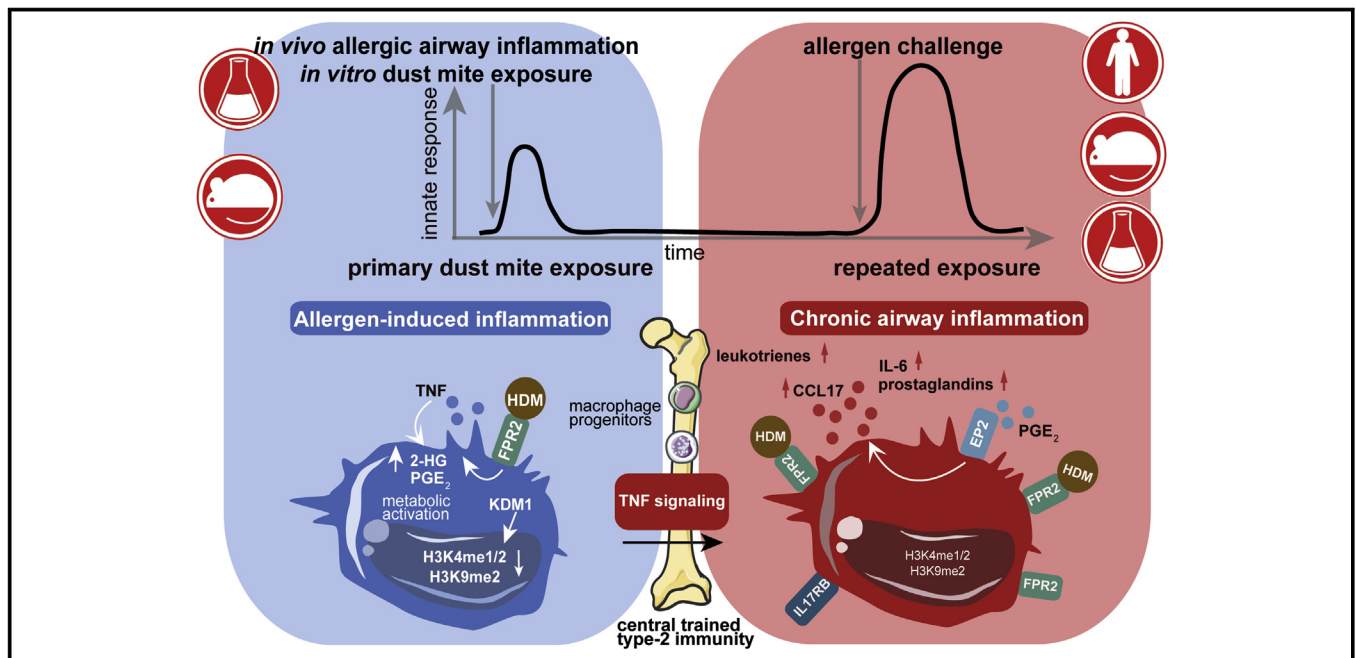


# Macrophages acquire a TNF-dependent inflammatory memory in allergic asthma



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## GRAPHICAL 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:** We sought 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 in mice, and an *in vitro* training setup to

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This study was supported by the German Research Foundation (DFG) (FOR2599, ES 471/3-1; ES 471/2-3), the Fritz Thyssen Stiftung (grant Az. 10.17.2.017MN), and a Helmholtz Young Investigator grant (VH-NG-1331) to J.E.v.B. C.O. is supported by the European Research Council (ERC starting grant project 716718) and the DFG (project 395357507-SFB1371 and grant OH 282/1-1 within FOR2599). C.S.W. receives grant support by the German Center for Lung Research (DZL;

82DZL00302). G.E.O., S.A.N., and M.S.D. are supported by grant 075-15-2019-1660 from the Ministry of Science and Higher Education of the Russian Federation. Disclosure of potential conflict of interest: C. B. Schmidt-Weber received grant support from Allergopharma, PLS Design, and Zeller AG; and received speaker honoraria from Allergopharma. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication February 13, 2021; revised October 18, 2021; accepted for publication November 26, 2021.

Available online December 30, 2021.

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0091-6749

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<https://doi.org/10.1016/j.jaci.2021.11.026>

analyze persistent changes in macrophage eicosanoid, cytokine, and chemokine production as well as the underlying metabolic and epigenetic mechanisms. Transcriptional and metabolic profiles of patient-derived and *in vitro* trained macrophages were assessed by RNA sequencing or metabolic flux analysis and liquid chromatography–tandem mass spectrometry 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- $\alpha$ , CCL17, leukotriene, PGE<sub>2</sub>, IL-6) responses upon stimulation. Macrophages from HDM-allergic mice initially exhibited a type 2 imprint, which shifted toward a classical inflammatory training over time. HDM-induced allergic airway inflammation 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–TNF–2-HG–PGE<sub>2</sub>/PGE<sub>2</sub> receptor 2 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. (J Allergy Clin Immunol 2022;149:2078-90.)

**Key words:** CCL17, chemokines, eicosanoids, lipid mediators, macrophages, trained immunity, type 2 inflammation

The prevalence of allergic asthma has constantly increased over the last 2 decades.<sup>1</sup> House dust mite (HDM) represents the most prominent aeroallergen, and approximately 50% of people with asthma are sensitized to it.<sup>2</sup> In addition to allergen-specific T cells, the innate immune system contributes to type 2 (T2) inflammation in allergy.<sup>3</sup> Macrophages play an important role in asthma, and asthma severity correlates with numbers of M2-polarized macrophages in the airways.<sup>4,5</sup> CCL17, a T<sub>H</sub>2-cell chemoattractant,<sup>6</sup> is overexpressed in alveolar macrophages from asthma patients and is involved in asthma exacerbations.<sup>7–10</sup> Eicosanoids are key mediators of T2 inflammation,<sup>11,12</sup> and airway macrophages (AMs) of asthma patients show an exaggerated production of proinflammatory leukotrienes (LTs).<sup>13</sup> LT production and recruitment of inflammatory monocytes are central for the development of allergic airway inflammation (AAI).<sup>14,15</sup> While other eicosanoid-producing myeloid cells (eg, eosinophils) are cleared from the lung after acute inflammation resolves,<sup>16</sup> macrophages persist.<sup>17</sup> 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.<sup>18–20</sup> This phenomenon, termed “trained immunity,” is not limited to tissue macrophages but extends to bone marrow progenitors that provide “central trained immunity.”<sup>21,22</sup> Respiratory viral infection can induce macrophage reprogramming and replacement, thus promoting or preventing asthma development.<sup>23–25</sup> However, if and how macrophage-trained immunity is triggered

#### Abbreviations used

2-HG:	2-Hydroxyglutarate
AAI:	Allergic airway inflammation
AM:	Airway macrophage
aMDM:	Alveolar-like monocyte-derived macrophage
BMDM:	Bone marrow–derived macrophage
cysLT:	Cysteinyl LT
EP2:	PGE <sub>2</sub> receptor 2
FPR2:	Formyl-peptide receptor 2
HDM:	House dust mite
HIF:	Hypoxia-inducible factor
KDM:	Lysine demethylase
LT:	Leukotriene
MDM:	Monocyte-derived macrophage
PGE <sub>2</sub> :	Prostaglandin E <sub>2</sub>
T2:	Type 2

during allergen-driven inflammation remained unclear. We found that monocyte-derived macrophages (MDM) or bone marrow–derived macrophages (BMDM) from HDM-allergic asthma patients or HDM-sensitized mice persistently upregulate inflammatory genes and T2-inflammatory chemokines and eicosanoids (CCL17, cysteinyl leukotrienes [cysLTs]). This inflammatory memory depended on FPR2 and TNF signaling, resulting in metabolic reprogramming and lysine demethylase (KDM) 1–mediated histone demethylation, thus representing a trained immunity program that may contribute to chronification and exacerbation of allergic asthma.

