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RESEARCH ARTICLE

The human MIF polymorphism $CATT_7$ enhances pro-inflammatory macrophage polarization in a clinically relevant model of allergic airway inflammation

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Abstract

High level expression of the pro-inflammatory cytokine macrophage migration inhibitory factor (MIF) has been associated with severe asthma. The role of MIF and its functional promotor polymorphism in innate immune training is currently unknown. Using novel humanized $CATT_7$ MIF mice, this study is the first to investigate the effect of MIF on bone marrow-derived macrophage (BMDM) memory after house dust mite (HDM) challenge. $CATT_7$ BMDMs demonstrated a significant primed increase in M1 markers following HDM and LPS stimulation, compared to naive mice. This M1 signature was found to be MIF-dependent, as administration of a small molecule MIF inhibitor, SCD-19, blocked the induction of this pro-inflammatory M1-like phenotype in BMDMs from $CATT_7$ mice challenged with HDM. Training naive BMDMs *in vitro* with HDM for 24 h followed by a rest period and subsequent stimulation with LPS led to significantly increased production of the pro-inflammatory cytokine TNFα in BMDMs from $CATT_7$ mice but not WT mice. Addition of the pan methyltransferase inhibitor MTA before HDM training significantly abrogated this effect in BMDMs from $CATT_7$ mice, suggesting that HDM-induced training is associated with epigenetic remodelling. These findings suggest that trained immunity induced by HDM is under genetic control, playing an important role in asthma patients with the high MIF genotypes ($CATT_{6/7/8}$).

KEYWORDS

bone marrow-derived macrophages, house dust mite, innate immunity, innate priming, innate training, macrophage migration inhibitory factor, polarization

Abbreviations: ANOVA, analysis of variance; Arg1, arginase-1; BALF, bronchoalveolar lavage fluid; BCG, bacillus calmette-guerin; BMDM, bone marrow-derived macrophage; CCL, C-C motif chemokine ligand; cDNA, complementary deoxyribonucleic acid; CT, cycle threshold; DMSO, dimethyl sulfoxide; ELISA, enzyme linked immunosorbent assay; FBS, fetal bovine serum; FIZZ1, found in inflammatory zone 1; HDM, house dust mite; HSPC, hematopoietic stem and progenitor cells; I.N., intranasal; I.P., intraperitoneal; I.V., intravenous; IL, interleukin; LPS, lipopolysaccharide; Mch, methacholine; M-CSF, macrophage colony stimulating factor; mg, milligram; MIF, macrophage migration inhibitory factor; ml, millilitre; mRNA, messenger ribonucleic acid; MTA, methylthioadenosine; N.D., not detected; ns, not significant; OVA, ovalbumin; PAS, periodic acid schiff; PBS, phosphate buffered solution; qRT-PCR, quantitative real time polymerase chain reaction; RBC, red blood cell; Retnla, resistin-like alpha; RNA, ribonucleic acid; RPMI, roswell park memorial institute; TNFa, tumor necrosis factor alpha; WGP, whole beta-glucan particles; WT, wildtype.

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1 | INTRODUCTION

Trained innate immunity has gained significant scientific attention in light of the recent SARS-CoV2 pandemic with vaccine efficacy, where this popular phenomenon illustrates the importance of immune cell memory and how this innate cell training is fundamental to an effective immunological response to pathogens.[1,2] A noteworthy example of this nonspecific trained immunity is demonstrated in the application of the Bacillus Calmette–Guérin (BCG) vaccine, originally developed to combat tuberculosis in 1928.[3] This vaccine is currently acknowledged to provide additional protection against other respiratory tract insults[4,5] including influenza[6,7] via trained innate immunity.

Macrophages play a dominant role in the innate immune system and the development of airway inflammation. Macrophage activation is dependent on their surrounding environment, where they can be classically activated to an M1 pro-inflammatory phenotype detected by increased TNFα, IL-6, IL-1β, and iNOS, or alternatively to an M2 anti-inflammatory phenotype detected by arginase-1 or Retnla (FIZZ1). M2 macrophages play a predominant role in asthma due to increased levels of IL-4 present, where these cells drive airway remodelling and wound healing within the lung.[8] As airway remodelling is a problematic feature of asthma, macrophages can accelerate and exacerbate this process.[8] However, mounting evidence indicates that macrophage activation is not a clear-cut process, with the M1-M2 axis being an ever-changing spectrum[9] with evidence for both M1 and M2 macrophages in asthma.[10]

An over-activated, trained immune response can be detrimental in the context of asthma, as the primed innate immune system is hyperresponsive to inhaled allergens, irritants, or environmental changes, resulting in frequent exacerbations.[11,12] In addition to playing a central role in host defence, macrophages are also a source of the pro-inflammatory cytokine macrophage migration inhibitory factor (MIF).[13] MIF is known to inhibit p53 in macrophages, thus promoting an accumulation of these cells.[14] As a result, MIF can drive macrophage-mediated inflammation. However, it is important to note, that MIF has also been documented to favour monocyte differentiation.[15,16] MIF is involved in the pathogenesis of a variety of inflammatory diseases including asthma; however, it remains to be resolved if MIF's role in disease is due to increased susceptibility or severity. MIF's involvement in asthma severity has been linked to a functional promotor polymorphism, where an increase in the number of repeats of a tetranucleotide sequence $(CATT)_n$, correlates with increased MIF expression.[17–19] Humans that possess a five-repeat allele of this MIF polymorphism have been noted to have a milder subtype of disease.[20] Interestingly, an association between severe human asthma and high expression MIF alleles has been documented,[21,22] with ~50% of asthma patients expressing the 6/7/8 CATT allele,[23] compared to 19%–20% of healthy controls.[24,25] Due to its enhanced presence in the bronchoalveolar lavage fluid (BALF) of asthmatic patients compared to nonatopic volunteers,[23] MIF has been shown to be an important player in asthma development. We have previously illustrated the role of the high expressing human $CATT_7$ MIF allele in a model of house dust mite (HDM)-induced allergic airway inflammation.[18,19]

This study is the first to characterize the macrophage phenotype in these novel human $CATT_7$ MIF expressing transgenic mice, comparing the macrophage activation status from both naive mice and those exposed to an acute model of HDM-induced asthma. Furthermore, this study is the first to identify the role of this high expressing human MIF polymorphism in the trained innate immune system of these mice after HDM exposure.

2 | MATERIALS AND METHODS

2.1 | Ethical approval and HPRA compliance

All procedures involving the use of animals were carried out by authorized personnel. Ethical approval for all work was granted by the ethics committee of Maynooth University (BRESC-2018-13). Project authorization was received from the HPRA (AE19124/P022), whereby the terms of the animal experiments within this project were outlined and adhered to in accordance with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) criteria.

