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Interleukin-1/inhibitory kappa B kinase epsilon-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.

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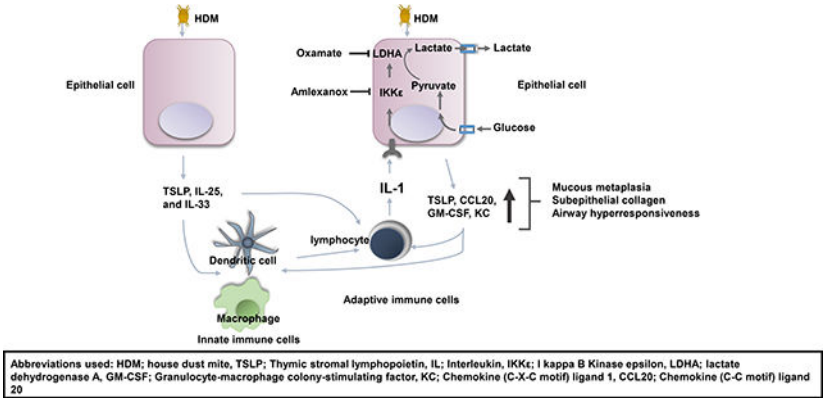
Results: In a murine model of allergic asthma, glycolysis was induced in the lungs in an IL-1-dependent manner. Furthermore, administration of IL-1β into airways stimulated lactate production and expression of glycolytic enzymes, with notable expression of lactate dehydrogenase A occurring in the airway 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.

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.

Graphical Abstract



Keywords

Asthma; house dust mite; glycolysis; interleukin-1; inhibitor of κB kinase ε; lactate; lactate dehydrogenase A

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, the end product of glycolysis, 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 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 ANCOVA. 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 (2XHDM, 5X HDM, HDM). These increases in lactate on days 20 and 30 corresponded with increases in total cells and notably increases in 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.

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 proinflammatory 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 *RagT*$^{-/-}$ 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).

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-1 α/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-1ß-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.

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 RN compared to saline-exposed mice, no further increases in RN 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.

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 Amlexamox 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 Amlexamox 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 Amlexamox in these settings (Figure 6F) may be attributable to TBK1.

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-1 β, 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.

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 μg/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.

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 patho-physiological 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 (32–37). 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 IKte 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 and 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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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

TGFβ	Transforming growth factor β
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
IFNγ	Interferon γ
NECs	Nasal epithelial cells
PKM2	Pyruvate kinase M2

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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 proinflammatory responses of airway epithelial cells.

