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MIF Licensing Enhances Mesenchymal Stromal Cell Efficacy in Allergic Asthma

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Doctor of Philosophy

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Declaration of Authorship

I certify that the work presented herein is, to the best of my knowledge, original, resulting from research performed by me, except where acknowledged otherwise (several data figures in Chapter 2 were generated in collaboration with Hazel Dunbar). This work has not been submitted in whole, or in part, for a degree at this or any other University.

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Date

Abstract

Human mesenchymal stromal cells (MSCs) rely on specific inflammatory disease microenvironments in order to carry out their anti-inflammatory actions *in vivo*. One of the barriers to the success of MSC therapy is the inability to identify potential responders. Macrophage migration inhibitory factor (MIF) has been identified to play a pivotal role in the pathogenesis of several inflammatory disorders including asthma. The aim of this thesis was to develop an understanding into the interaction between MIF and MSCs in a house dust mite (HDM) model of allergic asthma. Using humanised mice with either high- or low-expressing MIF promoter polymorphisms, we identified a dominant role of MIF allelic variants with the high expressing $CATT_7$ mice exhibiting a more severe asthma phenotype. The $CATT_7$ mice experienced significantly higher levels of eosinophilia, airway remodelling, and airway hyperresponsiveness. *In vitro* studies revealed the ability of high levels of hMIF to improve MSC migration, expansion and immunomodulation, thus, identifying MIF as a potential agent to enhance MSC therapeutic efficacy. High hMIF environments *in vivo* potentiated MSCs therapeutic effects with MSCs able to significantly attenuate airway inflammation and ameliorate airway remodelling in $CATT_7$ mice compared to $CATT_5$ or wildtype. Furthermore, we demonstrated that licensing MSCs with high levels of hMIF prior to administration can further enhance therapeutic efficacy through the upregulation of COX-2 expression. The data presented herein contributes to a broader understanding on how disease microenvironments can affect MSC therapeutic efficacy and identifies MIF as a potential biomarker for MSC success.

Publications Arising from this Thesis

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Publications

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*Awarded automatic free registration with same title and abstract as a result of distinguished poster award at the LSC

Abbreviations

AHE	*Aspergillus* hyphal extract
AHR	Airway hyperresponsiveness
$AlOH_3$	Aluminium hydroxide
Ang	Angiopoietin
ANOVA	Analysis of variance
APC	Allophycocyanin
ARDS	Acute respiratory distress syndrome
ASM	Airway smooth muscle
ATRA	All-trans retinoic acid
BAL	Bronchoalveolar lavage
bFGF	Basic fibroblast growth factor
BM	Bone marrow
BMDM	Bone marrow-derived macrophages
Breg	Regulatory B cell
$CATT_5$	Low-expressing MIF promoter polymorphism
$CATT_7$	High-expressing MIF promoter polymorphism
CD	Cluster of differentiation
cDC	Conventional dendritic cell
cDC2	Conventional dendritic cell type 2
CFSE	Carboxyfluorescein succinimidyl ester
CM	Conditioned media
COPD	Chronic obstructive pulmonary disease
COX-2	Cyclooxygenase-2
$cPLA_2$	Cytosolic phospholipase A2

CTL	Cytotoxic T cell
CXCL	C-X-C motif chemokine
CXCR	C-X-C chemokine receptor
DC	Dendritic cell
DCreg	Tolerogenic dendritic cell
Der f	*Dermatophagoides farinae*
Der p	*Dermatophagoides pteronyssinus*
DMEM	Dulbecco's Modified Eagles Media
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
ECP	Eosinophil cationic protein
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicines Agency
EP4	PGE2 receptor 4
EPA	Eicosapentaeoic acid
EPO	Eosinophil peroxidase
ERK	Extracellular signal-regulated kinase
EV	Extracellular vesicles
FasL	Fas ligand
FBS	Foetal bovine serum
FEV1	Forced expiratory volume in the first second of a forced breath
FOXP3	Forkhead box P3
FTVI	Fucosyltransferase VI
G	Tissue damping
GINA	Global initiative for asthma

GvHD	Graft versus host disease
H	Tissue elastance
H&E	Haematoxylin and eosin
HDM	House dust mite
HGF	Hepatocyte growth factor
HIF	Hypoxia inducible factor
HLA	Human leukocyte antigen
HLF	Human lung fibroblasts
HPRA	Health products regulatory agency
HRP	Horseradish peroxidase
i.n.	Intranasal
i.p.	Intraperitoneal
i.t.	Intratracheal
i.v.	Intravenous
IC_{50}	Half-maximal inhibitory concentration
ICAM-1	Intracellular adhesion molecule-1
IDO	Indolamine-2,3-dioxygenase
IFN	Interferon
Ig	Immunoglobulin
IL-	Interleukin-
IL-1RA	IL-1 receptor antagonist
IL-4Rα	IL-4 receptor α
IL-5R	IL-5 receptor
ILC2	Type 2 innate lymphoid cell
iPSC	Induced pluripotent stem cell
ISCT	International Society for Cellular Therapy

ISO-1	(S,R)-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester
JAK	Janus kinase
kDa	Kilodalton
Ki	Inhibitor constant
KO	Knockout
LPS	Lipopolysaccharide
M-CSF	Macrophage colony stimulating factor
MAPC	Multipotent adult progenitor cell
MAPK	Mitogen-activated protein kinase
MBP	Major basic protein
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MIF	Macrophage migration inhibitory factor
miRNA	MicroRNA
MKP-1	Mitogen-activated protein kinase phosphatase-1
MMP	Matrix metalloproteinase
MSC	Mesenchymal stromal cell
MT1	Membrane type 1
mTOR	Mammalian target of rapamycin
NK cell	Natural killer cell
NKT cell	Natural killer T cell
OVA	Ovalbumin
PAS	Periodic acid-Schiff
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor

PDL	Programmed death ligand
PE	Phycoerythrin
PerCP	Peridinin Chlorophyll Protein Complex
PET	Polyethylene terephthalate
PGE2	Prostaglandin E2
PI3K	Phosphatidylinositol 3-kinase
PMSF	Phenylmethylsulfonyl fluoride
PPAR	Peroxisome proliferator-activated receptor
PVDF	Polyvinylidene difluoride
RA	Rheumatoid arthritis
rh	Recombinant human
RIPA	Radioimmunoprecipitation assay
R_N	Airway resistance
RPMI	Roswell Park Memorial Institute
SCD-19	3-(29-methylphenyl)-isocoumarin
SDF	Stromal cell-derived factor
SDS-PAGE	Sodium dodecyl sulfate-poly-acrylamide gel electrophoresis
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism
ST2	IL-33 receptor
STAT1	Signal transducer and activator of transcription 1
STS	Staurosporine
TBST	Tris-buffered saline containing 0.1% Tween 20
TGF	Transforming growth factor
Th	T helper
Th2-Trm	T helper 2 resident memory T cell

TLR	Toll-like receptor
TNF	Tumour necrosis factor
Treg	Regulatory T cell
TSG-6	Tumour necrosis factor-inducible gene 6
TSLP	Thymic stromal lymphopoietin
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
VLA	Very late antigen
WT	Wild type

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Chapter 1 Introduction

1.1 Asthma

Asthma is a complex, coordinated, multifactorial inflammatory disorder characterised by chronic airway inflammation and structural remodelling. Asthma involves an orchestrated interplay between structural and inflammatory cells through direct contact and secreted molecules. Normal healthy lungs are dominated by structural mesenchymal and epithelial cells communicating under homeostasis. Allergic asthmatic lungs are dominated by inflammatory infiltrate in a landscape controlled by cluster of differentiation (CD)4^+ T helper 2 (Th2) cells (Vieira Braga et al., 2019). The global initiative for asthma (GINA) defines asthma as a heterogeneous disease, usually characterized by chronic airway inflammation. It is defined by the history of respiratory symptoms, such as wheeze, shortness of breath, chest tightness and cough, that vary over time and in intensity, together with variable expiratory airflow limitation (Reddel et al., 2022). Asthma affected an estimated 262 million people worldwide and caused 455 000 deaths in 2019, making it one of the most common chronic diseases (Vos et al., 2020). Asthma prevalence tends to be higher in developed western countries compared to lower income and more rural countries (To et al., 2012).

