ИММУНОМЕТАБОЛИЧЕСКИЕ ИЗМЕНЕНИЯ МАКРОФАГОВ В ОТВЕТ НА ЭКСТРАКТ ПЫЛЕВОГО КЛЕЩА

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Резюме. Молекулярные механизмы, лежащие в основе патогенеза астмы, одного из наиболее распространенных хронических заболеваний дыхательной системы, остаются не до конца изученными. Отсутствие специфичной и высокоэффективной терапии для некоторых подтипов этого гетерогенного заболевания требует поиска новых подходов к лечению. Один из таких подходов может заключаться в воздействии на иммунометаболические функции миелоидных клеток. Этот подход уже нашел свое применение в терапии рака и других заболеваний, в патогенезе которых важную роль играют макрофаги. Ранее было показано, что патогенез аллергической астмы, возникающей в ответ на распространенный сложный аллерген клеща домашней пыли, обусловлен метаболическим TNF-опосредованным перепрограммированием альвеолярных макрофагов. Это дает основания предполагать, что влияние на процесс продукции TNF или метаболические адаптации с помощью специфичных блокаторов могут также привести к ослаблению симптомов течения заболевания в целом. В данной работе было экспериментально проверено, сохраняется ли ранее описанный фенотип, возникающий у макрофагов в ответ на экстракт клеща домашней пыли (house dust mite, HDM) при культивировании в стандартной среде DMEM, в более физиологичных условиях – в среде близкой по составу к плазме крови. Также нами были проанализированы открытые базы данных секвенирования РНК из альвеолярных макрофагов, полученных от пациентов с астмой или из легких мышей в экспериментальной модели HDM-индуцированной астмы, с целью поиска и сравнения происходящих метаболических изменений. Было показано, что при культивировании в условиях близких к физиологическим, как и на классической среде, макрофаги в ответ на активацию HDM одновременно увеличивают показатели дыхания и гликолиза, а также продуцируют TNF. Наблюдаемый фенотип согласуется с данными транскриптомного анализа, выполненного на образцах человека и мыши, в которых было выявлено увеличение экспрессии генов, относящихся к гликолизу, окислительному фосфорилированию и сигнальному пути TNF. Таким образом, данные подтверждают релевантность

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фенотипа, полученного *in vitro*, изменениям происходящим в системах *in vivo*. На следующем этапе потребуется также функциональная верификация на уровне продуцируемых метаболитов, белков и изменений метаболической активности. Кроме того, в дальнейшем предстоит установить, как блокировка отдельных метаболических путей влияет на особенности функционального фенотипа макрофагов, возникающего в ответ на HDM, и способно ли данное воздействие облегчать симптомы астмы.

Ключевые слова: макрофаги, иммунометаболизм, HDM, аллергическая астма, TNF, поляризация макрофагов

IMMUNOMETABOLIC CHANGES IN MACROPHAGES IN RESPONSE TO HOUSE DUST MITE EXTRACT

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Abstract. To date, much remains unclear about the pathogenesis of asthma, one of the most common chronic and highly heterogenic diseases of the respiratory system. The lack of specific and highly effective therapy in case of certain asthma subtypes requires the search for new approaches to treatment. One possible approach would be to influence the metabolism and immune functions of myeloid cells. This approach finds its application in the treatment of cancer and other diseases in the pathogenesis of which macrophages play an important role. It was shown that the pathogenesis of allergic asthma in response to one of the most common allergens, house dust mite, is due to a metabolic TNF-mediated reprogramming of alveolar macrophages. This suggests that influencing the process of TNF production or metabolic adaptations with specific blockers may also lead to a reduction in the symptoms of the course of the disease as a whole. In this work, we experimentally tested whether the previously obtained phenotype that occurs in macrophages in response to HDM cultured in DMEM is preserved if cells are cultured under more physiologically relevant conditions: in a medium closely related in composition to blood plasma. We also analyzed open databases of alveolar macrophages sequencing obtained from patients with asthma or from the lungs of mice in an HDM-induced asthma model in order to correlate specific immunometabolic changes. It was found that macrophages cultured under conditions close to physiological, simultaneously increase the rates of respiration and glycolysis, and also produce TNF in response to HDM. The observed phenotype is consistent with transcriptomic analyzes performed on human and mouse samples, which revealed an increase in the expression of genes related to glycolysis, oxidative phosphorylation, and the TNF signaling pathway. Thus, the data confirm the relevance of the phenotype obtained *in vitro* to the changes occurring in the *in vivo* system. However, functional verification at the level of metabolites, proteins and changes in metabolic activity is also required. In addition, it remains to be established how the blocking of individual metabolic pathways affects the features of the functional macrophage phenotype that occurs in response to HDM, and whether this effect can alleviate asthma symptoms.

