2 levels were decreased in lung tissue of emphysema patients (238). In mice, *Nrf2* absence enhanced the susceptibility to smoke-induced emphysema (239), while activation of Nrf2 attenuated emphysema development (240). However, it is unknown if GSTs play a role herein. *Nrf2* deficient mice also show increased susceptibility to asthma, and cell-specific activation of Nrf2 in club cells significantly reduced allergen-induced oxidative stress, inflammation, mucus, and airway hyperresponsiveness (241), which is of particular interest given that *Gsta1*, *Gsta2,* and *Gstp1* are inducible by Nrf2 and all highly expressed in club cells (**Figure 3**). A disturbed detoxification may partially be caused by a lack of Nrf2-dependent upregulation of GSTs. This could be restored by dietary and natural compounds such as flavonoids, which have been shown to modulate the activity and/or expression of GSTs, and curcumin, which has been attributed antioxidant-like properties in part through activation of Nrf2 (242). Nonetheless, these compounds do not specifically induce GSTs via Nrf2.

*Contribution of GSTs through enzymatic activities and ligand binding*

In addition to the classical detoxification function of GSTs, alterations in GST expression and/or enzymatic activity, regardless of the isoform, may impact their ability to scavenge oxidants, regulate biological processes (e.g. protein-protein interaction), and affect redox signaling through modulation of PSSG (**Table I, II**). GSTs display an array of regulatory mechanisms of pathways involved in asthma and COPD. Since GST isoforms have diverging functions, the relative contribution of a given isoform to chronic lung disease may differ. For instance, lipid peroxidation, a consequence of oxidative stress, induces pulmonary inflammation, and is therefore believed to contribute to asthma and COPD pathophysiology (243, 244). Isoenzymes of mainly the GSTA, but also GSTM, and GSTT classes use lipid peroxidation products generated by ROS. The upregulation of *GSTA2* mRNA (and *GSTA1* mRNA; **Figure 2A**) in asthmatics as well as the upregulation of *GSTT* and *GSTM* isoenzymes in asthma and COPD (**Figure 2A, B**) could be a response to cope with the elevated level of

lipid peroxidation in these patients. GSTs also act as ligandins (non-enzymatic protein-protein interaction) regulating inflammation and (programmed) cell death. GSTM, GSTO, and GSTP regulate the activity of members of the MAPK pathway, especially JNK and TRAF2. The regulatory effect on JNK is of considerable interest as JNK has been shown to play a role in allergen-induced inflammation and remodeling associated with bronchial hyperresponsiveness (245). Moreover, activation of JNK signaling was present in lungs of patients with COPD and was shown to be involved in TNFα-driven extracellular matrix remodeling (246). The precise link between JNK and GSTP in the pathology of asthma and COPD remains to be examined, especially given that *GSTP* is differentially regulated in both diseases (decreased in COPD; increased in asthma) (**Figure 2, 4**). The Omega class of GSTs has furthermore been shown to enzymatically modulate ryanodine receptors, which may be a novel target to modulate airway reactivity in cigarette smoke linked diseases such as COPD, as exposure to acute cigarette smoke selectively altered small airway contraction and down-regulated ryanodine receptors in airway smooth muscle (247). Furthermore, the GSTO2 Asn142Asp genotype has been associated with age-related defects in smokers such as the development of cataracts due to inefficient ascorbate regeneration (248). Based on its function in β-oxidation, changes in GSTZ levels in the lung may regulate fatty acid metabolism and related mitochondrial (dys)function, which have previously been linked to chronic lung diseases such as asthma and COPD (249-251).

*Contribution of GSTs in PSSG*

Changes in redox balance may contribute to the pathogenesis of chronic lung diseases including asthma and COPD. Notably, dysregulation of reversible oxidation of protein cysteines including PSSG is thought to promote chronic lung diseases as it may affect cellular pathways which play an important role in the lung including cell proliferation, inflammation, metabolism and apoptosis. The importance of PSSG in particular the lung epithelium is beginning to be elucidated. Studies from our laboratory have shown that overall PSSG levels are increased in lungs of mice with allergic airways disease exposed to the house dust mite allergen compared to control lungs (252). On the other hand, PSSG levels were decreased in sputum samples from eosinophilic and neutrophilic asthmatics

compared to healthy controls, although this should be confirmed in a larger study (253). Others have moreover reported that the exposure to diesel exhaust co-administered with house dust mite allergen promotes PSSG in mice with allergic airways disease (254). Although PSSG levels have not yet been examined in COPD patients, exposure of lung epithelial cells to cigarette smoke extract increased PSSG (255), while PSSG was shown to be decreased in lungs of mice exposed to cigarette smoke extract (256). In BALF, as well as in macrophages isolated from smoke-exposed mice, an increase in PSSG was observed (255). PSSG seems thus to be distinctly regulated in different regions and cellular compartments of the lungs in response to cigarette smoke, which could be related to differences in GST expression and/or activity in these various compartments and cell types.

As previously mentioned, especially GSTP, and to a lesser extent GSTO and GSTM, have been shown to catalyze the forward PSSG reaction (50, 57, 69). GSTO has also been shown to deglutathionylate proteins, and the GSTO1 Ala140Asp polymorphism was found to have increased activity for the forward PSSG reactions, while deglutathionylation was repressed (69). The biological contribution of this polymorphism still has to be further confirmed, and so far, no association is found between this GSTO1 variant and asthma or COPD (**Table III**). With respect to specific targets, GSTP and GSTM can directly bind (enzymatically) and glutathionylate adenosine monophosphate activated protein kinase (AMPK), leading to its activation *in vitro* (57). AMPK is a key enzyme in the regulation of cellular energy homeostasis, and interestingly, also modulates inflammatory responses. AMPK has been shown to suppress airway smooth muscle cells thereby decreasing airway remodeling (257). Furthermore, AMPK has been shown to decrease lung inflammation and emphysema by reducing IL-8 production in airway cells (258). GSTP moreover promotes SRC-glutathionylation, which was essential for GSTP to inhibit SRC phosphorylation and activation (259). Glutathionylation of SRC regulates VE-cadherin stabilization, a key transmembrane adhesive protein in endothelium adherens junctions, thereby maintaining endothelial barrier function. SRC is a non-receptor tyrosine kinase protein which has been shown to play an essential role in mucin secretion induced by pathogens, and it can promote airway smooth muscle cell growth and migration which occur in airway remodeling found in asthma and COPD (260, 261). Furthermore, SRC has been shown to

regulate the allergic inflammatory response via epithelial growth factor receptor (EGFR) and subsequent downstream activation of multiple pathways including ERK1/2, PI3Kδ/AKT and NF-κB (262). These results suggest that GSTP (and GSTM)-induced PSSG of AMPK and SRC may act on airway remodeling and inflammation. Additionally, glutathionylation of actin and tubulin inhibits their polymerization, thereby altering cell structure and affecting morphological polarity and migration of neutrophils in response to chemotactic gradients or cell growth (263-265). Moreover, neutrophils from mice lacking *Glrx1* displayed impaired recruitment to sites of inflammation and reduced bacterial capability (265). It remains to be determined whether the GST-controlled forward reaction similarly regulates neutrophil migration.

Members of NF-κB, a family of transcription factors that promote the activation of pro-inflammatory responses (266), have been shown to be inhibited by glutathionylation including RelA, and p50 (267-269). Notably, we have shown that, upon LPS-induced lung inflammation, the activity of inhibitory kappa B kinase beta (IKKβ), a key activator of the NF-κB signaling pathway, was inhibited by GSTP-mediated PSSG, resulting in decreased levels of pro-inflammatory mediators in epithelial cells, which was rescued by GLRX1 (267). IL-1β is also a pro-inflammatory cytokine related to asthma pathology, and PSSG has been shown to protect inhibition of IL-1β by overoxidation thereby maintaining IL-1β activity (270). This is of interest as it has recently been reported that human nasal mucociliary epithelial cells exposed to $PM_{2.5}$ elicited a dose-dependent transcriptomic response with an upregulation of IL-1 (α and β) expression (271). Moreover, our laboratory has recently demonstrated that increases in IL-1β-dependent glycolysis (e.g. metabolic reprogramming) resulted in an induced inflammatory response in epithelial cells during allergic airways disease (272). We also showed that the induced pro-inflammatory signaling was in part driven by the glycolysis inactive form of Pyruvate Kinase M2 (PKM2) through, in part, phosphorylation of signal transducer and activator of transcription 3 (STAT3), an important protein in the regulation of cell proliferation, differentiation, apoptosis and inflammation (273). This is of considerable interest as metabolic proteins have been shown to be reversibly inactivated by PSSG, including glyceraldehyde-3-phosphate dehydrogenase

(GAPDH), a glycolysis enzyme, and α-ketoglutarate dehydrogenase (KGDH), a TCA cycle protein, as well Pyruvate Kinase in the liver (PKL) (274-276). Furthermore, STAT3 has been shown to be glutathionylated resulting in inhibition of its phosphorylation (277, 278). GSTP has already been shown to interact with STAT3, thereby inhibiting the STAT3-signaling pathway (279). The observed metabolic reprogramming during allergic airways disease may therefore be attributable to changes in (GSTP)-controlled PSSG.

Lastly, it has been shown that GSTP catalyzes the first step in the reduction of the sulfenylated peroxidatic cysteine of PRDX6 (1-cysPRX), a member of the peroxiredoxin family, by binding the sulfenic acid form of Cys47 and glutathionylating the active site cysteine (280, 281). PRDX6 is involved in redox regulation of the cell as it can for example reduce $H_2O_2$, short chain fatty acids and hydroperoxides. Oxidation of the catalytic cysteine of 1-cysPRX has been associated with loss of peroxidase activity (280, 282). Heterodimerization of 1-cysPRX with GSTP mediates the glutathionylation of the previously oxidized cysteine thus restoring its peroxidase activity. PRDX6 is unique since PRDX6 uses GSH as an electron donor, whereas PRDX1-5 used thioredoxin as an electron donor. Interestingly, PRDX6 is the highest expressed PRDX of the PRDX family in the epithelium of human lungs (283), and similarly GSTP is the most abundantly expressed GST in lung tissue, especially in the epithelium (125).

Given that GSTP is the most abundantly expressed GST in the lung epithelium, displaying differential expression in asthmatics and COPD patients compared to controls, coupled to its important role in PSSG chemistry, a prominent role for GSTP in lung epithelial pathology is suggested. However, the implications of PSSG, the proteins that are targeted via glutathionylation and the GSTs that control them in epithelia of asthmatics and COPD patients remains a much unexplored area of research.

**Conclusion and Future directions**

In this review we highlighted the current knowledge on GSTs in the susceptibility to and progression of asthma and COPD, as well as their contribution to lung growth and development. Despite poor reproducibility, genetic studies support the prevailing concept that GST deficiencies (notably the *GSTT* and *GSTM* null polymorphisms) are associated with an increased risk for the development of asthma and COPD, which could be related to adverse perinatal effects, as well as diminished lung development and growth. Gene-environment interactions, as well as epigenetic modulation likely contribute to these risks. In contrast, mRNA and/or protein expression of most GSTs are upregulated in asthma and COPD patients, as well as in smokers, compared to controls (**Figure 2, Table II)**. Although there are some exceptions, these expression data are in line with the notion that GSTs are upregulated during chronic lung diseases as part of an adaptive and protective response to the disease-triggering exposures. However, since GSTs have effects beyond detoxification, including scavenging of oxidants, ligand binding, and regulating redox signaling (**Table I**), this upregulation might not inherently be of a protective nature. Similarly, a downregulation of GSTs may have detrimental effects beyond attenuated detoxification. The contribution of GSTs to lung diseases through these alternative functions, which differ by (sub)class of GST, remain to be examined. For example, the most prominently expressed GST in human lung tissue, GSTP1, accounts for over 90% of the lung's activity towards CDNB, but also regulates various pathways that play a role in lung pathologies through S-glutathionylation, as well as by non-enzymatic protein-protein interactions. Generic knock-out studies at this point fail to unravel the contribution of each of these divergent functions of GSTP1 to the outcome in disease models. Future studies should therefore extend their analyses to include these alternative functions. Additionally, compounds should be developed that interfere with specific functions or disrupt the binding of GSTs with target proteins. Future studies could also include examining the effect of genetic polymorphisms of GSTs on their alternative functions in allergic asthma and COPD.

The purpose and importance of the differential and heterogeneous expression of GSTs in the lung remains to be further explored. However, single cell RNA sequencing

studies are on the rise and may help to provide further insight herein in health and disease. To this end, future studies could also focus on gene-editing of GSTs in specific target cells to examine and expand the knowledge on the precise role of different GSTs in a particular cell type during homeostasis, as well as in the responses to for example allergens, and inhaled pollutants.

In conclusion, in order to better understand the contribution of GSTs to (normal) lung growth and development, as well as to the onset of lung diseases at later age, more detailed insights are needed with respect to their complex regulation, their cellular distribution and versatility of effector functions. Only through such thorough and precise understanding can tailored therapeutic strategies be designed to affect specific functions of particular GSTs, that may help alleviate the burden, or even prevent the development, of chronic lung diseases.

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# 3

## IL-1/inhibitory κB kinase ε-induced glycolysis augment epithelial effector function and promote allergic airways disease

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## ABSTRACT

**Background:** Emerging studies suggest that enhanced glycolysis accompanies inflammatory responses. Virtually nothing is known about the relevance of glycolysis in allergic asthma.

**Objectives:** Here we sought to determine if glycolysis is altered in allergic asthma and to address its importance in the pathogenesis of allergic asthma.

**Methods:** We examined alterations in glycolysis in sputum samples from asthmatics and primary human nasal cells, and used murine models of allergic asthma as well as primary mouse tracheal epithelial cells to evaluate the relevance of glycolysis.

**Results:** In a murine model of allergic asthma, glycolysis was induced in the lungs in an IL-1-dependent manner. Furthermore, administration of IL-1β into airways stimulated lactate production and expression of glycolytic enzymes, with notable expression of lactate dehydrogenase A occurring in the airway epithelium. Indeed, exposure of mouse tracheal epithelial cells to IL-1β or IL-1α resulted in increased glycolytic flux, glucose usage, expression of glycolysis genes, and lactate production. Enhanced glycolysis was required for IL-1β- or IL-1α-mediated pro-inflammatory responses and the stimulatory effects of IL-1β on HDM-induced release of TSLP, and GM-CSF from tracheal epithelial cells. Inhibitor of κB kinase ε was downstream of house dust mite (HDM) or IL-1β, and was required for HDM-induced glycolysis and the pathogenesis of allergic airways disease. SiRNA-ablation of lactate dehydrogenase A attenuated HDM-induced increases in lactate and attenuated HDM-induced disease. Primary nasal epithelial cells from asthmatics intrinsically produced more lactate as compared to cells from healthy subjects. Lactate content was significantly higher in sputum supernatants from asthmatics, notably those patients with >61% neutrophils. A positively correlation was observed between sputum lactate and IL-1β, and lactate content negatively correlated with lung function.

**Conclusions:** Collectively, these findings demonstrate that IL-1β/IKKε signaling plays an important role in HDM-induced glycolysis and the pathogenesis of allergic airways disease.

**Key message:**

- Primary nasal epithelial cells from asthmatics intrinsically express more LDHA and produce more lactate as compared to healthy controls, and sputum lactate levels negatively correlate with lung function in asthmatics.
- The IL-1/IKKε signaling axis mediates HDM-induced glycolysis and allergic airways disease in mice.
- Increases in glycolysis are critical in the augmentation of HDM-triggered pro-inflammatory responses of airway epithelial cells.

**Capsule summary:**

IL-1 and IKKε play important roles in HDM-induced glycolysis and the pathogenesis of allergic airways disease, and lactate is a potential biomarker for increased glycolysis and IL-1-associated pro-inflammatory signals in airways of asthmatics.

**Keywords:** Asthma, house dust mite, glycolysis, interleukin-1, inhibitor of κB kinase ε, lactate, lactate dehydrogenase A

**Abbreviations:**

AHR: Airways hyperresponsiveness

LDHA: Lactate dehydrogenase A

HDM: House dust mite

WT: Wild-type

HK1: Hexokinase 1

HK2: Hexokinase 2

MTE cells: Mouse tracheal epithelial cells

ECAR: Extracellular acidification rates

OCR: Oxygen consumption rates

2-DG: 2-Deoxyglucose

IKK: Inhibitory kappa B kinase

IL1RI: Interleukin 1 receptor, type I

TLR4: Toll like receptor-4

TGF-β: Transforming growth factor β

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

IFNγ: Interferon γ

NECs: Nasal epithelial cells

PKM2: Pyruvate kinase M2

## INTRODUCTION

Asthma is a pulmonary disorder that is characterized by reversible airflow obstruction, chronic airway inflammation, airways hyperresponsiveness (AHR) and remodeling. Asthma affects nearly 10% of the US population and is increasing in prevalence, making it a major public health problem (1). Asthma is a complex and heterogeneous syndrome and has a number of different clinical phenotypes that are associated with distinct cellular and molecular mechanisms (2) controlled by innate and adaptive immune responses to allergens, which rely on both immune (such as DCs, Th2 and Th17 cells, and innate lymphoid cells) and structural cells that include airway epithelium (3, 4). The exact biochemical processes underlying the diverse phenotypes of asthma, and the precise contributions of lung structural and immune cells during asthma pathogenesis remain incompletely understood.

Changes in cellular metabolism, notably increases in glycolysis, accompany inflammatory responses (5). Glucose is taken up by cells through glucose transporters and subsequently undergoes glycolysis via a step-wise cascade to form pyruvate that can enter the mitochondria and undergo oxidative phosphorylation. Alternatively, pyruvate can be metabolized to lactate, via lactate dehydrogenase (LDHA). Aerobic glycolysis, the metabolism of glucose to form lactate in the presence of oxygen, is a feature of tumor or metabolically active cells, and is associated with increased glucose uptake and lactate over-production (6, 7). Aerobic glycolysis also generates NADPH that is important in protection against oxidative stress, and preserves the carbon backbone of glucose to fuel the synthesis of macromolecules (6, 7).

Glucose metabolism is implicated in immune activation, and increases in glycolysis regulate immune effector function through multiple mechanisms (8). For example, enhanced glycolysis has been shown to facilitate the polarization and/or activation of immune cells (9). Moreover, lactate accumulates at the sites of chronic inflammation (10, 11), and tumor microenvironments (12), indicative of increased glycolytic flux. Virtually nothing, however, is known about the glycolytic status in the setting of, and the relevance of deregulated

glycolysis in the pathogenesis of allergic airways diseases. A previous study demonstrated increases in lactate in serum of asthmatics compared to patients with COPD or healthy controls, and increases in lactate in proliferating CD4 T cells isolated from asthmatics compared to healthy subjects. The same authors demonstrated that intraperitoneal injection of dichloroacetate, an inhibitor of pyruvate dehydrogenase kinase, attenuated increases in lactate in proliferating CD4 T cells, and attenuated ragweed-induced allergic airways inflammation and airways hyperresponsiveness in mice (13). However, the extent of increases in glycolysis in airways of asthmatics remain unknown. Similarly, the signals that promote increases in glycolysis in allergically-inflamed lung tissue also remain elusive. It also is not clear whether increases in glycolysis occur in lung epithelial cells and affects the response of epithelial cells to house dust mite allergen. Therefore, the goal of the current study was to address some of these questions, using a mouse model of house dust mite (HDM)-induced allergic airways disease, nasal epithelial cells and sputum samples derived from asthmatics. Our results demonstrate that increases in glycolysis are a critical feature of allergic airways disease, controlled by an IL-1/IKKε signaling axis.

# MATERIALS AND METHODS

## Subject characteristics

The study population was enrolled at the asthma clinic in CHU Liege (Belgium). Healthy subjects were recruited at the hospital and University of Liege, Belgium. The study cohort consisted of healthy subjects (n = 20) and patients with asthma (n=94). The demographic and functional characteristics of the 114 subjects from the study cohort are shown in Table E1. The study was approved by the local ethics committee, University of Liege, Belgium, (reference 2005/181; conforming to the declaration of Helsinki).

Nasal epithelial cells were isolated from healthy subjects (n=6) or patients with allergic rhinitis and asthma asthmatics (n=7) enrolled at the University of Vermont Medical Center. Patient characteristics are provided in Table E2. The local IRB granted approval for all of the procedures involving human subjects (CHRMS 15-067). Additional details are provided as Online Supplementary Information.

## Mouse studies

Age-matched, 8- to 12-week-old mice were used (The Jackson Laboratory, Bar Harbor, ME) for all experiments. Wild-type (WT, C57BL6/NJ)), *Rag*$^{-/-}$ (C57BL6/J), or *Ikbke*$^{-/-}$ (C57BL6/J) mice along with their strain-matched controls were sensitized (Days 1 and 8), challenged (Days 15-19), and rechallenged (Days 29, 32, 36, and 39) with HDM extract as shown in Figure 1A. All animal experiments were approved by the Institutional Animal Care and Use Committee.

## Cell studies

Human nasal epithelial cells were isolated from healthy subjects or asthmatics. Cells were cultured and exposed to HDM for assessment of glycolysis proteins and lactate content in culture supernatants. Mouse tracheal epithelial cells were isolated from tracheas from WT mice of mice or mice lacking *Ikbke*. Cells were cultured and exposed to the indicated mediators, for the assessment of lactate in supernatants, glucose uptake, extracellular acidification rate, and cytokine levels in medium.

**Statistical analysis**

All data were evaluated using JMP Pro 10 software (SAS Institute, Cary, NC) and GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA). Cell culture and mouse data were compared with either one-way, or two-way ANOVA, followed by a Tukey post hoc test. Scoring of histological staining was analyzed by the Kruskal-Wallis test. Human demographic data were compared by Student T-test, Chi-squared test or Wilcoxon rank sum test. Human sputum data for lactate and IL-1β were log-transformed before being compared by Student T-tests. Comparisons of sputum data between asthmatic and healthy participants were adjusted for differences in BMI using ANOVA. *P* values less than 0.05 were considered statistically significant.

More detailed information on the materials and methods used in this study is available as supplemental information.

## RESULTS

**Increases in glycolysis in lungs from mice with house dust mite (HDM)-induced allergic airways disease.**

Low pH is a characteristic of chronic inflammatory sites (10, 14, 15) and results mainly from a metabolic shift to aerobic glycolysis and subsequent lactate over-production. Little is known about the glycolytic status in asthma. We therefore first determined whether glycolysis was affected in a HDM model of allergic airways disease (Figure 1A). No increases in lactate were observed acutely following HDM (Day 1 and 2, Figure 1B). Five consecutive daily exposures to HDM in week 3 without prior sensitization during week 1 and 2 (day 20: 2X saline, 5X HDM) also did not result in increases in lactate (Figure 1B). However, lactate levels were increased in the BAL and lung tissue homogenates of mice at Day 20 following 2 sensitizations and 5 challenges (Figure 1B). Significant increases in lactate production were also observed 24 h (Day 30) following HDM re-challenge on day 29, in mice previously sensitized and challenged with HDM (2X HDM, 5X HDM, HDM). These increases in lactate on days 20 and 30 corresponded with increases in total cells and notably increases in eosinophils and neutrophils in BAL (16) (Figure E1 A and B) and suggest that increases in lactate are a feature of the adaptive immune response. Increased expression of glycolysis proteins, including hexokinase 1 (HK1), HK2, and lactate dehydrogenase A (LDHA) were observed in lung tissue homogenates 20 or 29 days post HDM exposure, while they tended to decrease at day 30 (Figure 1C). LDHA preferentially converts pyruvate to lactate (17). Immunohistochemical analysis of LDHA in saline-exposed mouse lung tissues revealed that LDHA was constitutively expressed in bronchial epithelial and alveolar type II cells (Figure 1D). In response to HDM sensitization and challenge, widespread increases in expression of LDHA were apparent in lung tissue, with increases in immunoreactivity present not only in cells resembling infiltrating immune cells, consistent with the previously appreciated role of glycolysis in immune effector function (9), but also in bronchial epithelial cells (Figure 1D). These findings suggest that both structural and hematopoietic cells might be responsible for HDM-mediated increases in lactate production.

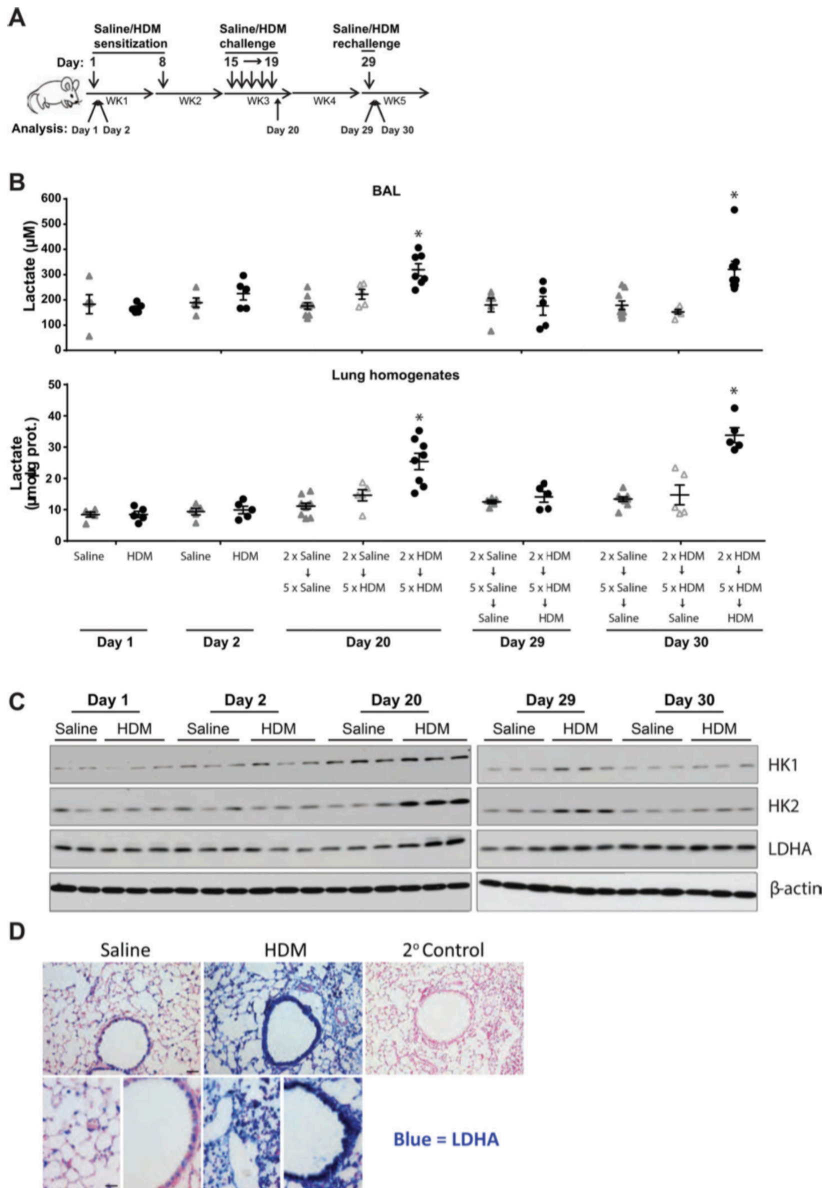


**Figure 1. Evaluation of glycolysis in the lung tissues of mice exposed to house dust mite (HDM). A,** Schematic depicting the dosing regimen of HDM. **B,** Lactate levels in BAL (top) and lung tissues (bottom) following a single or multiple exposures to HDM, according to the schematic in **A**. **P* < 0.05 (ANOVA) relative to the saline group (n=5-8 per group). **C,** Protein expression of glycolysis enzymes in lung lysates from saline- or HDM-challenged mice harvested at the indicated times. β-Actin = loading control. **D,** LDHA immunohistochemistry in lung tissues of HDM-sensitized and -challenged mice harvested at Day 20 (Top: scale bar, 50 µm; Bottom: scale bar, 25 µm). Blue = LDHA. 2°control; HDM-inflamed tissue wherein primary antibody was omitted as a negative control.

**An adaptive immune response and Interleukin-1 (IL1) signaling are required for increases in glycolysis in lungs of mice with HDM-induced allergic airways disease:**

To elucidate the mediators that cause glycolysis, we evaluated a number of pro-inflammatory mediators and assessed whether their levels correlated with increases in lactate. Levels of IL-1β, IL-6, and TNFα but not IL-1α and IL-17 were increased at times that roughly corresponded with increases in lactate (Figure 2A). We next sought to determine whether IL-1 signaling plays a causal role in the augmentation of glycolysis in HDM-induced disease. Neutralization of IL-1 with IL-1 trap (18) (Figure E2) attenuated the HDM-mediated lactate increases (Figure 2B) as well as expression of glycolytic enzymes HK2 and LDHA (Figure 2C), demonstrating the functional importance of IL-1 in augmenting glycolysis in HDM-exposed mice.

The delayed increases in lactate in BAL and lung tissues following HDM sensitization and challenge suggest the requirement of an adaptive immune response. To directly test whether IL-1-dependent increases in glycolysis in response to HDM were dependent on adaptive immunity, we assessed lactate levels in HDM-exposed WT and Rag1-/- mice which lack mature B and T lymphocytes (19). We previously published that Rag1−/− mice exhibited robust decreases in HDM-induced immune cell influx in BAL and IgG and IgE production (20). Strikingly, the HDM-mediated increases in lactate levels (Figure 2D) and IL-1β (Figure 2E) were completely inhibited in *Rag*$^{-/-}$ mice. Taken together, our results suggest that HDM-induced adaptive immunity is required for IL-1 signaling and resultant increases in glycolysis. In order to address whether IL-1β is sufficient to increase glycolysis, we directly administrated IL-1β into the airways of WT mice. IL-1β caused increases in lactate levels in BAL at 6 and 24 h post administration, and in lung tissue after 48 h post administration, and resulted in increases in BAL neutrophils, along with increases in the pro-inflammatory cytokines, CCL20, KC, GM-CSF and TSLP in lung tissue (Figure E3A-C). Increases in lactate were accompanied by increases in HK2 and LDHA in lung tissue (Figure 2G). Evaluation of LDHA by immunohistochemistry revealed increases in LDHA in bronchial epithelia 24 h post-administration of IL-1β (Figure 2H).

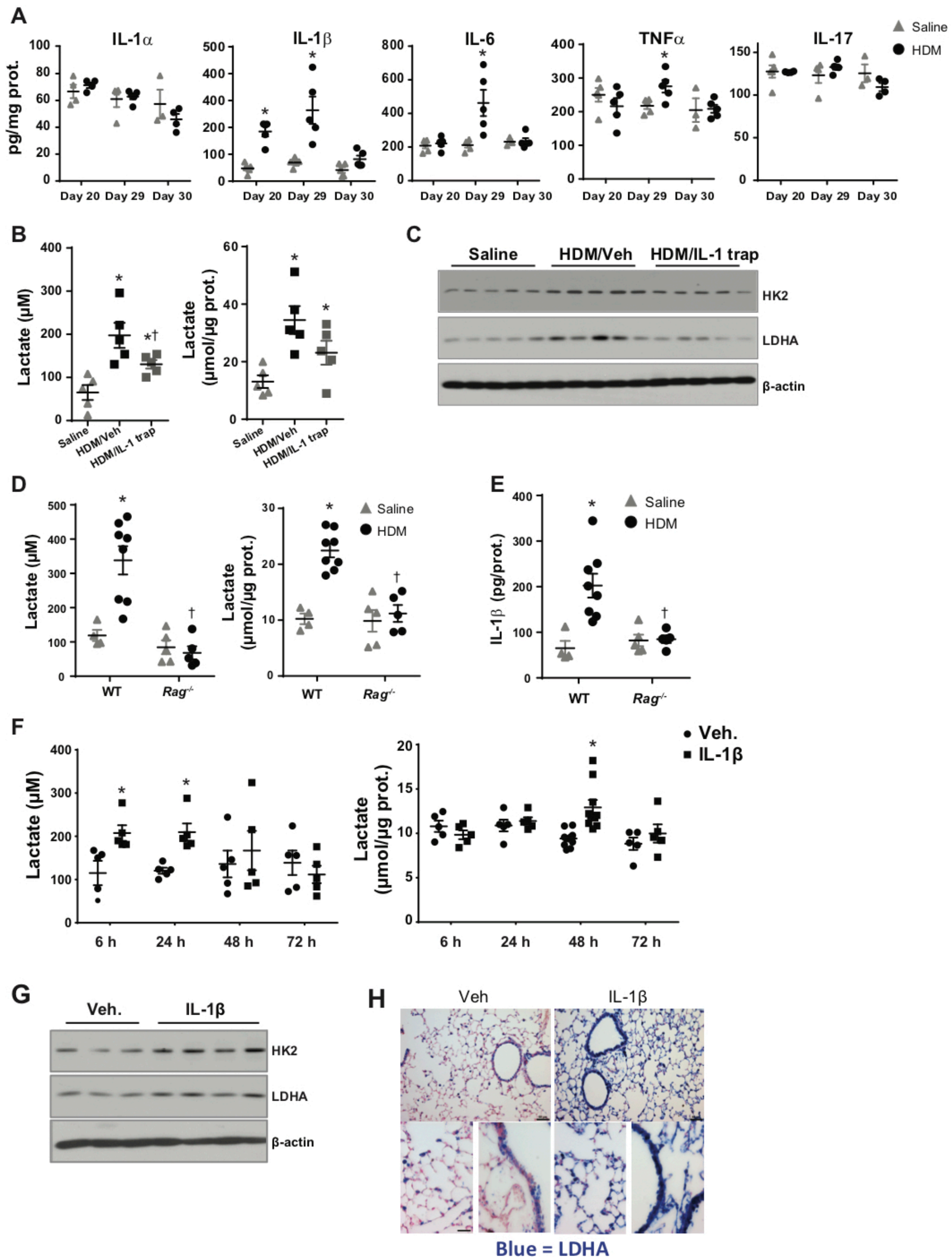


**Figure 2. House dust mite (HDM)-induced T and B-cell adaptive immune responses are required for IL-1β production and resultant increases in glycolysis in lung tissues. A,** Levels of pro-inflammatory cytokines in lung tissue of HDM-exposed mice at the times indicated. **P* < 0.05 compared to saline groups (ANOVA) (n=5 per group). **B-C,** Lactate levels in the bronchoalveolar lavage fluid (BAL) and homogenized lung tissues **(B)** and Western blot analysis of HK2 and LDHA in lung tissues **(C)** from saline-exposed mice or HDM-exposed mice treated with vehicle (Veh) or IL-1 TRAP. Mice were

harvested at day 20. *$P < 0.05$ compared to the saline group, †$P < 0.05$ compared to the HDM/Veh group (ANOVA) (n = 5 per group). Lactate levels in BAL fluid and lung tissues **(D)** and IL-1β levels in the lung tissues **(E)** from *Rag*$^{-/-}$ mice and WT mice exposed to saline or HDM. Mice were analyzed at Day 20. *$P < 0.05$ compared to the saline controls, †$P < 0.05$ compared to the respective WT group (ANOVA, n = 4–8 per group). **F,** Lactate levels in BAL fluid and lung tissues from the mice 6, 24, 48, and 72 h post intranasal administration of IL-1β. *$P < 0.05$ compared to Veh-exposed mice (ANOVA, n=5-8 per group). **G,** Western blotting of HK2 and LDHA in lung tissues from mice treated with recombinant IL-1β (1 µg/mouse) for 48 h. **H,** Immunohistochemical analysis of LDHA in lung tissues 24 h post administration of IL-1β or vehicle (Top: scale bar, 50 µm; Bottom: scale bar, 25 µm). Blue = LDHA.

