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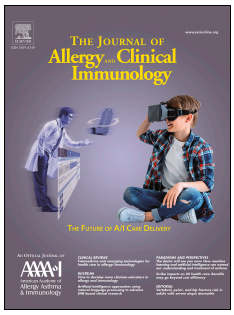

Macrophages acquire a TNF-dependent inflammatory memory in allergic asthma

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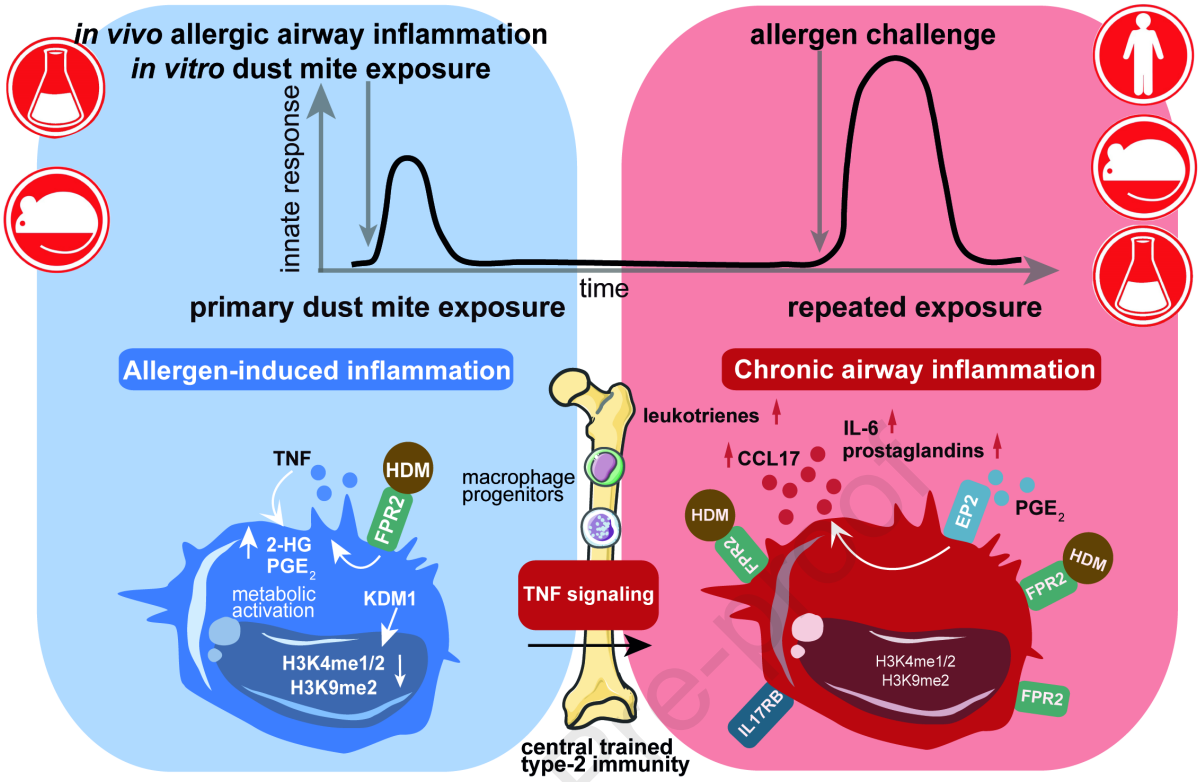

Macrophages acquire a TNF-dependent inflammatory memory in allergic asthma

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Abstract

Background: Infectious agents can reprogram or "train" macrophages and their progenitors to respond more readily to subsequent insults. However, whether such an inflammatory memory exists in type-2 inflammatory conditions such as allergic asthma was not known.

Objective: To decipher macrophage trained immunity in allergic asthma.

Methods: We used a combination of clinical sampling of house dust mite (HDM)-allergic patients, HDM-induced allergic airway inflammation (AAI) in mice and an *in vitro* training set-up to analyze persistent changes in macrophage eicosanoid-, cytokine- and chemokine production as well as underlying metabolic and epigenetic mechanisms. Transcriptional and metabolic profiles of patient-derived and *in vitro* trained macrophages were assessed by RNA sequencing or Seahorse and LC-MS/MS analysis, respectively.

Results: We found that macrophages differentiated from bone marrow- or blood monocyte-progenitors of HDM-allergic mice or asthma patients show inflammatory transcriptional reprogramming and excessive mediator (TNF-α, CCL17, leukotriene, PGE_2, IL-6) responses upon stimulation. Macrophages from HDM-allergic mice initially exhibited a type-2 imprint, which shifted towards a classical inflammatory training over time. HDM-induced AAI elicited a metabolically activated macrophage phenotype, producing high amounts of 2-hydroxyglutarate (2-HG). HDM-induced macrophage training *in vitro* was mediated by a formyl-peptide receptor 2 (FPR2)-TNF-2-HG-PGE_2/EP2-axis, resulting in an M2-like macrophage phenotype with high CCL17 production. TNF blockade by etanercept or genetic ablation of *Tnf* in myeloid cells prevented the inflammatory imprinting of bone marrow-derived macrophages from HDM-allergic mice.

Conclusion: Allergen-triggered inflammation drives a TNF-dependent innate memory, which may perpetuate and exacerbate chronic type-2 airway inflammation and thus represents a target for asthma therapy.

Key messages:

- Macrophages from HDM-allergic asthmatics show persistent inflammatory imprinting
- Inhibition of TNF signaling prevents macrophage trained immunity in allergic airway inflammation
- 2-hydroxyglutarate, PGE_2 and lysine demethylase 1 mediate allergen-driven metabolic and epigenetic macrophage reprogramming

Capsule summary: Macrophages and their progenitors develop a type-2 inflammatory memory in allergic asthma, which can be targeted by inhibiting tumor necrosis factor.

Keywords: CCL17, chemokines, eicosanoids, lipid mediators, macrophages, trained immunity, type 2 inflammation

Abbreviations:

AAI	Allergic airway inflammation
AM	Airway macrophage
aMDM	Alveolar-like monocyte-derived macrophage
BAL	Bronchoalveolar lavage
BMDM	Bone marrow-derived macrophage
cysLT	Cysteinyl leukotriene
DEG	Differentially expressed genes
FPR2	Formyl-peptide receptor 2
GM-CSF	Granulocyte-macrophage colony stimulating factor
HDM	House dust mite
HIF	Hypoxia-inducible factor
2-HG	2-hydroxyglutarate
IL	Interleukin
KDM	Lysine demethylase

LOX	Lipoxygenase
LSD1	Lysine demethylase 1
RNAseq	RNA sequencing
TGFβ	Transforming growth factor β
TLR	Toll-like receptor
TNF	Tumor necrosis factor

Introduction

The prevalence of allergic asthma has constantly increased over the last 2 decades (1). House dust mite (HDM) represents the most prominent aeroallergen and approximately 50% of asthmatics are sensitized to it (2). In addition to allergen-specific T cells, the innate immune system contributes to type-2 inflammation in allergy (3). Macrophages play an important role in asthma and asthma severity correlates with numbers of M2-polarized macrophages in the airways (4,5). CCL17, a T_H2-cell chemoattractant (6), is overexpressed in alveolar macrophages from asthmatic patients and involved in asthma exacerbations (7–10). Eicosanoids are key mediators of type-2 inflammation (11,12) and airway macrophages of asthmatic patients, show an exaggerated production of proinflammatory leukotrienes (LTs) (13). LT production and recruitment of inflammatory monocytes are central for the development of allergic airway inflammation (AAI) (14,15). While other eicosanoid-producing myeloid cells (e.g. eosinophils) are cleared from the lung after acute inflammation resolves (16), macrophages persist (17). Pathogen molecules or sterile inflammatory stimuli trigger bioenergetic and epigenetic reprogramming in monocytes and macrophages, which may result in persistently altered responsiveness and effector functions (18–20). This phenomenon, termed "trained immunity", is not limited to tissue macrophages but extends to bone marrow progenitors that provide "central trained immunity" (21,22). Respiratory viral infection can induce macrophage reprogramming and replacement, thus promoting or preventing asthma development (23–25). However, if and how macrophage trained immunity is triggered during allergen-driven inflammation remained unclear. We found that monocyte- or bone marrow-

derived macrophages from HDM-allergic asthmatics or HDM-sensitized mice persistently upregulate inflammatory genes and type-2-inflammatory chemokines and eicosanoids (CCL17, cysteinyl leukotrienes (cysLTs). This inflammatory memory depended on FPR2- and TNF-signaling resulting in metabolic reprogramming and KDM1-mediated histone demethylation, thus representing a trained immunity program that may contribute to chronification and exacerbation of allergic asthma.

Methods

For a more detailed description of the experimental procedures, see the Online Repository.