Keywords: macrophages, immunometabolism, HDM, allergic asthma, TNF, macrophage polarization

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Introduction

Bronchial asthma is one of the most common chronic respiratory diseases. The commonly used steroid-based therapy is not always effective in asthma due to the heterogeneity of the disease. In this regard, it remains relevant to study the mechanisms underlying the pathogenesis of asthma and to search for new therapeutic strategies. In recent studies the role of the innate immune system in the development and course of asthma was highlighted. Lung tissue-

resident macrophages are the first to encounter antigens that enter the respiratory tract, including common allergens such as household dust. House dust mite (HDM) causing allergic asthma in humans, and, in addition, it is used to model asthma in mice *in vivo*. It was demonstrated that the release of TNF, a key proinflammatory cytokine, by macrophages results in formation of innate immune memory and is crucial for HDM-induced asthma development in mice. Moreover, macrophage cellular metabolism affects TNF production in the context of HDM-induced asthma [4].

In our previous study we established that HDM induces distinct inflammatory cytokine profiles in bone marrow derived macrophages, dendritic cells and alveolar macrophages. In addition, we determined that these myeloid cell types acquire a specific metabolic phenotype characterized by a simultaneous increase in glycolysis and respiration in response to HDM activation *in vitro* associated with the alterations in the morphology of mitochondria [7]. In order to translate the relevance of our observations *in vitro* to future development of novel immunometabolism-based therapies of asthma in humans, we analyzed available transcriptomic data of human and mouse HDM-activated alveolar macrophages [1, 6]. Moreover, we confirmed HDM-induced macrophage metabolic profiling using cell culture medium that mimics human sera. In conclusion, **the purpose of this work** is to further elucidate the mechanisms underlying the metabolic reprogramming of macrophages in response to HDM.

Materials and methods

Mice

C57Bl/6 mice (6-8 weeks) were housed in SPF conditions at the Animal Facility of the Center for Precision Genome Editing and Genetic Technologies for Biomedicine, EIMB RAS (under contract № 075-15-2021-1067 with the Ministry of Science and Higher Education of the Russian Federation). All manipulations with animals were carried out in accordance with the protocol approved by the Bioethics Committee of the EIMB RAS (Protocol No. 3 from 27/10/22).

Primary cultures of bone marrow-derived macrophages (BMDM)

Bone marrow was isolated from tibias and femurs of C57Bl/6 mice. For BMDMs differentiation bone marrow cells were cultured on non-treated cell culture plates in Plasmax medium [2] containing 30% L929 conditioned medium as the source of M-CSF, 2% FBS (Capricorn Scientific), and antibiotics (Pen/Strep, ThermoFisher). Cells were harvested and passaged in fresh medium on day 5 and used for subsequent experiments on day 6.

BMDM activation

To analyze cytokine production, cells were seeded on a 96-well plate at a density of 10^5 cells per well in 100 μL of Plasmax medium 1 hour prior to the activation. Cells were activated for 4 hrs with LPS 10 ng/mL (Lipopolysaccharides from *Escherichia coli* O111:B4, catalog number L2630-100MG, Sigma) or HDM 10 μg/mL (*Dermatophagoides pteronyssinus* extract FD, catalog number 15G10, Citeq biologics).

