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

Cheryl van de Wetering

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

Dissertation

To obtain the degree of Doctor at Maastricht University,
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by

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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).

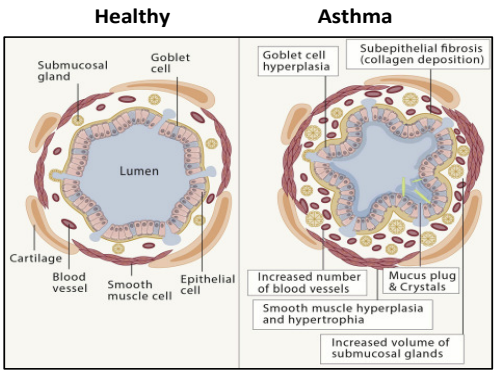

Figure 1: Schematic representation of histological characteristics of the airways in a lung of a healthy control subject (left) and the structural changes in a patient with severe asthma (right) (6, 7).

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.

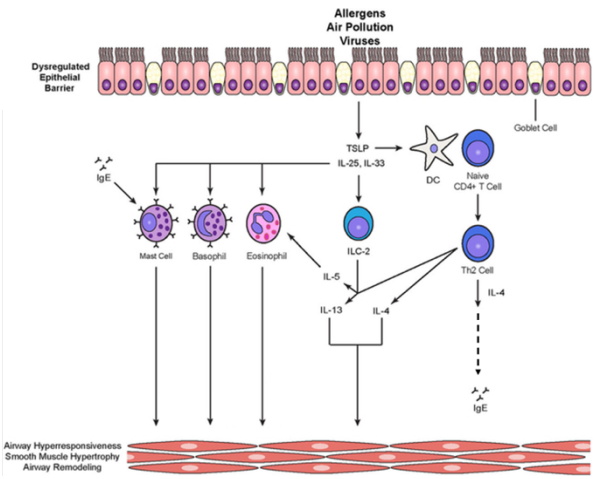

Figure 2: Schematic overview of the mechanism of type 2 inflammation in (severe) asthma, adapted from (11).

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).

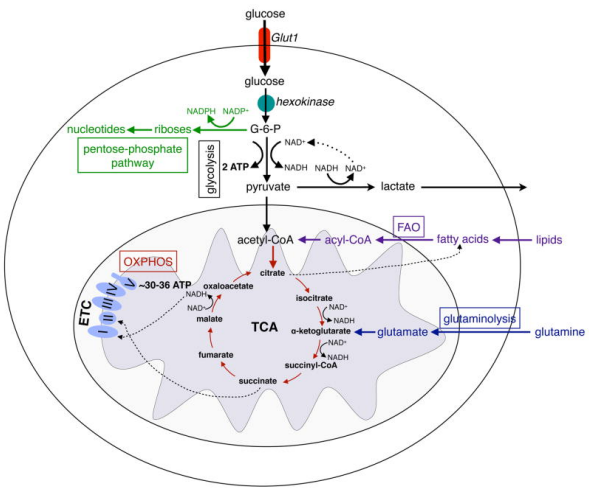

Figure 3: Schematic overview of major metabolic pathways in immune cells (25).

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).

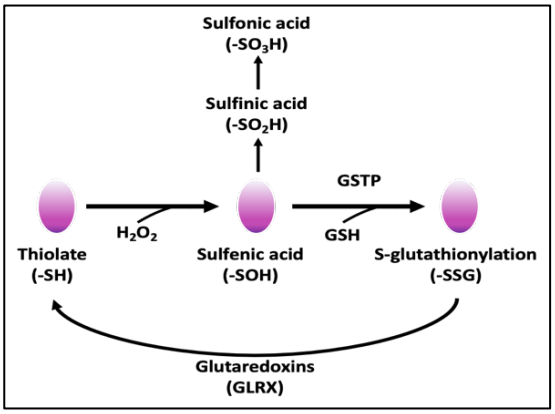

Figure 4: Schematic overview of the PSSG reaction, adapted from (50).

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.

REFERENCES

1. Eder W, Ege MJ, von Mutius E. The asthma epidemic. The New England journal of medicine. 2006;355(21):2226-35.
2. Fahy JV. Type 2 inflammation in asthma--present in most, absent in many. Nature reviews Immunology. 2015;15(1):57-65.
3. Reddel HK, Bateman ED, Becker A, Boulet LP, Cruz AA, Drazen JM, et al. A summary of the new GINA strategy: a roadmap to asthma control. The European respiratory journal. 2015;46(3):622-39.
4. Ray A, Raundhal M, Oriss TB, Ray P, Wenzel SE. Current concepts of severe asthma. J Clin Invest. 2016;126(7):2394-403.
5. Busse WW, Lemanske RF, Jr. Asthma. The New England journal of medicine. 2001;344(5):350-62.
6. Lambrecht BN, Hammad H, Fahy JV. The Cytokines of Asthma. Immunity. 2019;50(4):975-91.
7. Wadsworth S, Sin D, Dorscheid D. Clinical update on the use of biomarkers of airway inflammation in the management of asthma. J Asthma Allergy. 2011;4:77-86.
8. Caminati M, Pham DL, Bagnasco D, Canonica GW. Type 2 immunity in asthma. The World Allergy Organization journal. 2018;11(1):13.
9. Gregory LG, Lloyd CM. Orchestrating house dust mite-associated allergy in the lung. Trends Immunol. 2011;32(9):402-11.
10. Wynn TA. Type 2 cytokines: mechanisms and therapeutic strategies. Nature reviews Immunology. 2015;15(5):271-82.
11. Kuruvilla ME, Lee FE, Lee GB. Understanding Asthma Phenotypes, Endotypes, and Mechanisms of Disease. Clin Rev Allergy Immunol. 2019;56(2):219-33.
12. Morjaria JB, Kastelik JA. Unusual asthma syndromes and their management. Ther Adv Chronic Dis. 2011;2(4):249-64.
13. McGrath KW, Icitovic N, Boushey HA, Lazarus SC, Sutherland ER, Chinchilli VM, et al. A large subgroup of mild-to-moderate asthma is persistently noneosinophilic. American journal of respiratory and critical care medicine. 2012;185(6):612-9.
14. Moore WC, Meyers DA, Wenzel SE, Teague WG, Li H, Li X, et al. Identification of asthma phenotypes using cluster analysis in the Severe Asthma Research Program. American journal of respiratory and critical care medicine. 2010;181(4):315-23.
15. Sporik R, Holgate ST, Platts-Mills TA, Cogswell JJ. Exposure to house-dust mite allergen (Der p I) and the development of asthma in childhood. A prospective study. The New England journal of medicine. 1990;323(8):502-7.
16. Hammad H, Chieppa M, Perros F, Willart MA, Germain RN, Lambrecht BN. House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells. Nat Med. 2009;15(4):410-6.
17. Tully JE, Hoffman SM, Lahue KG, Nolin JD, Anathy V, Lundblad LK, et al. Epithelial NF-kappaB orchestrates house dust mite-induced airway inflammation, hyperresponsiveness, and fibrotic remodeling. J Immunol. 2013;191(12):5811-21.
18. Johnson JR, Wiley RE, Fattouh R, Swirski FK, Gajewska BU, Coyle AJ, et al. Continuous exposure to house dust mite elicits chronic airway inflammation and structural remodeling. American journal of respiratory and critical care medicine. 2004;169(3):378-85.
19. Cates EC, Fattouh R, Wattie J, Inman MD, Goncharova S, Coyle AJ, et al. Intranasal exposure of mice to house dust mite elicits allergic airway inflammation via a GM-CSF-mediated mechanism. J Immunol. 2004;173(10):6384-92.
20. Schramm CM, Puddington L, Wu C, Guernsey L, Gharaee-Kermani M, Phan SH, et al. Chronic inhaled ovalbumin exposure induces antigen-dependent but not antigen-specific inhalational tolerance in a murine model of allergic airway disease. The American journal of pathology. 2004;164(1):295-304.
21. Swirski FK, Gajewska BU, Alvarez D, Ritz SA, Cundall MJ, Cates EC, et al. Inhalation of a harmless antigen (ovalbumin) elicits immune activation but divergent immunoglobulin and cytokine activities in mice. Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology. 2002;32(3):411-21.
22. Swirski FK, Sajic D, Robbins CS, Gajewska BU, Jordana M, Stampfli MR. Chronic exposure to innocuous antigen in sensitized mice leads to suppressed airway eosinophilia that is reversed by granulocyte macrophage colony-

stimulating factor. J Immunol. 2002;169(7):3499-506.
23. Michaeloudes C, Bhavsar PK, Mumby S, Xu B, Hui CKM, Chung KF, et al. Role of Metabolic Reprogramming in Pulmonary Innate Immunity and Its Impact on Lung Diseases. J Innate Immun. 2020;12(1):31-46.
24. Vander Heiden MG, Cantley LC, Thompson CB. Understanding the Warburg effect: the metabolic requirements of cell proliferation. Science. 2009;324(5930):1029-33.
25. Ganeshan K, Chawla A. Metabolic regulation of immune responses. Annu Rev Immunol. 2014;32:609-34.
26. Mazurek S. Pyruvate kinase type M2: a key regulator of the metabolic budget system in tumor cells. Int J Biochem Cell Biol. 2011;43(7):969-80.
27. Christofk HR, Vander Heiden MG, Harris MH, Ramanathan A, Gerszten RE, Wei R, et al. The M2 splice isoform of pyruvate kinase is important for cancer metabolism and tumour growth. Nature. 2008;452(7184):230-3.
28. Anastasiou D, Yu Y, Israelsen WJ, Jiang JK, Boxer MB, Hong BS, et al. Pyruvate kinase M2 activators promote tetramer formation and suppress tumorigenesis. Nat Chem Biol. 2012;8(10):839-47.
29. Warburg O. On the origin of cancer cells. Science. 1956;123(3191):309-14.
30. Doherty JR, Cleveland JL. Targeting lactate metabolism for cancer therapeutics. J Clin Invest. 2013;123(9):3685-92.
31. Stincone A, Prigione A, Cramer T, Wamelink MM, Campbell K, Cheung E, et al. The return of metabolism: biochemistry and physiology of the pentose phosphate pathway. Biol Rev Camb Philos Soc. 2015;90(3):927-63.
32. Krawczyk CM, Holowka T, Sun J, Blagih J, Amiel E, DeBerardinis RJ, et al. Toll-like receptor-induced changes in glycolytic metabolism regulate dendritic cell activation. Blood. 2010;115(23):4742-9.
33. Everts B, Amiel E, Huang SC, Smith AM, Chang CH, Lam WY, et al. TLR-driven early glycolytic reprogramming via the kinases TBK1-IKKvarepsilon supports the anabolic demands of dendritic cell activation. Nature immunology. 2014;15(4):323-32.
34. Michalek RD, Gerriets VA, Jacobs SR, Macintyre AN, MacIver NJ, Mason EF, et al. Cutting edge: distinct glycolytic and lipid oxidative metabolic programs are essential for effector and regulatory CD4+ T cell subsets. J Immunol. 2011;186(6):3299-303.
35. Ostroukhova M, Goplen N, Karim MZ, Michalec L, Guo L, Liang Q, et al. The role of low-level lactate production in airway inflammation in asthma. American journal of physiology Lung cellular and molecular physiology. 2012;302(3):L300-7.
36. Caslin HL, Taruselli MT, Haque T, Pondicherry N, Baldwin EA, Barnstein BO, et al. Inhibiting Glycolysis and ATP Production Attenuates IL-33-Mediated Mast Cell Function and Peritonitis. Front Immunol. 2018;9:3026.
37. Zhang L, Ma C, Wang X, He S, Li Q, Zhou Y, et al. Lipopolysaccharide-induced proliferation and glycolysis in airway smooth muscle cells via activation of Drp1. J Cell Physiol. 2019;234(6):9255-63.
38. Winnica D, Corey C, Mullett S, Reynolds M, Hill G, Wendell S, et al. Bioenergetic Differences in the Airway Epithelium of Lean Versus Obese Asthmatics Are Driven by Nitric Oxide and Reflected in Circulating Platelets. Antioxidants & redox signaling. 2019;31(10):673-86.
39. Sies H, Jones DP. Reactive oxygen species (ROS) as pleiotropic physiological signalling agents. Nat Rev Mol Cell Biol. 2020.
40. Birben E, Sahiner UM, Sackesen C, Erzurum S, Kalayci O. Oxidative stress and antioxidant defense. The World Allergy Organization journal. 2012;5(1):9-19.
41. Dalle-Donne I, Rossi R, Giustarini D, Colombo R, Milzani A. S-glutathionylation in protein redox regulation. Free radical biology & medicine. 2007;43(6):883-98.
42. Gallogly MM, Mieyal JJ. Mechanisms of reversible protein glutathionylation in redox signaling and oxidative stress. Curr Opin Pharmacol. 2007;7(4):381-91.
43. Grek CL, Zhang J, Manevich Y, Townsend DM, Tew KD. Causes and consequences of cysteine S-glutathionylation. The Journal of biological chemistry. 2013;288(37):26497-504.
44. Klomsiri C, Karplus PA, Poole LB. Cysteine-based redox switches in enzymes. Antioxidants & redox signaling. 2011;14(6):1065-77.
45. Xiong Y, Uys JD, Tew KD, Townsend DM. S-glutathionylation: from molecular mechanisms to health outcomes. Antioxidants & redox signaling. 2011;15(1):233-70.
46. McIlwain CC, Townsend DM, Tew KD. Glutathione S-transferase polymorphisms: cancer incidence and therapy. Oncogene. 2006;25(11):1639-48.
47. Anttila S, Hirvonen A, Vainio H, Husgafvel-Pursiainen K, Hayes JD, Ketterer B. Immunohistochemical localization

of glutathione S-transferases in human lung. Cancer research. 1993;53(23):5643-8.
48. Adler V, Yin Z, Fuchs SY, Benezra M, Rosario L, Tew KD, et al. Regulation of JNK signaling by GSTp. The EMBO journal. 1999;18(5):1321-34.
49. Wu Y, Fan Y, Xue B, Luo L, Shen J, Zhang S, et al. Human glutathione S-transferase P1-1 interacts with TRAF2 and regulates TRAF2-ASK1 signals. Oncogene. 2006;25(42):5787-800.
50. Janssen-Heininger YM, Nolin JD, Hoffman SM, van der Velden JL, Tully JE, Lahue KG, et al. Emerging mechanisms of glutathione-dependent chemistry in biology and disease. Journal of cellular biochemistry. 2013;114(9):1962-8.
51. Hoffman S, Nolin J, McMillan D, Wouters E, Janssen-Heininger Y, Reynaert N. Thiol redox chemistry: role of protein cysteine oxidation and altered redox homeostasis in allergic inflammation and asthma. Journal of cellular biochemistry. 2015;116(6):884-92.
52. Hoffman SM, Qian X, Nolin JD, Chapman DG, Chia SB, Lahue KG, et al. Ablation of Glutaredoxin-1 Modulates House Dust Mite-Induced Allergic Airways Disease in Mice. American journal of respiratory cell and molecular biology. 2016;55(3):377-86.
53. Sohn SW, Jung JW, Lee SY, Kang HR, Park HW, Min KU, et al. Expression pattern of GSTP1 and GSTA1 in the pathogenesis of asthma. Experimental lung research. 2013;39(4-5):173-81.
54. Schroer KT, Gibson AM, Sivaprasad U, Bass SA, Ericksen MB, Wills-Karp M, et al. Downregulation of glutathione S-transferase pi in asthma contributes to enhanced oxidative stress. The Journal of allergy and clinical immunology. 2011;128(3):539-48.
55. Zhou J, Wolf CR, Henderson CJ, Cai Y, Board PG, Foster PS, et al. Glutathione transferase P1: an endogenous inhibitor of allergic responses in a mouse model of asthma. American journal of respiratory and critical care medicine. 2008;178(12):1202-10.

