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Mesenchymal stromal cells dampen trained immunity in house dust mite-primed macrophages expressing human MIF polymorphism

Hazel Dunbar¹², Ian J. Hawthorne¹², Courteney Tunstead¹², Eóin N. McNamee¹², Daniel J. Weiss³, Michelle E. Armstrong⁴, Seamas C. Donnelly⁴, Karen English¹²*

1. Department of Biology, Maynooth University, Maynooth, Co. Kildare, Ireland.
2. Kathleen Lonsdale Institute for Human Health Research, Maynooth University, Maynooth, Co. Kildare, Ireland
3. Department of Medicine, 226 Health Sciences Research Facility, Larner College of Medicine, University of Vermont, Burlington, VT 05405, USA
4. Department of Medicine, Trinity College Dublin and Tallaght University Hospital, Co. Dublin, Ireland

Running head: MSCs block HDM-induced pro-inflammatory macrophages

Abstract

Trained immunity (TI) results in long-term immunological memory, provoking a faster and greater immune response when innate immune cells encounter a secondary, often heterologous stimuli. We have previously shown that HDM-induced innate training was amplified in mice expressing the human macrophage migration inhibitory factor (MIF)
CATT7 functional polymorphism. This study investigated the ability of mesenchymal stromal cells (MSCs) to modulate MIF-driven trained immunity both in vitro and in vivo.

Compared to wildtype mice, in vivo house dust mite-primed bone marrow-derived macrophages (BMDMs) from CATT7 mice expressed significantly higher levels of M1 associated genes following LPS stimulation ex vivo. Transwell co-cultures of CATT7 BMDMs with MSCs suppressed this HDM-primed effect, with TNFα being significantly decreased in a COX-2 dependent manner. Interestingly, 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 CATT7 BMDMs when co-cultured at time of HDM stimulus on day 0, displaying their therapeutic efficacy in modulating an over-zealous, human MIF-dependent immune response. Utilising an in vivo model of HDM-induced trained immunity, MSCs administered systemically on day 10 and 11 suppressed this trained phenomenon, by significantly reducing TNFα, and reducing IL-6 and CCL17 production.

This novel study elucidates how MSCs can attenuate a MIF-driven, HDM-trained response in CATT7 mice, in a model of allergic airway inflammation.

Introduction

The concept of innate immune memory has significantly developed over the past decade. Innate immune cells including macrophages can undergo a process termed trained immunity (TI) 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 TI is associated with a rest period between the initial and secondary heterologous stimulus, inducing epigenetic remodelling (1). Although TI can boost the immune system and provide non-specific protection against pathogens, it can also be maladaptive, contributing to a hyperinflammatory state leading to autoimmunity and chronic inflammatory disease (2).

To investigate the pathophysiology of allergic asthma, preclinical models involving repetitive intranasal administration of the clinically relevant aeroallergen house dust mite (HDM) are
commonly used, as this model reproduces many aspects of human allergic asthma such as eosinophil infiltration and goblet cell hyperplasia (3-6). Recently, the capacity for HDM to induce TI in mouse and human macrophages has been identified, where macrophages from HDM-allergic mice and HDM-allergic asthma patients displayed a significantly elevated pro-inflammatory phenotype with epigenetic changes (3, 7-9).

The pro-inflammatory cytokine macrophage migration inhibitory factor (MIF) is associated with a range of inflammatory diseases including sepsis, autoimmune diseases and severe asthma (10-12). The MIF CATT7 allele correlates with high MIF expression (4, 13). We have demonstrated exacerbated HDM-induced airway inflammation in mice expressing the human MIF CATT7 allele (4, 6). Recently, using in vitro assays and an in vivo model of trained immunity, we have identified a novel role for the high expression MIF CATT7 allele in significantly enhancing HDM-induced TI in mouse BMDMs (5).