1.1.1 Aetiology

Risk for the development of asthma comes from a combination of genetic predisposition, immunological factors, age, and environmental factors including exposure to inhaled substances that provoke allergic reactions (Morales and Duffy, 2019). Asthma is a highly complex heterogeneous disease which can be split up into 2 groups, type 2-high asthma and type 2-low asthma (Peters et al., 2019). Type 2-low

asthma includes obesity-related asthma and neutrophilic asthma. Obesity-related asthma is an adult onset endotype which primarily occurs in women (Pakkasela et al., 2020). Elevated levels of tumour necrosis factor (TNF)-α, interleukin (IL)-1 and IL-6 is present in the lung and the chronic inflammation leads to tissue damage (Miethe et al., 2020). Although the link between obesity and asthma is not clear, the inflammation is ameliorated after weight loss or bariatric surgery (Peters et al., 2018). Neutrophilic asthma can be associated with inhalation of cigarette smoke and is characterised as sputum neutrophilia from the activation of a T helper 17 (Th17) immune response (Yamasaki et al., 2022). Neutrophilic asthma patients present with low levels of forced expiratory volume in the first second of a forced breath (FEV1), more air trapping and thicker airway walls.

Out of all asthma patients over 80% of children and most adults suffer from type 2-high asthma. The largest endotype of type 2-high asthma is allergic asthma which typically develops in childhood following sensitisation to aero-allergens and development of Th2 cell responses (Matucci et al., 2021). Such environmental allergens include those from dust mites, cockroaches, pollen, fungi and pet dander (Morales and Duffy, 2019). Th2 asthma can also be introduced later in life when challenged with new allergens. This is often associated with certain workplaces where exposure to chemicals, animals or microorganisms are common (Lummus et al., 2011). Up to 85% of asthma patients are sensitive to house dust mite (HDM) (Nelson et al., 1996). The two most studied species of HDM are *Dermatophagoides pteronyssinus* (Der p) and *Dermatophagoides farinae* (Der f). HDMs and their faecal pellets contributes to its allergenicity through their protease activity. They contain proteolytic enzymes such as cysteine protease which can cleave intracellular epithelial

tight junctions to aid delivery to subepithelial antigen-presenting cells to initiate immune response (Gregory and Lloyd, 2011).

1.1.2 Asthma Pathophysiology

1.1.2.1 Airway Inflammation

One of the main hallmarks of asthma pathophysiology is the presence of airway inflammation following challenge from environmental stimuli. This response is initiated when allergen specific immunoglobulins (Ig) of the IgE class bind to the surface of mast cells and basophils on the subepithelial layer of the airways (Murphy and O'Byrne, 2010). Upon exposure, these cells release mediators such as histamine, leukotrienes and prostaglandins which trigger bronchoconstriction, mucus secretion, and chemokine release (Liu et al., 1991). Epithelial-derived cytokines IL-25, IL-33, and thymic stromal lymphopoietin (TSLP) induce the recruitment of an inflammatory infiltrate comprised of Th2 lymphocytes, eosinophils, and macrophages (Holgate et al., 2015; Lambrecht et al., 2019). Sensitisation requires interaction between antigen presenting dendritic cells and naïve T cells. The initiation of Th2 response is mediated by a subset of dendritic cells (DCs) called conventional dendritic cell type 2s (cDC2s) which migrate to the draining lymph nodes to initiate Th2 cell differentiation following exposure to epithelial-derived cytokines (Deckers et al., 2017; Lamiable et al., 2020; Sakurai et al., 2021; Vroman et al., 2020). The presentation of antigens to T cells via the major histocompatibility complex (MHC) class II results in T cell differentiation into Th2 cells (Holgate et al., 2015). Th2 cells then orchestrate the

inflammatory cell profile through the release of prototypical Th2 cytokines IL-4, IL-5, and IL-13 (Hammad and Lambrecht, 2021; Nelson et al., 2020).

IL-4 is a key cytokine in the development of allergic airway inflammation. It has been shown that IL-4 is the main cytokine which promotes T cell differentiation into Th2 cells. $CD4^+$ T cells from IL-4 deficient mice fail to produce Th2 cytokines (Matucci et al., 2021). This makes IL-4 critical for promoting inflammation through the development of the inflammatory cascade. Additionally, IL-4 promotes airway inflammation through the regulation of IgE biosynthesis and lymphocyte trafficking. IL-4 mediates leukocyte trafficking through the upregulation of vascular cell adhesion molecule-1 (VCAM-1) on the endothelium thus promoting extravasation (Fukuda et al., 1996). These effects are also induced by IL-13 which binds to the IL-4 receptor (IL-4Rα) allowing it to cooperate with IL-4 to drive inflammation (Godar et al., 2018). Moreover, IL-13 induces airway hyperresponsiveness, aid the recruitment of leukocytes to the site of injury and increases mucus secretion through the promotion of goblet cell hyperplasia (Pelaia et al., 2022).

IL-5 plays a central role in the differentiation, recruitment, survival, and activation of eosinophils (Walsh, 2020). Eosinophil progenitors mature in the bone marrow and express IL-5 receptor (IL-5R) before being released into circulation following interaction with IL-5. IL-5 mobilises eosinophils from the bone marrow and drives activation, survival and proliferation in peripheral tissues (Pelaia et al., 2019). The recruitment of eosinophils and their presence within the airways is considered a key determinant in the development of allergic asthma. Following accumulation in the airways eosinophils degranulate releasing cytotoxic proteins [e.g. major basic protein (MBP), eosinophil cationic protein (ECP) and eosinophil peroxidase (EPO)], Th2

cytokines, and acute proinflammatory cytokines (e.g. TNF-α, IL-1β, IL-6, and IL-8). Such mediators contribute to several features of asthma by further exacerbating inflammation and inducing tissue damage (Bozza et al., 2020; Nelson et al., 2020).