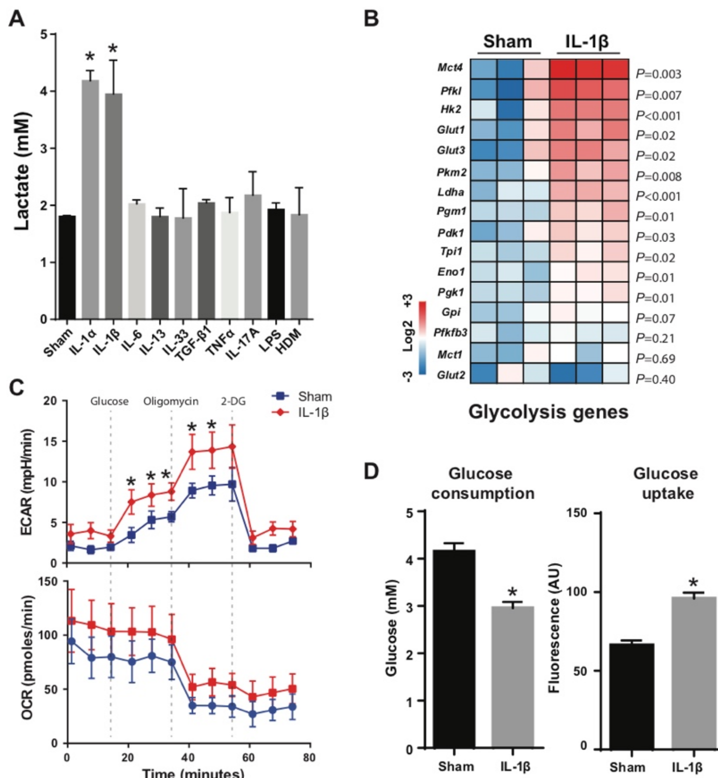


**Figure 3. IL-1α/β increase lactate production, glycolysis gene expression, glucose usage, and glycolytic flux rate in primary mouse tracheal epithelial (MTE) cells. A,** Lactate levels in the cell-culture supernatants of MTE cells following 24 h stimulation with IL-1α, IL-1β, IL-6, IL-13, IL-33, TGF-β1, TNFα, IL-17, LPS, or HDM. *$P < 0.05$ compared to the sham group (ANOVA). Representative results from one out three independent experiments are shown. **B**, mRNA expression of glycolysis-related genes in MTE cells treated with or without IL-1β (10 ng/mL). P values from Student's *t* test are indicated. **C**, ECAR and OCR of IL-1β- or sham-treated MTE cells, measured via a Seahorse Extracellular Flux (XF24) Analyzer. Glucose, oligomycin, and 2-DG were injected sequentially marked by the vertical lines. *$P < 0.05$ compared to the sham group (Student's *t* test). Representative results out three independent experiments were shown. **D,** glucose consumption (**left**) and uptake (**right**) in MTE cells 24 h post stimulation with IL-1β. *$P < 0.05$ compared to the sham group (Student's *t* test).

Increased glycolysis promotes Interleukin-1α- and Interleukin-1β-mediated pro-inflammatory responses in airway epithelial cells and augments release of pro-inflammatory mediators following subsequent exposure to house dust mite.

Our findings demonstrating that intranasal administration of IL-1β increases BAL lactate levels 6 h later, a time point prior to the recruitment of inflammatory cells (Figures E3B and E3C), suggest that IL-1β increases glycolysis in airway epithelial cells in settings of allergic airways disease. In order to directly address this possibility, we exposed mouse tracheal epithelial (MTE) cells to IL-1β or IL-1α for 24 h. Both cytokines resulted in significant increase in lactate levels in culture supernatants. No increases in lactate were observed 24 h after exposure to IL-6, IL-13, IL-33, TGF-β1, TNFα, IL-17, LPS, or HDM (Figure 3A), demonstrating notable selectivity of IL-1α/β in augmenting glycolysis in MTE cells in these experimental settings. Concomitant to increases in lactate, IL-1β significantly augmented expression of a number of genes in the glycolysis pathway (Figure 3B). IL-1β-treated MTE cells had higher basal extracellular acidification rates (ECAR) than vehicle-exposed cells, indicating a higher rate of release of lactate into the culture medium. In response to glucose injection, IL-1β-treated cells demonstrated higher rate of ECAR compared to control cells. IL-1β-treated cells were also more sensitive to the ATP synthesis inhibitor, oligomycin and maintained a higher ECAR (Figure 3C), revealing the higher glycolytic capacity of IL-1β-treated cells. Addition of 2-deoxyglucose (2-DG), a competitive inhibitor of glucose hexokinase, decreased ECAR to baseline levels, confirming that the observed ECAR is due to glycolysis (Figure 3C). In contrast to changes in ECAR, oxygen consumption rates (OCR) were similar in control and IL-1β-treated cells (Figure 3C). In line with these observations, glucose levels in the medium decreased and glucose uptake was increased in response to IL-1β (Figure 3D). These findings collectively demonstrate IL-1β (and IL-1α) as an inducer of glycolysis in lung epithelial cells, and that the ability of IL-1 to augment glycolysis in these experimental settings is not shared by other asthma-relevant mediators tested herein.

The importance of glycolysis in regulating immune effector function responses is well established (21, 22). It is not known whether glycolysis regulates pro-inflammatory responses in epithelial cells exposed to IL-1β. We therefore inhibited glycolysis by pre-

treating the MTE cells with the hexokinase inhibitor, 2-DG, or the LDHA inhibitor, oxamate, and assessed IL-1β-induced pro-inflammatory cytokines. As shown in Figure 4A, 2-DG completely blocked IL-1β-induced lactate production and strongly attenuated production of TSLP, GM-CSF, KC and CCL20 in response to IL-1β (Figure 4B). Similar inhibitory effects on IL-1β-induced lactate and pro-inflammatory cytokines were observed in cells treated with oxamate (Figure 4C and D). 2-DG or oxamate did not induce cell death (Figure E4A) demonstrating that decreases in cytokines observed are not due to a loss of survival. Similar to IL-1β, IL-1α also resulted in increases in the same cytokines which were also inhibited by 2-DG or oxamate (Figure E4B), suggesting that both interleukins trigger similar glycolysis-dependent pro-inflammatory responses in epithelial cells.

Results in Figures 2F and H demonstrate that IL1β was sufficient to increase lactate in lung tissues and expression of LDHA in bronchial epithelia. We next tested whether IL-1β-mediated increases in glycolysis in epithelial cells affected their subsequent response to HDM, in order to gain insights into the functional impact of enhanced glycolysis (which would be expected to occur in a setting wherein IL-1 is increased), for subsequent responses to allergens in airway epithelia. We treated MTE cells with 2-DG for one h or oxamate overnight, followed by treatment with IL-1β for 24 h. Cells were then washed and exposed to HDM for 2 h (Figure 4E and F). While IL-1β or HDM individually led to increases in pro-inflammatory cytokines, a strong synergy was observed in cells sequentially exposed to IL-1β and HDM. Importantly, inhibition of glycolysis with 2-DG strongly attenuated the IL-1β plus HDM-induced levels in TSLP or GM-CSF, and moderately decreased CCL-20 and KC (Figure 4E). Similar responses were observed with oxamate (Figure 4F) with the exception of KC which remained unaffected. Collectively, these data demonstrate that IL-1β-induced glycolysis augments the subsequent pro-inflammatory responses of epithelial cells to HDM.

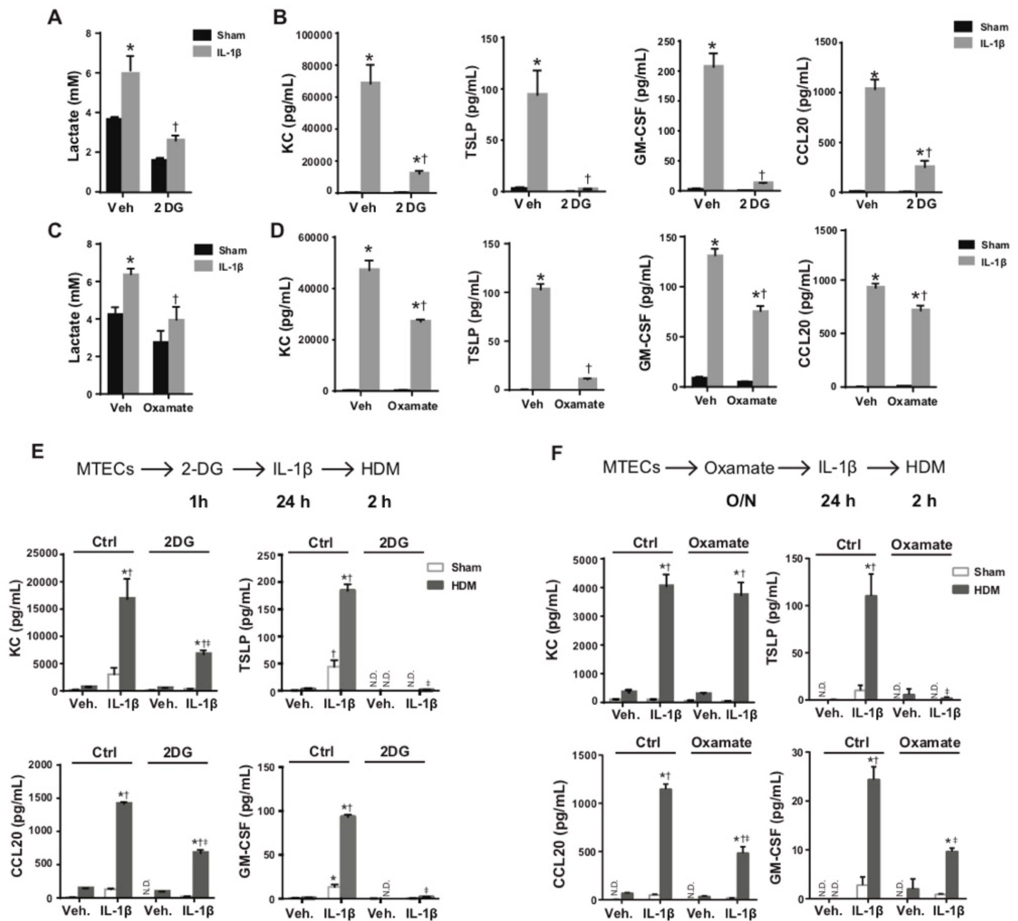


**Figure 4. Importance of glycolysis for IL-1β-induced pro-inflammatory responses and the IL-1β-mediated augmentation of HDM-induced innate cytokine responses in primary mouse tracheal epithelial (MTE) cells. A-D**, Lactate (**A&C**) and levels of pro-inflammatory mediators (**B&D**) in the cell culture supernatants of MTE cells. MTE cells were pre-treated with 2-Deoxyglucose (2-DG, 10 mM) (**A-B**), or oxamate (10 mM) (**C-D**), followed by stimulation with IL-1β (10 ng/mL) for 24 h. **E-F,** Importance of glycolysis in the IL-1β-mediated augmentation of HDM (50 µg/ml)-induced KC, CCL20, TSLP, and GM-CSF levels in culture supernatants. *$P$ < 0.05 compared to non-HDM exposed sham group, †$P$ < 0.05 compared to respective non-IL-1β treated vehicle group (Veh.), and ‡$P$ < 0.05 relative to non-2DG or non-oxamate treated control group (Ctrl) (two-way ANOVA).

**Inhibitory kappa B kinase-epsilon, (IKKε) promotes IL-1β-induced glycolysis in epithelial cells and HDM-induced allergic airways disease in mice**

The inhibitory kappa B kinase (IKK) family includes four kinase members, the canonical IKKα and IKKβ, as well as two non-canonical family members, IKKε and TBK1. We have previously shown that activation of IKKβ play a critical role in the pathogenesis of allergic airways disease (23, 24). Essentially no information exists about the role of other IKKs. IKKε is emerging as a critical regulator of Th17 maintenance, IL-17-induced airway neutrophilia (25), and glycolytic reprogramming in DCs (21). We therefore explored whether IKKε was increased during the pathogenesis of HDM-induced allergic airways disease, and whether IKKε contributed to IL-1β-induced glycolysis. In mice with HDM-induced disease, expression of IKKα and IKKβ increased in lung tissues (Figure 5A), consistent with our previous observations (16, 26). We also observed robust and prolonged increases in IKKε and TBK1 in lung tissues (Figure 5A). We next addressed the impact of *Ikbke* ablation (the gene encoding IKKε) (Figure 5B) on HDM-induced glycolysis and allergic airways disease. Ablation of *Ikbke* significantly attenuated the HDM-mediated increases in lactate (Figure 5C), suggesting the requirement of *Ikbke* in HDM-induced glycolysis. Assessment of HDM-induced airway inflammation revealed slight decreases in overall BAL cell counts in HDM-challenged *Ikbke*$^{-/-}$ mice compared to WT littermates, reflected by slight decreases in neutrophils (albeit not significant), significant decreases in eosinophils, and a lack of differences in macrophages or lymphocytes (Figure 5D). Similar to our previous studies (16) significant increases airway resistance ($R_N$) occurred in HDM-challenged WT mice compared to controls (Figure 5E). While HDM-exposed *Ikbke*$^{-/-}$ mice showed comparable increases in baseline $R_N$ compared to saline-exposed mice, no further increases in $R_N$ in response to increasing doses of methacholine were observed. No differences in tissue resistance (G) were observed between any of the groups. Converse to the attenuation of HDM-mediated increased in $R_N$ observed in HDM-exposed *Ikbke*$^{-/-}$ mice, tissue elastance was significantly elevated (Figure 5E), suggesting complex modulation of AHR in mice lacking *Ikbke*. In WT mice, HDM led to mucus metaplasia and increases in Muc5AC in BAL (Figure 5F-G), in association with increases in IL-33 and IL-13 in lung tissues (Figure 5H), consistent with a type 2, eosinophil-associated inflammatory response. In contrast, HDM-mediated increases

in mucus metaplasia, Muc5AC, IL-33, IL-13, and CCL-20 were strongly attenuated in *Ikbke*$^{-/-}$ mice (Figure 5F-H), suggesting that absence of *Ikbke* attenuates type 2 inflammation. Although levels of TSLP were constitutively lower in *Ikbke*$^{-/-}$ mice, compared to WT counterparts, no effect of HDM was observed at this time point (Figure 5H). No differences between HDM-mediated increases in IL-1β were observed between WT or *Ikbke*$^{-/-}$ mice (Figure 5H), suggesting that IL-1β is increased proximally to, or independently of, *Ikbke*. Because of these findings, the attenuation of HDM-induced lactate in lung tissues from *Ikbke*$^{-/-}$ mice, compared to WT littermates (Figure 5C), and the previously reported role of IKKε in glycolytic reprogramming of DCs (21), we next addressed the role of *Ikbke* in IL-1β-mediated increases in glycolysis in lung tissue. IL1β administration was sufficient to increase lactate in WT mice. The IL-1β-mediated increases in lactate were almost completely abolished in *Ikbke*$^{-/-}$ mice (Figure 5I), suggesting that *Ikbke* is required for IL-1β-induced glycolysis.

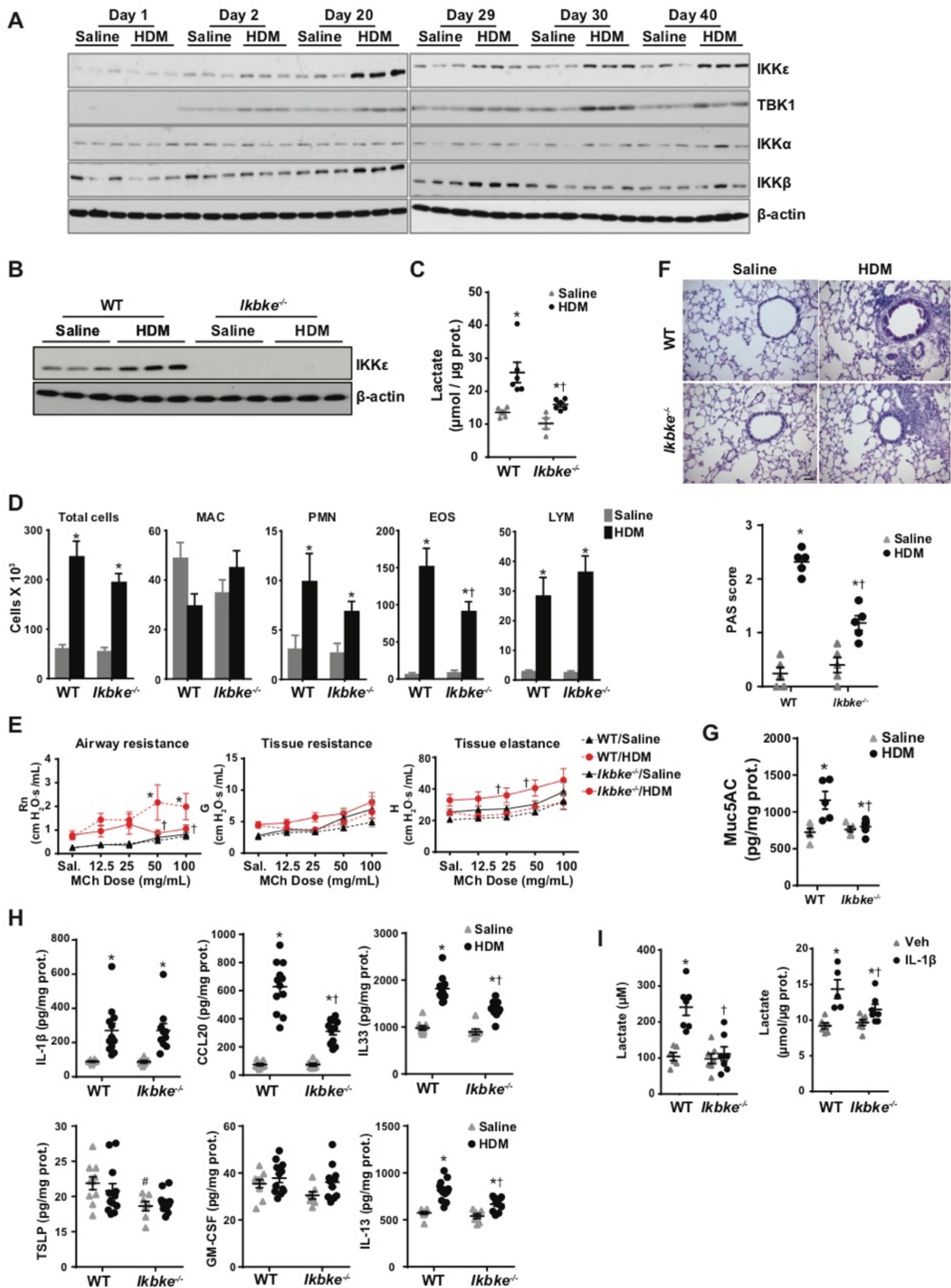

**Figure 5. A causal role for Inhibitory kappa B kinase ε (IKKε) in HDM- and IL-1β-mediated increases in glycolysis and the pathogenesis of allergic airways diseases. A,** Western blot analyses of IKKs in lung tissues of WT mice subjected to the HDM regimen for the indicated times. WT or *Ikbke -/-* mice were exposed as described in Fig. 1. Mice were euthanized at

day 20 for assessment of IKKε in lung tissue via Western blot analysis (**B**), lactate levels in lung tissues (**C**), total and differential cell counts in BAL fluid (**D**), and AHR (**E**). *$p < 0.05$ compared to the saline control group, †$p < 0.05$ compared to respective wild-type (WT) (ANOVA, n = 5-10 per group). **F,** Assessment of mucus metaplasia in WT or *Ikbke*$^{-/-}$ mice exposed to HDM or saline (scale bar, 50 µm) (**Top**). Quantification of airway mucus staining (PAS) intensity (**Bottom**). Data are expressed as means (±SEM) from five mice per group. *$P < 0.05$ compared with respective saline controls. †$P < 0.05$ compared with WT HDM groups (Kruskal-Wallis). Levels of Muc5AC (**G**) and pro-inflammatory mediators (**H**) in lung tissues of WT and *Ikbke*$^{-/-}$ mice exposed to HDM as described in B-E. **I,** BAL and lung lactate levels in WT and *Ikbke*$^{-/-}$ mice exposed to IL-1β for 24 h. *$p < 0.05$ relative to vehicle control group, †$p < 0.05$ relative to the respective wild-type (WT) group

Strong increases in IKKε immunolocalization were observed in bronchial epithelial cells in response to HDM or IL-1β (Figure 6A). We therefore explored the effect of IL-1β on IKKε expression and the role of IKKε in IL-1β-induced glycolysis. Exposure of MTE cells to IL-1β was sufficient to upregulate *Ikbke* mRNA (Figure 6B). IL-1β-mediated increases in lactate were attenuated in *Ikbke*$^{-/-}$ MTE cells (Figure 6C). Similarly, the IKKε/TBK1 inhibitor, Amlexanox resulted in a dose-dependent decrease in IL1β-induced lactate in MTE cells (Figure 6D), and abrogated IL1β-mediated increases in *Glut1*, *Hk2*, *Ldha*, and *Pkm2* mRNA (Figure 6E). The more potent effects of Amlexanox compared to *Ikbke* ablation are potentially due to Amlexanox targeting both IKKε and TBK1 (27). We next addressed the impact of IKKε/TBK1 on the IL-1β-mediated augmentation of HDM-induced pro-inflammatory responses. WT cells were treated with Amlexanox overnight, followed by treatment with IL-1β for 24 h. Cells were then washed and exposed to HDM for 2 h (Figure 6F). Similar to results in Figure 4E, prior exposure to IL-1β led to an augmentation of HDM-induced release of pro-inflammatory mediators from MTE cells (Figure 6F). Amlexanox ablated the IL-1β plus HDM-mediated increases in TSLP and GM-CSF, and attenuated CCL20 and KC (Figure 6F), identical to our findings with 2-DG (Figure 4E). Comparative evaluation of WT and *Ikbke*-/- epithelial cells demonstrated a strong attenuation of IL-1β/HDM-mediated increases of TSLP, and a modest attenuation of KC and GM-CSF, while CCL20 was increased equally in *Ikbke-/-* cells and WT cells in response to IL-1β/HDM. (Figure 6G). Overall, these data suggest that IKKε is a critical mediator in IL-1β-induced glycolysis and subsequent augmentation of HDM-mediated increases of TSLP in airway epithelial cells, and that the further decreases in CCL20, GM-CSF and KC observed in response to Amlexanox in these settings (Figure 6F) may be attributable to TBK1.

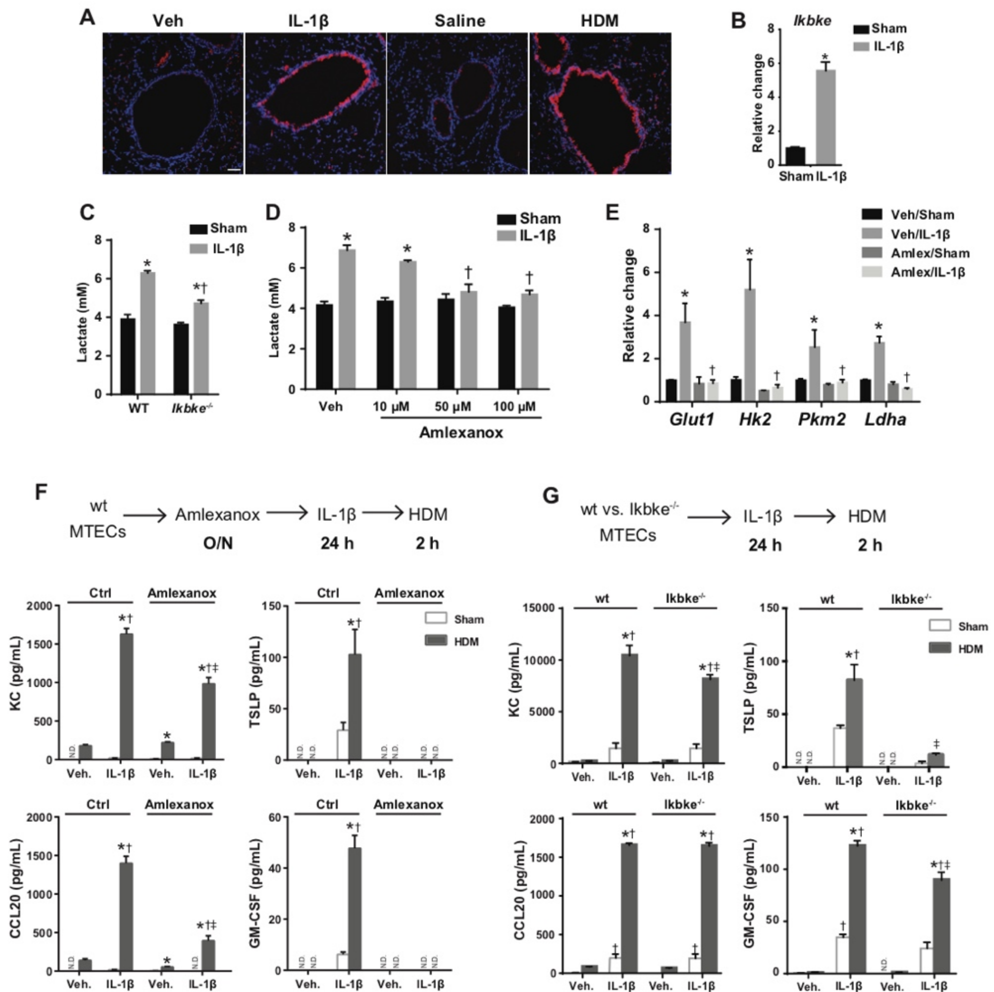

**Figure 6. Inhibitory kappa B kinase ε (IKKε) is required for IL-1β-mediated increases in glycolysis, and the IL-1β-mediated augmentation of HDM-induced innate cytokine responses in MTE cells. A,** Immunofluorescence analysis of IKKε in the lungs from HDM- or IL-1β-exposed mice. Red: IKK***ε***, Blue: DAPI counterstain (scale bar, 50 µm). **B,** mRNA expression of *Ikbke* in MTE cells exposed to IL-1β. *$P < 0.05$ relative to sham control (Student's *t* test). **C,** Lactate levels in supernatants of WT or *Ikbke*$^{-/-}$ MTE cells stimulated with IL-1β for 24 h. *$P < 0.05$ compared to sham controls, †$P < 0.05$ relative to respective WT (ANOVA). **D,** Lactate levels in cell culture supernatants of MTE cells treated with vehicle or amlexanox, at the indicated concentrations. *$P < 0.05$ compared to sham controls (Student's *t* test). **E,** Attenuation of IL-1β-induced expression of glycolysis genes in MTE cells pre-treated with 100 µM amlexanox. *$P < 0.05$ relative to the veh/sham group, †$P < 0.05$ relative to Veh/IL-1β (ANOVA). **F** MTE cells were pre-treated with 100 µM amlexanox, followed by stimulation of IL-1β for 24 h prior to exposure to HDM (50 µg/ml) for an additional 2 h according to the indicated schematic. KC, CCL20, TSLP and GM-CSF in the cell culture supernatants of mouse tracheal epithelial cells. **G** KC, CCL20, TSLP and GM-CSF levels in supernatants of WT or *Ikbke-/-* MTE cells sequentially exposed to IL-1β and HDM according to the schematic.

*$P < 0.05$ relative to non-HDM exposed sham group, †$P < 0.05$ compared to respective non-IL-1β treated vehicle group (Veh.), and ‡$P < 0.05$ relative to respective non-amlexanox treated control group (Figure F) or wt group (Figure G) (two-way ANOVA).

**Lactate dehydrogenase A (LDHA) augments lactate levels in lung tissues and contributes to HDM-induced allergic airways disease**

To address the functional importance of increased glycolysis, we administered *Ldha* siRNA in mice with pre-existing allergic airways disease (Figure 7A). SiRNA-mediated ablation of *Ldha* attenuated HDM-mediated increases in LDHA expression (Figure 7B) and lactate (Figure 7C), and markedly decreased HDM-mediated increases in airway inflammation (Figure 7D). *Ldha* siRNA attenuated tissue levels of IL-33, IL-13 and CCL-20, but did not affect GM-CSF, IL-1β, or TSLP (Figure 7E). *Ldha* siRNA decreased HDM-induced mucus metaplasia and diminished Muc5AC levels in BAL in HDM-exposed mice (Figure 7F-G), consistent with diminished type 2 inflammatory responses. Although siRNA-mediated ablation of *Ldha* did not affect Rn, it attenuated tissue resistance and elastance, compared to Ctrl siRNA HDM-exposed mice (Figure 7H). Collectively, these findings point to the functional relevance of LDHA-linked glycolysis in HDM-induced airways disease, and that increases in glycolysis are an important pro-inflammatory signal.

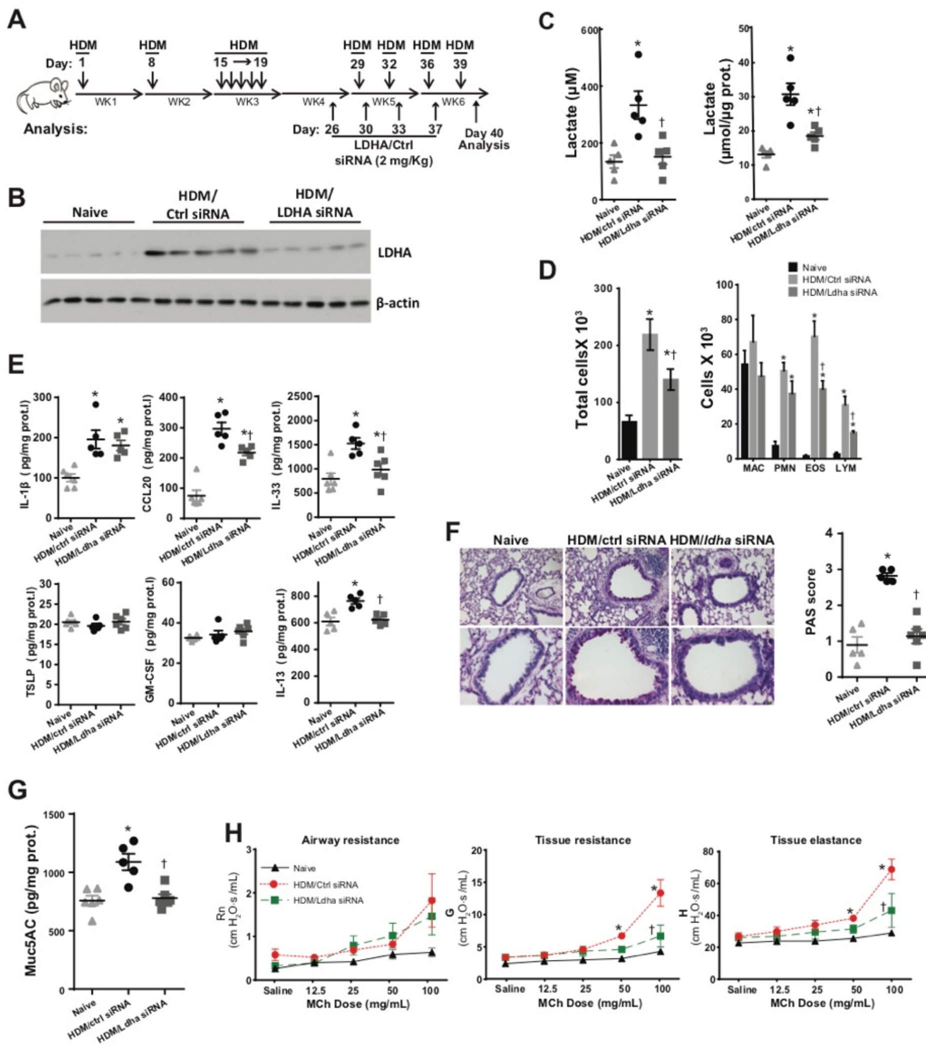

**Figure 7. siRNA-mediated knockdown of *Ldha* attenuates HDM-mediated increase in glycolysis, airway inflammation, and airways hyperresponsiveness. A,** Schematic depicting the dosing regimen of HDM, control (Ctrl) and *Ldha* siRNAs. At day 40, Saline-exposed mice or HDM-exposed mice treated with Ctrl siRNA or *Ldha* siRNA were harvested for the assessment of LDHA protein levels in the lung tissues via Western blot analyses (**B**) levels of lactate in BAL and lung tissue (**C**), total and differential cell counts in the BAL (**D**), levels of IL-1β, CCL20, IL-33, TSLP, GM-CSF, and IL-13 in the lung tissue (**E**). *$P < 0.05$ relative to the naive group, †$P < 0.05$ relative to the HDM/Ctrl siRNA group (ANOVA). **F,** Periodic acid Schiff (PAS) staining of airway mucus in saline- or HDM-exposed mice treated with Ctrl siRNA or *Ldha* siRNA (scale bar, 50 µm) (**Left**). Quantification of airway mucus staining (PAS) intensity (**Right**). Data are expressed as means (±SEM) from five-six mice per group. *$P < 0.05$ compared with naïve mice. †$P < 0.05$ compared to HDM/ctrl siRNA group (Kruskal Wallis) **G,** Measurement of muc5AC levels in the BAL from mice described in A-E. **H,** Assessment of AHR. *$P < 0.05$ relative to naive group, †$P < 0.05$ relative to HDM/Ctrl siRNA group (ANOVA).

**Evidence for increased glycolysis in human asthma in association with airway neutrophils**

In order to address the relevance of these findings for human asthma, we evaluated increases in glycolysis proteins and lactate in primary nasal epithelial cells (NECs). Protein levels of LDHA and pyruvate kinase M2 (PKM2) were constitutively increased in NECs from asthmatics as compared to controls (Figure 8A, Figure E5A), in association with increases in lactate (Figure 8B). In response to HDM, no further differences in expression of these mediators were observed. These findings suggest that asthmatic NECs show an intrinsic increase in glycolysis. Assessment of cell-free sputum samples of healthy subjects (n = 20) or asthmatics (n = 94) showed increased lactate levels in asthmatics as compared to controls (Figure 8C). Sputum lactate levels negatively correlated with %FEV1 in asthmatics but not in healthy individuals (Figure 8D). Although overall levels of IL-1β in sputum samples were not significantly higher in the overall asthmatic population, than those in controls (Figure 8E) a significant correlation was apparent between lactate and IL-1β in asthmatics (Figure 8F). Given the large fluctuations in levels of lactate (range 5.3-362.9 μM) and IL-1β (range 0.8 -262.8 pg/ml) in the asthmatic subjects we further investigated whether these parameters were related to specific clinical features. Lactate or IL-1β were not elevated in patients with eosinophilic asthma (>3% sputum eosinophils) compared to patients with low eosinophils (≤3% eosinophils, p= 0.81 and 0.57, respectively). Lactate was not different between atopic and non-atopic asthmatics (p=0.67). IL-1β levels trended towards being elevated in atopic compared to non-atopic asthmatics (p=0.07). Lactate and IL-1β values trended towards increases in asthmatic patients who received corticosteroids compared to the patients who did not (p = 0.09 and 0.07, respectively). Lactate and IL-1β were significantly elevated in neutrophilic asthmatics (>61% sputum neutrophils, Figure E5B) compared to patients with ≤61% neutrophils. Lactate levels (but not IL-1β) were significantly higher in patients whose asthma was uncontrolled (Figure E5B). The BMI was increased in asthmatics as compared to healthy subjects (Table E1). Adjustment for BMI still showed significant increases in lactate in asthmatics as compared to healthy subjects (p=0.0002). Collectively, these data suggest that IL-1-linked glycolysis is an important feature of allergic asthma.

