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Mesenchymal stromal cells dampen trained immunity in house dust mite-primed macrophages expressing human macrophage migration inhibitory factor polymorphism

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ABSTRACT

Background: Trained immunity results in long-term immunological memory, provoking a faster and greater immune response when innate immune cells encounter a secondary, often heterologous, stimulus. We have previously shown that house dust mite (HDM)-induced innate training is amplified in mice expressing the human macrophage migration inhibitory factor (MIF) $CATT_7$ functional polymorphism.
Aim: This study investigated the ability of mesenchymal stromal cells (MSCs) to modulate MIF-driven trained immunity both *in vitro* and *in vivo*.
Methods: Compared with wild-type mice, *in vivo* HDM-primed bone marrow-derived macrophages (BMDMs) from $CATT_7$ mice expressed significantly higher levels of M1-associated genes following lipopolysaccharide stimulation *ex vivo*. Co-cultures of $CATT_7$ BMDMs with MSCs suppressed this HDM-primed effect, with tumor necrosis factor alpha (TNF-α) being significantly decreased in a cyclooxygenase 2 (COX-2)-dependent manner. Interestingly, interleukin 6 (IL-6) was suppressed by MSCs independently of COX-2. In an *in vitro* training assay, MSCs significantly abrogated the enhanced production of pro-inflammatory cytokines by HDM-trained $CATT_7$ BMDMs when co-cultured at the time of HDM stimulus on day 0, displaying their therapeutic efficacy in modulating an overzealous human MIF-dependent immune response. Utilizing an *in vivo* model of HDM-induced trained immunity, MSCs administered systemically on day 10 and day 11 suppressed this trained phenomenon by significantly reducing TNF-α and reducing IL-6 and C-C motif chemokine ligand 17 (CCL17) production.
Conclusions: This novel study elucidates how MSCs can attenuate an MIF-driven, HDM-trained response in $CATT_7$ mice in a model of allergic airway inflammation.
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Introduction

The concept of innate immune memory has developed significantly over the past decade. Innate immune cells, including macrophages, can undergo a process called trained immunity, whereby various stimuli, including pathogenic agents such as β-glucan, can alter the cells to produce increased levels of pro-inflammatory cytokines in response to subsequent stimuli. This trained immunity is associated with a rest period between the initial and secondary heterologous stimulus, inducing epigenetic remodeling [1]. Although trained immunity can boost the immune system and provide non-specific protection against pathogens, it can also be maladaptive, contributing to a hyper-inflammatory state, leading to autoimmunity and chronic inflammatory disease [2].

To investigate the pathophysiology of allergic asthma, pre-clinical models involving repetitive intranasal administration of the clinically relevant aeroallergen house dust mite (HDM) are commonly used, as these models reproduce many aspects of human allergic asthma, such as eosinophil infiltration and goblet cell hyperplasia [3–6]. The capacity for HDM to induce trained immunity in mouse and human macrophages was recently identified, in which macrophages from HDM-allergic mice and HDM-allergic asthma patients displayed a significantly elevated pro-inflammatory phenotype with epigenetic changes [3,7–9].

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The pro-inflammatory cytokine macrophage migration inhibitory factor (MIF) is associated with a range of inflammatory diseases, including sepsis, autoimmune disease and severe asthma [10–12]. The MIF $CATT_7$ allele correlates with high MIF expression [4,13]. We have demonstrated exacerbated HDM-induced airway inflammation in mice expressing the human MIF $CATT_7$ allele [4,6]. Recently, using *in vitro* assays and an *in vivo* model of trained immunity, we identified a novel role for the high-expression MIF $CATT_7$ allele in significantly enhancing HDM-induced trained immunity in mouse bone marrow-derived macrophages (BMDMs) [5].

Mesenchymal stromal cells (MSCs) have demonstrated therapeutic efficacy in inflammatory diseases, such as in an HDM-induced model of allergic airway inflammation, in which we demonstrated that MSCs significantly attenuated airway inflammation, cellular infiltration and cytokine production in a cyclooxygenase 2 (COX-2)-dependent manner [6]. MSC therapeutic efficacy requires the presence of macrophages [14,15], in which complex bi-directional crosstalk between these cell populations can determine the inflammatory fate of their resident microenvironment. MSCs can modulate macrophages through active (secretion of TSG-6 and prostaglandin E2) and passive (being phagocytosed, secreting microRNA containing exosomes and mitochondrial transfer) means [16,17]. Moreover, MSCs are primarily known to polarize macrophages to an M2 (anti-inflammatory) phenotype [18–24], which in turn triggers macrophages to secrete mediators that can act to activate MSCs and further boost their immunomodulatory efficacy [16]. Conversely, there is also evidence that MSCs can polarize macrophages to an M1 phenotype [25,26]. However, to date we have not investigated the impact of MSCs on macrophages in high MIF-expressing $CATT_7$ mice challenged with HDM.