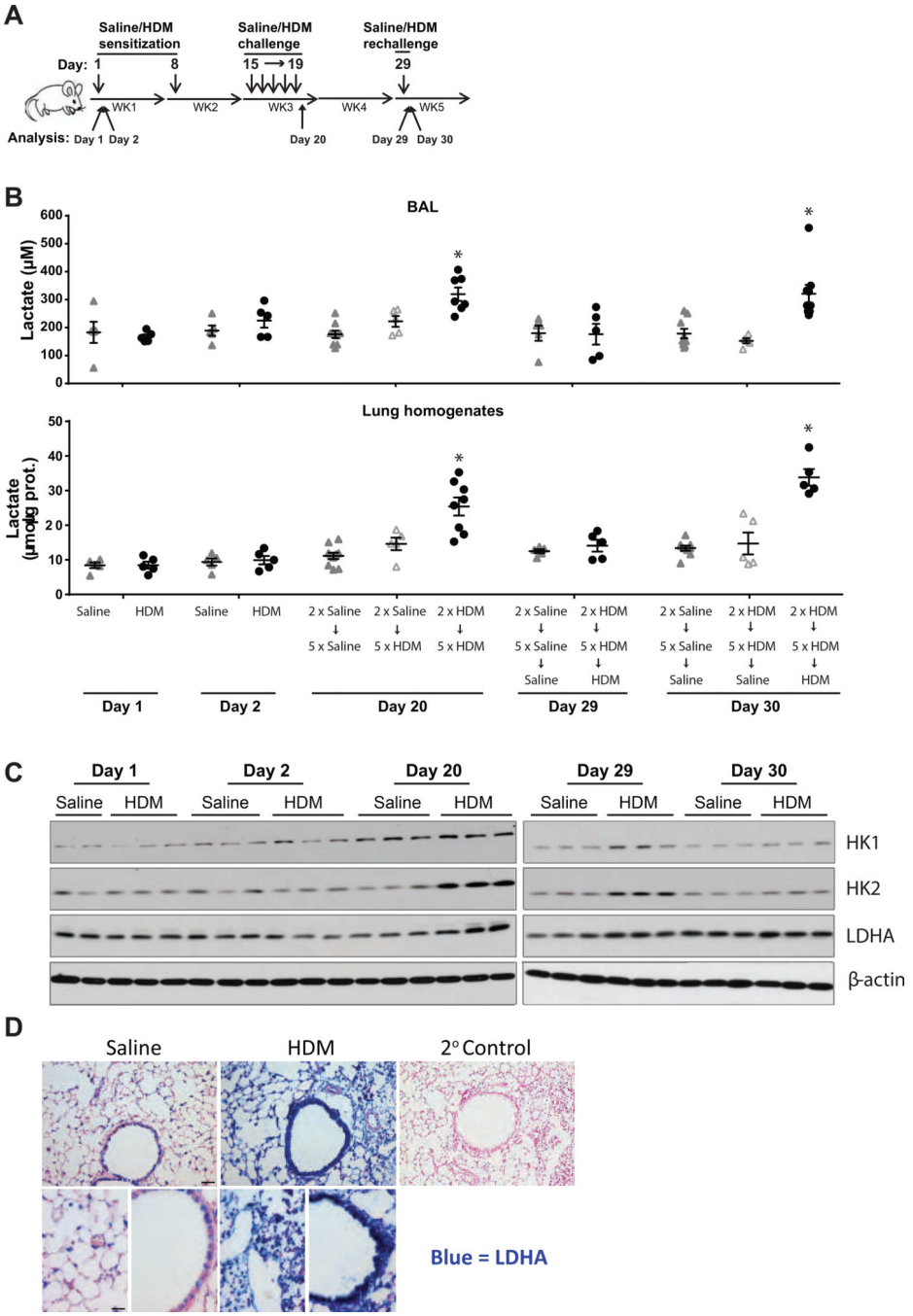

Figure 1. Evaluation of glycolysis in the lung tissues of mice exposed to house dust mite (HDM). **A,** Schematic depicting the dosing regimen of HDM (Detailed information is provided in the Supplemental Material). **B,** Lactate levels in BAL (top) and lung tissues (bottom) following a single or multiple 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.