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

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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.

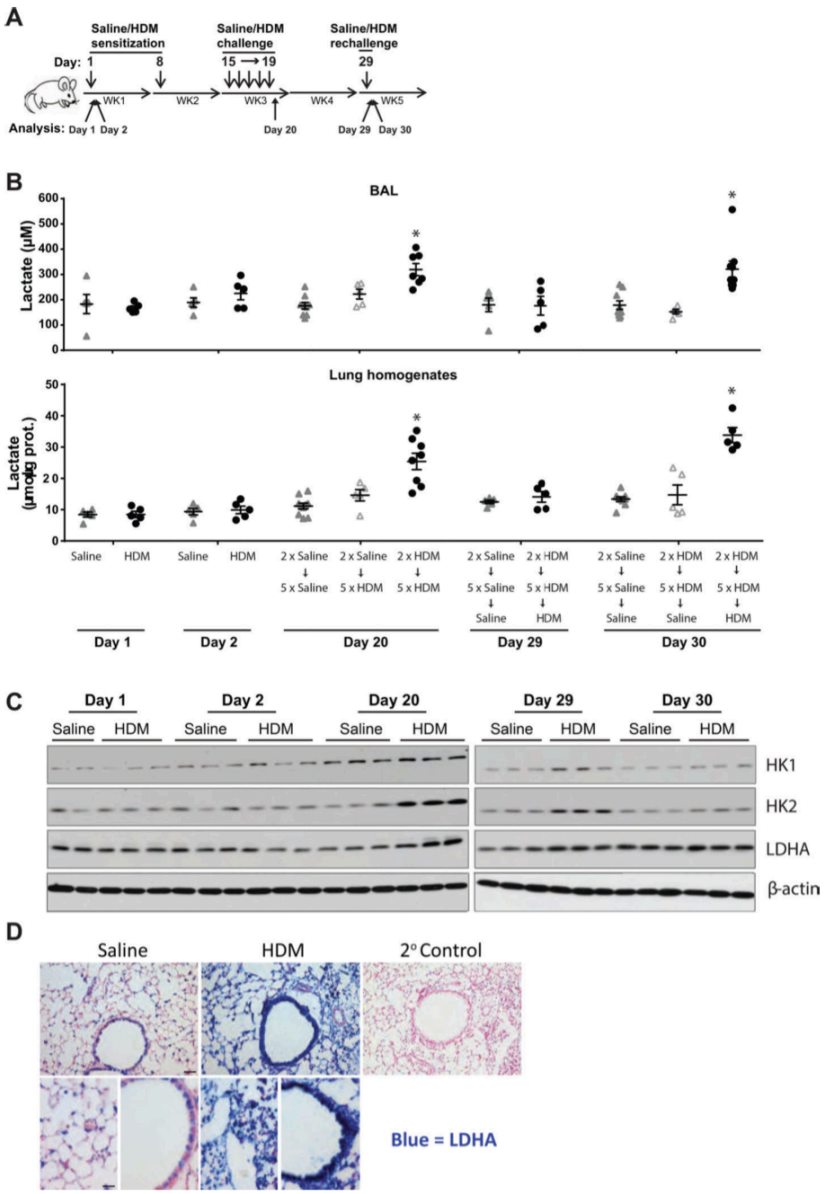


Figure 1. Evaluation of glycolysis in the lung tissues of mice exposed to house dust mite (HDM). A, Schematic depicting the dosing regimen of HDM. **B,** Lactate levels in BAL (top) and lung tissues (bottom) following a single or multiple exposures to HDM, according to the schematic in **A**. **P* < 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).

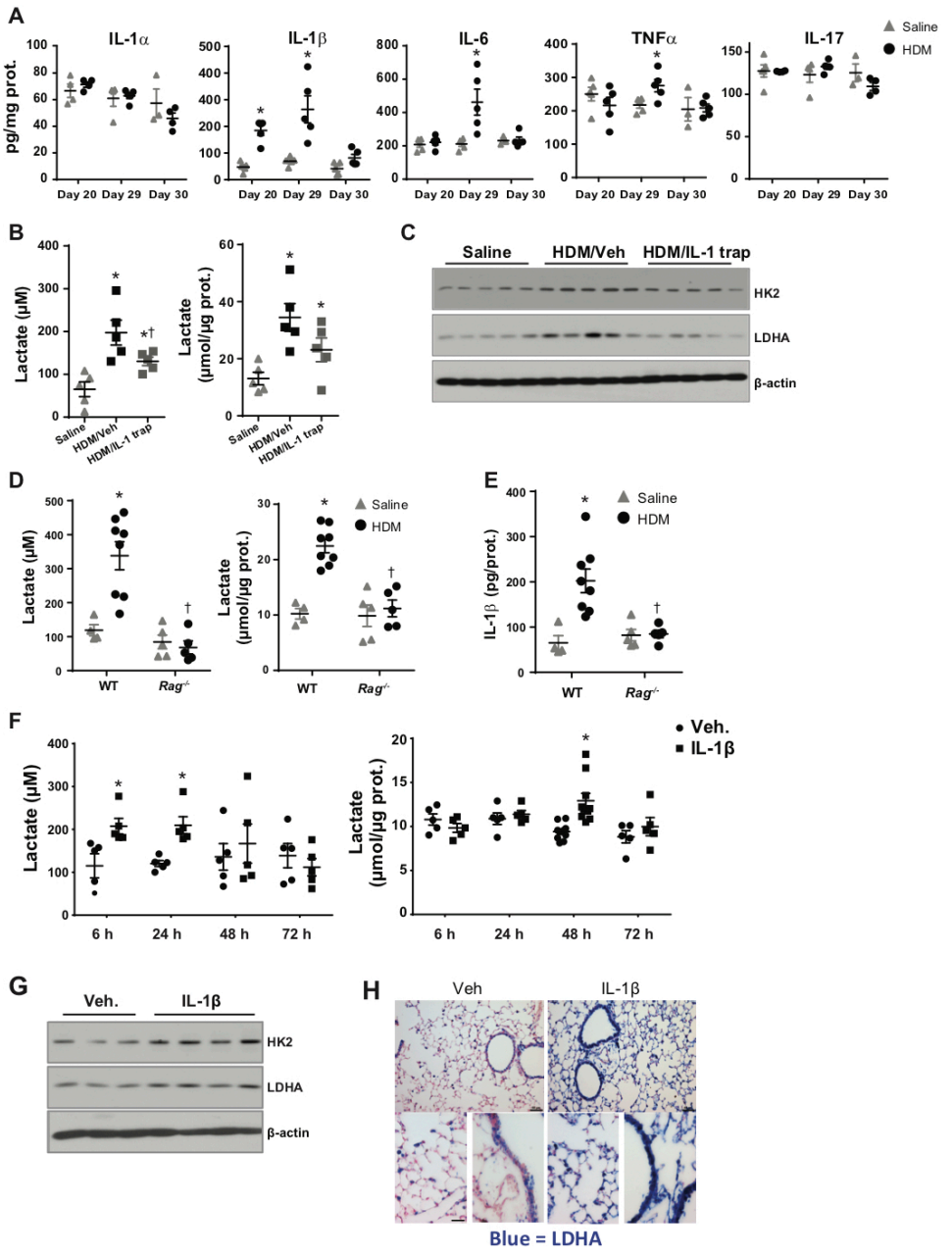


Figure 2. House dust mite (HDM)-induced T and B-cell adaptive immune responses are required for IL-1β production and resultant increases in glycolysis in lung tissues. A, Levels of pro-inflammatory cytokines in lung tissue of HDM-exposed mice at the times indicated. **P* < 0.05 compared to saline groups (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.

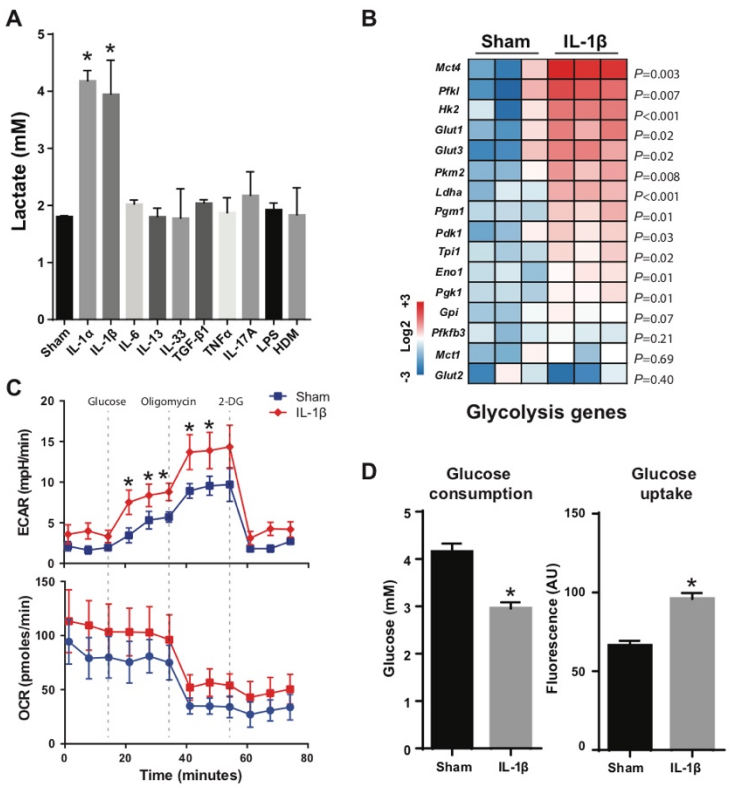


Figure 3. IL-1α/β increase lactate production, glycolysis gene expression, glucose usage, and glycolytic flux rate in primary mouse tracheal epithelial (MTE) cells. A, Lactate levels in the cell-culture supernatants of MTE cells following 24 h stimulation with IL-1α, IL-1β, IL-6, IL-13, IL-33, TGF-β1, 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.

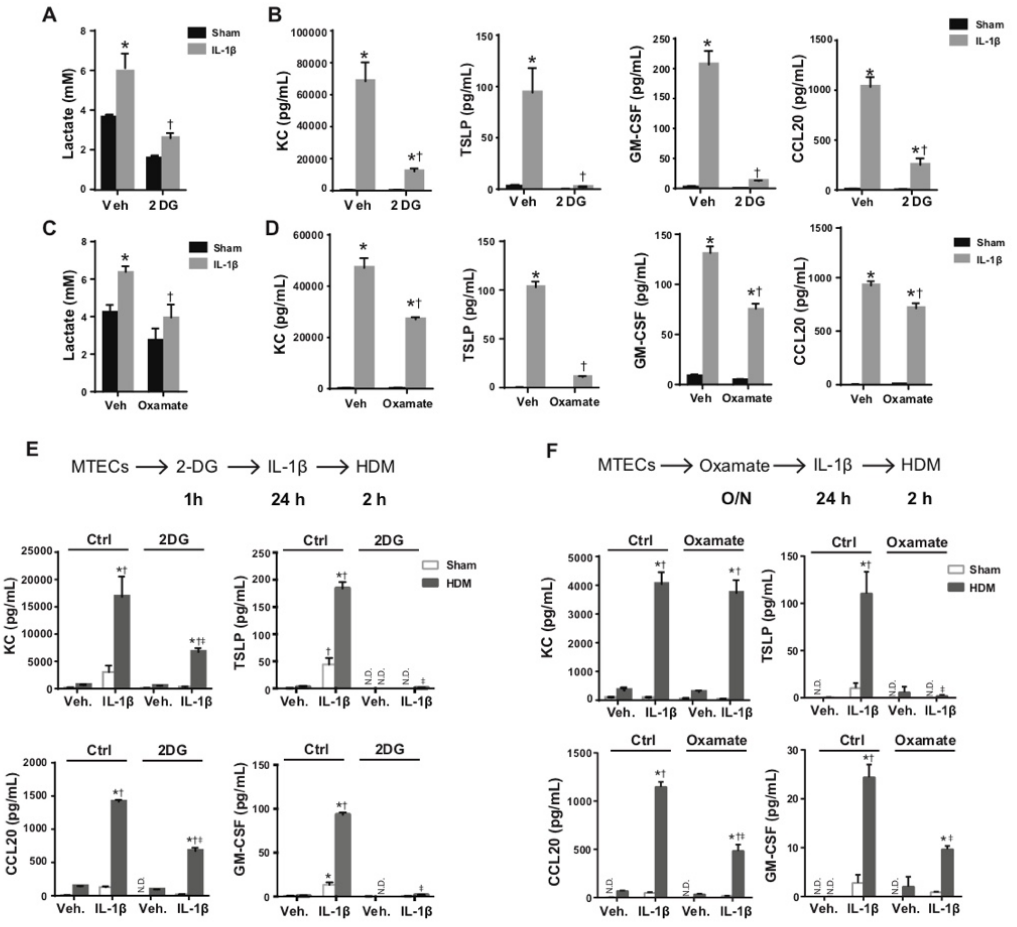


Figure 4. Importance of glycolysis for IL-1β-induced pro-inflammatory responses and the IL-1β-mediated augmentation of HDM-induced innate cytokine responses in primary mouse tracheal epithelial (MTE) cells. A-D, Lactate (**A&C**) and levels of pro-inflammatory mediators (**B&D**) in the cell culture 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.