This novel research describes the immunosuppressive capacity of human MSCs to block MIF-enhanced M1 priming in macrophages from HDM-challenged mice in a COX-2 partially dependent manner. Importantly, we show for the first time that MSCs can dampen HDM-induced trained immunity *in vitro* and *in vivo*.

Methods

Ethical approval and Health Products Regulatory Authority compliance

Ethical approval for all work was granted by the ethics committee of Maynooth University (BRESC-2018-13). Project Authorization was received from the Health Products Regulatory Authority (AE19124/P022), whereby the terms of the animal experiments within this project were outlined and adhered to.

Pre-clinical model of acute allergic airway inflammation

Human MIF-expressing $CATT_7$ mice and wild-type (WT) littermate controls were challenged with 25 μg of HDM intranasally as previously described [4,6].

Generation of L929-conditioned medium (macrophage colony-stimulating factor)

L929 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium with GlutaMAX (Thermo Fisher Scientific, Paisley, UK) supplemented with 10% heat-inactivated fetal bovine serum (Biosera, Cholet, France) and 10% penicillin/streptomycin (Sigma-Aldrich, Arklow, Wicklow, Ireland) (referred to as cRPMI) and incubated at 37°C in 5% carbon dioxide/20% oxygen for 7 days. Supernatant was collected, centrifuged and passed through a 0.2-μm filter and aliquoted and stored at −80°C. L929-conditioned medium will be referred to as macrophage colony-stimulating factor (M-CSF) throughout the article.

In vitro *innate priming assay (BMDMs)*

After HDM challenge *in vivo*, bone marrow was isolated from $CATT_7$ and WT mice 4 h after last challenge. A total of 1.5×10^6 bone marrow cells were seeded in cRPMI supplemented with 20% M-CSF in tissue culture grade six-well plates. Medium was changed on day 3 and day 6 to remove non-adherent cells. On day 7, differentiated BMDMs were stimulated for 24 h with lipopolysaccharide (LPS) (from *Escherichia coli* O111:B4) (100 ng/mL) (Sigma-Aldrich, Arklow, Wicklow, Ireland) to drive M1 polarization or murine interleukin (IL) 4 (10 ng/mL) (R&D Systems, Abingdon, UK) to drive M2 polarization. Cells and supernatants were harvested and stored at −20°C for RNA and protein analysis.

In vitro *innate training assay (hematopoietic stem and progenitor cells)*

Naive $CATT_7$ or WT bone marrow was isolated from femurs and tibiae and centrifuged at 300 × g for 5 min. Whole bone marrow containing hematopoietic stem and progenitor cells (HSPCs) was seeded at 1.5×10^6 cells per well in six-well non-tissue culture grade plates. After stimulating with 10 μg of HDM for 24 h, HDM was washed out. Cells were cultured in 20% M-CSF-supplemented cRPMI until day 6, when medium 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 harvested for enzyme-linked immunosorbent assay (ELISA).

Human bone marrow-MSC co-culture

Human bone marrow-MSCs (hBM-MSCs) (RoosterBio, Frederick, MD, USA) were expanded as previously described [6]. Afterward, MSCs were cultured and maintained in low-glucose Dulbecco's Modified Eagle's Medium (Sigma-Aldrich, Arklow, Wicklow, Ireland) supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) penicillin/streptomycin. For mechanistic experiments, MSCs (passage two to four) were incubated with 10 μM NS-398 (Sigma-Aldrich, Arklow, Wicklow, Ireland) or vehicle control for 24 h before being washed and seeded in 0.4-μM transwell inserts (Corning, Corning, NY, USA) and co-cultured with HSPCs from day 0 to day 6 for innate training assays or on day 7 for 24 h for priming assays.

In vivo *innate training assay*

$CATT_7$ and WT mice were challenged with 25 μg of the HDM allergen *Dermatophagoides pteronyssinus* (Greer Laboratories, Lenoir, NC, USA) on day 0, day 8, day 9, day 10 and day 11. A total of 5×10^5 hBM-MSCs were administered intravenously on day 10 and day 11. On day 18, bone marrow was isolated and BMDMs generated. Cells were stimulated with LPS (100 ng/mL) for 24 h on day 7.