Cytokine production

Cytokine release in culture supernatants of stimulated BMDM was measured by enzyme-linked immunosorbent assay (ELISA) using ready-made commercial kits "Mouse TNFalpha ELISA Ready-SET-Go" (ThermoFisher) according to the manufacturer's protocol.

Analysis of cellular metabolism

Cell metabolism experiments were carried out on Seahorse XFe24 Analyzer (Agilent) using an in-house validated protocol. The day prior to the experiment, cells were seeded in Seahorse 24-well plates at a density of $1{,}5 \times 10^5$ cells per well in 1 mL of complete Plasmax medium. Plates were placed overnight in an incubator 37 °C, 5% CO_2. Upon activation, cells were washed and incubated in Seahorse Assay Medium supplemented with 1 mM sodium pyruvate and 2 mM L-Glutamine at 37°C without CO_2 for 30-45 min. Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured during sequential addition of 10 mM glucose; 1 μM oligomycin; 2 μM fluoro-carbonyl cyanide phenylhydrazone (FCCP); and combination of 1 μM rotenone, 1 μM antimycin A and 50 mM 2-deoxy-glucose (2-DG). SeaHorse data were analyzed in accordance with the recommended protocol sing the Wave software (Agilent).

Statistical analysis

All experiments were performed at least 2 times. Data were analyzed using GraphPad Prism 7 software. Data were first tested for Gaussian distribution with the D'Agostino & Pearson omnibus normality test and then analyzed using Mann–Whitney test, 2-way ANOVA tests, followed by Sidak's or Tukey's post-test analysis for multiple comparisons. Results are displayed as mean ± SEM. Differences were considered significant when p values were below 0.05.

Differential gene expression analysis

Transcriptomic analysis of publicly available datasets (human data: GSE144576 [6]; mouse data:

GSE148590 [1]) was conducted and visualized using Phantasus resource (https://artyomovlab.wustl.edu/phantasus/). Data were log-transformed and genes below threshold expression level (median < 2) were excluded. Differential expression analysis was performed using limma algorithm. Genes from specific metabolic pathways were identified using Gene Ontology Browser from The Jackson Laboratory (https://www.informatics.jax.org/vocab/gene_ontology). P-values, adjusted for multiple comparisons, below 0.05 were considered statistically significant.

Results and discussion

Bone marrow-derived alveolar macrophages demonstrate HDM-specific metabolic phenotype

To examine whether HDM-induced immunometabolic phenotype observed under standard culture medium conditions (high glucose levels, lack of physiologic carbon sources) can be reproduced under more physiological conditions *in vitro*, BMDM were grown in Plasmax medium which is similar in composition to blood plasma. After six days in culture cells were activated with HDM or LPS followed by assessment of TNF production, as well as glycolysis and respiration rates (Figure 1A). BMDM grown in a physiological medium maintained a metabolic response to allergens similar to cells grown in classical medium. Activation of BMDM with LPS resulted in accelerated glycolysis (Figure 1E, F), while HDM promoted both glycolysis and oxidative phosphorylation (Figure 1C, D).

Interestingly, Plasmax-grown BMDM produced less TNF in response to HDM and LPS activation than DMEM-grown macrophages (Figure 1B). Despite the fact that the composition of the culture medium affected the TNF levels in activated macrophages, the overall immunometabolic phenotype in response to HDM was preserved. Therefore, we hypothesized that HDM-induced immunometabolic phenotype of alveolar macrophages observed *in vitro* can be translated *in vivo*.

Transcriptomic profiles of alveolar macrophages show increase in expression of a subset of 'Glycolysis', 'Electron transport chain' and 'TNF signaling' pathway genes

The study of specific expression profiles of cells derived from patients is a powerful tool for uncovering pathological mechanisms. To establish if metabolic pathways are altered in alveolar macrophages (AM) in response to HDM, we analyzed the expression of genes related to several metabolic pathways (glycolysis, Krebs cycle, electron transport chain (ETC), fatty acid oxidation (FAO) and synthesis (FAS), tryptophan metabolism, glutamine metabolism, and the pentose phosphate pathway) in open databases of Bulk RNA sequencing. Our analysis was based on two previously published studies. One study compared AMs, harvested via bronchoscopy from twelve patients with intermittent-to-mild asthma and HDM asthma and challenged with saline or HDM extract combined with LPS [6]. Another study compared AMs from C57Bl/6 mice during experimental allergic airway disease driven by repeated HDM inhalation and compared them to control AMs from na ve mice [1].