Mesenchymal stromal cells (MSCs) have demonstrated therapeutic efficacy in inflammatory diseases, such as in a HDM-induced model of allergic airway inflammation, where we have demonstrated that MSCs significantly attenuated airway inflammation, cellular infiltration and cytokine production in a COX-2 dependent manner (6). MSC therapeutic efficacy requires the presence of macrophages (14, 15), where a bi-directional, complex 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 PGE2) and passive (being phagocytosed, secreting miRNA containing exosomes and mitochondrial transfer) means (16, 17). Moreover, MSCs are primarily known to polarise macrophages to an M2, anti-inflammatory phenotype (18-24), which in turn, triggers macrophages to secrete mediators which can act to license MSCs and further boost their immunomodulatory efficacy (16). Conversely, there is also evidence that MSCs can polarise macrophages to an M1 phenotype (25, 26). However, to date we have not investigated the impact of MSCs on macrophages in the high MIF expressing CATT7 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 TI in vitro and in vivo.
Materials and Methods

Ethical approval and HPRA compliance

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

Preclinical model of acute allergic airway inflammation

Human MIF expressing CATT<sub>7</sub> mice and WT littermate controls were challenged with 25μg of HDM intranasally, as previously described (4, 6).

Generation of L929 conditioned media (M-CSF)

L929 cells were seeded in RPMI 1640 medium GlutaMAX supplemented with 10% heat inactivated fetal bovine serum (FBS) and 10% pen/strep (termed:cRPMI) (Gibco, Paisley, UK) and incubated at 37°C/5% CO<sub>2</sub>/20% O<sub>2</sub> for 7 days. Supernatant was collected, centrifuged and passed through a 0.2μm filter and aliquoted and stored at -80°C. L929 conditioned media will be referred to as macrophage-colony stimulating factor (M-CSF) throughout the text.

In vitro innate priming assay (BMDMs)

After HDM challenge in vivo, bone marrow was isolated from CATT<sub>7</sub> and WT mice 4 hours after last challenge. 1.5x10<sup>6</sup> bone marrow cells were seeded in cRPMI supplemented with 20% M-CSF into tissue culture grade 6 well plates. Media was changed on days 3 and 6 to remove non-adherent cells. On day 7, differentiated BMDMs were stimulated for 24hrs with LPS (from E. coli O111:B4) (100ng/ml) (Sigma-Aldrich, Wicklow, Ireland) to drive M1 polarisation, or murine IL-4 (10ng/ml) (R&D Systems, Abingdon, UK) to drive M2 polarisation. Cells and supernatants were harvested and stored at -20°C for RNA and protein analysis.

In vitro innate training assay (HSPCs)
Naive CATT7 or WT bone marrow was isolated from femurs and tibiae and centrifuged at 300g for 5 minutes. Whole bone marrow containing hematopoietic stem and progenitor cells (HSPCs) were seeded at 1.5x10^6 per well in 6 well non-tissue culture grade plates. After stimulating with 10μg of HDM for 24hr, HDM was washed out. Cells were cultured in 20% M-CSF supplemented cRPMI until day 6, when media was changed to non-supplemented cRPMI to facilitate a rest period. Differentiated macrophages were stimulated with 100ng/ml of LPS on day 10 for 24hrs. Supernatants were harvested for ELISA.

**Human bone marrow-mesenchymal stromal cell (hBM-MSC) co-culture**

hBM-MSCs (RoosterBio Frederick, MD 21703, United States) were expanded as previously described (6). Following this, MSCs were cultured and maintained in DMEM low glucose (Sigma-Aldrich, Arklow, Wicklow, Ireland) supplemented with 10% (v/v) FBS (BioSera, Cholet, France) and 1% (v/v) penicillin/streptomycin (Sigma-Aldrich). For mechanistic experiments MSCs (passage 2-4) were incubated with 10μM NS-398 (Sigma-Aldrich, Wicklow, Ireland) or vehicle control for 24hrs before being washed and seeded into 0.4μM transwell inserts (Greiner Bio One, UK) and co-cultured with HSPCs from day 0-6 for innate training assays, or on day 7 for priming assays for 24hrs.