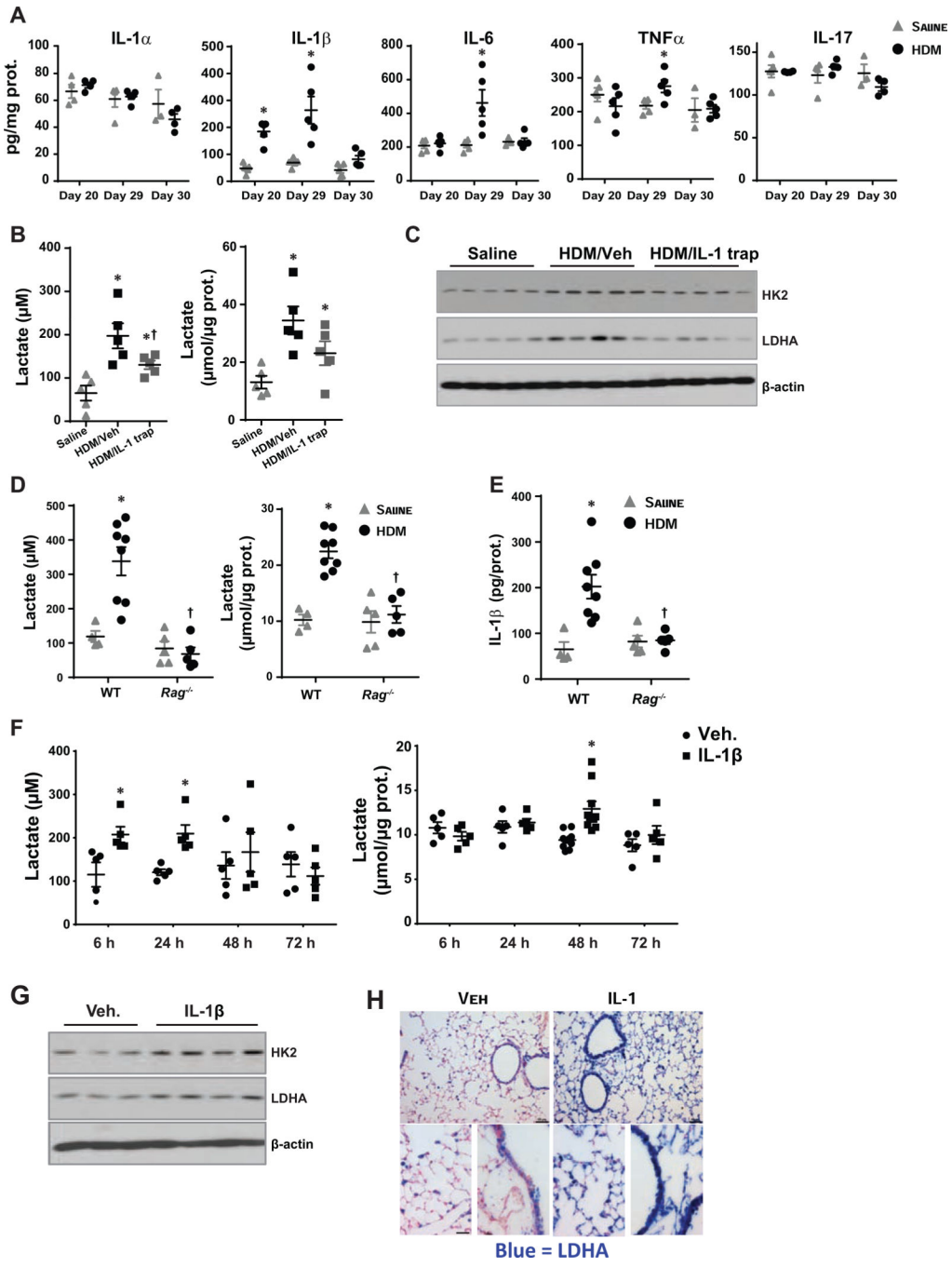

Figure 2. House dust mite (HDM)-induced T and B-cell adaptive immune responses are required for IL-1 β production and resultant increases in glycolysis in lung tissues.

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 broncho-alveolar 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.