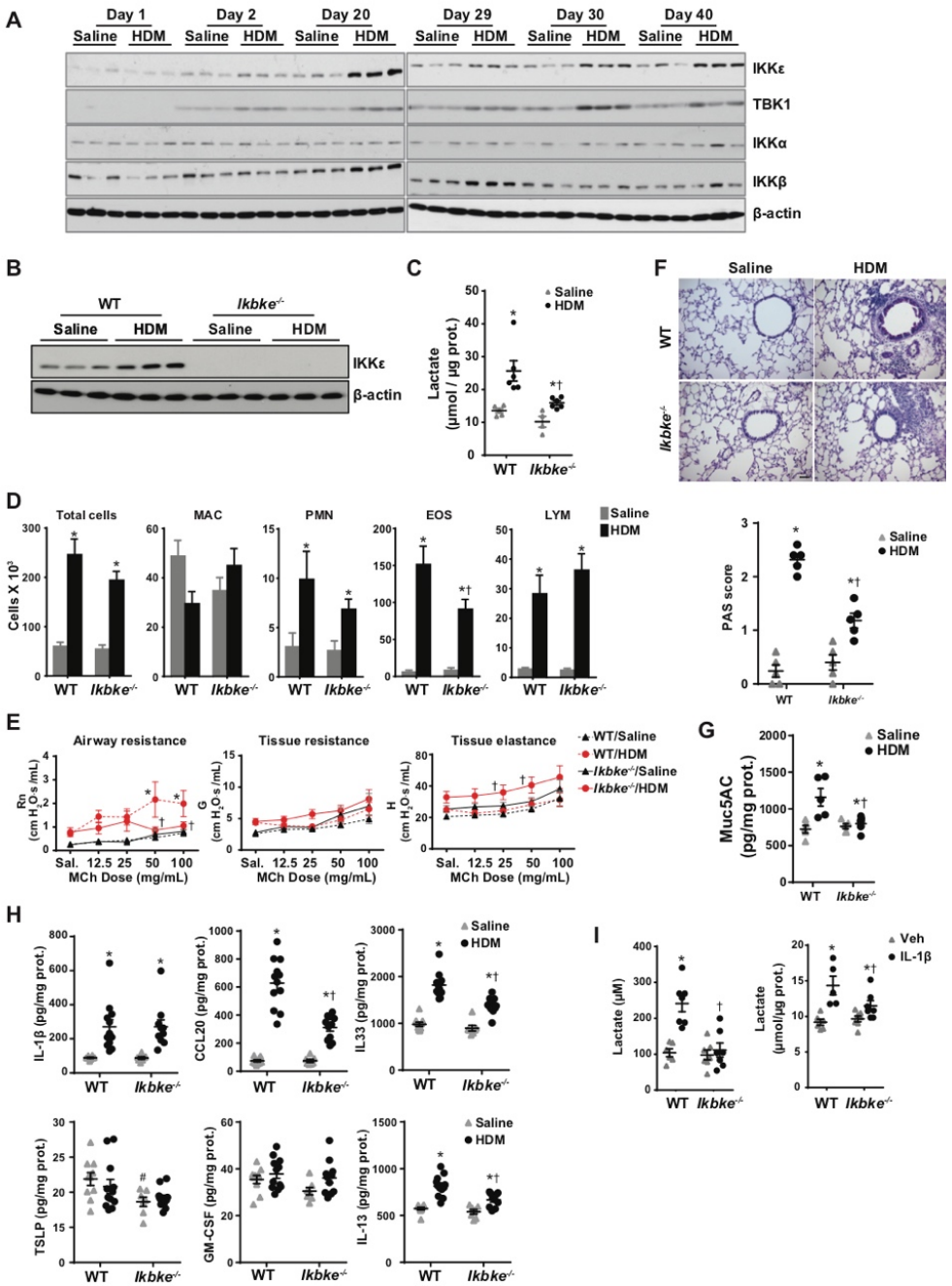


Figure 5. A causal role for Inhibitory kappa B kinase ε (IKKε) in HDM- and IL-1β-mediated increases in glycolysis and the pathogenesis of allergic airways diseases. A, Western blot analyses of IKKs in lung tissues of WT mice subjected to the HDM regimen for the indicated times. WT or *Ikbke -/-* mice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

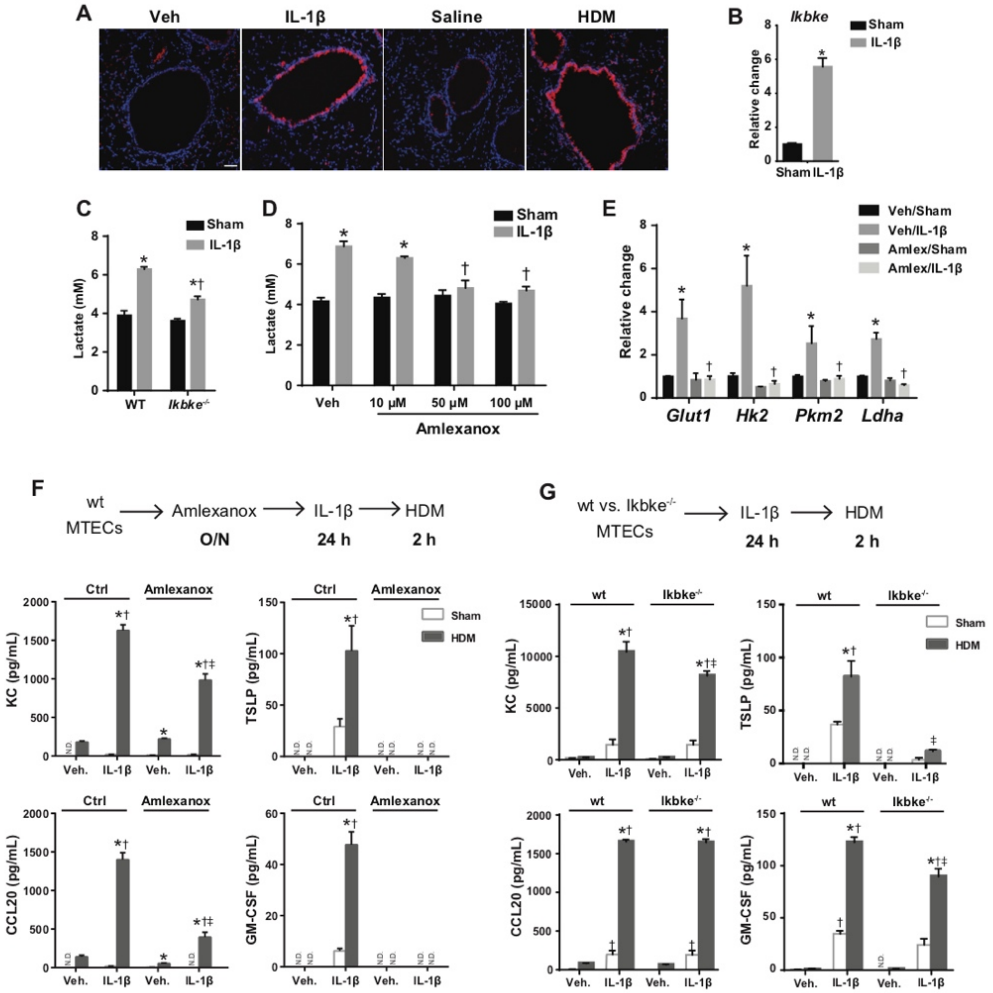


Figure 6. Inhibitory kappa B kinase ε (IKKε) is required for IL-1β-mediated increases in glycolysis, and the IL-1β-mediated augmentation of HDM-induced innate cytokine responses in MTE cells. A, Immunofluorescence analysis of IKKε in the lungs from HDM- or IL-1β-exposed mice. Red: IKK***ε***, Blue: DAPI 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.

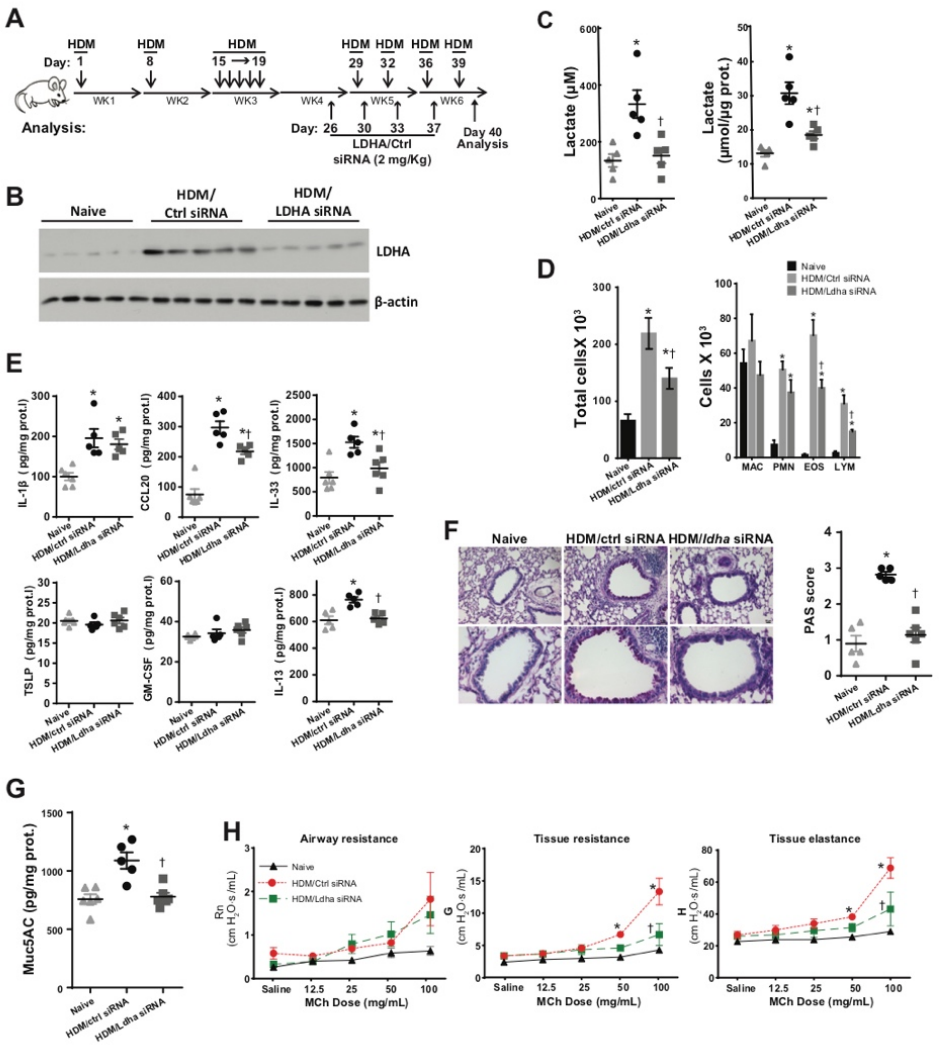


Figure 7. siRNA-mediated knockdown of *Ldha* attenuates HDM-mediated increase in glycolysis, airway inflammation, and airways hyperresponsiveness. A, Schematic depicting the dosing regimen of HDM, control (Ctrl) and *Ldha* siRNAs. At day 40, Saline-exposed mice or HDM-exposed mice treated with Ctrl siRNA 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.

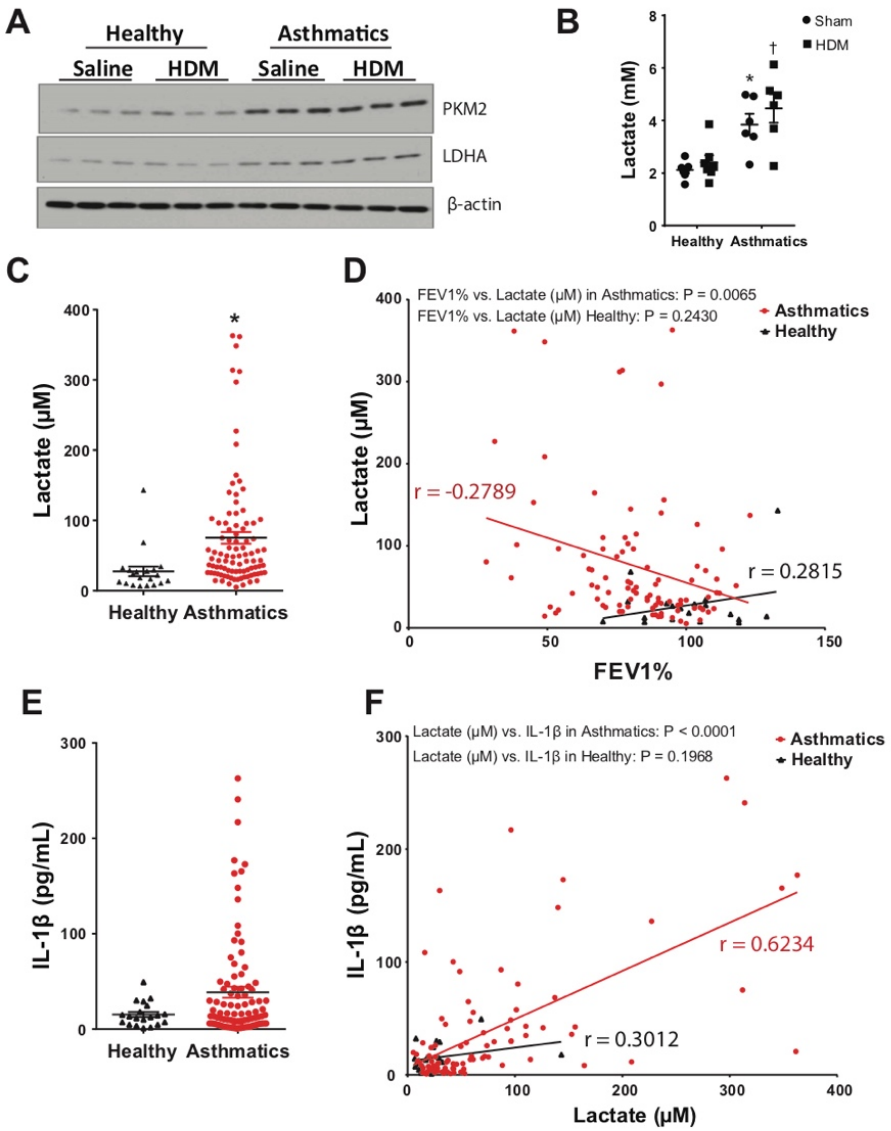


Figure 8. Evidence of increases in glycolysis in human asthma. A, Western blot analysis of PKM2 and LDHA, in saline or HDM-treated nasal cells isolated from asthmatics or healthy individuals. Data are representative of 6 healthy subjects, and 6 asthmatics **B,** Lactate content in culture supernatants 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.

REFERENCES

1. Moorman JE, Akinbami LJ, Bailey C, Zahran H, King M, Johnson C, et al. National surveillance of asthma: United States, 2001-2010. Vital & health statistics Series 3, Analytical and epidemiological studies/[US Dept of Health and Human Services, Public Health Service, National Center for Health Statistics]. 2012(35):1-67.
2. Gauthier M, Ray A, Wenzel SE. Evolving concepts of asthma. American journal of respiratory and critical care medicine. 2015;192(6):660-8.
3. Fahy JV. Type 2 inflammation in asthma [mdash] present in most, absent in many. Nature Reviews Immunology. 2015;15(1):57-65.
4. Erle DJ, Sheppard D. The cell biology of asthma. The Journal of cell biology. 2014;205(5):621-31.
5. Hotamisligil GS. Inflammation and metabolic disorders. Nature. 2006;444(7121):860-7.
6. Doherty JR, Cleveland JL. Targeting lactate metabolism for cancer therapeutics. The Journal of clinical investigation. 2013;123(9):3685-92.
7. Ward PS, Thompson CB. Metabolic reprogramming: a cancer hallmark even warburg did not anticipate. Cancer cell. 2012;21(3):297-308.
8. Nathan C, Ding A. Nonresolving inflammation. Cell. 2010;140(6):871-82.
9. O'Neill LA, Kishton RJ, Rathmell J. A guide to immunometabolism for immunologists. Nature Reviews Immunology. 2016.
10. Haas R, Smith J, Rocher-Ros V, Nadkarni S, Montero-Melendez T, D'Acquisto F, et al. Lactate regulates metabolic and pro-inflammatory circuits in control of t cell migration and effector functions. PLoS Biol. 2015;13(7):e1002202.
11. Leppänen O, Björnheden T, Evaldsson M, Borén J, Wiklund O, Levin M. ATP depletion in macrophages in the core of advanced rabbit atherosclerotic plaques in vivo. Atherosclerosis. 2006;188(2):323-30.
12. Colegio OR, Chu N-Q, Szabo AL, Chu T, Rhebergen AM, Jairam V, et al. Functional polarization of tumour-associated macrophages by tumour-derived lactic acid. Nature. 2014;513(7519):559-63.
13. Ostroukhova M, Goplen N, Karim MZ, Michalec L, Guo L, Liang Q, et al. The role of low-level lactate production in airway inflammation in asthma. American Journal of Physiology-Lung Cellular and Molecular Physiology. 2012;302(3):L300-L7.
14. Rajamäki K, Nordström T, Nurmi K, Åkerman KE, Kovanen PT, Öörni K, et al. Extracellular acidosis is a novel danger signal alerting innate immunity via the NLRP3 inflammasome. Journal of Biological Chemistry. 2013;288(19):13410-9.
15. Wike-Hooley J, Haveman J, Reinhold H. The relevance of tumour pH to the treatment of malignant disease. Radiotherapy and Oncology. 1984;2(4):343-66.
16. Tully JE, Hoffman SM, Lahue KG, Nolin JD, Anathy V, Lundblad LK, et al. Epithelial NF-κB orchestrates house dust mite–induced airway inflammation, hyperresponsiveness, and fibrotic remodeling. The Journal of Immunology. 2013;191(12):5811-21.
17. Doherty JR, Cleveland JL. Targeting lactate metabolism for cancer therapeutics. The Journal of clinical investigation. 2013;123(9):3685.
18. Ather JL, Foley KL, Suratt BT, Boyson JE, Poynter ME. Airway epithelial NF-κB activation promotes the ability to overcome inhalational antigen tolerance. Clinical & Experimental Allergy. 2015;45(7):1245-58.
19. Mombaerts P, Iacomini J, Johnson RS, Herrup K, Tonegawa S, Papaioannou VE. RAG-1-deficient mice have no mature B and T lymphocytes. Cell. 1992;68(5):869-77.
20. Hoffman SM, Chapman DG, Lahue KG, Cahoon JM, Rattu GK, Daphtary N, et al. Protein disulfide isomerase–endoplasmic reticulum resident protein 57 regulates allergen-induced airways inflammation, fibrosis, and hyperresponsiveness. Journal of Allergy and Clinical Immunology. 2016;137(3):822-32. e7.
21. Everts B, Amiel E, Huang SC-C, Smith AM, Chang C-H, Lam WY, et al. TLR-driven early glycolytic reprogramming via the kinases TBK1-IKK [epsiv] supports the anabolic demands of dendritic cell activation. Nature immunology. 2014;15(4):323-32.