In addition to the prototypical Th2 cytokines, IL-9 has been linked with type 2-high asthma with high levels detected in the bronchoalveolar lavage (BAL) fluid following local allergen challenge (Erpenbeck et al., 2003). IL-9 is produced by a range of cells including Th2 cells, eosinophils and neutrophils (Gounni et al., 2000; Seumois et al., 2020; B. Sun et al., 2018). Through the isolation of human peripheral blood mononuclear cells (PBMCs), a recent study identified greater numbers of IL-9 expressing HDM-reactive Th2 cells in asthmatics with HDM allergy compared to non-asthmatics with HDM-allergy (Seumois et al., 2020). Furthermore, IL-9 expressing Th cells have also been shown to be more pathogenic (Erpenbeck et al., 2003). Preclinical models of asthma have provided data on the role of IL-9 in the pathogenesis of asthma. Du et al. demonstrated the importance of IL-9 using IL-9 deficient mice. Following administration of IL-33, pathological changes such as immune cell infiltration, expression of cytokines, goblet cell hyperplasia, and collagen deposition were significantly attenuated in IL-9 deficient mice compared to wildtype (WT) control (Du et al., 2020). Additionally, the administration of IL-9 neutralising antibody reduced allergic inflammation in an aspergillus model of allergic asthma (Chakraborty et al., 2019).

In recent years type 2 innate lymphoid cells (ILC2s) have emerged as one of the main cellular players of asthma pathogenesis. ILCs reside in the mucosa and contribute to homeostasis and immunosurveillance (Halim, 2016). ILC2s are similar to Th2 cells as they also express Th2-associated transcription factor GATA3 and produce large

amounts of Th2 cytokines IL-5, IL-9 and IL-13 (Entwistle et al., 2020; Yu et al., 2014). Epithelial-derived cytokines IL-25, IL-33 and TSLP induce proliferation and activate ILC2s either directly or through the release of TGF-β1 (Denney et al., 2015). Higher numbers of ILC2s are detected in the blood and BAL fluid of asthma patients compared to healthy controls (Winkler et al., 2019). Furthermore, ILC2 numbers correlate to the scale of disease with higher numbers are detected in the sputum of patients with severe eosinophilic asthma compared to those with a mild form of the disease (Chen et al., 2017; Yu et al., 2018). The role of ILC2s has been demonstrated in asthmatic patients where significant increases in ILC2s secreting IL-5 and IL-13 are detected in the sputum within 24 hours post-allergen challenge (Chen et al., 2017). The large amounts of IL-5 and IL-13 produced by activated by ILC2s then further contribute to asthma pathogenesis. Furthermore, ILC2-derived IL-13 has also been shown to be critical in the migration of activated lung DCs in the draining lymph nodes where they primed naïve T cells thus promoting Th2 cell differentiation (Halim et al., 2016, 2014).

Th2 resident memory T (Th2-Trm) cells have also been identified to play a role in the pathogenesis of asthma. These cells reside in the lung long after the cessation of allergen exposure and retain an allergen memory (Rahimi et al., 2020). Th2-Trm cells have been detected in patients with type 2-high asthma and in the lungs of HDM-challenged mice (Hondowicz et al., 2016; Vieira Braga et al., 2019). Re-exposure to a known allergen results in rapid reactivation and the production of Th2 cytokines to promote eosinophil activation, mucus production and airway hyperresponsiveness (Rahimi et al., 2020). A recent study has suggested that Th2-Trm cells are directly affected by the epithelial-derived cytokine IL-33 as they showed that $CD69^{+}$IL-33 receptor $(ST2)^{+}$ Th2-Trm cells develop quickly after allergen exposure, yet

development of long term memory was abrogated in mice deficient in ST2 or IL-33, but not the TSLP receptor (Kobayashi et al., 2023). This shows that the ST2/IL-33 axis may play a role in the development of immune memory.

1.1.2.2 Airway Remodelling

Airway remodelling plays a vital role in the pathogenesis of asthma. Driven by the persistence of inflammation, airway remodelling represents the changes in structural cells and tissues in the lungs of asthmatic patients compared to healthy lungs (Fehrenbach et al., 2017). The main changes of airway remodelling include matrix changes which result in the thickening of the airway walls leading to airway narrowing and disruption of airflow, cellular changes such as epithelial cell damage which leads to barrier dysfunction, and goblet cell hyperplasia enhancing mucin secretion (Al-Muhsen et al., 2011). Post mortem studies show that airway remodelling affects both small and large airways (Elliot et al., 2015; James et al., 2012). Narrowing of the airway is a result of increased deposition of extracellular matrix (ECM) proteins in the reticular basement membrane, lamina propria and submucosa. The ECM is made up of structural proteins such as collagen and elastin, adhesion proteins, glycosaminoglycans and proteoglycans (Hough et al., 2020). Collagen, the most abundant component of the ECM, possesses a high tensile strength but low elasticity thus contributes to lung stiffness in asthma patients where collagen is overproduced. Resident airway smooth muscle (ASM) cells, epithelial cells and fibroblasts are thought to be the key drivers of asthma airway remodelling. Environmental stresses such as those seen during the exposure to environmental allergens drives cell death of epithelial cells and release of soluble factors such as transforming growth factor

(TGF)-β1. TGF-β1 is a profibrotic growth factor which drives airway remodelling by inducing proliferation and expression of contractile protein markers such as α-smooth muscle actin in ASM cells and drives the activation of fibroblasts (Halwani et al., 2011). Persistent activation of fibroblasts by TGF-β1 drives the fibroblast to myofibroblast transition via the TGF-β1/Smad2/3 pathway and differentiation of fibroblasts into myofibroblasts. Myofibroblasts cause the overproduction and deposition of extracellular proteins such as collagen and fibronectin into the subepithelial space which leads to subepithelial fibrosis (Gu et al., 2007; Wnuk et al., 2020). These structural changes to the airways result in airway hyperresponsiveness (AHR) and accelerate lung function decline. Alveolar macrophages and eosinophils also secrete TGF-β1 and thus contribute to airway remodelling in the asthmatic lung (Branchett et al., 2021; Kanda et al., 2020).

1.1.3 Current Treatments

Asthma in most patients is well managed under the use of inhaled corticosteroids and long acting β2-adenoceptor agonists. These drugs act to attenuate inflammation and slow the process of remodelling, however, many patients still experience severe exacerbations and accelerated decline in lung function (Imanirad and Tabatabaian, 2022). Around 4-5% of asthma patients have severe asthma with treatments in hospitals following exacerbations costing a total of €116 million in 2017 in Ireland ("Easing the Economic Burden of Asthma Report | Asthma Society of Ireland," n.d.; E. Wang et al., 2020). Novel biologics have been trialled in recent years to treat symptoms of asthma. These new therapeutics have been developed to specifically

target effector cells or cytokines involved in the pathogenesis of asthma. A range of monoclonal antibodies have been trialled in the last few years targeting IL-5. As previously mentioned, IL-5 is produced by Th2 and ILC2s to drive the activation and survival of eosinophils. Inhibiting IL-5 or the receptor has proven to be an effective strategy. The first biologic targeting IL-5 was the monoclonal antibody mepolizumab. Mepolizumab blocks the binding of IL-5 to the IL-5R therefore inhibiting eosinophilic inflammation. Treatment with mepolizumab has been shown to reduce exacerbations by 53% after 12 months in patients with severe eosinophilic asthma (Ortega et al., 2016). Benralizumab also targets the IL-5 pathway by binding to the α chain of the IL-5 receptor. Trials on severe eosinophilic asthma patients have shown benralizumab to significantly reduce exacerbation rates and improve FEV1 values (Bleecker et al., 2016; FitzGerald et al., 2016).