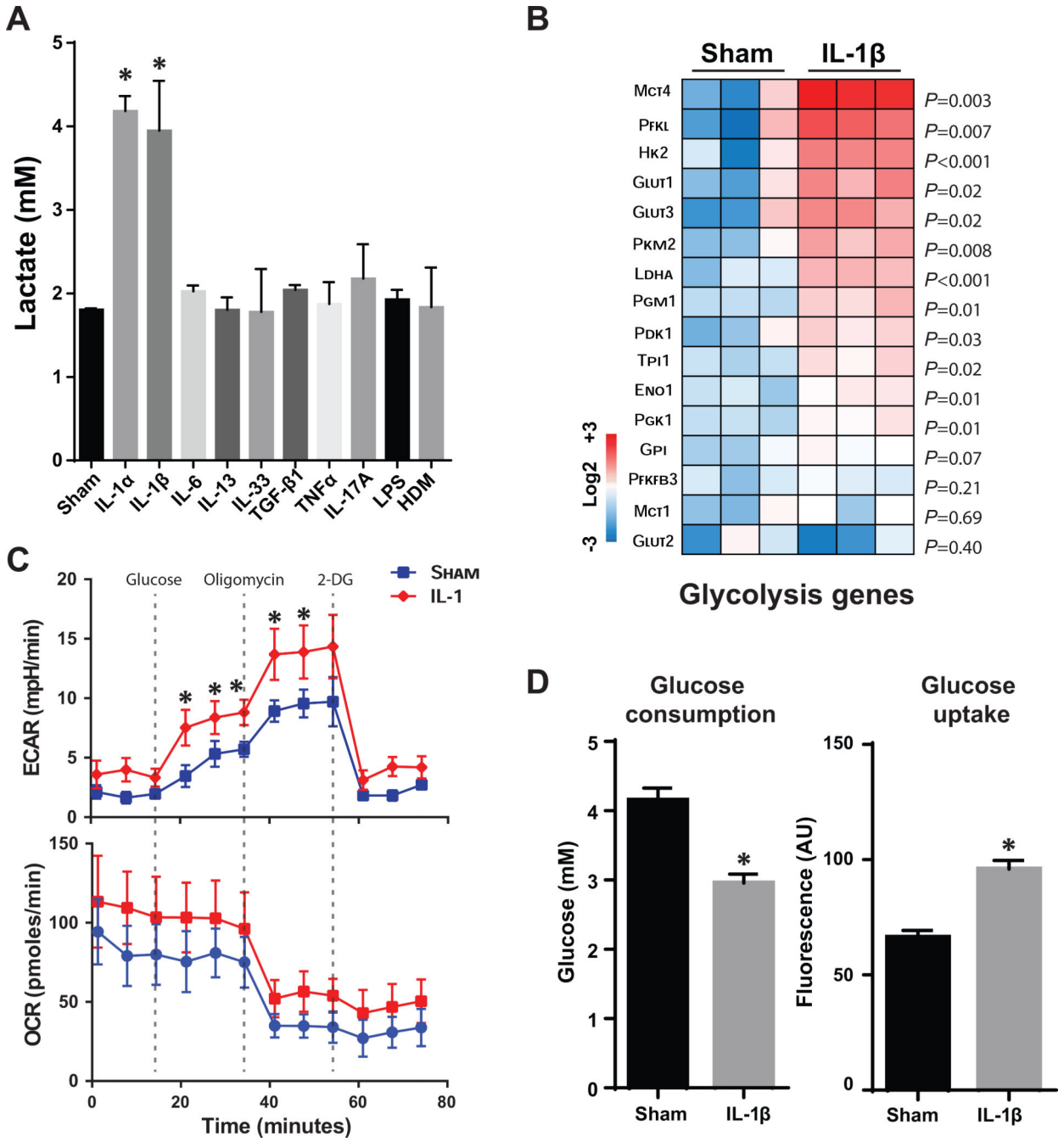

Figure 3. IL-1α/β increase lactate production, glycolysis gene expression, glucose usage, and glycolytic flux rate in primary mouse tracheal epithelial (MTE) cells.

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-1 β. *P < 0.05 compared to the sham group (Student's *t* test).