Cytokine quantification by ELISA

BMDM supernatants were analyzed for murine TNF-α, IL-6 and IL-1β (BioLegend, San Diego, CA, USA) as well as C-C motif chemokine ligand 17 (CCL17) (R&D Systems, Abingdon, UK) by ELISA. The absorbance (optical density) of the samples and standards was measured at 450 nm for all ELISAs using a microplate reader (CLARIOstar Plus; BMG Labtech, Bucks, UK).

Analysis of gene expression

Total RNA was extracted using TRIzol (Thermo Fisher Scientific, Cambridgeshire, UK). RNA concentrations were equalized to 100 ng/μL. Complementary DNA synthesis was performed using a qScript

cDNA Synthesis Kit (Quantabio, Beverly, MA, USA) according to the manufacturer's instructions. Real-time polymerase chain reaction was carried out using PerfeCTa SYBR Green FastMix (Quantabio, Beverly, 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.

Statistical analysis

Mice were randomized to control or treatment groups by a researcher blinded to the experimental protocol and endpoints. Observers assessing endpoints were blinded to group assignment. Data for individual animals and independent experiments are presented as individual symbols. All data are presented as mean ± standard error of the mean. Results of two or more groups were compared by one-way analysis of variance followed by post-hoc Tukey multiple comparison test. Prism (GraphPad Software, San Diego, CA, USA) was used for all statistical analyses.

Results

HDM challenge in CATT$_7$ mice enhances pro-inflammatory macrophage polarization

Previously, we elucidated the inflammatory status of WT and CATT$_7$ mice exposed to HDM or phosphate-buffered saline (PBS) control, measuring cytokine levels in lung homogenates [5]. However, to capture the effects of HDM training on the bone marrow niche, we focused on BMDMs for this study. CATT$_7$ mice challenged with HDM three times a week for 3 weeks exhibited a boosted M1 phenotype, with significantly elevated expression of genes associated with classically activated M1 macrophages (Figure 1). Compared with naive BMDMs, *tnfα* expression was significantly upregulated in both WT and CATT$_7$ BMDMs challenged with HDM *in vivo* and stimulated with LPS *in vitro* (Figure 1B). Interestingly, BMDMs from HDM-CATT$_7$ mice demonstrated significantly elevated *il-6* (Figure 1C), *il-1β* (Figure 1D) and *nos2* (Figure 1E) expression compared with naive CATT$_7$ mice after a secondary