2.2 | Transgenic humanized MIF mice

A C57BL/6 mouse strain expressing the human high expression $CATT_7$ MIF allele (*MIF*CATT7 [(C57BL/6NTac-Miftm3884.1(MIF)Tac-Tg(CAG-Flpe)2Arte)]) was created using vector-based recombinant replacement of murine MIF by Taconic Biosciences (Rensselaer, NY). Validation of human but not murine MIF mRNA expression was verified by qPCR, and -794 CATT length-dependent stimulated MIF production was confirmed *in vivo*.[26]

2.3 | Preclinical model of acute allergic airway inflammation

$CATT_7$ and WT mice (6–18 weeks old; male and female) were challenged with 25 μg of house dust mite allergen,

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Dermatophagoides pteroyssinus with an average endotoxin content of 9937.5 EU/vial (Greer Labs, Lenoir, NC, USA) or PBS control intranasally (I.N.) under isoflurane anesthesia, on Days 0, 2, 4, 7, 9, 11, 14, 16, and 18. After each challenge, mice were returned to their cage and monitored closely. An animal welfare score sheet was utilized throughout the study, where examining features included: weight loss, activity, general appearance, and clinical signs.

2.4 | MIF inhibitor

SCD-19 (3-(2-methylphenyl)-1H-isochromen-1-one) (Specs.net, Netherlands), a small molecular weight inhibitor of MIF biological activity was used in the house dust mite model of acute allergic asthma. SCD-19 or vehicle control (35 mg/kg) (70% ethanol in PBS) was administered intraperitoneally (I.P.) twice weekly for 3 weeks; Days 0, 4, 7, 11, 14, and 18.

2.5 | Histological analysis

On Day 21 of the model, lungs were harvested, processed, and sectioned. Tissue sections (5 μm) were stained for Masson's Trichrome (Sigma-Aldrich, Wicklow, Ireland) and Periodic Acid Schiff (Abcam, Cambridge, UK). Samples were air-dried and a coverslip was mounted with DPX mounting media (Sigma-Aldrich, Wicklow, Ireland). Images (4× and 20×) were taken using an Olympus BX51 light microscope. Following staining, slides were coded without reference to prior treatment and examined in a blind manner. For PAS, images were scored by counting the number of PAS-positive (magenta) mucin-producing goblet cells present within the airway, relative to the diameter to the airway. For Masson's Trichrome, the percentage of subepithelial collagen was quantified using Fiji software with a Trainable Weka Segmentation plugin.

2.6 | Bronchoalveolar lavage fluid (BALF) retrieval and analysis

Mice were euthanized by lethal overdose of sodium pentobarbital via I.P. injection on Day 18 of the model, 4 h after last challenge. A tracheostomy and cannulation was performed, where a 27-gauge cannula was secured in place with sutures. Cold endotoxin-free PBS (1 mL) (Sigma-Aldrich, Wicklow, Ireland) was infused into the lungs through the cannula using a 1 mL syringe for three gentle instillations. BALF was kept on ice before being centrifuged at 300 **g** for 5 min at 4°C. Cells were isolated from BALF via centrifugation and resuspended in 100 μL of endotoxin-free PBS for counting. Cytospin funnels were pre-wet by spinning with 300 μL of PBS onto glass slides at 600 rpm for 5 min. 1×10^5 cells in a volume of 300 μL of PBS were spun onto fresh labelled glass slides at 600 rpm for 10 min using a RotoFix 32 cytocentrifuge (Hettich Zentrifugen). Slides were air-dried before being stained with Kwik- Diff™ Stain (Richard-Allan Scientific, Kalamazoo, MI, USA); 25 s in fixative, 15 s in solution I (eosin), and 15 s in solution II (methylene blue). Slides were imaged on an Olympus BX51 light microscope until 300 cells could be counted. Cells were identified using the following morphological characteristics: neutrophils (nucleus containing 2–5 lobes), eosinophils (bi-lobed nucleus and cytoplasmic granules), macrophages (single nucleus, cell large in size), or lymphocytes (single nucleus, cell small in size). Cells populations were divided by the number of total cells counted and normalized to the volume of BALF collected.

2.7 | Cytokine and protein quantification

Lungs were harvested from $CATT_7$ and WT mice 4 h after last PBS or HDM administration of the HDM-induced model of allergic airway inflammation. Tissue was snap frozen in liquid nitrogen, before being homogenized in PBS supplemented with 1× protease inhibitor (Roche). Samples were centrifuged at 300 **g** for 5 min and supernatants were collected and stored at −20°C. Lung homogenates were analyzed for murine cytokines TNFα, IL-6, and IL-1β, IL-4, IL-5, (Biolegend, San Diego, CA, USA), CCL17 (R&D Systems, Abingdon, UK), and IL-13 (eBioscience, San Diego, CA, USA) or human MIF (R&D Systems, Abingdon, UK) by ELISA. The absorbance optical density (OD) of the samples and standards were measured at 450 nm for all ELISAs using a microplate reader (Clariostar Plus, BMG Labtech, Bucks, UK).

2.8 | FlexiVent® lung function

Mice were anesthetized with 150 mg/kg ketamine and 2 mg/kg medetomidine via subcutaneous injection and the surgical plane of anaesthesia was reached. A tail vein catheter was inserted. Tracheostomy and cannulation was carried out and the subject was placed close to the FlexiVent® FX system (SCIREQ, Emka Technologies, Paris, France) and mechanical ventilation was initiated by selecting a predefined ventilation. Every 6 min, alfaxan and 0.5 mg/kg atracurium besilate, a neuromuscular blocking agent (NMBA), was administered through

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the tail vail catheter to ensure passive breathing. The measurement of lung function was initiated and approximately 100 μL of PBS or the bronchoconstrictor methacholine (MCh) (25 mg/mL) (Sigma-Aldrich, Wicklow, Ireland) was loaded into the nebulizer. Upon completion of lung function measurements at baseline and following aerosolized methacholine challenges, the ventilator was stopped and the mouse was euthanized using either I.P. injection of sodium pentobarbital or via cervical dislocation.

2.9 | Generation of L929 conditioned media (M-CSF)

NCTC clone 929 (L929) cells were thawed, seeded in complete Roswell Park Memorial Institute (RPMI) 1640 medium GlutaMAX (Gibco, Paisley, UK) and incubated at 37°C/5% CO_2/20% O_2 for 7 days. Supernatant was collected and centrifuged at 300 **g** for 5 min to remove cell debris. After being passed through a 0.2 μm filter for sterilization, conditioned media containing M-CSF was aliquoted and stored at −80°C. L929-conditioned media will be referred to as M-CSF throughout the text.

2.10 | Isolation of bone marrow containing hematopoietic stem and progenitor cells (HSPCs)

Mice were humanely sacrificed using cervical dislocation and the bone marrow was isolated from femur and tibia and centrifuged at 300 **g** for 5 min. Red blood cells were lysed using 1X RBC lysis buffer (eBioscience, San Diego, CA, USA) for 5 min. Cells were centrifuged at 300 **g** for 5 min before being counted with a hemocytometer and seeded out at 1.5×10^6 per well of 6-well non-tissue culture grade plate in complete cRPMI supplemented with 10% heat inactivated fetal bovine serum (FBS) and 20% L929-conditioned media (M-CSF).