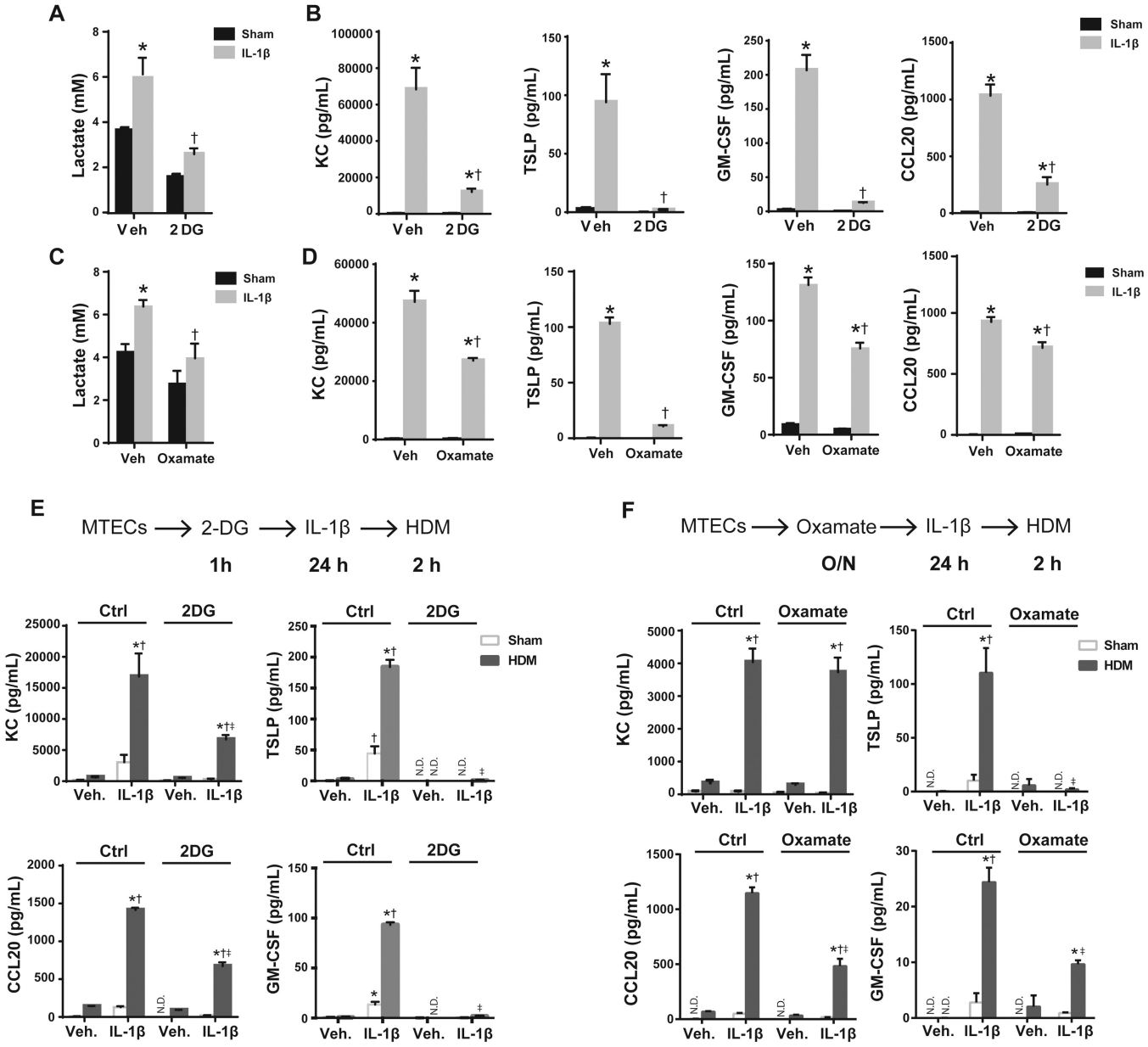

Figure 4. Importance of glycolysis for IL-1β-induced pro-inflammatory responses and the IL-1β-mediated augmentation of HDM-induced innate cytokine responses in primary mouse tracheal epithelial (MTE) cells.

A-D, Lactate (**A&C**) and levels of proinflammatory 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).