Human study participants

The ethics committee of the Technical University of Munich approved the study (internal reference: 422/16). HDM-allergic patients and healthy subjects (see Table 1 for patient characteristics) were recruited at the Allergy Section, Otolaryngology Department, TUM School of Medicine. All participants gave informed written consent in accordance with the Declaration of Helsinki before sampling. The study visit consisted of questionnaires (SNOT22, MiniRQLQ, PSQ20), blood- and sputum collection. The clinical diagnostic laboratory of the hospital assessed differential blood cell counts, specific mite IgE and total IgE. Sputum induction and sputum cell isolation was performed as previously described (26).

Murine model of allergic airway inflammation

6-8 weeks old mice (wildtype C57BL/6J, *Tnf* $^{fl/fl}$ and LysM-cre *Tnf* $^{fl/fl}$) were intranasally sensitized and challenged with HDM extract as previously described (11) (Fig.E1). Analysis was performed on bronchoalveolar lavage, lung tissue, airway macrophages and bone marrow cells, comparing mice sensitized and challenged to PBS or HDM.

In vitro macrophage differentiation and culture

Monocyte- or bone marrow-derived macrophages were generated as previously described (11,12). Supernatants were analyzed by liquid-chromatography tandem-mass spectrometry or ELISA after stimulation with ionophore A23187 (5 µmol/L, Merck) for 10 min, and cell pellets were analyzed via western blot, qPCR or RNA sequencing.

Metabolic flux analysis

5 x 10^4 MDM or BMDM were plated per well on a Seahorse Miniplate (Agilent) and cultured for training (aMDM) or stimulated overnight (BMDM) before mitochondrial stress test (Agilent).

Metabolomics analysis

5 x 10^5 aMDM or BMDM were pelleted for targeted metabolomics. Metabolite quantification by LC-MS was performed at the Metabolomics Core Facility of the Max Planck Institute for Immunobiology and Epigenetics in Freiburg, Germany.

Statistical analysis

Data were analyzed using Graphpad Prism 9 (Graphpad, San Diego, CA, USA). T-test or Mann-Whitney test were used to compare two populations depending on normal distribution. For comparison of more groups, Friedmann test, one-way or two-way ANOVA was used with correction for multiple comparisons as indicated in the figure legends. P values<0.05 were considered statistically significant. See figure legends for details of statistical tests and sample size. Heatmaps were generated using Morpheus software (Broad Institute).

Results

Macrophages from HDM-allergic patients show transcriptional reprogramming and enhanced production of type-2 inflammatory mediators

Macrophages represent key regulators of lung homeostasis and immunity and they govern airway inflammation by producing eicosanoids and chemokines (15,27). We recently described stable differences in gene expression and metabolite profiles in macrophages from patients with NSAID-exacerbated respiratory disease (N-ERD) (26), a non-allergic chronic type-2 inflammatory condition. To study a potential macrophage memory in allergic asthma, we generated macrophages (aMDM) from monocytes of HDM-allergic or healthy donors (Table 1) (Fig.E1 A). RNA sequencing (RNAseq) analysis yielded 88 genes differentially expressed between aMDM from HDM-allergic compared to non-allergic donors (28 up, 60 down) (Fig.1 A, B, Supplementary Data file 1), indicating stable transcriptional reprogramming that persisted throughout *ex vivo* differentiation. *S100P*, *TNFSF10* (TRAIL), *CLEC4D* (dectin-3), *LGALS12* (galectin-3) and *IL12RB1*, all implicated in macrophage activation (28–32), were upregulated in aMDM of allergic donors while immunoregulatory genes such as *MERTK* and *CD84* (33,34) were downregulated (Fig.1 A, B). *CD84* and *MERTK* gene expression correlated negatively while *ITGA1* and *S100P* correlated positively with disease scores MiniRQLQ and SNOT-22 (Fig.E2 A). Several of the DEGs identified in aMDM of HDM-allergic asthmatics (e.g. *S100P*, *ITGA1*, *TNSF10*, *MERTK*, *CD84*), are regulators or downstream targets of TNF-signaling. *In vitro* HDM exposure resulted in enhanced production of TNF, IL-12 p70, CXCL2, S100P and IL-1β from patient-derived aMDM, while IL-10 induction tended to be reduced (Fig.1 C, Fig. E2 B). However, CCL5, CCL11 and IL-18 production was similar in aMDM from HDM-allergic and healthy subjects (Fig. E2 C), suggesting that the enhanced HDM response of patient aMDMs was dominated by TNF. Unstimulated aMDM, but not airway macrophages (AM) from HDM-allergic individuals produced exaggerated amounts of cysLTs, important mediators of type-2 inflammation (14) as well as further 5-lipoxygenase (5-LOX)-derived eicosanoids (Fig.1 D, E, Fig.E2 D, E). In addition, CCL17, a driver of the Th2 response in asthma (7,35) tended to be increased in aMDM and AM of HDM-allergic asthmatics (Fig.1 D, E). Thus,

aMDM from allergic asthmatics exhibited inflammatory imprinting and type-2-driving mediator profiles at baseline and enhanced TNF-dominated HDM responses.

HDM-induced allergic airway inflammation induces a persistent inflammatory imprint in the bone marrow

Similar to their human counterpart, murine macrophages (BMDM) differentiated for 7 days from bone marrow progenitors of HDM-sensitized mice (Fig.E1 B) showed an elevated production of cysLTs and enhanced *Ccl17* expression compared to PBS-sensitized mice (Fig.2 A, Fig.E2 F), which was reflected in airway macrophages of HDM-sensitized mice (Fig.2 B). In contrast to cysLTs, 5-LOX-derived mediators were not generally increased in AM (Fig.E2 G). Seven days post-challenge, HDM-induced AAI as well as type-2 cytokine expression in the bone marrow had mostly resolved (Fig.2 C, Fig.E2 H). However, AM and BMDM maintained their elevated production of CCL17 (Fig.2 D). Additionally, BMDM upregulated classical trained immunity genes (*Il6* and *Ptgs2*) (Fig.2 E, Fig.E2 I). Genes differentially regulated in aMDM from HDM-allergic donors (Fig.1 A, B), *Cd84, Mertk, Clec4d, Itga1* and *Tnfsf10* showed a similar pattern in BMDM from HDM-sensitized mice (Fig.2 E, E2 J). Together this suggested that allergic airway inflammation leaves an innate memory both locally and in bone marrow progenitors.

HDM-training elicits exaggerated cysLT and CCL17 responses and transcriptional reprogramming in human aMDM

To study whether *in vivo* reprogramming of HDM-experienced macrophages could be mimicked *in vitro* (Fig.E1 C), aMDM were stimulated ("trained") with HDM on day 7 of differentiation, re-stimulated after a 5-day wash-out period and harvested 24h later for eicosanoid, gene expression and cytokine analyses. *In vitro* HDM-trained and re-stimulated

aMDM escaped HDM-induced, TLR4-dependent cysLT-suppression (12) resulting in high amounts of cysLTs (Fig.3 A, Fig.E3 A), resembling the exaggerated cysLT production in aMDM or BMDM from HDM-sensitized humans or mice (Fig.1 D, 2 A). HDM-training of aMDM *in vitro* also resulted in an increased CCL17 production in response to HDM challenge (Fig.3 B), reminiscent of enhanced CCL17 production of airway macrophages from HDM-allergic patients or mice (Fig.1 E, 2 B, D). The primed CCL17 response was evident already before challenge (Fig.E3 B), dose-dependent (Fig.E3 C) and not evoked by β-glucan (BGP), a classical trigger of trained immunity (20) (Fig.E3 D). HDM-training did not affect macrophage viability (Fig.E3 E) and training with purified allergens (*Der f1* or *Der f2)* did not enhance macrophage inflammatory responsiveness (Fig.E3 F). RNAseq analysis of HDM-trained macrophages with or without HDM re-stimulation (Fig.E1 C) identified 166 DEGs in HDM-trained macrophages 6 days after HDM exposure compared to control macrophages (139 up, 27 down) and 304 DEGs between previously HDM-trained and "naïve" macrophages 24h after HDM challenge (159 up, 143 down) (Fig.3 C-F). HDM-trained macrophages exhibited an increased expression of genes involved in M2 polarization (e.g. *IRF4*, *CD163*, *IL4I1*, *VEGFA*) and chemokine/cytokine signaling (*CCL17*, *CCL18*, *CXCL9*) (Fig.3 C, E, Supplementary Data file 2), while the HDM-driven induction of interferon-induced genes, (e.g. *OASL*, *OAS2*/*3*, *ISG15*/*20*, *USP18*, *CMPK2*) was reduced compared to "naïve" HDM-stimulated aMDM (Fig.3 D, F). TNF-signaling (Fig.E3 G) as well as cytokine-cytokine receptor interaction and chemokine-signaling (Fig.E3 G, H) were enriched in HDM-trained macrophages. Inflammatory gene expression was paralleled by metabolic activation of HDM-trained macrophages (Fig.3 G-I), suggesting that metabolic reprogramming persisted following wash-out of HDM. *IL17RB* (the receptor subunit binding IL-25 (36)) was upregulated in ~~both~~ *in vitro* trained and patient-derived aMDM (Fig.1 A, B, Supplementary Data file 1, Fig.3 J) and exposure to IL-25 resulted in increased CCL17 and cysLT production in allergen-trained compared to control aMDM (Fig.3 K, L), suggesting heightened responsiveness to epithelial cues. Conversely, supernatants

from HDM-trained and challenged macrophages upregulated CXCL8 in human bronchial epithelial cells (Fig.3 M). Thus, *in vitro* HDM-training induced transcriptional and metabolic reprogramming and reproduced hallmarks of the inflammatory memory in asthma patients' macrophages with functional consequences on the airway epithelium.