We found that in response to HDM in AMs, both from patients with allergic asthma and those obtained in mouse asthma model, the expression of glycolysis (Figure 2A, B) and ETC (Figure 2C, D) genes increased. In particular, there is an increase in expression of genes encoding 6-phosphofructokinase 1 (PFKM, Pfk1, Pfkp), enolase (ENO1, Eno2), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phosphoglycerate kinase (Pgk1) and hexokinase (Hk1). In addition, the expression of a number of genes encoding the synthesis of complex I (NDUFS8, NDUFA9, NDUFB11, NDUFS7, NDUFS5, NDUFA3, Ndufc1, Ndufa13, Ndufb6), complex III (UQCRC1, Uqcrc10, Uqcrq, CYC1) and cytochrome c (COX8A, COX7A2, COX6A1, Cox7b, Cox5b, Cox7a2) increased. These data are consistent with the previously observed functional increase in glycolysis and respiration in HDM-activated macrophages [7].

In addition, HDM stimulates an increase in the genes involved in fatty acid synthesis and the production of prostaglandins, which play an important role in the pathogenesis of asthma. Our search did not reveal a significant differential expression of genes associated with Krebs cycle, FAO, tryptophan metabolism, glutamine metabolism, or the pentose phosphate pathway (data not shown). TNF-induced metabolic reprogramming plays an important role in HDM dependent asthma [4]. According to evidence of TNF participation in asthma pathogenesis, analyzed data also shown an increase in genes involved in the TNF signaling pathway (Figure 2E, F). However, it was previously shown that changes in immunometabolic functions can procced independently of transcriptional programs, since the regulation of many metabolism-associated factors rely on posttranslational modifications or substrates availability [3]. Thus, the multi-omics approach will allow learning more about the relationship between

immunity and metabolism in patients or *in vivo* animal models.

Altogether, the HDM-induced phenotype of alveolar macrophages observed *in vitro* (Figure 1) and *in vivo* (Figure 2) may suggest a major reorganization of metabolism in response to activation. On the other hand, it remains unclear, how simultaneous upregulation of glycolysis and oxidative phosphorylation influence the severity of asthma. We can hypothesize that high levels of respiration may promote the production of mitochondrial ROS, thus ROS play an important role in serve asthma pathogenesis. The activity of OXPHOS is associated with the Krebs cycle, which uses not only pyruvate as substrates, but also fatty acid oxidation products and glutamine. In this regard, it is important to study which substrates are involved in the development of increased respiration in macrophages activated by HDM.

The search for specific metabolic pathways involved in the formation of metabolic adaptations and TNF production by macrophages in response to HDM and their subsequent blocking seems to be a promising and interesting direction for finding new approaches to asthma therapy. For example, TNF blocking was effective in the context of a mouse model of asthma [4]. A deeper understanding of the TNF-dependent metabolic rearrangement will allow to improve this approach and make therapy more specific. Given that metabolic changes can be associated not only with the transcriptional level, RNAseq data have limited predictive value for the analysis of functional changes. However, our analysis allowed us to confirm the existence of general trends at different levels: *in vitro*, *in vivo* mouse models and clinical specimens, and the significance of the observed macrophage immunometabolic phenotype for HDM-induced asthma (Figure 3).