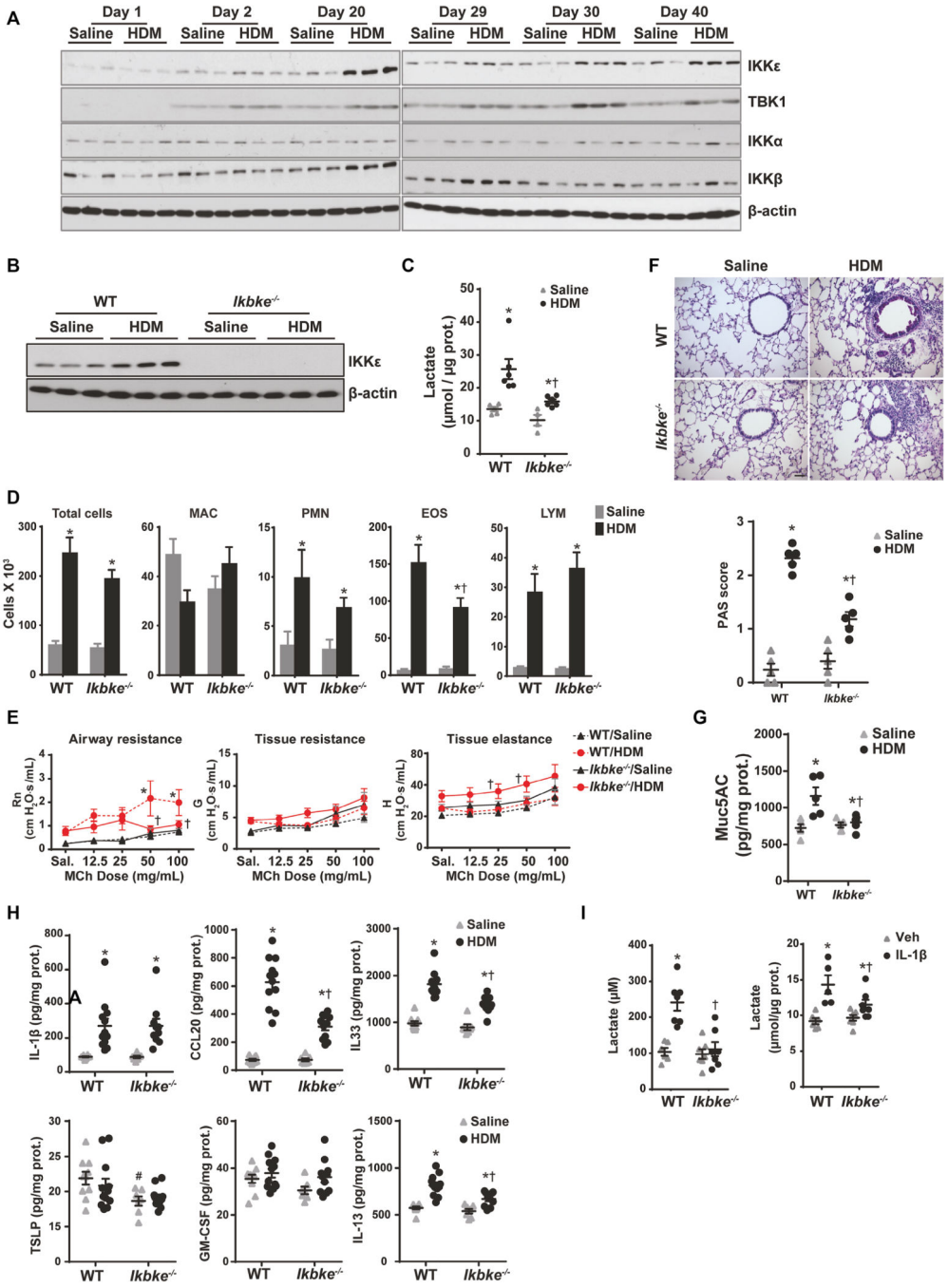

Figure 5. A causal role for Inhibitory kappa B kinase ε (IKKε) in HDM- and IL-1β-mediated increases in glycolysis and the pathogenesis of allergic airways diseases.

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 wildtype (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 Veh (vehicle) control group, †$P < 0.05$ relative to the respective wild-type (WT) group (ANOVA, n = 5–10 per group).