FPR2- and TNF- signaling mediate HDM-induced macrophage reprogramming

We next sought to identify mechanisms underlying macrophage reprogramming by HDM. The formyl peptide rector 2 (FPR2), implicated in HDM sensing (37,38), was persistently upregulated in HDM-trained macrophages (Supplementary Data files 2,3), and induced by HDM stimulation (Fig.4 A). Blocking FPR2-signaling by a pharmacological inhibitor (PBP10) during HDM training suppressed the enhanced CCL17 response (Fig.4 B) and prevented the induction of TNF (Fig.4 C), suggesting FPR2 as a major HDM receptor involved in HDM-driven macrophage reprogramming. Since TNF-signaling was reported to initiate CCL17-mediated inflammation (39) and as it was enriched in aMDM of asthmatic patients or following *in vitro* HDM training (Fig.1, Fig.4 D, Fig.E3 G), we neutralized TNF during HDM-training, which resulted in suppression of the enhanced CCL17 response in HDM re-stimulated aMDM (Fig.4 E). *In vitro* cysLT responses were not affected by inhibition of TNF or FPR2 (Fig.E4 A, B). Treatment with the FPR2 inhibitor or TNF-neutralizing antibody alone did not influence macrophage HDM-responses on day 13 (Fig.E4 C, D). To test the relevance of TNF signaling *in vivo*, we injected HDM-sensitized mice with etanercept (a TNFR2-based fusion protein which neutralizes TNF and lymphotoxin a) during sensitization and challenge (Fig.4 F upper panel). Etanercept treatment did not influence HDM-induced AAI at 72h or 7 days post-challenge (Fig.E4 E, F) (Fig.4 G). However, etanercept treatment attenuated the increased CCL17 release by BMDM from HDM-sensitized mice (Fig.4 H, left). During *in vitro* HDM re-stimulation, the enhanced CCL17 and IL-6 response of BMDM from HDM-sensitized mice

was prevented by etanercept treatment during HDM-induced AAI (Fig.4 H, right, Fig.4 I). Sensitization and challenge of mice with a myeloid deficiency in TNF (LysM-cre $Tnf^{fl/fl}$) (40) (Fig.4 F lower panel) resulted in reduced airway eosinophilia (Fig.4 J) as well as decreased CCL17 production by BMDM at baseline and following IL-4 stimulation (Fig.4 K), supporting a role for myeloid-derived TNF in type-2 imprinting in the bone marrow during HDM-induced AAI. Together, this suggested that autocrine TNF signaling, induced via FRP2, drives the proinflammatory macrophage memory during allergen-driven inflammation.

2-hydroxyglutarate and lysine demethylase-1 drive inflammatory macrophage reprogramming

Based on the observed metabolic reprogramming of *in vitro* trained macrophages (Fig.3 G, H, I), we performed a targeted metabolomic analysis, quantifying amino acid- and TCA-cycle metabolites. BMDM from HDM-sensitized mice showed an increased output of amino acids and TCA-cycle intermediates (Fig.5 A), including metabolites involved in LT biosynthesis, M2 activation and type-2 immunity (Fig.5 A-C) (41–43). 2-hydroxyglutaric acid (2-HG), a modulator of α-ketoglutarate-dependent dioxygenase activity (44) was increased (Fig.5 D), while bioenergetic parameters indicative of glycolysis (ECAR) or mitochondrial respiration (OCR) were unaltered in HDM-sensitized compared to mock-sensitized BMDM (Fig.E5 A, B). Similarly, baseline expression of M2 markers in BMDM and genes related to the glycolytic pathway were unchanged (Fig.E5 C). M2 markers were not generally affected by inhibition or myeloid deficiency of TNF (Fig.E5 D, E), however Arginase-1 (*Arg1*) expression in BMDM was increased (Fig.5 E, F), suggesting a suppressive role of TNF on negative regulators of type-2 inflammation (45). In line with increased 2-HG in HDM-sensitized BMDM, acute HDM exposure upregulated 2-HG in human aMDM (Fig.5 G). Replacement of HDM by 2-HG during training resulted in an enhanced CCL17 but not cysLT response to HDM challenge (Fig.5 H,

Fig.E5 F), partially mimicking HDM-induced training. When added during acute activation of macrophages with LPS, 2-HG potentiated induction of *CCL17*, *IL1B* and *PTGS2* (Fig.5 I), indicating that 2-HG can enhance the inflammatory activation of aMDM. In BMDM, addition of 2-HG increased PGE_2 and CCL17 production (Fig.5 J), suggesting an involvement of 2-HG in type-2 imprinting. 2-HG promotes HIF-1α activation by inhibiting its degradation by prolyl-hydroxylases and *Hif1a* was upregulated in BMDM from HDM-sensitized mice (Fig.E5 C). HIF1α-target genes (*VEGFA*, *MMP2*, *PLOD2*, *EGR1*, *VLDLR*, *RBP1*, *PPFIA4*) (46–51) as well as *HIF1A* transcription were induced by HDM ~~-training~~ in human macrophages (Fig.3 E, F, Fig.5 K), but inhibiting HIF1α during HDM-training only partially abrogated the enhanced CCL17 response (Fig.5 L) and glycolysis (Fig.E5 G). 2-HG also modulates the activity of histone demethylases, e.g. lysine demethylase (KDM) families 2-8 (52) and *KDM6B* (JMJD3) is implicated in M2 macrophage activation (53). Genes related to M2 activation and IL-4 signaling were enriched in HDM-trained macrophages (Fig.E5 H), but *KDM6B* was suppressed in HDM-trained macrophages (Fig.E5 I) and inhibition of KDM6B during HDM-training did not affect enhanced mediator responses (Fig.E5 J, K). Instead, a screen of different histone 3 modifications in HDM-trained aMDM (Table 2) revealed less abundant H3K4 mono- and tri-methylation as well as H3K9 di-methylation, modifications induced by family 1 KDMs, e.g. *KDM1A* (LSD1) (54). Application of the KDM1A inhibitor pargyline during training suppressed CCL17 and cysLT responsiveness upon HDM-challenge (Fig.5 M), suggesting KDM1A-mediated reprogramming as the epigenetic mechanism underlying HDM-training.

HDM-induced macrophage training is distinct from classical trained immunity and driven by prostaglandin E_2/EP2-signaling

To further identify downstream mediators of TNF-driven metabolic and epigenetic macrophage reprogramming, we performed targeted LC-MS/MS and multiplex cytokine analyses for HDM-

trained aMDM immediately after allergen-training (day 8), after 5 days of rest (day13) and 24h post-HDM challenge (day 14). Except for CCL17, HDM-training evoked a transient increase of cytokines and eicosanoids which had returned to baseline after the resting phase (Fig.6 A). After HDM re-stimulation, most cytokines and chemokines were similar between HDM-trained and acutely stimulated macrophages, except for CCL17 and IL-6, which were increased in trained macrophages after HDM challenge (Fig. 3 B-F, 6 B, C). HDM-trained aMDM also synthesized high amounts of prostanoids upon challenge (Fig.6 D) and enzymes involved in the production of PGE_2, particularly mPGES1, were persistently induced by HDM training and challenge (Fig.6 E, F). Together with HDM-induced cyclooxygenase-2 (12) this likely explains augmented HDM-triggered PGE_2 production in HDM-experienced human and murine macrophages (Fig.6 G, H). Reduced HDM-triggered COX-2 (*Ptgs2*) induction following etanercept treatment (Fig.6 I) further implicated the COX-2/PGE_2 pathway in TNF-driven reprogramming. PGE_2 receptor 2 (EP2)-deficient BMDM showed an intact HDM-triggered TNF response, but a reduced CCL17 response compared to wildtype BMDM (Fig.6 J, K), suggesting that enhanced PGE_2 synthesis by macrophages represents a downstream mechanism of TNF-mediated innate immune training. Thus, the increased arachidonic acid metabolism of HDM-trained macrophages contributes to TNF-mediated trained type-2 immunity. Together, these data identify a metabolic-epigenetic circuit leading to persistent type-2 inflammatory macrophage reprogramming in allergic asthma.