2.11 | Generation and polarization of bone marrow-derived macrophages (BMDMs)

Cells were isolated from the bone marrow of naive $CATT_7$ and WT mice as described. 1.5×10^6 cells were seeded in cRPMI supplemented with 20% M-CSF into tissue culture grade 6-well plates (Sarstedt, Nümbrecht, Germany). Media was changed on Days 3 and 6 to remove non-adherent cells. On Day 7, media was replenished and cells were stimulated for 24 h with either LPS (from *E. coli* O111:B4) (100 ng/mL) (Sigma-Aldrich, Wicklow, Ireland) to drive M1 polarization, or murine IL-4 (10 ng/mL) (R&D Systems, Abingdon, UK) to drive M2 polarization.

2.12 | In vitro innate priming assay

After the HDM-induced allergic airway inflammation model (primary stimulus), bone marrow was isolated from $CATT_7$ and WT mice 4 h after last challenge. BMDMs were differentiated as described. On Day 7, BMDMs received a secondary heterologous stimulus of LPS (100 ng/mL) or mIL-4 (10 ng/mL) for 24 h. Cells and supernatants were harvested and stored at −20°C for RNA and protein analysis.

2.13 | In vitro innate training assay

For *in vitro* innate training assays, HSPCs were isolated from the bone marrow of naive $CATT_7$ or WT mice. A methyltransferase inhibitor, methylthioadenosine (MTA) (1 mM) (Sigma-Aldrich, Wicklow, Ireland) or a DMSO vehicle control was added to freshly isolated whole bone marrow cells containing HSPCs 30 min prior to any stimulus. The cells were then stimulated with 10 μg of HDM and media was supplemented with 20% M-CSF. After 24 h, cells were washed in warm PBS to remove HDM stimulus. Cells were cultured in 20% M-CSF supplemented cRPMI to facilitate macrophage differentiation, until Day 6, when media was changed to non-supplemented cRPMI to facilitate a rest period. Differentiated macrophages were stimulated with 100 ng/mL of LPS on Day 10 for 24 h. Supernatants were collected and analyzed for protein production.

2.14 | Analysis of gene expression

Total RNA was extracted using TRIzol (Ambion Life Sciences, Cambridgeshire, UK) according to manufacturer's instructions. RNA concentrations were measured using a spectrophotometer (Nanodrop 2000, Thermo Scientific, Wilmington, DE, USA) and were equalized to 100 ng/μl before cDNA synthesis. cDNA synthesis was performed using manufacturer's instructions (Quantabio cDNA synthesis kit). Real-time polymerase chain reaction (RT-PCR) was carried out for several genes (detailed in Table S1) using PerfeCta SYBR Green FastMix (Quantabio, MA, USA). Expression was quantified in relation to the housekeeper gene HPRT using the ΔCT method. The fold change in the relative gene expression was determined by calculating the $2^{-\Delta\Delta CT}$ values.

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2.15 | Statistical analysis

Mice were randomized. Observers assessing end-points were blinded to group assignment. Data for individual animals and independent experiments are presented as individual symbols. All data are presented as mean ± SEM. Results of two or more groups were compared by one-way or two-way analysis of variance (ANOVA) followed by the post hoc Tukey's multiple comparison test. GraphPad Prism (GraphPad Software Inc, San Diego, CA, USA) was used for all statistical analyses.

3 | RESULTS

3.1 | CATT$_7$ human MIF polymorphism drives pathological severity in HDM-induced allergic airway inflammation model

Novel transgenic humanized MIF mice were generated to investigate the translational relevance of high levels of human MIF expression, under the functional promotor polymorphism. C57BL/6 mice were humanized by replacing the murine MIF gene with the human counterpart. Within this human MIF gene, 794 downstream of the promotor region, the number of tetranucleotide repeats correlates with MIF allele expression,[17] where seven repeats of this tetranucleotide sequence "CATT" generated CATT$_7$ mice, containing the high expressing MIF allele.[18,19] To characterize pathological differences associated with the expression of the human MIF polymorphism after intranasal administration of HDM or PBS control in a model of allergic airway inflammation (Figure 1A), cell populations in the BALF were identified by carrying out differential cell counts and identifying infiltrated immune cells based on their morphology. BALF from HDM-challenged CATT$_7$ mice exhibited significantly elevated numbers of immune cells, predominately eosinophils rather than lymphocytes, neutrophils, or macrophages, compared to WT mice (Figure 1B). Airway remodelling was evident in CATT$_7$ mice after HDM challenge. High expression of human MIF was found to further exacerbate airway goblet cell hyperplasia, and increased subepithelial collagen deposition compared to WT mice and PBS controls (Figure 1C). In response to nebulization of the bronchoconstrictor methacholine, HDM-challenged CATT$_7$ mice that received 25 mg/mL of methacholine had significantly increased airway resistance (R^N) compared to HDM-challenged CATT$_7$ mice that received a PBS control. Moreover, HDM-challenged CATT$_7$ mice that received 25 mg/mL of nebulized methacholine had a significantly elevated percentage airway resistance compared to WT mice that also received 25 mg/mL of nebulized methacholine (Figure 1D). The percentage change from baseline measurements of lung mechanics for tissue elastance (H) (Figure 1D) and tissue damping (G) were increased in HDM-challenged CATT$_7$ mice compared to WT mice. To profile the inflammatory status of the lung after WT and CATT$_7$ mice received HDM or PBS control, lung homogenates were analyzed for target genes of interest: Type 1: *tnfα*, *il-6*, *il-1β*, and *nos2* or Type 2: *arg1* and *retnla* (Figure 1E). Lower mean cycle threshold (CT) values[23–30] were observed for the Type 2 inflammatory genes *arg1* and *retnla* compared to higher CT values[31–35] for Type 1 *tnfα*, *il-6*, *il-1β*, and *nos2* genes, indicating that HDM challenge facilitates higher Type 2 and lower Type 1 inflammation in the lung of WT and CATT$_7$ mice (Figure 1E). Lung homogenates from CATT$_7$ and WT mice that received intranasal administration of HDM or PBS were analyzed for Type 1 (TNFα, IL-6, and IL-1β) and Type 2 (IL-4, IL-5, IL-13, and CCL17) cytokine or chemokine production (Figure 1F). HDM challenge increased IL-13 and CCL17 in both WT and CATT$_7$ mice; however, this was not significant. IL-4 and IL-5 were significantly increased in lung homogenates in HDM-CATT$_7$ mice but not HDM-WT controls. Interestingly, the type 1 cytokine IL-6 was significantly increased in CATT$_7$ mice but not in WT mice following HDM challenge. While there was no difference in TNFα or IL-1β in WT mice challenged with HDM or PBS, there was a trending increase in TNFα and IL-1β in HDM-CATT$_7$ mice (Figure 1F).