## METHODS

A detailed description of the experimental procedures is provided in this article’s Online Repository available at [www.jacionline.org](http://www.jacionline.org).

### Human study participants

The ethics committee of the Technical University of Munich approved the study (approval 422/16). HDM-allergic patients and healthy subjects were recruited at the Allergy Section, Otolaryngology Department, Technical University of Munich School of Medicine (Table 1). All participants provided informed written consent in accordance with the Declaration of Helsinki before sampling. The study visit consisted of questionnaires including the Sinusnasal Outcome Test 22, the Mini Rhinoconjunctivitis Quality of Life Questionnaire, and the Perceived Stress Questionnaire 20; and collection of blood and sputum. The clinical diagnostic laboratory of the hospital assessed differential blood cell counts, specific mite IgE, and total IgE. Sputum induction and sputum cell isolation were performed as previously described.<sup>26</sup>

### Murine model of AAI

Mice aged 6 to 8 weeks (wild-type C57BL/6J, *Tnf<sup>fl/fl</sup>* and *LysM-cre Tnf<sup>fl/fl</sup>*) were intranasally sensitized and challenged with HDM extract as previously described (Fig E1 in the Online Repository available at [www.jacionline.org](http://www.jacionline.org)).<sup>11</sup> Analysis was performed on bronchoalveolar lavage, lung tissue, AMs, and bone marrow cells, comparing mice sensitized and challenged to PBS or HDM.

### *In vitro* macrophage differentiation and culture

MDM or BMDM were generated as previously described.<sup>11,12</sup> Supernatants were analyzed by liquid chromatography–tandem mass spectrometry or ELISA after stimulation with ionophore A23187 (5  $\mu$ mol/L, Merck,

**TABLE I.** Clinical characteristics of healthy and HDM-allergic subjects

Characteristic	Healthy	Allergic	P value
Age (years)	28.8 ± 2.2	30.0 ± 7.7	.7473
Sex (F/M)	5/0	4/2	
Body mass index (kg/m <sup>2</sup> )	21.4 ± 2.6	25.7 ± 4.8	.1170
MiniRQLQ score	6.6 ± 6.5	24.4 ± 5.9	.0020
SNOT-22 score	5.6 ± 2.7	25.0 ± 14.1	.0163
PSQ-20 score	49.0 ± 3.9	48.0 ± 3.1	.4654
Total IgE (kU/L)	10.8 ± 8.4	241.6 ± 328.0	.0079
Der p IgE (kU/L)	0	13.4 ± 16.9	.0079
Der f IgE (kU/L)	0.1 ± 0.3	15.4 ± 20.3	.0079
Eur m IgE (kU/L)	0	3.1 ± 4.0	.0079
Blood monocytes, no. (%)	7.6 ± 0.5	7.2 ± 2.2	.7937
Blood eosinophils, no. (%)	2.8 ± 2.5	5.0 ± 1.6	.1339

Data are presented as means ± SDs unless otherwise indicated. *MiniRQLQ*, Mini Rhinoconjunctivitis Quality of Life Questionnaire; *PSQ-20*, Perceived Stress Questionnaire 20; *SNOT-22*, Sinusitis Outcome Test 22.

Darmstadt, Germany) for 10 minutes, and cell pellets were analyzed via Western blot analysis, quantitative PCR, or RNA sequencing.

### Metabolic flux analysis

A total of  $5 \times 10^4$  MDM or BMDM were plated per well on a Seahorse Miniplate (Agilent Technologies, Santa Clara, Calif) and cultured for training (alveolar-like monocyte-derived macrophage, aMDM) or stimulated overnight (BMDM) before mitochondrial stress test (Agilent).

### Metabolomics analysis

A total of  $5 \times 10^5$  aMDM or BMDM were pelleted for targeted metabolomics. Metabolite quantification by liquid chromatography–tandem mass spectrometry was performed at the Metabolomics Core Facility of the Max Planck Institute for Immunobiology and Epigenetics in Freiburg, Germany.

### Statistical analysis

Data were analyzed by GraphPad Prism 9 software (GraphPad Software, La Jolla, Calif). The *t* test or Mann-Whitney test were used to compare 2 populations depending on normal distribution. For comparison of more groups, Friedman test, 1-way ANOVA, or 2-way ANOVA was used with correction for multiple comparisons, as indicated in the figures.  $P < .05$  was considered statistically significant. Details of statistical tests and sample size are provided in the figures. Heat maps were generated by the Broad Institute's Morpheus software (software.broadinstitute.org/morpheus/).

## RESULTS

### Macrophages from HDM-allergic patients show transcriptional reprogramming and enhanced production of T2 inflammatory mediators

Macrophages represent key regulators of lung homeostasis and immunity, and they govern airway inflammation by producing eicosanoids and chemokines.<sup>15,27</sup> We recently described stable differences in gene expression and metabolite profiles in macrophages from patients with nonsteroidal anti-inflammatory drug–exacerbated respiratory disease,<sup>26</sup> a nonallergic chronic T2 inflammatory condition. To study a potential macrophage memory in allergic asthma, we generated macrophages (aMDM) from monocytes of HDM-allergic or healthy donors (Table I) (Fig E1, A). RNA sequencing analysis yielded 88 genes differentially expressed between aMDM from HDM-allergic compared to nonallergic donors (28 up, 60 down) (Fig 1, A and B, Data File