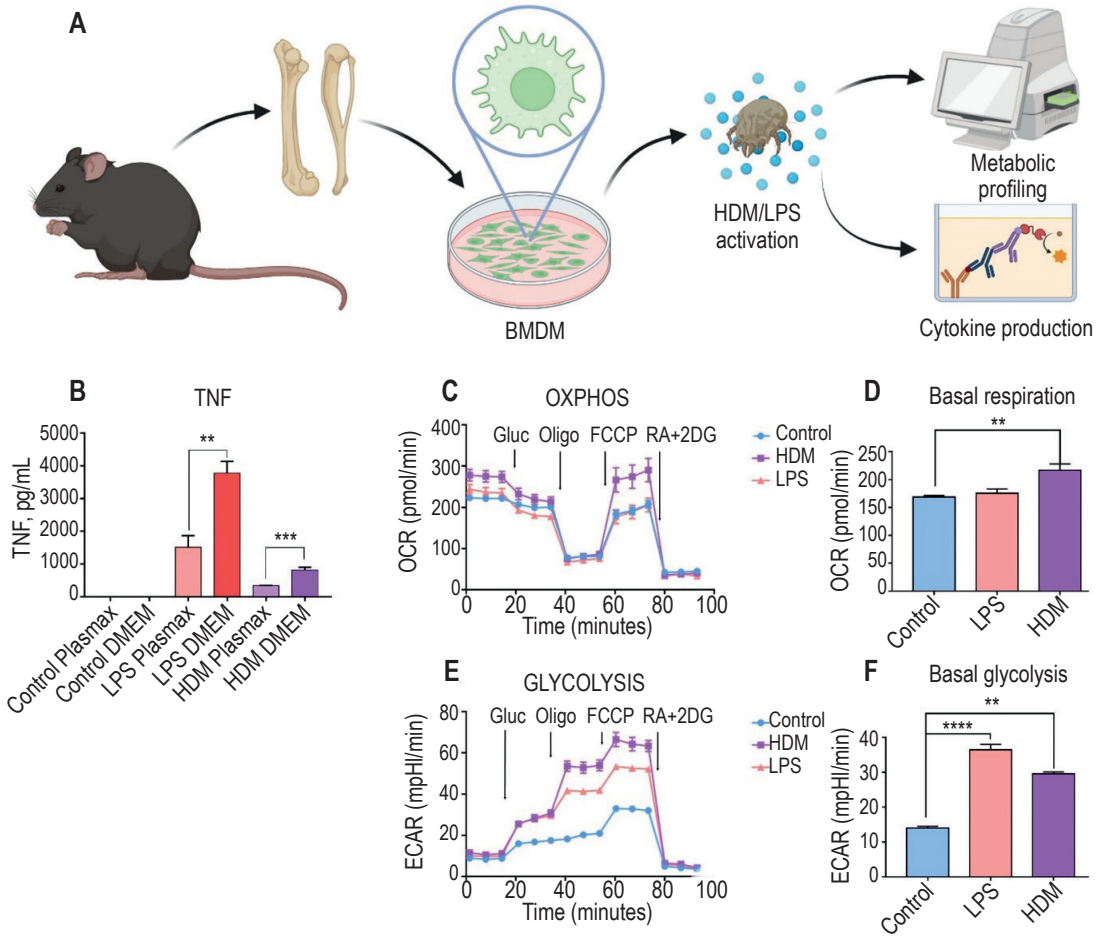

Figure 1. Bone marrow-derived alveolar macrophages demonstrate HDM-specific metabolic phenotype in Plasmax

Note. Bone marrow cells were differentiated in DMEM or Plasmax into macrophages with L929-conditioned medium; stimulated with HDM, LPS or unstimulated control for 24 h (A). Concentration of TNF in supernatants was measured by ELISA (B). OCR and ECAR of BMDM (C and E) were analyzed using Seahorse. Basal respiration was calculated based on OCR (D). Basal glycolysis was calculated based on ECR (F). Gluc, glucose; Oligo, oligomycin; FCCP, fluoro-carbonyl cyanide phenylhydrazone; RA + 2DG, combination of rotenone, antimycin A and 2-deoxy-glucose. Data are presented as mean ± SEM. TNF production data was analyzed using Mann–Whitney test. Seahorse data were analyzed using two-way ANOVA with Tukey's multiple comparisons test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