3.2 | In vivo HDM challenge primes CATT$_7$ mouse BMDMs, boosting their M1 response to a heterologous secondary stimuli in a MIF-dependent manner

The immune system can be primed by an initial stimulus (e.g., HDM), leading to an activation of gene transcription. Upon encountering a secondary stimulus, often heterologous, this results in an additive or synergistic immune response.[1] In the lung, the predominant macrophage genes expressed were of the M2 phenotype following HDM challenge in both WT and CATT$_7$ mice (Figure 1E). Next, we directly compared bone marrow-derived macrophages (BMDMs) from naive CATT$_7$ and WT mice polarized toward an M1-like phenotype using LPS stimulation or an M2-like phenotype using an IL-4 stimulation (Figure 2). As expected LPS stimulation significantly increased expression of M1 associated genes *tnfα*, *il-6*, *il-1b*, *nos2*, and *il-10* in WT and CATT$_7$ BMDMs (Figure 2A–E). CATT$_7$ BMDMs expressed significantly higher *tnfα* than WT BMDMs in response to LPS stimulation and this was also observed at the protein level (Figure 2A). While CATT$_7$

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FIGURE 1 Characterization of WT and $CATT_7$ mice after HDM exposure. (A) Preclinical mouse model of acute allergic airway inflammation. Mice received 25 μg of house dust mite or PBS control intranasally three times for 3 weeks. BALF and lung homogenates were collected on Day 18, 4 h after last challenge, or lungs were taken for histology on Day 21. (B) Cells infiltrated into the BALF were analyzed using differential stained cytospin slides. 300 cells were counted and identified as eosinophils, macrophages, lymphocytes or neutrophils based on their morphology, and cell numbers were normalized to the volume of BALF collected. (C) Representative images of lung tissue from WT and $CATT_7$ mice after HDM or PBS challenge (I.N.). Tissue sections were stained with Periodic Acid Schiff (Goblet cell hyperplasia) at 20× magnification or Masson's Trichrome (subepithelial fibrosis) at 4× magnification. Scale bar = 20 μm. (D) 24 h after last HDM challenge, the lung function of WT and $CATT_7$ mice was measured using a FlexiVent® instrument (SCIREQ) in response to PBS or methacholine (25 mg/mL). Parameters include airway resistance (R_N), tissue damping (G), and tissue elasticity (H). Data are presented as peak response normalized to the baseline and expressed as % increase over baseline. (E) Average CT values for M1 and M2 macrophage markers from WT and $CATT_7$ lung homogenate after HDM challenge. (F) Pro-inflammatory status of lung homogenates from WT and $CATT_7$ mice after HDM or PBS challenge were analyzed by ELISA. Th2 markers include IL-4, IL-5, IL-13, and CCL17. Th1 markers include TNFα, IL-6, and IL-1β. Data are presented as mean ± SEM; N = 3–6 mice per group. ns, nonsignificant, *$p < .05$, **$p < .01$, ***$p < .001$, ****$p < .0001$.

BMDMs stimulated with LPS expressed significantly lower levels of *il-1b* mRNA, there was no difference in IL-1B protein production (Figure 2C). However this difference of IL-1β between mRNA and protein levels may be due to our 24 h timepoint, as *il-1β* mRNA has been shown to peak at 5 h after LPS stimulation.[27] There were no significant differences in *il-6*, *nos2*, or *il-10* (Figure 2B,D,E) between $CATT_7$ and WT BMDMs stimulated with LPS. Similarly, IL-4 stimulation led to significant increases in the expression of the M2 associated genes *arg-1* or *retnla* in both $CATT_7$ and WT BMDMs however, there were no significant differences between $CATT_7$ and WT BMDMs (Figure 2F,G). Additionally, LPS stimulation led to a significant increase in human MIF production from $CATT_7$ BMDMs, however IL-4 stimulation had no effect (Figure S1A).

The priming effect of HDM was also observed in BMDMs from HDM-treated WT mice, but to a lesser extent than $CATT_7$ BMDMs. HDM challenge *in vivo* significantly increased the production of human MIF from $CATT_7$ BMDMs *in vitro* (Figure 3). As expected WT BMDMs isolated from naive or HDM-challenged WT mice do not produce human MIF (indicated by not detected (N.D.)) (Figure 3). Furthermore, it has been demonstrated that $CATT_7$ mice do not produce mouse MIF.[24] Next, we investigated the role of MIF in immune priming by HDM, using a clinically relevant HDM-induced allergic airway inflammation model (Figure 4A), including the use of a small molecular weight inhibitor of MIF, SCD-19, which alters MIF's conformationally sensitive tautomerase active site and impairs its interaction with other molecules and thus, its downstream biological effects.[18,28,29] Administration of SCD-19 but not vehicle control significantly blocked the primed immune response observed in BMDMs from $CATT_7$ mice. BMDMs from $CATT_7$ mice had significantly lower expression of all M1-associated genes, including *tnfα* (Figure 4B), *il-6* (Figure 4C), *il-1β* (Figure 4D), *nos2* (Figure 4E), and *il-10* (Figure 4F) compared to $CATT_7$ mice that received the vehicle control. Furthermore, protein levels of TNFα, IL-6, and IL-1β were also significantly decreased in BMDMs from HDM-$CATT_7$ that received SCD-19 compared to vehicle control (Figure 4B–D). Importantly, BMDMs from WT mice that received SCD-19 had no significant differences in the expression of these markers, with the exception of TNFα, which was significantly reduced compared to the vehicle control after LPS stimulation (Figure 4B). SCD-19 had no effect on HDM-$CATT_7$ BMDM expression of the M2 markers *arg1* or *retnla* compared to vehicle control (Figure 4G,H). However administration of SCD-19 to HDM-WT mice resulted in a significant increase in *arg1* and a significant decrease in *retnla* gene expression in BMDMs compared to HDM-WT mice that received the vehicle control (Figure 4G,H). LPS led to a significant increase in human MIF production from HDM-challenged $CATT_7$ BMDMs (Figure S1B); however, similar to naive $CATT_7$ BMDMs, IL-4 stimulation had no effect (Figure S1B). Interestingly, administration of SCD-19 reversed this effect, dampening LPS-induced human MIF production in $CATT_7$ BMDMs (Figure S1B).

3.3 | HDM increases innate immune training in $CATT_7$ mice, which can be blocked using the pan-methyltransferase inhibitor MTA

This study has found that novel transgenic humanized MIF $CATT_7$ mice can be primed by HDM challenge, resulting in a paradigm shift, increasing M1 activation in BMDMs (Figure 4). This priming effect was also found to be dependent on human MIF expression (Figure 4).