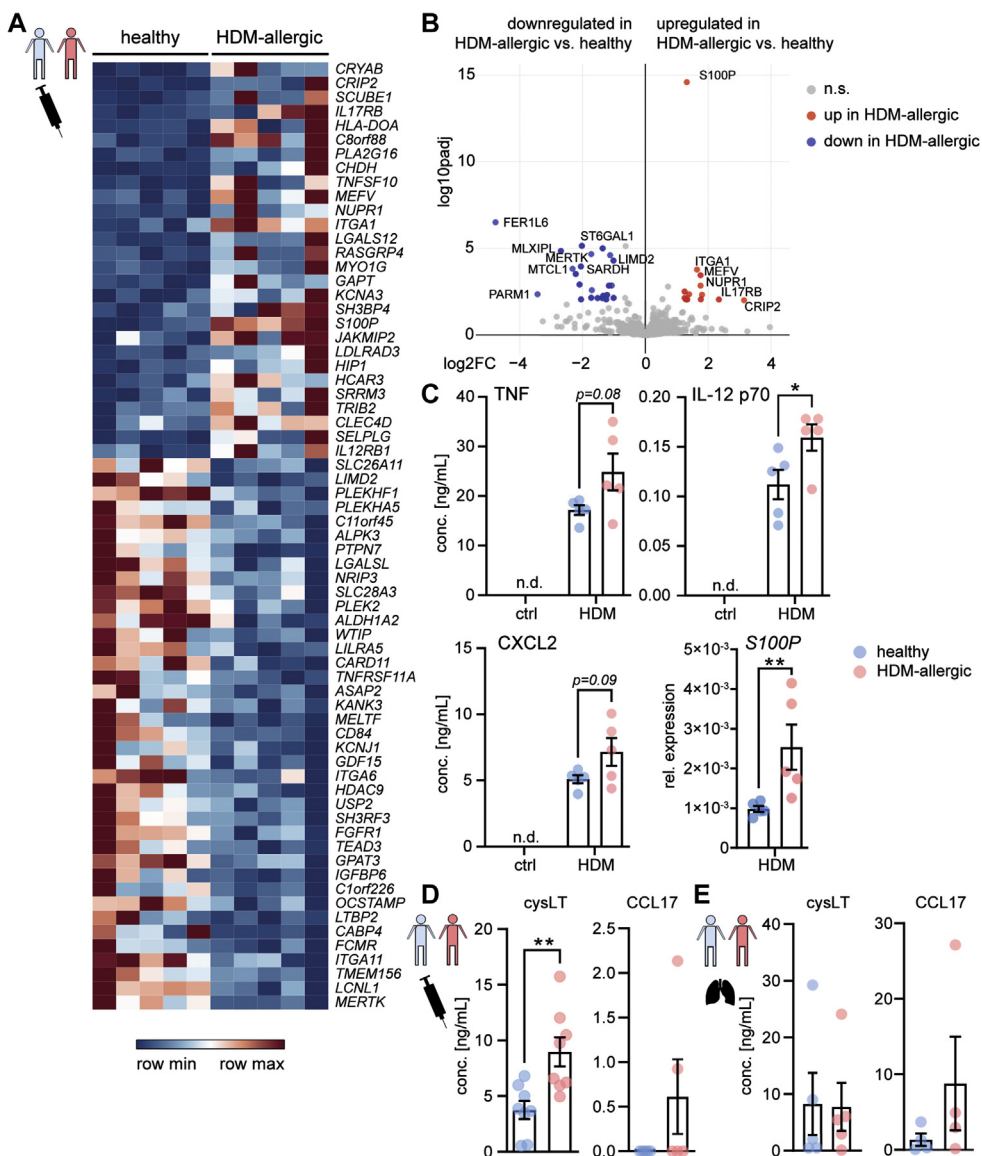
E1 in the Online Repository available at [www.jacionline.org](http://www.jacionline.org)), indicating stable transcriptional reprogramming that persisted throughout *ex vivo* differentiation. *S100P*, *TNFSF10* (TRAIL), *CLEC4D* (dectin-3), *LGALS12* (galectin-3), and *IL12RB1*, all implicated in macrophage activation,<sup>28–32</sup> were upregulated in aMDM of allergic donors while immunoregulatory genes such as *MERTK* and *CD84*<sup>33,34</sup> were downregulated (Fig 1, A and B). *CD84* and *MERTK* gene expression correlated negatively while *ITGA1* and *S100P* correlated positively with disease scores on the Mini Rhinoconjunctivitis Quality of Life Questionnaire and the Sinusitis Outcome Test 22 (Fig E2, A, in the Online Repository available at [www.jacionline.org](http://www.jacionline.org)). Several of the differentially expressed genes identified in aMDM of HDM-allergic asthma patients (eg, *S100P*, *ITGA1*, *TNFSF10*, *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-1 $\beta$  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 AMs, from HDM-allergic individuals produced exaggerated amounts of cysLTs, important mediators of T2 inflammation<sup>14</sup> as well as further 5-lipoxygenase–derived eicosanoids (Fig 1, D and E, Fig E2, D and E). In addition, CCL17, a driver of the T<sub>H</sub>2 response in asthma,<sup>7,35</sup> tended to be increased in aMDM and AM of HDM-allergic asthma patients (Fig 1, D and E). Thus, aMDM from allergic asthma patients exhibited inflammatory imprinting and T2-driving mediator profiles at baseline and enhanced TNF-dominated HDM responses.

### HDM-induced AAI induces a persistent inflammatory imprint in 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 AMs of HDM-sensitized mice (Fig 2, B). In contrast to cysLTs, 5-lipoxygenase–derived mediators were not generally increased in AM (Fig E2, G). Seven days after challenge, HDM-induced AAI as well as T2 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 and B)—*Cd84*, *Mertk*, *Clec4d*, *Irgal1*, and *Tnfsf10*—showed a similar pattern in BMDM from HDM-sensitized mice (Fig 2, E, Fig E2, J). Together, this suggested that AAI leaves an innate memory both locally and in bone marrow progenitors.

### HDM training elicits exaggerated cysteinyl leukotriene and CCL17 responses and transcriptional reprogramming in human aMDM

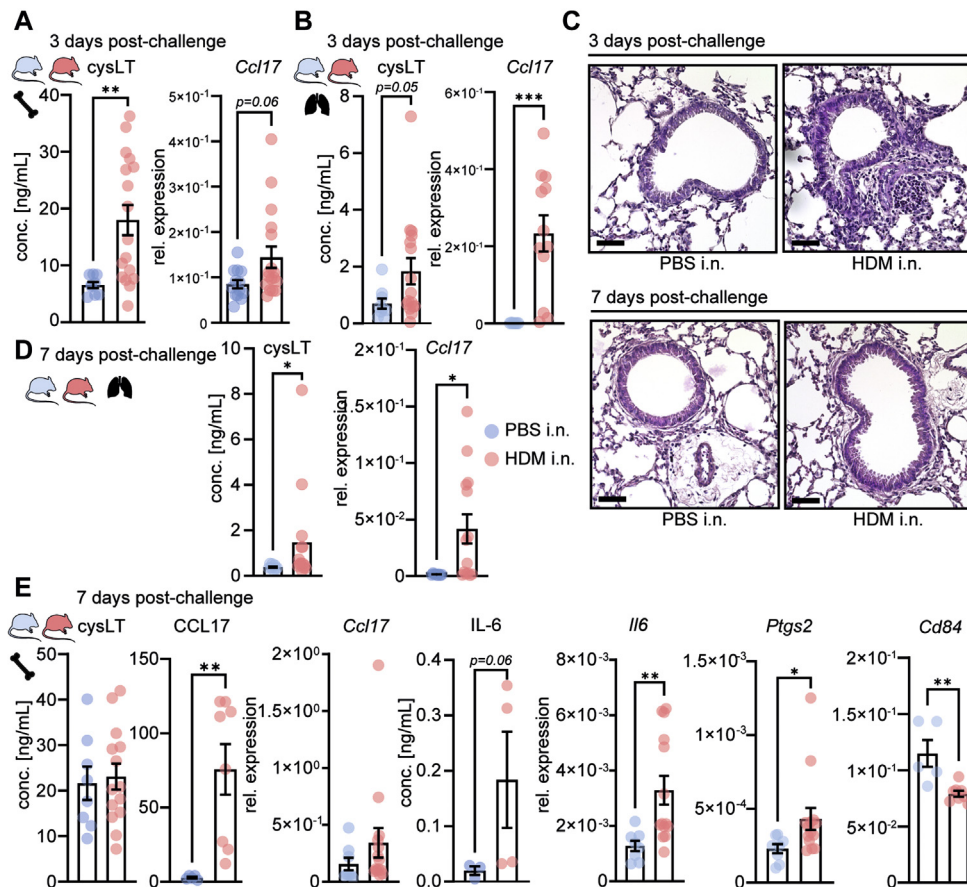
To study whether *in vivo* reprogramming of HDM-experienced macrophages could be mimicked *in vitro* (Fig E1, C), aMDM were stimulated (trained) with HDM on day 7 of differentiation,