Dupilumab, an anti-IL4 receptor α monoclonal antibody has also shown therapeutic efficacy. Both IL-4 and IL-13 bind to IL-4Rα making it an attractive target. Dupilumab has been found to be effective in moderate-severe asthma patients by reducing exacerbations by 47%, improve lung function, and reduce asthma symptoms (Corren et al., 2020; Witt et al., 2022). However, some patients experienced temporary rises in blood eosinophils following subcutaneous administration of the therapy (Castro et al., 2018).

In addition to Th2 cytokine inhibitors, Tezepelumab, an anti-TSLP monoclonal antibody has also been trialled. Tezepelumab has shown to reduce exacerbations, improve FEV1 values, control symptoms, and improve quality of life in moderate-severe asthma patients (Corren et al., 2017). Tezepelumab has also shown to reduce airway hyperresponsiveness and airway inflammation compared to placebo (Sverrild

et al., 2021). Additionally, a recent publication reported that Tezepelumab treatment is well tolerated for up to 2 years and maintained efficacy in reduced numbers of exacerbations in patients with severe uncontrolled asthma (Menzies-Gow et al., 2023).

Despite these interventions, asthma-related morbidity and mortality persist. Currently, there are no therapeutic treatments for asthma which reverse airway remodelling, therefore, there is a clear need for novel strategies to attenuate inflammation and repair damaged tissue (Varricchi et al., 2022). Furthermore, not all type 2-high patients respond to these biologics, therefore, identification of novel biomarkers through omic analysis is needed to improve patient responses. Additionally, biologics such as dupilumab require frequent administrations as many as once every 2 weeks (Castro et al., 2018; Corren et al., 2020; Wenzel, 2012). Taken together, these data demonstrate a need for long-acting therapeutics for the treatment of severe type 2-high asthma patients.

1.1.4 Mouse Models of Allergic Asthma

Mouse models are a very useful tool in preclinical research as they can mimic physiological conditions seen in human disease to allow greater understanding of pathophysiology. However, current mouse models fail to encompass all hallmark features of complex diseases such as asthma. Mice do not spontaneously develop asthma, so measures have to be employed to mimic asthma like features and a Th2 immune response (Kips et al., 2003). There are many factors that can influence the Th2 phenotype that results from a preclinical asthma model including animal strain,

allergen type, and method of sensitisation and challenge. The most commonly used mouse strain used in allergic asthma research is BALB/c as they elicit a strong Th2 biased immune response and have the ability to develop airway hyperresponsiveness. Other strains such as C57BL/6 mice are used successfully however they present more of a bias towards Th1 immunity compared to the more Th2 BALB/c strain (Schulte et al., 2008). Ovalbumin (OVA) is derived from chicken eggs and despite being rarely implicated in human asthma is the most common allergen used in mouse models of allergic asthma. OVA requires an initial sensitisation intraperitoneally alongside an adjuvant such as aluminium hydroxide ($AlOH_3$) to elicit a Th2 response when re-exposed to the allergen (Casaro et al., 2019). Following sensitisation mice are challenged with OVA intranasally to induce allergic reaction. OVA can reproduce many features observed in asthma including airway eosinophilia, airway hyperresponsiveness and airway remodelling. However, recent studies have opted for different allergens as the systemic delivery of OVA during the sensitisation phase lacks clinical relevance. More environmentally relevant allergens such as fungi (*Alternaria alternata, Aspergillus fumigatus*), cockroach extracts, helminth parasites (*Ascaris, Schistoma*), ragweed, and HDM (Der p, Der f) which possess immunogenic properties without the need of an adjuvant are used (Aun et al., 2017).

In addition to allergen type, route of sensitisation and challenge can also be a source of variation of Th2 phenotype. Intraperitoneal (i.p.) sensitisation such as in OVA models require few doses however lacks similarity with human sensitisation and usually requires the use of an adjuvant (Aun et al., 2017). The intranasal (i.n.) route of administration is most similar to that of human asthma, does not require the use of adjuvants and can be used in chronic models without the induction of immune tolerance. However, sedation and many instillations are required in order to induce

airway inflammation (Alessandrini et al., 2020). Aerosol challenge also mimics human asthma and does not require sedation, however, high doses are required. Intratracheal (i.t.) challenge has an advantage of only requiring low doses of allergen which can be driven directly into the lower airways to induce an immune response. However, this invasive method can be very time consuming as it requires a deeper plane of anaesthesia compared to i.n. challenge (Kim et al., 2019).

1.2 Macrophage Migration Inhibitory Factor (MIF)

Macrophage migration inhibitory factor (MIF) is a pleiotropic cytokine implicated in the pathogenesis of many inflammatory disorders (Bernhagen et al., 1993; Foote et al., 2004; Leech et al., 1999; Mikulowska et al., 1997). Although first described in the 1960s as a soluble factor produced by activated T cells that inhibits the migration of macrophages, it was not until the 1990s when its role as a pro-inflammatory cytokine was elucidated (Bernhagen et al., 1993; Bloom and Bennett, 1966). MIF is a highly conserved protein with different ancient homologues present in plants, protozoans, nematodes and invertebrates (Sparkes et al., 2017). It is composed of 115 amino acids with a molecular weight of 12.5 kilodaltons (kDa) and is encoded by a single gene located on chromosome 22q11.2 made up of three exons interspaced by two introns (Budarf et al., 1997; Weiser et al., 1989). MIF is produced by a range of cells in the body including epithelial, endothelial, and immune cells such as T cells, macrophages and eosinophils (Calandra et al., 1994; Grieb et al., 2010). MIF is stored preformed in intracellular pools to aid rapid release into the surrounding microenvironment (Nishino et al., 1995). Rapid MIF release promotes the production of other pro-inflammatory cytokines to further potentiate inflammation. The role of MIF as a pro-

inflammatory cytokine was first explored in a lethal endotoxaemia model where it was shown to be a major secreted factor in response to lipopolysaccharide (LPS) stimulation which could override the anti-inflammatory effects of glucocorticoids (Bernhagen et al., 1993; Calandra et al., 1995). Since then MIF has been shown to be an important mediator in a plethora of pro-inflammatory diseases such as rheumatoid arthritis (RA) (Leech et al., 1999), systemic lupus erythematosus (SLE) (Foote et al., 2004), multiple sclerosis (Benedek et al., 2017), severe acute pancreatitis (Sakai et al., 2003), and autoimmune kidney disease (H. Y. Lan et al., 1997).

1.2.1 Mode of Action of MIF

As a cytokine, MIF has been reported to interact with cell surface receptors. MIF classical receptor is CD74, however, MIF is also a non-cognate ligand of C-X-C chemokine receptor type 2 (CXCR2), CXCR4, and CXCR7 (Alampour-Rajabi et al., 2015; Bernhagen et al., 2007; Leng et al., 2003). CD74 is a type 2 transmembrane protein known as the MHC class II chaperone invariant chain (Stumptner-Cuvelette and Benaroch, 2002).