Discussion

Previous studies have shown that innate memory responses on the level of ILC2s and epithelial stem cells can contribute to type-2 inflammation in the context of allergic airway inflammation and nasal polyposis (55,56). Here, we describe an allergen-driven trained immunity program in macrophages that drives the production of key mediators involved in asthma. Macrophages

derived from allergic asthma patients, HDM-sensitized mice or trained with HDM extract *in vitro* produced high amounts of CCL17 and cysLTs, both potent mediators of type-2 immunity and therapeutic targets in asthma (14,35). Trained type-2 immunity was associated with an increased arachidonic acid metabolism and prostaglandin signaling perpetuated inflammatory macrophage reprogramming. This identifies an unprecedented role for eicosanoids in trained immunity and highlights leukotrienes and prostaglandins as promising targets for preventing the chronification or exacerbation of allergen-induced airway inflammation. The heightened cysLT response of asthma patient macrophages was mimicked by HDM-training and re-exposure of macrophages *in vitro*, where it depended on TLR4 and KDM1A. KDM1A demethylates histones (particularly H3K4 and H3K9), but it has not been previously implicated in trained immunity. We found reduced H3K4 tri- and mono-methylation and reduced H3K9 di-methylation in HDM-trained vs. control macrophages, suggesting a role for KDM1 in removing repressive marks to enhance type-2 inflammatory mediator responses (57). As KDM1A activity is necessary for hematopoietic stem cell differentiation (58), its role in reprogramming of bone marrow cells and macrophage progenitors in asthma warrants further investigation. The exaggerated CCL17 and LT response of HDM-trained macrophages and macrophages from asthmatics appears to be a hallmark of allergen-induced training that drives a chronic pathologic type-2 immune bias. However, gene expression profiles of HDM-trained and challenged macrophages from healthy blood donors minimally overlapped with profiles of macrophages from HDM-allergic patients. This may be due to high experimental doses of HDM *in vitro* while *in vivo*, macrophages are exposed to lower HDM doses but over a longer time span and within a complex tissue milieu. While *in vitro* trained aMDM exhibited an M2-like transcriptional profile, allergic aMDM showed a downregulation of immunoregulatory genes (e.g. *MERTK* and *CD84*), suggesting that tolerogenic pathways may be defective in macrophages from allergic individuals. However, upregulation of *IL17RB* was evident in both allergic aMDM as well as after *in vitro* HDM-training and challenge, similar to murine ILC2

memory of allergic inflammation (55) suggesting heightened IL-25 responsiveness as a feature of the innate memory in allergic asthma. In murine BMDM, no clear M2-like phenotype was observed as *Arg1* was less induced in BMDM from HDM-sensitized compared to control mice which could result in prolonged type-2 inflammation as *Arg1* suppresses pathological Th2 responses (45). While we did not observe heightened baseline CCL17 expression in aMDM from allergic donors, sputum-derived airway macrophages cultured *ex vivo* released high levels of CCL17 compared to aMDM or compared to airway macrophages from healthy controls. This suggests that aberrant CCL17 responses depend on tissue priming of monocytes/macrophages in the lung. HDM-trained macrophages did not generally increase their production of proinflammatory cytokines, but specifically induced cysLTs and CCL17, which elicit type-2 immune responses. Thus, allergen-induced trained type-2 immunity appears to be distinct from trained immunity programs driven by microbial products, despite some overlapping features such as increased IL-6 responses (19,22). The transient upregulation of IL-4 and IL-13 in the BM following HDM challenge may contribute to the time dependent shift from type-2 to classical imprinting of macrophage progenitors. HDM-training also transiently induced TNF in an FPR2 dependent fashion, suggesting that the HDM components Der p13 and Blo t13, recently identified ligands of SAA-1-mediated FPR2 activation, mediate TNF-driven macrophage imprinting (38). TNF functions as a negative regulator of M2 polarization in cancer or infectious diseases (59–61). In arthritis, in contrast, TNF signaling is important at early time points, while TNF-induced CCL17 appears as a late mediator (39), mirroring the kinetics of HDM training in macrophages. CD84, which was significantly downregulated in patient-derived macrophages, predicts the response to etanercept in rheumatoid arthritis patients (62), suggesting TNF-mediated downregulation of CD84 as a mechanism of aberrant macrophage activation in type-2 inflammation. In the trained type-2 immunity pathway we uncovered, TNF acted as an early initiator of type-2 inflammatory macrophage activation. These data argue that TNF has a complex effect on M2 myeloid pathways that require further analyses. One

prediction emerging from our work is that TNF may have differential inhibitory or enhancing effects depending on timing and signaling via the two TNF receptors. Importantly, altered expression of TNF-response genes and type-2-inducing effector functions persisted during macrophage differentiation from bone marrow- or monocyte progenitors isolated from HDM-sensitized mice or HDM-allergic patients. Thus, HDM exposure does not only trigger local inflammatory responses, but results in a persistent reprogramming of myeloid progenitors or monocytes giving rise to macrophages with elevated inflammatory effector functions.

The induction of a trained CCL17 response by 2-HG, a modulator of histone demethylase and prolyl hydroxylase activity, suggests the involvement of histone modifications and HIF-1α in TNF-mediated trained type-2 immunity (11,63). However, how 2-HG production and HIF-1α activation are elicited downstream of FPR2 and TNF, remains to be determined. Our data suggest that 2-HG promotes COX-2 expression and PGE_2 production downstream of HDM-induced TNF, thus driving M2-like reprogramming and enhanced CCL17 production. Future studies should assess sites of differential histone methylation in HDM-experienced macrophages and define how individual modifications regulate CCL17 and cysLT responses, respectively. Based on our study design, we cannot discern whether HDM itself or the type-2 inflammation triggered by HDM is responsible for macrophage training *in vivo*. The finding that HDM-training of macrophages *in vitro* resulted in exaggerated CCL17 and cysLT responses upon challenge suggests that resident macrophages in the airways can be directly trained by HDM. In contrast, central trained type-2 immunity on the level of myeloid progenitors in the bone marrow may be evoked by the inflammatory response to HDM and our findings implicate TNF-signaling in this process. Similar to clinical trials failing to show efficacy of etanercept in asthmatic patients (64), airway inflammation was unchanged in etanercept-treated HDM-sensitized mice. However, inflammatory imprinting in bone marrow progenitors was attenuated by TNF blockade, which may prevent asthma progression or exacerbation. As TNF inhibition possesses the risk of increased infection susceptibility, it will

be necessary to understand the role of TNF-induced trained immunity in distinct human asthma endotypes. (65). It will be important to further decipher innate memory responses in allergic asthma since inflammatory reprogramming of myeloid cells may contribute to the chronification, exacerbation or even transmission of type-2 airway inflammation.

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Tables

Table 1. Clinical characteristics of healthy and HDM-allergic probands

	Healthy (SDV)		**Allergic (SDV)**		***P value***
Age [years]	28.8	2.2	30.0	7.7	0.7473
Sex [f/m]	5/0		4/2		
BMI [kg/m^2]	21.4	2.6	25.7	4.8	0.1170
MiniRQLQ	6.6	6.5	24.4	5.9	0.0020
SNOT22	5.6	2.7	25.0	14.1	0.0163
PSQ20	49.0	3.9	48.0	3.1	0.4654
Total IgE [kU/L]	10.8	8.4	241.6	328.0	0.0079
Der p IgE [kU/L]	0.0	0.0	13.4	16.9	0.0079
Der f IgE [kU/L]	0.1	0.3	15.4	20.3	0.0079
Eur m IgE [kU/L]	0.0	0.0	3.1	4.0	0.0079
Blood monocytes [%]	7.6	0.5	7.2	2.2	0.7937
Blood eosinophils [%]	2.8	2.5	5.0	1.6	0.1339

Data are presented as mean. SDV=standard deviation; F=female; m=male; BMI=body mass index; MiniRQLQ= Mini Rhinoconjunctivitis Quality of Life Questionnaire; SNOT22=Sino-nasal Outcome Test; PSQ20=Perceived Stress Questionnaire; Der p=Dermatophagoides pteronyssinus; Der f= Dermatophagoides farinae; Eur m=Euroglyphus maynei

607 **Table 2.** Histone 3 modification screen in trained vs. macrophages

Histone 3 modification	HDM-trained vs. control
H3K14ac	--
H3K18ac	=
H3K27me1	=
H3K27me2	+
H3K27me3	=
H3K36me1	=
H3K36me2	-
H3K36me3	-
H3K4me1	--
H3K4me2	=
H3K4me3	--
H3K56ac	-
H3K79me1	=
H3K79me2	=
H3K79me3	=
H3K9ac	=
H3K9me1	=
H3K9me2	--
H3K9me3	+
H3ser10P	-
H3ser28P	-