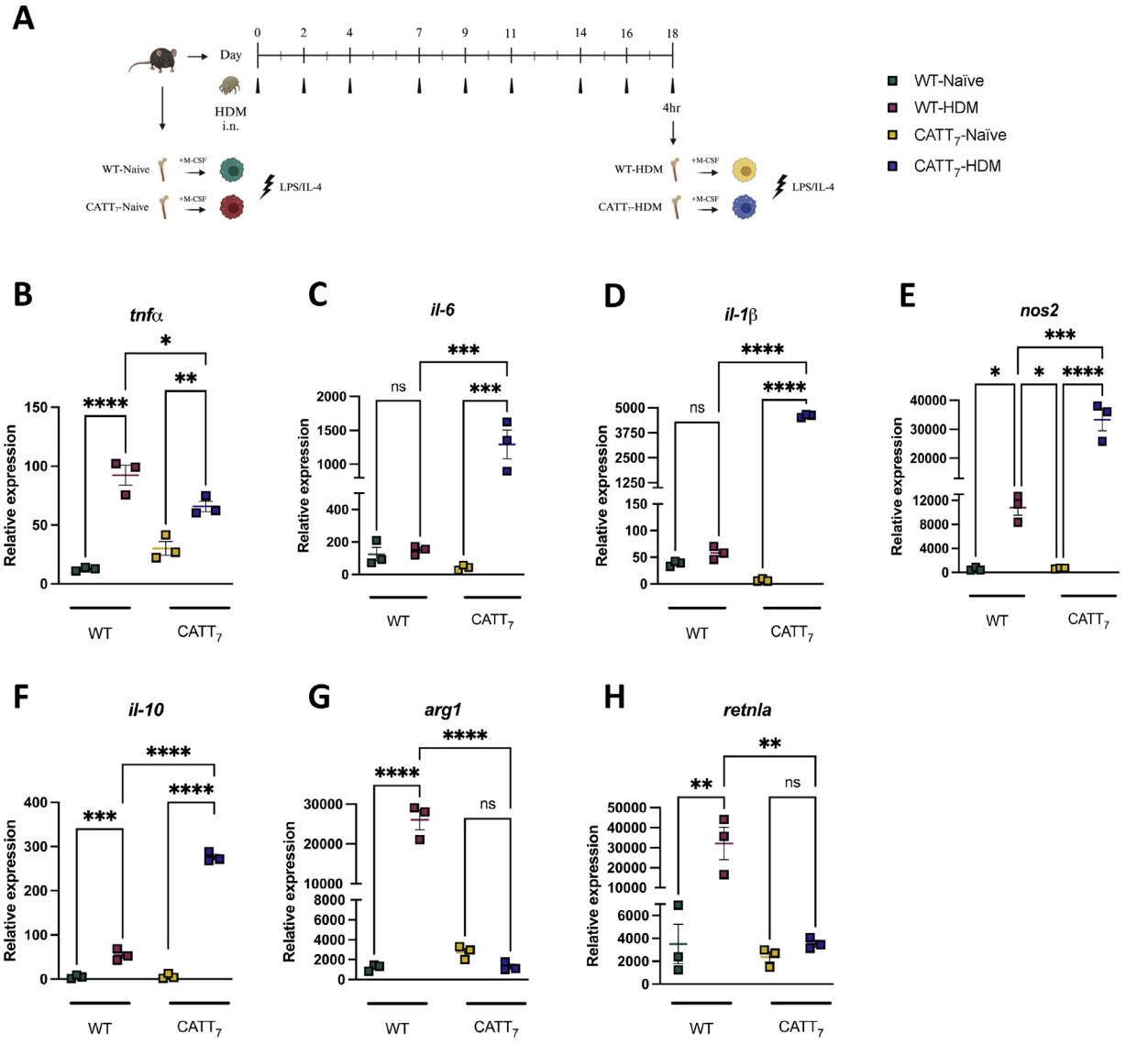

Fig. 1. HDM primes BMDMs from CATT$_7$ mice, increasing their relative M1 gene expression. (A) Model of allergic airway inflammation to investigate HDM-induced trained immunity. Bone marrow was isolated from naive or HDM-challenged WT and CATT$_7$ mice 4 h after last challenge and differentiated with M-CSF over 7 days. (B–F) M1 and (G,H) M2 macrophage marker expression in BMDMs polarized with LPS or IL-4 on day 7 for 24 h was measured by PCR. Each data point represents a single animal. Data are presented as mean ± SEM (n = 3 mice per group). ns, $P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. PCR, polymerase chain reaction; SEM, standard error of the mean; i.n., intranasal. (Color version of figure is available online.)

heterologous LPS stimulation. In HDM-challenged WT BMDMs, *nos2* was significantly increased but approximately 4-fold less than in HDM-CATT$_7$ BMDMs (Figure 1E). This effect for other M1 genes was not observed in BMDMs from HDM-WT mice, as no significant difference in *il-6* (Figure 1C) or *il-1β* (Figure 1D) was observed. Although not strictly associated with the M1 phenotype, *il-10* was significantly increased in HDM-challenged, LPS-stimulated WT BMDMs, albeit approximately 5-fold lower than in CATT$_7$ BMDMs (Figure 1F). By contrast, M2 polarization induced by IL-4 stimulation led to significantly increased levels of *arg1* and *retnla* in HDM-challenged WT BMDMs but not in CATT$_7$ BMDMs (Figure 1G,H).

hBM-MSCs block HDM macrophage priming effect in a COX-2 partially dependent manner

MSCs are known for their ability to calm an overzealous immune response through the secretion of paracrine immunomodulatory factors [17,27–30]. Here we investigated the ability of MSCs to block HDM priming of an M1 phenotype in BMDMs from CATT$_7$ mice (Figure 2A). Using a transwell system, hBM-MSCs co-cultured with differentiated CATT$_7$ BMDMs on day 7 significantly reduced M1 marker gene expression after LPS stimulation (Figure 2B–F). MSCs significantly decreased *tnfα* gene expression in CATT$_7$ and WT mice. By contrast, MSCs significantly decreased TNF-α protein production in CATT$_7$ mice but not in WT mice (Figure 2B). Furthermore, when co-cultured with BMDMs from CATT$_7$ mice, MSCs significantly reduced *il-6* (Figure 2C) and *il-1β* (Figure 2D) gene expression and protein production in CATT$_7$ BMDMs but not in WT BMDMs. Following this trend, MSCs significantly decreased *nos2* expression in CATT$_7$ mice; however, MSCs had no significant effect on the expression of *nos2* in BMDMs from WT mice (Figure 2E). MSCs also significantly reduced *il-10* expression in CATT$_7$ BMDMs but not in WT BMDMs (Figure 2F).