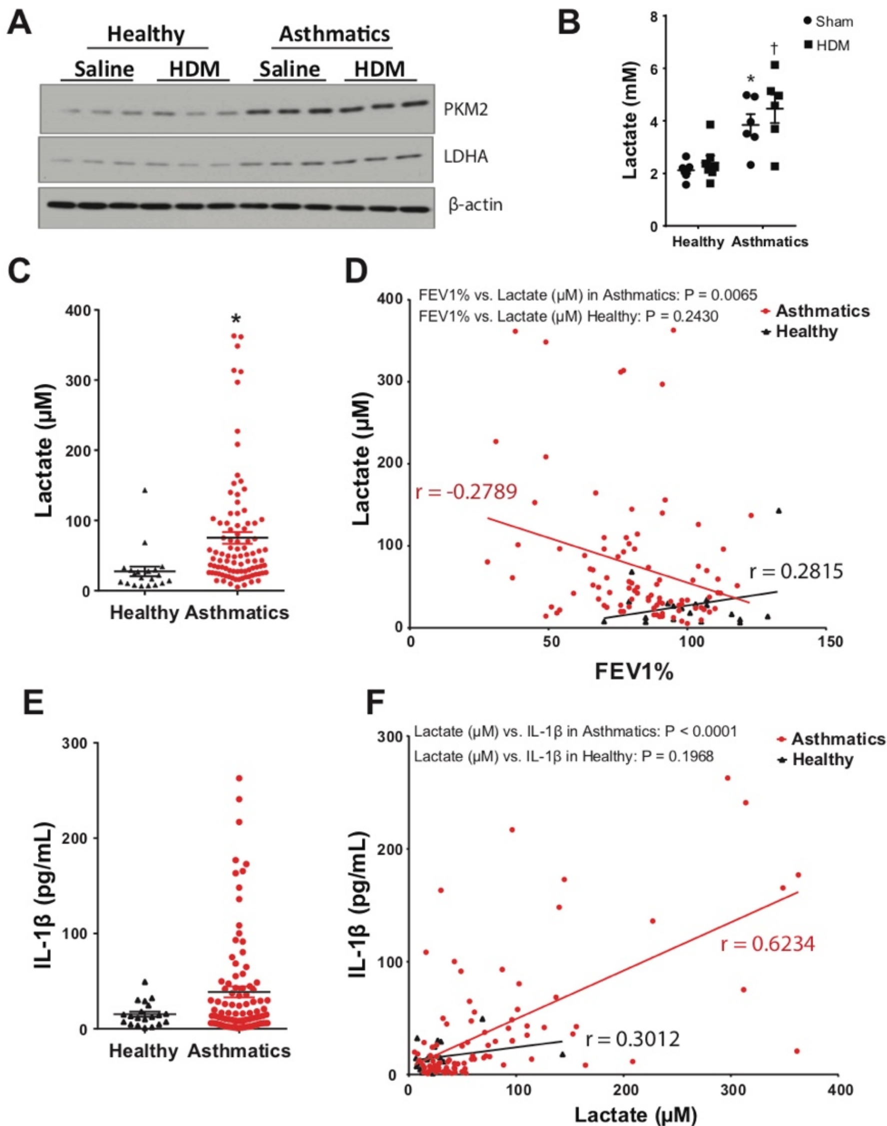


**Figure 8. Evidence of increases in glycolysis in human asthma. A,** Western blot analysis of PKM2 and LDHA, in saline or HDM-treated nasal cells isolated from asthmatics or healthy individuals. Data are representative of 6 healthy subjects, and 6 asthmatics **B,** Lactate content in culture supernatants of cells shown in A. **P* < 0.05 compared to cells from healthy controls not exposed to HDM, †*P* < 0.05 compared to cells from healthy controls exposed to HDM, (ANOVA). **C-F,** Lactate (**C**) and IL-1β levels (**E**) in the sputum supernatants from healthy subjects (n=20) or asthmatics (n=94). Correlations between lactate content and forced expiratory volume in 1 s percentage predicted (FEV1%) (**D**) or IL-1β levels (**F**) in asthmatics and healthy subjects. Correlation analyses were performed via Spearman rank correlation coefficients.

## DISCUSSION

Perturbations in glycolysis are implicated in the pathogenesis of several chronic inflammatory diseases (10, 28). However, the role of dysregulated glycolysis in allergic asthma is not well appreciated. Herein, we discovered that in mice with HDM-induced allergic airways disease, glycolysis was increased in association with HDM-induced inflammation, mucus metaplasia, and AHR. Our results also illuminated that IL-1- and IKKε-dependent signals are important in augmenting glycolysis in HDM-exposed mice, and in enhancing HDM-induced pro-inflammatory signals in epithelial cells. Importantly, inhibition of glycolysis via administering *Ldha* siRNA in mice with pre-existing allergic airways disease attenuated the pathophysiological manifestations of allergic airways disease. These findings have potential relevance to human asthma given the robust increases in expression of LDHA and increased levels of lactate in primary human NECs and cell culture supernatants, respectively, and the observed positive correlation between lactate and IL-1β in asthmatic sputum samples.

In the present study we demonstrated the importance of IL-1 signaling in mediating HDM-induced glycolysis. This claim is based upon findings demonstrating that increases in IL-1β levels were temporally correlated with increases in lactate in response to HDM, that neutralization of IL-1 significantly attenuated HDM-induced glycolysis, and that administration of IL-1β into airways or to MTE cells was sufficient to increase glycolysis. These findings are consistent with earlier studies showing that IL-1 signaling increases glycolysis during Th17 cell differentiation (29), and in mesangial cells (30). IL-1α and IL-1β share biological activity by acting exclusively on Interleukin 1 receptor, type I (IL1RI) (31), and various studies suggest that both cytokines play critical roles in asthma (32-27). Neutralizing IL-1α during allergic sensitization to HDM resulted in strongly attenuated Th2 inflammation (36). Although we did not detect increases in IL-1α at times that corresponded with increases in lactate, we cannot rule out the possibility that IL-1α may be involved in increased glycolysis in settings of allergic airways disease. As IL-1β and IL-1α both activate IL-1RI, and increase glycolysis in epithelial cells (Figure 3) and IL1-Trap lowered both IL-1β and IL-1α in lung tissue (Figure E2), their relative contributions in

mediating HDM-induced glycolysis need to be further dissected. Lastly, we also report that HDM-induced adaptive immunity is required for the observed increases in IL-1β and associated increases in lactate.

Alterations in cellular metabolism are known to affect function of immune cells (9), and increases in glycolysis have been shown to regulate immune effector function (21, 22, 38-41). Despite these studies, the role of enhanced glycolysis in structural cells such as airway epithelium and implications for their innate effector function has remained unknown. Here, we demonstrate that inhibition of glycolysis via targeting HK or LDHA markedly dampened IL-1α- or IL-1β-induced pro-inflammatory responses, and strongly attenuated the ability of IL-1β to augment HDM-induced innate cytokine responses in MTE cells. Overall these findings suggest that enhanced glycolysis is important for the amplification of allergen-induced pro-inflammatory responses. However, further mechanistic studies will be required to unravel how glycolysis modulates pro-inflammatory responses in epithelial cells. Rapid ATP generation during glycolysis is required for immediate energy demand during immune cell proliferation and activation (9). We and others have shown that extracellular ATP activates purinergic receptors, leading to release of IL-33 from epithelial cells (42), suggesting a potential mechanism whereby increased glycolysis augments epithelial effector function.

In the present study we also demonstrate that IKKε expression is increased in bronchial epithelium in response to HDM or IL-1β, and that it promotes glycolysis and pro-inflammatory responses in epithelial cells and contributes to HDM-induced allergic airways disease. However, the molecular details whereby IL-1 and IKKε enhance glycolysis remain unknown. IL-1 has recently been shown to activate IKKε and subsequent AKT-mTOR signaling pathway, leading to Th17 cell maintenance (25); and, AKT or mTOR, when activated, are known to induce glycolysis (21, 43), suggesting the potential role of AKT-mTOR signaling in HDM/IL-1/IKKε-induced glycolysis.

The connection of IL-1 signaling and glycolysis described herein in the murine model of allergic airway disease is corroborated by our findings in samples from asthmatics. As was mentioned above, lactate was significantly higher in sputum samples of asthmatics. Furthermore, lactate and IL-1β level were positively correlated in asthmatic sputum supernatants, and lactate negatively correlated with lung function. Lactate and IL-1β were notably increased in patients with neutrophilic asthma (≥61% neutrophils), whereas no correlations between these parameters and eosinophils were observed. IL-1β has implicated in a number of pulmonary diseases (44-46). Although increases in IL-1β observed herein are not specific to only patients with asthma, IL-1β is emerging as a key cytokine relevant to the pathogenesis of asthma (47). IL-1β has been linked to severe, neutrophilic, steroid insensitive asthma in a mouse model (48). In contrast to the present data, a recent study suggested a critical role for the IL-1β pathway in patients with $T_H2/T_H17$-predominant asthma (having 4% of BAL neutrophils) whereas IL-1α was linked to neutrophilic asthma (having 16% BAL neutrophils) (49). The discrepancy between these findings may be associated with differences in patient characteristics, sampling (sputum as compared to BAL analyses) and illuminates the complexities among the various asthma subtypes. Therefore, additional studies will be essential to unravel the contributions of IL-1α, IL-1β and activation of glycolysis pathways in the asthma subtypes. Excessive β-agonist administration has been associated with elevated plasma lactate levels (50-52). We believe it is unlikely that salbutamol used to induce sputum in our study contributed to the increased level of sputum lactate because a low dose of salbutamol (400 µg) was used, and both healthy and asthmatic patients received salbutamol. This notion is also backed by our findings that asthmatic NECs expressed more LDHA and produced more lactate as compared to controls, in the absence of exposure to β-agonists (Figure 8). The latter findings also suggests that human NECs from asthmatics are intrinsically different from their counterparts derived from healthy individuals. Considering that IL-1 proteins can be produced by epithelial cells (36), it will be interesting to elucidate whether epithelial IL-1 and IL-1RI signaling form an autocrine loop that sustains the constitutive over-production of lactate observed in NECs derived from asthmatics. Furthermore, an epigenetic mechanism may also be involved in this process, as a recent study discovered that, during

Th1 cell differentiation, LDHA-mediated increases in glycolysis maintain a high concentration of acetyl-coenzyme A that in turn enhances histone acetylation (53).

In summary, the present study demonstrates the importance of glycolysis in the pathophysiology of allergic airways disease, and suggests that targeting glycolysis (6, 54, 55) may ultimately provide a new approach in the treatment of asthma. Additional studies will be required to elucidate the cell types wherein enhanced glycolysis occurs in settings of asthma. Similarly, the molecular details whereby changes in glycolysis regulate the effector function of epithelial and other cell types also warrant further investigation.

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## SUPPLEMENTAL MATERIAL AND METHODS

### Sputum induction

Sputum was induced and processed, as described previously (1-3). Prior to sputum induction, subjects inhaled 400 µg salbutamol using a metered-dose inhaler (+spacer). Sputum was induced using an ultrasonic nebulizer (ultra-Neb 2000, Devilbiss; output set at 0.9 ml/min). Subjects inhaled hypertonic saline (NaCl 5%) when FEV1 post salbutamol was ≥ 65% predicted and isotonic saline (NaCl 0.9%) when FEV1 was <65% predicted. The aerosol was inhaled for three consecutive periods of 5 min. FEV1 was monitored every 5 minutes for safety reasons, and when FEV1 dropped to 80% of the post-bronchodilator values, the induction procedure was stopped. The whole sputum was weighted and three volumes of PBS were added. After homogenizing by manual agitation for 30 sec and centrifugation (800 g) for 10 min at 4°C, the cell pellet and supernatant were separated. Cells were treated with Sputolysin® 0.1% (Calbiochem, Germany), washed with PBS and resuspended in 1 ml. Total cell counts, % squamous cells and cell viability (trypan blue staining) were determined with a manual hemocytometer. Sputum cell differentials were determined by counting 500 cells non squamous cells on Cytospin samples that were stained with RAPI-DIFF II stain (Atom Scientific, Manchester, United Kingdom).

### Cell culture and treatments

Primary human nasal epithelial cells (NECs) were isolated from 6 healthy volunteers and 6 patients with allergic rhinitis and asthma by gentle stroking of the inferior turbinate surface with a Rhino-Probe curette and cultured as recently described (4) in bronchial epithelial cell growth medium (Lonza). Atopy was confirmed by positive skin tests and elevated serum IgE (>100 IU/ml), and asthma was diagnosed by physicians, confirmed by positive response to bronchodilator (≥200 cc and 12% improvement in FEV1 and/or FVC) or a positive methacholine challenge test (PC20 < 8 mg/ml), and had rhinitis with a sinonasal questionnaire (SNQ) score (5) of greater than 1. Healthy volunteers had no history of rhinitis or asthma, negative skin tests, negative methacholine challenge tests, and a SNQ score of less than 1. For experiments, NECs were plated on collagen-coated 12-well plates at a

density of 2 × $10^5$ cells/well and cultured in a 1:1 mixture of bronchial epithelial cell basic medium and DMEM-H with SingleQuot supplements (Cambrex), bovine pituitary extract (13 mg/ml), bovine serum albumin (1.5 µg/ml), and nystatin (20 units). Following 2 h starvation in basal medium, NECs were treated with 50ug/ml of HDM D. pteronyssinus; GREER, Lenoir, NC; 144.9 endotoxin units/mg protein, lot 290903) for 24 h. Primary mouse tracheal epithelial (MTE) cells were isolated from wild-type (WT) C57BL/6 mice or C57BL/6 mice lacking the Inhibitor of κB kinase ε gene (referred to herein as *Ikbke*$^{-/-}$) and cultured as previously described (6, 7). After reaching confluence, MTE cells were incubated for 16 h in serum-free medium. Cells were stimulated with IL-1α (10 ng/mL), IL-1β (10 ng/mL), IL-6 (10 ng/mL), IL-13 (5 ng/mL), IL-33 (5 ng/mL), TGF-β1 (5 ng/mL), TNFα (5 ng/mL), IL-17 (20 ng/mL), lipopolysaccharide (LPS, 1 µg/mL), or HDM (10 µg/mL or 50 µg/mL as indicated in the Figure Legend) for 2 or 24 hr.

To address the importance of glycolysis or IKKε/TBK1 in IL-1β induced pro-inflammatory responses in MTE cells, cells were pre-treated with the hexokinase inhibitor, 2-Deoxyglucose (2-DG, 10 mM) for 1 h prior to exposure to IL-1β for 24 h. Alternatively, cells were incubated with the lactate dehydrogenase A inhibitor, oxamate (10 mM), or the IKKε/TBK1 inhibitor, Amlexanox (10-100 µM, Tocris) overnight, followed by the stimulation IL-1β (10 ng/mL) for 24 h. To determine whether IL-1β-induced glycolysis or IKKε/TBK1 augmented the subsequent response to HDM, in select experiments, cells were washed post inhibitor/IL-1β treatment, incubated with DMEM/F12 medium for 2 h before stimulation with HDM for an additional 2 hours according the schematic illustrations shown in the relevant figures. To address the role of glycolysis in IL-1α-dependent pro-inflammatory responses, MTE cells were pre-incubated with 10 mM 2-DG for 1 h or 10 mM oxamate for 16 h prior to exposure to IL-1α for 24 h and subsequent assessment of pro-inflammatory mediators in supernatants. Note that absolute values of KC vary between studies due to freezing of some supernatants.

**Mouse studies**

Age-matched, 8- to 12-week-old mice were used (*The Jackson Laboratory, Bar Harbor, ME*) for all experiments. Wild-type (WT, C57BL6/NJ), *Rag*$^{-/-}$ (C57BL6/J), or *Ikbke*$^{-/-}$ (C57BL6/J) mice along with their strain-matched controls were sensitized (Days 1 and 8), challenged (Days 15-19), and rechallenged (Days 29, 32, 36, and 39) with HDM extract containing 10 µg protein (GREER, Lenoir, NC; 144.9 endotoxin units/mg protein, lot 290903) as shown in Figure 1A. Mice were euthanized and analyzed at different end points at Days 1 (2 h post HDM sensitization), 2 (24 h post HDM sensitization), 20 (24 h post the last challenge of 5 consecutive HDM challenge), 29 (2 h post the first HDM re-challenge), 30 (24 h post the first HDM rechallenge), and/or 40 (24 h post the last HDM rechallenge). The control group was subjected to saline as a vehicle control. In the *Ldha* siRNA knockdown studies, WT C57BL6/NJ mice were anesthetized with isoflurane and subjected to 10 mg/kg of siRNA targeting *Ldha* or scrambled small interfering siRNA oropharyngeally on days 26, 30, 33, and 37 post-initiation of the HDM exposure regimen, and mice were harvested in day 40 (24h post the last HDM re-challenge). In the IL-1 neutralization experiments, WT C57BL6/J mice were challenged with HDM on days 15, 16, 17, and 18, and analyzed on day 20. Mice received 5 mg/kg of IL-1 Trap (Regeneron Pharmaceuticals, Tarrytown, NY) on Day 14 and Day 17 by i.p. injections, based upon a previous study demonstrating that this dosing regimen of IL-1 Trap attenuated cardiac remodeling after experimental acute myocardial infarction in mice (8). In select experiments, 1 µg of IL-1β (R&D Systems, resuspended in 0.1% BSA in PBS) was directly administered intranasally.

**Assessment of airway hyperresponsiveness**

Following completion of the HDM protocol, mice were anesthetized with intraperitoneal pentobarbital sodium (90 mg/kg), tracheotomized, and mechanically ventilated at 200 breaths/min. Mice were subjected to increasing doses of methacholine (0, 12.5, 25, 50, and 100 mg/mL) administered via ultrasonic nebulization, and respiratory mechanics were assessed using a forced oscillation technique on a computer-controlled small animal ventilator (SCIREQ, QC, Canada), as previously described (9, 10). Parameters of Newtonian resistance (Rn), tissue resistance (G) and elastance (H) were calculated and quantified by

averaging the three highest measurements obtained at each incremental methacholine dose for each mouse (9, 10).

**Assessment of mucus metaplasia**

Airway mucus was stained via Periodic acid Schiff (PAS) and the staining intensity was evaluated by scoring of slides by two independent blinded investigators (11). Levels of MUC5AC were evaluated in lung tissue or BAL via ELISA (My Biosource).

**Bronchoalveolar lavage fluid processing**

After mice were euthanized, bronchoalveolar lavage (BAL) was performed using 1 ml PBS. BAL was collected and total cell counts were determined using an Advia 120 Automated Hematology Analyzer. BAL was spun down at 1200xg for 5 min. Cells were transferred to slides using a cytospin, fixed in methanol and stained using the Hema3 kit (Fisher Scientific, Kalamazoo, MI) and analyzed by counting a minimum of 300 cells per mouse, as described elsewhere (11). Supernatants were flash frozen in liquid nitrogen and stored at -80 °C until analysis.

**Enzyme-Linked Immunosorbent Assay (ELISA)**

IL-1α, IL-1β, IL-6, TNFα, IL-17, GM-CSF, and CCL20 were detected by ELISA in lung homogenates (normalized for protein) or supernatants from cell culture, according to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

**Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)**

RNA was extracted using miRNeasy columns (Qiagen, Valencia, CA) as directed by the manufacturer. One µg of RNA was reverse transcribed to cDNA for gene analysis using SYBR Green (Bio-Rad; Hercules, CA, USA) to assess expression of *Mct4*, monocarboxylate transporter 4; *Pfkl*, phosphofructokinase, live type; *Hk2*, Hexokinase 2; *Glut1*, glucose transporter 1; *Glut3*, glucose transporter 3; *Pkm2*, pyruvate kinase isoenzyme type M2; *Ldha*, lactate dehydrogenase A; *Pgm1*, phosphoglucomutase 1; *Pdk1*, pyruvate dehydrogenase kinase 1; *Tpi1*, triosephosphate isomerase 1; *Eno1*, enolase 1; *Pgk1*,

phosphoglycerate kinase 1; *Gpi*, glucose-6-phosphate isomerase; *Pfkfb3*, 6-Phosphofructo-2-Kinase/Fructose-2,6-Biphosphatase 3; *Mct1*, monocarboxylate transporter 1; *Glut2*, glucose transporter 2; and *Ikbke*, inhibitory kappa B kinase ε. Expression values were normalized to the house keeping gene cyclophilin. Detailed primer sequences are provided in the online supplement, Table E3.

**Bioenergetics**

The extracellular acidification rate (ECAR) was measured using the Seahorse Extracellular Flux (XF24) Analyzer (Agilent Technologies). MTE cells were seeded onto 24-well seahorse plate at a density of 50,000 cells per well and cultured with or without 10 ng/mL IL-1β for 24 h. Cells were then washed 3 times with Seahorse stress test glycolysis assay media (DMEM without glucose, L-glutamine, phenol red, sodium pyruvate, and sodium bicarbonate [Sigma-Aldrich] supplemented with 1.85 g/l sodium chloride, 2mM L-glutamine, and 3 mg/l phenol red [GlycoStress Assay], pH 7.35). The plate was incubated in a 37°C non-$CO_2$ incubator for 1 h. The plate was then transferred to the Seahorse XF24 Analyzer for analysis and subjected to ECAR measurements followed by successive treatments with glucose (10 mM), oligomycin (0.25 µM), and 2-deoxyglucose (100 mM).

**Glucose measurements**

Glucose consumption and uptake in MTE cells were measured 24 h post stimulation with IL-1β, by measuring glucose concentration in the cell culture supernatants (Eton Bioscience) and cellular incorporation of fluorescent glucose analog [2-NBDG, 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose, Life technology] using a plate reader (Biotek, Winooski, VT).

**Immunohistochemistry**

Fixed sections were prepared for immunostaining by deparaffinizing with xylene and rehydrating through a series of ethanol. For antigen retrieval, slides were heated for 20 min in 95°C citrate buffer (pH 6.0), then rinsed in distilled water. Sections were then blocked for 1 h in blocking serum as per manufacturer's instructions (Vectastain Alkaline Phosphatase

Universal, Vector). Slides were then washed in TBS with 0.1% TWEEN-20 3×5 min, followed by incubation with primary antibody for lactate dehydrogenase A overnight at 4°C. Sections were washed again and incubated with a biotinylated universal secondary antibody (Vectastain Alkaline Phosphatase Universal, Vector) for 30 min at room temperature. Slides were washed and incubated with the Vectastain ABC-AP reagent (prepared as per manufacturer's instructions) for 30 min at room temperature. Sections were then incubated with Vector Red/Vector Blue Alkaline Phosphatase Substrate Kit I (Vector) for 10 min at room temperature, rinsed with tap water, and counterstained with Mayer's Hematoxylin.

**Immunofluorescence**

Following euthanization, left lobes were fixed with 4% paraformaldehyde, stored at 4°C overnight for fixation of the tissue, mounted in paraffin, and 5 µm sections were affixed to glass microscope slides for histopathology as previously described (12). For antigen retrieval, slides were heated for 20 min in 95°C citrate buffer (pH 6.0) with 0.05% TWEEN-20 then rinsed in distilled water. Sections were then blocked for 1 h in 1% bovine serum albumin (BSA) in PBS, followed by incubation with primary antibody for IKBKE (Cell Signaling Technology, Danvers, MA) at 1:100, overnight at 4°C. Slides were then washed 3x5min in PBS, incubated with Alexafluor 647, and counterstained with DAPI in PBS for nuclear localization. Sections were imaged using a Zeiss 510-META confocal laser scanning microscope.

**Lactate assay**

The concentration of lactate in the medium, BAL, and lung homogenates was assessed with a Lactate Assay Kit (Eton Bioscience) according to each manufacturer's recommendations.

# SUPPLEMENTAL DATA

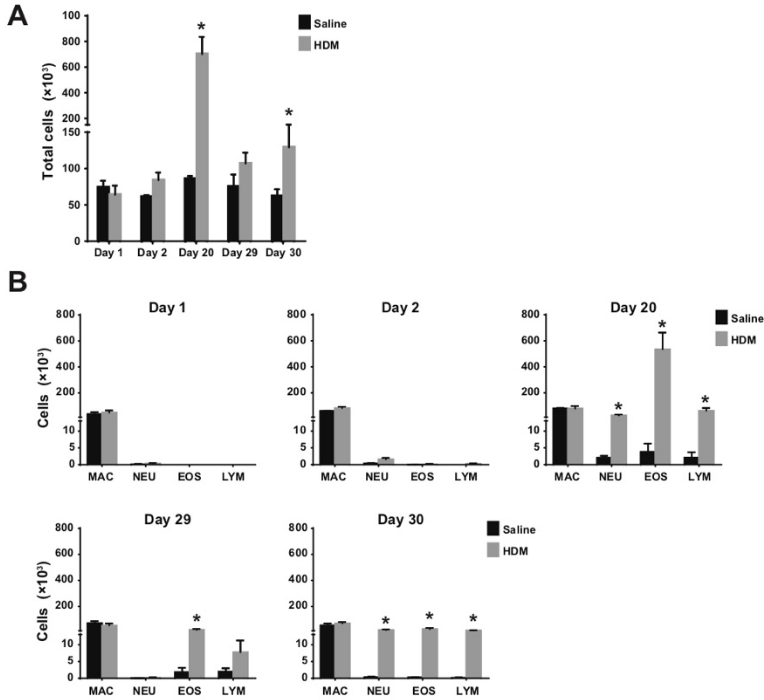



**Figure E1:** Airway inflammation in mice exposed to house dust mite (HDM). (**A**) Total and (**B**) differential cell counts in bronchoalveolar lavage (BAL) in response to saline or HDM, (see schematic in **Figure 1A)**. Data are expressed as means (± SEM) (n = 5 mice per group). *p < 0.05 (Student's t test) compared with saline controls.

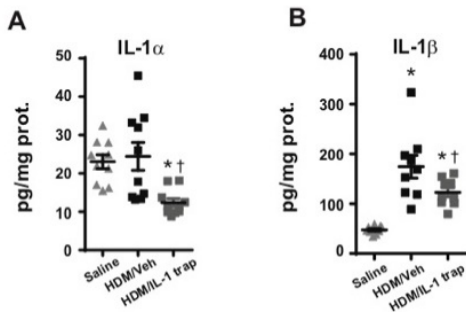



**Figure E2:** Assessment of levels of IL-1α and IL-1β in lung tissues from mice subjected to vehicle control or IL1 Trap. Mice were challenged with HDM on days 15, 16, 17, and 18, and received 5 mg/kg of IL-1 Trap or vehicle on days 14 and 17 intraperitoneally. Levels of IL-1α and IL-1β in lung tissues homogenates were evaluated on day 20 via ELISA. *P < 0.05 compared to saline control, † p < 0.05 compared to HDM/Veh group (ANOVA).

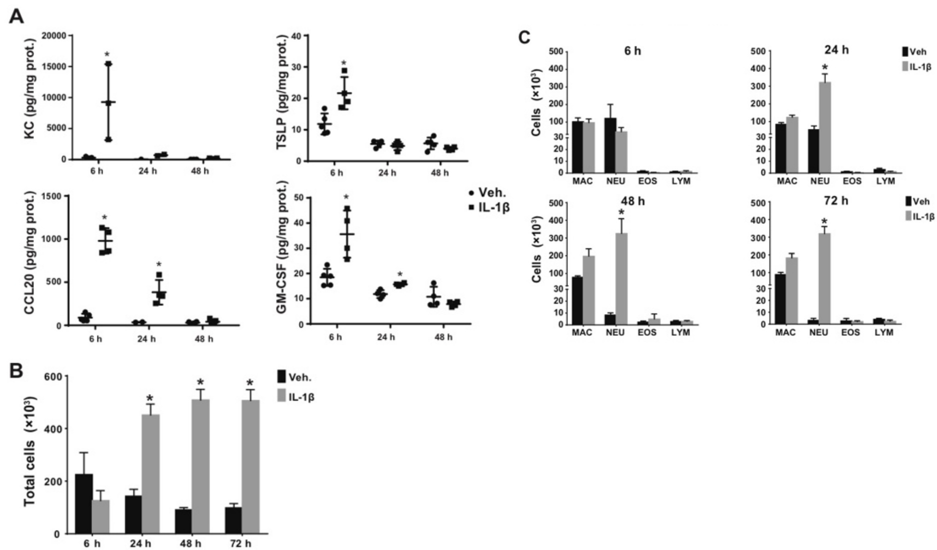



**Figure E3:** Evaluation of pro-inflammatory mediators and airway inflammation in mice exposed to interleukin (IL)-1β. **A**: Lung tissue levels of KC, TSLP, CCL20 and GM-CSF following intranasal administration of IL-1β or vehicle. Total (**B**) and differential cell counts (**C**) in BAL from the mice at multiple time points post intranasal administration of vehicle or IL-1β. Data are expressed as means (± SEM) (n = 5 mice per group). *p < 0.05 (ANOVA) compared with Vehicle controls.

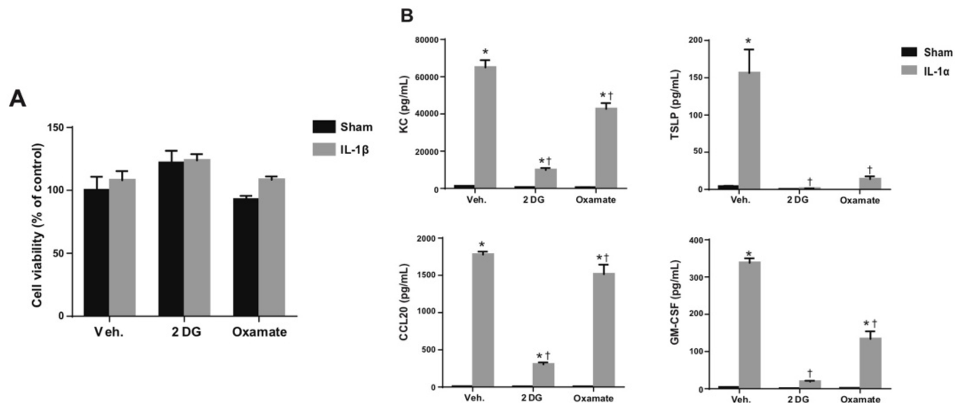
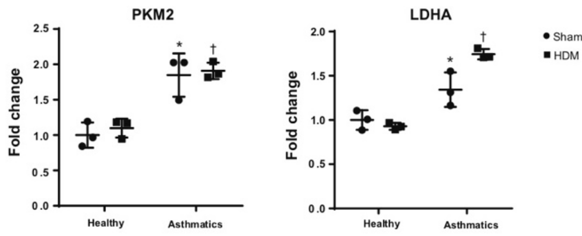



**Figure E4: A:** Assessment of viability following exposure to MTE cells to oxamate or 2-deoxyglucose. Epithelial cells were exposed to IL-1β in the presence or absence of inhibitors. Cell survival was evaluated via crystal violet staining of cells. Results were expressed as % survival compared to untreated control cultures. **B:** Impact of 2-DG or oxamate on IL1α-mediated increases in lactate and the indicated pro-inflammatory mediators measured 24 hr post exposure to IL-1α. *P < 0.05 (ANOVA) compared to the sham group. †p < 0.05 compared to the IL-1β treated Vehicle group (ANOVA).

**A**




**B**

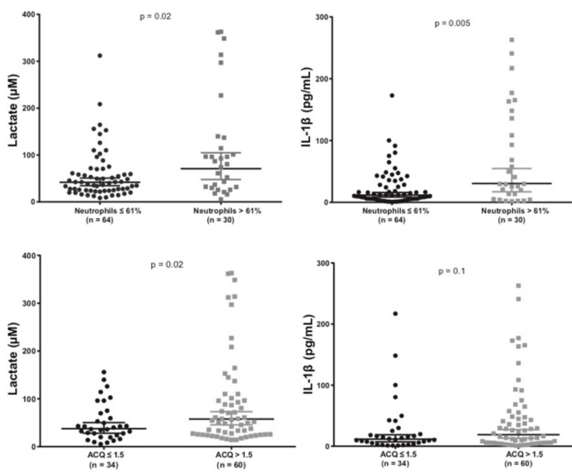



**Figure E5: A:** Quantification of Western blots shown in Figure 8A. Data reflects n=3 healthy subjects and n=3 asthmatics. Data were normalized to β-actin and are expressed as fold change from healthy sham controls *$P < 0.05$ compared to the sham healthy group. †$P < 0.05$ compared to HDM healthy group (ANOVA). **B:** Sub-analysis of sputum lactate and IL-1β in asthmatics with normal neutrophils (cut off ≤ 61%) or high neutrophils (cut off > 61%), controlled (ACQ ≤ 1.5) or uncontrolled asthma (ACQ > 1.5). p-values (ANOVA or Wilcoxon rank sum test) are provided in each of the figures.

**Supplementary table I.** Demographic, functional, and inflammatory characteristics of the study cohort

| | Healthy subjects | Asthmatic patients | *P* value |
|---|---|---|---|
| n | 20 | 94 | |
| Age (yr) | 53 ± 3 | 52 ± 2 | 0.86 |
| BMI (kg/m2) | 24.30 ± 1.05 | 27.28 ± 0.48 | 0.01 |
| Age asthma onset (range) | - | 41 (12-54) | - |
| Gender (M/F) | 10/10 | 43/51 | 0.3 |
| Atopy*, no. (%) | 4 (20) | 42 (45) | 0.03 |
| Positive Dpt*, no. (% atopy) | 3 (75) | 25 (60) | |
| FEV1 % predicted | 101 ± 4.62 | 81.56 ± 2.07 | 0.0001 |
| FEV1/FVC ratio | 77.68 ± 2.51 | 72.41 ± 1.13 | 0.0291 |
| FENO (ppb), median (IQR) | 20 (17.3-26.8) | 17.5 (10-36.25) | 0.13 |
| Eosinophils (%), median (IQR) | 0.2 (0-0.9) | 0.9 (0.2-5.45) | 0.03 |
| **Medication use** | | | |
| Not treated | - | 5 (5) | - |
| ICS/LABA, no. (%) | - | 58 (62) | - |
| eq Beclomethasone µg/ml | - | 1000 (400-2000) | |
| ICS alone, no. (%) | - | 2 (2) | - |
| OCS, no. (%) | - | 3 (3) | - |
| SABA, no. (%) | - | 64 (68) | - |
| SABA only, no. (%) | - | 19 (20) | - |
| LTRA, no. (%) | - | 24 (26) | - |

Data are expressed as means ± SDs, or medians with interquartile range (IQR). P values are based on the student's t test (mean ± SD), the $\chi 2$ test for proportions (sex), the Wilcoxon rank sum test (median [range]), or Poisson regression (atopy). Atopy is defined as positive test results for at least 1 specific IgE to common aeroallergens. FENO: Fraction of exhaled nitric oxide. * Dpt.: Dermatophagoid pteronyssinus. ICS: inhaled corticosteroid. LABA: long acting beta agonist. OCS: oral corticosteroids. SABA: Short acting beta agonist. LTRA: leukotriene receptor agonists. Ethnicity: All subjects are Caucasian except for 2 African subjects.

**Supplementary table II.** Demographic, functional, and inflammatory characteristics of the study cohort enrolled at the University of Vermont Medical Center

| | Healthy subjects | Asthmatic patients |
|---|---|---|
| n | 7 | 6 |
| Age (range) | 23 (19-27) | 23 (19-45) |
| Gender (M/F) | 0/6 | 3/4 |
| Age asthma onset (range) | - | 4 (1-18) |
| BMI | 23.2 (19.8-26.3) | 28.6 (22.0-35.0) |
| Atopy*, no. (%) | - | 7 (100) |
| **Medication use** | | |
| ICS_LABA | - | 3 |
| ICS | - | 3 |
| SABA only | - | 1 |
| SNQ | 0.4 (0-0.8) | 1.4 (1.2-1.6) |

Data are expressed as median (range). ICS_LABA: inhaled corticosteroid_long acting beta agonist. ICS: inhaled corticosteroid. SABA: short acting beta agonist. SNQ: sinonasal questionnaire. *All participants were allergic to house dust mite as determined either by positive skin prick test to D. pteronyssinus or positive serum IgE to D. pteronyssinus. All subject are Caucasian.