**In vivo innate training assay**

CATT7 or WT mice were challenged with 25μg of house dust mite allergen, *Dermatophagoides pteronyssinus* (Greer Labs, Lenoir, NC, USA) on day 0, 8, 9, 10, 11. 5x10^5 hBM-MSCs were administered intravenously (i.v.) on day 10 and 11. On day 18, bone marrow was isolated and BMDMs generated. Cells were stimulated with LPS (100ng/ml) for 24hr on day 7.

**Cytokine quantification by ELISA**

BMDM supernatants were analysed for murine TNFα, IL-6, and IL-1β, (Biolegend, San Diego, CA, USA) and CCL17 (R&D Systems, Abingdon, UK) by ELISA. The absorbance (optical density (O.D)) of the samples and standards were 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 (Ambion Life Sciences, Cambridgeshire, UK). RNA concentrations were equalised to 100ng/μl. cDNA synthesis was performed using manufacturer’s instructions (Quantabio cDNA synthesis kit). Real Time-Polymerase Chain Reaction (RT-PCR) was carried out 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.

**Statistical Analysis**

Mice were randomised to control or treatment groups by a researcher blinded to the experimental protocol and end-points. 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 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.

**Data Availability Statement**

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

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**Author’s Contributions**

HD performed research, data analysis, study design and wrote the manuscript. IJH performed research, data analysis and study design. CT performed research. ENMcN provided reagents and contributed to study design. DJW contributed to study design and interpretation of the data. SCD & MEA 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.

**Declaration of Interest**

The authors declare no conflict of interest.

**Keywords:** Mesenchymal Stromal Cells, Macrophage Migration Inhibitory Factor, Bone Marrow-Derived Macrophages, House Dust Mite, Innate Priming, Polarisation, Innate Immunity, Innate Training, Cyclooxygenase

**Results**

**HDM-challenge in CATT<sub>7</sub> mice enhances pro-inflammatory macrophage polarisation**

Previously, we elucidated the inflammatory status of WT and CATT<sub>7</sub> mice exposed to HDM or 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<sub>7</sub> mice challenged with HDM three times a week for three weeks exhibited a boosted M1 phenotype, with significantly elevated expression of genes associated with classically activated M1 macrophages (Figure 1). Compared to naive BMDMs, m<sub>fα</sub> expression was significantly upregulated in both WT and CATT<sub>7</sub> BMDMs challenged with HDM in vivo and stimulated with LPS in vitro (Figure 1B). Interestingly, BMDMs from HDM-CATT<sub>7</sub> mice demonstrated significantly elevated il-6 (Figure 1C), il-1β (Figure 1D) and nos2 (Figure 1E) expression compared to naive CATT<sub>7</sub> mice, after a secondary heterologous LPS stimulation. In HDM-challenged WT BMDMs, nos2 was significantly increased but ~4 fold less than that of HDM-CATT<sub>7</sub> 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 ~5 fold lower than in CATT<sub>7</sub> BMDMs (Figure 1F). In contrast, M2 polarisation induced by IL-4 stimulation led to significantly increased levels of arg1 and retnla in HDM-challenged WT BMDMs, but not in CATT<sub>7</sub> 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 over-zealous immune response through the secretion of paracrine immunomodulatory factors (17, 27-30). Here we investigated the capacity for MSCs to block HDM-priming of a M1 phenotype in BMDMs from CATT7 mice (Figure 2A). Using a transwell system, hBM-MSCs co-cultured with differentiated-CATT7 BMDMs on day 7 significantly reduced M1 marker gene expression after LPS stimulation (Figure 2B-F). MSCs significantly decreased \( \text{mif} \) gene expression in CATT7 and WT mice. However MSCs significantly decreased TNF\( \alpha \) protein production in CATT7 mice, but not WT mice (Figure 2B). Furthermore, when co-cultured with BMDMs from CATT7 mice, MSCs significantly reduced \( \text{il-6} \) (Figure 2C) and \( \text{il-1\beta} \) (Figure 2D) gene expression and protein production in CATT7 BMDMs, but not in WT BMDMs. Following this trend, MSCs significantly decreased \( \text{nos2} \) expression in CATT7 mice, however MSCs had no significant effect on the expression of \( \text{nos2} \) in BMDMs from WT mice (Figure 2E). MSCs also significantly reduced \( \text{il-10} \) expression in CATT7, but not WT BMDMs (Figure 2F).