22. Krawczyk CM, Holowka T, Sun J, Blagih J, Amiel E, DeBerardinis RJ, et al. Toll-like receptor–induced changes in glycolytic metabolism regulate dendritic cell activation. Blood. 2010;115(23):4742-9.
23. Pantano C, Ather JL, Alcorn JF, Poynter ME, Brown AL, Guala AS, et al. Nuclear factor-κB activation in airway epithelium induces inflammation and hyperresponsiveness. American journal of respiratory and critical care medicine. 2008;177(9):959-69.
24. Ather JL, Hodgkins SR, Janssen-Heininger YM, Poynter ME. Airway epithelial NF-κB activation promotes allergic sensitization to an innocuous inhaled antigen. American journal of respiratory cell and molecular biology. 2011;44(5):631-8.
25. Gulen MF, Bulek K, Xiao H, Yu M, Gao J, Sun L, et al. Inactivation of the enzyme GSK3α by the kinase IKKi promotes AKT-mTOR signaling pathway that mediates interleukin-1-induced Th17 cell maintenance. Immunity. 2012;37(5):800-12.
26. Tully JE, Nolin JD, Guala AS, Hoffman SM, Roberson EC, Lahue KG, et al. Cooperation between classical and alternative NF-κB pathways regulates proinflammatory responses in epithelial cells. American journal of respiratory cell and molecular biology. 2012;47(4):497-508.
27. Reilly SM, Chiang S-H, Decker SJ, Chang L, Uhm M, Larsen MJ, et al. An inhibitor of the protein kinases TBK1 and IKK-[epsiv] improves obesity-related metabolic dysfunctions in mice. Nature medicine. 2013;19(3):313-21.
28. Shirai T, Nazarewicz RR, Wallis BB, Yanes RE, Watanabe R, Hilhorst M, et al. The glycolytic enzyme PKM2 bridges metabolic and inflammatory dysfunction in coronary artery disease. The Journal of experimental medicine. 2016;213(3):337-54.
29. Park M-J, Lee SH, Lee S-H, Lee E-J, Kim E-K, Choi JY, et al. IL-1 Receptor Blockade Alleviates Graft-versus-Host Disease through Downregulation of an Interleukin-1-Dependent Glycolytic Pathway in Th17 Cells. Mediators of inflammation. 2015;2015.
30. Taneja N, Coy PE, Lee I, Bryson JM, Robey RB. Proinflammatory interleukin-1 cytokines increase mesangial cell hexokinase activity and hexokinase II isoform abundance. American Journal of Physiology-Cell Physiology. 2004;287(2):C548-C57.
31. Garlanda C, Dinarello CA, Mantovani A. The interleukin-1 family: back to the future. Immunity. 2013;39(6):1003-18.
32. Broide DH, Campbell K, Gifford T, Sriramarao P. Inhibition of eosinophilic inflammation in allergen-challenged, IL-1 receptor type 1–deficient mice is associated with reduced eosinophil rolling and adhesion on vascular endothelium. Blood. 2000;95(1):263-9.
33. Schmitz N, Kurrer M, Kopf M. The IL-1 receptor 1 is critical for Th2 cell type airway immune responses in a mild but not in a more severe asthma model. European journal of immunology. 2003;33(4):991-1000.
34. Nakae S, Komiyama Y, Yokoyama H, Nambu A, Umeda M, Iwase M, et al. IL-1 is required for allergen-specific Th2 cell activation and the development of airway hypersensitivity response. International immunology. 2003;15(4):483-90.
35. Whelan R, Kim C, Chen M, Leiter J, Grunstein M, Hakonarson H. Role and regulation of interleukin-1 molecules in pro-asthmatic sensitised airway smooth muscle. European Respiratory Journal. 2004;24(4):559-67.
36. Willart MA, Deswarte K, Pouliot P, Braun H, Beyaert R, Lambrecht BN, et al. Interleukin-1α controls allergic sensitization to inhaled house dust mite via the epithelial release of GM-CSF and IL-33. The Journal of experimental medicine. 2012;209(8):1505-17.
37. Johnson VJ, Yucesoy B, Luster MI. Prevention of IL-1 signaling attenuates airway hyperresponsiveness and inflammation in a murine model of toluene diisocyanate–induced asthma. Journal of allergy and clinical immunology. 2005;116(4):851-8.
38. Jantsch J, Chakravortty D, Turza N, Prechtel AT, Buchholz B, Gerlach RG, et al. Hypoxia and hypoxia-inducible factor-1α modulate lipopolysaccharide-induced dendritic cell activation and function. The Journal of Immunology. 2008;180(7):4697-705.
39. Donnelly RP, Loftus RM, Keating SE, Liou KT, Biron CA, Gardiner CM, et al. mTORC1-dependent metabolic reprogramming is a prerequisite for NK cell effector function. The Journal of Immunology. 2014;193(9):4477-84.

40. Michalek RD, Gerriets VA, Jacobs SR, Macintyre AN, MacIver NJ, Mason EF, et al. Cutting edge: distinct glycolytic and lipid oxidative metabolic programs are essential for effector and regulatory CD4+ T cell subsets. The Journal of Immunology. 2011;186(6):3299-303.
41. Doughty CA, Bleiman BF, Wagner DJ, Dufort FJ, Mataraza JM, Roberts MF, et al. Antigen receptor–mediated changes in glucose metabolism in B lymphocytes: role of phosphatidylinositol 3-kinase signaling in the glycolytic control of growth. Blood. 2006;107(11):4458-65.
42. Kouzaki H, Iijima K, Kobayashi T, O'Grady SM, Kita H. The danger signal, extracellular ATP, is a sensor for an airborne allergen and triggers IL-33 release and innate Th2-type responses. The Journal of Immunology. 2011;186(7):4375-87.
43. Moon J-S, Hisata S, Park M-A, DeNicola GM, Ryter SW, Nakahira K, et al. mTORC1-Induced HK1-Dependent glycolysis regulates NLRP3 inflammasome activation. Cell reports. 2015;12(1):102-15.
44. Pugin J, Ricou B, Steinberg KP, Suter PM, Martin TR. Proinflammatory activity in bronchoalveolar lavage fluids from patients with ARDS, a prominent role for interleukin-1. Am J Respir Crit Care Med. 1996;153(6 Pt 1):1850-6.
45. Pauwels NS, Bracke KR, Dupont LL, Van Pottelberge GR, Provoost S, Vanden Berghe T, et al. Role of IL-1alpha and the Nlrp3/caspase-1/IL-1beta axis in cigarette smoke-induced pulmonary inflammation and COPD. The European respiratory journal. 2011;38(5):1019-28.
46. Rogliani P, Calzetta L, Ora J, Matera MG. Canakinumab for the treatment of chronic obstructive pulmonary disease. Pulmonary pharmacology & therapeutics. 2015;31:15-27.
47. Peebles RS, Jr. Is IL-1beta inhibition the next therapeutic target in asthma? The Journal of allergy and clinical immunology. 2017.
48. Kim RY, Pinkerton JW, Essilfie AT, Robertson AA, Baines KJ, Brown AC, et al. Role for NLRP3 Inflammasome-mediated, IL-1beta-dependent Responses in Severe, Steroid-resistant Asthma. Am J Respir Crit Care Med. 2017.
49. Liu W, Liu S, Verma M, Zafar I, Good JT, Rollins D, et al. Mechanism of TH2/TH17-predominant and neutrophilic TH2/TH17-low subtypes of asthma. The Journal of allergy and clinical immunology. 2017;139(5):1548-58 e4.
50. Rodrigo G, Rodrigo C. Elevated plasma lactate level associated with high dose inhaled albuterol therapy in acute severe asthma. Emergency Medicine Journal. 2005;22(6):404-8.
51. Lewis LM, Ferguson I, House SL, Aubuchon K, Schneider J, Johnson K, et al. Albuterol administration is commonly associated with increases in serum lactate in patients with asthma treated for acute exacerbation of asthma. CHEST Journal. 2014;145(1):53-9.
52. Dodda V, Spiro P. Albuterol, an uncommonly recognized culprit in lactic acidosis. CHEST Journal. 2011;140(4_MeetingAbstracts):183A-A.
53. Peng M, Yin N, Chhangawala S, Xu K, Leslie CS, Li MO. Aerobic glycolysis promotes T helper 1 cell differentiation through an epigenetic mechanism. Science. 2016;354(6311):481-4.
54. Baggstrom MQ, Qi Y, Koczywas M, Argiris A, Johnson EA, Millward MJ, et al. A phase II study of AT-101 (Gossypol) in chemotherapy-sensitive recurrent extensive-stage small cell lung cancer. Journal of Thoracic Oncology. 2011;6(10):1757-60.
55. Polański R, Hodgkinson CL, Fusi A, Nonaka D, Priest L, Kelly P, et al. Activity of the monocarboxylate transporter 1 inhibitor AZD3965 in small cell lung cancer. Clinical Cancer Research. 2014;20(4):926-37.

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

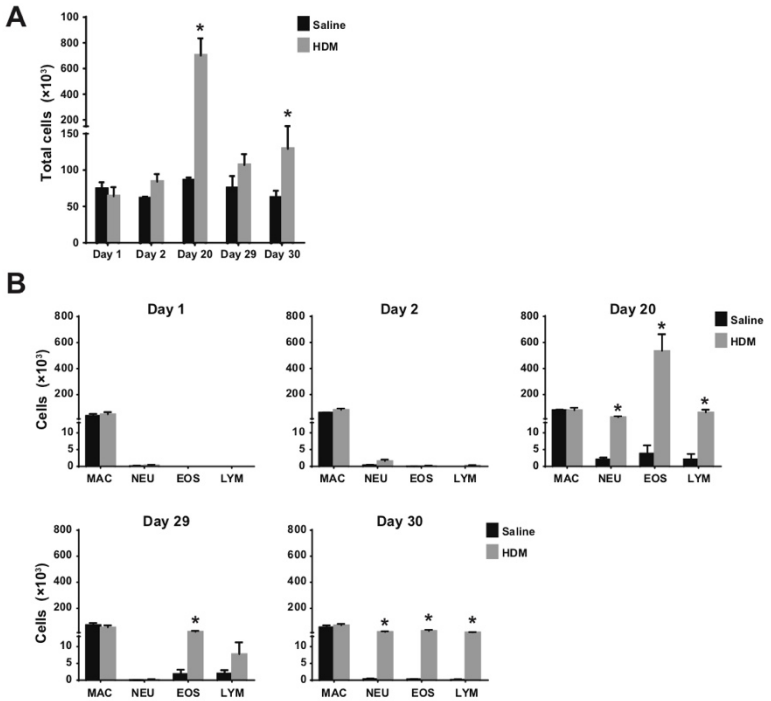


Figure E1: Airway inflammation in mice exposed to house dust mite (HDM). (**A**) Total and (**B**) differential cell counts in bronchoalveolar lavage (BAL) in response to saline or HDM, (see schematic in **Figure 1A)**. Data are expressed as means (± SEM) (n = 5 mice per group). *p < 0.05 (Student's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

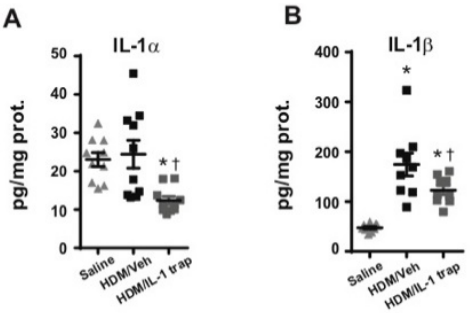


Figure E2: Assessment of levels of IL-1α and IL-1β in lung tissues from mice subjected to vehicle control or IL1 Trap. Mice were challenged with HDM on days 15, 16, 17, and 18, and received 5 mg/kg of IL-1 Trap or vehicle on days 14 and 17 intraperitoneally. Levels of IL-1α and IL-1β in lung tissues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

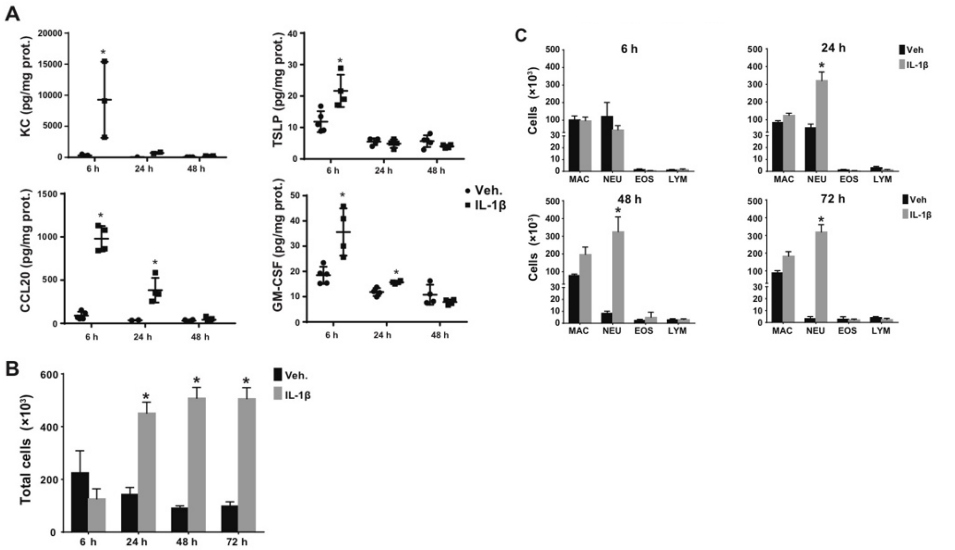


Figure E3: Evaluation of pro-inflammatory mediators and airway inflammation in mice exposed to interleukin (IL)-1β. **A**: Lung tissue levels of KC, TSLP, CCL20 and GM-CSF following intranasal administration of IL-1β or vehicle. Total (**B**) and differential cell counts (**C**) in BAL from the mice 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.