Priming occurs at the individual cell level (e.g., macrophage), whereas the concept of innate immune training occurs within the bone marrow niche in the hematopoietic stem and progenitor cells (HSPCs). Furthermore, innate immune training has been associated with epigenetic reprogramming, such as the methylation of DNA controlled

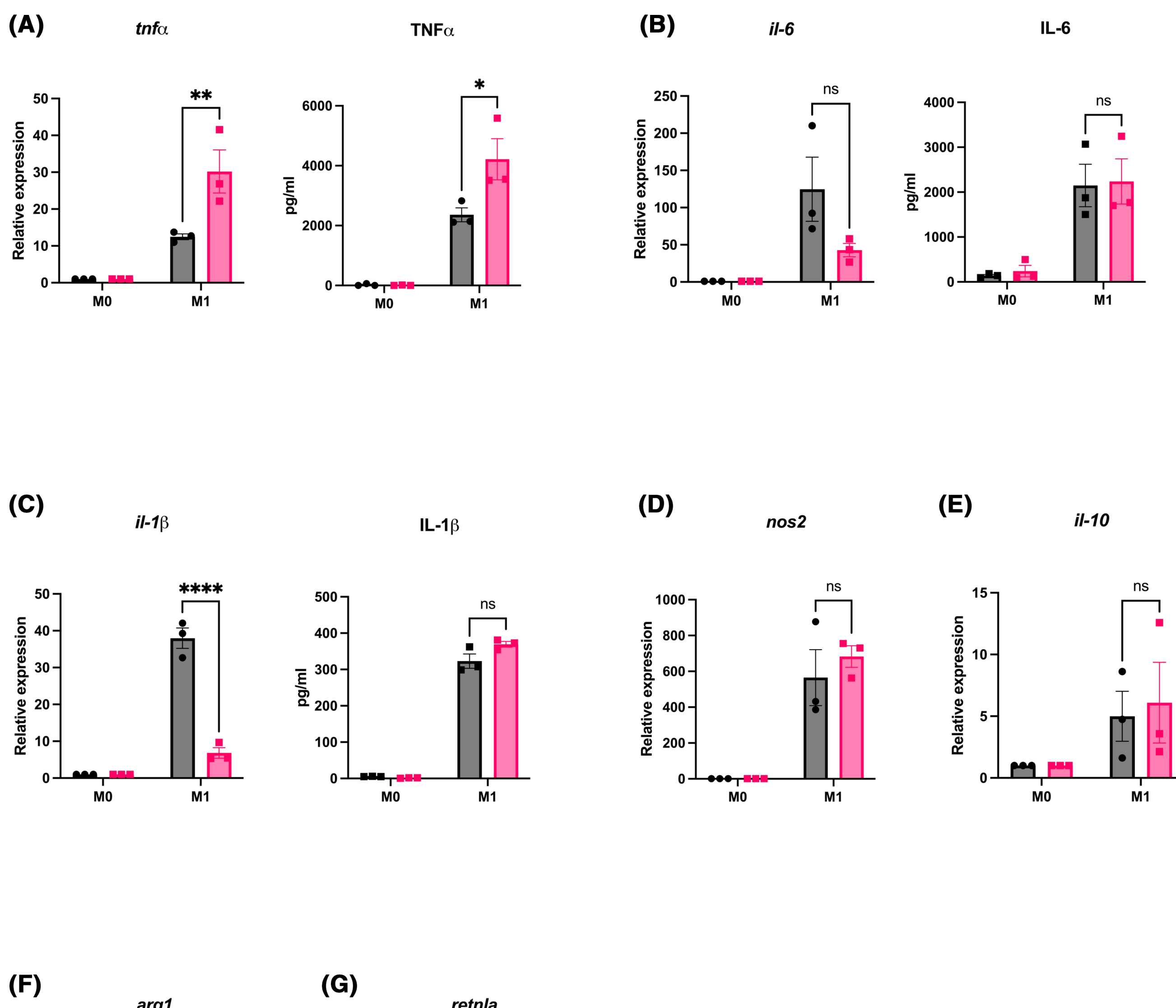

FIGURE 2 Polarization of BMDMs from naive $CATT_7$ mice and WT mice are similar under steady state conditions. HSPCs are isolated from WT and $CATT_7$ mice and differentiated by culturing in M-CSF supplemented media for 7 days. BMDMs from naive WT and $CATT_7$ mice were stimulated with polarizing conditions; LPS or IL-4 for 24 h. Gene expression and protein production of M1 macrophage markers A *tnfα*, B *il-6*, C *il-1β*, D *nos2*, E *il-10* or M2 markers F *arg1* and G *retnla* were analyzed using RT-PCR. Data are presented as mean ± SEM; $N = 3$ mice per group. ns, nonsignificant, *$p < .05$, **$p < .01$, ****$p < .0001$.

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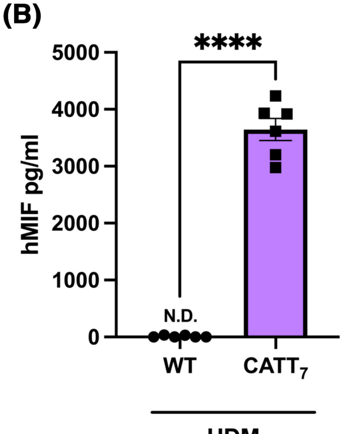

FIGURE 3 HDM challenge *in vivo* significantly increases human MIF production by BMDMs from $CATT_7$ mice. Bone marrow cells were isolated from naive or HDM challenged (25 μg of house dust mite intranasally three times for 3 weeks) WT and $CATT_7$ mice. BMDMs were generated and the concentration of human MIF released into the supernatant from A naive or B $CATT_7$ BMDMs was measured by ELISA. Data are presented as mean ± SEM; $N = 4–6$ per group. ND, not detected, $^{**}p < .01$; $^{****}p < .0001$.

by a methyltransferase enzyme.[30,31] Thus, we used a methyltransferase inhibitor known as methylthioadenosine (MTA) in naive $CATT_7$ whole bone marrow cells containing HSPCs, prior to the introduction of a stimulus, to investigate a role for epigenetic reprogramming following HDM training *in vitro* (Figure 5A). After the application of MTA for 30 min, whole bone marrow cells were stimulated with HDM *in vitro* for 24 h, before being washed in PBS and cultured in M-CSF supplemented media to allow for macrophage differentiation. Importantly, differentiated macrophages were then cultured in a stimuli-free environment of cRPMI media alone to facilitate the rest period. On day 10, differentiated macrophages were stimulated with a secondary heterologous stimulus, LPS, for 24 h before TNFα and IL-6 protein content were measured by ELISA (Figure 5A).