MSC co-culture with CATT7 BMDMs had no significant effect on \( \text{arg1} \) expression after IL-4 stimulation (Figure 2G). MSCs co-cultured with HDM-WT BMDMs significantly decreased \( \text{arg1} \) gene expression to levels comparable to those expressed by HDM-CATT7 BMDMs (Figure 2G). Lastly, as CATT7 BMDMs did not exhibit increased expression of \( \text{retnla} \) after HDM priming, it is 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 \( \text{retnla} \) expression (Figure 2H).

We next sought to elucidate the mechanism by which MSCs suppressed the pro-inflammatory signature in HDM-CATT7 BMDMs. As cyclo-oxygenase (COX) is known to facilitate MSC’s immunosuppressive capabilities (6), we investigated the role of these enzymes in MSC suppression of CATT7 BMDM pro-inflammatory cytokine production (Figure 2A). COX-2 was found to have a mechanistic role in MSC-mediated suppression of TNF\( \alpha \), as MSCs pre-treated with the COX-2 inhibitor NS-398 prior to co-culture with CATT7 BMDMs could no longer significantly suppress TNF\( \alpha \) production (Figure 2I). However other factors may play a role in the suppression of CATT7 pro-inflammatory markers, as COX-2 inhibition had no effect on MSC suppression of IL-6 production by CATT7 BMDMs (Figure 2J).
hBM-MSCs block HDM-induced trained immunity in macrophage from CATT7 mice in \textit{vitro} and \textit{in vivo}

We used an \textit{in vitro} HDM-induced model of TI to investigate if MSCs could also block TI in HSPCs within whole bone marrow cells (Figure 3A). Bone marrow cells from CATT7 mice trained with HDM \textit{in vitro} on day 0 followed by a rest period and subsequent stimulation with LPS produced significantly increased levels of TNF\(\alpha\) (Figure 3B) and higher levels of IL-6 (Figure 3C) compared to cells exposed to HDM alone or LPS alone, indicative of a TI-phenotype. Importantly, we have previously demonstrated that human MIF expression in the CATT7 mice plays a key role in enhancing HDM-induced TI with evidence of epigenetic remodelling (5). Strikingly, CATT7 BMDMs derived from HSPCs that were co-cultured with MSCs on day 0, illustrated a significant reduction in TNF\(\alpha\) production (Figure 3B). MSC transwell co-culture also decreased the level of IL-6 protein production by trained CATT7 BMDMs, however this was not statistically significant (Figure 3C). MSCs had no significant effects on TNF\(\alpha\) and IL-6 production by CATT7 BMDMs that only received 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 \textit{in vitro} assay.

Next we translated these findings in an \textit{in vivo} model of HDM-induced innate immune training in humanised MIF CATT7 mice. CATT7 mice were HDM-challenged on day 0, 8, 9, 10 and 11. MSCs were administered i.v. day 10 and 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\(\alpha\) production between groups (Figure 3E). However BMDMs from HDM-CATT7 had significantly increased levels of IL-6 production after LPS stimulation, compared to PBS-CATT7 BMDMs (Figure 3F). Moreover, MSC administration significantly decreased levels of IL-6 production after LPS stimulation, compared to those that did not receive MSC (Figure 3F). CCL17, a known marker of HDM-induced TI (7), increased (although not significantly) in BMDMs from HDM-CATT7 mice after LPS stimulation, compared to PBS control mice (Figure 3G). MSCs decreased CCL17 production in HDM-CATT7 BMDMs compared to those that did not received MSC treatment (Figure 3G).