**FIG 1.** Monocyte-derived macrophages from HDM-allergic asthma patients show persistent inflammatory gene expression and exaggerated production of inflammatory mediators. **A**, Heat map of 28 significantly upregulated and 39 downregulated differentially expressed genes (DEGs) in aMDM from HDM-allergic donors versus healthy donors ( $n = 5$  per group, DeSeq2). **B**, Volcano plot of DEGs (fold change  $> 2$ ,  $P_{\text{adj}} < .05$ ) in aMDM from HDM-allergic versus healthy donors ( $n = 5$  per group). **C**, TNF, IL-12 p70, and CXCL2 production and S100P expression of aMDM from HDM-allergic donors versus healthy donors after 24-hour HDM exposure *in vitro* ( $n = 5$  per group, repeated measures 2-way ANOVA, unpaired *t* test). **D**, Baseline cysLT and CCL17 production of aMDM from healthy versus 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 versus HDM-allergic human donors (normalized to RNA concentration,  $n = 5$  per group, Mann-Whitney test). Data are presented as z scores transformed (heat map) or means  $\pm$  SEMs. \* $P < .05$ , \*\* $P < .01$ . *n.d.*, Not detected.

restimulated after a 5-day washout period, and collected 24 hours later for eicosanoid, gene expression, and cytokine analyses. *In vitro* HDM-trained and restimulated aMDM escaped HDM-induced, TLR4-dependent cysLT suppression<sup>12</sup> resulting in high amounts of cysLTs (Fig 3, A, and Fig E3, A, in the Online Repository available at [www.jacionline.org](http://www.jacionline.org)), resembling the exaggerated cysLT production in aMDM or BMDM from HDM-sensitized humans or mice (Fig 1, D, Fig 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 AMs from HDM-allergic patients or mice (Fig 1, E, Fig 2, B and D). The primed CCL17 response was evident already before challenge (Fig E3, B), was dose dependent (Fig E3, C), and was not evoked by  $\beta$ -glucan, a classical trigger of trained immunity<sup>20</sup> (Fig E3, D). HDM training did not affect macrophage viability (Fig E3, E), and training with purified allergens (*Derf1* or *Derf2*) did not enhance macrophage inflammatory responsiveness (Fig E3, F). RNA sequencing



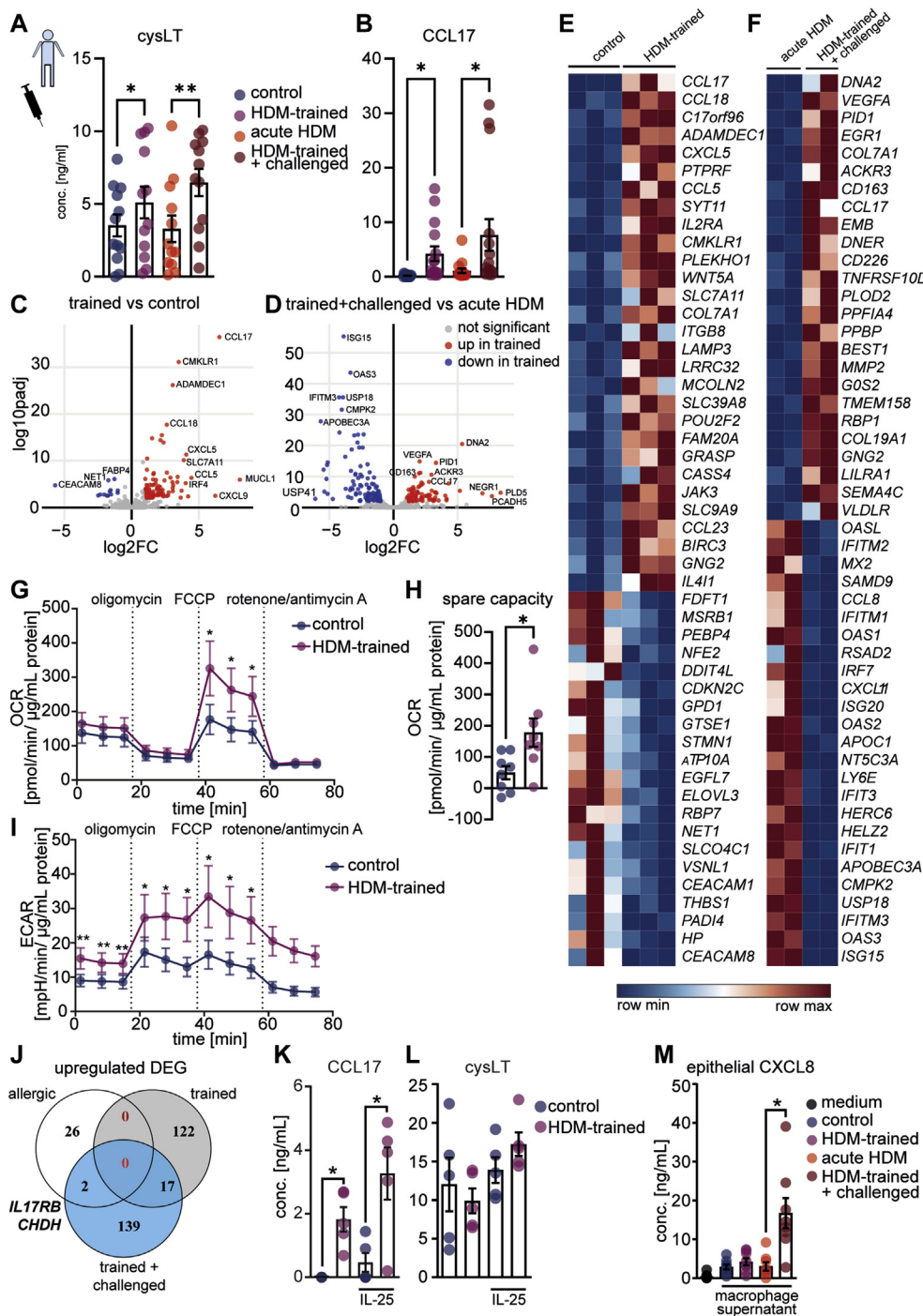
**FIG 2.** HDM-induced airway inflammation induces a T2 imprint in murine peripheral and AMs, which shifts toward classical central trained immunity. **A** and **B**, CysLT production and *Ccl17* expression in BMDM (**A**) or BAL AM (**B**) from PBS- versus HDM-sensitized mice 3 days after challenge; Mann-Whitney test,  $n = 13-17$  (**A**) or unpaired *t* test,  $n = 9-16$  (**B**) per group. **C**, Representative images of lung histology of PBS- versus HDM-sensitized mice 3 and 7 days after challenge (hematoxylin and eosin staining). Bars, 50  $\mu\text{m}$ . **D**, Baseline cysLT (normalized to RNA) production of and *Ccl17* gene expression of BALF macrophages from PBS- versus HDM-sensitized mice collected 7 days after 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- versus HDM-sensitized mice collected 7 days after challenge ( $n = 10-15/n = 4-8$ , unpaired *t* test/Mann-Whitney test). Data are presented as means  $\pm$  SEMs. \* $P < .05$ , \*\* $P < .01$ . BAL, Bronchoalveolar lavage; BALF, BAL fluid; *i.n.*, intranasal administration.