**Supplementary table III. The primers used in this study**

| Genes | Forward | Reverse |
|---|---|---|
| ***Mct4*** | 5'-ATCGTGGGCACTCAGAAGTT-3' | 5'-CGCCAGGATGAACACATACTT-3' |
| ***Pfkl*** | 5'-CATATATGTGGGGGCCAAAG-3' | 5'-GACACACAGGTTGGTGATGC-3' |
| ***Hk2*** | 5'-GGGACGACGGTACACTCAAT-3' | 5'-GCCAGTGGTAAGGAGCTCTG-3' |
| ***Glut1*** | 5'-TCTCTGTCGGCCTCTTTGTT-3' | 5'-CCAGTTTGGAGAAGCCCATA-3' |
| ***Glut2*** | 5'-GCCTGTGTATGCAACCATTG-3' | 5'-GAAGATGGCAGTCATGCTCA-3' |
| ***Glut3*** | 5'-TGTCACAGGAGAAGCAGGTG-3' | 5'-GCTCCAATCGTGGCATAGAT-3' |
| ***Pkm2*** | 5'-CTGCAGGTGAAGGAGAAAGG-3' | AGATGCAAACACCATGTCCA-3' |
| ***Ldha*** | 5'-GGAAGGAGGTTCACAAGCAG-3' | 5'-ACCCGCCTAAGGTTCTTCAT-3' |
| ***Pgm1*** | 5'-TCAGGCCATTGAGGAAAATC-3' | 5'-CGAACTTCACCTTGCTCTCC-3' |
| ***Pdk1*** | 5'-GGCGGCTTTGTGATTTGTAT-3' | 5'-ACCTGAATCGGGGGATAAAC-3' |
| ***Tpi1*** | 5'-CCTGGCCTATGAACCTGTGT-3' | 5'-CAGGTTGCTCCAGTCACAGA-3' |
| ***Eno1*** | 5'-CTGCCTCCGAGTTCTACAGG-3' | 5'-CGCTTAGGGTTGGTCACTGT-3' |
| ***Pgk1*** | 5'-CAAGGCTTTGGAGAGTCCAG-3' | 5'-TGTGCCAATCTCCATGTTGT-3' |
| ***Gpi*** | 5'-GTGGTCAGCCATTGGACTTT-3' | 5'-CTGGAAATAGGCAGCAAAGC-3' |
| ***Pfkfb3*** | 5'-CAGCTACCAGCCTCTTGACC-3' | 5'-AACTTCTTGCCTCTGCTGGA-3' |
| ***Mct1*** | 5'-TCCAGTAATGATCGCTGGTG-3' | 5'-AGTTGAAAGCAAGCCCAAGA-3' |
| ***Ikbke*** | 5'-CTGGATGTCCCAAAGTTCGT-3' | 5'-AGGCTGCTGCTGAGGTAGAG-3' |

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# 4

# Activation of Pyruvate Kinase M2 attenuates expression of pro-inflammatory mediators in house dust mite-induced allergic airways disease

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## ABSTRACT

Asthma is a chronic disorder characterized by inflammation, mucus metaplasia, airway remodeling and hyperresponsiveness. We recently showed that interleukin-1 (IL-1)-induced glycolytic reprogramming contributes to allergic airway disease using a murine house dust mite (HDM) model. Moreover, levels of pyruvate kinase M2 (PKM2) were increased in this model as well as in nasal epithelial cells from asthmatics as compared to healthy controls. While the tetramer form of PKM2 converts phosphoenolpyruvate to pyruvate, the dimeric form of PKM2 has alternative, non-glycolysis functions as a transcriptional co-activator to enhance the transcription of several pro-inflammatory cytokines. In the present study, we examined the impact of PKM2 on the pathogenesis of HDM-induced allergic airways disease in C57Bl/6NJ mice. We report here that activation of PKM2, using the small molecule activator, TEPP46, augmented PKM activity in lung tissues and attenuated airway eosinophils, mucus metaplasia, and subepithelial collagen. TEPP46 attenuated IL-1β-mediated airway inflammation and expression of pro-inflammatory mediators. Exposure to TEPP46 strongly decreased the IL-1β-mediated increases in thymic stromal lymphopoietin (TSLP) and granulocyte macrophage colony stimulating factor (GM-CSF), in primary tracheal epithelial cells isolated from C57Bl/6NJ mice. We also demonstrate that IL-1β-mediated increases in nuclear phospho-STAT3 were decreased by TEPP46. Lastly, STAT3 inhibition attenuated the IL-1β-induced release of TSLP and GM-CSF, suggesting that the ability of PKM2 to phosphorylate STAT3 contributes to its pro-inflammatory function. Collectively, these results demonstrate that the glycolysis-inactive form of PKM2 plays a crucial role in the pathogenesis of allergic airways disease by increasing IL-1β-induced pro-inflammatory signaling, in part through phosphorylation of STAT3.

**Key Points:**

- A small molecular activator of PKM2 attenuates allergic airways disease in mice.
- Activation of PKM2 decreases IL-1β-induced airway inflammation.
- PKM2 activation decreases IL-1β-induced nuclear phosphorylation of STAT3.

**Keywords:**
Asthma, Pyruvate Kinase M2, Interleukin-1β, TEPP46, Thymic stromal lymphopoietin, Granulocyte macrophage colony stimulating factor

**Abbreviations**

BAL: bronchoalveolar lavage; EGF: epidermal growth factor; EGFR: EGF receptor; GLUT-1: glucose transporter 1; HDM: house dust mite; HIF-1α: hypoxia-inducible factor 1α; IKKε: inhibitory k B kinase ε; MTE: mouse tracheal epithelial; PEP: phosphoenolpyruvate; PK: pyruvate kinase; PKM1: PK muscle isozyme M1; PKM2: PK muscle isozyme M2; α-SMA: α–smooth muscle actin; TEPP46: 6-[(3-Aminophenyl)methyl]-4,6-dihydro-4-methyl-2(methylsulfinyl)-5H-Thieno[29,39:4,5]pyrrolo[2,3-d]pyridazin-5-one; TSLP: thymic stromal lymphopoietin; WT: wild-type.

## INTRODUCTION

Asthma is a complex pulmonary disorder that is characterized by mucus metaplasia, airways hyperresponsiveness (AHR) and remodeling, and is accompanied by a chronic inflammatory process controlled by cells of the innate and adaptive immune system (1). The precise metabolic alterations that are induced in structural or immune cells which promote the disease processes remain incompletely understood. However, glycolytic reprogramming has been shown to be important in the regulation of immune cell activation and differentiation (1, 2). Our laboratory recently described that interleukin-1 (IL-1)-induced glycolytic reprogramming contributes to allergic inflammation, airway remodeling and AHR in a mouse model of house dust mite (HDM)-induced allergic airway disease (3). Moreover, enhanced glycolysis was shown to be required for the IL-1β-mediated release of the pleiotropic cytokines thymic stromal lymphopoietin (TSLP) and granulocyte macrophage colony stimulating factor (GM-CSF), two major epithelium-derived inflammatory mediators implicated in the pathogenesis of asthma. Levels of lactate were also increased in sputum of asthmatics, and significant correlations were observed between lactate and IL-1β. Moreover, lactate levels were elevated in subjects with neutrophilic asthma who had poor disease control (3), suggesting that increased glycolysis may be feature of severe asthma.

During glycolysis, glucose is converted into pyruvate which can be further metabolized in the mitochondria to produce adenosine triphosphate (ATP) via oxidative phosphorylation. Pyruvate kinase (PK) catalyzes the final, rate-limiting step in glycolysis, the formation of pyruvate from phosphoenolpyruvate (PEP) while generating two molecules of ATP per glucose molecule. Pyruvate can also be converted into lactate under hypoxic conditions (anaerobic glycolysis), or in the presence of oxygen (aerobic glycolysis) in metabolically active cells such as cancer cells (4, 5). The PK family consists of four isoforms, which are encoded by two distinct genes. The Pkrl gene encodes the isoforms PKL and PKR, which are expressed in the liver and red blood cells respectively, and the PK muscle isozymes M1 and M2 (PKM1 and PKM2) which are derived from alternative splicing of the PKM gene (6, 7). PKM1 naturally occurs in a highly active tetrameric form, and is expressed in many differentiated tissues such as the muscle and the brain (8), whereas PKM2 can adopt

monomer, dimer or tetramer structural forms that dictate its intracellular function (9, 10). PKM2 is highly expressed during embryonic development as well as in proliferating cells (9). Tetrameric PKM2 has a high binding affinity to its substrate, PEP, prompting PKM2 glycolytic activity (11). In contrast, PKM2 in its dimer form has a low binding affinity to PEP, and can translocate into the nucleus where it acts as a transcriptional co-activator to enhance transcription of multiple pro-inflammatory cytokines (12). PKM2 has been shown to phosphorylate signal transducer and activator of transcription 3 (STAT3), which in turn augments its transcriptional activity (13). PKM2-linked STAT3 activation was recently shown to contribute to LPS-induced lung injury (14).

We previously showed that in mice with HDM-induced airway disease, levels of pyruvate kinase M2 were increased, compared to controls. Similarly, primary nasal epithelial cells derived from asthmatics also displayed increased PKM2 protein levels, compared to cells from healthy controls. These observations of increases in PKM2 in settings of allergic airway disease along with its dichotomous role as a glycolysis enzyme (glycolytic kinase) or pro-inflammatory mediator, led us to investigate whether a small molecule activator of PKM2, which stabilizes tetrameric PKM2 to promote conversion of PEP to pyruvate, affects HDM-induced allergic airways disease and IL-1β-induced inflammation.

Here we show that activation of the glycolysis function of PKM2 with the small molecule activator, TEPP46, exerts an anti-inflammatory effect in models of HDM- or IL-1β-induced lung inflammation in association with diminished activation of STAT3.

## MATERIALS AND METHODS

### Reagents and antibodies

All reagents were from Sigma-Aldrich unless otherwise noted.

### Mouse studies

Age-matched 8-10 weeks old male and female wild-type C57Bl/6NJ mice (WT mice) were bred at the University of Vermont. All animal experiments were approved by the Institutional Animal Care and Use Committee. To induce allergic airways disease, mice were sensitized intranasally with 10 µg of HDM (GREER, Lenoir, NC, XPB70D3A2.5, lot 348718; volume: 2.5 mL/vial; endotoxin: 1140 EU/vial; Der p 1 levels: 144.9 mcg/vial; dry weight: 17 mg/vial; protein 2.92 mg/vial) in week 1 (day 1), re-sensitized in week 2 (day 8) followed by 5 consecutive challenges in week 3 (day 15-19). Moreover, mice were intraperitoneally (i.p.) injected once per day with 25 mg/kg or 50 mg/kg TEPP46 (6-[(3-Aminophenyl)methyl]-4,6-dihydro-4-methyl-2-(methylsulfinyl)-5H-Thieno[2',3':4,5]pyrrolo[2,3-d]pyridazin-5-one, Cayman Chemicals) on days 14-19. HDM was dissolved in saline, whereas TEPP46 was dissolved in 100% DMSO and further diluted 1:1 in a 0.5% carboxy methyl cellulose solution in water. Therefore, the vehicle control groups were exposed to saline and received DMSO (50%) in carboxy methyl cellulose solution (50%). Mice were harvested on day 20, 24 hours after the last HDM installation.

In separate experiments, WT mice were intraperitoneal injected with 50 mg/kg TEPP46 at day 1, followed by a second injection after 24 hours of 50 mg/kg TEPP46 at day 2, together with intranasal administration of 1 µg of IL-1β (R&D Systems). IL-1β was dissolved in 0.1% Bovine Serum Albumin (BSA) in Phosphate Buffered Saline (PBS). Mice were harvested 6 or 24 hours after the IL-1β administration.

### Bronchoalveolar lavage (BAL) fluid processing

Mice were euthanized, and BAL was performed using 1 mL PBS. BAL was collected and total cells were counted manually using a hemocytometer. BAL was centrifuged at 500xg for 10 minutes at 4°C. Supernatant was stored at -80°C for further analysis. The cells were

resuspended in 5% BSA in PBS and subsequently transferred to slides using a cytospin, fixed in 100% methanol for 5 minutes and stained with the Hema3 kit (Fisher Scientific, Kalamazoo, MI). Total macrophages, neutrophils, eosinophils, and lymphocytes were analyzed by counting a total of 300 cells per slide by two independent investigators blinded to the identity of the samples.

**Cell culture**

Primary mouse tracheal epithelial cells (MTE) were isolated from WT C57BL/6NJ mice (purchased from The Jackson Laboratory, Bar Harbor, ME) and cultured as previously described (15, 16). WT MTE cells were grown on 12-well transwell inserts to confluency, followed by overnight starvation, and pre-treated with 100 µM TEPP46 (Cayman Chemicals) for 1 hour prior to stimulation with 10 ng/mL IL-1β (R&D Systems) for 24 hrs. In all cell experiments, MTE cells were treated at the apical and basolateral side. In HDM-treated MTE cell experiments, cells were washed 24 hours after IL-1β treatment, incubated with DMEM/F12 medium for 2 hours before stimulation with 50 µM HDM (GREER, Lenoir, NC, XPB70D3A2.5, lot 348718; volume: 2.5 mL/vial; endotoxin: 1140 EU/vial, Der p 1 levels: 144.9 mcg/vial; dry weight: 17 mg/vial; protein 2.92 mg/vial) for an additional 2 hours. Cells were harvested for protein or mRNA, and medium was collected for analysis of lactate and cytokine levels. To examine the contribution of phosphorylation of STAT3, MTE cells were treated with Stattic (17) (Abcam, Cambridge, UK) at the indicated concentrations for 1 hour prior to IL-1β treatment for 24 hrs.

**Assessment of mucus metaplasia and collagen deposition**

Left lung lobes were fixed in 4% paraformaldehyde in PBS, embedded in paraffin, and sectioned. Airway mucus was analyzed via Periodic acid Schiff (PAS) stain. Collagen deposition was assessed via Masson's trichrome stain. The intensity of the staining was evaluated by scoring of slides by two independent blinded investigators.

### Western blotting

Protein concentrations in cell and tissue lysates were determined by Bio-Rad DC Protein Assay Kit (Bio-Rad, Hercules, CA). Proteins were resolved using reducing sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, then transferred to polyvinylidene difluoride (PVDF) membranes followed by incubation with the indicated primary antibody. PKM1 (#7067), PKM2 (#4053), p-STAT3 (#8119), STAT3 (#4904), IKKε (#3416) and histone H3 (#4499) antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). β-actin antibody was acquired from Sigma-Aldrich. Subsequently, membranes were incubated with peroxidase-conjugated secondary antibodies and visualized using chemiluminescence (Pierce, Rockford, IL, USA). Non-reducing gel electrophoresis assays in the presence of the disuccinimidyl suberate (DSS) crosslinker (Thermo Scientific, MA, USA) were performed to evaluate the formation of tetrameric PKM2. Densitometric analyses were performed using Image J Software. Values were normalized to corresponding β-actin bands.

### PKM activity assay

PKM activity assay was performed using a pyruvate kinase activity kit according to the manufacturer's protocol (BioVision, CA, USA). Briefly, lung tissues or MTE cells were homogenized in PBS and lysates were normalized to equal protein concentrations. Equal volumes (total volume of 50 µl) of normalized lung tissue or cell lysates were used in the assay. The relative fluorescence units (RFU), which displays the rate of pyruvate yield, was normalized and expressed as RFU per minute per µg of protein.

### Preparation of nuclear extracts

For fractionation, MTE cells were stimulated with 10 ng/mL IL-1β (R&D Systems) for 24 hours. Fractionation of nuclear and cytosolic extracts was performed by using the Nuclear and Cytoplasmic Extraction kit (Thermo Scientific, MA, USA) according to manufacturer's protocol followed by western blotting.

**Lactate measurements**

Lactate levels were measured in cell culture medium with a lactate assay kit (Eton BioScience) according to manufacturer's instructions. Equal volumes of cell culture medium was used in 10 kDa Amicon Ultra centrifugal filters (EMD-Millipore). Samples were deproteinized by centrifugation for 1 hour at 14000xg at 4°C.

**Cell viability assay**

MTE cells were gently washed twice in ice cold PBS and stained with a Crystal Violet dye (0.5% Crystal Violet solution in 20% methanol) for 20 minutes at room temperature. After incubation, the staining solution was carefully removed and the cells were washed 4 times with distilled water. Subsequently, 10% acetic acid was added to the cells for 30 seconds while shaking. Lastly, 100 µl of the acetic acid solution per well was transferred to a 96 wells plate and the optical density was measured at a wavelength of 595 nm. In addition, a Calcein AM assay kit (Cayman Chemicals) was used according to manufacturer's instructions. Cell survival was expressed as percentage of survival compared to untreated control cultures.

**Real-Time quantitative PCR (Q-PCR)**

Total RNA was extracted using miRNeasy columns (Qiagen, Valencia, CA) according to the manufacturer's protocol. First-strand cDNA was synthesized from 1 µg RNA and reverse transcribed for gene analysis using SYBR Green (Bio-rad, Hercules, CA). cDNA from the samples were amplified by real-time quantitative PCR (Q-PCR) with specific primers for *Tslp*, *Csf2*, *Cxcl1*, *Ccl20*, *Muc5AC*, and *Col1a1*. The data was normalized to *Ppia* (also known as cyclophilin A). The primer sequences are listed in supplementary table I.

**Enzyme-linked immunosorbent assay (ELISA)**

CCL20, TSLP, GM-CSF, KC, IL-33, and IL-1β were detected by enzyme-linked immunosorbent assay (ELISA) kits (R&D systems, Minneapolis, MN) in normalized lung tissue lysates, or supernatants from cell cultures according to the manufacturer's instructions.

**Statistical analysis**

Data are expressed as means±SEM. All cell experiments were performed at least 3 times with n=3 per group. Significant differences between groups were determined using the GraphPad Prism software (Graphpad) by two-way ANOVA with a Tukey post hoc test for multiple comparisons. P values lower than 0.05 were accepted as significant.

## RESULTS

**Activation of the glycolysis function of PKM2 with TEPP46 attenuates airway inflammation, mucus metaplasia, and subepithelial collagen in mice with HDM-induced allergic airways disease**

To investigate the role of PKM2 in the pathogenesis of HDM-induced allergic airways disease, C57BL6/NJ mice were sensitized with HDM once in week 1, and in week 2, followed by 5 consecutive challenges in week 3 to induce allergic airways disease (Figure 1A). To promote PKM2 glycolytic activity, mice were injected intraperitoneally once per day with TEPP46 during the HDM challenges (days 14-19), starting the day prior to the HDM challenges in week 3 (Figure 1A). Administration of TEPP46 resulted in elevated activity of PKM in lung tissue from saline control animals and further increases in PKM activity were observed in lungs from mice exposed to HDM, indicating that TEPP46 augmented PKM2 activity (Figure 1B). While protein levels of PKM1 did not differ between the groups, we observed slight increases in PKM2 expression in HDM-exposed mice (Figure 1C), consistent with our previous observations, and these increases in PKM2 expression were no longer observed in mice receiving TEPP46. No statistically significant differences in overall PKM activity were observed in lungs from saline or HDM-treated mice in the absence of TEPP46 (Figure 1B, vehicle groups), despite observed increases in PKM2 expression in mice exposed to HDM (Figure 1C), suggesting that the increased expression of PKM2 does not contribute to its enhanced activity as a glycolysis enzyme, converting PEP to pyruvate. Next, we assessed the extent of inflammation by total and differential immune cell counts in the BALF. Activation of PKM2 by TEPP46 attenuated the HDM-mediated increases in total cells in the BALF, reflected by a decrease in eosinophils (Figure 1D, E), while the number of neutrophils, macrophages and lymphocytes were comparable between the HDM-treated groups. To further investigate the impact of activation of PKM2 on the extent of HDM-induced allergic airway inflammation, we evaluated protein levels of various cytokines in lung tissue homogenates. HDM-mediated increases of the cytokines CCL20 and KC, were attenuated upon PKM2 activation (Figure 1F). Small, but not statistically significant, decreases in IL-33 were observed in HDM exposed mice that received TEPP46, compared

to the respective control group while IL-1β levels did not change (Figure 1F). Together, these results show that PKM2 activation, using TEPP46, attenuates HDM-induced inflammatory cytokines in the lung.

We next evaluated the impact of PKM2 activation on airway remodeling, by assessing mucus metaplasia, subepithelial collagen and alpha smooth muscle actin (α-SMA). Results in Figure 2A-E demonstrate that administration of TEPP46 attenuated HDM-mediated increases in mucus metaplasia, subepithelial collagen and α-SMA content, and decreased expression of Muc5AC, and Col1a1 mRNAs. These results demonstrate that activation of PKM2 by TEPP46 attenuates airway remodeling in mice with HDM-induced allergic airways disease.

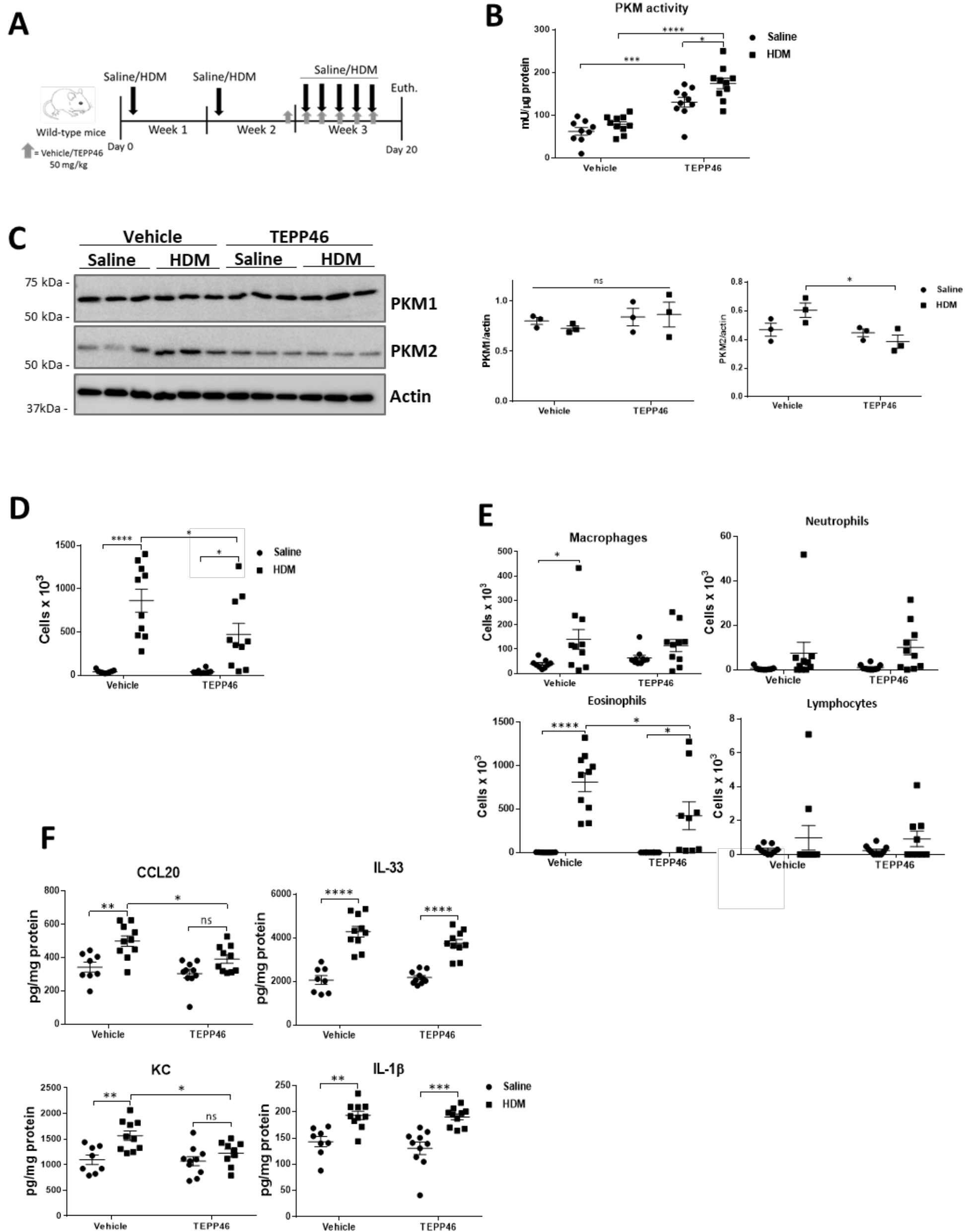


**Figure 1: Activation of PKM2 by TEPP46 attenuates pro-inflammatory cytokines in mice with HDM-induced allergic airway disease. A,** Schematic depicting the exposure regimen. Mice were sensitized twice with 10 μg of HDM or saline on days 1, and 8. Mice were treated with 50 mg/kg TEPP46 intraperitoneally daily, starting on day 14. Mice were challenged with HDM on days 15-19 and euthanized 24 hours after the final HDM challenge. **B,** Assessment of PKM activity in lung tissue homogenates. **C,** Representative western blots and quantification for total PKM1, and PKM2 levels. β-actin; loading control. n=3-6 per group. **D and E,** Total and differential cell counts in bronchoalveolar lavage fluid. **F,** Measurements of CCL20, IL-33, KC, and IL-1β in lung tissue homogenates by ELISA. For A-B, D-F; n=8-10 per group. *P < 0.05; **P < 0.01; ***P < 0.001; **** P < 0.0001.

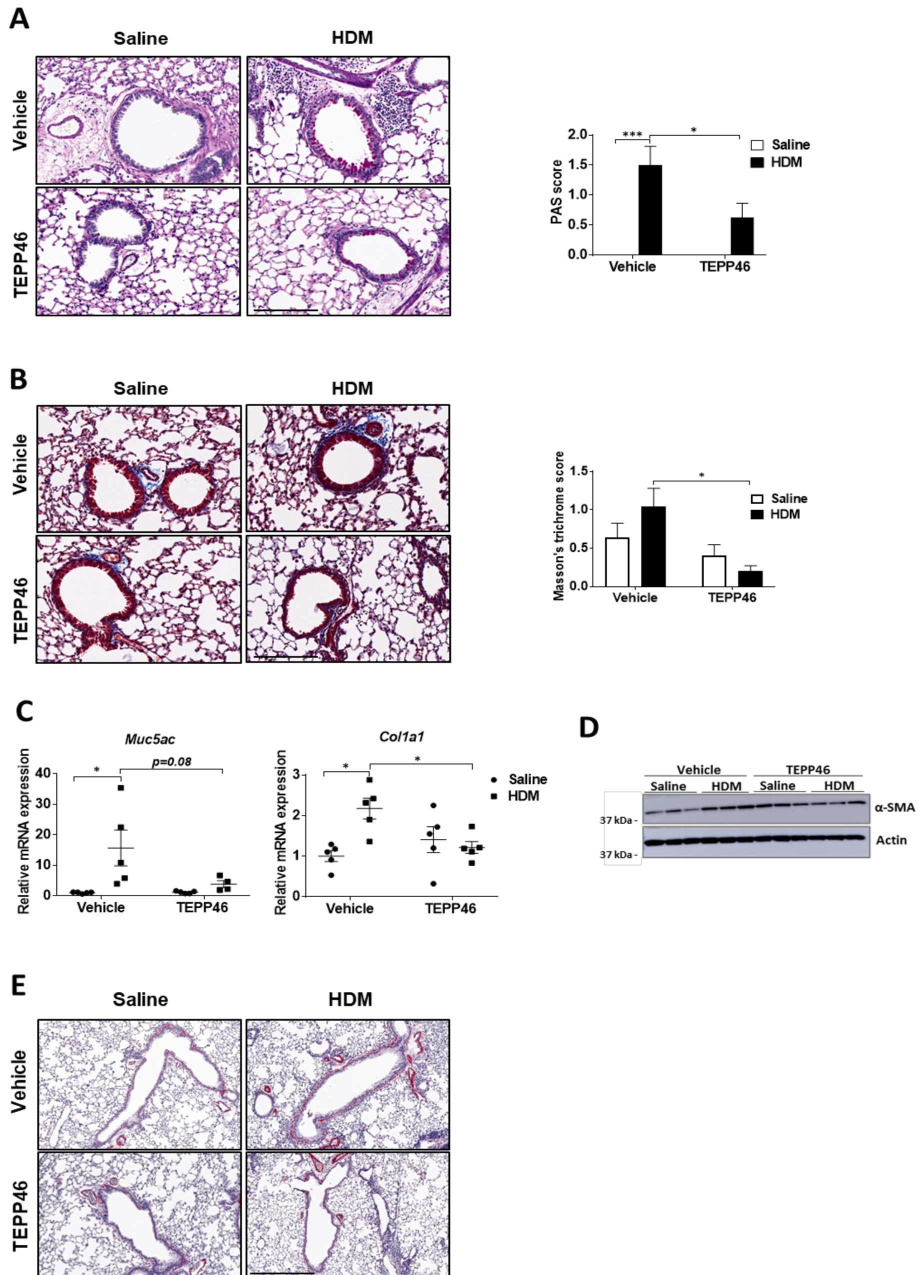


**Figure 2: Activation of PKM2 by TEPP46 attenuates mucus metaplasia, subepithelial collagen, and markers of airway remodeling in mice with HDM-induced allergic airway disease. A,** Assessment and quantification of mucus metaplasia by PAS staining intensity and **B,** Collagen deposition by Masson's trichrome staining. Scale bars: 200 µm. **C,** mRNA expression of *Muc5AC*, and *Col1a1,* normalized to *Ppia*. **D,** Representative western blots for α-smooth muscle actin (SMA) levels and the loading control β-actin. **E,** Assessment of SMA staining around large airways. Scale bar: 300 µm. n=5 per group. *P < 0.05; ***P < 0.001.

**Activation of PKM2 by TEPP46 attenuates IL-1β-mediated pro-inflammatory responses in mouse lungs**

We have previously shown that increases in glycolysis promote pro-inflammatory responses in airway epithelial cells exposed to IL-1β by increasing the production of the pro-inflammatory cytokines TSLP, GM-CSF, KC and CCL20 (3). Results in Figure 1F demonstrate similar increases in IL-1β levels in lung tissue from HDM-exposed mice receiving vehicle or TEPP46, suggesting that TEPP46 does not regulate expression of IL-1β. To examine whether PKM2 activity affects the responsiveness of lungs to IL-1β, WT mice were intraperitoneally injected with TEPP46 prior to intranasal IL-1β instillation for either 6 or 24 hours (Figure 3A). TEPP46 increased the total PKM activity in WT mice, with no further increases being observed in response to IL-1β (Figure 3B) after 6 hours and similar results were observed after 24 hours (data not shown). As expected, IL-1β elicited increases in total cell counts, reflected by neutrophils 24 hours post intranasal administration, without affecting airway eosinophils, macrophages, and lymphocytes (Figure 3C and D). In animals receiving TEPP46, the IL-1β-mediated increases in total cell counts and neutrophils were diminished (Figure 3C and D). In agreement with these findings, mRNA expression levels of *Csf2* and *Ccl20* and the respective protein levels of GM-CSF, and CCL20, were significantly attenuated in mice treated with TEPP46 prior to IL-1β instillation for 6 hours (Figure 3E, F), while there was less to no effect on *Cxcl1* and *Tslp* mRNA and protein levels (KC and TSLP). Doses of 25 mg/kg or 50 mg/kg of TEPP46 were similar in their ability to induce PKM activity and dampen pro-inflammatory responses (Figure 3B, 3E, and 3F). Collectively, these results demonstrate that PKM2 activation decreases select IL-1β-induced inflammatory responses in the lung.

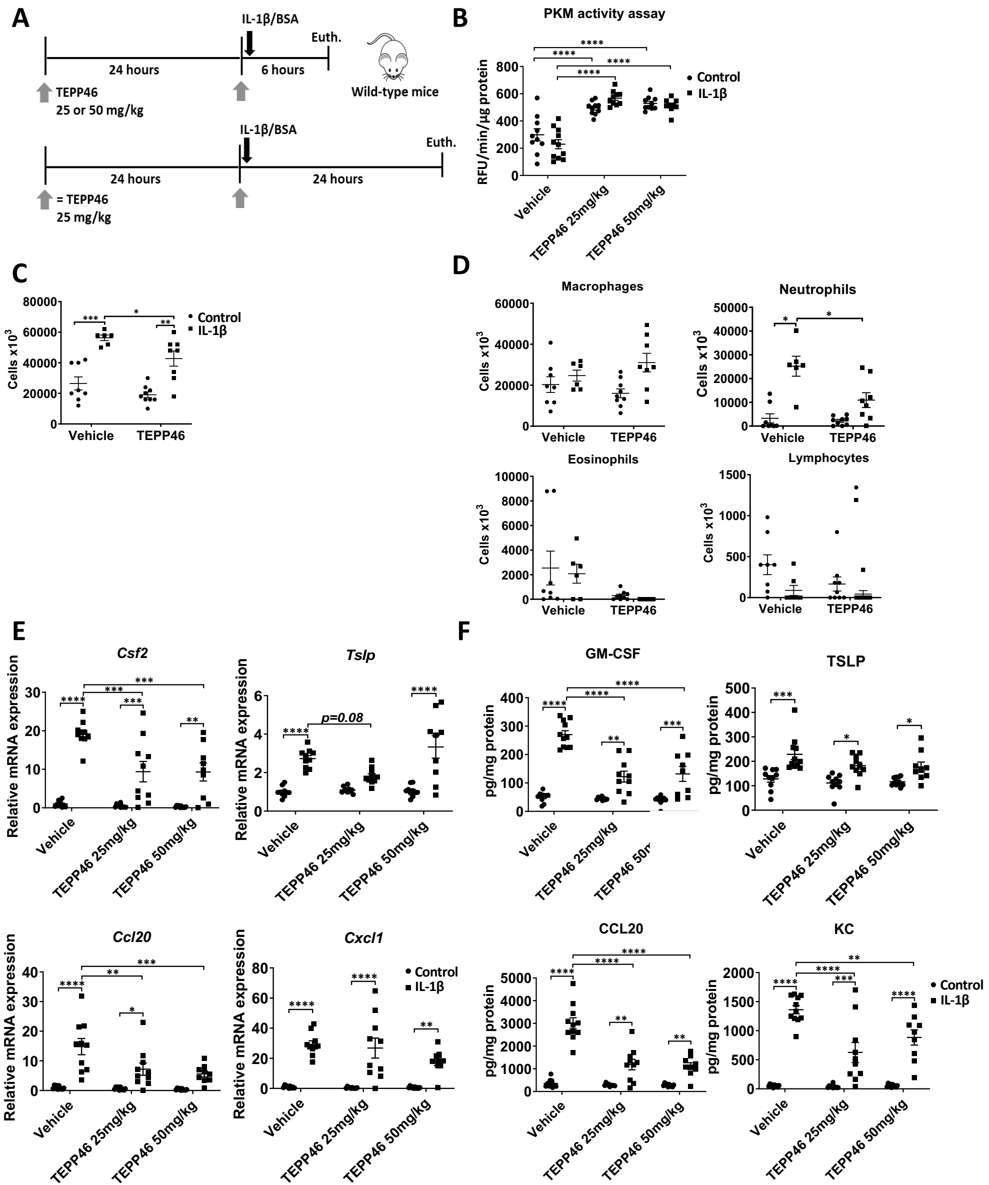


**Figure 3: PKM2 activation attenuates the release of pro-inflammatory cytokines following intranasal administration of IL-1β. A,** Schematic depicting the pre-treatment with 25 or 50 mg/kg TEPP46 prior to intranasal administration of 1 μg of IL-1β for either 6 or 24 hours. The total cell count and cell differentials in the BAL fluid reflect 24 hours post IL-1β treatment, the other results shown are obtained 6 hours post IL-1β. **B,** assessment of PKM activity in lung tissue homogenates. **C,** and **D,** Total and differential cell counts in BAL fluid. **E,** mRNA expression of pro-inflammatory cytokine genes in lung tissue homogenates. Results were normalized to the house keeping gene, *Ppia*. **F,** Levels of pro-inflammatory mediators TSLP, GM-CSF, KC, and CCL20 in lung tissue homogenates by ELISA. n=6-11 per group. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

**TEPP46 decreases nuclear translocation of PKM2 and dampens IL-1β-mediated pro-inflammatory responses in mouse tracheal basal cells**

Airway epithelial cells are important contributory cells to allergic airway disease, as these cells release a number of mediators that promote innate and adaptive immune responses (18, 19). We previously demonstrated that IL-1β-induced glycolysis is critical for the release of TSLP and other asthma-relevant cytokines by epithelial cells, and that IL-1β-induced glycolysis also primes these cells to elicit augmented pro-inflammatory responses to HDM (3). PKM2 in its dimer form has a low binding affinity to PEP, and can translocate into the nucleus where it acts as a transcriptional co-activator to enhance transcription of multiple pro-inflammatory cytokines. We therefore next determined whether IL-1β affects the status and/or nuclear presence of PKM2 in epithelial cells, and whether PKM2 activation affects the response to IL-1β. Primary MTE cells were pre-treated with TEPP46 for 1 hour, prior to IL-1β stimulation for 24 hours. This experimental regimen did not result in apparent changes in expression levels in PKM1 and PKM2 (Figure 4A). However, TEPP46 augmented overall PKM glycolytic activity in control cells, and a further enhancement of PKM activity occurred when cells were treated with TEPP46 in combination with IL-1β (Figure 4B). PKM2 is active as a glycolysis enzyme in its tetramer form, and loses its activity as a glycolytic kinase in the dimer form (11). Instead, dimeric PKM2 has been shown to translocate into the nucleus where it acts as a protein kinase to induce phosphorylation of STAT3, augmenting STAT3 transcriptional activity, leading to increased expression of pro-inflammatory mediators and increased expression of glycolysis enzymes including glucose transporter 1 (GLUT-1), thereby promoting glycolytic reprogramming (20). IL-1β led to a slight attenuation of PKM2 tetramers in MTE cells, while TEPP46 increased PKM2 tetramers in both control and IL-1β-treated cells, relative to the respective vehicle groups (Figure 4C). We did not observe an increase in nuclear PKM2 24 hrs post-administration of IL-1β. However, TEPP46 diminished the nuclear presence of PKM2 and increased its cytoplasmic localization, in both control and IL-1β-stimulated cells (Figure 4D). Consistent with the attenuation of glycolytic reprogramming, TEPP46 administration led to a decrease in IL-1β-mediated lactate secretion (Figure 4E).