One of the initial observations of MIF binding was its ability to regulate glucocorticoid immunosuppression. Glucocorticoids have anti-inflammatory effects through the regulation the immune cell trafficking, inhibition of adhesion molecule expression and the promotion of leukocyte apoptosis (Coutinho and Chapman, 2011). MIF is released in response to glucocorticoid release allowing counter regulation and control of immune cell activation (Bernhagen et al., 1993). *In vitro*, MIF is shown to counteract glucocorticoid mediated suppression of pro-inflammatory secretion from LPS-stimulated macrophages (TNF, IL-1, IL-6, IL-8) (Calandra et al., 1995). *In vivo*, the

administration of recombinant MIF together with dexamethasone in LPS-treated mice abrogated the protective effects of dexamethasone on LPS lethality. Furthermore, MIF has been shown to override glucocorticoid inhibition of T cell proliferation (Bacher et al., 1996). Inhibition of mitogen-activated protein kinase phosphatase-1 (MKP-1) has been proposed to be the mechanism involved in MIF suppression of glucocorticoid action (Roger et al., 2005). MKP-1 is upregulated in the presence of glucocorticoids to inhibit mitogen-activated protein kinase (MAPK) signalling (Roger et al., 2005).

Further evidence of the role of MIF as a master regulator of host inflammatory responses is demonstrated through its ability to promote the production of other inflammatory mediators. MIF has been shown to amplify the expression of TNF, (IFN)-γ, IL-1β, IL-2, IL-6, IL-8 from immune cells (Bacher et al., 1996; Calandra et al., 1995, 1994; Donnelly et al., 1997). This augmentation of immune signals contributes to MIF mediated pathogenesis by acting as a mediator to sustain the inflammatory response. This has been shown in a range of inflammatory diseases where the absence of MIF is associated with lower levels of pro-inflammatory cytokines resulting in reduced pathology. For example, the use of anti-MIF antibodies results in reduced Th2 cytokines in models of allergic airway inflammation (Amano et al., 2007). MIF knockdown mice produce lower levels of TGF-β1, TNF-α, and IL-17 in bleomycin-induced pulmonary fibrosis (Luo et al., 2021). Additionally, lower levels of circulating TNF and IL-1 are detected in rats treated with anti-MIF antibodies in a model of immunologically induced kidney disease (Hui Y. Lan et al., 1997). These results demonstrate the pivotal role MIF plays in the host immune response through the regulation of pro-inflammatory mediator release.

Upon binding to CD74, MIF has been purported to initiate downstream signalling pathways involved in cell survival and proliferation. In order to activate signalling pathways CD74 complexes with CD44 to initiate the signalling cascade (Meyer-Siegler et al., 2006; Shi et al., 2006). As a pro-inflammatory cytokine, MIF plays a pivotal role in sustaining immune cell survival to maintain tissue inflammation. Mitchell at al. demonstrated the role of MIF in macrophage survival. Initially they showed a reduction in macrophage viability in MIF deficient mice following exposure to LPS *in vivo*. They expanded on these results showing that MIF suppresses macrophage apoptosis by suppressing P53 via the induction of cyclooxygenase-2 (COX-2) (Mitchell et al., 2002). MIF is also shown to induce B cell survival following interaction with CD74 (Gore et al., 2008). MIF signalling in B cells drives cell proliferation and survival and in a CD74-CD44 receptor complex dependent manner (Gore et al., 2008). The role of MIF in the early inflammatory response has also been shown through the inhibition of neutrophil apoptosis (Schindler et al., 2021).

In addition to MIFs role as an inflammatory mediator, MIF has also been shown to play a role in resolution and repair. MIF has been shown to be involved in haemostasis through its ability to promote survival of platelets (Chatterjee et al., 2014). MIF can also promote survival of structural cell types including placental cells under stress in order to maintain trophoblast homeostasis (Costa et al., 2016; Ietta et al., 2018). MIF's cytoprotective ability has also been demonstrated within the heart by promoting cell survival of cardiac myofibroblasts and cardiac stem cells as well as inducing endothelial cell differentiation following ischemia/reperfusion (Cui et al., 2016; Soppert et al., 2018). MIF can also be renoprotective in models of kidney disease where a decrease in renal MIF is associated with fibrosis and the inhibition of MIF *in vitro* reduces tubular cell proliferation (Djudjaj et al., 2017). Moreover, the ability to

enhance survival and promote proliferation has been demonstrated in neural stem/progenitor cells, cells involved in neurogenesis and protection (Ohta et al., 2012). In contrast to its role in maintaining homeostasis, the ability of MIF to promote cell survival can also be implicated in the pathogenesis of inflammatory diseases. MIF has been shown to regulate proliferation and cell survival of fibroblast-like synoviocytes which are involved in synovial tissue expansion which mediates the progressive destruction of cartilage and bone in RA (Leech et al., 2003). Reduced severity of disease is observed in MIF knockout mice in animal models of RA and was associated with increased levels of p53 and apoptosis in the synovium (Leech et al., 2003). MIF induced cytoprotection has also been implicated in playing a role in the pathogenesis of cancer through the direct inhibition of p53 (Fukaya et al., 2016; Lue et al., 2007).

Many studies have explored the pathways involved in MIF-directed cell survival. Following binding to CD74, MIF mediates the formation of the CD74-CD44 receptor complex to initiate signal transduction. MIF activation of the phosphatidylinositol 3-kinase (PI3K) Akt signalling pathway has been shown to play a pivotal role in the pro-survival features of MIF (Costa et al., 2016; Cui et al., 2016; Gore et al., 2008; Mitchell et al., 2002; Soppert et al., 2018). Additionally, MIF inhibits activation induced apoptosis by promoting p53 and MAPK extracellular signal-regulated kinase (ERK)1/2 signalling pathways (Fallica et al., 2014; Fukaya et al., 2016; Gore et al., 2008; Jankauskas et al., 2019; Leech et al., 2003; Mitchell et al., 2002).

In addition to its ability to modulate glucocorticoids, induce pro-inflammatory cytokine production, promote cell survival and proliferation, MIF possesses chemokine-like characteristics through its ability to interact with chemokine receptors CXCR2, CXCR4, and CXCR7. The promotion of migration allows MIF to further

potentiate its pro-inflammatory effects by driving immune cells to the site of injury. MIF has been shown to promote the chemotaxis of T cells, B cells, monocytes, neutrophils, and DCs through the engagement of one or several of its receptors (Alampour-Rajabi et al., 2015; Bernhagen et al., 2007; de Souza et al., 2015; Ives et al., 2021; Klasen et al., 2014; Santos et al., 2011). MIF driven chemotaxis has been shown to play a central role in the development of atherosclerosis. Bernhagen et al. demonstrated that MIF drives the recruitment of mononuclear cells through binding to either CXCR2 or CXCR4 (Bernhagen et al., 2007). Moreover, MIF has been shown to contribute to the pathogenesis of eosinophilic esophagitis through the promotion of eosinophil chemotaxis through the binding to the CXCR4 receptor (de Souza et al., 2015). De Souza et al. showed that treatment of mice with an anti-MIF antibody or CXCR4 inhibitor prevented eosinophil accumulation and tissue remodelling in a preclinical model of eosinophilic esophagitis. Conversely, recombinant MIF exacerbated the pathogenesis in WT mice.

The mechanism involved in MIF chemotaxis appears to be cell specific. CXCR2 appears to drive the migration of monocytes, myeloid derived suppressor cells, and NKT cells (Bernhagen et al., 2007; Hsieh et al., 2014; Xu et al., 2015; Zhang et al., 2017). Whilst, CXCR4 mediates MIF driven migration of T cells, B cells, and eosinophils (Bernhagen et al., 2007; de Souza et al., 2015; Klasen et al., 2014). CXCR7 has also been shown to mediate B cell chemotaxis (Alampour-Rajabi et al., 2015). In order to mediate MIF-specific signalling, CD74 is required to bind with the chemokine receptor forming a heteromeric receptor complex. The cooperative engagement of receptors initiates signal transduction induce MIF-directed recruitment (Hoffmann et al., 2020; Ives et al., 2021; Klasen et al., 2014; Schwartz et al., 2009).