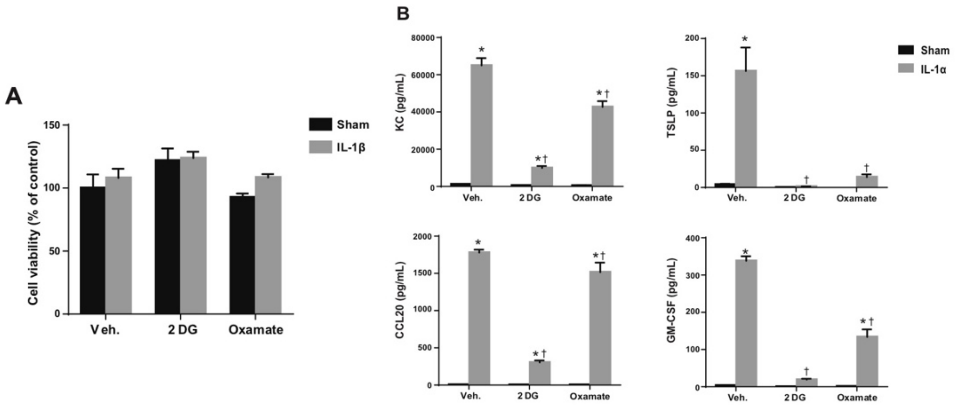
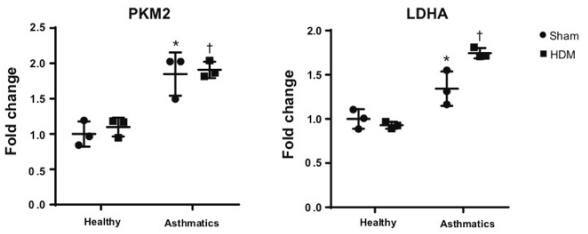


Figure E4: A: Assessment of viability following exposure to MTE cells to oxamate or 2-deoxyglucose. Epithelial cells were exposed to IL-1β in the presence or absence of inhibitors. Cell survival was evaluated via crystal violet staining of cells. Results were expressed as % survival compared to untreated 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

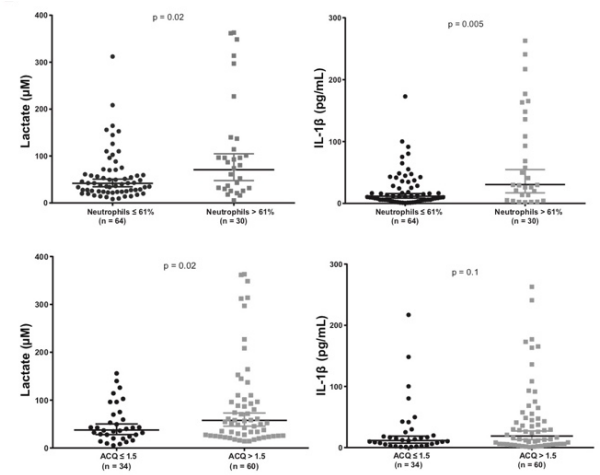


Figure E5: A: Quantification of Western blots shown in Figure 8A. Data reflects n=3 healthy subjects and n=3 asthmatics. Data were normalized to β-actin and are expressed as fold change from healthy sham controls *P < 0.05 compared to the sham healthy group. †P < 0.05 compared to HDM healthy group (ANOVA). **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'

REFERENCES

1. Manise M, Holtappels G, Van Crombruggen K, Schleich F, Bachert C, Louis R. Sputum IgE and cytokines in asthma: relationship with sputum cellular profile. PloS one. 2013;8(3):e58388.
2. Delvaux M, Henket M, Lau L, Kange P, Bartsch P, Djukanovic R, et al. Nebulised salbutamol administered during sputum induction improves bronchoprotection in patients with asthma. Thorax. 2004;59(2):111-5.
3. Maes T, Cobos FA, Schleich F, Sorbello V, Henket M, De Preter K, et al. Asthma inflammatory phenotypes show differential microRNA expression in sputum. Journal of Allergy and Clinical Immunology. 2016;137(5):1433-46.
4. Müller L, Brighton LE, Carson JL, Fischer WA. Culturing of human nasal epithelial cells at the air liquid interface. Journal of visualized experiments: JoVE. 2013(80).
5. Dixon AE, Sugar EA, Zinreich SJ, Slavin RG, Corren J, Naclerio RM, et al. Criteria to screen for chronic sinonasal disease. CHEST Journal. 2009;136(5):1324-32.
6. Wu R, Smith D. Continuous multiplication of rabbit tracheal epithelial cells in a defined, hormone-supplemented medium. In vitro. 1982;18(9):800-12.
7. Alcorn JF, Guala AS, van der Velden J, McElhinney B, Irvin CG, Davis RJ, et al. Jun N-terminal kinase 1 regulates epithelial-to-mesenchymal transition induced by TGF-β1. Journal of cell science. 2008;121(7):1036-45.
8. Van Tassell BW, Varma A, Salloum FN, Das A, Seropian IM, Toldo S, et al. Interleukin-1 trap attenuates cardiac remodeling after experimental acute myocardial infarction in mice. Journal of cardiovascular pharmacology. 2010;55(2):117-22.
9. Tomioka S, Bates JH, Irvin CG. Airway and tissue mechanics in a murine model of asthma: alveolar capsule vs. forced oscillations. Journal of Applied Physiology. 2002;93(1):263-70.
10. Riesenfeld E, Allen GB, Bates JH, Poynter ME, Wu M, Aimiand S, et al. The temporal evolution of airways hyperresponsiveness and inflammation. Journal of allergy & therapy. 2012;1(5):1.
11. Tully JE, Hoffman SM, Lahue KG, Nolin JD, Anathy V, Lundblad LK, et al. Epithelial NF-κB orchestrates house dust mite–induced airway inflammation, hyperresponsiveness, and fibrotic remodeling. The Journal of Immunology. 2013;191(12):5811-21.
12. Anathy V, Aesif SW, Hoffman SM, Bement JL, Guala AS, Lahue KG, et al. Glutaredoxin-1 attenuates S-glutathionylation of the death receptor fas and decreases resolution of Pseudomonas aeruginosa pneumonia. American journal of respiratory and critical care medicine. 2014;189(4):463- 74.

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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.

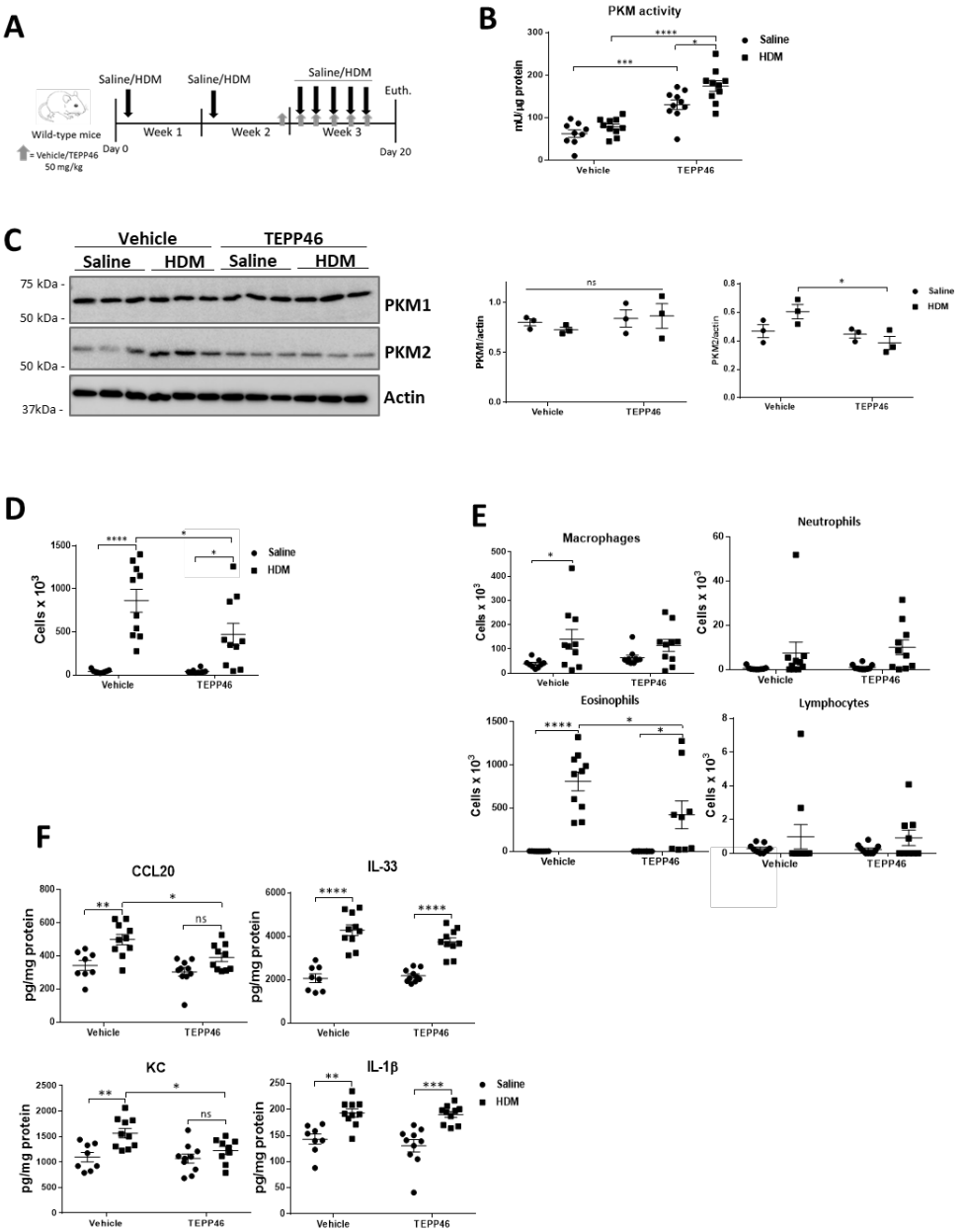

Figure 1: Activation of PKM2 by TEPP46 attenuates pro-inflammatory cytokines in mice with HDM-induced allergic airway disease. A, Schematic depicting the exposure regimen. Mice were sensitized twice with 10 µg of HDM or saline on days 1, and 8. Mice were treated with 50 mg/kg TEPP46 intraperitoneally 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.

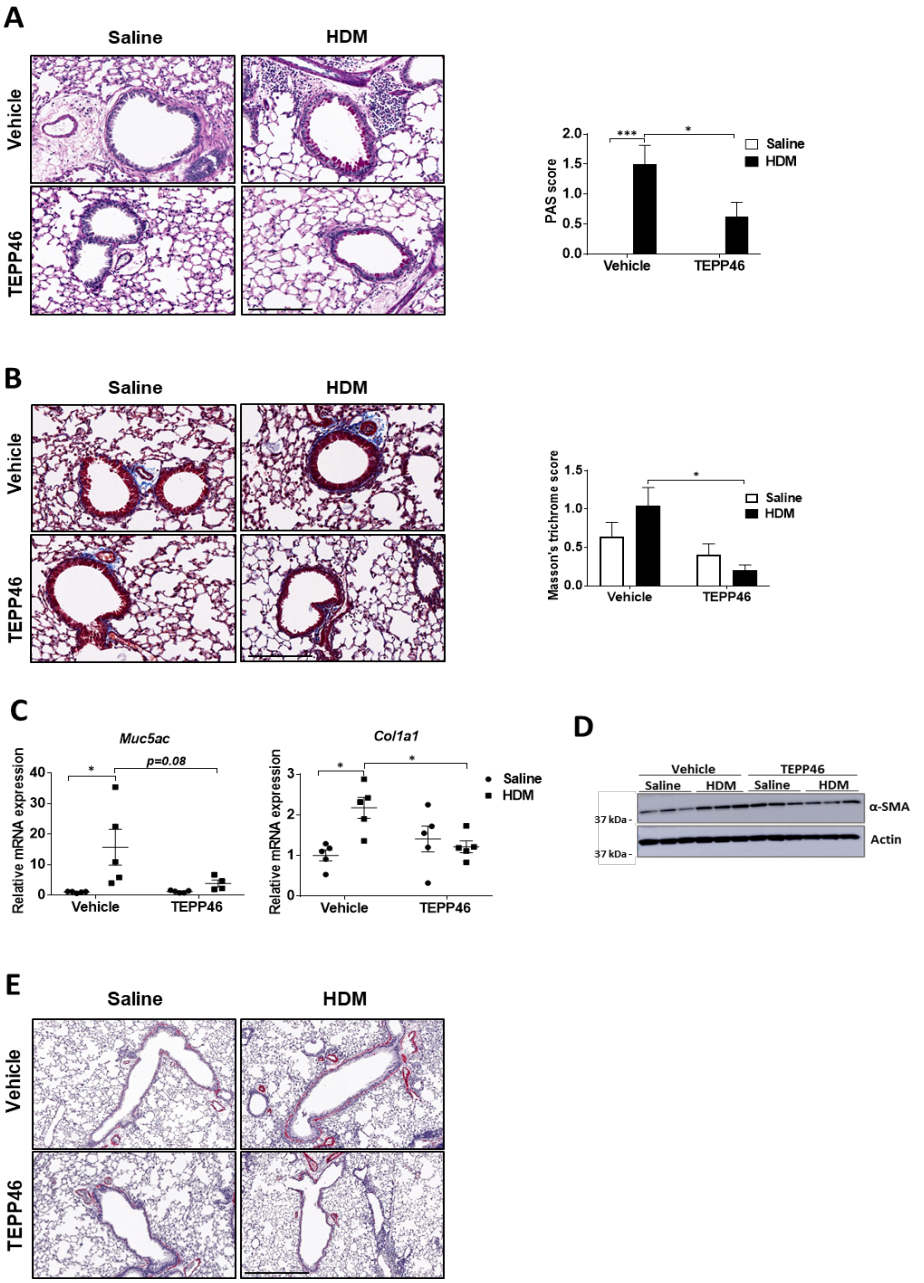

Figure 2: Activation of PKM2 by TEPP46 attenuates mucus metaplasia, subepithelial collagen, and markers of airway remodeling in mice with HDM-induced allergic airway disease. A, Assessment and quantification of mucus metaplasia by PAS staining intensity and **B,** Collagen deposition by Masson's trichrome 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.