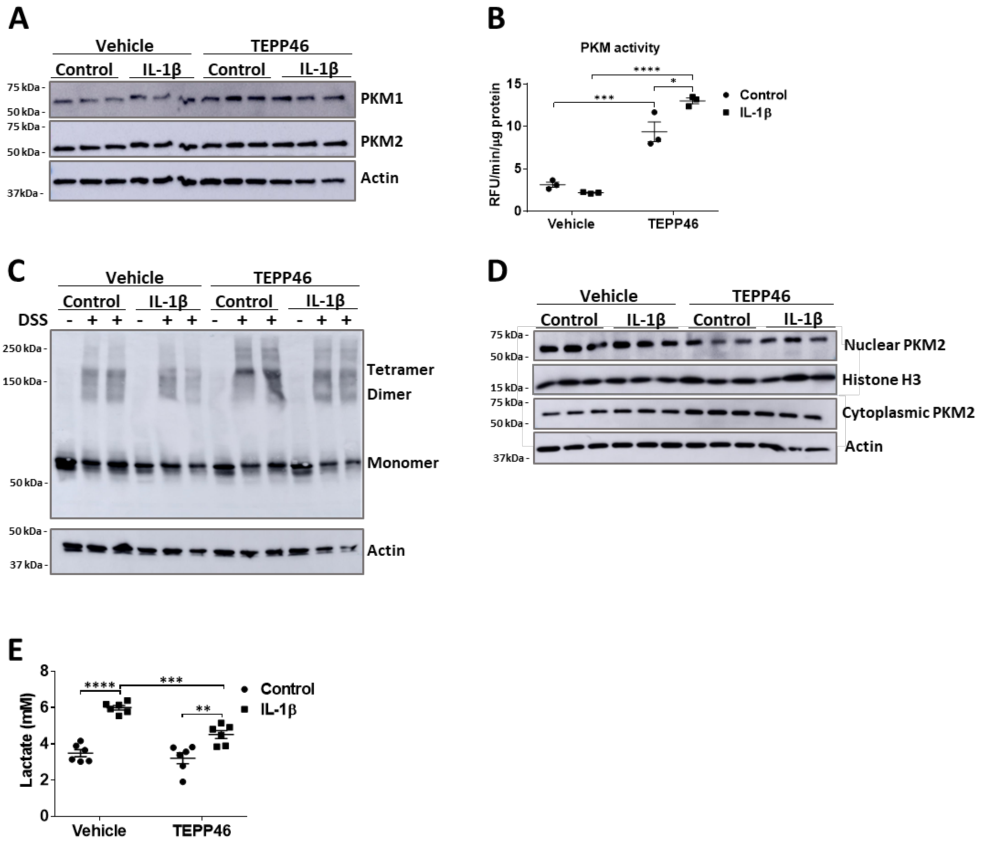


**Figure 4: TEPP46 augments PKM activity, and PKM2's cytosolic presence and attenuates interleukin-1β-mediated lactate secretion in primary MTE cells.** MTE cells were treated with 100 µM TEPP46 for 1 hour prior to stimulation with 10 ng/mL IL-1β for 24 hours. **A,** Representative western blot of total PKM1 and PKM2 levels, and β-actin. **B,** PKM activity assay in MTE cells and **C,** representative western blot for tetrameric, dimeric and monomeric PKM2 and the loading control β-actin. MTE cells were incubated in the presence or absence (first lane of each condition) of the DSS crosslinker to evaluate the formation of the isoforms of PKM2. **D,** Representative western blots of nuclear and cytosolic extracts of PKM2. (n=3 per group). **E,** Lactate levels in supernatants of MTE cells. Experiments were performed at least 3 times. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

To investigate whether activation of PKM2 attenuates pro-inflammatory cytokine release from airway epithelial cells, MTE cells were pre-treated with TEPP46 followed by stimulation with IL-1β (Figure 5A). Strikingly, PKM2 activation strongly attenuated the IL-1β-induced mRNA and protein levels of Tslp (TSLP) and Csf2 (GM-CSF), respectively, while it modestly or did not affect Ccl20 (CCL20) or Cxcl1 (KC) (Figure 5B, C). As was stated earlier, exposure to IL-1β primes MTE cells to subsequent stimulation with HDM, leading to augmented release of pro-inflammatory cytokines. We therefore pre-treated primary MTE cells with TEPP46, followed by stimulation with IL-1β for 24 hours. Cells were then washed and exposed to HDM for 2 hours (Figure 5D). In agreement with our previous observations, prior exposure to IL-1β leads to potent HDM-stimulated release of TSLP, GM-CSF, KC and CCL20. TEPP46 almost completely abolished TSLP and GM-CSF in this sequential exposure regimen, and significantly decreased KC and CCL20 (Figure 5E). TEPP46 treatment alone or in combination with IL-1β or IL-1β+HDM did not induce cell death (Figure 5F), demonstrating that the decreased cytokine production is not due to a loss of cell survival. Collectively, these data demonstrate that TEPP46 diminishes IL-1β and HDM-mediated pro-inflammatory responses in epithelial cells, in association with increases in PKM2 cytosolic presence and enhanced PKM glycolytic activity.

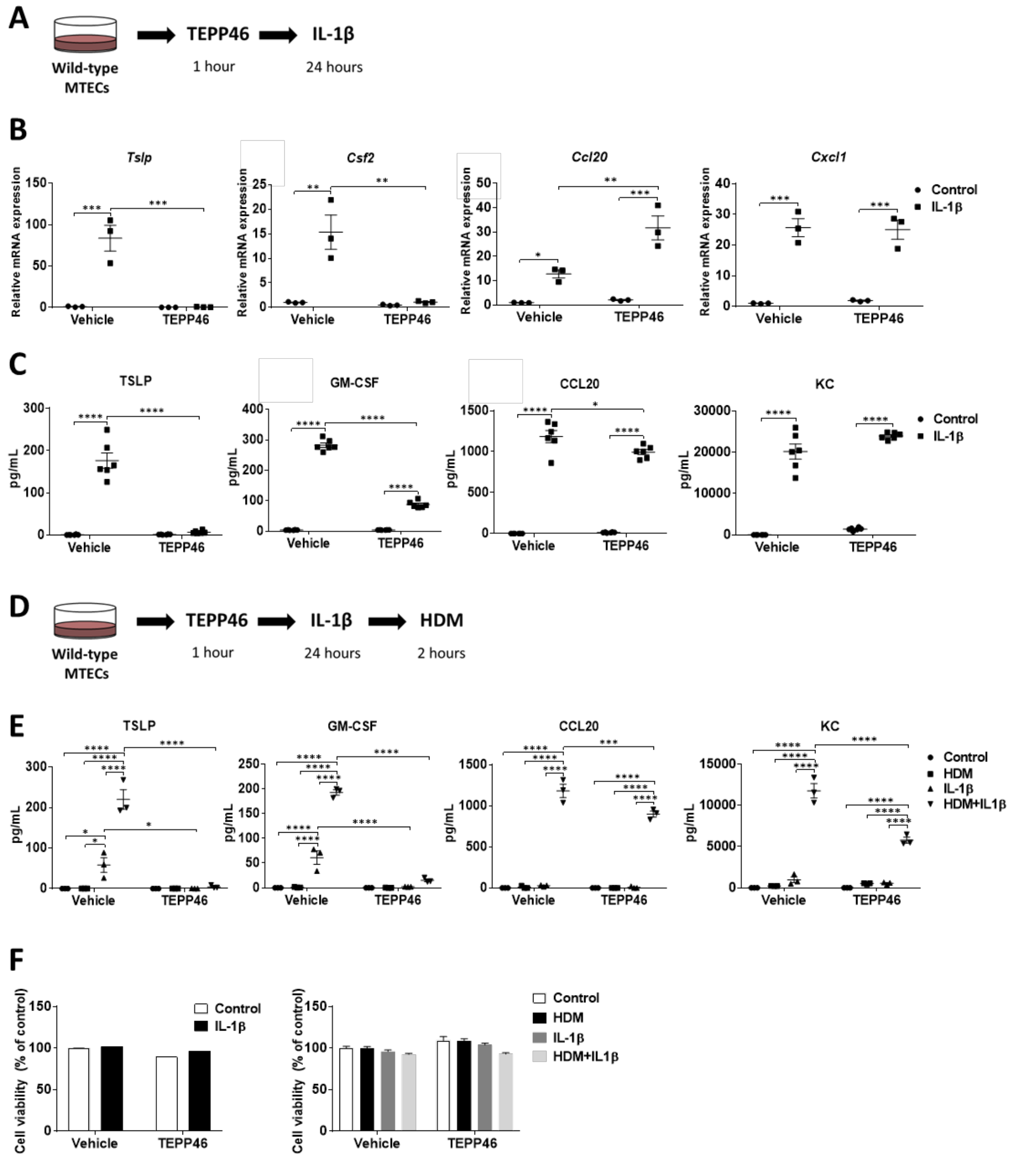


**Figure 5: Activation of PKM2 attenuates IL-1β-mediated pro-inflammatory responses in primary MTE cells and the release of pro-inflammatory mediators following subsequent exposure to HDM. A,** Schematic depicting the pre-treatment with 100 μM TEPP46 followed by stimulation of 10 ng/mL IL-1β for 24 hours. **B,** mRNA expression of *Tslp*, *Csf2*, *Cxcl1* and *Ccl20* in MTE cells. *Ppia* is used as housekeeping gene. **C,** Pro-inflammatory cytokine mediators TSLP, GM-CSF, KC, and CCL20 in cell culture supernatants of MTE cells were detected by ELISA**. D,** Schematic depicting the pre-treatment with 100 μM TEPP46 followed by stimulation of 10 ng/mL IL-1β for 24 hours. Media was replaced and exposed to HDM (50 μg/mL) for an additional 2 hours. **E,** Pro-inflammatory cytokine mediators TSLP, GM-CSF, KC, and CCL20 in cell culture supernatants of MTE cells**. F,** Cell survival was evaluated by crystal violet staining (left) and Calcein AM assay (right) in MTE cells. n=3-6 per group. Experiments were performed at least 3 times. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

**PKM2-mediated phosphorylation of STAT3 contributes to IL-1β-mediated pro-inflammatory signaling in epithelial cells**

It has been previously described that nuclear PKM2 phosphorylates STAT3, thereby augmenting the production of pro-inflammatory cytokines, including IL-6 and IL-1β (11, 20). We therefore addressed whether PKM2 contributed to STAT3 activation, and in turn whether STAT3 promoted IL-1β-induced pro-inflammatory signaling in mouse epithelial cells. Despite the lack of observed increases in nuclear PKM2 in response to IL-1β (Figure 4D), IL-1β elicited strong increases in nuclear pSTAT3 using an antibody directed against phosphorylation of tyrosine 705, the residue known to be phosphorylated by PKM2 (11) (Figure 6A). Total content of STAT3 in the nucleus was also increased in epithelial cells exposed to IL-1β (Figure 6A). Exposure to TEPP46 led to a strong diminution of nuclear pSTAT3 (Figure 6A), consistent with the aforementioned role of PKM2 as a STAT3 kinase (20). Similarly, phosphorylation of nuclear STAT3 was also increased in lung tissues from mice with HDM-induced allergic airways disease, and was diminished in mice also treated with TEPP46 (Figure 6B). We previously showed data suggesting that inhibitory kappa B kinase epsilon (IKKε) is a critical mediator in IL-1β-induced glycolysis. Here we show that the IL-1β-induced expression levels of IKKε were unaffected when MTE cells were pre-treated with TEPP46, suggesting that the effect of TEPP46 on diminishing STAT3 phosphorylation may be downstream or independent of IKKε (Figure 6A). To further corroborate the role of STAT3 in promoting IL-1β-induced pro-inflammatory responses, we used the STAT3 inhibitor, Stattic (17, 20), in vitro. Concentrations greater than 1 µM Stattic caused marked epithelial cell death (Figure 6C). Nonetheless, a concentration of 0.5 µM Stattic diminished IL-1β-mediated phosphorylation of STAT3 in whole cell lysates (Figure 6D), and attenuated the IL-1β-mediated increases of TSLP, and GM-CSF without affecting the other cytokines (Figure 6E). All together, these data suggest that activation of PKM2 as a glycolytic kinase by TEPP46 diminishes the pro-inflammatory responses induced by IL-1β in lung epithelial cells or in mice with allergic airway disease, and that the increased kinase activity of PKM2 towards STAT3 in these settings may in part contribute to PKM2-linked inflammation.

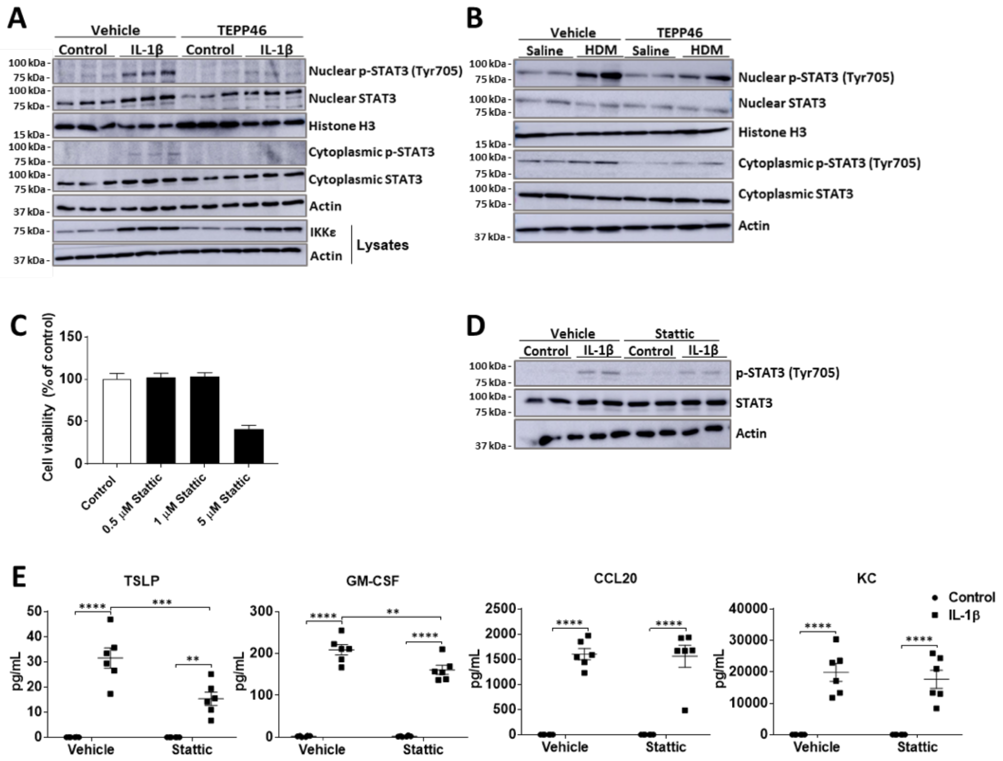


**Figure 6: PKM2-mediated phosphorylation of STAT3 contributes to IL-1β-mediated pro-inflammatory signaling in epithelial cells. A,** Representative western blots of total and phosphorylated STAT3 in nuclear and cytosolic extracts from MTE cells, and total IKKε levels in whole cell lysates. **B,** Representative western blots of total and phosphorylated STAT3 in nuclear and cytosolic extracts from HDM- or saline-treated lung tissues. **C,** Impact of Stattic on survival of MTE cells was evaluated by a Calcein AM assay. **D,** Representative western blots of total and phosphorylated STAT3 in whole cell lysates from control or IL-1β stimulated MTE cells pre-treated with Stattic or vehicle control. **E,** Pro-inflammatory mediators TSLP, GM-CSF, KC, and CCL20 in cell culture supernatants of MTE cells, after treatment for 1 hour with 0.5 μM Stattic, followed by stimulation of 10 ng/mL IL-1β for 24 hours**.** Experiments were conducted at least 3 times. **P < 0.01; ***P < 0.001; ****P < 0.0001.

## DISCUSSION

Allergic airway disease is associated with chronic inflammation and airway remodeling, processes that are metabolically demanding. During glycolysis, some of the carbons derived from glucose are used to allow for biosynthetic processes. In addition, glycolysis has also been linked to pro-inflammatory responses in immune cells (21, 22). Our laboratory has previously shown that glycolysis is a feature of allergic asthma in association with neutrophilic inflammation and steroid-resistant disease, and that IL1 is an important driver of glycolysis in settings of allergic airways disease in mice (3). In addition to these observations, increases in aerobic glycolysis have been shown to promote T cell activation (23) and to promote T cell effector function (21). Increases in glycolysis also have been implicated in lipopolysaccharide (LPS)-induced airway smooth muscle cell proliferation (24) and in IL-33-mediated increases in cytokine production in mast cells (25). Asthma-associated single-nucleotide polymorphisms within the orosomucoid-like 3 (ORMDL3) locus have been implicated in disease susceptibility. A recent study showed that ablation of ORMDL3 attenuated IL1-mediated endoplasmic reticulum stress and cytokine responses in A549 lung epithelial cells, in association with alterations in glycolysis and glucose metabolism genes (26) indicating a potential link between glycolysis and asthma susceptibility. Increases in glycolysis, basal and maximal respiration, and oxidative stress were demonstrated in airway epithelial cells and platelets from obese asthmatics (who tend to have more severe disease), in comparison to lean asthmatics and healthy subjects (27). Notably, increases in airway lactate were demonstrated in asthmatics with a high fraction of exhaled nitric oxide (FeNO), in association with elevated expression of inducible nitric oxide synthase and arginase 2, and suggests a link between enhanced glycolysis, arginine metabolism and a high FeNO asthma phenotype (28). Nonetheless, the precise signals and settings that elicit the glycolysis-associated pro-inflammatory responses in lung epithelial cells remain unclear. In the present study we demonstrate the importance of the glycolytic enzyme PKM2 in promoting inflammation and airway remodeling in mice with HDM-induced allergic airway disease. PKM2 has generated substantial interest due to its impact on glycolytic reprogramming in activated immune cells and tumor cells, and its emerging

role as a pro-inflammatory mediator (9, 12). Herein we demonstrate that activation of the glycolysis function of PKM2 with TEPP46, augments pyruvate kinase activity in lung tissue and airway epithelial cells, and dampens inflammation, evidenced by attenuated airway eosinophilia and airway remodeling in mice with HDM-induced allergic airways disease. Moreover, administration of TEPP46 attenuated IL-1β-induced airway neutrophilia in mice and significantly reduced IL-1β-mediated expression of pro-inflammatory cytokines and lactate. These results, in addition to our previous results (3), show that enhanced glycolysis is important for the amplification of allergen-induced pro-inflammatory responses and show the importance that PKM2 plays in regulating this process.

Results herein point to the importance of glycolysis in the secretion of specific pro-inflammatory mediators from airway epithelial cells, notably TSLP and GM-CSF. Activation of PKM2 with TEPP46 almost completely abolished expression of both cytokines, while Stattic also attenuated the IL-1β-mediated release of these cytokines (Figure 5C, and 6E). These findings are in line with our previous study wherein we demonstrated that inhibition of inhibitory kappa B kinase epsilon (IKBKE) or TANK-binding kinase 1 (TBK1), two kinases critical in promoting IL-1β-induced glycolysis also virtually abolished secretion of TSLP and GM-CSF (3). The importance of TSLP in asthma has been extensively studied (22, 29-32). TSLP is primarily expressed in epithelial cells and acts on both innate and adaptive immune cells thereby promoting T-helper 2 immunity and steroid resistance (33). Overexpression of TSLP results in the development of severe airway inflammation and airway hyper responsiveness (29, 32). The USA Food and Drug Administration (FDA) has granted Breakthrough Therapy Designation for tezepelumab, a TSLP-blocker, in patients with severe asthma. Blocking TSLP may prevent the release of other pro-inflammatory cytokines by immune cells resulting in the prevention of asthma exacerbations and improved asthma control (34). Similarly, GM-CSF has been shown to activate macrophages and promote eosinophil migration, differentiation and survival, in addition to its function in the differentiation and maturation of dendritic cells (35). Our present data showing that activation of PKM2 with TEPP46, preferentially attenuates TSLP and GM-CSF, while modestly or not affecting CCL20 and KC, suggest that avenues to attenuate glycolysis, or to

activate PKM2 in an environment where IL-1 signaling is operative will be attractive strategies to dampen TSLP and GM-CSF.

The pro-inflammatory role of PKM2 has been extensively studied in tumor and immune cells, and a number of transcription factors have been demonstrated to mediate the pro-inflammatory effect of PKM2 (36-38). In macrophages and tumor cells, LPS induces nuclear binding of PKM2 to hypoxia-inducible factor 1-alpha (HIF-1α) (20, 39). In tumor cells, an interaction between PKM2 and Jumonji C domain-containing dioxygenase (JMJD5) also has been shown (37). These interactions with PKM2 have been linked to the transcription of glycolysis genes including LDHA and GLUT-1. Moreover, nuclear PKM2 was also shown to phosphorylate STAT3 (13, 36), which in turn augments IL-1β and IL-6 production (20). In line with the latter findings, the present observations that IL-1β or HDM led to increases in phosphorylation of STAT3, which were attenuated by TEPP46, suggest the importance of PKM2 in promoting STAT3 phosphorylation. Our findings that inhibition of STAT3 attenuated release of TSLP and GM-CSF in airway basal cells, strongly suggest that the ability of PKM2 to phosphorylate STAT3 contributes to its pro-inflammatory function. Findings from the present study using an activator of PKM2 are in line with another study showing that activation of the glycolysis function of PKM2 attenuated the pro-inflammatory phenotype of macrophages from patients with atherosclerotic coronary artery disease (20), and inhibited the PKM2-HIF-1α complex (39) leading to decreased IL-1β production and glycolysis and pro-inflammatory genes (38, 39). Moreover, it has been shown that inhibition of STAT3, by Stattic, attenuated inflammatory injury in LPS-challenged mice (14).

PKM2 and PKM1 are both encoded by the same PKM gene, however they represent different splicing products (exon 9 for PKM1, exon 10 for PKM2). Unlike PKM1, PKM2 is not a constitutive stable tetrameric enzyme, and can be allosterically regulated by fructose-1,6-bisphosphate (FBP) to enhance tetramer formation. The PKM2 tetramer can be converted to dimers following a number of post translational modifications that include phosphorylation (40, 41), acetylation (42, 43), oxidation (44), hydroxylation (37, 38), ubiquitination (45), glycosylation (46), methylation (47), and sumoylation (48) in response to various stimuli. Interestingly, epidermal growth factor (EGF)-activated ERK2 binds

directly to PKM2 and can induce phosphorylation of PKM2 at serine 37, in association with its nuclear translocation and increases in transcriptional activation of GLUT-1, and LDHA (49, 50). The EGF receptor (EGFR) is of notable interest due to its role of type 2 inflammatory responses in allergic airways disease, including mucus metaplasia (51-53). Additional studies will be required to elucidate whether EGFR activation contributed to phosphorylation and subsequently the inactivation of PKM2 that was observed in the present study. Other studies have demonstrated that PKM2 can be inactivated following oxidation of cysteine 358 (44). Changes in the oxidative environment and notably cysteine oxidations accompany allergic airway disease and lead to activation of EGFR in cells (51). Similarly, changes in the oxidative environment also control dendritic cell activation and T-cell subsets (54). Additional studies will also be required to address whether oxidative events regulate PKM2 activity herein.

Small molecule activators of PKM2 such as TEPP46 (also known as ML265) and DASA-58 have been developed to stabilize PKM2 in the tetramer configuration. TEPP46 activates PKM2 by binding to the dimer-dimer interface between two subunits of PKM2, which stabilizes tetrameric PKM2 to promote conversion of PEP to pyruvate, hence increasing its glycolytic activity. TEPP46 is highly selective in its ability to activate PKM2 (55), since it does not affect recombinant PKM1 in vitro (10) and has no significant effect in PKM2 knockout models (39, 56). These observations suggest that the effects observed by TEPP46 herein are due to the activation of the glycolysis function of PKM2, and not due to off target effects, although additional studies will be required to corroborate the lack of off target effects. Paradoxically, while activation of PKM2 dampened inflammation, ablation of PKM2 also elicited anti-inflammatory effects. As discussed earlier, the TEPP46-induced PKM2 tetramer inhibited LPS-induced expression of *IL-1β* and other *HIF-1α*-dependent genes in macrophages (20, 39), while macrophages lacking PKM2 also showed reduced expression of *IL-1β* and *Ldha* mRNAs in response to LPS. These findings suggest that dimeric PKM2 has a pro-inflammatory gain of function, and that strategies to either remove PKM2 altogether, or to promote its glycolysis kinase function elicit similar anti-inflammatory effects. Another limitation of the current manuscript is that experiments were performed in mice only.

Further studies using human samples will be required to fully understand the contribution of PKM2 to pro-inflammatory responses in epithelial cells or airways from asthmatics.

Altogether, our results demonstrate that the glycolysis-inactive form of PKM2 plays a crucial role in the pathogenesis of allergic airway disease in association with enhancing IL-1β-induced pro-inflammatory signaling, in part through phosphorylation of STAT3, and notably the upregulation of *Tslp* and *Csf2* genes. PKM2 therefore could be a novel potential target for the development of anti-inflammatory therapies for the treatment of IL1 high, glycolysis-associated asthma.

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**Supplemental Table I: primer sequences used in this study**

| GENES | FORWARD | REVERSE |
|---|---|---|
| ***Tslp*** | TGAGAGCAAGCCAGCTTGTC | GTGCCATTTCCTGAGTACCG |
| ***Csf2*** | ATGCCTGTCACGTTGAATGA | CCGTAGACCCTGCTCGAATA |
| ***Cxcl1*** | TGCGAAAAGAAGTGCAGAGA | TACAAACACAGCCTCCCACA |
| ***Ccl20*** | AAGACAGATGGCCGATGAAG | AGCCCTTTTCACCCAGTTCT |
| ***Muc5ac*** | GCACAGGAGGAAAGAGCATC | AACTTTGCCGAAAACCACAT |
| ***Col1a1*** | CACCCTCAAGAGCCTGAGTC | AGACGGCTGAGTAGGGAACA |
| ***Ppia*** | AACTTTGCCGAAAACCACAT | GCACAGGAGGAAAGAGCATC |

# 5

## Glutathione-S-transferase P promotes Interleukin-1β-mediated pulmonary inflammation and airway remodeling in mice with house dust mite-induced allergic airways disease in association with S-glutathionylation of Pyruvate Kinase M2

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## ABSTRACT

Alterations in metabolism and associated changes in redox homeostasis have been shown to be an important feature of chronic lung diseases. Notably, protein S-glutathionylation (PSSG), an oxidative modification of protein cysteines, has been implicated in regulating pro-inflammatory signals. Glutathione-S-transferase P (GSTP) can catalyse the forward PSSG reaction, and has been linked to asthma. We recently demonstrated that increased Interleukin-1 (IL-1) signaling promotes glycolytic reprogramming in a model of house dust mite (HDM)-induced allergic airways disease in mice. Moreover, diminished glycolytic activity of pyruvate kinase M2 (PKM2) promotes HDM-induced allergic airways disease, in association with phosphorylation of STAT3. Herein, we sought to elucidate whether GSTP-linked PSSG promotes allergic airways disease and IL-1β-induced pulmonary inflammation. Our results show that genetic ablation of *Gstp* decreased PSSG and attenuated HDM-induced mucus metaplasia, airway remodeling, and airway hyperresponsiveness. Although ablation of *Gstp* did not clearly impact HDM-induced inflammation, *Gstp* ablation attenuated IL-1β-mediated airway inflammation and lactate levels in the bronchoalveolar lavage. *Gstp* deletion or inhibition using a clinically relevant inhibitor of GSTP, TLK199, decreased the IL-1β-mediated increases in thymic stromal lymphopoietin (TSLP) and granulocyte macrophage colony stimulating factor (GM-CSF), and the secretion of lactate by primary tracheal epithelial cells. Direct exposure of recombinant PKM2 to GSSG resulted in a loss of its glycolytic activity. Moreover, GSTP interacted with PKM2 in lungs and in epithelial cells. Additional computational studies suggest that GSTP-PKM2 tetramer is more likely to complex than GSTP-PKM2 dimer, and that Cys423 and Cys424 on PKM2 can be redox active for GSH transfer. Lastly, we demonstrate that HDM, or IL-1β-mediated increases in phosphorylation of STAT3 were decreased in *Gstp*$^{-/-}$ mice, and that TLK199 decreases the IL-1β-mediated increases of STAT3 phosphorylation in tracheal epithelial cells. Collectively, these results show the importance of GSTP-controlled S-glutathionylation of PKM2 in the promotion of pro-inflammatory signaling in epithelial cells that are known to be relevant to allergic airway remodeling.

**Keywords:** Asthma, Glutathione-S-transferase P, S-glutathionylation, glycolysis, Pyruvate Kinase M2, Interleukin-1β

**Abbreviations:** GSH: Glutathione; GST-π/GSTP: Glutathione-S-transferase P; Glrx: Glutaredoxin; AHR: Airways hyperresponsiveness; HDM: House dust mite; PSSG: S-Glutathionylation; IL-1β: Interleukin 1β; PKM2: Pyruvate kinase M2; NADPH: Nicotinamide-adenine-dinucleotide phosphate; WT: Wild-type; MTE cells: Mouse tracheal epithelial cells; BSA: Bovine serum albumin; BALF: Bronchoalveolar lavage fluid; HK2: Hexokinase II; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; α-SMA: Alpha smooth muscle actin; TSLP: Thymic stromal lymphopoietin; GM-CSF: Granulocyte-macrophage colony-stimulating factor; CCL20: Chemokine (C-C motif) ligand 20; CXCL1: Chemokine (C-X-C motif) ligand 1; IKKε: Inhibitory kappa B kinase epsilon; STAT3: Signal transducer and activator of transcription 3

## INTRODUCTION

Asthma is a chronic inflammatory disease characterized by various degrees of airflow limitation, airway remodelling, and airway hyperresponsiveness (1). We recently showed that changes in cellular metabolism, notably increases in the glycolysis pathway, are an important feature of allergic airways disease and contribute to enhanced pro-inflammatory signalling in airway epithelial cells (2,3). The underlying mechanism that drives glycolytic reprogramming during allergic airways disease is still an unexplored area of research. However, changes in metabolism are shown to be regulated by redox perturbations including oxidation reactions (4, 5). Recent studies highlight the importance of oxidants in (patho)physiological processes through oxidation of reactive cysteines in target proteins. Notably, post-translational S-glutathionylation (PSSG) is a process that affects protein cysteine residues by the conjugation of glutathione (GSH), and affects protein structure and function (6). Glutathione-S-transferases (GSTs), in particular the Pi class, can catalyze forward PSSG reactions, whereas glutaredoxins (GLRXs), under physiological conditions, reverse PSSG and thereby restoring the protein sulfhydryl group (7, 8). Glutathione-S-transferase Pi (GSTP), a phase II detoxifying enzyme that is highly expressed in airway epithelial cells, is thought to bind its target proteins, which in turn activates GSTP to induce PSSG (9-11). Interestingly, GSTP has already been linked to asthma, as polymorphisms in the *GSTP* gene have been associated with an increased susceptibility to develop asthma in children and adults (12-15), although substantial disagreement between studies exists (16). We have previously shown that PSSG was decreased in sputum samples from eosinophilic and neutrophilic asthmatics compared to healthy controls (17). In contrast, in lungs of mice exposed to the asthma-relevant allergen, house dust mite (HDM), PSSG was increased (18). Although these data implicate a role for PSSG in allergic airways disease, it is unknown whether GSTP-catalyzed PSSG is involved in the pathophysiology of allergic airways disease, and which processes are regulated by this post-translational modification.

Redox perturbations have been shown to underlie adaptations in cellular metabolism, including changes in glycolysis. In turn, glycolytic reprogramming has been shown to be important in the regulation of pro-inflammatory signaling in immune cells (19, 20). During

glycolytic reprogramming, cells shift their metabolism from catabolic mitochondrial oxidative phosphorylation to glycolysis and other anabolic pathways. Glycolytic reprogramming occurs in metabolically active or proliferating cells with a high demand for energy, and is accompanied with increases in lactate production (21). Our laboratory recently demonstrated that Interleukin (IL)-1 signaling increases glycolytic reprogramming in epithelial cells in association with neutrophilic inflammation and steroid resistance during allergic airways disease (2). Moreover, increases in glycolysis promote IL-33-dependent cytokine production (22), and LPS-induced airway smooth muscle cell proliferation (23).

The last, rate-limiting step of glycolysis is the conversion of phosphoenolpyruvate (PEP) to pyruvate, catalyzed by Pyruvate Kinase M (PKM). The PKM gene consists of two isozymes, PKM2 and PKM1. PKM1 is constitutively active and occurs as a tetramer with high binding affinity for the PEP substrate. PKM2 on the other hand, can also exist as dimers or monomers, which have low binding affinity to PEP (24-26), but can translocate into the nucleus to function as a transcriptional coactivator of pro-inflammatory cytokines. For example, PKM2 has been shown to phosphorylate Signal transducer and activator of transcription 3 (STAT3), thereby augmenting the production of the pro-inflammatory cytokines IL-1β and IL-6 (27, 28). Our laboratory has recently shown that PKM2 protein levels were intrinsically increased in nasal epithelial cells from asthmatics as compared to healthy controls (2). We also showed that a small molecule activator of PKM2 enhanced PKM activity, and decreased inflammation in mice with HDM-induced allergic airways disease, in association with diminished activation of STAT3 (3). The glycolytic activity of PKM2 can be regulated by numerous post-translational modifications including phosphorylation, acetylation, methylation, and oxidation (29-33). Oxidation of PKM2 on Cys358 inhibits its tetramer formation and glycolytic activity, and promotes monomeric/dimeric PKM2 which can lead to transcriptional activation and inflammation. Moreover, PKM2 oxidation allows for diversion of glucose-6-phosphate into the pentose phosphate pathway (26), which in turn leads to synthesis of NADPH, which is critical for generating reduced GSH (34, 35). Altogether, these observations suggest that PKM2 may

be a potential target for PSSG, resulting in altered glycolytic function. Therefore, we aimed to investigate whether GSTP, by catalyzing PSSG, regulates glycolysis and plays a role in the pathogenesis of HDM-induced allergic airways disease. We also investigated to what extent GSTP-controlled PSSG chemistry contributes to IL-1-induced metabolic reprogramming and subsequent pro-inflammatory signaling in epithelial cells.

## MATERIALS & METHODS

### Mouse studies

All mice used in this study were age-matched 8-12 weeks old male and female wild-type (WT) C57Bl6/NJ mice (purchased from The Jackson Laboratory, Bar Harbor, ME) or global *Gstp*$^{-/-}$ mice (mice deficient in both *Gstp1* and *Gstp2*) bred at the University of Vermont. All animal experiments were approved by the Institutional Animal Care and Use Committee. To induce allergic airways disease, mice were sensitized intranasally (i.n) with HDM (10 µg protein GREER, Lenoir, NC; Lot 290903, endotoxin level 927.5 units/vial, protein 6.4 mg/vial, Der p 1 level 287.96 mcg/vial) in week 1 (day 1), re-sensitized in week 2 (day 8) followed by 5 consecutive challenges (i.n.) in week 3 (day 15-19). HDM was dissolved in saline, which was therefore used as a vehicle control. Mice were euthanized at day 20, 24 hours after the final HDM instillation.

In separate experiments, WT and *Gstp*$^{-/-}$ mice received an intranasal administration of 1 µg of IL-1β (R&D Systems). Control groups received 0.1% Bovine Serum Albumin (BSA) in Phosphate Buffered Saline (PBS), which served as the solvent for IL-1β. Mice were harvested 6 or 24 hours after IL-1β administration.