$CATT_7$ BMDMs that received the primary stimulus HDM alone produced low levels of TNFα and IL-6. Stimulation with LPS alone increased TNFα and IL-6 production. Interestingly, $CATT_7$ cells that received a primary stimulus of HDM on Day 0 *in vitro* followed by a secondary heterologous stimulus of LPS on Day 10 produced the highest levels of TNFα and IL-6 (Figure 5B,C), indicating an innate immune training signature in these novel transgenic mice. Exposing the progenitor cells at time 0 to a methyltransferase inhibitor (MTA) but not the vehicle control DMSO for 30 min prior to addition of the primary stimulus (HDM) significantly reduced TNFα production in response to the secondary heterologous stimulus (LPS) (Figure 5B) due to the blocking of methylation of $CATT_7$ DNA and thus preventing any histone modifications occurring at the epigenetic level. A similar but non-significant result was observed in IL-6 production where bone marrow cells were incubated with MTA on Day 0 (Figure 5C). This reduction in protein markers associated with innate immunity was not seen when bone marrow cells were treated with a DMSO vehicle control on Day 0.

4 | DISCUSSION

The concept of immunological memory was first thought to be exclusive to the adaptive immune system, however evidence over the last decade demonstrates that innate immune cells including macrophages can develop a heterologous memory phenotype.[31] We have previously elucidated the important role of high levels of human MIF expression in the development and maintenance of asthma severity *in vivo*.[18] Along with investigating the dynamic interplay between the two theoretically opposing concepts of Type 1 and Type 2 inflammation within our model of acute HDM-induced asthma in novel humanized MIF $CATT_7$ mice, this study is the first to elucidate the role of MIF in enhanced training of the innate immune system, demonstrating that HDM can prime and train human MIF expressing $CATT_7$ macrophages and their HSPC precursors, resulting in a heightened inflammatory response to a secondary heterologous insult.

Macrophages are known to be predominately M2 in allergic asthma due to increased levels of IL-4 present, where these cells drive airway remodelling and wound healing within the lung.[8] However, evidence also shows that both M1 and M2 macrophage subtypes can coexist in the context of both mouse and human asthma, with an imbalance between the two being a key driver of disease.[9,32] For this study, a representative panel of pro-and anti-inflammatory macrophage markers was generated to depict and decipher a clear profile of

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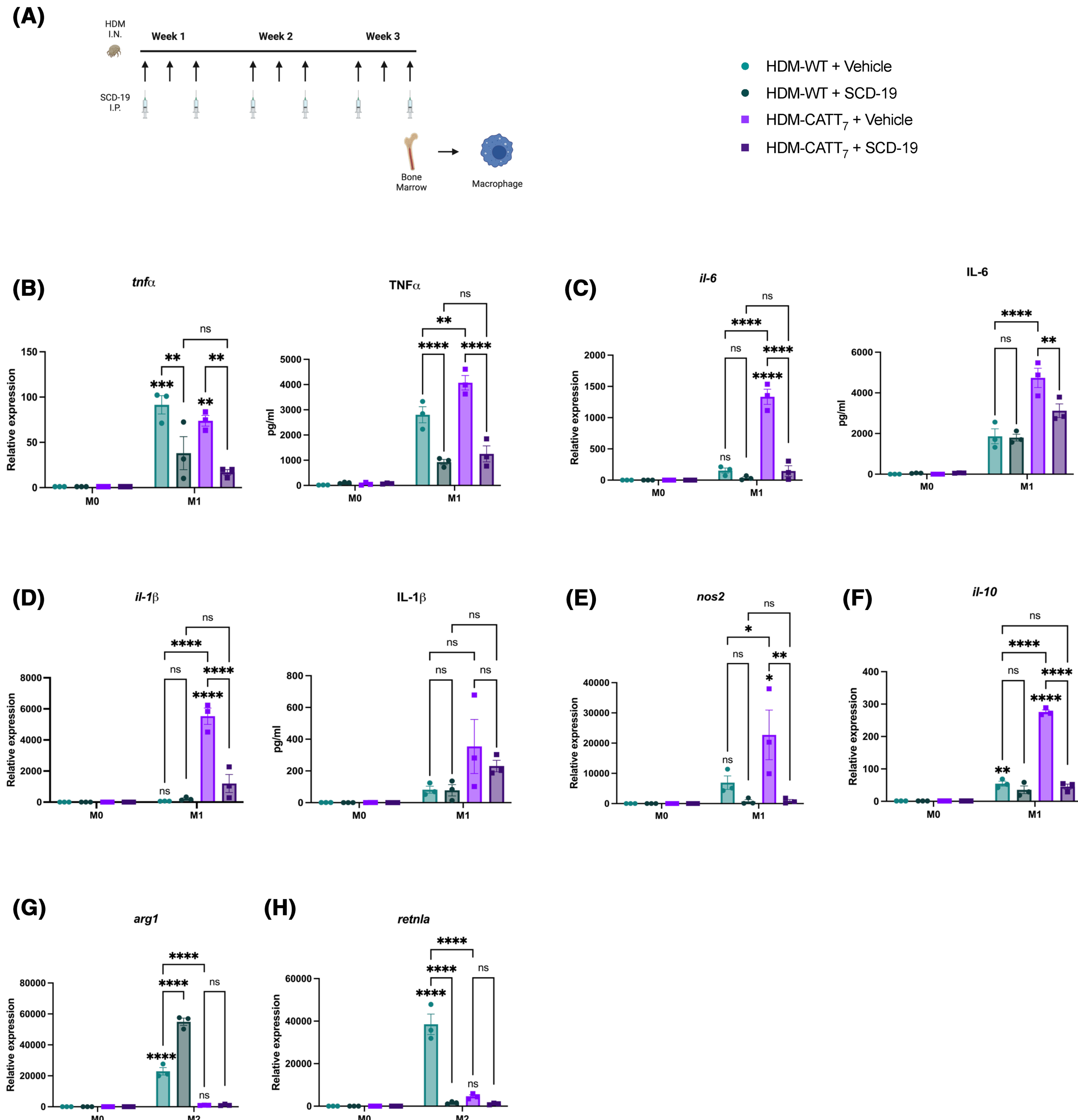

FIGURE 4 Enhanced pro-inflammatory priming in BMDMs from humanized MIF mice following HDM challenge is MIF specific (Blockade via SCD-19). (A) MIF antagonist SCD-19 is administered intraperitoneally twice a week for three weeks in a HDM-induced model of allergic asthma. Bone marrow cells containing HSPCs were isolated from WT and $CATT_7$ on Day 18, 4h after last challenge. BMDMs were generated by culturing cells in M-CSF supplemented cRPMI for 7 days. After being stimulated with polarizing conditions; LPS (M1) or IL-4 (M2) for 24h, (B–F) M1 and (G,H) M2 gene expression and/or protein production was analyzed by RT-PCR and ELISA. Data are presented as mean ± SEM; $N = 3$ mice per group. ns, nonsignificant, *$p < .05$, **$p < .01$, ***$p < .001$, ****$p < .0001$.