MSC co-culture with CATT$_7$ BMDMs had no significant effect on *arg1* expression after IL-4 stimulation (Figure 2G). MSCs co-cultured with HDM-WT BMDMs significantly decreased *arg1* gene expression to levels comparable to those expressed by HDM-CATT$_7$ BMDMs. Finally, as CATT$_7$ BMDMs did not exhibit increased expression of *retnla* after HDM priming, it was not surprising that no effect was seen with this gene after MSC co-culture (Figure 2H). MSC co-culture with HDM-WT BMDMs had no significant effect on *retnla* expression.

We next sought to elucidate the mechanism by which MSCs suppressed the pro-inflammatory signature in HDM-CATT$_7$ BMDMs. As cyclooxygenase is known to facilitate the immunosuppressive capabilities of MSCs [6], we investigated the role of this enzyme in MSC suppression of CATT$_7$ BMDM pro-inflammatory cytokine production (Figure 2A). COX-2 was found to have a mechanistic role in MSC-mediated suppression of TNF-α, as MSCs pre-treated with the COX-2 inhibitor NS-398 prior to co-culture with CATT$_7$ BMDMs could no longer significantly suppress TNF-α production (Figure 2I). However, other factors may play a role in the suppression of CATT$_7$ pro-inflammatory markers, as COX-2 inhibition had no effect on MSC suppression of IL-6 production by CATT$_7$ BMDMs (Figure 2J).

hBM-MSCs block HDM-induced trained immunity in macrophages from CATT$_7$ mice in vitro *and* in vivo

We used an *in vitro* HDM-induced model of trained immunity to investigate whether MSCs could also block trained immunity in HSPCs within whole bone marrow cells (Figure 3A). Bone marrow cells from CATT$_7$ mice trained with HDM *in vitro* on day 0 followed by a rest period and subsequent stimulation with LPS produced significantly increased levels of TNF-α (Figure 3B) and higher levels of IL-6 (Figure 3C) compared with cells exposed to HDM alone or LPS alone, indicative of a trained immunity phenotype. Importantly, we have previously demonstrated that human MIF expression in CATT$_7$ mice plays a key role in enhancing HDM-induced trained immunity, with evidence of epigenetic remodeling [5]. Strikingly, CATT$_7$ BMDMs derived from HSPCs that were co-cultured with MSCs on day 0 illustrated a significant reduction in TNF-α production (Figure 3B). MSC transwell co-culture also decreased the level of IL-6 protein production by trained CATT$_7$ BMDMs; however, this was not statistically significant (Figure 3C). MSCs had no significant effect on TNF-α and IL-6 production by CATT$_7$ BMDMs that received only LPS stimulation, proving the importance of having both a primary HDM stimulus on day 0 and a rest period followed by a secondary LPS stimulus in this innate immunity *in vitro* assay.

Next, we translated these findings in an *in vivo* model of HDM-induced innate immune training in humanized MIF CATT$_7$ mice. CATT$_7$ mice were HDM-challenged on day 0, day 8, day 9, day 10 and day 11. MSCs were administered intravenously on day 10 and day 11 (Figure 3D). After a rest period of 7 days, bone marrow was harvested on day 18 and BMDMs were differentiated as described. No significant differences were seen in TNF-α production between groups (Figure 3E). However, HDM-CATT$_7$ BMDMs had significantly increased levels of IL-6 production after LPS stimulation compared with PBS-CATT$_7$ BMDMs (Figure 3F). MSC administration significantly decreased levels of IL-6 production after LPS stimulation compared with those that did not receive MSC treatment. CCL17, a known marker of HDM-induced trained immunity [7], increased (although not significantly) in BMDMs from HDM-CATT$_7$ mice after LPS stimulation compared with PBS control mice (Figure 3G). Administration of MSCs decreased CCL17 production in HDM-CATT$_7$ BMDMs compared with those that did not receive MSC treatment.

Discussion

Previously, we demonstrated the immunomodulatory effects of hBM-MSCs in a model of HDM-induced airway inflammation [6]. In this study, we illustrated the ability of MSCs to modulate HDM-induced trained immunity *in vitro* and *in vivo*. hBM-MSCs co-cultured with CATT$_7$ BMDMs using transwells significantly suppressed the M1 pro-inflammatory signature after HDM priming. Furthermore, MSCs significantly reduced M2 marker *arg1* in BMDMs from WT mice. When co-cultured with CATT$_7$ BMDMs, MSCs had no significant effect on this M2 marker. COX-2 inhibition abolished the ability of MSCs to significantly suppress TNF-α production. Interestingly, MSC-COX-2 activity was not involved in MSC suppression of BMDM IL-6 production, suggesting that other unidentified soluble factors may be involved in MSC suppression of other M1-associated pro-inflammatory cytokines produced by BMDMs.