analysis of HDM-trained macrophages with or without HDM re-stimulation (Fig E1, C) identified 166 differentially expressed genes in HDM-trained macrophages 6 days after HDM exposure compared to control macrophages (139 up, 27 down) and 304 differentially expressed genes between previously HDM-trained and naive macrophages 24 hours after HDM challenge (159 up, 143 down) (Fig 3, C-F). HDM-trained macrophages exhibited an increased expression of genes involved in M2 polarization (eg, *IRF4*, *CD163*, *IL4I1*, *VEGFA*) and chemokine/cytokine signaling (*CCL17*, *CCL18*, *CXCL9*) (Fig 3, C and E, and Data File E2 in the Online Repository available at [www.jacionline.org](http://www.jacionline.org)), while the HDM-driven induction of interferon-induced genes, (eg, *OASL*, *OAS2/3*, *ISG15/20*, *USP18*, *CMPK2*) was reduced compared to naive HDM-stimulated aMDM (Fig 3, D and F). TNF signaling (Fig E3, G) as well as cytokine–cytokine receptor interaction and chemokine signaling (Fig E3, G and 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 after washout of HDM. *IL17RB* (the receptor subunit binding IL-25<sup>36</sup>) was upregulated in *in vitro* trained and patient-derived aMDM (Fig 1, A and B, Data File E1, 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 and 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 receptor 2



**FIG 3.** HDM training of differentiated human macrophages drives a T2 promoting and metabolically activated phenotype. **A** and **B**, cysLT (**A**) or CCL17 (**B**) production of control and HDM-trained aMDM (day [D] 14,  $n = 12/n = 15$ , repeated measures 1-way ANOVA with Geisser-Greenhouse correction, Holm-Sidak multiple comparisons test). **C** and **D**, Volcano plots of differentially expressed genes (DEGs; fold change  $> 2$ ,  $P_{adj} < .05$ ) in HDM-trained versus control (**C**) or HDM-trained and challenged versus acutely HDM-exposed aMDM (**D**) on D14 ( $n = 3/n = 2$ ). **E** and **F**, Heat maps of DEGs in HDM-trained versus control (**E**) or HDM-trained and challenged versus acutely HDM-exposed (**F**) aMDM (D14,  $n = 3/n = 2$ ). **G-I**, Oxygen consumption rate (OCR) (**G**) and spare respiratory capacity (**H**), and (**I**) extracellular acidification rate (ECAR) of control and HDM-trained aMDM ( $n = 7-8$ , paired  $t$  test). **J**, Venn diagram of upregulated DEGs in trained/control, trained and challenged/acute HDM, and HDM-allergic/healthy aMDM. **K** and **L**, CCL17 (**K**) or cysLT (**L**) production by control and HDM-trained aMDM with or without IL-25 ( $n = 5$ , repeated measures 1-way ANOVA, Sidak multiple comparisons test). **M**, CXCL8 production by normal human bronchial epithelial cells, with or without medium or supernatants from control or HDM-trained aMDM ( $n = 8$ , Friedman test, Dunn multiple comparisons test). Data are presented as means  $\pm$  SEMs or z scores transformed. \* $P < .05$ , \*\* $P < .01$ .

(FPR2), implicated in HDM sensing,<sup>37,38</sup> was persistently upregulated in HDM-trained macrophages (Data Files E2 and E3 in the Online Repository available at [www.jacionline.org](http://www.jacionline.org)) and was induced by HDM stimulation (Fig 4, A). Blocking FPR2 signaling by a pharmacologic inhibitor (PBP10) during HDM training suppressed the enhanced CCL17 response (Fig 4, B) and prevented the induction of TNF (Fig 4, C), suggesting FPR2 may be a major HDM receptor involved in HDM-driven macrophage reprogramming. Because TNF signaling was reported to initiate CCL17-mediated inflammation,<sup>39</sup> and was enriched in aMDM of asthma patients or after *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-restimulated aMDM (Fig 4, E). *In vitro* cysLT responses were not affected by inhibition of TNF or FPR2 (Fig E4, A and B, in the Online Repository available at [www.jacionline.org](http://www.jacionline.org)). Treatment with the FPR2 inhibitor or TNF-neutralizing antibody alone did not influence macrophage HDM responses on day 13 (Fig E4, C and D). To test the relevance of TNF signaling *in vivo*, we injected HDM-sensitized mice with etanercept (a TNFR2-based fusion protein that neutralizes TNF and lymphotoxin) during sensitization and challenge (Fig 4, F, top). Etanercept treatment did not influence HDM-induced AAI at 72 hours or 7 days after challenge (Fig E4, E and 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 restimulation, 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<sup>fl/fl</sup>*)<sup>40</sup> (Fig 4, F, bottom) resulted in reduced airway eosinophilia (Fig 4, J) as well as decreased CCL17 production by BMDM at baseline and after IL-4 stimulation (Fig 4, K), supporting a role for myeloid-derived TNF in T2 imprinting in the bone marrow during HDM-induced AAI. Together, this suggested that autocrine TNF signaling, induced via FPR2, drives the proinflammatory macrophage memory during allergen-driven inflammation.

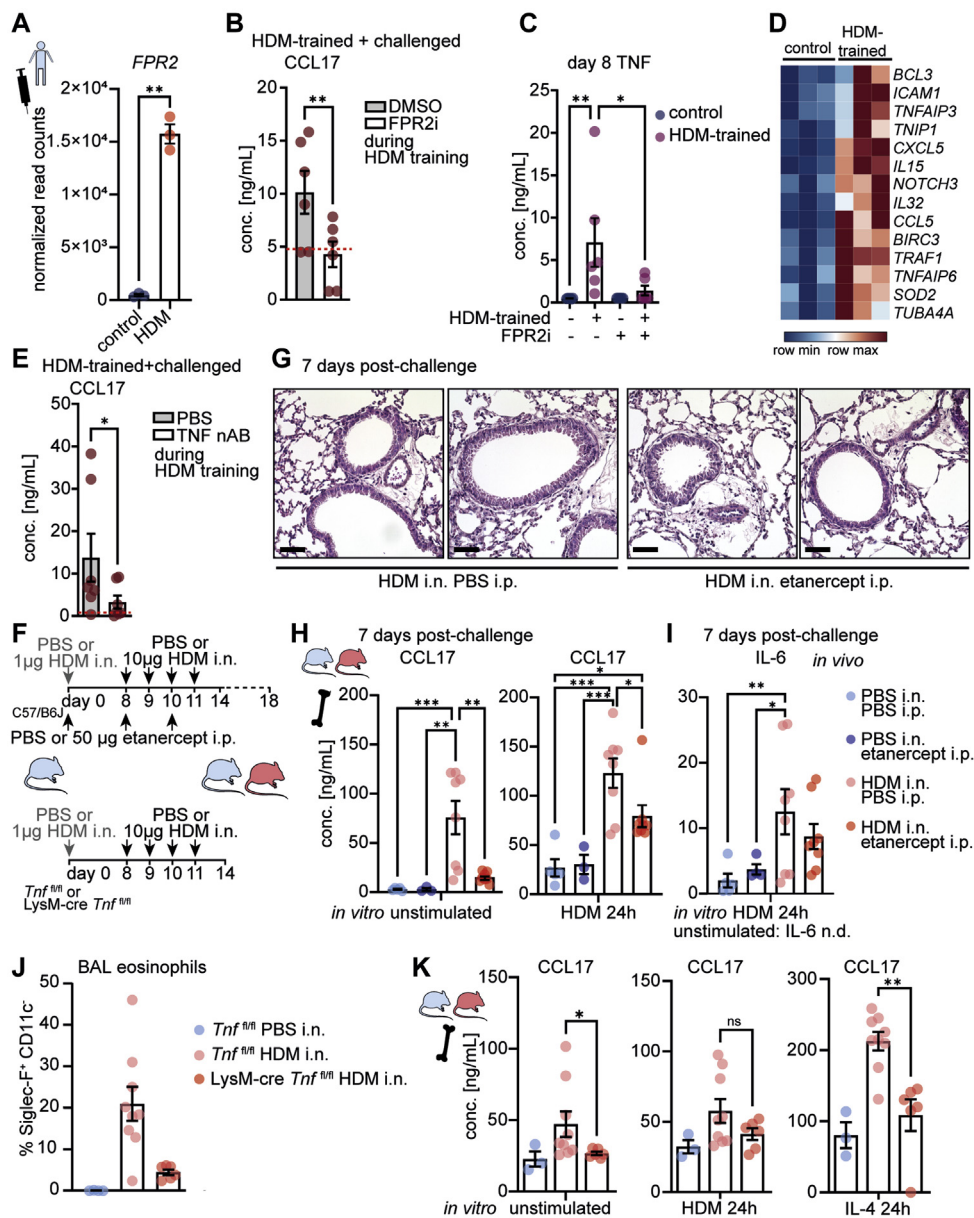
## 2-Hydroxyglutarate and lysine demethylase-1 drive inflammatory macrophage reprogramming