macrophage activation, where each marker plays a pivotal role in macrophage function. In allergic asthma, TNFα and IL-1β are induced after LPS-stimulation of monocyte-derived macrophages,[33] with macrophages previously shown to acquire a TNF-dependent memory in the Type 2 inflammatory asthmatic environment.[12] Furthermore, when macrophages are classically activated, IL-6 is known to be upregulated.[12] Type 2 nitric oxide synthase, referred to as *nos2* or inducible NOS, is highly upregulated in classically activated M1 macrophages, documented to play a defensive role in innate immunity.[34,35] Both macrophage phenotypes can act

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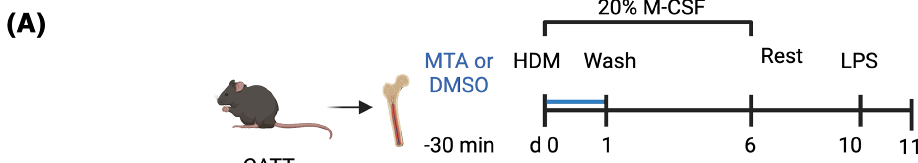

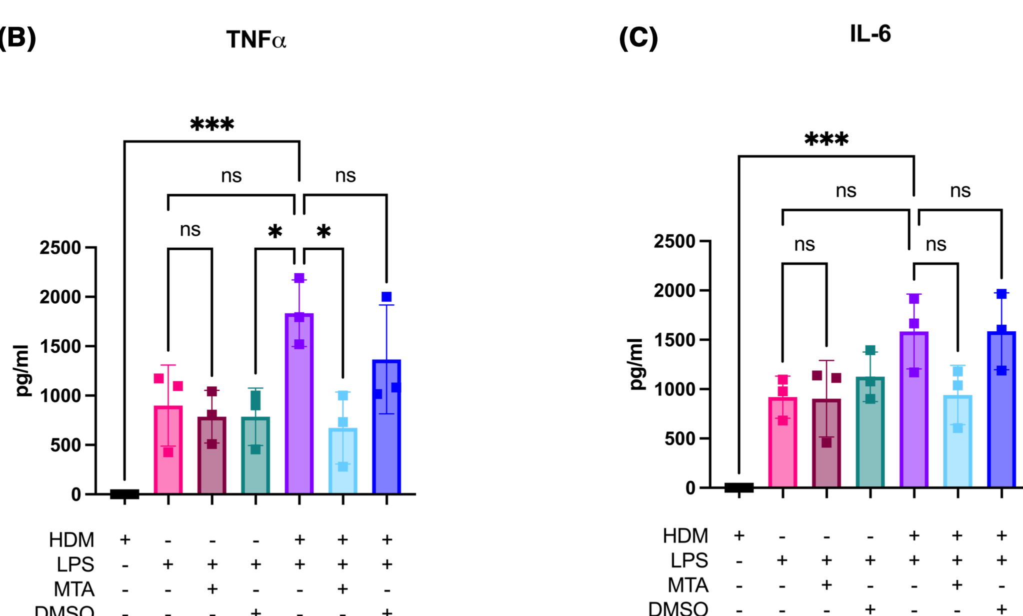

FIGURE 5 Methyltransferase inhibition blocks HDM-induced innate immune training effect in HSPCs from CATT$_7$ mice. (A) Experimental design. After isolation from the bone marrow of CATT$_7$ mice, bone marrow cells containing HSPCs were exposed to MTA or vehicle control 30 min prior to HDM challenge on day 0. HDM was washed out after 24 h. Cells were allowed a rest period from Days 6 to 10, before being stimulated with LPS for 24 h. (B) TNFα and (C) IL-6 protein production in differentiated BMDM supernatants were analyzed by ELISA. Data are presented as mean ± SEM; $N = 3–6$ mice per group. ns, nonsignificant, $^*p < .05$, $^{***}p < .001$.

in a pro-inflammatory or anti-inflammatory manner depending on their surrounding microenvironment, causative allergen (i.e. OVA or HDM), mouse strain (i.e. C57BL/6 or BALB/c), or even disease severity.[32,36–38] However, M2 alternatively activated macrophages tend to sway toward a more anti-inflammatory function, indicated by an increase in *arg1* and *retnla*. Found in inflammatory zones 1 (FIZZ1), also known as RELMα or *retnla* (resistin-like alpha) was first discovered to have an association with pulmonary inflammation in 2000.[39] Moreover, *retnla* does not have a human homolog and thus is a signature marker of M2 alternatively-activated murine macrophages.[40] Investigating these markers after polarizing conditions, naive BMDMs from WT and high human MIF expressing CATT$_7$ mice did not show evidence of a significant activation trend (Figure 2). Notably, at both the mRNA and protein level, high human MIF CATT$_7$ BMDMs had significantly increased levels of TNFα and human MIF following LPS stimulation. IL-4 stimulation did not increase human MIF production. However this was expected, as a profound correlation between LPS-induced macrophage TNFα production and MIF expression is documented, where LPS-induced TNFα production from peripheral blood mononuclear cells (PBMCs) from cystic fibrosis patients was attenuated in the presence of the MIF inhibitor ISO-1.[41] Similarly, in a model of septic shock and also *Salmonella typhiurium* infection, MIF mediated increased TNFα production.[42,43]

Interestingly, BMDMs from high human CATT$_7$ mice challenged with HDM had a significantly enhanced M1 profile, with increased levels of *il-6*, *il-1β*, and *nos2*

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compared to naive $CATT_7$ mice in the vehicle control group (Figure 4) which correlated with significantly increased human MIF production following M1 polarization with LPS. Conversely, WT mice (administered the vehicle control) that do not express human MIF had lower levels of M1 markers, and BMDMs from these mice had significantly elevated levels of the M2 markers, *arg1* and *retnla* following IL-4 (M2) polarization. This was expected as in diseases such as allergic asthma that have a well-documented Type 2 inflammatory profile, M2 macrophages are known to have a central impact after HDM challenge, suspected to be orchestrated by the ormyl peptide receptor 2–TNF–2-HG– PGE2/PGE2 receptor 2 axis.[12] $CATT_7$ BMDMs did not show an increase in relative *arg1* expression after HDM challenge and subsequent M2 polarization with IL-4, maintaining low levels of this enzyme in both the lung (Figure 1E) and bone marrow (Figure 4G). In addition to their striking M1 phenotype and associated elevated human MIF production, $CATT_7$ BMDMs have demonstrated a significant increase in *il-10* expression after HDM challenge and LPS stimulation *in vitro* (Figure 4F). WT BMDMs also exhibited significantly increased *il-10* expression in response to LPS stimulation, although approximately sixfold lower than in $CATT_7$ BMDMs (Figure 4F). This is likely associated with a negative feedback loop whereby elevated TNFα induced IL-10 to promote the resolution of inflammation.[44]