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Figure Legends

Fig. 1: Monocyte-derived macrophages from HDM-allergic asthma patients show persistent inflammatory gene expression and exaggerated production of inflammatory mediators

A: Heatmap of 28 significantly upregulated and 39 downregulated DEG in aMDM from HDM-allergic donors versus healthy donors (n=5 per group, DeSeq2) **B**: Volcano plot of DEG (fold change>2, p_{adj} <0.05) in aMDM from HDM-allergic versus healthy donors (n=5 per group) **C**: TNF, IL-12 p70, CXCL2 production and *S100P* expression of aMDM from HDM-allergic donors versus healthy donors, after 24h HDM exposure *in vitro* (n=5 per group, RM two-way ANOVA, Sidak's multiple comparisons test) **D**: Baseline cysLT and CCL17 production of aMDM from healthy vs. HDM-allergic human donors (ELISA, n=4-8 per group, Mann-Whitney or unpaired t-test) **E**: Baseline cysLT and CCL17 production of sputum-derived macrophages from healthy vs. HDM-allergic human donors (normalized to RNA concentration, n=5 per group, Mann-Whitney test). Data are presented as z-score transformed (heatmap) or mean + SEM. *p<0.05, **p<0.01

Fig. 2: HDM-induced airway inflammation induces a type-2 imprint in murine peripheral and airway macrophages, which shifts towards classical central trained immunity

A, B: CysLT production and *Ccl17* expression in BMDM (A) or BAL AM (B) from PBS- vs HDM-sensitized mice 3 days post-challenge (Mann-Whitney test, n=13-17 (A)/ unpaired t- test, n=9-16 (B) per group), **C**: Representative images of lung histology of PBS- vs HDM-sensitized mice, 3 and 7 days post-challenge (Hematoxylin and eosin staining). Bars indicate 50 µm. **D**: Baseline cysLT (normalized to RNA) production of, and *Ccl17* gene expression of BALF macrophages from PBS- vs. HDM-sensitized mice, harvested 7 days post-challenge (n=8-14 per group, unpaired t- test). **E:** Baseline cysLT, CCL17 and IL-6 production, and *Ccl17, Il6, Ptgs2* and *Cd84* gene expression of BMDM of PBS- vs. HDM-sensitized mice, harvested 7

days post-challenge (n=10-15/n=4-8, unpaired t-test/Mann-Whitney test). Data are presented as mean + SEM. *p<0.05, **p<0.01. i.n.=intranasal administration, BALF=bronchoalveolar lavage fluid

Fig. 3: HDM training of differentiated human macrophages drives a type-2 promoting and metabolically activated phenotype

A,B: cysLT (A) or CCL17 (B) production of control and HDM-trained aMDM (D14, n=12/ n=15, RM one-way ANOVA with Geisser-Greenhouse correction, Holm-Sidak's multiple comparisons test) **C, D**: Volcano plots of DEG (FC>2, p_{adj} <0.05) in HDM-trained versus control (C) or HDM-trained and challenged versus acutely HDM-exposed aMDM (D) on day 14 (n=3/ n=2) **E, F**: Heatmaps of DEG in HDM trained versus control (E) or HDM trained and challenged versus acutely HDM-exposed (F) aMDM (D14, n=3/ n=2) **G**: Oxygen consumption rate (OCR) and **H**: Spare respiratory capacity, and **I**: Extracellular acidification rate (ECAR) of control and HDM-trained aMDM (n=7-8, paired t-test) **J**: Venn diagram of upregulated DEG in trained/control, trained+challenged/acute HDM and HDM-allergic/healthy aMDM **K, L**: CCL17 (K) or cysLT (L) production by control and HDM-trained aMDM ± IL-25 (n=5, RM one-way ANOVA, Sidak's multiple comparisons test) **M**: CXCL8 production by normal human bronchial epithelial cells, ± medium or supernatants from control or HDM-trained aMDM (n=8, Friedmann test, Dunn's multiple comparisons test). Data are presented as mean + SEM or z-score transformed. *p<0.05, **p<0.01.

Fig. 4: Autocrine TNF signaling mediates HDM-driven type-2 imprinting *in vitro* and *in vivo*.

A: Normalized read counts for *FPR2* in aMDM (n=3 healthy donors), ± 24h HDM (padj, DeSeq2) **B**: CCL17 production by challenged HDM-trained aMDM ± Formyl peptide receptor

2 inhibitor (FPR2i) during training (D14, n=6, paired t-test). Dotted line: CCL17 production by aMDM + 24h HDM. **C**: TNF production of control and HDM-trained aMDM ± FPR2i during training (n=6, Friedmann test, Dunn's multiple comparisons test) **D**: Genes related to TNF signaling enriched in HDM-trained versus control aMDM (n=3) **E**: CCL17 production by challenged HDM-trained aMDM ± TNF neutralizing antibody (nAB) during training (D14, n=7, paired t test). **F**: Experimental scheme for HDM-induced AAI ± TNF inhibition (upper), or in mice deficient in myeloid *Tnf* (lower) **G**: Representative histology images of lung tissues of HDM-sensitized mice ± etanercept treatment. Scale bar: 50 µm. **H, I**: CCL17 (H) or IL-6 (I) production by BMDM from PBS- or HDM-sensitized mice ± etanercept treatment ± 24h *ex vivo* HDM (n=3-8, two-way ANOVA, Tukey's multiple comparisons test). **J, K**: BAL eosinophils (J) or *ex vivo* BMDM CCL17 production (K) for HDM-sensitized $Tnf^{fl/fl}$ or LysM-cre $Tnf^{fl/fl}$ mice**.** Data are presented as mean + SEM or z-score transformed. *p<0.05, **p<0.01, ***p<0.001. n.d.=not detected.

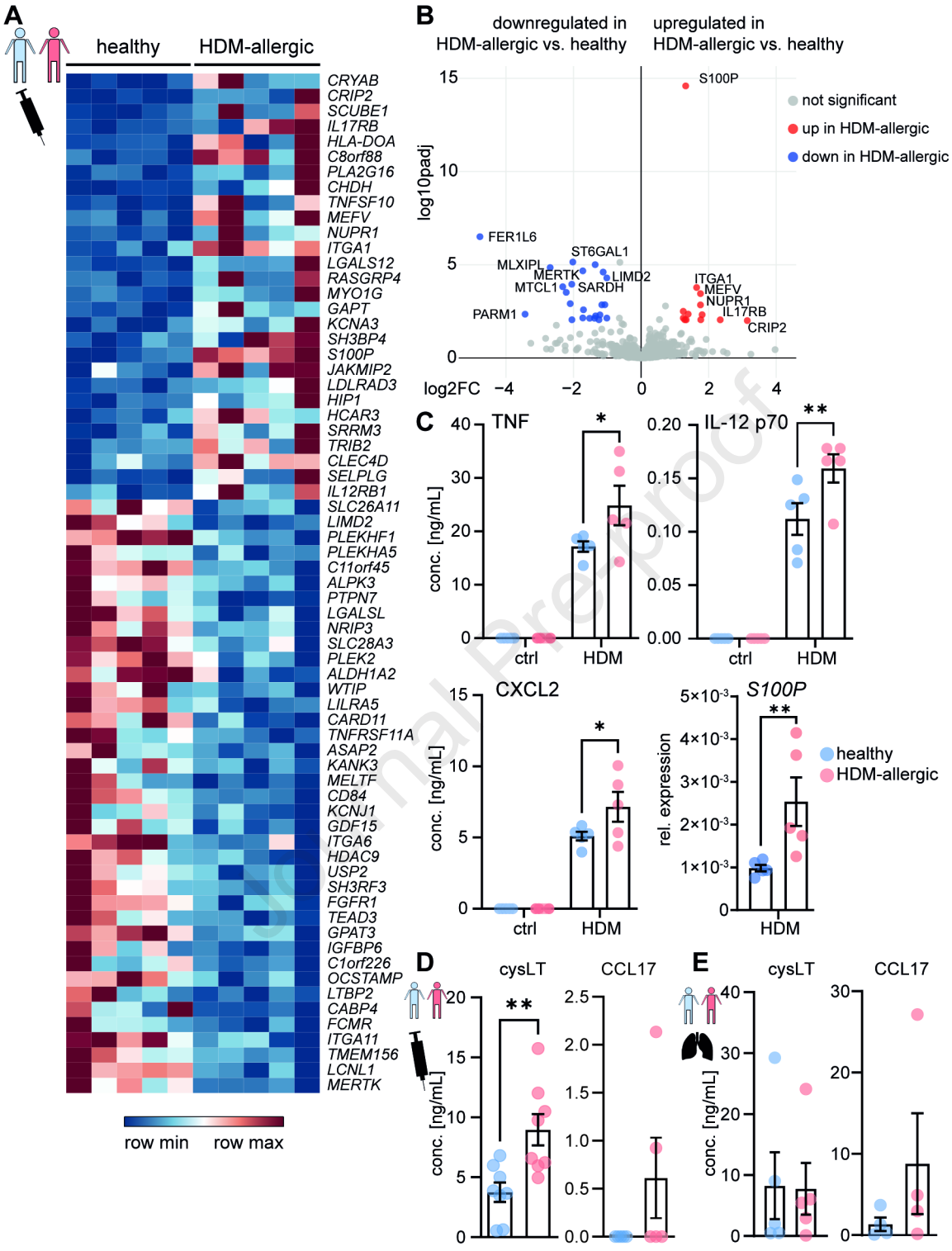
Fig. 5: A metabolic-epigenetic crosstalk via 2-hydroxyglutarate and KDM1A contributes to HDM-induced macrophage hyperresponsiveness