### Measurement of airway hyperresponsiveness

To assess airway hyper responsiveness, mice were anesthetized with 10% pentobarbital in water, mechanically ventilated at 200 breaths per minute, and exposed to incremental doses of aerosolized methacholine (0, 12.5, 25, 50 and 100 mg/mL) in saline. Respiratory mechanics were evaluated as previously described (2, 36). For each mouse, all measurements were averaged at each methacholine dose and the Newtonian resistance (Rn), tissue resistance (G) and elastance (H) were quantified as previously described (36).

### Tissue harvest - Bronchoalveolar lavage (BAL) fluid processing and fixation

Mice were euthanized, and bronchoalveolar lavage (BAL) was performed using 1 ml PBS. Total cells were counted manually using Crystal Violet solution and a hemocytometer. The remaining BAL was collected and centrifuged at 500x*g* for 5 minutes at 4°C and the supernatant was flash frozen in liquid nitrogen and stored at -80°C for further analysis. Cell

differentials were determined as previously described (3). Right lung lobes were frozen for detection of protein and mRNA analyses, and left lobes were fixed in 4% paraformaldehyde in PBS, followed by paraffin embedding and sectioned for immunohistochemical analyses.

**Cell experiments**

Primary mouse tracheal epithelial (MTE) cells were isolated from C57BL6/NJ (WT) or *Gstp*$^{-/-}$ mice and cultured as previously described (37). MTE cells were grown to confluency on 6- or 12-wells transwell inserts (Corning, Corning, NY). Cells were starved overnight in plain DMEM/F12 phenol red free media, supplemented with penicillin (50U/mL)-streptomycin (50 μg/mL (P/S), and treated with 1 ng/ml of IL-1β (R&D Systems) at indicated timepoints.

To examine the effect of *Gstp* inhibition, WT MTE cell were pre-treated with 50 μM of TLK199 (Sigma-Aldrich) for 1 hour prior to stimulation with 1 ng/ml of IL-1β (R&D Systems) and re-treated with TLK199 8 hours post IL-1β treatment. TLK199 was dissolved in DMSO and IL-1β in 0.1% BSA in PBS. MTE cells were treated at the apical and basolateral side in all experiments. Medium was collected to assess pro-inflammatory cytokine and lactate levels, and cells were harvested for protein and biochemical analyses.

**Western blotting and antibodies**

Cell and tissue lysates were prepared in RIPA buffer containing protease and phosphatase inhibitors (Thermo Fisher Scientific, MA, USA). Protein concentrations were determined with the Bio-Rad DC Protein Assay kit (Bio-Rad, Hercules, CA). For western blot analysis, 20 μg protein was used and resolved using reducing sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, then transferred to polyvinylidene difluoride (PVDF) membranes followed by incubation with primary antibodies. PKM2 (#4053), LDHA (#2012), Hexokinase 2 (HK2) (#2867), inhibitory kappa B kinase epsilon (IKKε) (#2905), STAT3 (#4368), phospho-STAT3 (Tyr705) (#4074), and TANK-binding Kinase 1 (TBK1) (#3504) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). The GSTP antibody was purchased from MBL International (MBL312), and the β-actin antibody from Sigma-Aldrich. Subsequently, membranes were incubated with peroxidase-conjugated secondary antibodies and visualized using chemiluminescence (Pierce, Rockford, IL, USA).

**Immunoprecipitation**

To assess the interaction between GSTP and PKM2, MTE cells were pre-treated with 50 µM of TLK199 for 1 hour followed by treatment with IL-1β for 2 hours. Lysates were prepared in buffer containing 20 mM Tris, pH 7.4, 150 mM NaCl, 10% glycerol, and 0.5% NP-40 with protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, MA, USA). 300 µg of protein was used for immunoprecipitation using 1 µg/µl anti-GSTP antibody with recombinant protein A agarose beads (Invitrogen), and PKM2 was detected by SDS-PAGE and probing of the western blots.

For detection of protein S-glutathionylation, lung tissues were homogenized in buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, 0.25% SDS, 0.5% CHAPS, 1% NP-40, 20 mM N-ethylmaleimide (NEM), and protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, MA, USA). Samples were immunoprecipitated using 1 µg/µl anti-glutathione antibody (Virogen, MA, USA) and protein G agarose beads overnight at 4°C. Precipitated proteins were separated using polyacrylamide gel electrophoresis and subsequent immunoblotting with PKM2 antibody.

**Histology and immunohistochemistry**

Lung tissue sections were deparaffinized and airway mucus was assessed via Periodic acid Schiff (PAS) stain, and Masson's trichrome stain was used to analyze collagen deposition. The intensity of the staining was evaluated by scoring of slides by two independent investigators blinded to the identity of the samples.

For the GSTP immunofluorescence, lung tissue sections were deparaffinized followed by antigen retrieval for 40 min at 95°C in 10 mM Sodium Citrate buffer (pH 6.0) with 0.1% Trixon-X100 in PBS and permeabilization in 1x PBS with 0.1% Triton-X100 and 1% SDS for 20 min. Slides were blocked and incubated with primary antibody against GSTP (#MBL312, MBL International) overnight at 4°C. After three washes, slides were incubated with 10 µg/ml goat anti rabbit Alexa Fluor 647 secondary antibody (Thermo Fisher Scientific, MA, USA) for 1 hour in the dark. DAPI was applied to stain nuclei. Lastly, the slides were mounted with AquaPoly mount. In between each step, slides were washed in 0.05%

Tween20 in PBS. HDM-inflamed tissues wherein primary antibody was omitted were used as a negative control.

**Enzyme-Linked Immunosorbent Assays (ELISAs)**

In cell culture supernatants, or in lung tissue lysates containing equal protein concentrations (prepared in 0.5% Triton X-100 in PBS), IL-1β, IL-33, CCL20, CXCL1, TSLP, and GM-CSF were detected by enzyme-linked immunosorbent assay (ELISA, Duoset kits, R&D Systems, MN, USA). Muc5ac levels in BALF were analyzed using a Muc5ac ELISA kit according to the manufacturer's instructions (My BioSource, CA, USA).

**Lactate assay**

Equal volumes of lung tissue lysates normalized for protein content, BALF, or cell culture medium were used in 10 kDa Amicon Ultra centrifugal filters (MilliporeSigma, USA), to deproteinize samples by centrifugation for a minimum of 1 hour at 12000x*g* at 4°C. Lactate levels were measured in these deproteinized lung tissue lysates, BAL, or cell culture medium using a lactate assay kit (Eton BioScience, CA, USA) according to manufacturer's instructions. Data from lung tissues was expressed as micromole per μg of protein.

**PKM activity assay**

PKM activity was assessed using a kit according to the manufacturer's protocol (BioVision, CA, USA).. The relative fluorescence units (RFU), which displays the rate of pyruvate yield, were expressed as RFU per minute per μg of protein.

**Cell viability assay**

To measure cell viability of MTE cells, a Calcein AM assay kit (Cayman Chemicals, MI, USA) was used according to manufacturer's instructions. Cell survival was expressed as percentage of survival compared to untreated control cultures.

**RT-qPCR**

Total RNA was extracted from lung tissue using the RNeasy mini kit (Qiagen, Valencia, CA) following the manufacturer's instructions. RNA was reverse transcribed to cDNA using the miScript Reverse Transcription kit (Bio-Rad, CA, USA) according to the manufacturer's instructions. qPCR was performed using primers pairs (see supplemental table I) and SYBR

Green Universal Taq Mastermix (Bio-Rad, CA, USA). PCR data were analyzed using the $\Delta\Delta C_T$ method and the expression levels were normalized to the house-keeping gene *Ppia* (known as cyclophilin).

**Computer-simulated docking studies**

Protein-Protein docking was performed with the software program Megadock v4.1.3 (38) to identify potential interfaces between GSTP and PKM2 with GSTP in the dimer form (PDB ID: 1AQW), and PKM2 in either the dimer or the tetramer form (PDB ID: 1T5A). Next, the top 5 complex models were selected and simulated in solution with molecular dynamics (MD) for 15 ns. These models were solvated with SPC water models in cubic simulation boxes (15 nm x 15 nm x 15 nm with periodic boundary condition) and prepared by System Builder in the Maestro program (2019-4 release (39)). Moreover, parameters from the OPLS3e force field were used (40). Each construct went through minimization, equilibration, and 15 ns NPT simulations (300K, 1 atm). In particular, an extended simulation was carried out with a model of GSTP-PKM2 tetramer for 150 ns to identify potential cysteine residues in PKM2 in the proximity of GSH from GSTP. Systems that have been tested are shown in supplemental table 2.

**Statistical analysis**

All cell experiments were performed at least three times with n=3 per group. Significant differences between groups were evaluated by two-way ANOVA with a Tukey post-hoc test for multiple comparison using GraphPad Prism software 8.0 (GraphPad, Inc., La Jolla, CA). Data are shown as means $\pm$ S.E.M. Values were considered statistically significant when $P < 0.05$.

## RESULTS

**GSTP expression in the airway epithelium of HDM-induced allergic airways disease mice**

To investigate the role of GSTP in the pathogenesis of HDM-induced allergic airways disease, C57Bl6/NJ WT and *Gstp*$^{-/-}$ mice were sensitized and challenged with HDM (Figure 1A). First, we assessed whether HDM changed protein and mRNA expression of GSTP/*Gstp* (Figure 1B, 1C and 1D). No significant differences in GSTP protein expression were observed between lungs from saline and HDM-treated WT mice (Figure 1B, C). GSTP has been reported to be highly expressed in the lung epithelium (9), and indeed, immunofluorescence staining confirmed that GSTP was mainly expressed in the airway epithelium in saline- and HDM-exposed lung tissues (Figure 1C). In contrast to protein levels, *Gstp* mRNA expression decreased in WT mice subjected to HDM compared to saline controls (Figure 1D). We next examined whether compensatory changes in the expression of other *Gst* isoforms occurred in settings of *Gstp* deficiency. No differences in mRNA expression of other *Gst* genes occurred in the lungs of WT or *Gstp*$^{-/-}$ mice compared with their respective exposure groups, with the exception of a decrease in the mRNA levels of *Gst-theta* (*Gstt)* in HDM-exposed *Gstp*$^{-/-}$ mice compared to HDM-exposed WT mice (Figure 1D). Similar to *Gstp*, mRNA expression levels of *Gst-mu* (*Gstm*) and *Gstt* were also decreased in HDM-exposed mouse lungs compared to saline controls, while *Gst-omega* (*Gsto*) was unaffected. We furthermore examined the mRNA expression of glutaredoxins, (*Glrx's)* enzymes involved in deglutathionylation, as well as thioredoxin (*Txn*), a major reducing enzyme of protein disulfides by cysteine thiol-disulfide exchange. Increases in glutaredoxin 1 (*Glrx*) were observed after HDM exposure in both WT and *Gstp*$^{-/-}$ mice, whereas *Glrx3* was only decreased in HDM-exposed lungs in WT mice, and *Glrx2* and *Txn* mRNAs were decreased in HDM-exposed lungs from *Gstp*$^{-/-}$ mice compared to HDM-exposed lungs from WT mice (Figure 1E).

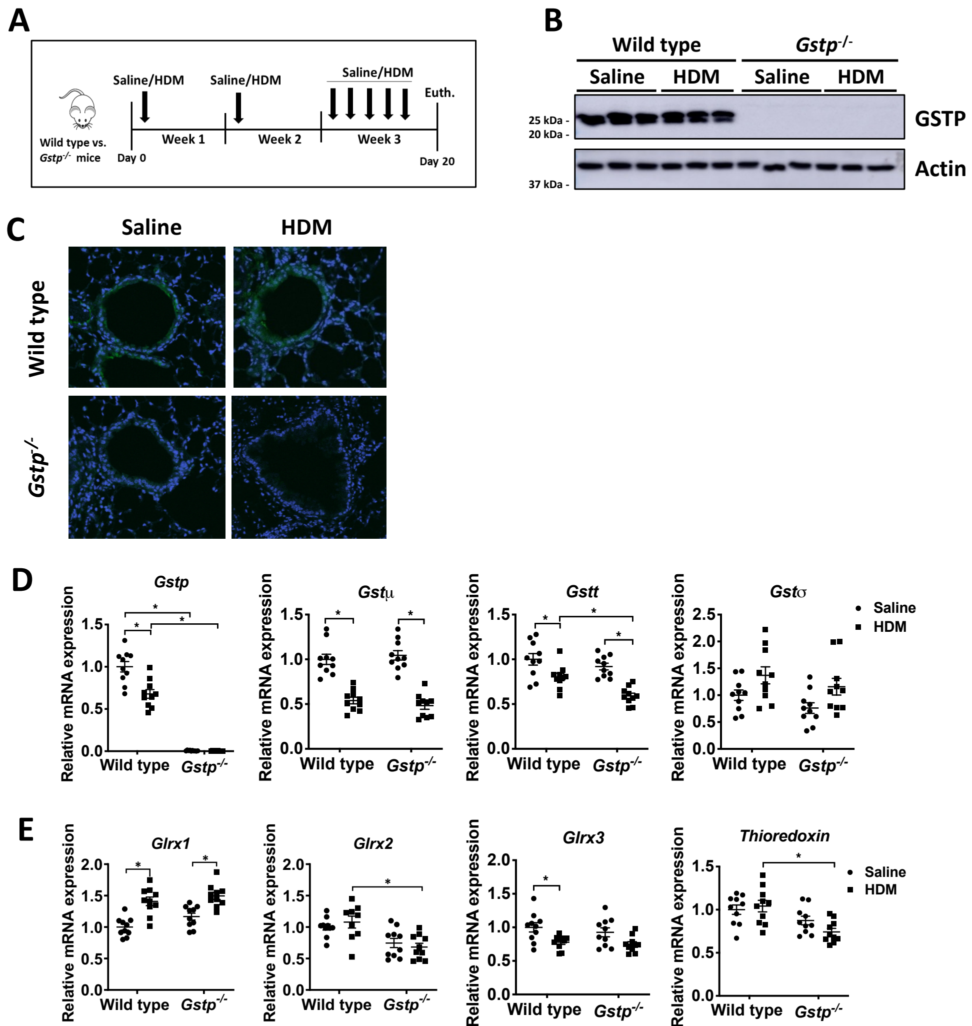


**Figure 1: GSTP expression in the airway epithelium of mice exposed to HDM. A,** Schematic depicting the exposure regimen. Wild-type and *Gstp*$^{-/-}$ mice were sensitized twice with 10 μg of HDM or saline on days 1, and 8, followed by 5 HDM challenges on days 15-19 and euthanized 24 hours after the final HDM challenge. **B,** Representative western blots for total GSTP levels. β-actin; loading control. **C,** Assessment of GSTP by immunofluorescent staining in lung tissues. Green = GSTP; Blue = Dapi (nuclei). **D,** mRNA expression of *Gstp, Gstt, Gsto, Gstμ*, and **E,** *Glrx1, Glrx2, Glrx3, Txn* normalized to *Ppia*. n=10 per group. *p < 0.05 analyzed by two-way ANOVA.

**Genetic ablation of *Gstp* attenuates mucus metaplasia, subepithelial collagen, markers of airway remodeling, and airway hyperresponsiveness in mice with HDM-induced allergic airway disease.**

Next, we assessed if genetic ablation of *Gstp* had an impact on airway remodeling and airway hyperresponsiveness. *Gstp* deficiency reduced HDM-mediated increases in mucus metaplasia, MUC5AC levels in the BALF, and subepithelial collagen deposition, as well as attenuated mRNA expression of markers of airway remodeling, collagen 1a1 (*Col1a1),* fibronectin 1 (*Fn1*) and alpha smooth muscle actin (*Acta2)* compared to WT counterparts (Figure 2A-D). Moreover, mice sensitized with HDM displayed increased airway resistance (Rn), tissue elastance (G), and tissue resistance (H) compared to saline control mice (Figure 2E). Interestingly, HDM treated *Gstp*$^{-/-}$ mice displayed significantly less tissue elastance (G) and resistance (H) compared to WT mice exposed to HDM, while the absence of *Gstp* did not affect central airway resistance (Rn) (Figure 2E).

We next assessed the magnitude of airway inflammation in WT and *Gstp*$^{-/-}$ mice exposed to HDM by total and differential cell counts in the BALF. HDM-exposed WT and *Gstp*$^{-/-}$ mice showed similar increases in total cell counts compared to their saline controls (Figure 2F), reflected mainly by increases in eosinophils (Figure 2G). Together, these results show that GSTP promotes mucus metaplasia, subepithelial collagen, and airway hyperresponsiveness, but has no major impact on inflammation in HDM-induced allergic airways disease.

**Genetic ablation of *Gstp* attenuates HDM-mediated lactate secretion**

We recently showed that glycolytic reprogramming is an important feature of HDM-induced allergic airways disease that contributes to airway remodeling and AHR (2). As ablation of *Gstp* diminished remodeling and AHR, we next assessed if GSTP absence also dampened glycolytic reprogramming. Results in Figure 2 indicate that absence of *Gstp* attenuated the HDM-mediated increases in lactate in BALF (Figure 3A), but not in lung tissue (Figure 3B). In agreement with previous results (2), HDM increased protein expression of main glycolysis enzymes including PKM2, HK2, LDHA, as well as IKKε, and TBK1, kinases involved in HDM-induced glycolytic reprogramming (Figure 3C). However, no

differences in expression of these proteins in lung tissues were found between HDM-exposed WT and *Gstp*$^{-/-}$ mice (Figure 3C).

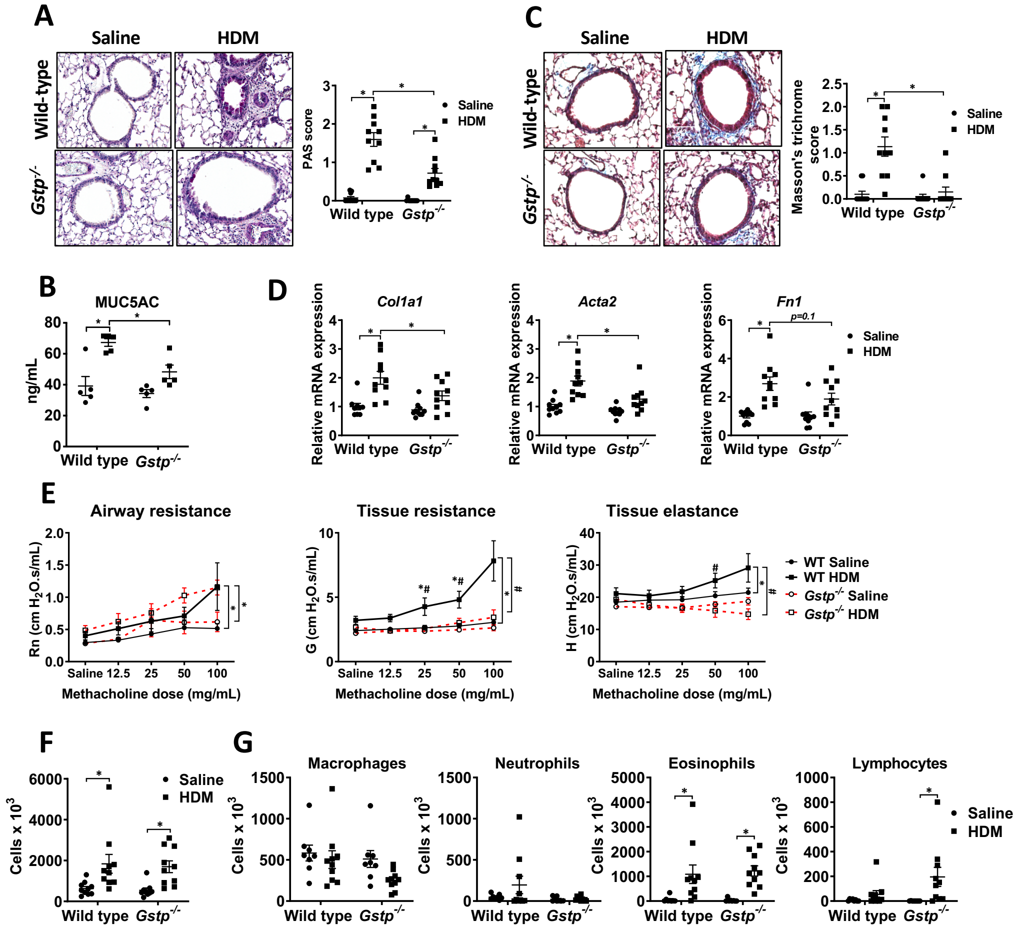


**Figure 2: Genetic ablation of *Gstp* attenuates mucus metaplasia, subepithelial collagen, markers of airway remodeling, and airway hyperresponsiveness in mice with HDM-induced allergic airway disease. A,** Assessment and quantification of mucus metaplasia by PAS staining intensity. **B,** MUC5AC protein expression in BAL fluid. n=5 per group. **C,** Assessment and quantification of collagen deposition by Masson's trichrome staining. **D,** mRNA expression of *Col1a1, Fn1,* and *Acta2* (α-smooth muscle actin) normalized to *Ppia.* **E,** Assessment of airway hyperresponsiveness using a Flexivent small animal ventilator. * respective saline vs HDM group ; # WT HDM vs *Gstp*$^{-/-}$ HDM. n=10 per group. **F,** Total, and **G,** Differential cell counts in BAL fluid. n=8-10 per group unless otherwise noted. */# $p < 0.05$ analyzed by two-way ANOVA.

IL-1ß is a major driver of glycolysis and increases in lactate levels in the BAL were previously linked to increases in IL-1ß levels and other pro-inflammatory cytokines (2). However, IL-1ß levels slightly increased in *Gstp*$^{-/-}$ mice exposed to HDM compared to WT mice (Figure 3D). Furthermore, *Gstp* ablation did not affect levels of CCL20 or IL-33 in lung tissue homogenates in response to HDM (Figure 3D).

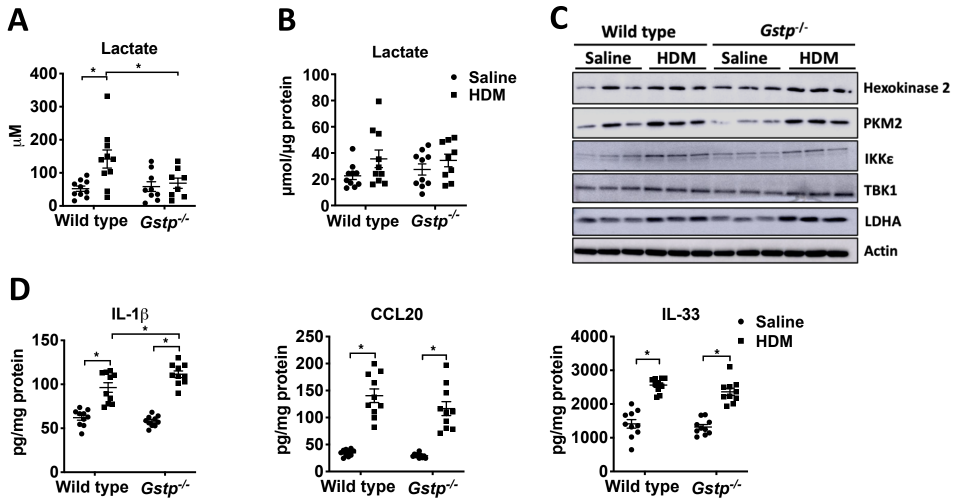


**Figure 3: Genetic ablation of *Gstp* attenuates HDM-mediated lactate secretion.** Measurements of lactate levels in **A,** BAL fluid and **B,** lung tissue. **C,** Representative western blot analyses for Hexokinase 2, PKM2, LDHA, TBK1, IKKε. β-actin is used as the loading control. **D,** Measurements of IL-1β, CCL20, and IL-33 in lung tissue homogenates by ELISA. n=9-10 per group. *$p < 0.05$ analyzed by two-way ANOVA.

## Absence of *Gstp* results in attenuated release of pro-inflammatory cytokines and decreases airway neutrophilia following intranasal administration of IL-1ß

Results in Figure 3 showed decreased lactate levels in the BALF in HDM-exposed *Gstp*$^{-/-}$ mice compared to WT mice, while ablation of *Gstp* did not attenuate IL-1ß levels in lung tissues. These results suggest that the attenuating effect of *Gstp* ablation on airway lactate may occur independently or downstream of IL-1ß signaling. To further examine this, we administered IL-1β intranasally to WT or *Gstp*$^{-/-}$ mice and assessed lactate or cytokines in BALF 6 or 24 h later (Figure 4A). In WT mice, GSTP protein expression was slightly decreased in lungs from IL-1ß-treated vs control mice after 6h (Figure 4B). The IL-1β-induced cell

counts were attenuated (albeit not-significantly) in *Gstp* deficient mice compared to WT counterparts, due to a significant decrease in neutrophil counts 24 h after intranasal administration, while macrophages, eosinophils, and lymphocytes were unaffected (Figure 4C, D). Moreover, the IL-1β-induced release of pro-inflammatory cytokines TSLP, GM-CSF, and CXCL1 were all markedly attenuated in *Gstp*$^{-/-}$ mice compared to WT mice 6 h post IL-1β instillation, while increases in CCL20 were not affected (Figure 4E). Lastly, ablation of *Gstp* also abrogated the IL-1β-mediated increases in lactate in the BALF (Figure 4F). Collectively, these results demonstrate that GSTP is a critical mediator that promotes IL-1β-induced pro-inflammatory signals in the lung as well as IL-1β-mediated lactate secretion.

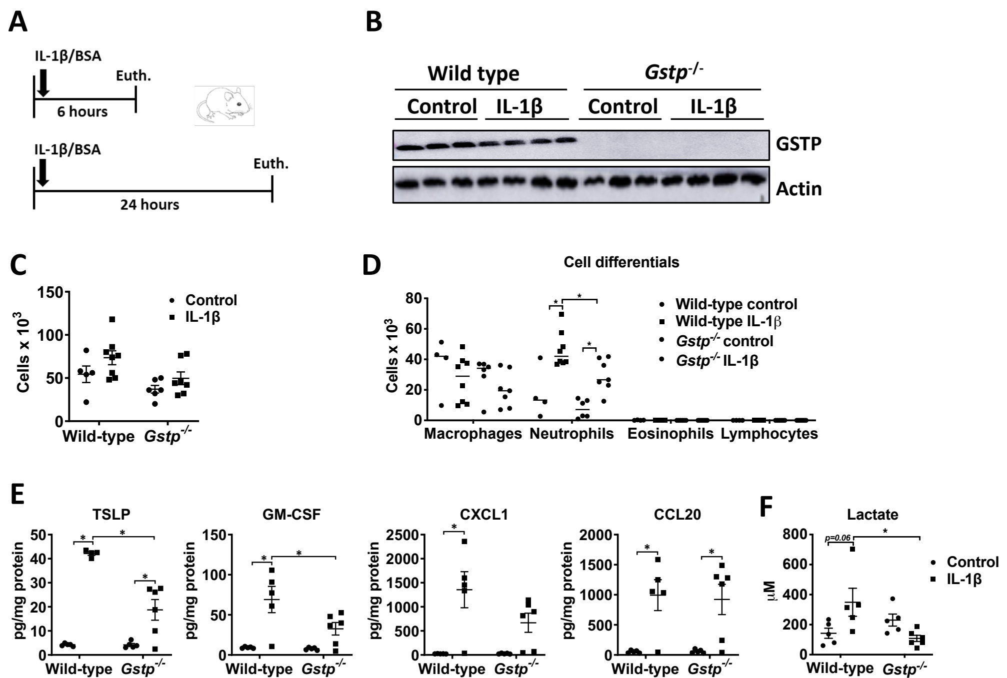


**Figure 4: *Gstp* absence attenuates the release of pro-inflammatory cytokines and decreases airway neutrophilia following intranasal administration of IL-1β. A,** Schematic depicting the intranasal administration of 1 μg of IL-1β for either 6 or 24 hours. The total cell count and cell differentials in the BAL fluid reflect 24 hours post IL-1β treatment, the other results shown are obtained 6 hours post IL-1β administration. **B,** Representative western blots for total GSTP levels. β-actin; loading control. **C,** Total, and **D,** Differential cell counts in BAL fluid. **E,** Levels of the pro-inflammatory cytokines TSLP, GM-CSF, CXCL1 and CCL20 in lung tissue homogenates by ELISA. **F,** Measurements of lactate levels in BAL fluid. n=4-8 per group. * $p < 0.05$ analyzed by two-way ANOVA.

## Ablation or TLK199-mediated inhibition of *Gstp* attenuates IL-1β-induced lactate and pro-inflammatory mediators in primary MTE cells

To examine whether GSTP also promotes IL-1(β)-induced lactate and proinflammatory mediator release from airway epithelial cells, WT and *Gstp*$^{-/-}$ MTE cells were cultured and treated with 1 ng/mL IL-1β for time periods ranging from 1 hour to 24 hours (Supplemental Figure 1). IL-1β increased lactate levels and pro-inflammatory cytokine secretion in a time-dependent manner, with maximal responses observed after 24 h (Supplemental Figure 1). *Gstp* deficiency attenuated the IL-1β-induced lactate secretion, as well as the release of TSLP and GM-CSF, while CCL20, and CXCL1 were unaffected (Figure 5A, B). Similar results were obtained when GSTP was pharmacologically inhibited with the selective GSTP inhibitor TLK199, prior to IL-1β exposure (Figure 5C, D). Pre-treatment of MTE cells with TLK199 did not induce cell death, demonstrating that the results described were not due to a loss of cell survival (Supplemental Figure 1).

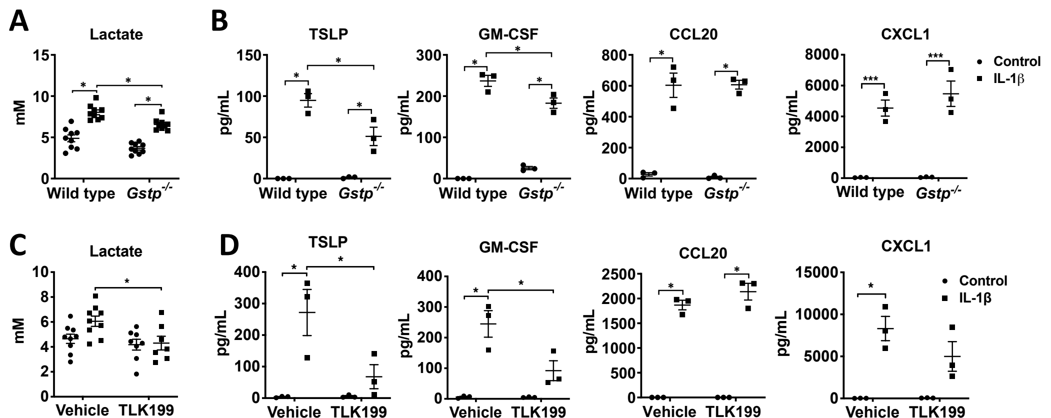


**Figure 5: Ablation or inhibition of *Gstp,* by TLK199, attenuates IL-1β-induced lactate and pro-inflammatory responses in primary MTE cells. A,** Measurements of lactate levels and **B,** levels of pro-inflammatory cytokine mediators TSLP, GM-CSF, CXCL1, and CCL20 in cell culture supernatants of wild-type and *Gstp*$^{-/-}$ MTE cells**. C,** Wild-type MTE cells were pre-treated with 50 μM TLK199 for 1 hour followed by stimulation of 1 ng/mL IL-1β for 24 hours and re-treatment with TLK199. Lactate levels, and **D,** levels of pro-inflammatory cytokines in cull culture supernatants. n=3-9 per group. *p < 0.05 analyzed by two-way ANOVA.

**GSTP-linked S-glutathionylaton of PKM2 attenuates its activity as a glycolysis enzyme**

As stated earlier, GSTP catalyzes the forward PSSG reaction, by conjugating GSH to selective proteins, thereby changing their structure and function (6). Our laboratory has shown that total PSSG was increased in lungs of mice exposed to HDM (18). We therefore investigated whether GSTP contributes to the increases in PSSG in HDM-induced allergic airways disease. As expected, HDM led to increases in PSSG in WT mice. In contrast, PSSG was attenuated in HDM-exposed lungs from *Gstp*$^{-/-}$ mice compared to the respective WT group (Figure 6A). Based upon our prior findings showing that small molecule-mediated activation PKM2 dampens allergic airway inflammation in mice (3) and previous observations that PKM2 contains reactive cysteines that upon oxidation cause a decrease in its activity as a glycolysis enzyme (32), we next addressed whether PKM2 could be S-glutathionylated in mice exposed to HDM. Immunoprecipitation of S-glutathionylated proteins followed by western blotting of PKM2 showed increases in S-glutathionylation of PKM2 (PKM2-SSG) in HDM-exposed mouse lungs (Figure 6B). Direct exposure of recombinant PKM2 to oxidized GSH (GSSG), a condition that induces PSSG, was sufficient to cause a decrease of PKM2 enzymatic activity (Figure 6C). To examine whether GSTP can directly interact with PKM2, protein-protein docking was performed with crystal structures of PKM2 and GSTP, and the complex stability was confirmed by conventional molecular dynamics (MD) simulations (Figure 6D, Supplemental Figure 2). Protein-protein docking studies and binding affinity calculations predict that both dimeric and tetrameric PKM2 can interact with GSTP, with a more likely interactions between GSTP with tetrameric PKM2 as compared to dimeric PKM2, due to the larger interface and more contacts (Supplemental Figure 2). To gain a better understanding of the most redox active Cys on PKM2, we carried out 150 ns MD simulations. The MD simulations of GSTP-PKM2 tetramer suggests that Cys423 and Cys424 can be redox active for GSH transfer (Figure 6E) as they are in the closest distance, 15.3 and 14.1 Å respectively, compared to the other Cys residues of PKM2. These predictions were corroborated by co-immunoprecipitation of GSTP and PKM2 from lung tissue, which reveal increased interaction between GSTP and PKM2 in IL-1ß-exposed mouse lungs (6 hours), as well as in IL-1ß-treated MTE cells compared to the control groups (Figure 6F, G). Interestingly, the GSTP inhibitor TLK199 tended to decrease the interaction between GSTP

and PKM2 (Figure 6G). Altogether, these data suggest a model wherein GSTP binds to PKM2 resulting in its S-glutathionylation, thereby decreasing its activity.

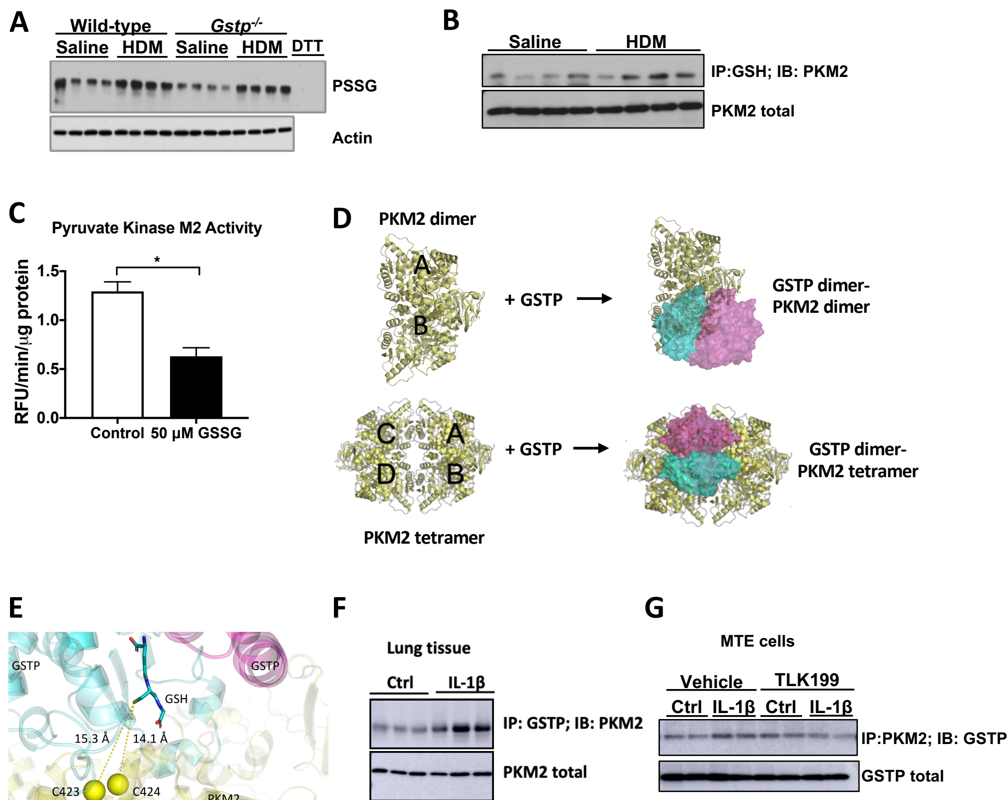


**Figure 6: GSTP-linked S-glutathionylation of Pyruvate Kinase M2 attenuates its glycolysis activity. A,** Representative western blot of total PSSG in lung tissue from HDM-exposed WT and *Gstp*$^{-/-}$ mice. **B,** Representative western blot of PKM2-SSG in saline vs HDM-exposed mouse lungs. **C,** Pyruvate kinase enzymatic activity assay of 10 ng recombinant PKM2 incubated with 50 μM oxidized glutathione (GSSG). **D,** Computer simulated docking analysis models of the most likely formed and stable interaction between dimeric or tetrameric PKM2 with GSTP. Available crystal structures: PKM2 PDB ID: 1T5A; GSTP dimer: 1AQW. All predicted models can be found in the supplemental information. **E,** GSTP-PKM2 tetramer model showing that GSH (stick model) may potentially be transferred to Cys423 or Cys424 (alpha carbon shown as yellow spheres) based on the proximity (16.7 Å from the S atom in GSH to the alpha carbon in C424). **F,** Representative western blots of GSTP-PKM2 interaction via co-immunoprecipitation and total PKM2 levels in IL-1β-treated mouse lung tissues. **G,** Representative western blot of PKM2-GSTP blots (IP: PKM2; IB; GSTP) and total PKM2 levels in wild type MTE cells pre-treated with 50 μM TLK199 for 1 hour followed by stimulation with 1 ng/ml IL-1β for 2 hours. *p < 0.05 analyzed by two-way ANOVA.