MSCs can communicate with HSPCs within the bone marrow niche not only through extracellular soluble mediators and exosomes [31,32] but also by intracellular means through organelles called migrasomes [33]. Therefore, hBM-MSCs were co-cultured with HSPCs from CATT$_7$ mice using transwell inserts to elucidate whether these immunomodulatory cells could block or suppress HDM-induced trained immunity in macrophages. Strikingly, MSCs were able to significantly reduce TNF-α and reduce IL-6 production by CATT$_7$ BMDMs trained with HDM when co-cultured with HSPCs from day 0. MSCs had no significant effect on HSPC-derived BMDMs that received only the secondary LPS stimulus. It is important to note that MSCs are present in the transwell co-culture with HSPCs trained with HDM from day 0 to day 6; however, the LPS stimulus is added on day 10 in the absence of MSCs. These data clearly illustrate the ability of MSCs to block HDM imprinting on HSPCs on day 0 and leave a lasting immunomodulatory memory, seen in HSPC-derived macrophages on day 11 [34]. Other groups have illustrated the ability of MSCs to block trained immunity in models of ischemic stroke [35] and pulmonary bacterial infection [36]; however, this study is the first to demonstrate the ability of MSCs to block this HDM-induced innate training *in vivo* in mice expressing the human MIF CATT$_7$ polymorphism.