\textbf{Discussion}
Previously, we have demonstrated the immunomodulatory effects of hBM-MSCs in a model of HDM-induced airway inflammation (6). In this study, we illustrate the ability of MSCs to modulate HDM-induced trained immunity in vitro and in vivo. hBM-MSCs co-cultured with CATT7 BMDMs using transwells significantly suppressed the M1 pro-inflammatory signature after HDM priming. Furthermore, MSCs significantly reduced M2 marker \textit{arg1} in BMDMs from WT mice. When co-cultured with CATT7 BMDMs, MSCs had no significant effect on these M2 markers. COX-2 inhibition abolished MSCs’ ability to significantly suppress TNF\(\alpha\) 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 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 CATT7 mice using transwell inserts to elucidate if these immunomodulatory cells could block or suppress HDM-induced TI in macrophages. Strikingly, MSCs were able to significantly reduce TNF\(\alpha\) and reduce IL-6 production by CATT7 BMDMs trained with HDM, when co-cultured with HSPCs from day 0. MSCs had no significant effect in HSPC-derived BMDMs that only received 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 MSCs ability to block TI in models of ischemic stroke (35) and pulmonary bacterial infection (36), however this manuscript is the first to demonstrate the ability of MSCs to block this HDM-induced innate training in vivo in mice expressing the human MIF 7-7 polymorphism.

The potential ability of MSCs to inhibit epigenetic modifications in response to HDM training within CATT7 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 characterised 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, where they can not only calm an established immune response (6), but also prophylactically prevent HDM-induced epigenetic re-wiring, thus modulating the immune response against future infection or immunological insults.

Figure Legends
Figure 1. HDM primes BMDMs from CATT7 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 CATT7 mice 4hr after last challenge, and were differentiated with M-CSF over 7 days. B-F M1 and G-H M2 macrophage marker expression in BMDMs polarised with LPS or IL-4 on day 7 for 24hr was measured by PCR. Data are presented as mean ± SEM; N=3 mice per group, with each data point representing a single animal. ns = non significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.
Figure 2. hBM-MSCs block HDM-induced priming effect in CATT_7 mice, decreasing their M1 macrophage gene expression. A Bone marrow cells containing HSPCs were isolated from HDM-challenged WT and CATT_7 mice 4hr after last challenge, and differentiated with M-CSF over 7 days. On day 7, hBM-MSCs were co-cultured with BMDMs using 0.4μM transwells at time of polarisation with LPS or IL-4, for 24hr. B-F Gene expression and/or 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 MSCs’ ability to suppress trained immunity in CATT7 BMDMs, MSCs were pre-exposed to a COX-2 inhibitor NS-398 or vehicle control for 24hr prior to co-culture with BMDMs in transwells. TNFα and IL-6 protein production in supernatants from CATT7 BMDMs co-cultured with MSCs (+/- NS-398 or vehicle control) were measured by ELISA. Data are presented as mean ± SEM; N=3 mice per group, with each data point representing a single animal. Ns = non significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Figure 3. hBM-MSCs block HDM-induced innate immune training effect in HSPCs from CATT7 mice in vitro and in vivo. A Experimental protocol for in vitro trained immunity assay. Bone marrow containing HSPCs were isolated on day 0 and co-cultured with MSCs in transwells during training window with HDM (24hr). 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 hr, where cells and supernatants were harvested for gene expression and protein analysis. B TNFα and C IL-6 protein production in differentiated-BMDM supernatants were analysed by ELISA. D In vivo model of HDM-induced innate training, where mice received HDM challenge (i.n.) on day 0, 8, 9, 10, 11. MSCs were administered (i.v.) on days 10, 11. After a rest period of 7 days, bone marrow was isolated, BMDMs were differentiated over 7 days with M-CSF before receiving a second stimulus of LPS on day 7 for 24 hr. E TNFα F IL-6 and G CCL17 protein production in differentiated-BMDM supernatants stimulated with LPS for 24 hr were analysed by ELISA. Data are presented as mean ± SEM; N=3-6 mice per group. B, C Each data point represents pooled triplicate samples per animal. E, F, G Each data point represents a single animal. Ns = non significant, *p<0.05, **p<0.01, ****p<0.0001.