***Gstp*** **ablation or inhibition reduces phosphorylation of STAT3**

The monomer/dimer form of PKM2 that is inactive as a glycolysis kinase converting PEP to pyruvate has alternative functions, for example by acting as a transcriptional activator. It has been described that nuclear PKM2 is able to phosphorylate STAT3, thereby promoting inflammation (27, 28). We recently showed that STAT3 contributes to the IL-1ß-induced inflammatory response promoted by PKM2 (3). We next addressed whether GSTP indirectly affects phosphorylation of STAT3. Consistent with prior data (3), HDM and IL-1β exposures increased phosphorylation of STAT3 in lung tissue as well as in MTE cells (Supplemental Figure 3). Ablation of *Gstp* almost completely attenuated the HDM- (Supplemental Figure 3A) or IL-1β-induced (Supplemental Figure 3B) phosphorylation of STAT3 in the lungs of mice. Inhibition of GSTP with TLK199 also decreased the IL-1β-induced phosphorylation of STAT3 in MTE cells (Supplemental Figure 3C). Collectively, these results show that STAT3 is one of the downstream targets that promotes airway inflammation following GSTP-mediated glutathionylation.

## DISCUSSION

Enhanced glycolysis is a process that accompanies allergen-induced allergic airways disease and promotes airway remodeling. Changes in the redox environment have been shown to promote glycolysis and to accompany asthma (18, 41, 42). In the present study we point to a link between glycolysis and redox changes by demonstrating that GSTP, an enzyme that promotes S-glutathionylation, contributes to HDM-induced airway remodeling, and AHR, in part by promoting glycolytic reprogramming. Specifically we show herein that GSTP contributes to IL-1-induced glycolysis and pro-inflammatory signaling in epithelial cells and that GSTP interacts with PKM2, leading to its S-glutathionylation and diminished glycolytic activity. Together with our previous observations showing that activation of the glycolytic function of PKM2 attenuates HDM-induced allergic airway disease (3), these finding point to a GSTP-PKM2 redox axis in the pathogenesis of HDM-induced allergic airways disease.

The exact mechanism whereby S-glutathionylation leads to inactivation of PKM2 and the cysteine target(s) in PKM2 will require further study. As described before, PKM1 is constitutively active in its tetramer form, while PKM2 can also adopt monomer and dimer forms. PKM2 contains 10 cysteines (Cys152, Cys165, Cys326, Cys358, Cys316, Cys423, Cys424, Cys31, Cys474, and Cys49), of which 9 are conserved between PKM2 and PKM1 (43). Cys424 is the only cysteine unique to PKM2, which is of interest as modeling studies indicate that Cys424 in PKM2 is the most likely cysteine to be redox active for GSH transfer from GSTP to PKM2 (Figure 6E). The protein-protein interaction modeling presented herein also indicates that GSTP is more likely to bind tetrameric PKM2 than dimeric PKM2. These results are of interest as cysteine oxidation of PKM2 is thought to dictate its structure and activity. Notably, oxidation of Cys358 of PKM2 has been implicated in the dissociation of the tetramer form to the less glycolytic active dimer form in response to increased intracellular concentrations of ROS (32). In support of this, a serine mutant of Cys358 was shown to stabilize the tetramer form of PKM2 (43). Recently, others have also shown that replacement of Cys424 with Leucine 424 protected against the inhibitory effects on PKM2 by oxidative stress, and that Cys424 is the cysteine that determines the different catalytic and modulatory properties between PKM1 and PKM2 (44). Cysteine residues can also be

modified by S-nitrosation, involving the addition of an NO moiety to a reactive protein cysteine. PKM1 and PKM2 can both be S-nitrosated, although PKM2 to a greater extent than PKM1 (43). Cys326 seems to be a critical target of S-nitrosation in PKM2, and Cys326 oxidation by either a sulfenic acid or nitroso group was shown to interfere the (re)formation of the tetramer. Cys326 is located at the A-A interface, the large interface on which the PKM2 monomers bind, of PKM2 and is therefore buried in the PKM2 tetramer, while in the PKM2 monomer this cysteine is solvent-exposed and accessible to oxidizing agents. The cysteines of PKM1 are protected in a stable tetramer, and therefore less exposed to oxidizing conditions. As our modeling studies indicate that GSTP will more favorably bind to tetrameric PKM2 than to dimeric PKM2, and that PSSG decreases the glycolytic activity of PKM2, this could imply that S-glutathionylation of Cys424 by GSTP may dissociate the tetramer form instead of hindering tetramer (re)formation. However, further computational modeling and *in vitro* assays should be performed to corroborate this.

Herein, we also investigated the extent to which GSTP contributes to IL-1ß-induced pro-inflammatory signaling in epithelial cells. Although GSTP does not appear to affect HDM-induced lung inflammation, our data shows that GSTP promotes IL-1ß-induced airway neutrophilia in mice, as well as IL-1ß-mediated expression of pro-inflammatory cytokines, especially TSLP and GM-CSF, in airways and epithelial cells. We recently demonstrated that activation of PKM2 with the small molecule activator TEPP46 elicited similar responses and attenuated HDM and IL-1ß-mediated allergic airways disease and inflammation, respectively (3). These prior results along with the present study support the scenario that GSTP-induced S-glutathionylation of PKM2 promotes allergic airways disease and IL-1ß-induced pro-inflammatory signaling. Our data also shows that deletion of *Gstp* or TLK199-mediated inhibition of GSTP decreases phosphorylation of STAT3 in mouse lungs and airway epithelial cells. As previously reported, nuclear PKM2 can phosphorylate STAT3 (27, 28), and our previous findings indicated that the ability of PKM2 to phosphorylate STAT3 contributes to its pro-inflammatory function in airway epithelial cells (3). The decrease of IL-1-induced pro-inflammatory mediators and STAT3 phosphorylation in the absence of *Gstp* or upon inhibition of its catalytic activity, are similar to the effects of activating PKM2

with TEPP46. The possibility that TEPP46 protects PKM2 from inactivation caused by GSTP-mediated S-glutathionylation awaits further study.

Of the cytosolic GST family, GSTP is the highest expressed in the airway epithelium (9). Our results demonstrate that the protein expression of GSTP in whole lung lysates, as well as specifically in the airway epithelium of mice, was not or marginally affected by HDM exposure (Figure 1B, C), while *Gstp* mRNA expression decreased in WT mice subjected to HDM compared to saline controls. The latter is in line with earlier published results showing that *Gstp* transcript levels were downregulated in lung tissues from WT mice challenged with HDM (45), as well as in an ovalbumin (OVA) model of allergic airways disease (46) and in nasal epithelial cells from asthmatic children (45). However, increases in *Gstp* mRNA expression in allergic disease models in mice have also been reported (47). Despite the lack of increases in GSTP's expression level in lung tissues, here we show that GSTP nonetheless contributes to the pathogenesis of HDM-induced allergic airways disease. Our results demonstrating that GSTP promotes mucus metaplasia, airway remodeling, and AHR is in contrast with another study demonstrating that acute injections of OVA (i.p. sensitization, followed by after 12 days, aerosol challenges with OVA for 8 days) in *Gstp*$^{-/-}$ mice on a B57BL/C background, showed enhanced AHR, eosinophilia, and goblet cell hyperplasia (46). However, these results were not found in mice on a BALB/c background with the exception of enhanced eosinophilia after acute injection. Interestingly, a recent study reported that GSTP increases the proteolytic activity of the protease Der p1 in HDM (48), providing an additional mechanism whereby GSTP contributes to HDM-induced allergic disease. The discrepancy in data on GSTP expression levels and contribution to disease from mouse studies is likely attributable to differences in the allergen challenge regimen, the allergen itself, and differences in strains of mice used. Most studies investigating the role of GSTP during asthma, utilized a global *Gstp* knock-out mouse model. As GSTP is highly expressed in the airway epithelium, specific epithelial-targeted ablation would be insightful, especially given that online database containing RNA-sequencing data from controls and asthmatics reveal that GSTP is highly expressed in different bronchial and alveolar epithelial cell subtypes (49). Therefore, to elucidate the contribution of GSTP in allergic lung diseases, is it important to consider the cell type, allergen and mouse models used.

Although our study shows that GSTP contributes to HDM-induced allergic airways disease, human samples will be required to fully understand the contribution of GSTP-controlled S-glutathionylation of PKM2 in promoting pro-inflammatory responses in epithelial cells or airways from asthmatics. In addition to the mechanism shown here, other targets for GSTP-mediated S-glutathionylation could also potentially contribute to allergic airways disease. For example, GSTP has been shown to glutathionylate AMPK and SRC, leading to decreases of their activity (50, 51). Our laboratory has also demonstrated that GSTP is able to glutathionylate a key activator of the NF-κB signaling pathway, inhibitory kappa B kinase beta (IKKβ), thereby inhibiting its function, leading to attenuated inflammation in epithelial cells (52). It is worthy of mention that IL-1ß itself is a target for S-glutathionylation which enhances its biological activity (53). Additional studies will be required to assess whether GSTP contributes to S-glutathionylation of IL-1ß. Moreover, GSTs regulate protein function following binding to client proteins. One binding partner of GSTP with potential relevance to asthma is c-Jun N-terminal kinase (JNK), which has been shown to play a role in allergen-induced inflammation and remodeling associated with bronchial hyperresponsiveness (54). Binding of GSTP to JNK results in inhibition of its kinase activity (55). Further studies to unravel the proteins that GSTP interacts with in settings of allergic inflammation, the cell types wherein this occurs, along with the targets of S-glutathionylation will provide a more complete description of the proteins and processes affected by GSTP in lungs of mice with allergic airways disease.

Altogether, the data presented herein demonstrate that GSTP promotes airway remodeling and AHR during HDM-induced allergic airways disease, in association with enhanced glycolysis. Moreover, our findings also indicate that an important target of GSTP-controlled glutathionylation is PKM2, a major regulator of glycolytic programming. Lastly, the demonstration that GSTP increases IL-1-induced pro-inflammatory signaling, combined with the role of IL-1ß in neutrophilic severe asthma (2, 56, 57) suggest that targeting the GSTP/PKM2 axis may offer potential novel treatment strategies for certain difficult to treat asthma endotypes.

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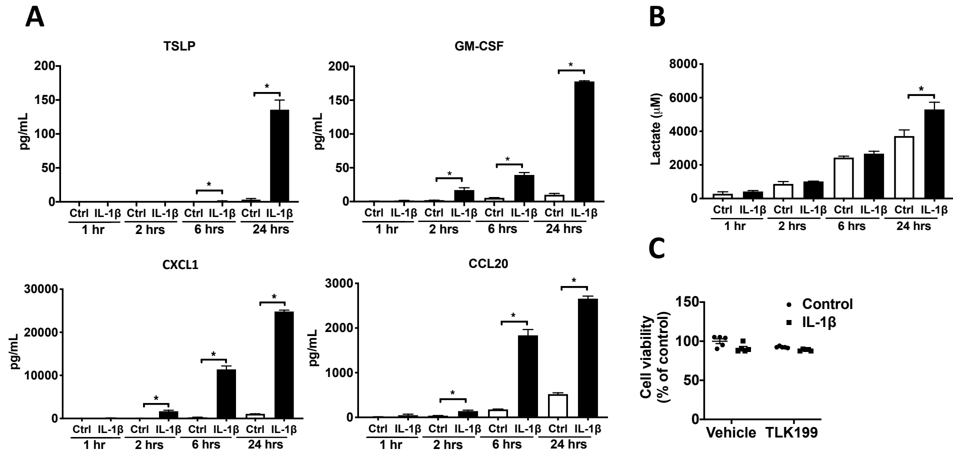
## SUPPLEMENTAL DATA

**Supplemental table 1**: primer sequences used in this study

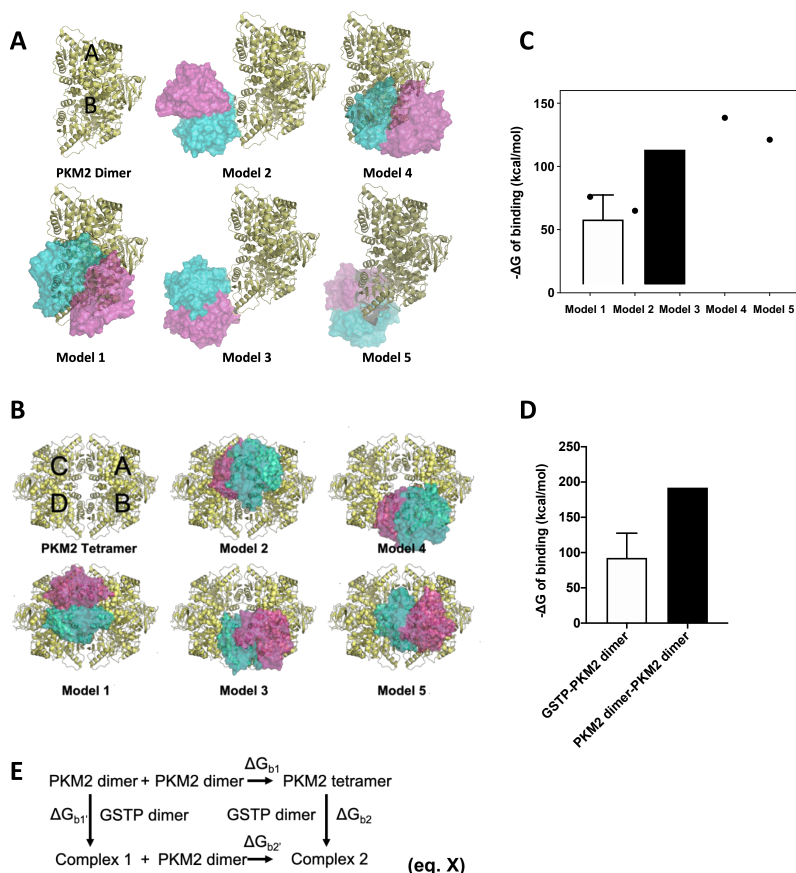
| | Forward | Reverse |
|---|---|---|
| ***Gstp*** | AGCAGGCATGCCACCATA | GCTGCCCATACAGACAAGTG |
| ***Gsto1*** | AGCTAGAGGAGGGCATGGAT | GAGCTCCAATGCTTCCAGTC |
| ***Gstt1*** | ATATCCCGTTCCAGATGCAC | CATCATGGCTGGTACCCTCT |
| ***Gstm*** | GCACAACCTGTGTGGAGAGA | GTAGCAAACCATGGCCAACT |
| ***Glrx1*** | AACAACACCAGTGCGATTCA | ATCTGCTTCAGCCGAGTCAT |
| ***Glrx2*** | GTTTCAAGATGCGCTTCACA | TGTGAAGCCTGTGAGTGTCC |
| ***Glrx3*** | AGTCGACCGGTTAGATGGTG | GTCAGCTTTTTCAGGCGAAG |
| ***Txn*** | AGCTGATCGAGAGCAAGGAA | TGATCATTTTGCAAGGTCCA |
| ***Col1a1*** | CACCCTCAAGAGCCTGAGTC | AGACGGCTGAGTAGGGAACA |
| ***Acta2*** | CGCTGTCAGGAACCCTGAGA | CGAAGCCGGCCTTACAGA |
| ***Fn*** | AATGGAAAAGGGGAATGGAC | CTCGGTTGTCCTTCTTGCTC |
| ***Ppia*** | AACTTTGCCGAAAACCACAT | GCACAGGAGGAAAGAGCATC |

**Supplemental table 2**: Systems that have been tested *In Silico*

| **Systems** | **Protein-protein docking** | **MD simulations** | **Notes** |
|---|---|---|---|
| GSTP dimer -> PKM2 dimer | PPI Score = 6.8 | 15 ns | Top five complex models were selected (Supplemental Figure 2) |
| GSTP dimer -> PKM2 tetramer | PPI Score = 5.8 | 15 ns | Top five complex models were selected (Supplemental Figure 2) |
| PKM2 dimer -> GSTP dimer-PKM2 dimer complex (model 1) | PPI Score = 11.1 | 15 ns | PKM2 tetramer can still form, even when GSTP is already bound to a PKM2 dimer |
| PKM2 dimer -> GSTP dimer-PKM2 dimer complex (model 2) | PPI Score = 7.3 | 15 ns | Likely PKM2 tetramer will form, even with the presence of GSTP bound to one PKM2 dimer |
| **Benchmarking**: | | | |
| PKM2 monomer -> PKM2 monomer | PPI Score = 32.5 | | The software program correctly predicted the monomer-monomer interface for the PKM2 dimer. High PPI score suggests a high tendency for PKM2 dimerization. |
| PKM2 dimer -> PKM2 dimer | PPI Score = 13.35 | | The software program correctly predicted the dimer-dimer interface for the PKM2 tetramer. |

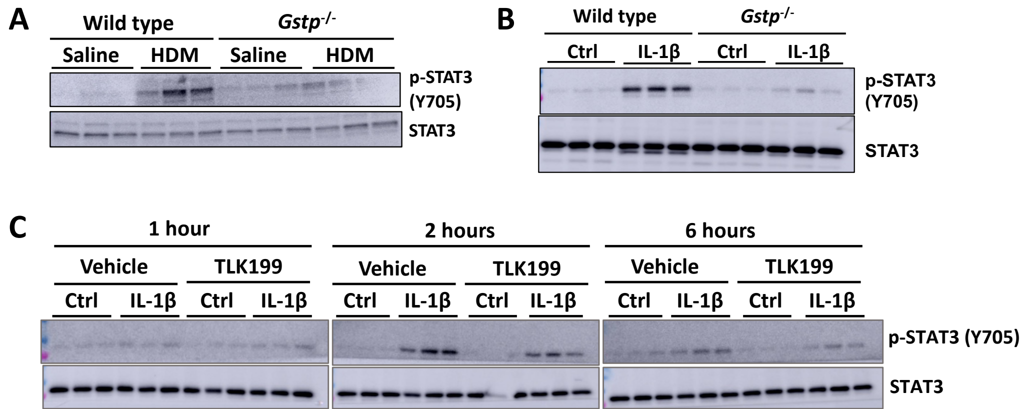



**Supplemental Figure 1: Time-course IL-1ß.** MTE cells were treated with 1 ng/mL IL-1β for the indicated time points. **A,** Pro-inflammatory cytokine mediators TSLP, GM-CSF, CXCL1, and CCL20, and **B,** Lactate levels in cell culture supernatants of MTE cells. *p < 0.05 analyzed by student's t-test for each time point. **C,** Cell survival was evaluated by Calcein AM assay in MTE cells. n=3 per group.




**Supplemental Figure 2: GSTP-PKM2 modeling. A,** The five most likely models of GSTP (dimer, cyan and magenta surface representations) in complex with PKM2 dimer (chains A and B from the tetramer model, yellow cartoon). Models 1 and 4 show GSTP not at the PKM2 dimer-dimer interface, in contrast to Models 2 and 3. Model 5 is between these two, with GSTP partially bound to the PKM2 dimer-dimer interface. In the consequent 15 ns MD simulations, model 4 (RMSD=2.9 Å) seem to be more stable than the models with GSTP binding at the PKM2 dimer-dimer interface (Model 2, RMSD=3.5 Å and Model 3, RMSD=4.4 Å; larger RMSD means likely lower stability). **B,** The five most likely models of GSTP in complex with the PKM2 tetramer. Models 1, 2, 3 and 5 show contacts between GSTP and all monomers of PKM2. **C,** ΔG of binding of PKM2 dimer with GSTP (Model 1-5 from protein-protein docking). **D,** Comparison of binding affinity of PKM2 with PKM2 dimer vs. PKM2 dimer with GSTP. PKM2 dimer presents a binding affinity of 194.5 kcal/mol ($\Delta G_{b1}$ on Eq. X, shown in **E**) with another PKM2 dimer which is approximately 100 kcal/mol higher than the average ΔG of the binding of a PKM2 dimer with GSTP (($\Delta G_{b1'}$ on Eq. X, shown in **E**). As a higher binding affinity is correlated to stronger binding, this reflects a higher tendency for the PKM2 tetramer to form before GSTP binds. The error bar and mean have been calculated from the 5 points in panel C. **E,** The binding free energy $\Delta G_b$ was estimated using the VSGB implicit solvent model in the Prime-

MMGBSA v3.0 program (58), where the conformation of complex 1 was extracted from the final snapshot of the 15-ns MD simulations. All the conformations were optimized in Prime before the free energy calculations.




**Supplemental Figure 3: *Gstp* ablation or inhibition reduces phosphorylation of STAT3. A,** Representative western blot of total and phosphorylated PKM2 and STAT3 in lung tissue lysates from wild type and *Gstp*$^{-/-}$ mice exposed to HDM. **B,** Representative western blot of total and phosphorylated STAT3 in lung tissue from wild-type and *Gstp*$^{-/-}$ mice 6 hours post IL-1β administration. **C,** Representative western blot of total and phosphorylated STAT3 in MTE cells pre-treated with TLK199 for 1 hour prior to IL-1β administration for 1, 2, or 6 hours. n=3 per group.

# 6

# General discussion

The aim of this thesis was to examine if altered cellular metabolism contributes to asthma pathology and whether these metabolic changes are regulated by redox perturbations, notably by the protein oxidation reaction protein S-glutathionylation. The most essential findings of this thesis are summarized and discussed in this chapter, as well as the limitations and implications for further research are included.

## Implications for asthma pathology

Asthma is one of the most common chronic inflammatory diseases and affects approximately 300 million people worldwide (1). Current therapies target specific symptoms, but there remains a significant population for whom these treatments are not effective (2-4). As described earlier, different endotypes, and phenotypes based on distinct molecular mechanisms are taken into account to define patients with asthma (5). In the majority of asthmatics, inflammation is driven by allergen-induced Th2 immunity and eosinophilic inflammation, however non-atopic patients can also develop a similar type of inflammation (6). Moreover, some patients show non-atopic, non-eosinophilic inflammation but display neutrophilic inflammation in association with more severe disease. Anti-inflammatory therapies targeting Th2 cytokines of the well-defined Th2-high endotype demonstrated beneficial effects, and a small number of relevant biomarkers have been identified in this endotype such as high-exhaled nitric oxide (FeNO), elevated periostin in serum, IgE levels, and eosinophil counts (7-9). Only a few biomarkers have emerged to define the Th2-low endotype including neutrophil counts and IL-17 or IL-8, but lack specificity (9). The absence of relevant biomarkers for patients with severe disease has made it difficult to identify and characterize subjects into distinct phenotypes. Therefore, a better understanding of the underlying molecular mechanisms of asthma pathophysiology will help to better classify the current endotypes and accompanying biomarkers in order to personalize treatment for severe asthma patients.

### *IL-1ß as biomarker of neutrophilic asthma*

Results in this thesis highlight that increases in the glycolysis pathway are a critical feature of the pathophysiological manifestations underlying asthma. Our results moreover demonstrate that IL-1(ß) is an important signal that drives the increased glycolytic response

that enhances allergen-induced proinflammatory responses in mice with HDM-induced allergic airways disease as well as in epithelial cells. In the introduction it has been briefly described that Th2 cells are predominately responsible for the development of asthma. However, naïve Th cells, once activated, divide and can give rise to different Th $CD4^+$ effector cells including Th type-1 (Th1), type 2 (Th2) or type 17 (Th17). Different molecular mechanisms determine the differentiation into a Th1, Th2, or Th17 cell to trigger the appropriate immune response for a specific pathogen (6). Th17 cytokines lead to pulmonary neutrophil recruitment amongst others. IL-1ß is a proinflammatory cytokine that plays an important role in immune responses and has been identified to drive Th17 polarization (10). IL-1ß is known to be important in a number of severe inflammatory diseases including cancer, type II diabetes and autoimmune diseases. Moreover, IL-1ß (in serum) is associated with promoting inflammation in patients with severe persistent allergic rhinitis (11), IL-1ß is upregulated in COPD small airway epithelial cells, and IL-1ß and IL-17 are shown to be the mediators of neutrophilic airway inflammation in (exacerbating) COPD patients (12). The IL-1 receptor has emerged as a marker of neutrophilic inflammation and airflow obstruction in sputum of patients with asthma (13), and IL-1ß itself has also been linked to severe, neutrophilic asthma, and prevention of IL-1 signaling in sensitized-challenged mouse models of asthma demonstrated decreased AHR and inflammation (14, 15).

Older asthmatics displayed higher number of neutrophils and eosinophils than the younger subjects (16). Moreover, in the older population, sputum concentrations of IL-6 and CCL20 (chemokine produced by Th17 cells) were associated with worsened asthma control and increased sputum levels of IL-1ß, IL-6 and CCL20 were associated with hospitalization. However, targeting IL-6 has not been effective in asthmatic patients so far and inhibition of IL-17 signaling with an IL-17 receptor antagonist did not improve asthma outcome (17). It appears that the role of IL-17 in asthma differs among various patient subgroups, and the precise function of IL-17 in the pathology of asthma is still elusive as some studies showed that IL-17 also has protective effects in allergic airways disease. Indeed, a clinical study showed that patients with Th2/Th17-predominant asthma displayed severe disease, and using an antagonist of the IL-1ß receptor, anakinra, inhibited the development of Th2/Th17

cells, which suggests that targeting IL-1(ß) in patients with severe asthma is more potent (18). The clinical potential of anakinra is still unknown, but it can reduce airway neutrophils in a mouse model of asthma (19). Our results show that IL-1β exerts a major role in the innate immune response in asthma. As just mentioned, IL-1β has been identified as an important neutrophil activator (20), and our data indeed demonstrate that administration of mice with IL-1β was associated with an influx of neutrophils rather than eosinophils, and show that both lactate and IL-1β levels were elevated in patients with neutrophilic asthma, but not in eosinophilic asthmatics (**Chapter 3, 4**). It has also been shown that IL-1 signaling increases glycolysis during Th17 cell differentiation (10), which is in line with our results showing that neutralization of IL-1 attenuated HDM-induced glycolysis. The underlying mechanism in other (lung) diseases associated with neutrophilic airway inflammation could therefore involve IL-1 driven glycolysis. Our results also display that IL-1β strongly increases the expression of the epithelial cell-derived cytokine TSLP in primary epithelial cells. TSLP promotes Th2 inflammation and steroid resistance, and TSLP overexpression is associated with severe airway inflammation and airway hyper responsiveness, highlighting the potential therapeutic relevance of epithelial driven allergic airways disease. Together, these results demonstrate that increased IL-1ß levels in serum or sputum is potentially useful as a non-invasive biomarker for increases in glycolysis and IL-1 associated inflammation and subsequently neutrophilic allergic disease, and should be investigated as a therapeutic target in asthma and other (inflammatory) lung diseases.

*Lactate is not just a waste product*

Our findings in **Chapter 3** show that primary nasal epithelial cells isolated from asthmatics intrinsically showed an increased expression of PKM2 as well as LDHA protein, the enzyme that catalyzes the conversion from pyruvate to lactate, and produce more lactate as compared to controls. Moreover, sputum samples of asthmatics displayed increased levels of lactate compared to healthy individuals, which correlated negatively with lung function (FEV1%). In healthy individuals, lactate is mainly metabolized in organs with a high metabolic rate such as muscle and liver. For a long time, lactate was considered a waste product of glycolysis as the result of hypoxia in contracting skeletal muscle. However, we now know that lactate is a carbon fuel source (21), and it is even suggested that during

metabolic reprogramming, the contribution of glucose to the TCA cycle is mostly through circulating lactate, which involves high LDH activity and rapid lactate transport (22). Indeed, the metabolic role of lactate (shuttling) has been recognized as fuel for tissue and tumors, and may be one of the most prominent respiratory fuels in non-small cell lung cancer (23). Lactate is actively oxidized at all times, and sequestration and oxidation of lactate to pyruvate affects the cellular redox state (by the conversion of $NAD^+$/NADH), both promoting energy flux and signaling cellular events. In a healthy lung, the net balance of lactate is almost zero (24). Similar to our results displaying increased lactate levels during asthma pathogenesis, studies have shown that during acute lung injury and sepsis, the net production of lactate in the lung increases and correlates with the severity of lung injury (25, 26). In patients with cystic fibrosis (CF), chronic airway inflammation and tissue remodeling are thought to be caused by neutrophils and in a study of CF patients with pulmonary exacerbations, an association was shown between the sputum neutrophil count and sputum lactate levels (27). A recent study moreover showed that sputum lactate levels were correlated with neutrophil accumulation/invasion in the lungs of patients with acute lower respiratory tract infection (28), and metabolic analysis of BALF from mice with OVA-induced allergic airways disease revealed increases in the energy-related metabolites including lactate compared to controls (29). Moreover, lactate has been shown as a pro-fibrotic mediator and is an important intermediate metabolite in the process of wound repair and regeneration. Previous studies have already shown that lung inflammation triggers increased lactate production (30), and our results showing that IL-1β signaling induced pro-inflammatory mediators in association with increases in lactate (and lactate transporters), demonstrates that the production of lactate is due to amplification of cytokine effects on epithelial and inflammatory cells (31). In addition, our data showed that lactate levels were significantly higher in neutrophilic asthmatics as well as in patients whose asthma was uncontrolled (**Chapter 3**). Sputum lactate levels also positively correlated with IL-1β, but negatively correlated with reduced lung function in asthmatics, which again fits with the thought that patients with neutrophilic asthma are the subgroup that often display poor controlled and more severe disease. An interesting note is that the population of asthmatics in **Chapter 3** displayed higher BMI, and the FeNO levels were not

different between asthmatics and controls, which may suggest that this population is not the typical Th2-high subgroup of asthmatics. Lactate should therefore be further evaluated as a potential marker of increased glycolysis and associated (neutrophilic) inflammation in airways of patients with lung diseases.

*PKM2 as therapeutic target*

The importance of alterations in the glycolysis pathway in allergic airways disease have been further emphasized by the results in **Chapter 4** demonstrating that activation of PKM2, the rate-limiting enzyme in the glycolysis pathway, with TEPP46 attenuates subepithelial fibrosis, airway remodeling, and allergen induced inflammation. PKM2 plays an important role in glycolytic reprogramming in activated immune cells and tumor cells and upregulation of PKM2 has been linked to a variety of chronic inflammatory diseases. Our results describe that PKM2 is upregulated in settings of asthma and promotes inflammation in lungs and in epithelial cells (**Chapter 3, 4)**.

Glycolytic reprogramming e.g. increased glucose uptake and production of lactate and the induction of metabolic enzymes including the oncoprotein PKM2 has been best described in cancer models to fulfill the metabolic demands of the tumor. As described before, PKM2 can adopt multiple isoforms, which dictate its kinase function. The activity of PKM2 is controlled at the (post)transcriptional, and metabolic level; the glycolytic activity of PKM2 is controlled by allosteric regulation and post-translation modifications, whereas metabolically inactive PKM2 can translocate to the nucleus to function as protein kinase stimulating transcription (32, 33). For instance, nuclear PKM2 has been shown to phosphorylate STAT3, thereby promoting inflammation and glycolytic reprogramming, and our results further confirm that the ability of PKM2 to phosphorylate STAT3 contributes to its proinflammatory function in epithelial cells (**Chapter 4**). Preclinical studies have shown that PKM2 limits the T cell responses against tumors (34), and modulates T inflammatory cell metabolic reprogramming contributing to inflammation. Studies targeting PKM2 with small molecular activators, such as TEPP46, to stabilize the active tetramer as well as to inhibit PKM2 activity directly have been performed which showed a reduction of tumor growth and metabolic stress. However, inhibition of PKM2 will also suppress the immune

response as immune cells require PKM2 for their activation and proliferation, and it influences the glycolytic pathway globally which may hamper the process in which PKM2 plays a major role. Our results show that TEPP46-mediated activation of PKM2 attenuated IL-1(β)-induced secretion of lactate and pro-inflammatory mediators in mouse airways and epithelial cells (**Chapter 4**). Additionally, in **Chapter 5** we describe that PKM2 can be S-glutathionylated, which inhibits its glycolytic activity. It remains unclear whether TEPP46 can protect PKM2 from inactivation caused by GSTP-mediated S-glutathionylation. As glycolytic reprogramming is critical in the pathogenesis of allergic airways disease, clinical studies should be performed to examine and develop potential therapeutic activators of PKM2 in asthma patients who display increased glycolysis in the lungs. It is important to include patients with different (carefully characterized) endotypes in such studies, as well as to closely monitor factors that can affect outcome results including diet, age, weight, sex, urbanization, etcetera.

*GSTP in allergic airways disease*

Protein-S-glutathionylation (PSSG) has been shown to be increased in a mouse model of allergic airways disease contributing to disease pathogenesis (35). As thoroughly described in **Chapter 2**, GSTP is abundantly expressed in the airway epithelium, and displays multiple functions that may impact the susceptibility to and progression of asthma pathogenesis. These functions include detoxification, scavenging of oxidants, catalyzing protein S-glutathionylation, and GSTP regulates signaling pathways by the interaction of proteins such as members of the MAPK pathway including JNK. In addition, we demonstrate in **Chapter 5** that GSTP promotes HDM-induced allergic airways disease by increasing airway remodeling and AHR in association with increases in glycolysis. GSTP was shown to interact with PKM2, which affected its glycolytic activity, and interestingly, activation of PKM2 as well as ablation of *Gstp* attenuated the IL-1ß-induced expression of inflammatory cytokines (TSLP and GM-CSF).

GSTP is overexpressed in a wide variety of tumors, and was thought to be involved in the resistance (detoxification) to several anticancer drugs. Moreover, the interaction of GSTP with JNK resulting in inhibition of apoptosis, which all led to the synthesis of a significant

number of GST(P) inhibitors (36-39). An example of a clinically relevant GSTP inhibitor is TLK199 (or Ezatiostat hydrochloride, TER199, Telintra), a small peptide GSH analogue molecule that selectively inhibits GSTP acting on MAPK signaling pathway. TLK199 undergoes esterase hydrolysis when it enters the cell, which then releases TLK117, its activated form that has anti-GSTP activity. TLK117 has binding affinity greater than GSH itself and has a 50 fold greater selectivity for GSTP than other GSTs (40). TLK199 prevents the binding of GSTP and JNK, thereby leading to activation of JNK and subsequent increased tumor cell apoptosis. Moreover, TLK199 is able to promote hematopoietic progenitor cell maturation, and inhibits myeloproliferative disease. TLK199 is therefore currently in use for treatment of myelodysplastic syndrome (41). TLK117 was shown to halt the progression of fibrosis in mice, in association with decreased levels of PSSG (42), and our results in **Chapter 5** demonstrate that TLK199 attenuated the production of IL-1-induced lactate and proinflammatory mediators in epithelial cells, which seems promising for its use in chronic lung diseases. Clinical studies utilizing a GSTP inhibitor such as TLK199 or a variant thereof would provide valuable insights into the importance of GSTP-controlled-PSSG in the pathogenesis and progression of asthma.