1.2.2 Functional Polymorphisms

In terms of genetic regulation, two functional polymorphisms have been identified within the promoter region at positions -173, single nucleotide polymorphism (SNP) (G to C), and at -794, tetranucleotide repeat sequence CATT (5-8) (Baugh et al., 2002; Donn et al., 2002). The -173*C SNP has been shown to be associated with susceptibility to diseases such as psoriasis (Donn et al., 2004), juvenile idiopathic arthritis (De Benedetti et al., 2003; Donn et al., 2002, 2001), breast cancer (Avalos-Navarro et al., 2020), SLE (De la Cruz-Mosso et al., 2014), and pneumococcal meningitis (Savva et al., 2016).

The CATT MIF polymorphism in the promoter region comprises of 5-8 repeats of the CATT sequence and has been found to be important in a range of inflammatory diseases. Functionally, the CATT polymorphism affects the activity of the MIF promoter where the shorter number of CATT repeats correlates with lower transcriptional activity, whilst longer numbers of CATT repeats within promoter region of the MIF gene correlates with more severe disease, particularly among the 7/7 and 8/8 haplotypes. This was first discovered by Baugh and colleagues in RA patients where patients with the low-expressing $CATT_5$ repeat allele experienced low disease severity (Baugh et al., 2002). Using gene reporter assays they showed that $CATT_5$ repeat promoter constructs has the lowest levels of basal and stimulated MIF promoter activity compared to the other haplotypes. Moreover, analysis of the development of atopy within a Japanese population showed an association between MIF polymorphisms and susceptibility to atopy development. They found that carriers of the $CATT_7$ repeat to have a higher risk of atopy compared to the $CATT_5$ haplotype

(Hizawa et al., 2004). Furthermore, associations between the CATT polymorphism and asthma incidence and severity have been observed (Mizue et al., 2005). A study of 151 asthma patients and 164 healthy controls showed a significant association between mild asthma and the low-expressing $CATT_5$ allele. $CATT_5$ carriers experienced less severe clinical disease with higher FEV1 and fewer hospital admissions. Additionally, high levels of MIF as a result of longer CATT repeats such as $CATT_7$ have been shown to increase severity in a range of diseases including severe anaemia (Awandare et al., 2009), pneumococcal meningitis (Savva et al., 2016), multiple sclerosis (Benedek et al., 2017), tuberculosis (Liu et al., 2018) and COVID-19 (Shin et al., 2022). Not only do these studies show the pivotal role that MIF plays in pro-inflammatory diseases they also affirm the importance of differences in the MIF CATT polymorphism.

1.2.3 MIF in Asthma

The role of MIF in asthma was first investigated in the 1990s by Rossi and colleagues. They discovered that human circulating eosinophils contained preformed MIF which was released upon stimulation (Rossi et al., 1998). By sampling BAL fluid from asthma patients they detected significantly higher levels of MIF compared to a non-atopic population. These observations were supported by Yamaguchi and colleagues when they took sputum and serum samples from asthma patients (Yamaguchi et al., 2000). They detected significantly higher levels of MIF in the serum of asymptomatic patients compared to healthy controls. Symptomatic patients also displayed significantly higher levels of MIF compared to asymptomatic patients suggesting MIF

levels mirror the severity of the disease. These data provided further evidence for the importance of MIF in allergic asthma.

The involvement of MIF in asthma has also been investigated in animal models though various means to manipulate MIF expression or binding to the classical MIF receptor CD74. Summary of effects of manipulation on asthma pathogenesis is outlined in figure 1.1. MIF knockout ($MIF^{-/-}$) mice display a less severe phenotype when exposed to OVA compared to control mice (Das et al., 2011; Li et al., 2021; Magalhães et al., 2007; Mizue et al., 2005). They display decreased AHR and lower inflammatory cell infiltration into the lungs. $MIF^{-/-}$ mice also express a reduced Th2 cytokine profile and goblet cell metaplasia. Blocking studies using anti-MIF antibodies mirrored the results observed in the knockout models. Reducing inflammatory infiltrates in OVA challenged mice, however, no changes were detected in levels of Th2 cytokines in the BAL fluid (Amano et al., 2007; Kobayashi et al., 2006; Magalhães et al., 2007). Furthermore, the use of the MIF inhibitor (S,R)-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester (ISO-1) also improved features of asthma with the treated groups displaying lower airway inflammation, subepithelial fibrosis and goblet cell metaplasia in a chronic OVA model (Chen et al., 2010).

Despite these studies showing MIF is important in allergic asthma, the specific mechanisms involved are still poorly understood. It is suggested that MIF may regulate asthma pathogenesis through the modulation of eosinophil activation and biogenesis of leukotriene C_4-synthesising lipid bodies (Vieira-de-Abreu et al., 2011). Leukotrienes released by eosinophils contribute to vascular permeability, airway hyperresponsiveness and airway remodelling. Intrapleural injection of recombinant murine MIF promotes eosinophil activation and production of leukotriene C_4-

synthesising lipid bodies. These effects were abrogated in the presence of CD74 neutralising antibody (Vieira-de-Abreu et al., 2011). Moreover, MIF has been shown to play a role in IL-5 driven maturation of eosinophils and eosinophilia following *Schistosoma mansoni* infection (Magalhães et al., 2009). MIF has also been shown to regulate the adaptive immune response in asthma models. Das and colleagues observed that $MIF^{-/-}$ mice produce lower levels of Th2 cytokines following antigen challenge (Das et al., 2011). They go on to show CD74 deficient mice fail to produce an inflammatory response following epicutaneous challenge of OVA. Furthermore, they observe that adoptive transfer of *in vitro* sensitised T cells expressing a Th2 phenotype into KO mice failed to induce eosinophilia compared to transfer into wildtype mice (Das et al., 2011). More recently, a study by Allam et al. suggests that MIF impairs glucocorticoid-mediated resolution of the neutrophilic response by inhibiting annexin-A1 (Allam et al., 2022). They showed that treatment with ISO-1 increases sensitivity to one dose of the glucocorticoid dexamethasone in a murine model of severe asthma. Taken together these results highlight the important role MIF plays in asthma pathogenesis through the regulation of both the innate and adaptive immune system.