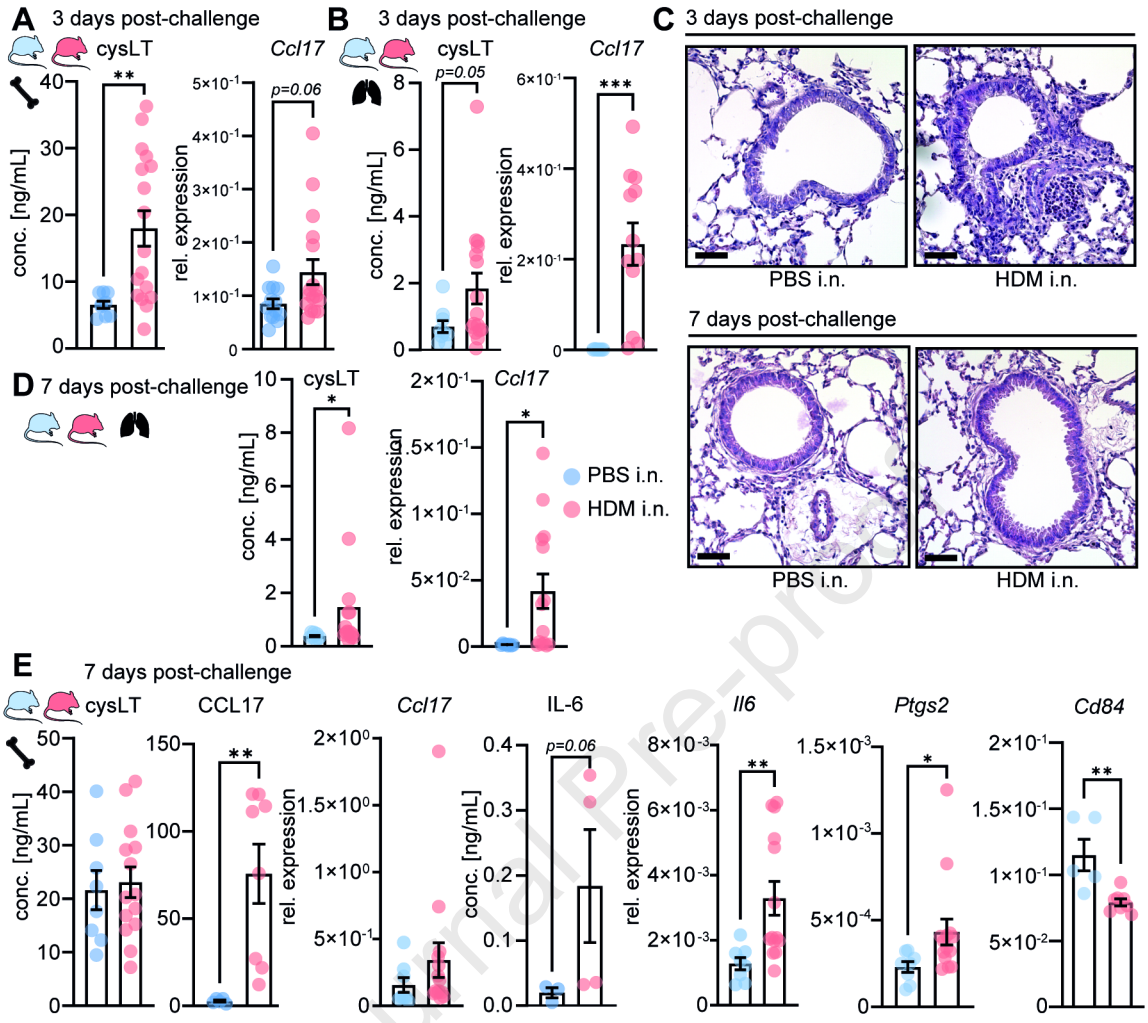
A: Targeted metabolomics, and histograms for **B**: glutathione, **C**: adenosine, and **D**: 2-hydroxyglutarate (2-HG) of BMDM from PBS- vs. HDM-sensitized mice (n=3 per group, paired t-test) **E**: *Arg1* expression in BMDM of PBS- or HDM-sensitized $Tnf^{fl/fl}$ or LysM-cre $Tnf^{fl/fl}$ mice ± 24h IL-4 (n=4-9) (E) or from PBS- or HDM-sensitized mice ± etanercept treatment (n=5-8) (F), E,F: two-way ANOVA, Sidak's multiple comparisons test **G**: 2-HG in MDM from healthy donors ± 24h HDM (n=7, paired t test) **H**: CCL17 production by control or 2-HG-trained macrophages ± HDM challenge (D14, n=3, RM one-way ANOVA, Sidak's multiple comparisons test) **I**: LPS versus control, fold change of *CCL17*, *IL1B* and *PTGS2* ± 2-HG (n=6, paired t test) Dotted lines: fold change=1. **J**: PGE_2 and CCL17 production of BMDM ± 2-HG (n=5, Mann-Whitney test) **K**: *HIF1A* expression in control and HDM-trained human

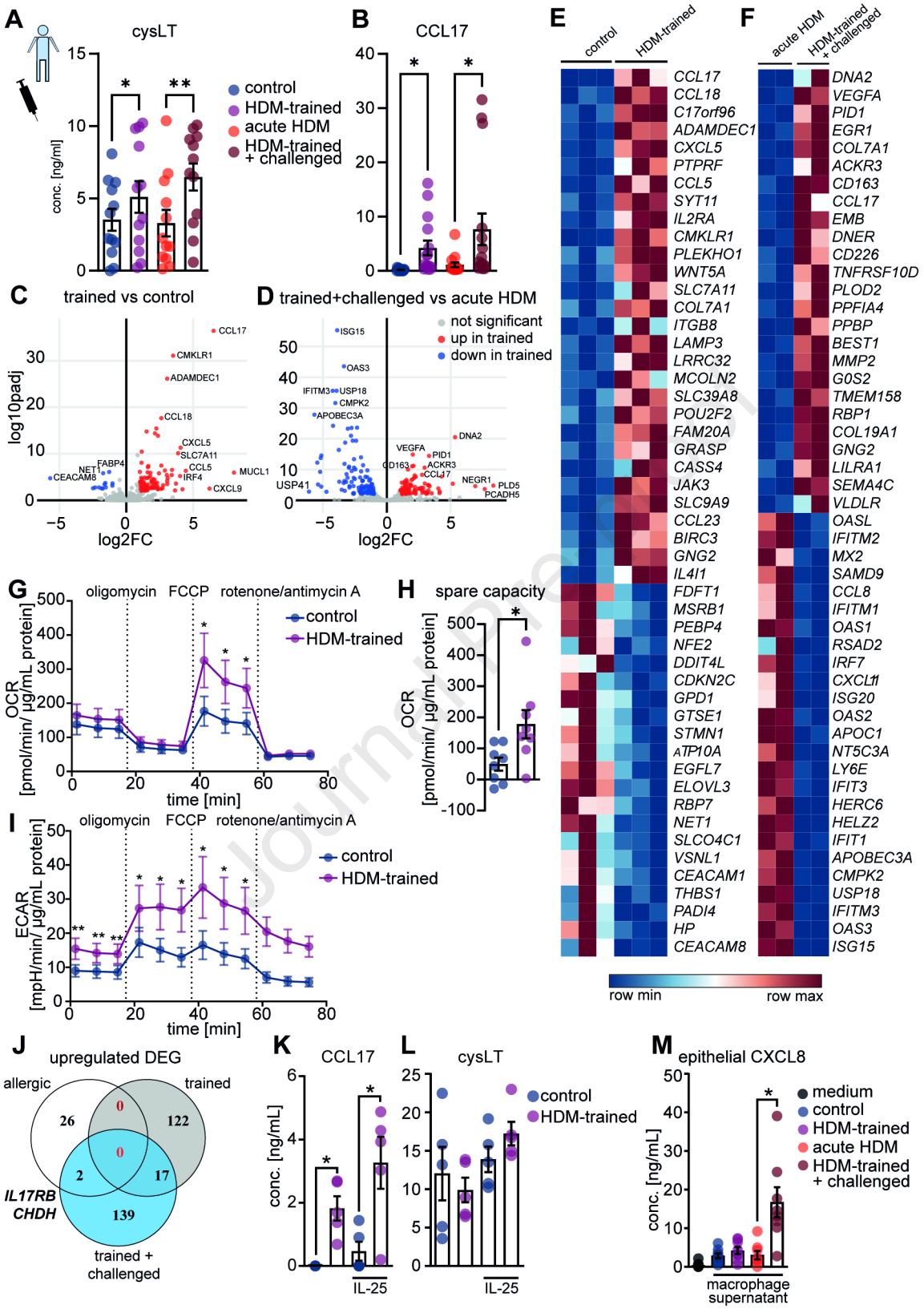
macrophages (n=10) **L**: CCL17 production by HDM-trained human macrophages, ± HIF1α inhibition during training (D14, n=5). **M**: CCL17 and cysLT production by challenged HDM-trained macrophages, ± KDM1A inhibition during training, (D14, n=8/n=5, Wilcoxon test). L, M: Dotted line: CCL17 or cysLT in aMDM + 24h HDM. Data are presented as z-score transformed or mean + SEM. *p<0.05, **p<0.01, ***p<0.001. AUC=area under curve.