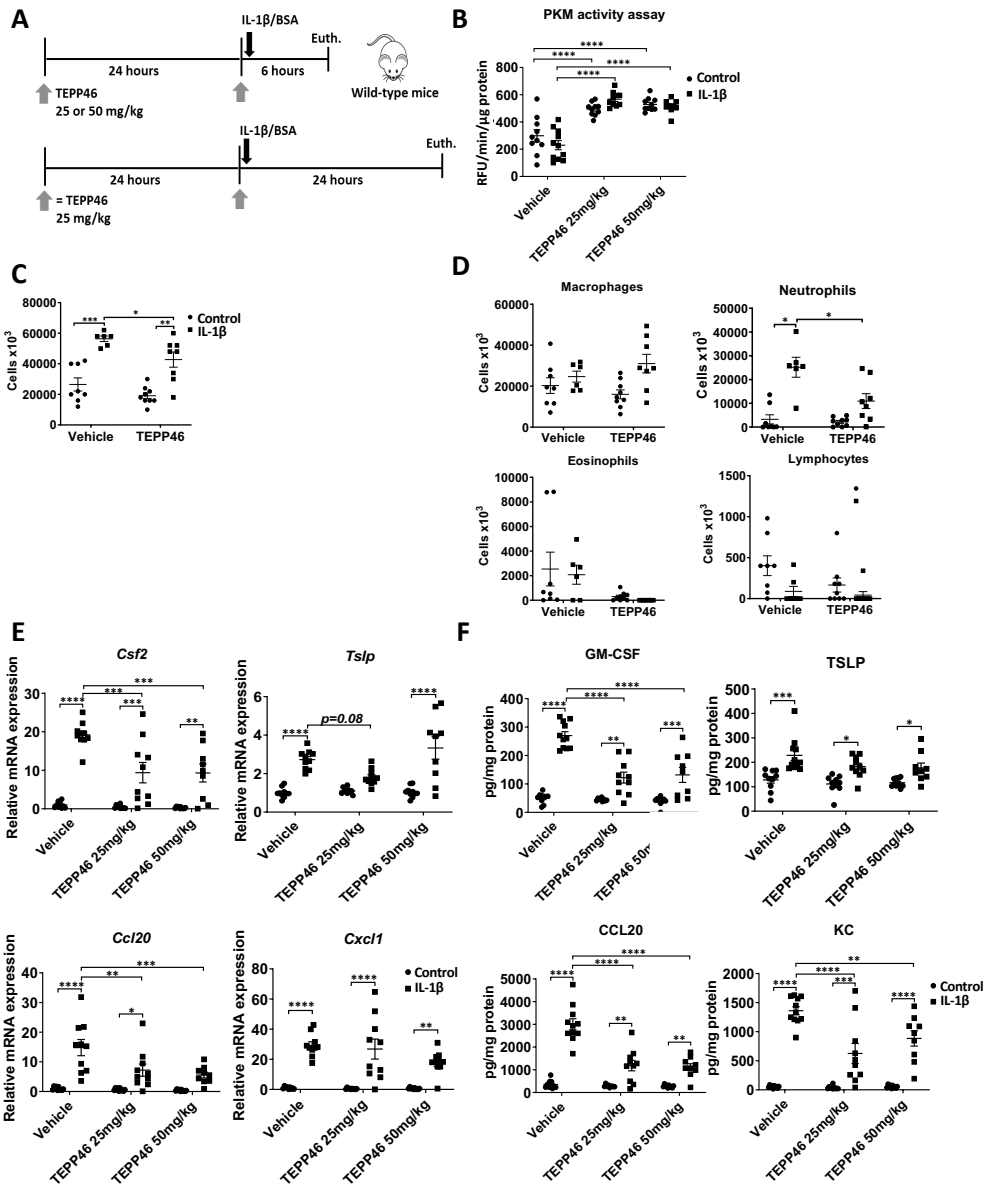

Figure 3: PKM2 activation attenuates the release of pro-inflammatory cytokines following intranasal administration of IL-1β. **A,** Schematic depicting the pre-treatment with 25 or 50 mg/kg TEPP46 prior to intranasal administration of 1 µg of IL-1β for either 6 or 24 hours. The total cell count and cell 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).

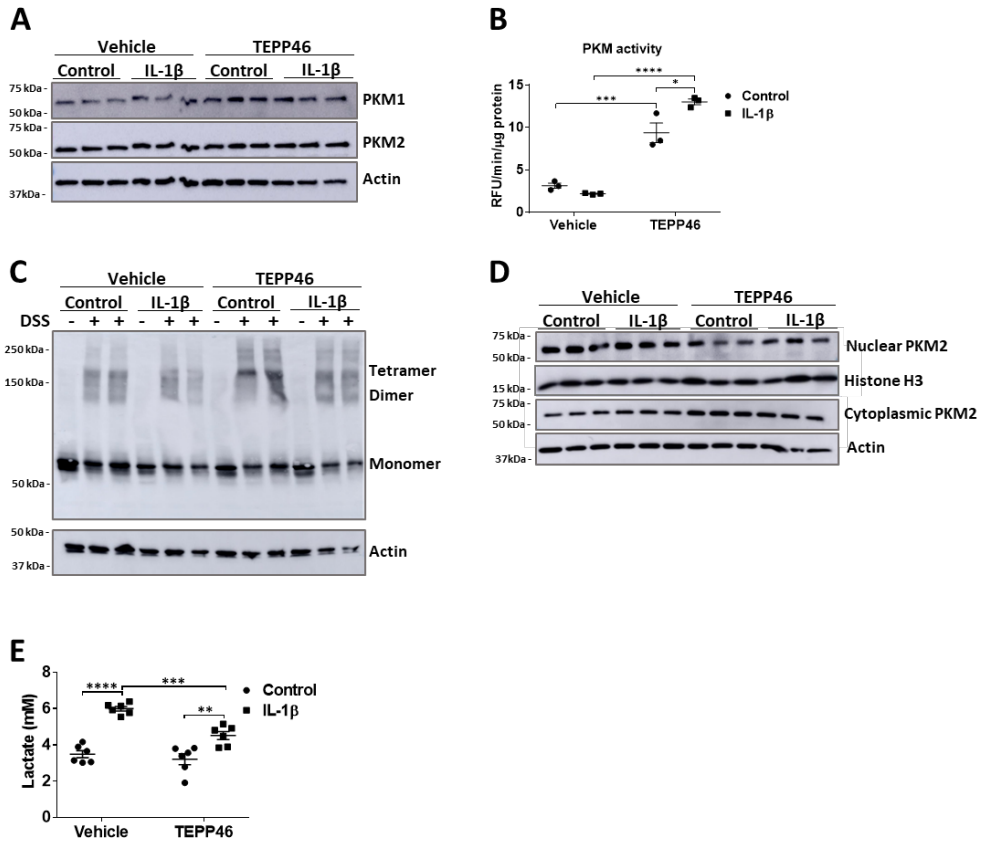

Figure 4: TEPP46 augments PKM activity, and PKM2's cytosolic presence and attenuates interleukin-1β-mediated lactate secretion in primary MTE cells. MTE cells were treated with 100 μM TEPP46 for 1 hour prior to stimulation with 10 ng/mL IL-1β for 24 hours. **A,** Representative western blot of total 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.

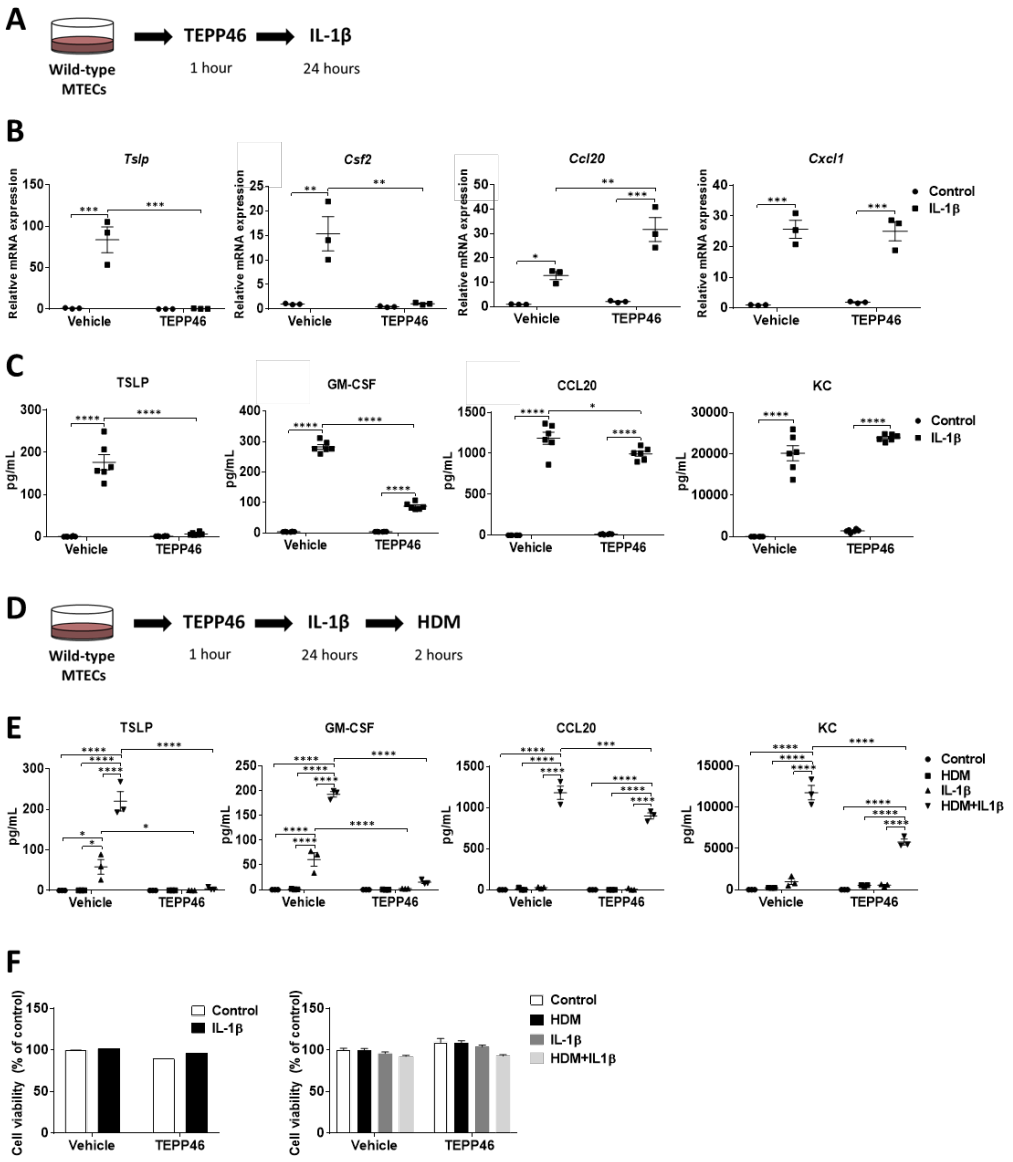

Figure 5: Activation of PKM2 attenuates IL-1β-mediated pro-inflammatory responses in primary MTE cells and the release of pro-inflammatory mediators following subsequent exposure to HDM. A, Schematic depicting the pre-treatment with 100 μM TEPP46 followed by stimulation of 10 ng/mL IL-1β for 24 hours. **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.

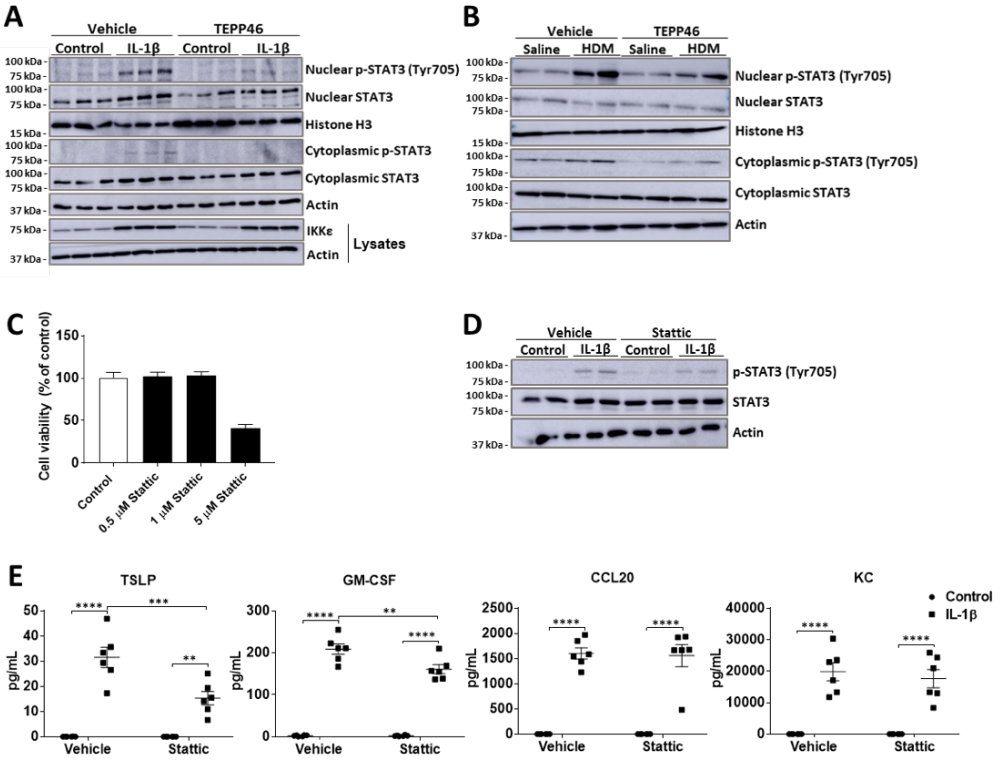

Figure 6: PKM2-mediated phosphorylation of STAT3 contributes to IL-1β-mediated pro-inflammatory signaling in epithelial cells. **A,** Representative western blots of total and phosphorylated STAT3 in nuclear and cytosolic extracts from MTE cells, and total IKKε levels in whole cell lysates. **B,** Representative 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.