On the basis of the observed metabolic reprogramming of *in vitro* trained macrophages (Fig 3, G-I), we performed a targeted metabolomic analysis, quantifying amino acid- and tricarboxylic acid-cycle metabolites. BMDM from HDM-sensitized mice showed an increased output of amino acids and tricarboxylic acid-cycle intermediates (Fig 5, A), including metabolites involved in LT biosynthesis, M2 activation, and T2 immunity (Fig 5, A-C).<sup>41-43</sup> 2-Hydroxyglutarate (2-HG), a modulator of  $\alpha$ -ketoglutarate-dependent dioxygenase activity,<sup>44</sup> was increased (Fig 5, D), while bioenergetic parameters indicative of glycolysis (extracellular acidification rate) or mitochondrial respiration (oxygen consumption rate) were unaltered in HDM-sensitized compared to mock-sensitized BMDM (Fig E5, A and B, in the Online Repository available at [www.jacionline.org](http://www.jacionline.org)). 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 and E); however, arginase-1 (*Arg1*) expression in BMDM was increased (Fig 5, E and F), suggesting a

suppressive role of TNF on negative regulators of T2 inflammation.<sup>45</sup> 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 prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and CCL17 production (Fig 5, J), suggesting an involvement of 2-HG in T2 imprinting. 2-HG promotes hypoxia-inducible factor (HIF)-1 $\alpha$  activation by inhibiting its degradation by prolyl-hydroxylases, and *Hif1a* was upregulated in BMDM from HDM-sensitized mice (Fig E5, C). HIF-1 $\alpha$  target genes (*VEGFA*, *MMP2*, *PLOD2*, *EGR1*, *VLDLR*, *RBP1*, *PPF1A4*)<sup>46-51</sup> as well as *HIF1A* transcription were induced by HDM in human macrophages (Fig 3, E and F, Fig 5, K), but inhibiting HIF-1 $\alpha$  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, as in KDM families 2-8,<sup>52</sup> and *KDM6B* (JMJD3) is implicated in M2 macrophage activation.<sup>53</sup> 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 and K). Instead, a screen of different histone 3 modifications in HDM-trained aMDM (Table II) revealed less abundant H3K4 mono- and trimethylation as well as H3K9 dimethylation, modifications induced by family 1 KDMs such as *KDM1A* (lysine demethylase 1, LSD1).<sup>54</sup> Application of the KDM1A inhibitor pargyline during training suppressed CCL17 and cysLT responsiveness upon HDM challenge (Fig 5, M), suggesting LSD1-mediated reprogramming as the epigenetic mechanism underlying HDM training.

## HDM-induced macrophage training is distinct from classical trained immunity and driven by PGE<sub>2</sub>/PGE<sub>2</sub> receptor 2 signaling

To further identify downstream mediators of TNF-driven metabolic and epigenetic macrophage reprogramming, we performed targeted liquid chromatography–tandem mass spectrometry and multiplex cytokine analyses for HDM-trained aMDM immediately after allergen training (day 8), after 5 days of rest (day 13), and 24 hours after HDM challenge (day 14). Except for CCL17, HDM training evoked a transient increase of cytokines and eicosanoids that had returned to baseline after the resting phase (Fig 6, A). After HDM restimulation, 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, Fig 6, B and C). HDM-trained aMDM also synthesized high amounts of prostanoids upon challenge (Fig 6, D), and enzymes involved in the production of PGE<sub>2</sub>, particularly microsomal prostaglandin E synthase 1, were persistently induced by HDM training and challenge (Fig 6, E and F). Together with HDM-induced cyclooxygenase-2,<sup>12</sup> this likely explains augmented HDM-triggered PGE<sub>2</sub> production in HDM-experienced human and murine macrophages (Fig 6, G and H). Reduced HDM-triggered COX-2 (*Ptgs2*) induction after