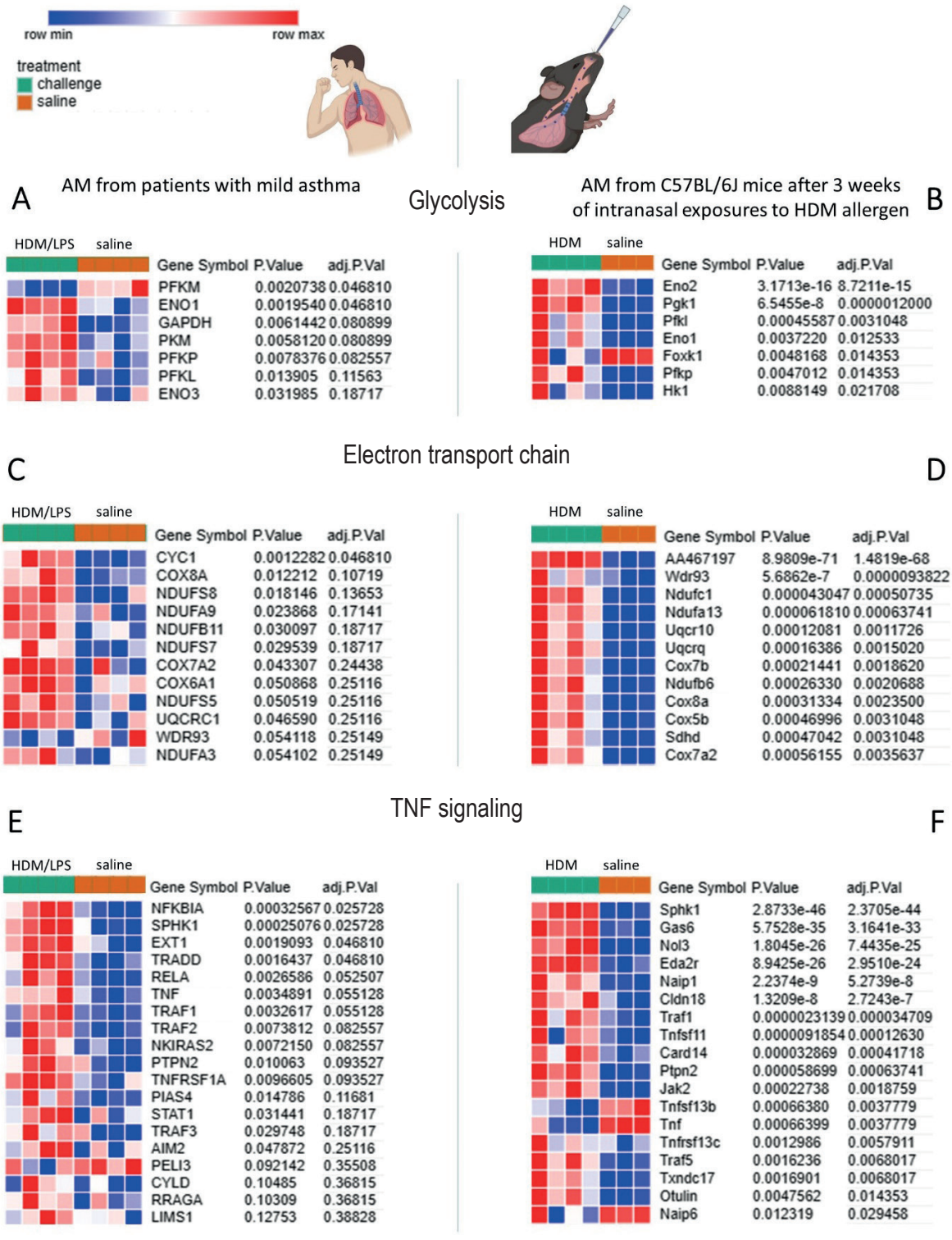

Figure 2. HDM-induced increase in expression of genes, associated with glycolysis and cellular respiration in human and murine alveolar macrophages

Note. Increased expression of representative genes from "Glycolysis" pathway in human AMs HDM/LPS compared to control AMs (A) and in mice AMs HDM compared to control AMs (B). Expression of representative genes for "Oxidative Phosphorylation and electron transport chain" subset in human AMs HDM/LPS compared to control AMs (C) and in mice AMs HDM compared to control AMs (D). Decreased expression of representative genes from "TNF signaling" pathway in human AMs HDM/LPS compared to control AMs (E) and in mice AMs HDM compared to control AMs (F). Heatmaps show row-centred gene expression values (n = 4 patients, PBS; n = 4 patients, HDM/LPS; n = 3 mice, PBS; n = 4 mice, HDM).