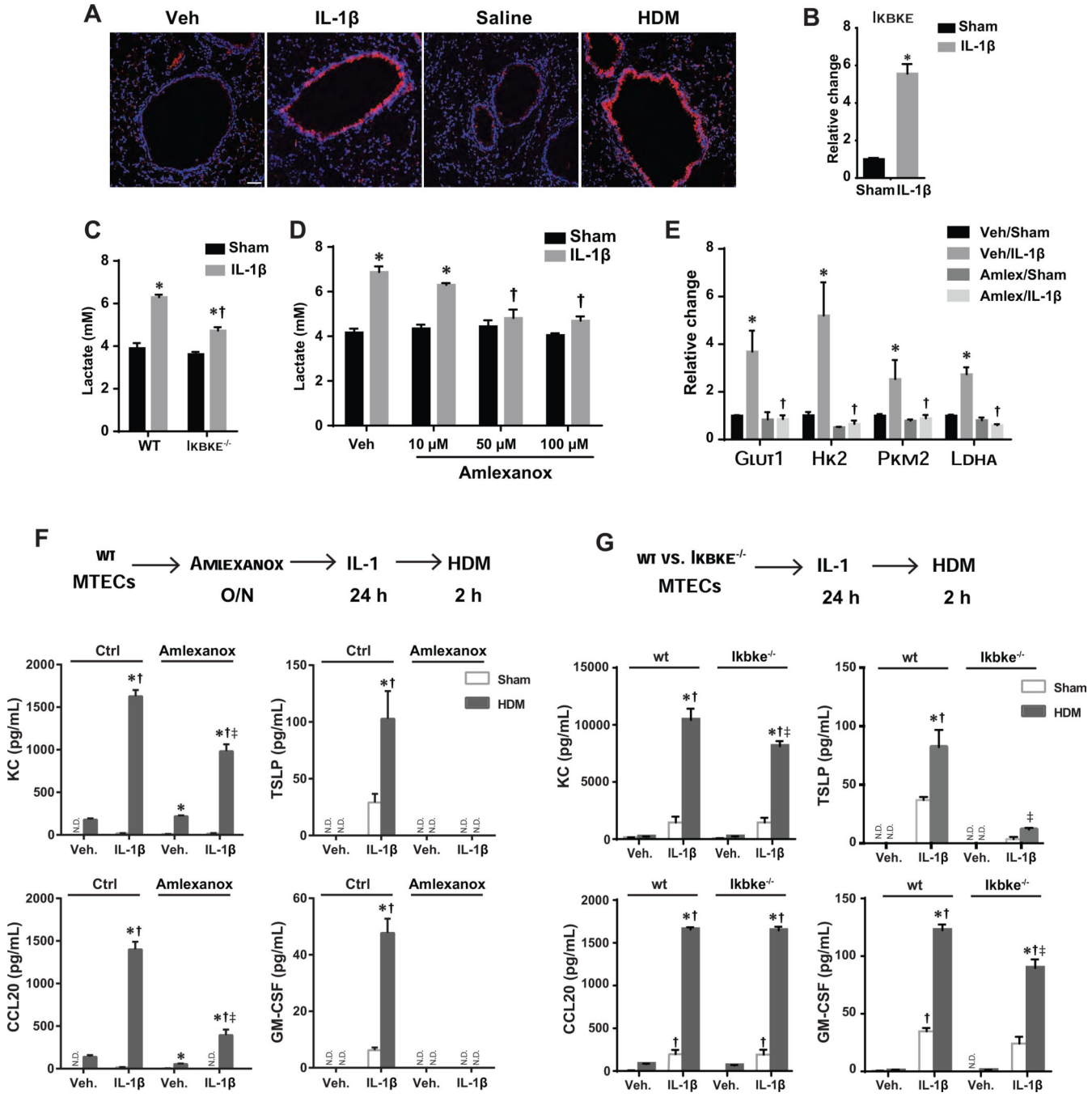

Figure 6. Inhibitory kappa B kinase ε (IKKε) is required for IL-1β-mediated increases in glycolysis, and the IL-1β-mediated augmentation of HDM-induced innate cytokine responses in MTE cells.

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, $\dagger P < 0.05$ 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, †$P < 05$ 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).

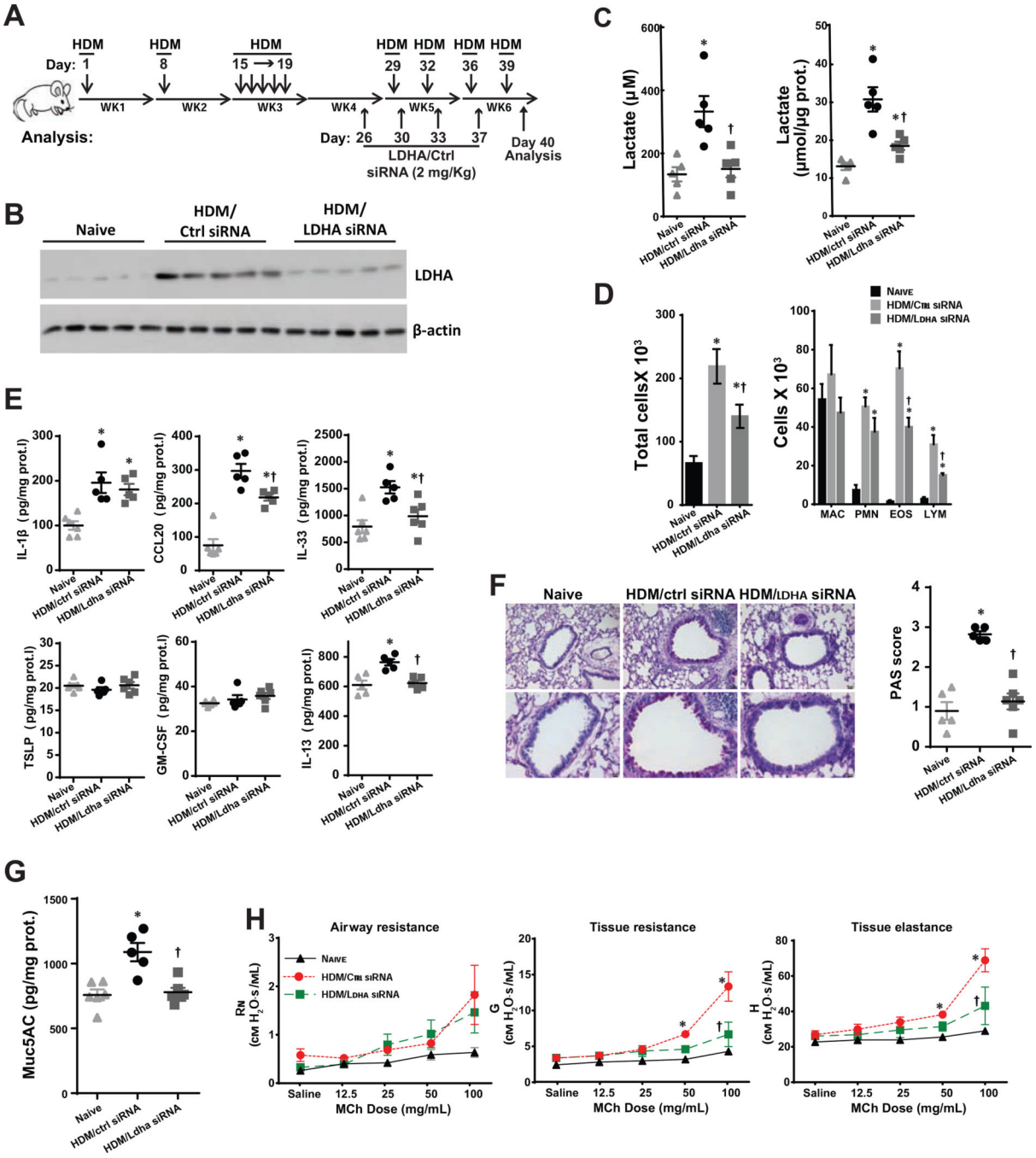

Figure 7. siRNA-mediated knockdown of *Ldha* attenuates HDM-mediated increase in glycolysis, airway inflammation, and airways hyperresponsiveness.