REFERENCES

1. Fahy, J. V. 2015. Type 2 inflammation in asthma--present in most, absent in many. Nature reviews. Immunology 15: 57-65.
2. Domblides, C., L. Lartigue, and B. Faustin. 2018. Metabolic Stress in the Immune Function of T Cells, Macrophages and Dendritic Cells. Cells 7.
3. Qian, X., R. Aboushousha, C. van de Wetering, S. B. Chia, E. Amiel, R. W. Schneider, J. L. J. van der Velden, K. G. Lahue, D. A. Hoagland, D. T. Casey, N. Daphtary, J. L. Ather, M. J. Randall, M. Aliyeva, K. E. Black, D. G. Chapman, L. K. A. Lundblad, D. H. McMillan, A. E. Dixon, V. Anathy, C. G. Irvin, M. E. Poynter, E. F. M. Wouters, P. M. Vacek, M. Henket, F. Schleich, R. Louis, A. van der Vliet, and Y. M. W. Janssen-Heininger. 2018. IL-1/inhibitory kappaB kinase epsilon-induced glycolysis augment epithelial effector function and promote allergic airways disease. The Journal of allergy and clinical immunology 142: 435-450.e410.
4. Doherty, J. R., and J. L. Cleveland. 2013. Targeting lactate metabolism for cancer therapeutics. The Journal of clinical investigation 123: 3685-3692.
5. Ward, P. S., and C. B. Thompson. 2012. Metabolic reprogramming: a cancer hallmark even warburg did not anticipate. Cancer cell 21: 297-308.
6. Noguchi, T., H. Inoue, and T. Tanaka. 1986. The M1- and M2-type isozymes of rat pyruvate kinase are produced from the same gene by alternative RNA splicing. The Journal of biological chemistry 261: 13807-13812.
7. Noguchi, T., K. Yamada, H. Inoue, T. Matsuda, and T. Tanaka. 1987. The L- and R-type isozymes of rat pyruvate kinase are produced from a single gene by use of different promoters. The Journal of biological chemistry 262: 14366-14371.
8. Mazurek, S. 2011. Pyruvate kinase type M2: a key regulator of the metabolic budget system in tumor cells. The international journal of biochemistry & cell biology 43: 969-980.
9. Christofk, H. R., M. G. Vander Heiden, M. H. Harris, A. Ramanathan, R. E. Gerszten, R. Wei, M. D. Fleming, S. L. Schreiber, and L. C. Cantley. 2008. The M2 splice isoform of pyruvate kinase is important for cancer metabolism and tumour growth. Nature 452: 230-233.
10. Anastasiou, D., Y. Yu, W. J. Israelsen, J. K. Jiang, M. B. Boxer, B. S. Hong, W. Tempel, S. Dimov, M. Shen, A. Jha, H. Yang, K. R. Mattaini, C. M. Metallo, B. P. Fiske, K. D. Courtney, S. Malstrom, T. M. Khan, C. Kung, A. P. Skoumbourdis, H. Veith, N. Southall, M. J. Walsh, K. R. Brimacombe, W. Leister, S. Y. Lunt, Z. R. Johnson, K. E. Yen, K. Kunii, S. M. Davidson, H. R. Christofk, C. P. Austin, J. Inglese, M. H. Harris, J. M. Asara, G. Stephanopoulos, F. G. Salituro, S. Jin, L. Dang, D. S. Auld, H. W. Park, L. C. Cantley, C. J. Thomas, and M. G. Vander Heiden. 2012. Pyruvate kinase M2 activators promote tetramer formation and suppress tumorigenesis. Nature chemical biology 8: 839-847.
11. Gao, X., H. Wang, J. J. Yang, X. Liu, and Z. R. Liu. 2012. Pyruvate kinase M2 regulates gene transcription by acting as a protein kinase. Molecular cell 45: 598-609.
12. Alves-Filho, J. C., and E. M. Palsson-McDermott. 2016. Pyruvate Kinase M2: A Potential Target for Regulating Inflammation. Frontiers in immunology 7: 145.
13. Demaria, M., and V. Poli. 2012. PKM2, STAT3 and HIF-1alpha: The Warburg's vicious circle. Jak-stat 1: 194-196.
14. Hu, K., Y. Yang, L. Lin, Q. Ai, J. Dai, K. Fan, P. Ge, R. Jiang, J. Wan, and L. Zhang. 2018. Caloric Restriction Mimetic 2-Deoxyglucose Alleviated Inflammatory Lung Injury via Suppressing Nuclear Pyruvate Kinase M2-Signal Transducer and Activator of Transcription 3 Pathway. Frontiers in immunology 9: 426.
15. Wu, R., and D. Smith. 1982. Continuous multiplication of rabbit tracheal epithelial cells in a defined, hormone-supplemented medium. In Vitro 18: 800-812.
16. Alcorn, J. F., A. S. Guala, J. van der Velden, B. McElhinney, C. G. Irvin, R. J. Davis, and Y. M. Janssen-Heininger. 2008. Jun N-terminal kinase 1 regulates epithelial-to-mesenchymal transition induced by TGF-beta1. J Cell Sci 121: 1036-1045.
17. McMurray, J. S. 2006. A new small-molecule Stat3 inhibitor. Chemistry & biology 13: 1123-1124.
18. Holtzman, M. J., D. E. Byers, J. Alexander-Brett, and X. Wang. 2014. The role of airway epithelial cells and innate immune cells in chronic respiratory disease. Nature reviews. Immunology 14: 686-698.
19. Hahn, C., A. P. Islamian, H. Renz, and W. A. Nockher. 2006. Airway epithelial cells produce neurotrophins and promote the survival of eosinophils during allergic airway inflammation. The Journal of allergy and clinical immunology 117: 787-794.
20. Shirai, T., R. R. Nazarewicz, B. B. Wallis, R. E. Yanes, R. Watanabe, M. Hilhorst, L. Tian, D. G. Harrison, J. C.

Giacomini, T. L. Assimes, J. J. Goronzy, and C. M. Weyand. 2016. The glycolytic enzyme PKM2 bridges metabolic and inflammatory dysfunction in coronary artery disease. J Exp Med 213: 337-354.
21. Michalek, R. D., V. A. Gerriets, S. R. Jacobs, A. N. Macintyre, N. J. MacIver, E. F. Mason, S. A. Sullivan, A. G. Nichols, and J. C. Rathmell. 2011. Cutting edge: distinct glycolytic and lipid oxidative metabolic programs are essential for effector and regulatory CD4+ T cell subsets. Journal of immunology (Baltimore, Md. : 1950) 186: 3299-3303.
22. Lambrecht, B. N., H. Hammad, and J. V. Fahy. 2019. The Cytokines of Asthma. Immunity 50: 975-991.
23. Ostroukhova, M., N. Goplen, M. Z. Karim, L. Michalec, L. Guo, Q. Liang, and R. Alam. 2012. The role of low-level lactate production in airway inflammation in asthma. American journal of physiology. Lung cellular and molecular physiology 302: L300-307.
24. Zhang, L., C. Ma, X. Wang, S. He, Q. Li, Y. Zhou, Y. Liu, M. Zhang, X. Yu, X. Zhao, F. Li, and D. L. Zhu. 2019. Lipopolysaccharide-induced proliferation and glycolysis in airway smooth muscle cells via activation of Drp1. Journal of cellular physiology 234: 9255-9263.
25. Caslin, H. L., M. T. Taruselli, T. Haque, N. Pondicherry, E. A. Baldwin, B. O. Barnstein, and J. J. Ryan. 2018. Inhibiting Glycolysis and ATP Production Attenuates IL-33-Mediated Mast Cell Function and Peritonitis. Frontiers in immunology 9: 3026.
26. Zhang, Y., S. A. G. Willis-Owen, S. Spiegel, C. M. Lloyd, M. F. Moffatt, and W. Cookson. 2019. The ORMDL3 Asthma Gene Regulates ICAM1 and Has Multiple Effects on Cellular Inflammation. Am J Respir Crit Care Med 199: 478-488.
27. Winnica, D., C. Corey, S. Mullett, M. Reynolds, G. Hill, S. Wendell, L. Que, F. Holguin, and S. Shiva. 2019. Bioenergetic Differences in the Airway Epithelium of Lean Versus Obese Asthmatics Are Driven by Nitric Oxide and Reflected in Circulating Platelets. Antioxidants & redox signaling.
28. Xu, W., S. A. A. Comhair, A. J. Janocha, A. Lara, L. A. Mavrakis, C. D. Bennett, S. C. Kalhan, and S. C. Erzurum. 2017. Arginine metabolic endotypes related to asthma severity. PLoS One 12: e0183066.
29. West, E. E., M. Kashyap, and W. J. Leonard. 2012. TSLP: A Key Regulator of Asthma Pathogenesis. Drug discovery today. Disease mechanisms 9.
30. Smelter, D. F., V. Sathish, M. A. Thompson, C. M. Pabelick, R. Vassallo, and Y. S. Prakash. 2010. Thymic stromal lymphopoietin in cigarette smoke-exposed human airway smooth muscle. Journal of immunology (Baltimore, Md. : 1950) 185: 3035-3040.
31. Demehri, S., M. Morimoto, M. J. Holtzman, and R. Kopan. 2009. Skin-derived TSLP triggers progression from epidermal-barrier defects to asthma. PLoS Biol 7: e1000067.
32. Zhou, B., M. B. Headley, T. Aye, J. Tocker, M. R. Comeau, and S. F. Ziegler. 2008. Reversal of thymic stromal lymphopoietin-induced airway inflammation through inhibition of Th2 responses. Journal of immunology (Baltimore, Md. : 1950) 181: 6557-6562.
33. Ziegler, S. F., F. Roan, B. D. Bell, T. A. Stoklasek, M. Kitajima, and H. Han. 2013. The biology of thymic stromal lymphopoietin (TSLP). Advances in pharmacology (San Diego, Calif.) 66: 129-155.
34. Gauvreau, G. M., P. M. O'Byrne, L. P. Boulet, Y. Wang, D. Cockcroft, J. Bigler, J. M. FitzGerald, M. Boedigheimer, B. E. Davis, C. Dias, K. S. Gorski, L. Smith, E. Bautista, M. R. Comeau, R. Leigh, and J. R. Parnes. 2014. Effects of an anti-TSLP antibody on allergen-induced asthmatic responses. The New England journal of medicine 370: 2102-2110.
35. Shi, Y., C. H. Liu, A. I. Roberts, J. Das, G. Xu, G. Ren, Y. Zhang, L. Zhang, Z. R. Yuan, H. S. Tan, G. Das, and S. Devadas. 2006. Granulocyte-macrophage colony-stimulating factor (GM-CSF) and T-cell responses: what we do and don't know. Cell research 16: 126-133.
36. Yang, P., Z. Li, H. Li, Y. Lu, H. Wu, and Z. Li. 2015. Pyruvate kinase M2 accelerates pro-inflammatory cytokine secretion and cell proliferation induced by lipopolysaccharide in colorectal cancer. Cellular signalling 27: 1525-1532.
37. Wang, H. J., Y. J. Hsieh, W. C. Cheng, C. P. Lin, Y. S. Lin, S. F. Yang, C. C. Chen, Y. Izumiya, J. S. Yu, H. J. Kung, and W. C. Wang. 2014. JMJD5 regulates PKM2 nuclear translocation and reprograms HIF-1alpha-mediated glucose metabolism. Proceedings of the National Academy of Sciences of the United States of America 111: 279-284.
38. Luo, W., H. Hu, R. Chang, J. Zhong, M. Knabel, R. O'Meally, R. N. Cole, A. Pandey, and G. L. Semenza. 2011. Pyruvate kinase M2 is a PHD3-stimulated coactivator for hypoxia-inducible factor 1. Cell 145: 732-744.
39. Palsson-McDermott, E. M., A. M. Curtis, G. Goel, M. A. Lauterbach, F. J. Sheedy, L. E. Gleeson, M. W. van den Bosch, S. R. Quinn, R. Domingo-Fernandez, D. G. Johnston, J. K. Jiang, W. J. Israelsen, J. Keane, C. Thomas, C. Clish,

M. Vander Heiden, R. J. Xavier, and L. A. O'Neill. 2015. Pyruvate kinase M2 regulates Hif-1alpha activity and IL-1beta induction and is a critical determinant of the warburg effect in LPS-activated macrophages. Cell Metab 21: 65-80.
40. Hitosugi, T., S. Kang, M. G. Vander Heiden, T. W. Chung, S. Elf, K. Lythgoe, S. Dong, S. Lonial, X. Wang, G. Z. Chen, J. Xie, T. L. Gu, R. D. Polakiewicz, J. L. Roesel, T. J. Boggon, F. R. Khuri, D. G. Gilliland, L. C. Cantley, J. Kaufman, and J. Chen. 2009. Tyrosine phosphorylation inhibits PKM2 to promote the Warburg effect and tumor growth. Science signaling 2: ra73.
41. Christofk, H. R., M. G. Vander Heiden, N. Wu, J. M. Asara, and L. C. Cantley. 2008. Pyruvate kinase M2 is a phosphotyrosine-binding protein. Nature 452: 181-186.
42. Lv, L., D. Li, D. Zhao, R. Lin, Y. Chu, H. Zhang, Z. Zha, Y. Liu, Z. Li, Y. Xu, G. Wang, Y. Huang, Y. Xiong, K. L. Guan, and Q. Y. Lei. 2011. Acetylation targets the M2 isoform of pyruvate kinase for degradation through chaperone-mediated autophagy and promotes tumor growth. Molecular cell 42: 719-730.
43. Bhardwaj, A., and S. Das. 2016. SIRT6 deacetylates PKM2 to suppress its nuclear localization and oncogenic functions. Proceedings of the National Academy of Sciences of the United States of America 113: E538-547.
44. Anastasiou, D., G. Poulogiannis, J. M. Asara, M. B. Boxer, J. K. Jiang, M. Shen, G. Bellinger, A. T. Sasaki, J. W. Locasale, D. S. Auld, C. J. Thomas, M. G. Vander Heiden, and L. C. Cantley. 2011. Inhibition of pyruvate kinase M2 by reactive oxygen species contributes to cellular antioxidant responses. Science 334: 1278-1283.
45. Liu, K., F. Li, H. Han, Y. Chen, Z. Mao, J. Luo, Y. Zhao, B. Zheng, W. Gu, and W. Zhao. 2016. Parkin Regulates the Activity of Pyruvate Kinase M2. The Journal of biological chemistry 291: 10307-10317.
46. Wang, Y., J. Liu, X. Jin, D. Zhang, D. Li, F. Hao, Y. Feng, S. Gu, F. Meng, M. Tian, Y. Zheng, L. Xin, X. Zhang, X. Han, L. Aravind, and M. Wei. 2017. O-GlcNAcylation destabilizes the active tetrameric PKM2 to promote the Warburg effect. Proceedings of the National Academy of Sciences of the United States of America 114: 13732-13737.
47. Liu, F., F. Ma, Y. Wang, L. Hao, H. Zeng, C. Jia, Y. Wang, P. Liu, I. M. Ong, B. Li, G. Chen, J. Jiang, S. Gong, L. Li, and W. Xu. 2017. PKM2 methylation by CARM1 activates aerobic glycolysis to promote tumorigenesis. Nature cell biology 19: 1358-1370.
48. Spoden, G. A., D. Morandell, D. Ehehalt, M. Fiedler, P. Jansen-Durr, M. Hermann, and W. Zwerschke. 2009. The SUMO-E3 ligase PIAS3 targets pyruvate kinase M2. Journal of cellular biochemistry 107: 293-302.
49. Yang, W., Y. Xia, H. Ji, Y. Zheng, J. Liang, W. Huang, X. Gao, K. Aldape, and Z. Lu. 2011. Nuclear PKM2 regulates beta-catenin transactivation upon EGFR activation. Nature 480: 118-122.
50. Yang, W., Y. Zheng, Y. Xia, H. Ji, X. Chen, F. Guo, C. A. Lyssiotis, K. Aldape, L. C. Cantley, and Z. Lu. 2012. ERK1/2-dependent phosphorylation and nuclear translocation of PKM2 promotes the Warburg effect. Nature cell biology 14: 1295-1304.
51. Hristova, M., A. Habibovic, C. Veith, Y. M. Janssen-Heininger, A. E. Dixon, M. Geiszt, and A. van der Vliet. 2016. Airway epithelial dual oxidase 1 mediates allergen-induced IL-33 secretion and activation of type 2 immune responses. The Journal of allergy and clinical immunology 137: 1545-1556.e1511.
52. Burgel, P. R., and J. A. Nadel. 2008. Epidermal growth factor receptor-mediated innate immune responses and their roles in airway diseases. The European respiratory journal 32: 1068-1081.
53. Hamilton, L. M., C. Torres-Lozano, S. M. Puddicombe, A. Richter, I. Kimber, R. J. Dearman, B. Vrugt, R. Aalbers, S. T. Holgate, R. Djukanovic, S. J. Wilson, and D. E. Davies. 2003. The role of the epidermal growth factor receptor in sustaining neutrophil inflammation in severe asthma. Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology 33: 233-240.
54. Tan, P. H., P. Sagoo, C. Chan, J. B. Yates, J. Campbell, S. C. Beutelspacher, B. M. Foxwell, G. Lombardi, and A. J. George. 2005. Inhibition of NF-kappa B and oxidative pathways in human dendritic cells by antioxidative vitamins generates regulatory T cells. Journal of immunology (Baltimore, Md. : 1950) 174: 7633-7644.
55. Boxer, M. B., J. K. Jiang, M. G. Vander Heiden, M. Shen, A. P. Skoumbourdis, N. Southall, H. Veith, W. Leister, C. P. Austin, H. W. Park, J. Inglese, L. C. Cantley, D. S. Auld, and C. J. Thomas. 2010. Evaluation of substituted N,N'-diarylsulfonamides as activators of the tumor cell specific M2 isoform of pyruvate kinase. Journal of medicinal chemistry 53: 1048-1055.
56. Qi, W., H. A. Keenan, Q. Li, A. Ishikado, A. Kannt, T. Sadowski, M. A. Yorek, I. H. Wu, S. Lockhart, L. J. Coppey, A. Pfenninger, C. W. Liew, G. Qiang, A. M. Burkart, S. Hastings, D. Pober, C. Cahill, M. A. Niewczas, W. J. Israelsen, L. Tinsley, I. E. Stillman, P. S. Amenta, E. P. Feener, M. G. Vander Heiden, R. C. Stanton, and G. L. King. 2017. Pyruvate kinase M2 activation may protect against the progression of diabetic glomerular pathology and mitochondrial dysfunction. Nat Med 23: 753-762.