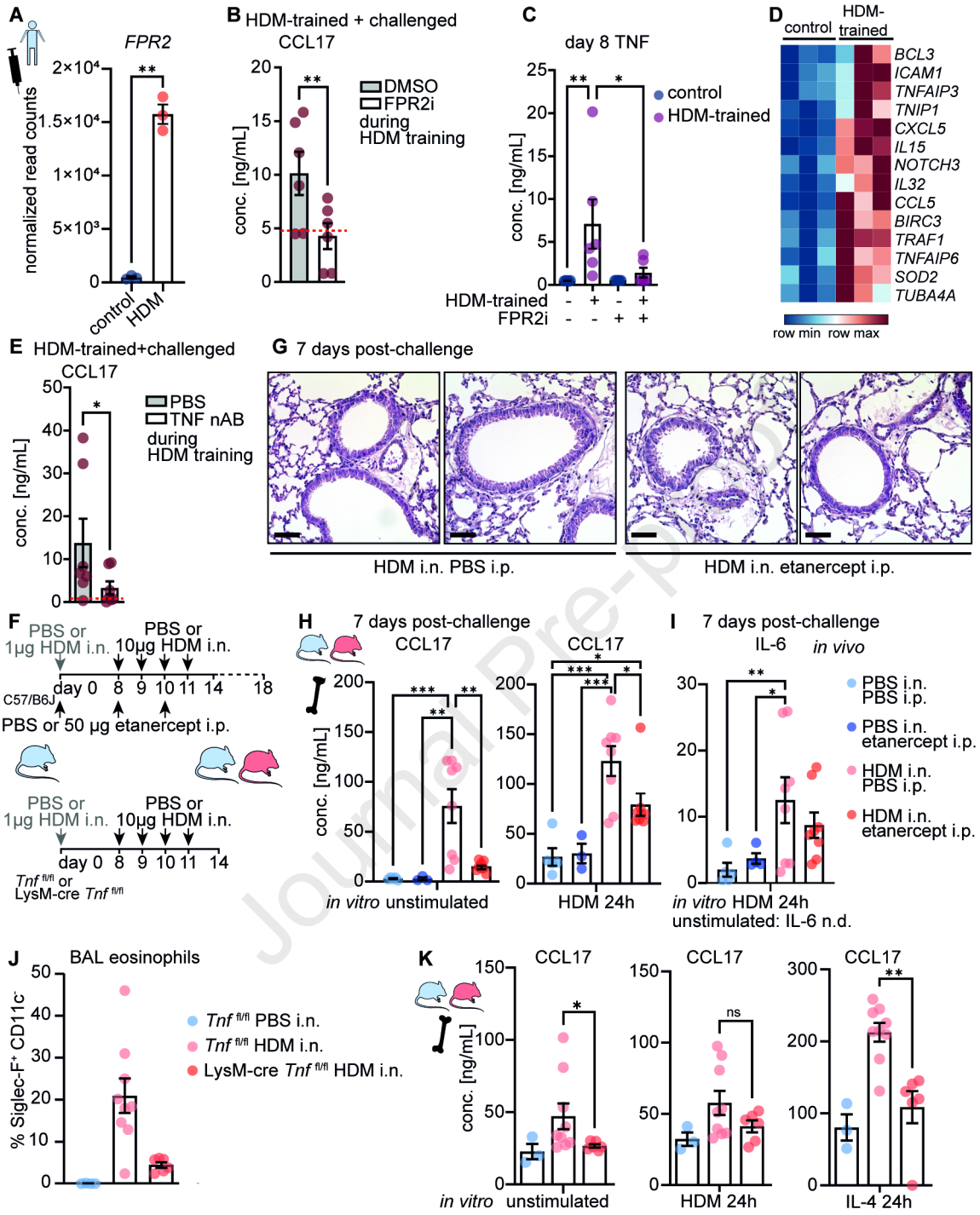
Fig. 6: HDM-induced macrophage training is distinct from classical trained immunity and driven by prostaglandin E_2/EP2-signaling

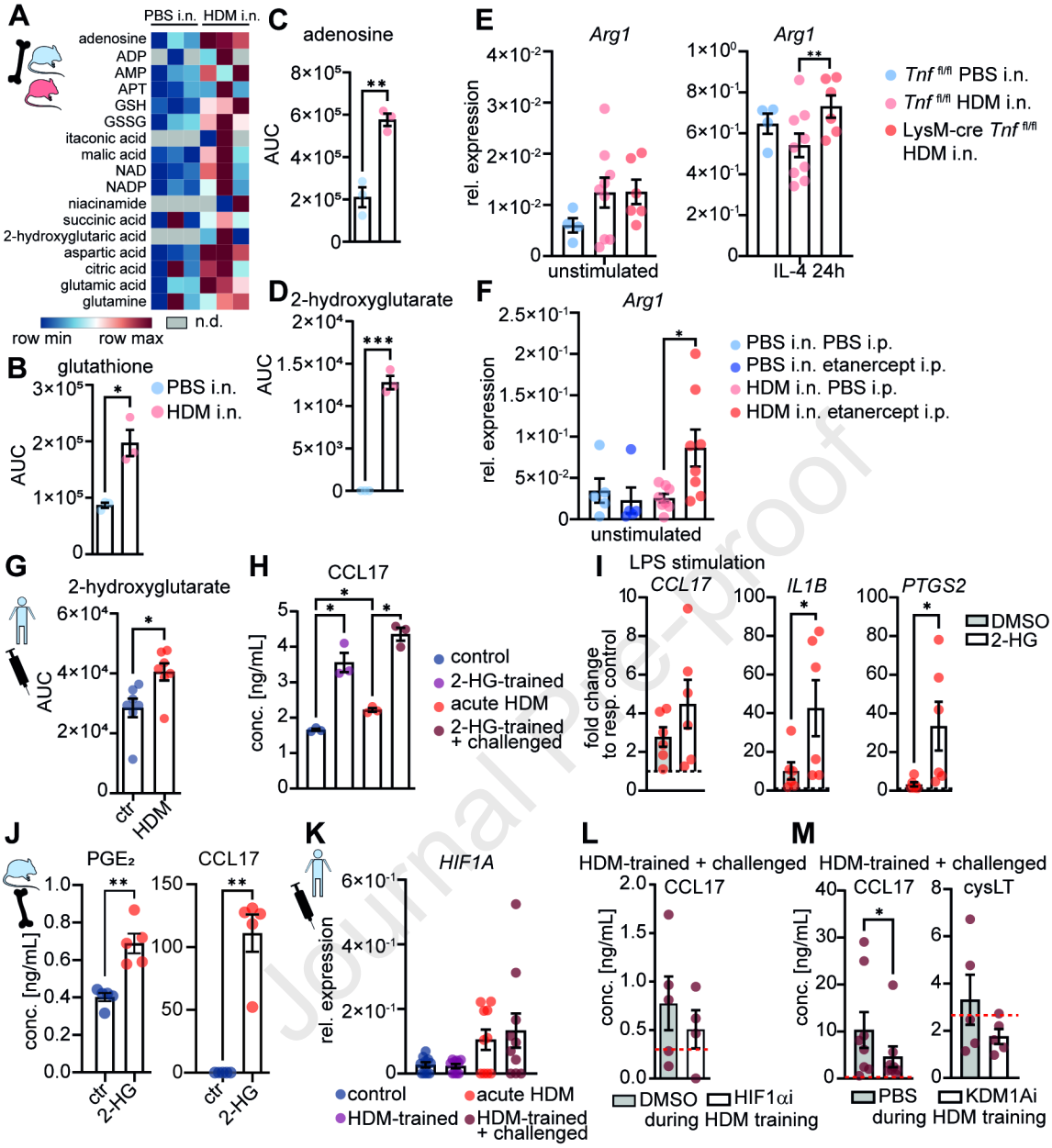
A,B: Mediator production of HDM-trained aMDM on D8 and D13 (A) or D14, after HDM restimulation) **C**: IL-6 production of control and HDM-trained aMDM, after 1h, 8h or 24h of HDM restimulation (n=4) **A-C:** RM two-way ANOVA, Sidak's multiple comparisons test) **D**: Eicosanoid production by control or HDM-trained human macrophages (n=11) **E**: Normalized read counts (RNAseq) of eicosanoid metabolism genes in control and HDM-trained aMDM (n=2) **F**: mPGES1 protein levels for control and HDM-trained aMDM, (n=5, Friedmann test, Dunn's multiple comparisons test) and representative western blot **G, H**: PGE_2 production by aMDM (G) or BMDM (H) from healthy or HDM-allergic donors or mice, ± 24h HDM (n=5 /n=8-9 per group) **I**: *Ptgs2* expression in from PBS- or HDM-sensitized mice ± etanercept treatment ± 24h HDM (n=5-8, RM two-way ANOVA) G, H, I: RM two-way ANOVA, Sidak's multiple comparisons test **J, K**: TNF (J) or CCL17 (K) production of wildtype or EP2 KO BMDM, ± 24h HDM exposure (n=7, Mann-Whitney test). Data are presented as z-score transformed or mean + SEM. n.d.=not detected, EP2 KO=*Ptger2* knockout. *p<0.05, *p<0.01.