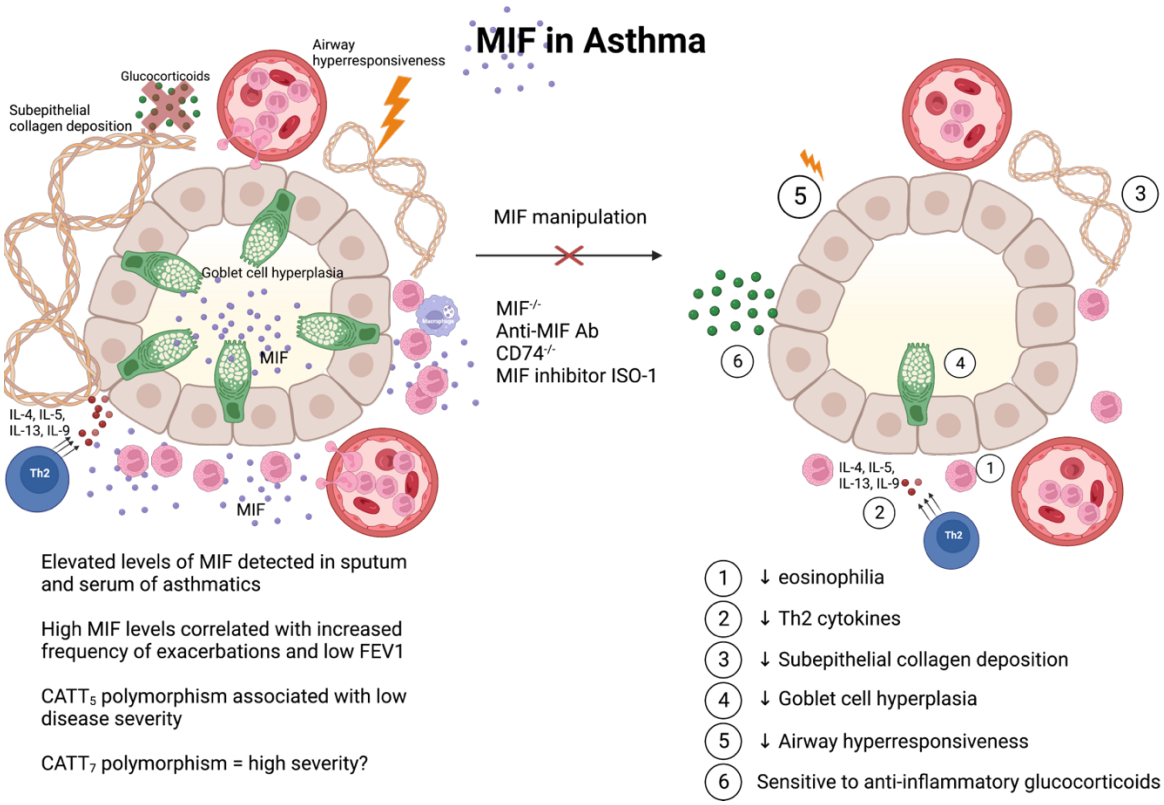

Figure 1.1. Effect of blocking MIF on asthma pathogenesis. The crucial role of MIF in asthma has been previously demonstrated in studies involving knocking out MIF or blocking MIF signalling pathways. MIF inhibition has been shown to ameliorate asthma pathogenesis with reduced cellular infiltration, lower levels of Th2 cytokines and attenuated airway remodelling and hyperresponsiveness. Blocking MIF has also been shown to restore glucocorticoid responsiveness.

1.3 Mesenchymal Stromal Cells

Mesenchymal stromal cells (MSCs) are a heterogenous population of non-haematopoietic multipotent progenitor cells that display regenerative and immunomodulatory properties. MSC research first emerged in the 1970s when Friedenstein and colleagues discovered a population of bone marrow stroma-derived fibroblastic cells that formed plastic adherent colonies *in vitro (Friedenstein et al., 1974, 1970)*. In the years following, these cells were shown to be capable of self-renewal and differentiate into several tissue types *in vitro* (Ashton et al., 1980; Bab et al., 1986; Castro-Malaspina et al., 1980). Dr Arnold Caplan first adopted the term "mesenchymal stem cells" to describe this diverse multipotent population and recognised MSC therapeutic potential to regenerate bone, cartilage and ligaments in animal and clinical studies (Caplan, 1991; Caplan and Dennis, 2006). The nomenclature was further refined in 2005 by the International Society for Cellular Therapy (ISCT) MSC committee clarifying that mesenchymal stem cells and mesenchymal stromal cells are not equivalent or interchangeable (Horwitz et al., 2005). They outlined that mesenchymal stem cells refer to a stem cell population with progenitor functionality of self-renewal and differentiation. Whilst the term mesenchymal stromal cell should only refer to a heterogenous population with secretory, immunomodulatory, and homing properties. The ISCT issued three main criteria to define MSCs: (i) adherence to plastic in standard culture conditions, (ii) the expression of CD73, CD90 and CD105, the lack of expression of the haematopoietic and endothelial markers CD11b, CD14, CD19, CD34, CD45, CD79a and human leukocyte antigen (HLA)-DR, (iii) capable to differentiate *in vitro* into adipocyte, chondrocyte and osteoblast lineages (Dominici et al., 2006; Viswanathan et al., 2019). In addition, the MSC committee of the ISCT recommends to support the minimal

phenotypical characterisation with functional assays to confirm hallmark properties of MSCs such as the secretion of indolamine-2,3-dioxygenase (IDO) upon *in vitro* licensing with IFN-γ and the ability to regulate immune cell responses (Krampera et al., 2013). Furthermore, it has been recommended to include TF/CD142 expression and haemocompatibility testing to the criteria when MSCs are intended for intravascular use (Moll et al., 2022). MSCs can be isolated from various tissues of the body, however, most studies utilise MSCs from locations where they can be readily isolated such as the bone marrow, adipose tissue (Zuk et al., 2002), umbilical cord tissue (Erices et al., 2000) and placenta (In 't Anker et al., 2004). The most prevalent source in studies is bone marrow followed by adipose and umbilical cord tissue due to their ease of harvesting cells after surgery or birth where the tissue would otherwise be discarded (Markov et al., 2021).

MSCs have also been shown to possess a range of immunoregulatory abilities and are capable of affecting the functionality of both innate and adaptive immune cells. MSCs are plastic in nature and display both pro-inflammatory and anti-inflammatory effects based on stimuli from the surrounding microenvironment (Dunbar et al., 2021; Liu et al., 2022; Luk et al., 2017). Immunomodulation occurs when MSCs are licensed by inflammation where they require threshold levels of pro-inflammatory factors to potentiate this immunosuppression (Boland et al., 2018; Carty et al., 2021; Corbett et al., 2021; Dunbar et al., 2021; English et al., 2007). Most studies have focussed on MSC immunosuppression through the paracrine secretion of cytokines, morphogens, growth factors and cargo-bearing exosomes which are able to modulate the surrounding microenvironment and affect the adjacent and distant responding immune cells (Chang et al., 2021). The MSC secretome contains a plethora of soluble mediators that can carry out immunomodulatory, pro-reparative and pro-survival

functions. In terms of immunomodulation MSCs secrete prostaglandin E2 (PGE2), IDO, hepatocyte growth factor (HGF), tumour necrosis factor-inducible gene 6 protein (TSG-6), IL-10, and TGF-β. PGE2 is produced by MSCs when COX-2 catalyses the conversion of arachidonic acid to prostaglandin G2 then prostaglandin H2. Prostaglandin H2 is then converted to PGE2 by microsomal prostaglandin E synthase-1 (Greenhough et al., 2009). PGE2 is considered one of the most important MSC mediators as it can modulate the maturation of dendritic cells, suppress proliferation of activated lymphocytes, and drive the differentiation of macrophages into M2 phenotype (English et al., 2009, 2007; Le Blanc and Mougiakakos, 2012; Spaggiari et al., 2009, 2008; Vasandan et al., 2016). IDO secretion is enhanced by IFN-γ stimulation. IDO catalyses the metabolism of the essential amino acid tryptophan into kynurenine (Jones et al., 2013). Metabolites of kynurenine are immunomodulatory and have been shown to suppress the proliferation of T cells in MSC: PBMC co-culture (Corbett et al., 2021; English et al., 2007). Moreover, MSCs secretome contains growth factors such as vascular endothelial growth factor (VEGF), and basic fibroblast growth factor (bFGF) which possess angiogenic, proliferative and anti-apoptotic properties (Healy et al., 2015; Nawrocka et al., 2017; Tao et al., 2016).