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

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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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.

REFERENCES

1. Disease GBD, Injury I, Prevalence C. Global, regional, and national incidence, prevalence, and years lived with disability for 328 diseases and injuries for 195 countries, 1990-2016: a systematic analysis for the Global Burden of Disease Study 2016. Lancet (London, England). 2017;390(10100):1211-59.
2. Ray A, Raundhal M, Oriss TB, Ray P, Wenzel SE. Current concepts of severe asthma. J Clin Invest. 2016;126(7):2394-403.
3. Marcia Regina Piuvezam LKDPF, Talissa Mozzini Monteiro, Giciane Carvalho Vieira and Claudio Roberto Bezerra-Santos Severe Asthma: Updated Therapy Approach Based on Phenotype and Biomarker, Asthma Diagnosis and Management - Approach Based on Phenotype and Endotype, Kuan-Hsiang Gary Huang and Chen Hsuan Sherry Tsai, IntechOpen, DOI: 10.5772/intechopen.74775. . (July 4th 2018).
4. Reddel HK, Bateman ED, Becker A, Boulet LP, Cruz AA, Drazen JM, et al. A summary of the new GINA strategy: a roadmap to asthma control. The European respiratory journal. 2015;46(3):622-39.
5. Fajt ML, Wenzel SE. Asthma phenotypes and the use of biologic medications in asthma and allergic disease: the next steps toward personalized care. The Journal of allergy and clinical immunology. 2015;135(2):299-310; quiz 311.
6. Fahy JV. Type 2 inflammation in asthma--present in most, absent in many. Nature reviews Immunology. 2015;15(1):57-65.
7. Shimoda T, Obase Y, Nagasaka Y, Asai S. Phenotype classification using the combination of lung sound analysis and fractional exhaled nitric oxide for evaluating asthma treatment. Allergol Int. 2018;67(2):253-8.
8. Nagasaki T, Matsumoto H, Izuhara K, Ki HACRMG. Utility of serum periostin in combination with exhaled nitric oxide in the management of asthma. Allergol Int. 2017;66(3):404-10.
9. Berry A, Busse WW. Biomarkers in asthmatic patients: Has their time come to direct treatment? The Journal of allergy and clinical immunology. 2016;137(5):1317-24.
10. Park MJ, Lee SH, Lee SH, Lee EJ, Kim EK, Choi JY, et al. IL-1 Receptor Blockade Alleviates Graft-versus-Host Disease through Downregulation of an Interleukin-1beta-Dependent Glycolytic Pathway in Th17 Cells. Mediators of inflammation. 2015;2015:631384.
11. Han MW, Kim SH, Oh I, Kim YH, Lee J. Serum IL-1beta can be a biomarker in children with severe persistent allergic rhinitis. Allergy Asthma Clin Immunol. 2019;15:58.
12. Yi G, Liang M, Li M, Fang X, Liu J, Lai Y, et al. A large lung gene expression study identifying IL1B as a novel player in airway inflammation in COPD airway epithelial cells. Inflamm Res. 2018;67(6):539-51.
13. Evans MD, Esnault S, Denlinger LC, Jarjour NN. Sputum cell IL-1 receptor expression level is a marker of airway neutrophilia and airflow obstruction in asthmatic patients. The Journal of allergy and clinical immunology. 2018;142(2):415-23.
14. Wang CC, Fu CL, Yang YH, Lo YC, Wang LC, Chuang YH, et al. Adenovirus expressing interleukin-1 receptor antagonist alleviates allergic airway inflammation in a murine model of asthma. Gene Ther. 2006;13(19):1414-21.
15. Johnson VJ, Yucesoy B, Luster MI. Prevention of IL-1 signaling attenuates airway hyperresponsiveness and inflammation in a murine model of toluene diisocyanate-induced asthma. The Journal of allergy and clinical immunology. 2005;116(4):851-8.
16. Busse PJ, Birmingham JM, Calatroni A, Manzi J, Goryachokovsky A, Fontela G, et al. Effect of aging on sputum inflammation and asthma control. The Journal of allergy and clinical immunology. 2017;139(6):1808-18 e6.
17. Hynes GM, Hinks TSC. The role of interleukin-17 in asthma: a protective response? ERJ Open Res. 2020;6(2).
18. Liu W, Liu S, Verma M, Zafar I, Good JT, Rollins D, et al. Mechanism of TH2/TH17-predominant and neutrophilic TH2/TH17-low subtypes of asthma. The Journal of allergy and clinical immunology. 2017;139(5):1548-58 e4.
19. Ather JL, Ckless K, Martin R, Foley KL, Suratt BT, Boyson JE, et al. Serum amyloid A activates the NLRP3 inflammasome and promotes Th17 allergic asthma in mice. J Immunol. 2011;187(1):64-73.
20. Prince LR, Allen L, Jones EC, Hellewell PG, Dower SK, Whyte MK, et al. The role of interleukin-1beta in direct and toll-like receptor 4-mediated neutrophil activation and survival. The American journal of pathology. 2004;165(5):1819-26.

21. Gladden LB. Lactate metabolism: a new paradigm for the third millennium. J Physiol. 2004;558(Pt 1):5-30.
22. Hui S, Ghergurovich JM, Morscher RJ, Jang C, Teng X, Lu W, et al. Glucose feeds the TCA cycle via circulating lactate. Nature. 2017;551(7678):115-8.
23. Faubert B, Li KY, Cai L, Hensley CT, Kim J, Zacharias LG, et al. Lactate Metabolism in Human Lung Tumors. Cell. 2017;171(2):358-71 e9.
24. Mitchell AM, Cournand A. The fate of circulating lactic acid in the human lung. J Clin Invest. 1955;34(3):471-6.
25. De Backer D, Creteur J, Zhang H, Norrenberg M, Vincent JL. Lactate production by the lungs in acute lung injury. American journal of respiratory and critical care medicine. 1997;156(4 Pt 1):1099-104.
26. Brown SD, Clark C, Gutierrez G. Pulmonary lactate release in patients with sepsis and the adult respiratory distress syndrome. J Crit Care. 1996;11(1):2-8.
27. Bensel T, Stotz M, Borneff-Lipp M, Wollschlager B, Wienke A, Taccetti G, et al. Lactate in cystic fibrosis sputum. J Cyst Fibros. 2011;10(1):37-44.
28. Fredman G, Kolpen M, Hertz FB, Petersen PT, Jensen AV, Baunbaek-Egelund G, et al. The inflamed sputum in lower respiratory tract infection: l-lactate levels are correlated to neutrophil accumulation. APMIS. 2019;127(2):72-9.
29. Ho WE, Xu YJ, Xu F, Cheng C, Peh HY, Tannenbaum SR, et al. Metabolomics reveals altered metabolic pathways in experimental asthma. American journal of respiratory cell and molecular biology. 2013;48(2):204-11.
30. Wolak JE, Esther CR, Jr., O'Connell TM. Metabolomic analysis of bronchoalveolar lavage fluid from cystic fibrosis patients. Biomarkers. 2009;14(1):55-60.
31. Iscra F, Gullo A, Biolo G. Bench-to-bedside review: lactate and the lung. Crit Care. 2002;6(4):327-9.
32. Gao X, Wang H, Yang JJ, Liu X, Liu ZR. Pyruvate kinase M2 regulates gene transcription by acting as a protein kinase. Mol Cell. 2012;45(5):598-609.
33. Alves-Filho JC, Palsson-McDermott EM. Pyruvate Kinase M2: A Potential Target for Regulating Inflammation. Front Immunol. 2016;7:145.
34. Palsson-McDermott EM, Dyck L, Zaslona Z, Menon D, McGettrick AF, Mills KHG, et al. Pyruvate Kinase M2 Is Required for the Expression of the Immune Checkpoint PD-L1 in Immune Cells and Tumors. Front Immunol. 2017;8:1300.
35. Hoffman SM, Qian X, Nolin JD, Chapman DG, Chia SB, Lahue KG, et al. Ablation of Glutaredoxin-1 Modulates House Dust Mite-Induced Allergic Airways Disease in Mice. American journal of respiratory cell and molecular biology. 2016;55(3):377-86.
36. Allocati N, Masulli M, Di Ilio C, Federici L. Glutathione transferases: substrates, inihibitors and pro-drugs in cancer and neurodegenerative diseases. Oncogenesis. 2018;7(1):8.
37. Townsend DM, Tew KD. The role of glutathione-S-transferase in anti-cancer drug resistance. Oncogene. 2003;22(47):7369-75.
38. Tentori L, Dorio AS, Mazzon E, Muzi A, Sau A, Cuzzocrea S, et al. The glutathione transferase inhibitor 6-(7-nitro-2,1,3-benzoxadiazol-4-ylthio)hexanol (NBDHEX) increases temozolomide efficacy against malignant melanoma. Eur J Cancer. 2011;47(8):1219-30.
39. Federici L, Lo Sterzo C, Pezzola S, Di Matteo A, Scaloni F, Federici G, et al. Structural basis for the binding of the anticancer compound 6-(7-nitro-2,1,3-benzoxadiazol-4-ylthio)hexanol to human glutathione s-transferases. Cancer research. 2009;69(20):8025-34.
40. Morgan AS, Ciaccio PJ, Tew KD, Kauvar LM. Isozyme-specific glutathione S-transferase inhibitors potentiate drug sensitivity in cultured human tumor cell lines. Cancer Chemother Pharmacol. 1996;37(4):363-70.
41. Raza A, Galili N, Smith S, Godwin J, Lancet J, Melchert M, et al. Phase 1 multicenter dose-escalation study of ezatiostat hydrochloride (TLK199 tablets), a novel glutathione analog prodrug, in patients with myelodysplastic syndrome. Blood. 2009;113(26):6533-40.
42. McMillan DH, van der Velden JL, Lahue KG, Qian X, Schneider RW, Iberg MS, et al. Attenuation of lung fibrosis in mice with a clinically relevant inhibitor of glutathione-S-transferase pi. JCI Insight. 2016;1(8).

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).

REFERENCES

1. Disease GBD, Injury I, Prevalence C. Global, regional, and national incidence, prevalence, and years lived with disability for 328 diseases and injuries for 195 countries, 1990-2016: a systematic analysis for the Global Burden of Disease Study 2016. Lancet (London, England). 2017;390(10100):1211-59.
2. Reddel HK, Bateman ED, Becker A, Boulet LP, Cruz AA, Drazen JM, et al. A summary of the new GINA strategy: a roadmap to asthma control. The European respiratory journal. 2015;46(3):622-39.
3. Croisant S. Epidemiology of asthma: prevalence and burden of disease. Adv Exp Med Biol. 2014;795:17-29.
4. Nunes C, Pereira AM, Morais-Almeida M. Asthma costs and social impact. Asthma Res Pract. 2017;3:1.
5. Kuruvilla ME, Lee FE, Lee GB. Understanding Asthma Phenotypes, Endotypes, and Mechanisms of Disease. Clin Rev Allergy Immunol. 2019;56(2):219-33.
6. Michaeloudes C, Bhavsar PK, Mumby S, Xu B, Hui CKM, Chung KF, et al. Role of Metabolic Reprogramming in Pulmonary Innate Immunity and Its Impact on Lung Diseases. J Innate Immun. 2020;12(1):31-46.
7. McGeachie MJ, Dahlin A, Qiu W, Croteau-Chonka DC, Savage J, Wu AC, et al. The metabolomics of asthma control: a promising link between genetics and disease. Immun Inflamm Dis. 2015;3(3):224-38.
8. Reinke SN, Gallart-Ayala H, Gomez C, Checa A, Fauland A, Naz S, et al. Metabolomics analysis identifies different metabotypes of asthma severity. The European respiratory journal. 2017;49(3).
9. Carraro S, Bozzetto S, Giordano G, El Mazloum D, Stocchero M, Pirillo P, et al. Wheezing preschool children with early-onset asthma reveal a specific metabolomic profile. Pediatric allergy and immunology : official publication of the European Society of Pediatric Allergy and Immunology. 2018;29(4):375-82.
10. Prince LR, Allen L, Jones EC, Hellewell PG, Dower SK, Whyte MK, et al. The role of interleukin-1beta in direct and toll-like receptor 4-mediated neutrophil activation and survival. The American journal of pathology. 2004;165(5):1819-26.
11. Townsend DM, Manevich Y, He L, Hutchens S, Pazoles CJ, Tew KD. Novel role for glutathione S-transferase pi. Regulator of protein S-Glutathionylation following oxidative and nitrosative stress. The Journal of biological chemistry. 2009;284(1):436-45.
12. Global Initiative for Asthma (GINA). Global strategy for asthma management and prevention. 2019. https://ginasthma.org/wp-content/uploads/2019/06/GINA-2019-main-report-June-2019-wms.pdf.
13. Xie N, Tan Z, Banerjee S, Cui H, Ge J, Liu RM, et al. Glycolytic Reprogramming in Myofibroblast Differentiation and Lung Fibrosis. American journal of respiratory and critical care medicine. 2015;192(12):1462-74.
14. Han MW, Kim SH, Oh I, Kim YH, Lee J. Serum IL-1beta can be a biomarker in children with severe persistent allergic rhinitis. Allergy Asthma Clin Immunol. 2019;15:58.
15. Yi G, Liang M, Li M, Fang X, Liu J, Lai Y, et al. A large lung gene expression study identifying IL1B as a novel player in airway inflammation in COPD airway epithelial cells. Inflamm Res. 2018;67(6):539-51.
16. McElvaney OJ, McEvoy NL, McElvaney OF, Carroll TP, Murphy MP, Dunlea DM, et al. Characterization of the Inflammatory Response to Severe COVID-19 Illness. American journal of respiratory and critical care medicine. 2020;202(6):812-21.
17. Peters U, Dixon AE, Forno E. Obesity and asthma. The Journal of allergy and clinical immunology. 2018;141(4):1169-79.
18. Enquobahrie DA, Rice K, Williams OD, Williams MA, Gross MD, Lewis CE, et al. IL1B genetic variation and plasma C-reactive protein level among young adults: the CARDIA study. Atherosclerosis. 2009;202(2):513-20.
19. Dinarello CA. Interleukin 1 and interleukin 18 as mediators of inflammation and the aging process. Am J Clin Nutr. 2006;83(2):447S-55S.
20. Aberle MR, Burkhart RA, Tiriac H, Olde Damink SWM, Dejong CHC, Tuveson DA, et al. Patient-derived organoid models help define personalized management of gastrointestinal cancer. Br J Surg. 2018;105(2):e48-e60.

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List of publications

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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

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.