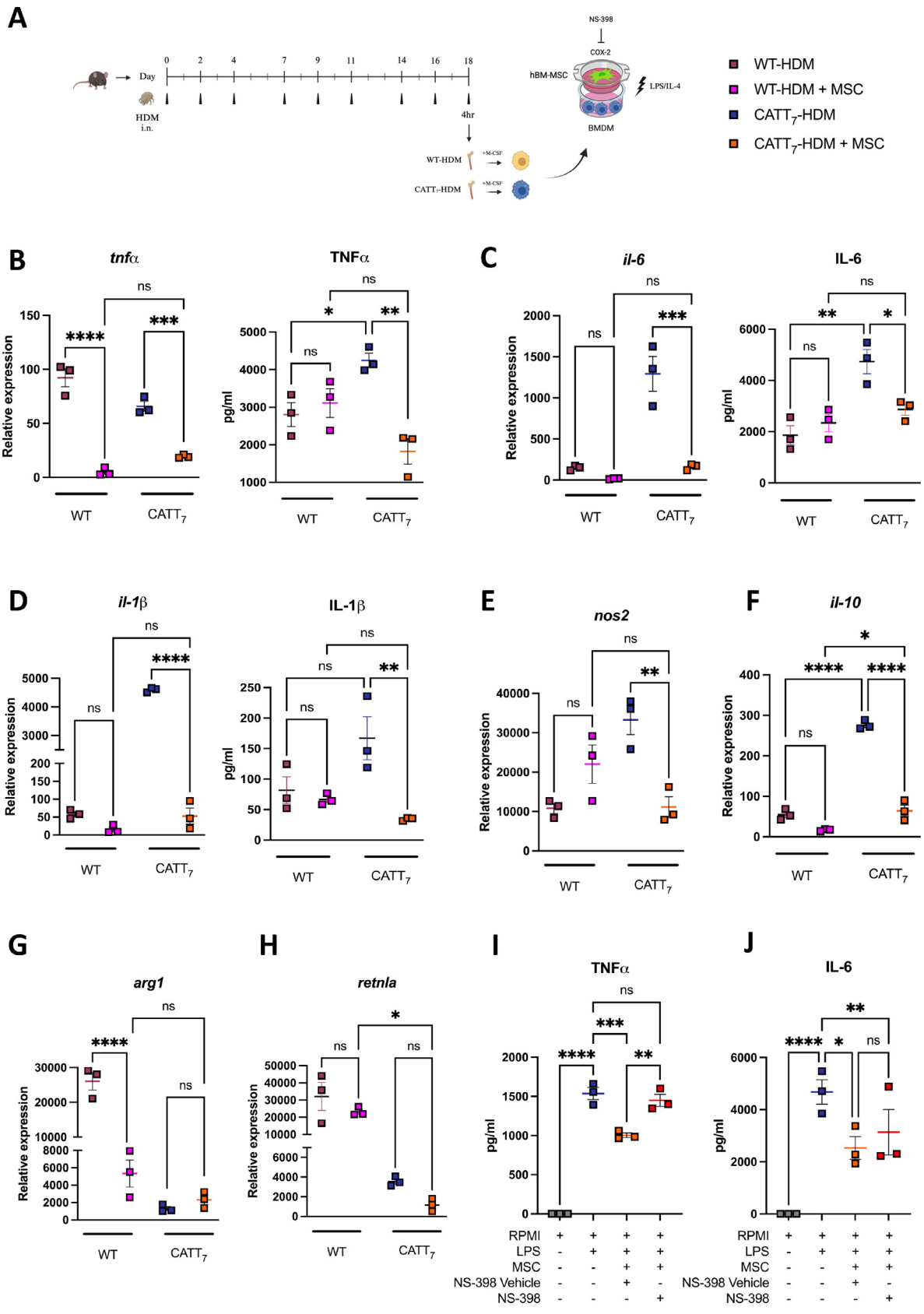

Fig. 2. hBM-MSCs block HDM-induced priming effect in $CATT_7$ mice, decreasing M1 macrophage gene expression. (A) Bone marrow cells containing HSPCs were isolated from HDM-challenged WT and $CATT_7$ mice 4 h after last challenge and differentiated with M-CSF over 7 days. On day 7, hBM-MSCs were co-cultured with BMDMs using 0.4-μM transwells at time of polarization with LPS or IL-4 for 24 h. (B–F) Gene expression and protein production of M1 macrophage markers in BMDMs measured by PCR or ELISA. (G,H) Gene expression of M2 markers *arg1* and *retnla*. (I,J) To investigate the role of COX-2 in the ability of MSCs to suppress trained immunity in $CATT_7$ BMDMs, MSCs were pre-exposed to the COX-2 inhibitor NS-398 or vehicle control for 24 h prior to co-culture with BMDMs in transwells. TNF-α and IL-6 protein production in supernatants from $CATT_7$ BMDMs co-cultured with MSCs ± NS-398 or vehicle control was measured by ELISA. Each data point represents a single animal. Data are presented as mean ± SEM (n = 3 mice per group). ns, $P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. PCR, polymerase chain reaction; SEM, standard error of the mean; i.n., intranasal. (Color version of figure is available online.)