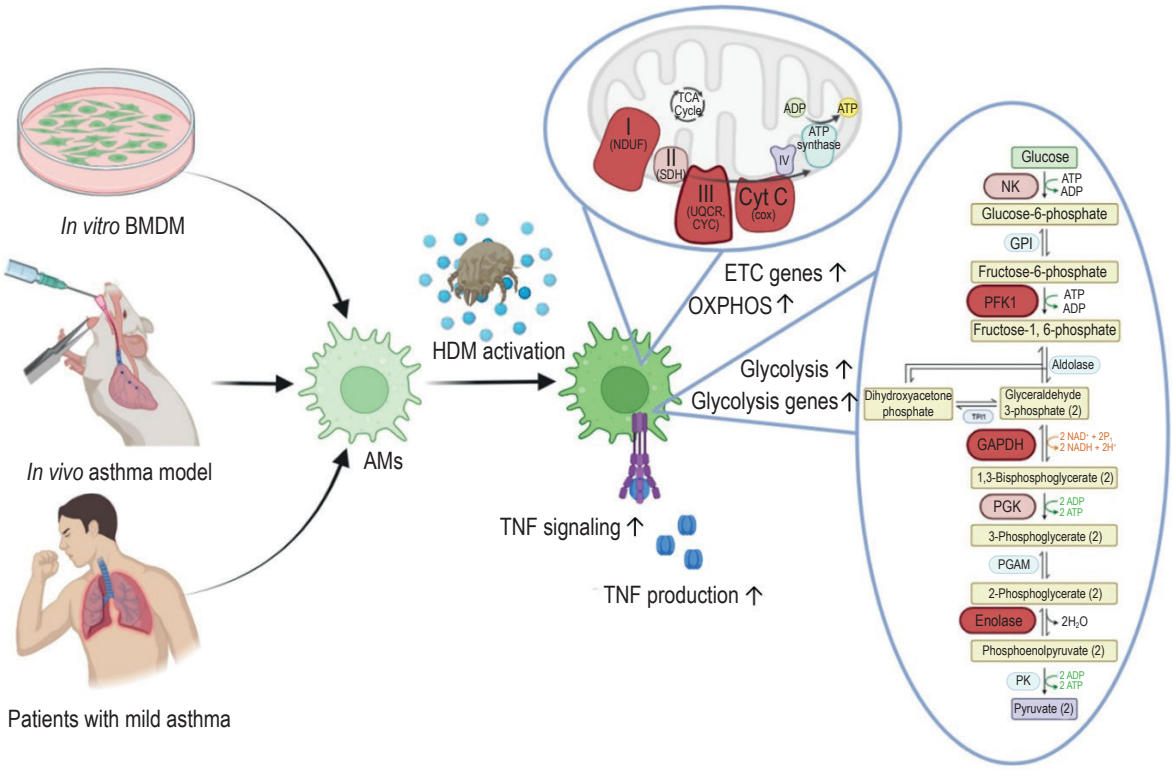

Figure 3. Development of novel immunometabolism-based therapies of asthma in humans

Conclusion

The strategy for immunometabolism intervention is currently being studied predominantly in the context of cancer, but there is growing evidence suggesting that it may have wider applications. Although targeting metabolic pathways is a promising strategy for the treatment of inflammatory diseases, this approach has many limitations, including the low specificity of metabolic inhibitors. Many questions remain to be addressed, however, a comprehensive view on the metabolic pathway system, an identification of molecules associated with dynamic changes in macrophage activation, and a better understanding of their interactions are critical for understanding the molecular basis of disease progression and for development of new therapeutic strategies targeting macrophages.

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