Importantly we demonstrated that this robust pro-inflammatory response after HDM challenge was MIF-dependent by administering the potent MIF inhibitor SCD-19 in the allergic asthma model (Figure 4). Given that our model involves expression of human MIF in a mouse host, this raises the question of interspecies ligand/receptor functionality. Importantly several studies have demonstrated that human MIF can signal in mice through murine CD74.[45–50] Furthermore, all mammalian MIFs (human, mouse, rat, and cattle) have ~90% homology, with this conservation of MIF across species indicating that MIF might have important biological functions and the ability to signal interchangeably through species-specific MIF receptors.[51,52]

In addition to increased inflammatory cytokine production in response to a secondary heterologous stimulus, the other key features of trained immunity are epigenetic and metabolic remodelling.[53,54] In contrast to innate priming, innate immune training results in long-term memory, provoking a faster and greater immune response when the macrophage encounters future, often heterologous stimuli.[1,2,30,31] Thus, epigenetic control of histone modifications leading to long-term opening of chromatin is the basis of trained immunity.[30] Importantly, it has been shown that primed microglia do not exhibit chromatin remodelling in typical histone modifications.[36] Moreover, repressive marks, H3K9me3 and H3K27me3, showed no dynamics during the first 24 h, indicating little role in the early, priming phase of innate immune memory.[55] Together, these studies confirm that epigenetic reprogramming occurs in the bone marrow after "training," but not "priming."

As this *in vitro* assay was carried out utilizing differentiated macrophages that were cultured in M-CSF supplemented media for the duration of the assay, this data can only be classified as innate "priming." Therefore, we set out to investigate if this robust increase in macrophage activation and thus MIF-mediated production of pro-inflammatory cytokines (Figure 4) was evidence of true innate immune training. Central innate immune training occurs in the hematopoietic stem and progenitor cells (HSPCs),[1,2,30,31] pre-cursors of our previously analyzed BMDMs. Bone marrow cells containing HSPCs were isolated from naive mice and stimulated with HDM on Day 0 *in vitro*. A hallmark indicator of innate immunity is epigenetic reprogramming through histone modification, such as DNA methylation.[30,31]

A methyltransferase inhibitor, MTA, was added to the bone marrow cells containing HSPCs 30 min prior to the addition of the primary stimulus (HDM). Importantly, differentiated macrophages derived from these HSPCs were rested prior to receiving a secondary heterologous insult of LPS, allowing their active gene transcription to resume to basal levels. We found that $CATT_7$ HSPC-derived macrophages that received both a primary (HDM) and secondary (LPS) stimulus had increased TNFα and IL-6 production compared to those that only received the first or secondary stimulus alone (Figure 5). This illustrates that *in vitro* HDM exposure on Day 0 of this assay trained HSPCs from $CATT_7$ mice. Importantly, we saw no significant signs of trained immunity in BMDMs from WT mice (Figure S2), demonstrating that this process was human MIF-dependent. Furthermore, innate immunity was confirmed when DNA methylation in these $CATT_7$ HSPCs was blocked upon receiving a methyltransferase inhibitor MTA, as they had significantly reduced TNFα production, and reduced IL-6 production compared to those that received a vehicle control. The use of MTA not only confirmed that innate immune training was occurring, but that the primary stimulus of HDM was reprogramming $CATT_7$ HSPCs at an epigenetic level.

These novel findings demonstrate evidence of enhanced innate immune priming and training occurring in the bone marrow niche of transgenic high human MIF expressing $CATT_7$ mice suggesting that trained immunity may be associated with genetic expression of human MIF. Others have published that a single-nucleotide polymorphism in the promoter region of the gene encoding IL-1β

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(rs16944) can affect the induction of trained immunity by the BCG vaccine[56]; however, we are the first to illustrate a novel role for the $CATT_7$ human MIF polymorphism in driving innate immunity. Utilizing the aeroallergen HDM, an evident driver of allergic airway inflammation as a primary stimulus to induce innate immune training, it would be beneficial to investigate if alveolar macrophages are also reprogrammed in the same manner. However, from an experimental perspective, low cell numbers of alveolar macrophages are obtained from the BALF. Thus, we chose to work with BMDMs due to their ease of isolation, high cell return and also in the interest of reducing the number of animals used. Importantly, we did see the same polarization trends in the lung and bone marrow, with WT mice having an increased M2 phenotype compared to $CATT_7$ mice (Figure 1E). Moreover, MIF has also been proven to drive innate immunity elicited by other pathogens such as whole beta-glucan particles (WGP),[57] demonstrating that the data described in this manuscript is not only important when investigating HDM-specific trained immunity, but trained immunity as a concept in these novel humanized mice expressing the 7-7 functional polymorphism, which is found in the human population and associated with inflammatory disease.[20,26,58–62]

Our data demonstrates that high expression of human MIF can favour, and amplify a pro-inflammatory response driven by macrophages in a disease setting. Thus, we are the first to present evidence of HDM training in humanized $CATT_7$ MIF mice, while also documenting the immunological response to a secondary LPS stimulation *in vitro*. First, this is important as trained immunity characteristically facilitates an immune response to heterogenous stimuli (i.e., HDM and LPS). Second, these data are not only relevant in the context of allergic asthma, but also for bacterial infections such as sepsis, as it indicates that asthmatics that possess the $CATT_7$ allele may have an amplified immune response to microbial infection. This research is also the first to demonstrate that the inhibition of methyltransferase enzymes using methylthioadenosine (MTA) blocks this HDM-training imprint in HSPCs from $CATT_7$ mice, strengthening this evidence of HDM-induced epigenetic reprogramming in HSPCs from the bone marrow of $CATT_7$ mice. Overall, these data unravel a novel story elucidating the impact of human MIF on macrophage polarization and priming in naive and HDM-challenged mice. Furthermore, this research reveals a novel finding regarding the MIF-specific macrophage activation profile in the context of allergic asthma, where this study demonstrated a distinct, amplified M1 pro-inflammatory signature in bone marrow-derived macrophages from humanized $CATT_7$ mice. This is important given that the bone marrow is the major source of monocytes that are recruited to the lung following inflammatory insults to provide a source of alveolar macrophages.[63] This novel information can be applied translationally to humans, perhaps uncovering why specific subsets of people may exhibit an exacerbated immune response to environmental triggers, whilst demonstrating that trained innate immunity is influenced by genetic expression of the high expression MIF allele $CATT_7$.

AUTHOR CONTRIBUTIONS

Hazel Dunbar performed research, data analysis, study design, and wrote the manuscript. Ian J. Hawthorne performed research, data analysis, and study design. Eóin N. McNamee provided reagents and contributed to study design. Seamas C. Donnelly and Michelle E. Armstrong provided reagents, contributed to study design and data analysis. KE designed and supervised the study and wrote the manuscript. All authors approved the final manuscript.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author.

DISCLOSURES

The authors declare no conflict of interest.

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Additional supporting information can be found online in the Supporting Information section at the end of this article.

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