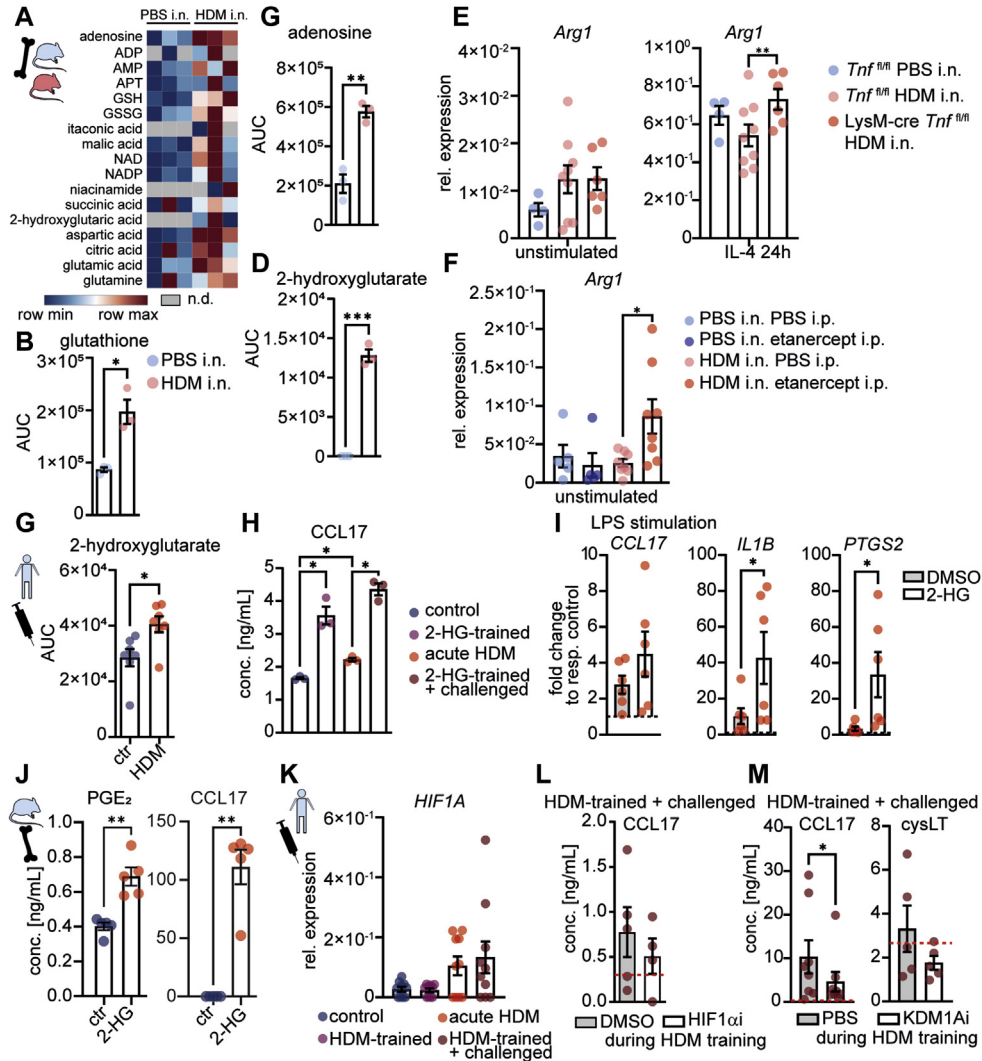


**FIG 4.** Autocrine TNF signaling mediates HDM-driven T2 imprinting *in vitro* and *in vivo*. **A**, Normalized read counts for *FPR2* in aMDM ( $n = 3$  healthy donors), with or without 24-hour HDM (padj, DeSeq2). **B**, CCL17 production by challenged HDM-trained aMDM with or without *FPR2* inhibitor (*FPR2i*) during training (day [D] 14,  $n = 6$ , paired *t* test). Dotted line indicates CCL17 production by aMDM plus 24-hour HDM. **C**, TNF production of control and HDM-trained aMDM with or without *FPR2i* during training ( $n = 6$ , Friedman test, Dunn 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 with or without TNF neutralizing antibody (nAB) during training (D14,  $n = 7$ , paired *t* test). **F**, Experimental scheme for HDM-induced AAI with or without TNF inhibition (top) or in mice deficient in myeloid *Tnf* (bottom). **G**, Representative histology images of lung tissues of HDM-sensitized mice with or without etanercept treatment. Scale bar, 50  $\mu$ m. **H** and **I**, CCL17 (**H**) or IL-6 (**I**) production by BMDM from PBS- or HDM-sensitized mice with or without etanercept treatment and with or without 24-hour *ex vivo* HDM ( $n = 3-8$ , 2-way ANOVA, Tukey multiple comparisons test). **J** and **K**, Bronchoalveolar lavage (BAL) eosinophils (**J**) or *ex vivo* BMDM CCL17 production (**K**) for HDM-sensitized *Tnf<sup>fl/fl</sup>* or *LysM-cre Tnf<sup>fl/fl</sup>* mice. Data are presented as means  $\pm$  SEMs or z scores transformed. \* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .001$ . nd, Not detected.

etanercept treatment (Fig 6, I) further implicated the COX-2/PGE<sub>2</sub> pathway in TNF-driven reprogramming. PGE<sub>2</sub> receptor 2 (EP2)-deficient BMDM showed an intact HDM-triggered TNF response but a reduced CCL17 response compared to wild-type

BMDM (Fig 6, J and K), suggesting that enhanced PGE<sub>2</sub> synthesis by macrophages represents a downstream mechanism of TNF-mediated innate immune training. Thus, the increased arachidonic acid metabolism of HDM-trained macrophages contributes





**FIG 5.** Metabolic-epigenetic cross talk via 2-HG and KDM1A contributes to HDM-induced macrophage hyperresponsiveness. Targeted metabolomics (A) and histograms for (B) glutathione, (C) adenosine, and (D) 2-HG of BMDM from PBS- versus HDM-sensitized mice ( $n = 3$  per group, paired  $t$  test). E and F, *Arg1* expression in BMDM of PBS- or HDM-sensitized *Tnf<sup>fl/fl</sup>* or *LysM-cre Tnf<sup>fl/fl</sup>* mice with or without 24-hour IL-4 ( $n = 4-9$ ) (E) or from PBS- or HDM-sensitized mice with or without etanercept treatment ( $n = 5-8$ ) (F). Two-way ANOVA, Sidak multiple comparisons test (E and F). G, 2-HG in MDM from healthy donors with or without 24-hour HDM ( $n = 7$ , paired  $t$  test). H, CCL17 production by control or 2-HG-trained macrophages with or without HDM challenge (day [D] 14,  $n = 3$ , repeated measures 1-way ANOVA, Sidak multiple comparisons test). I, LPS versus control, fold change of *CCL17*, *IL1B*, and *PTGS2* with or without 2-HG ( $n = 6$ , paired  $t$  test). Dotted lines indicate fold change = 1. J, PGE<sub>2</sub> and CCL17 production of BMDM with or without 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, with or without HIF-1 $\alpha$  inhibition during training (D14,  $n = 5$ ). M, CCL17 and cysLT production by challenged HDM-trained macrophages, with or without KDM1A inhibition during training (D14,  $n = 8/n = 5$ , Wilcoxon test). Dotted line in (L) and (M) indicates CCL17 or cysLT in aMDM with 24-hour HDM. Data are presented as z score transformed or means  $\pm$  SEMs. \* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .001$ . AUC, Area under curve.

to TNF-mediated trained T2 immunity. Together, these data identify a metabolic-epigenetic circuit leading to persistent T2 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 T2

inflammation in the context of AAI and nasal polyposis.<sup>55,56</sup> 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, or from HDM-sensitized mice, or macrophages trained with HDM extract *in vitro* produced high amounts of CCL17 and cysLTs, both potent mediators of T2 immunity and therapeutic targets in asthma.<sup>14,35</sup> Trained T2 immunity was associated with an

**TABLE II.** Histone 3 modification screening

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	-

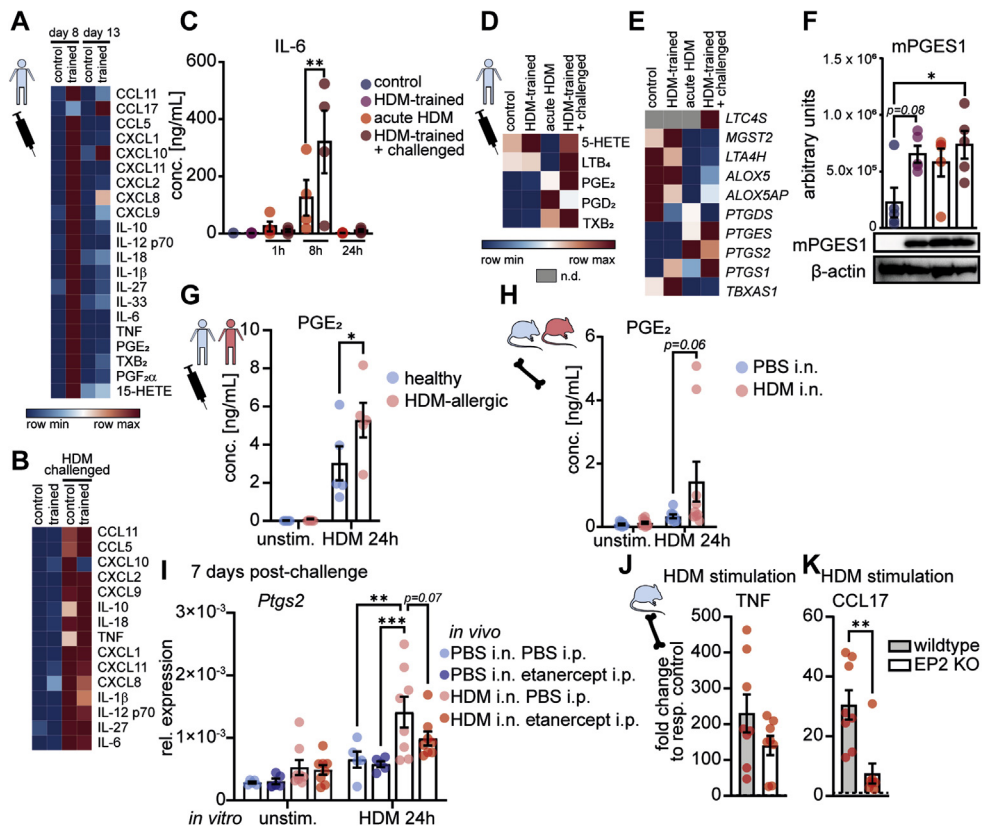
\*– decreased, - tendency to decreased, = unchanged, + increased.