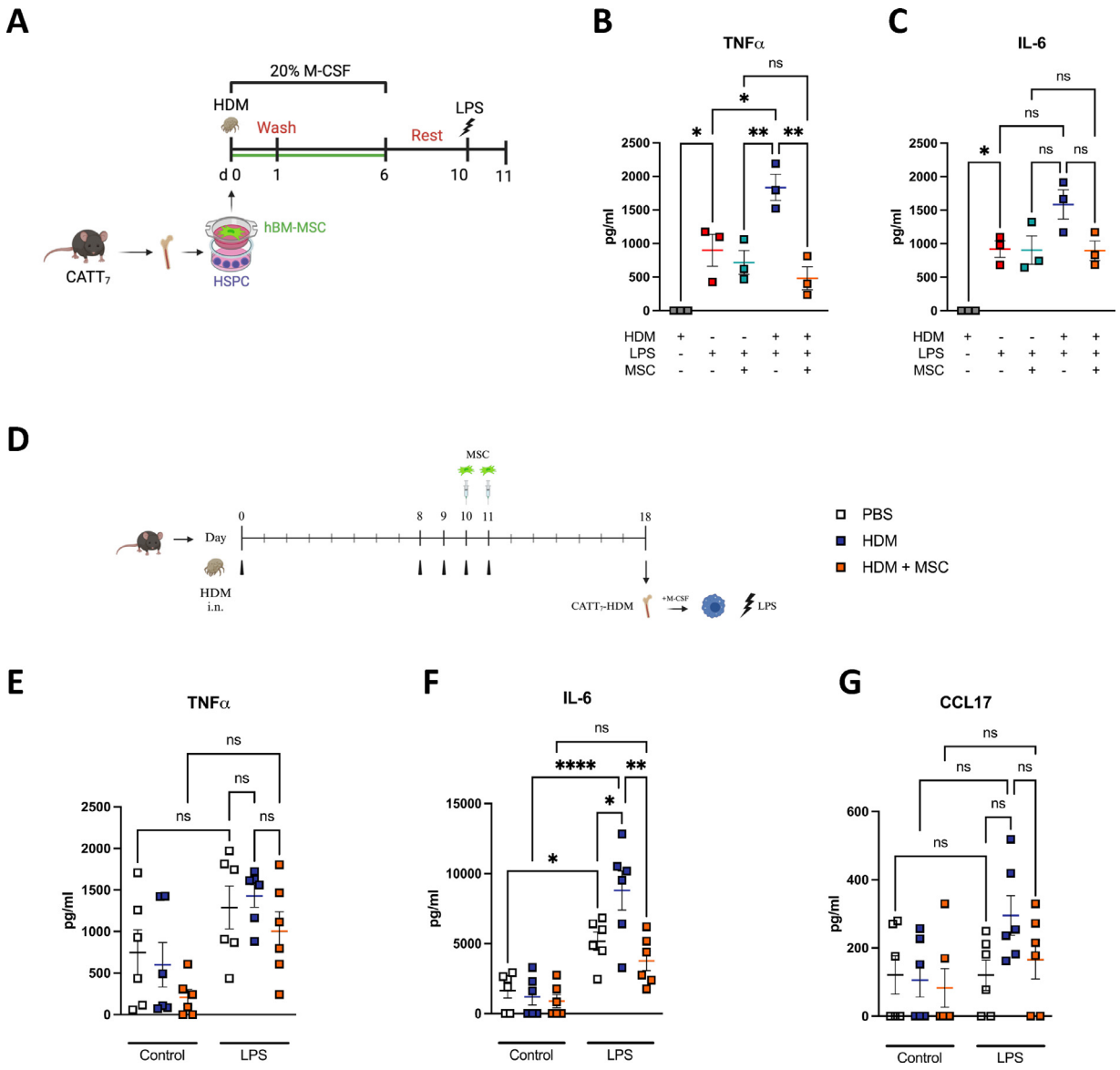

Fig. 3. hBM-MSCs block HDM-induced innate immune training effect in HSPCs from $CATT_7$ mice *in vitro* and *in vivo*. (A) Experimental protocol for *in vitro* trained immunity assay. Bone marrow containing HSPCs was isolated on day 0 and co-cultured with MSCs in transwells during training window with HDM (24 h). HDM stimulus was washed out on day 1. On day 6, MSC transwells were removed from co-culture and differentiated BMDMs entered a rest period. BMDMs received a second stimulus of LPS on day 10 for 24 h, in which cells and supernatants were harvested for gene expression and protein analysis. (B) TNF-α and (C) IL-6 protein production in differentiated BMDM supernatants was analyzed by ELISA. Each data point represents pooled triplicate samples per animal. (D) *In vivo* model of HDM-induced innate training in which mice received HDM challenge (i.n.) on day 0, day 8, day 9, day 10 and day 11. MSCs were administered intravenously on day 10 and day 11. After a rest period of 7 days, bone marrow was isolated and BMDMs were differentiated over 7 days with M-CSF before receiving a second stimulus of LPS on day 7 for 24 h. (E) TNF-α, (F) IL-6 and (G) CCL17 protein production in differentiated BMDM supernatants stimulated with LPS for 24 h was analyzed by ELISA. Each data point represents a single animal. Data are presented as mean ± SEM (n = 3–6 mice per group). ns, $P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$. d, day; SEM, standard error of the mean; i.n., intranasal. (Color version of figure is available online.)

The potential ability of MSCs to inhibit epigenetic modifications in response to HDM training within $CATT_7$ HSPCs has clinical implications. By disrupting the epigenetic reprogramming of immune cells, MSCs can exert immunomodulatory effects and reduce amplified inflammatory responses associated with a trained immune system. These findings strengthen the argument that MSC-based therapies could be beneficial for individuals with conditions characterized by these aberrant immune responses, such as asthma, specifically in the context of our HDM-induced model of allergic airway inflammation, with 50–85% of asthmatics allergic to HDM [3,9,37–39]. These data further demonstrate the universal therapeutic efficacy of MSCs at different time points of disease progression, in which they can not only calm an established immune response [6] but also prophylactically prevent HDM-induced epigenetic rewiring, thus modulating the immune response against future infection or immunological insults.

Declaration of Competing Interest

The authors have no commercial, proprietary or financial interest in the products or companies described in this article.

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Author Contributions

Conception and design of the study: HD, IJH, ENM, DJW, SCD, MEA and KE. Acquisition of data: HD, IJH and CT. Analysis and interpretation of data: HD, IJH, DJW, SCD and MEA. Drafting or revising the manuscript: HD and KE. All authors have approved the final article

Data Availability Statement

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

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