## Future directions

As described before, asthma pathogenesis is driven by low-grade persistent inflammation due to constant immune responses that produce mediators resulting in airway remodeling and subsequent clinical symptoms of asthma. Airway epithelial cells are critical in this process as they produce mediators to recruit and activate immune cells but they also respond to subsequent mediators produced by immune cells and other lung structural cells. We display the importance of glycolytic reprogramming in epithelial cells and demonstrate that GSTP-controlled PSSG of PKM2 promotes allergic airways disease and IL-1-induced inflammation by utilizing a global *Gstp*$^{-/-}$ mice. As GSTP is highly abundant in epithelial cells, but also expressed in immune cells (**Chapter 2**), we are unable to conclude that the attenuated effect of ablation of *Gstp* on HDM-induced airway remodeling and AHR is due to a deletion of *Gstp* in epithelial cells only. Our results in *Gstp*$^{-/-}$ mouse tracheal epithelial cells confirm the importance of epithelial cells in asthma pathogenesis, but an inducible

transgenic mouse to delete *Gstp* specifically in lung epithelial cells will help to clarify how GSTP within the epithelial cells regulates HDM-induced allergic airways disease. In addition, global knockout models of proteins may impact the ability of other functions of that specific protein and can thereby affect other signaling pathways. Moreover, although we did not see compensation of other GSTs or GLRXs in lungs of the *Gstp*$^{-/-}$ mice, other antioxidant systems could still compensate for the loss of GSTP.

We moreover show that increased glycolysis is important for the amplification of allergen-induced proinflammatory responses and show the regulatory roles of PKM2 and GSTP herein. Although lactate is an indicator for glycolytic reprogramming, metabolomic experiments by the use of carbon (13C) labeling would provide a more complete description of the metabolites affected by IL-1-induced glycolysis during allergic airways disease. Moreover, although the field of metabolomics is still progressing, the translatability of metabolomic research appears promising. Metabolomic profiling in humans is capable of distinguishing diseased patients from non-diseased patients, and may be a promising field that can result in the discovery of potential new biomarkers, and eventually therapeutics, especially when data will be integrated with other omics fields (genomics, transcriptomics, proteomics). Further mechanistic studies are also required to unravel how glycolysis regulates inflammatory responses in the epithelium.

Here, we focus on PKM2 as target for GSTP-controlled PSSG because of its function as a glycolytic enzyme, but as we discussed in **Chapter 2 and 6**, other targets are likely to contribute to allergic airways disease as well. Future studies should include a GSTP interactome in combination with a glutathionylated proteome to provide a better description of the proteins affected by GSTP during allergic airways disease. For example, a protocol that utilizes methyl metanethoisulfonate (MMTS), a small compound that reversibly blocks cysteines and other sulfhydryl groups, and the use of GLRX1 to selectively reduce proteins followed by labelling and mass spectrometry would provide further insight into the effect of oxidative modification events in the pathogenesis of allergic airways disease. Also, our modeling results identify cysteine 423, and 424 of PKM2 as likely cysteines for GSH binding. However, PKM2 contains a total of 10 cysteines, and other

cysteines, such as Cys358, have already been described to be also prone for oxidation. It is therefore important to follow up with *in vitro* and *in vivo* experiments to determine which cysteines of PKM2 are affected by protein S-glutathionylation by replacing prone cysteines with non-oxidizable amino acids and examine the importance of the cysteine oxidation on HDM-induced allergic airways disease.

Non-specific quenching of ROS by the use of (low molecular mass) antioxidant compounds has been shown unsuccessful in counteracting disease progression in clinical trials. Therefore, controlling specific signaling pathways by selective targeting offers a perspective for a future of more refined redox medicine. Studies could yield successful outcomes when focused more on selective inhibition of interaction of GSTP with target proteins inducing pathways involved in inflammation, metabolism, as well as lipid peroxidation, and cell death regulation. Studies performed in human lung samples will be required to determine the contribution of PKM2 and GSTP respectively in promoting asthma pathogenesis. The consideration of clinical studies using TLK199, or a variant thereof that selectively inhibits PSSG, without affecting the detoxification function of GSTs is of high importance. As described in **chapter 2**, polymorphisms in the *GSTP* gene can effects its enzyme activity, such as the Ile105Val polymorphism resulted in a decreased enzyme activity towards CDNB, and therefore polymorphic screening should be monitored when selecting patients for clinical trials. Moreover, clinical trials are needed to examine PKM2 activation on the pathogenesis of asthma in patients with different endotypes, and should take into account factors that can affect these outcomes including diet, age, weight, sex, urbanization.

## Conclusion

The results in this thesis demonstrate that alterations in cellular metabolism are regulated by redox perturbations that contribute to the pathogenesis of asthma. Understanding the metabolic regulation of (innate) immune responses and in lung epithelial cells during asthma support the identification of new therapeutic targets for controlling inflammation and/or biomarkers for disease phenotyping and predicting therapeutic response. Here we show that IL-1ß is a critical signal that drives glycolytic reprogramming contributing to asthma pathogenesis by increasing inflammation, airway remodeling, and AHR. We

discussed that lactate could be a potential biomarker for asthma patients who display increases in glycolysis and IL-1-associated inflammation. Moreover, IL-1ß levels in the sputum of asthmatics could be an indicator of increased neutrophilic asthma, which may help to tailor more personalized treatment. Furthermore, this thesis increases the knowledge on the contribution of redox regulation during allergic airways disease, in particular the importance of GSTP-controlled protein-S-glutathionylation chemistry in association with its regulation of the glycolysis pathway. The importance of the glycolytic pathway in asthma has been further emphasized by our results indicating that activation of PKM2 attenuated airway remodeling and inflammation during allergic airways disease. Therefore, PKM2 could be a novel potential target for the development of anti-inflammatory therapies for the treatment of asthmatics with high IL-1 levels and increased glycolysis. In conclusion, cellular metabolism is a new avenue that should be considered in the clinic to better characterize asthma patients and to provide more tailored treatment that may improve clinical symptoms and their quality of life.

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# Summary
# Nederlandse Samenvatting

The results described in this thesis highlight that asthma is a complex pulmonary disease characterized by glycolytic reprogramming accompanied by elevated lactate levels and increases in protein-S-glutathionylation. **The general aim of this thesis was to examine the importance of an altered cell metabolism during allergic airways disease and whether these metabolic changes may be regulated by redox perturbations.**

Following the introduction and overall aim of this thesis in **Chapter 1, Chapter 2** provides an overview of the specific functions of mammalian cytosolic GSTs that may impact the pathogenesis of chronic lung diseases. In particular, the contribution of GSTs and their genetic variants to normal lung growth and development as well as their implication in the susceptibility to and progression of asthma and COPD have been described. Moreover, the expression profile of the GSTs in the lung as well as in epithelial cells specifically in healthy subjects versus asthmatics are discussed.

**Chapter 3** describes that increases in glycolysis are a critical feature of house dust mite-induced allergic airways disease, mediated by the Interleukin-1/IKKε signaling axis. Moreover, the increases in glycolysis are required to augment the house dust mite-induced pro-inflammatory responses of airway epithelial cells. Furthermore, primary nasal epithelial cells from asthmatics intrinsically produce more lactate as compared to healthy controls, and sputum lactate levels positively correlated with IL-1β, and negatively correlated with lung function in asthmatics. Lactate could therefore be a potential biomarker for increased glycolysis and IL-1-associated pro-inflammatory signals in airways of asthmatics.

**Chapter 4** describes that PKM2 promotes the pathogenesis of house dust mite-induced allergic airways disease. Activation of the glycolysis function of PKM2, with TEPP46, resulted in a diminished phenotype of disease. Pyruvate Kinase M2 activation moreover decreases Interleukin-1β-mediated expression of pro-inflammatory mediators in the lung and in epithelial cells, in part through nuclear phosphorylation of Signal Transducer and Activator of Transcription 3 (STAT3).

We demonstrate in **Chapter 5** that GSTP, which catalyzes the forward protein S-glutathionylation reaction, promotes house dust mite-induced allergic airways disease. GSTP also promotes the Interleukin-1(β)-induced secretion of lactate and pro-inflammatory mediators in lungs and primary tracheal epithelial cells from mice. Redox proteomic screens in combination with computational simulating modelling as well as the use of recombinant protein assays identified PKM2 as target for protein S-glutathionylation, and confirm the interaction between GSTP and PKM2, as well as the impact of GSTP-controlled protein S-glutathionylation on the glycolytic activity of PKM2.

In **Chapter 6**, the main findings are summarized and elaborately discussed. In conclusion, this thesis contributes to a better understanding of the pathology of asthma. The results herein demonstrate the importance of an altered metabolism, in particular increases in glycolysis, and the role of the glycolytic enzyme PKM2, during the development of allergic airways disease. Furthermore, this thesis increases the knowledge on the contribution of redox regulation during allergic airways disease, in particular the importance of GSTP-controlled protein-S-glutathionylation chemistry in association with its regulation of the glycolysis pathway. Our results indicate and further confirm the need for personalized treatment. PKM2 could be a novel potential target for the development of anti-inflammatory therapies for the treatment of asthmatics with high Interleukin-1 levels and increased glycolysis.

De resultaten beschreven in dit proefschrift tonen aan dat astma een complexe longaandoening is dat wordt gekarakteriseerd door glycolytische reprogrammering, vergezeld door verhogingen in lactaat levels en totaal eiwit glutathionylatie. **Het doel van dit proefschrift was om het belang van een verstoord metabolisme bij allergische longziekte te onderzoeken en of deze metabolische veranderen worden gereguleerd door redox verstoringen.**

In navolging van de inleiding en algemene doelen van dit proefschrift in **hoofdstuk 1**, **hoofdstuk 2** omvat een literatuuroverzicht over de specifieke functies van cytosolische Glutathione-S-transferases (GST's) in zoogdieren en hun bijdrage aan de pathogenese van chronische longziekten. Met name de bijdrage van GST's en hun genetische varianten aan de normale groei en ontwikkeling van de longen wordt beschreven, evenals de implicatie van GST's voor de gevoeligheid voor en progressie van astma en COPD. Bovendien worden de expressieprofielen van de verschillende GST's in de longen en in epitheelcellen specifiek bij gezonde proefpersonen versus astmapatiënten behandeld.

**Hoofdstuk 3** beschrijft dat een toename in glycolyse een cruciaal kenmerk is van door huisstofmijt veroorzaakte allergische luchtwegaandoeningen, dat wordt gemedieerd door Interleukine-1(β)/IKKε signalering. Daarnaast is de verhoging in glycolyse vereist om de door huisstofmijt geïnitieerde pro-inflammatoire responsen te versterken in luchtweg epitheelcellen. Bovendien produceerden primaire nasale epitheelcellen van astmapatiënten intrinsiek meer lactaat in vergelijking met gezonde proefpersonen, en hebben we aangetoond dat in sputum lactaatlevels positief correleren met IL-1β, en negatief correleren met longfunctie in astmapatiënten. Lactaat kan een potentiële biomarker zijn voor verhoogde glycolyse en IL-1 geassocieerde inflammatie signalen in luchtwegen van astmapatiënten.

**Hoofdstuk 4** toont aan dat PKM2 de pathogenese van door huisstofmijt veroorzaakte allergische luchtwegaandoeningen bevordert. Activering van de glycolysefunctie van PKM2 met TEPP46, resulteerde in een verminderd fenotype van ziekte. PKM2 activatie verminderd bovendien de door Interleukin-1β gemedieerde expressie van pro-

inflammatoire mediatoren in de longen en in epitheelcellen, gedeeltelijk door fosforylering van signaaltransducer en activator van transcriptie 3 (STAT3) in de nucleus.

We tonen in **hoofdstuk 5** aan dat GSTP, een katalyse van de eiwit glutathionylatie reactie, bijdraagt aan huisstofmijt veroorzaakte allergische luchtwegaandoeningen. GSTP bevordert ook de door IL-1(β)-geïnduceerde secretie van lactaat en pro-inflammatoire mediatoren in longen en tracheale epitheelcellen van de muis. Redox proteomic screens in combinatie met computationele simulatiemodellering en het gebruik van recombinante proteïne assays identificeerden PKM2 als doelwit voor eiwit S-glutathionylering, en bevestigen de interactie tussen GSTP en PKM2, evenals de impact van GSTP-gecontroleerde proteïne S-glutathionylering op de glycolytische activiteit van PKM2.

In **hoofdstuk 6** worden de belangrijkste bevindingen samengevat en besproken. Concluderend draagt dit proefschrift bij aan een beter begrip van de pathologie van astma. De resultaten hierin tonen het belang aan van een veranderd metabolisme, in het bijzonder toenames in glycolyse, en de rol van het glycolytische enzyme PKM2, tijdens de ontwikkeling van allergische luchtwegaandoeningen. Bovendien vergroot dit proefschrift de kennis over de bijdrage van redoxregulatie tijdens allergische luchtwegaandoeningen, in het bijzonder het belang van GSTP-gecontroleerde eiwit-S-glutathionylatiechemie in combinatie van de regulering van de glycolyse-pathway. Bovendien geven onze resultaten de behoefte aan een gepersonaliseerde behandeling aan. PKM2 kan mogelijk een nieuw potentieel doelwit zijn voor de ontwikkeling van ontstekingsremmende therapieën voor de behandeling van astmapatiënten met hoge Interleukin-1-spiegels en verhoogde glycolyse.

# Impact

## Background

Asthma is defined as a heterogeneous pulmonary disease, characterized by chronic inflammation, which affects more than 300 million people worldwide (1). The pathophysiology of asthma consists of structural changes in the airways that may induce symptoms including chest tightness, frequent coughs and wheezes as well as airway obstruction together with variable airflow limitation (2). These typical asthma symptoms vary over time and intensity and can affect daily life activities and reduce the quality of life in asthmatic patients. Asthma is generally not seen as a disease with high mortality, but according to the WHO, at least 350,000 deaths are attributed to asthma annually (3, 4). Current available therapeutics target symptoms but do not cure disease, and unfortunately, a significant patient population remains for whom these treatments are not effective. The fundamental causes of asthma are still not fully understood but are likely a combination of genetic profile and external factors. Moreover, it has to be taken into account that every individual is different in terms of their physiological and genetic profile. To better categorize asthmatic patients, several subtypes of asthma exist called endotypes and phenotypes, although it is hard to define patients into endotypes and phenotypes as overlap exists (5). Especially patients with severe asthma do not respond to current treatment, have uncontrolled disease and are hard to define into subgroups. To obtain better therapeutics, it is critical to understand the fundamental causes and the underlying (molecular) mechanisms of asthma pathophysiology. This way, asthmatics can be better classified in order to personalize treatment.

## Research and relevance

Abnormal cellular metabolism is implicated in the pathogenesis of several diseases, including diabetes, cancer as well as multiple chronic lung diseases (6). Metabolomic approaches on blood, and urine revealed that fatty acid and lipid metabolism were affected in asthmatics (7-9), but the metabolic alterations in lung tissues and specifically in epithelial cells, that drive the inflammatory response in asthmatics, remained largely unexplored. The first main objective of this thesis was to examine if alterations in cellular metabolism contribute to asthma pathology and secondly, whether metabolic changes are regulated by changes in the oxidative environment, notably by redox perturbations. The main results of

this thesis demonstrate that increases in the glycolysis pathway, which is a process that involves the breakdown of glucose to extract energy for cell metabolism, contribute to pathophysiological manifestations underlying asthma including increased lung inflammation and worsened lung function. Moreover, our results demonstrate that a pro-inflammatory mediator, Interleukin-1ß (IL-1β), is an important signal that induces these increases in glycolysis during asthma which corresponded with increased levels of lactate, which is an indicative of disturbed cell metabolism and worsened disease. Interestingly, our data displayed that IL-1β was associated with neutrophilic asthma (has been linked to more severe disease), rather than eosinophilic asthma, which is in line with published results showing that IL-1β is an important neutrophil activator (10). Additionally, IL-1β increases the expression of the epithelial cell-derived cytokine TSLP in epithelial cells, which is linked to steroid resistance and severe airway inflammation. This thesis moreover highlights the importance of the glycolysis pathway during asthma by showing that the key, rate-limiting, enzyme of the glycolysis pathway, called Pyruvate Kinase M2, contributes to asthma pathology. Our second objective was focused on Glutathione-S-transferase Pi (GSTP), a redox-based enzyme that is highly expressed in the lung and in epithelial cells, which also functions in cellular detoxification by neutralizing toxic and carcinogenic compounds in our body. GSTP catalyzes an oxidative process that changes the function of proteins, thereby affecting processes including metabolic pathways (11). Interestingly, our results displayed that GSTP worsens asthma by changing cellular metabolism, notably by affecting the glycolysis enzyme Pyruvate Kinase M2.

As mentioned, asthmatics with severe disease often do not respond to current treatments. Severe asthma is associated with increased mortality and hospitalization, reduced quality of life and increased health care costs, and accounts for approximately 5-10% of all confirmed asthma cases in developed countries (12). As this thesis highlights the importance of cellular metabolism in asthma, monitoring changes in cellular metabolism in patients (with severe disease) in the clinic could be useful to gain better insights into the underlying mechanisms and may lead to new and better therapeutics. Results in this thesis showed that the pro-inflammatory mediator IL-1β drives increases in glycolysis in association with increased lactate levels in sputum of asthmatic patients. Therefore, lactate

as well as IL-1β levels in sputum as well as in blood could be non-invasive, quick and reliable biomarkers to identify patients with altered cell metabolism (glycolysis) and improve diagnostics and characterization of patients into endotypes and phenotypes.

Our results moreover highlight the importance of the key glycolysis enzyme Pyruvate Kinase M2 in asthma pathology, and showed that its function can be altered by the redox-based enzyme GSTP. Inhibition of GSTP moreover displayed reduced lactate levels and inflammation from epithelial cells. GSTP and PKM2 are also shown to be present in extracellular fluids including bronchoalveolar lavage and sputum. Therefore, Pyruvate Kinase M2 and/or GSTP could be potential targets for the development of anti-inflammatory therapies and inhibition of glycolysis for the treatment of asthmatics with high IL-1β levels and increased glycolysis.

**Target groups and activity**

Screening for alterations in cellular metabolism will not only be beneficial for asthmatics. It has for instance been shown that inhibition of glycolysis may have therapeutic benefit in lung fibrosis in animal models (13). Inhibition of glycolysis has also been tested in other metabolic disorders such as cancer and inflammatory diseases, and increased IL-1ß levels have been associated with more inflammation in patients with severe persistent allergic rhinitis as well as in COPD patients (14, 15). Moreover, it was found that patients with COVID-19 displayed higher IL-1ß levels in plasma than healthy subjects (16). Moreover, lung immune cells (that also play important roles in asthma) from COVID-19 patients displayed altered metabolism, increased levels of Pyruvate Kinase M2, as well as lactate levels. It is thought that these increases in cellular metabolism contribute to disease pathology, and severity of disease (e.g. more symptoms and hospitalization) and underline the importance of this research.

While there is debate whether asthma cases are increasing or not, it is worrisome that asthma is often underdiagnosed as well as undertreated. The development of asthma is complicated since it is often related to other factors including immunological factors, age, gender and obesity. Obesity-related asthma is linked to more severe disease and up to 30% of obese asthma patients do not respond to steroids (17). According to the CDC, the

prevalence of obesity is increasing and is currently approximately observed in 42,4% of the global population. Aging is another factor that is increasing, and asthma in elderly patients presents itself differently, with higher mortality rates. Therefore, deaths or incidents due to obesity or aging are not always directly linked to asthma. IL-1ß has already emerged as a biomarker in obesity (18), and it is suggested that IL-1ß participates in fundamental inflammatory processes that increase during the aging process (19).

It is important that target groups will be informed about research findings to incorporate potential novel and improved strategies in the clinic. Results from this thesis will be published in scientific journals which are available to a broad spectrum of people. However, patients without a background in science or healthcare rarely have access to or read these articles and they should be informed as well. The connection and communication between scientists and other healthcare professionals including medical doctors and nurses is essential. Moreover, patients including their families, friends and/or caretakers should be informed about novel research in an understandable way via healthcare professionals, health magazines or via (social) media sources. Important is that people with scientific backgrounds communicate the findings on these platforms, as data often is misunderstood or miscommunicated via the media. The Longdagen ('Days of the Lungs') is a conference held in the Netherlands where scientists, doctors, nurses and patients come together and discuss the latest findings regarding lung diseases. More gatherings like these should be organized and advertised as well as routine social meetings with patients, families and/or caretakers and pulmonary professionals including doctors and scientists would be essential to share most valuable information regarding better treatment strategies. Social media platforms such as twitter, linkedIN, and TED talks nowadays also share research outcomes, and improve the dissemination of research. Research funders can be of help to research organizations to develop knowledge uptake skills and promote research communication (20).

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# List of publications

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# Grants/awards

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## LIST OF PUBLICATIONS

Yvonne M. Janssen-Heininger, Charles M. Kinsey, Shi B. Chia, David J. Seward, **Cheryl van de Wetering**, Reem Aboushousha, Evan Elko, Allison M. Manuel, Cuixia Erickson, Kelly J. Butnor, Zhihua Peng, Iris van Gerwen, Jos L. van der Velden. *Increased survival in KRASG12D-induced tumorigenesis with a clinically relevant inhibitor of Glutathione-S-transferase P*. In preparation

**Cheryl van de Wetering**, Allison M. Manuel, Mona Sharafi, Xi Qian, Cuixia Erickson, Maximilian B. MacPherson, Reem Aboushousha, Jos van der Velden, Jianing Li, Emiel F. Wouters, Niki L. Reynaert, Yvonne M. Janssen-Heininger. *Glutathione-S-transferase P promotes IL-1β-induced pulmonary inflammation in mice with house dust mite-induced allergic airways disease.* Submitted to Am J Respir Crit Care Med.

Allison M. Manuel* **Cheryl van de Wetering***, Jos van der Velden, Maximilian B. MacPherson, Cuixia Erickson, Anne E. Dixon, Matt Poynter, Charles G. Irvin, Yvonne M. Janssen-Heininger. *Dimethyl Fumarate Reduces House Dust Mite-Induced Glycolytic reprogramming and Inflammation in Mice with Allergic Airways Disease.* In revision at AJP Lung. * Equal authorship.

Mahyar Aghapour*****, Sara Cuevas-Ocaña*****, Declan F. Doherty*****, **Cheryl van de Wetering***, Agnes Boots, Aurelie Fabre, Catherine M. Greene, Irene H. Heijink, Silke Meiners, Niki D. Ubags. *ERS International Congress 2020 virtual: highlights from the Basic and Translational Science Assembly.* In revision at ERJ OR. * Equal authorship.

**Cheryl van de Wetering**, Evan Elko, Marijn Berg, Caspar H.J. Schiffers, Vasili Stylianidis Maarten van den Berge, Martijn C. Nawijn, Emiel F.M. Wouters, Yvonne M.W. Janssen-Heininger, Niki L. Reynaert. *Glutathione-S-transferases and their implications in the lung disease asthma and Chronic Obstructive Pulmonary Disease: early life susceptibility?* **Redox Biology**. 2021 10.1016/j.redox.2021.101995.

Reem Aboushousha*, Evan Elko*, Shi Chia, Allison Manuel, **Cheryl van de Wetering**, Jos van der Velden, Max MacPherson, Cuixia Erickson, Julie Reisz, Angelo D'Alessandro, Emiel Wouters, Niki Reynaert, Ying-Wai Lam, Vikas Anathy, Albert van der Vliet, David Seward[#], Yvonne Janssen-Heininger[#]. *Glutathionylation chemistry promotes Interleukin 1-Beta-*

*mediated metabolic reprogramming and pro-inflammatory signaling in lung epithelial cells.* **FASEB J**. 2021 May;35(5):e21525. * Equal authorship. # co-corresponding authors.

Allison M. Manuel, **Cheryl van de Wetering**, Maximilian B. MacPherson, Cuixia Erickson, Caliann Murray, Reem Aboushousha, Jos van der Velden, Anne E. Dixon, Matt Poynter, Charles G. Irvin, Douglas J. Taatjes, Albert van der Vliet, Vikas Anathy, Yvonne M. Janssen-Heininger. *Dysregulation of pyruvate kinase M2 promotes inflammation in a mouse model of obese allergic asthma.* **Am J Respir Cell Mol Biol**. 2021 Mar.

Caspar Schiffers, **Cheryl van de Wetering**, Robert Bauer, Aida Habibovic, Milena Hristova, Christopher Dustin, Sara Lambrichts, Pamela Vacek, Emiel Wouters, Niki Reynaert, Albert van der Vliet. *Downregulation of Epithelial Dual Oxidase 1 (DUOX1) in Chronic Obstructive Pulmonary Disease Contributes to Disease Pathogenesis.* **JCI Insight**. 2020 Dec 10;142189.

**Cheryl van de Wetering***, Reem Aboushousha*, Allison M. Manuel, Shi B. Chia, Cuixia Erickson, Maximilian B. MacPherson, Jos L. van der Velden, Vikas Anathy, Anne E. Dixon, Charles G. Irvin, Matthew E. Poynter, Albert van der Vliet, Emiel F.M. Wouters, Niki L. Reynaert, Yvonne M.W. Janssen-Heininger. *Activation of Pyruvate Kinase M2 attenuates expression of pro-inflammatory mediators in house dust mite-induced allergic airways disease.* **J Immunol.** 2020 Feb 15;204(4):763-774. * Equal authorship.

Shi Chia, Evan Elko, Reem Aboushousha, Allison Manuel, **Cheryl van de Wetering**, Joseph Druso, Jos van der Velden, David Seward, Vikas Anathy, Charles Irvin, Ying Lam, Albert van der Vliet, and Yvonne Janssen-Heininger. *Dysregulation of the glutaredoxin/S-glutathionylation redox axis in lung diseases.* **Am J Physiol Cell Physiol.** 2020 Feb 1;318(2):C304-C327.

Elko EA, Cunniff B, Seward DJ, Chia SB, Aboushousha R, **van de Wetering C**, van der Velden J, Manuel A, Shukla A, Heintz NH, Anathy V, van der Vliet A, Janssen-Heininger YMW. *Peroxiredoxins and Beyond; Redox Systems Regulating Lung Physiology and Disease.* **Antioxid Redox Signal.** 2019 Nov 10;31(14):1070-1091.

Qian X, Aboushousha R*****, **van de Wetering C***, Chia SB, Amiel E, Schneider RW, van der Velden JLJ, Lahue KG, Hoagland DA, Casey DT, Daphtary N, Ather JL, Randall MJ, Aliyeva M, Black KE, Chapman DG, Lundblad LKA, McMillan DH, Dixon AE, Anathy V, Irvin CG, Poynter

ME, Wouters EFM, Vacek PM, Henket M, Schleich F, Louis R, van der vliet A, Janssen-Heininger YMW*. IL-1/inhibitory κB kinase ε-induced glycolysis augment epithelial effector function and promote allergic airways disease.* **J Allergy Clin Immunol.** 2018 Aug;142(2):435-450.e10.   * Equal authorship.

Irene M.J. Eurlings, Niki L. Reynaert, **Cheryl van de Wetering**, Scott W. Aesif, Evi M. Mercken, Rafael de Cabo, Jos L. van der Velden, Yvonne M. Janssen-Heininger, Emiel F.M. Wouters, Mieke A. Dentener. *Involvement of JNK in TNFα Driven Remodelling.* **Am J Respir Cell Mol Biol.** 2017 Mar;56(3):393-401.

## PRESENTATIONS

**Cheryl van de Wetering**, Allison Manuel, Mona Sharafi, Jianing Li, Emiel Wouters, Niki Reynaert, Yvonne Janssen-Heininger. *Glutathione-S-transferase P Promotes HDM-induced Allergic Airway Disease in Association with Enhanced Glycolysis via Glutathionylation of PKM2.* Society for Redox Biology and Medicine. 2020, November, Virtual Conference. (*oral presentation*)

**Cheryl van de Wetering,** Emiel Wouters, Niki Reynaert, and Yvonne Janssen-Heininger. Glutathione-S-transferase P promotes Interleukin-1β-induced inflammation and metabolic reprogramming in mice with allergic airways disease. European Respiratory Society International Congress, 2020, September, Virtual Conference. (*oral presentation*)

**Cheryl van de Wetering,** Xi Qian, Allison Manuel, Reem Aboushousha, Jianing Li, Garrett Chan, Emiel Wouters, Niki Reynaert, and Yvonne Janssen-Heininger. Novel role for Glutathione-S-transferase P in asthma. Gordon Research Conference Oxygen Radicals, 2020, February, Ventura, CA, USA. (*1-minute oral presentation and poster presentation*)

**Cheryl van de Wetering**, Reem Aboushousha, Allison Manuel, Cuixia Erickson, Albert van der Vliet, Emiel Wouters, Niki Reynaert, Yvonne Janssen-Heininger. Glutathione-S-transferase P promotes interleukin-1β-induced pulmonary inflammation in association with S-glutathionylation of the glycolysis regulator, Pyruvate kinase M2. Society for Redox Biology and Medicine, 2019, November, Las Vegas, NV, USA. (*oral presentation*)

**C. van de Wetering**, A. Manuel, X. Qian, R. Aboushousha, S.B. Chia, C. Erickson, J. Bates, C. Irvin, A. van der Velden, A. van der Vliet, N. Reynaert, E. Wouters, Y. Janssen Heininger. Glutathione-S-transferase P promotes interleukin-1β-induced pulmonary inflammation in association with S-glutathionylation of the glycolysis regulator, Pyruvate kinase M2. American Thoracic Society, 2019, May, Dallas, TX, USA. (*poster presentation*)

**Cheryl van de Wetering**, Reem Aboushousha, Shi Biao Chia, Allison Manuel, Jason Bates, Jos van der Velden, Niki Reynaert, Albert van der Vliet, Emiel Wouters, Albert van der Vliet, Yvonne Janssen-Heininger. Glutathione-S-transferase P promotes interleukin-1β-induced

pulmonary inflammation in association with S-glutathionylation of the glycolysis regulator, Pyruvate kinase M2. Society for Redox Biology and Medicine, 2018, November, Chicago, IL, USA. (*oral presentation*)

**Cheryl van de Wetering,** Niki Reynaert, Emiel Wouters, Yvonne Janssen-Heininger. Ablation of Glutathione-S-transferase P Attenuates Glycolysis in House Dust Mite-Induced Allergic Airways Disease. Research Day, 2018, October, Burlington, USA. (*1-minute pitch*)

**Cheryl van de Wetering**, Reem Aboushousha, Wyatt Chia, Xi Qian, Lennart Lundblad, Niki Reynaert, Emiel Wouters, Albert van der Vliet, and Yvonne Janssen-Heininger. Ablation of Glutathione-S-Transferase P attenuates glycolysis in house dust mite-induced allergic airways disease. Gordon Research Seminar and Conference Oxygen Radicals, 2018, February, Ventura, CA, USA. (*oral and poster presentation at both seminar and conference*)

**C. van de Wetering**, N. Reynaert, E. Wouters, Y. Janssen-Heininger. The use of decellularized lung scaffolds to study cell-matrix interactions in COPD. Nutrim day, 2016, December, Maastricht, The Netherlands. (*poster presentation*)

**Cheryl van de Wetering**, Mieke A. Dentener, Niki L. Reynaert, Yvonne M. Janssen-Heininger, Emiel. F. M. Wouters. Pulmonary epithelium as a central player in lung pathology; Use of the Primary Lung Culture facility to study the role of EMT and Redox biology in COPD. Science Day, 2016, June, Maastricht, the Netherlands. (*oral presentation*)

**Cheryl van de Wetering**, Irene M. J. Eurlings, Niki L. Reynaert, Scott W. Aesif, Evi M. Mercken, Rafael de Cabo, Jos L. van der Velden, Yvonne M. Janssen-Heininger, Emiel. F. M. Wouters, Mieke A. Dentener. Involvement of JNK in TNFα-driven remodeling. Longdagen (Days of the lung), 2016, April, Ermelo, The Netherlands. (*poster presentation*)

**Cheryl van de Wetering**, Ramon Langen, Jos van der Velden, Marco Kelders, Annemie Schols. Tumor derived muscle atrophy signaling in a model of lung cancer cachexia. Longdagen (Days of the lung), 2016, April, Ermelo, The Netherlands. (*poster presentation*)

## AWARDS AND GRANTS

| | |
|---|---|
| 2020 | **Trainee Award** for top selected abstract and oral presentation at the Society for Redox Biology and Medicine, Nov 18-20, 2020, Virtual Conference |
| 2020 | **Disease Models and Mechanisms (DMM) Conference Travel Grant** by the Company of Biologists for attending the Gordon Research Conference Oxygen Radicals Feb 2-Feb 7, 2020 in Ventura, USA |
| 2019 | **Young Investigator Award** for oral presentation at the Society for Redox Biology and Medicine, Nov 20-23, 2019, Las Vegas, USA |
| 2019 | **Young Investigator Travel Grant** by the Netherlands Respiratory Society (NRS) to attend the Society for Redox Biology and Medicine, Nov 20-23, 2019, Las Vegas, USA |
| 2019 | **Better Together Award** by the University of Vermont, for outstanding teamwork in the process of human lung autopsies and the collection of lung specimens |
| 2019 | **Travel award** by Cayman Chemical, towards the costs of attending the American Thoracic Society (ATS) May 17-22, 2019, Dallas, USA |
| 2014 | **Travel scholarship KWF Kankerbestrijding** (Dutch Cancer Society) to support Senior Practical Training at the University of Vermont, USA |
| 2014 | **FreeMover grant** from Maastricht University to support Senior Practical Training at the University of Vermont, Burlington, USA |

## PROFESSIONAL SERVICE

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| 2019- Present | European Respiratory Society (ERS) Early Career Member of Assembly 3 (basic and translational sciences) and volunteer translator for the European Lung Foundation (ELF) |
| 2020 | Conference Chair of the 2020 Oxygen Radicals Gordon Research Seminar, CA, USA. *Supporting grants/funds*: National Institutes of health (NIH), Cayman Chemicals, Society for Free Radical Research (SFFR) Europe, the Company of Biologists, Society of Toxicology, New England Biolabs, University of Pittsburgh |
| 2019 | Co-Chair of the Opening Doors Event: Professionalism – Building Success In Science at the Society for Redox Biology and Medicine, Las Vegas, NV, USA |

## CURRICULUM VITAE

Cheryl van de Wetering was born on May 30, 1991 in Brunssum, the Netherlands. She acquired her secondary school diploma (VWO) in 2010. She graduated her bachelor education in Biomedical Sciences at Maastricht University in 2013 and subsequently studied the master Biomedical Sciences. She conducted her senior master internship at the University of Vermont under supervision of Dr. J. van der Velden, where she worked on the project ‘Tumor derived muscle atrophy signaling in a model of lung cancer cachexia’, for which she received a travel scholarship and travel grant. She received her master’s degree with the specialization Clinical Molecular Sciences in September 2015. One day later she started her pre-PhD year at the Department of Respiratory Medicine and School of Nutrition and Translational Research in Metabolism (NUTRIM) at Maastricht University Medical center+ under the supervision of Prof. Dr. E.F.M. Wouters, Prof. Dr. Y.M.W. Janssen-Heininger and Dr. N.L. Reynaert. During this year she worked on the project ‘Activation of the JNK1-SMAD3 signaling axis in the airway epithelium contributes to the pathogenesis of sub-epithelial fibrosis in COPD patients’. In January 2017, Cheryl moved to Vermont in the United States to start working on her PhD project ‘Redox regulation of metabolism in asthma’ in the Janssen-Heininger laboratory. During her PhD trajectory, Cheryl teached and supervised several students in the laboratory. She presented her work at multiple international conferences, for which she received multiple travel grants and awards for her oral presentations. Moreover, she was elected as chair to organize the Gordon Research Seminar Oxygen Radicals in 2020. The results obtained and published during her PhD trajectory are described in this thesis. Cheryl will continue her scientific career as a post-doctoral candidate at the St. Anna Children’s Cancer Research Institute in Vienna, Austria.