increased arachidonic acid metabolism– and prostaglandin signaling–perpetuated inflammatory macrophage reprogramming. This identifies an unprecedented role for eicosanoids in trained immunity and suggests that leukotrienes and prostaglandins are promising targets for preventing the chronification or exacerbation of allergen-induced airway inflammation. The heightened cysLT response of asthma patients' macrophages was mimicked by HDM training and reexposure 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 mono- and trimethylation and reduced H3K9 dimethylation in HDM-trained versus control macrophages, suggesting a role for KDM1A in removing repressive marks to enhance T2 inflammatory mediator responses.<sup>57</sup> Because KDM1A activity is necessary for hematopoietic stem cell differentiation,<sup>58</sup> 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 asthma patients appears to be a hallmark of allergen-induced training that drives a chronic pathologic T2 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*, whereas *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 (eg, *MERTK*, *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,<sup>55</sup> 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 T2 inflammation as *Arg1* suppresses pathologic T<sub>H</sub>2 responses.<sup>45</sup> While we did not observe heightened baseline CCL17 expression in aMDM from allergic donors, sputum-derived AMs cultured *ex vivo* released high levels of CCL17 compared to aMDM or compared to AMs 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 they specifically induced cysLTs and CCL17, which elicit T2 immune responses. Thus, allergen-induced trained T2 immunity appears to be distinct from trained immunity programs driven by microbial products, despite some overlapping features such as increased IL-6 responses.<sup>19,22</sup> The transient upregulation of IL-4 and IL-13 in the bone marrow after HDM challenge may contribute to the time-dependent shift from T2 to classical imprinting of macrophage progenitors. HDM training also transiently induced TNF in an FPR2-dependent fashion, suggesting that the HDM components *Der p 13* and *Blo t 13*, recently identified ligands of serum amyloid 1–mediated FPR2 activation, mediate TNF-driven macrophage imprinting.<sup>38</sup> TNF functions as a negative regulator of M2 polarization in cancer or infectious diseases.<sup>59-61</sup> In arthritis, in contrast, TNF signaling is important at early time points, while TNF-induced CCL17 appears as a late mediator,<sup>39</sup> 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,<sup>62</sup> suggesting TNF-mediated downregulation of CD84 as a mechanism of aberrant macrophage activation in T2 inflammation. In the trained T2 immunity pathway we uncovered, TNF acted as an early initiator of T2 inflammatory macrophage activation. These data suggest 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 2 TNF receptors. Importantly, altered expression of TNF-response genes and T2-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 also 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-1 $\alpha$  in TNF-mediated trained T2 immunity.<sup>11,63</sup> However, how 2-HG production and HIF-1 $\alpha$  activation are elicited downstream of FPR2 and TNF remains to be determined. Our data suggest that 2-HG promotes COX-2 expression and PGE<sub>2</sub> 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. On the basis of our study design, we cannot discern whether HDM itself



**FIG 6.** HDM-induced macrophage training is distinct from classical trained immunity and is driven by PGE<sub>2</sub>/EP2 signaling. **A** and **B**, Mediator production of HDM-trained aMDM on day (D) 8 and D13 (**A**) or D14 after HDM restimulation. **C**, IL-6 production of control and HDM-trained aMDM after 1, 8, or 24 hours of HDM restimulation (n = 4). **A-C**, Repeated measures 2-way ANOVA, Sidak multiple comparisons test. **D**, Eicosanoid production by control or HDM-trained human macrophages (n = 11). **E**, Normalized read counts (RNA sequencing) of eicosanoid metabolism genes in control and HDM-trained aMDM (n = 2). **F**, Microsomal prostaglandin E synthase 1 (mPGES1) protein levels for control and HDM-trained aMDM (n = 5, Friedman test, Dunn multiple comparisons test) and representative Western blot. **G** and **H**, PGE<sub>2</sub> production by aMDM (**G**) or BMDM (**H**) from healthy or HDM-allergic donors or mice, with or without 24-hour HDM (n = 5/n = 8-9 per group). **I**, *Ptg2* expression in from PBS- or HDM-sensitized mice with or without etanercept treatment with or without 24-hour HDM (n = 5-8, repeated measures 2-way ANOVA). **G-I**, Repeated measures 2-way ANOVA, Sidak multiple comparisons test. **J** and **K**, TNF (**J**) or CCL17 (**K**) production of wild-type or EP2 KO BMDM, with or without 24-hour HDM exposure (n = 7, Mann-Whitney test). Data are presented as z score transformed or means ± SEMs. *EP2 KO*, *Ptger2* knockout; *nd*, not detected. \**P* < .05, \*\**P* < .01.

or the T2 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 T2 immunity on the level of myeloid progenitors in 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 asthma patients,<sup>64</sup> 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. Because TNF inhibition results in increased infection susceptibility, it will be necessary to understand the role of

TNF-induced trained immunity in distinct human asthma endotypes.<sup>65</sup> It will be important to further decipher innate memory responses in allergic asthma because inflammatory reprogramming of myeloid cells may contribute to the chronification, exacerbation, or even transmission of T2 airway inflammation.

We thank the animal caretakers at the Helmholtz Center Animal Facility, Sebastian Kotz and Jana Hartmann (ENT Department, TUM Medical School) for support with patient sampling, and all volunteers for participation in this study. We thank Sonja Schindela, Johanna Grosch, Sandra Riemer, Lara Paulini (Center of Allergy and Environment), Olga Namakanova, and Marina Bondareva (Engelhardt Institute of Molecular Biology) for excellent technical assistance; Jan P. Böttcher (Institute for Molecular Immunology, Technical University of Munich) for providing bone marrow of EP2-knockout mice; and Shu-Hung Wang, Caroline Pilz, Felix Lauffer (Center of Allergy and Environment), and many friendly MD students for blood collection.

### Key messages

- Macrophages from HDM-allergic asthma patients show persistent inflammatory imprinting.
- Inhibition of TNF signaling prevents macrophage-trained immunity in AAI.
- 2-HG, PGE<sub>2</sub>, and lysine demethylase 1 mediate allergen-driven metabolic and epigenetic macrophage reprogramming.

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