A
healthy
HDM-allergic
CRYAB
CRIP2
SCUBE1
IL17RB
HLA-DOA
C8orf88
PLA2G16
CHDH
TNFSF10
MEFV
NUPR1
ITGA1
LGALS12
RASGRP4
MYO1G
GAPT
KCNA3
SH3BP4
S100P
JAKMIP2
LDLRAD3
HIP1
HCAR3
SRRM3
TRIB2
CLEC4D
SELPLG
IL12RB1
SLC26A11
LIMD2
PLEKHF1
PLEKHA5
C11orf45
ALPK3
PTPN7
LGALSL
NRIP3
SLC28A3
PLEK2
ALDH1A2
WTIP
LILRA5
CARD11
TNFRSF11A
ASAP2
KANK3
MELTF
CD84
KCNJ1
GDF15
ITGA6
HDAC9
USP2
SH3RF3
FGFR1
TEAD3
GPAT3
IGFBP6
C1orf226
OCSTAMP
LTBP2
CABP4
FCMR
ITGA11
TMEM156
LCNL1
MERTK
row min
row max
B
downregulated in HDM-allergic vs. healthy
upregulated in HDM-allergic vs. healthy
log10padj
log2FC
S100P
not significant
up in HDM-allergic
down in HDM-allergic
FER1L6
ST6GAL1
MLXIPL
MERTK
LIMD2
MTCL1
SARDH
PARM1
ITGA1
MEFV
NUPR1
IL17RB
CRIP2
C
TNF
IL-12 p70
CXCL2
S100P
conc. [ng/mL]
rel. expression
ctrl
HDM
healthy
HDM-allergic
D
cysLT
CCL17
E
cysLT
CCL17




A
cysLT
conc. [ng/ml]
control
HDM-trained
acute HDM
HDM-trained + challenged
B
CCL17
C
trained vs control
log10padj
log2FC
D
trained+challenged vs acute HDM
not significant
up in trained
down in trained
E
control
HDM-trained
CCL17
CCL18
C17orf96
ADAMDEC1
CXCL5
PTPRF
CCL5
SYT11
IL2RA
CMKLR1
PLEKHO1
WNT5A
SLC7A11
COL7A1
ITGB8
LAMP3
LRRC32
MCOLN2
SLC39A8
POU2F2
FAM20A
GRASP
CASS4
JAK3
SLC9A9
CCL23
BIRC3
GNG2
IL4I1
FDFT1
MSRB1
PEBP4
NFE2
DDIT4L
CDKN2C
GPD1
GTSE1
STMN1
ATP10A
EGFL7
ELOVL3
RBP7
NET1
SLCO4C1
VSNL1
CEACAM1
THBS1
PADI4
HP
CEACAM8
F
acute HDM
HDM-trained + challenged
DNA2
VEGFA
PID1
EGR1
COL7A1
ACKR3
CD163
CCL17
EMB
DNER
CD226
TNFRSF10D
PLOD2
PPFIA4
PPBP
BEST1
MMP2
G0S2
TMEM158
RBP1
COL19A1
GNG2
LILRA1
SEMA4C
VLDLR
OASL
IFITM2
MX2
SAMD9
CCL8
IFITM1
OAS1
RSAD2
IRF7
CXCL11
ISG20
OAS2
APOC1
NT5C3A
LY6E
IFIT3
HERC6
HELZ2
IFIT1
APOBEC3A
CMPK2
USP18
IFITM3
OAS3
ISG15
row min
row max
G
oligomycin
FCCP
rotenone/antimycin A
control
HDM-trained
OCR
[pmol/min/ µg/mL protein]
time [min]
H
spare capacity
I
ECAR
[mpH/min/ µg/mL protein]
J
upregulated DEG
allergic
trained
trained + challenged
IL17RB
CHDH
26
122
2
17
139
K
CCL17
conc. [ng/mL]
IL-25
L
cysLT
M
epithelial CXCL8
medium
control
HDM-trained
acute HDM
HDM-trained + challenged
macrophage supernatant


A
FPR2
normalized read counts
control
HDM
B HDM-trained + challenged
CCL17
conc. [ng/mL]
DMSO
FPR2i during HDM training
C
day 8 TNF
conc. [ng/mL]
control
HDM-trained
HDM-trained
FPR2i
D
control
HDM-trained
BCL3
ICAM1
TNFAIP3
TNIP1
CXCL5
IL15
NOTCH3
IL32
CCL5
BIRC3
TRAF1
TNFAIP6
SOD2
TUBA4A
row min row max
E HDM-trained+challenged
CCL17
conc. [ng/mL]
PBS
TNF nAB during HDM training
G 7 days post-challenge
HDM i.n. PBS i.p.
HDM i.n. etanercept i.p.
F
PBS or 1µg HDM i.n.
PBS or 10µg HDM i.n.
day 0 8 9 10 11 14 18
C57/B6J
PBS or 50 µg etanercept i.p.
PBS or 1µg HDM i.n.
PBS or 10µg HDM i.n.
day 0 8 9 10 11 14
Tnf fl/fl or LysM-cre Tnf fl/fl
H 7 days post-challenge
CCL17
CCL17
conc. [ng/mL]
in vitro unstimulated
HDM 24h
I 7 days post-challenge
IL-6 in vivo
PBS i.n. PBS i.p.
PBS i.n. etanercept i.p.
HDM i.n. PBS i.p.
HDM i.n. etanercept i.p.
in vitro HDM 24h
unstimulated: IL-6 n.d.
J
BAL eosinophils
% Siglec-F+ CD11c-
Tnf fl/fl PBS i.n.
Tnf fl/fl HDM i.n.
LysM-cre Tnf fl/fl HDM i.n.
K
CCL17
CCL17
CCL17
conc. [ng/mL]
ns
in vitro unstimulated
HDM 24h
IL-4 24h


A
PBS i.n.
HDM i.n.
adenosine
ADP
AMP
APT
GSH
GSSG
itaconic acid
malic acid
NAD
NADP
niacinamide
succinic acid
2-hydroxyglutaric acid
aspartic acid
citric acid
glutamic acid
glutamine
n.d.
row min
row max
B
glutathione
AUC
PBS i.n.
HDM i.n.
C
adenosine
AUC
D
2-hydroxyglutarate
AUC
E
Arg1
rel. expression
unstimulated
Arg1
IL-4 24h
Tnf fl/fl PBS i.n.
Tnf fl/fl HDM i.n.
LysM-cre Tnf fl/fl HDM i.n.
F
Arg1
rel. expression
unstimulated
PBS i.n. PBS i.p.
PBS i.n. etanercept i.p.
HDM i.n. PBS i.p.
HDM i.n. etanercept i.p.
G
2-hydroxyglutarate
AUC
ctr
HDM
H
CCL17
conc. [ng/mL]
control
2-HG-trained
acute HDM
2-HG-trained + challenged
I
LPS stimulation
CCL17
IL1B
PTGS2
fold change to resp. control
DMSO
2-HG
J
PGE₂
CCL17
conc. [ng/mL]
ctr
2-HG
K
HIF1A
rel. expression
control
acute HDM
HDM-trained
HDM-trained + challenged
L
HDM-trained + challenged
CCL17
conc. [ng/mL]
DMSO
HIF1αi
during HDM training
M
HDM-trained + challenged
CCL17
cysLT
conc. [ng/mL]
PBS
KDM1Ai
during HDM training