A, Schematic depicting the dosing regimen of HDM, control (Ctrl) and *Ldha* siRNAs. At day 40, Salineexposed 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 naive 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).

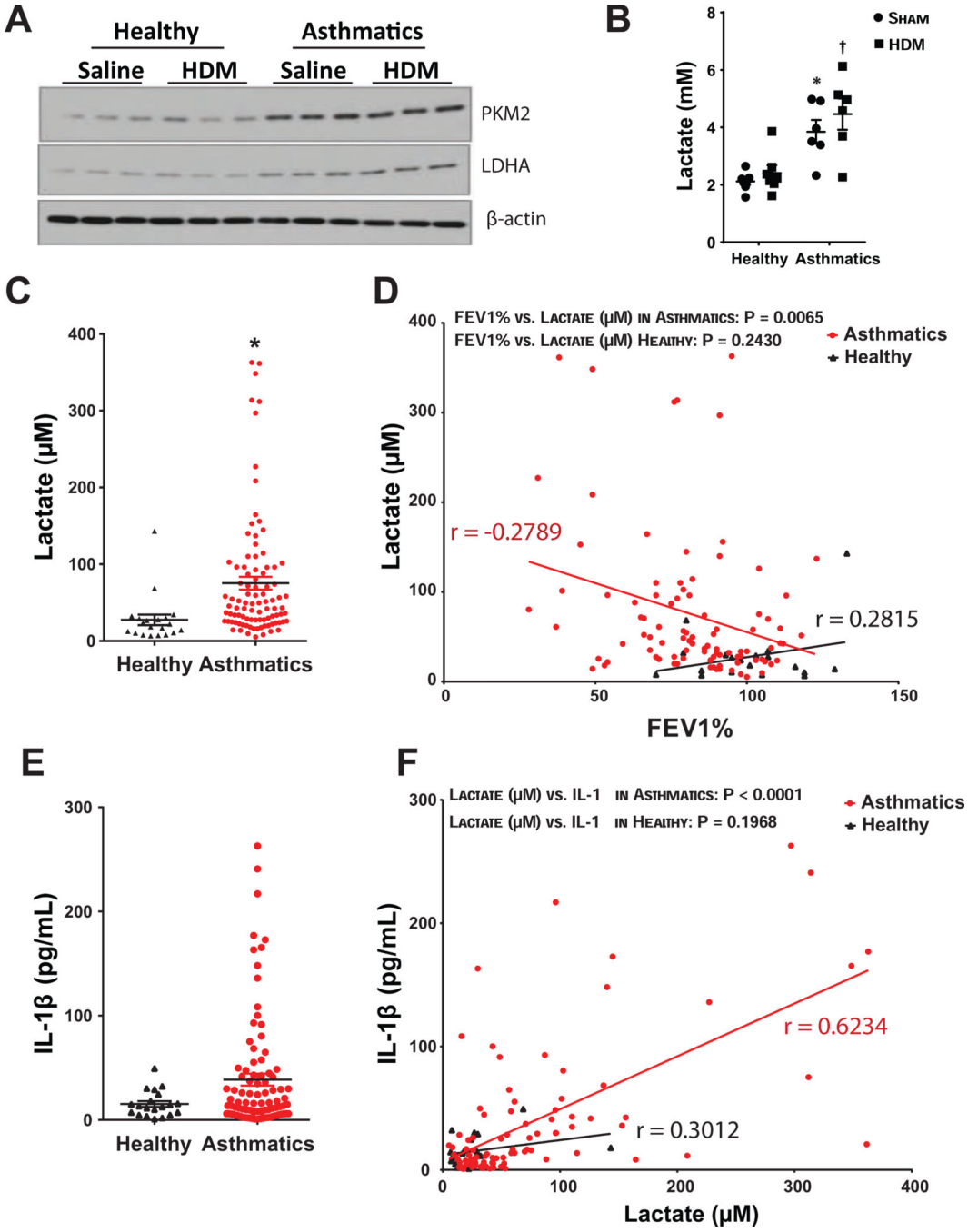

Figure 8. Evidence of increases in glycolysis in human asthma.

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.