1.3.1 MSCs and the innate immune system

1.3.1.1 MSC influence on Macrophages

Macrophages are an important mediator of the innate immune system playing a crucial role in the inflammatory response and the tissue regeneration that follows (Davies et al., 2013). Macrophage plasticity allows them to maintain tissue homeostasis as they

can be polarised depending on the microenvironmental stimuli into a pro-inflammatory M1 type or the anti-inflammatory M2 (Italiani and Boraschi, 2014). Generally, M1 macrophages are classically activated through stimulation by IFN-γ produced by Th1 cells, cytotoxic T cells or natural killer (NK) cells. Additionally, they can also be stimulated by LPS. M1 macrophages possess antimicrobial activity and are capable of producing pro-inflammatory factors such as TNF, IL-6, and IL-12 to initiate an immune response (Chávez-Galán et al., 2015). Whereas stimuli from IL-4, IL-13, IL-10, TGF-β, and toll-like receptor (TLR) ligands induces alternative activation of M2 macrophages. M2 macrophages resolve inflammation through the release of anti-inflammatory mediators such as IL-10 and enhanced phagocytosis of apoptotic cells (Yunna et al., 2020). This M1/M2 paradigm is considered to be too simplistic considering the complexity of the process. Instead, the activation process is much more dynamic with M1 and M2 displaying overlapping effects. Rather than existing as distinct populations, M1 and M2 signatures can coexist resulting in a mixed phenotype which can be altered based on the tissue microenvironment (Martinez and Gordon, 2014).

MSCs have been shown to mediate macrophage polarisation from the pro-inflammatory M1-like phenotype to the anti-inflammatory M2-like phenotype (Liao et al., 2020; Luz-Crawford et al., 2017; Németh et al., 2009; Wang et al., 2021). One of the earlier studies on MSCs influence on macrophage polarisation and reprogramming was demonstrated in a murine model of sepsis (Németh et al., 2009). The authors show that treatment with MSCs promotes survival through the reprogramming of tissue monocytes and macrophages. *Ex vivo* analysis of pulmonary macrophages showed that macrophages from MSC treated mice produce greater amounts of IL-10. Moreover, lower levels of circulating TNF-α and IL-6 were

detected in MSC treated mice compared to the untreated control. Increased levels of IL-10 produced by the repolarised macrophages led to reduced neutrophil recruitment into the inflamed tissue thus preventing further damage. They showed that the polarisation toward the M2 phenotype was mediated by the PGE2 from the MSCs acting on the macrophages thus stimulating IL-10 release (Németh et al., 2009). The role of PGE2 in the polarisation of macrophages toward an anti-inflammatory phenotype has been further described in by multiple authors. Chemical inhibition of PGE2 with indomethacin and COX-2 knockdown MSCs fail to modulate the phenotype and function of M1 polarised macrophages (Ortiz-Virumbrales et al., 2020; Vasandan et al., 2016). Moreover, Wang et al. demonstrated that PGE2 mediated re-polarisation is abrogated in the presence of a PGE2 receptor 4 (EP4) antagonist (Wang et al., 2021). These data show that PGE2 secreted from MSCs suppress inflammation by binding to the EP4 receptor on macrophages to induce polarisation towards an M2-like phenotype.

In addition to PGE2, other soluble factors secreted by MSCs have been shown to modulate macrophages. Coculture of MSCs and RAW264.7 murine macrophages stimulated with LPS has been shown to polarise macrophages towards the M2-like phenotype (Liu et al., 2019). Polarisation was abrogated in the presence of the TGF-β receptor inhibitor LY2109761. IDO has also been implicated in the polarisation toward the M2-like phenotype (François et al., 2012). MSCs have also shown to induce a suppressive macrophage population through the secretion of TSG-6. Studies have shown that MSCs from TSG-6 knockdown mice fail to ameliorate disease in preclinical models of autoimmune uveitis and colitis (Ko et al., 2016; Sala et al., 2015).

MSCs have also been shown to modulate macrophage activity and function through the release of extracellular vesicles (EV). EVs consist of the lipid bilayer with transmembrane proteins and contain cellular components such as lipids, cytosolic proteins, DNA, RNA, and microRNAs (miRNAs). EVs serve as a means of cellular communication through the transfer of cellular components between cells (Maas et al., 2017). MSC-derived EVs containing miRNAs have been shown to ameliorate lung injury by transfer to alveolar macrophages to regulate polarisation (Su et al., 2022; J. Wang et al., 2020). However, particular interest has garnered around EV-mediated mitochondrial transfer from MSCs to macrophages. Morrison et al. demonstrated that MSCs promote metabolic reprogramming toward M2-like phenotype with enhanced phagocytic activity through EV-mediated mitochondrial transfer (Morrison et al., 2017). The transfer of mitochondria resulted in enhanced bioenergetics in macrophages by promoting oxidative phosphorylation. Adoptive transfer of alveolar macrophages pre-treated with MSC-derived EVs elicited protection by reducing inflammation and lung injury in an LPS-model of acute respiratory distress syndrome (ARDS) (Jackson et al., 2016; Morrison et al., 2017).

In addition to soluble factor-dependent mechanism, MSC regulation of macrophages can also be mediated through cell-cell contact. De Witte and colleagues revealed that MSCs activate and polarise monocytes towards the non-classical $CD14^{+}CD16^{+}CD206^{+}$ M2-like phenotype after being phagocytosed. The phagocytosis of MSCs promoted the expression of programmed death ligand (PDL)-1, production of IL-10 and the reduction of TNF-α (Witte et al., 2018). The authors also showed that phagocytosis by monocytes results in a different macrophage phenotype than culture with MSC conditioned medium. They observed a higher frequency of $CD163^{+}CD206^{+}$ monocytes after phagocytosis of MSCs compared to culturing in MSC conditioned

medium (Witte et al., 2018). This demonstrated that in addition to MSCs modulating the inflammatory environment in a paracrine manner through the secretion of soluble factors, they can also induce phenotypical and functional changes in monocytes/macrophages after being phagocytosed to subsequently modulate immune cells indirectly. de Witte et al. previously alluded to the fact that the majority of MSCs administered intravenously die within 24 hours. Death of MSCs through apoptosis has been shown to be important in MSC-education of macrophages following uptake by macrophages. Administration of apoptotic MSCs have been shown to ameliorate eosinophilic infiltration in OVA model of allergic asthma (Galleu et al., 2017; Pang et al., 2021). Apoptotic MSCs have been shown to be efferocytosed by alveolar macrophages leading to the induction of IDO production of the recipient phagocytes (Galleu et al., 2017). Efferocytosis of apoptotic MSCs causes alterations in the immunometabolism of alveolar macrophages leading to decreased expression of genes known to drive asthma and lung inflammation. Moreover, efferocytosis of MSCs also mediated changes in metabolic pathways within alveolar macrophages with the change in expression in genes in phosphorylation, fatty acid metabolism and glycolysis (Pang et al., 2021). These studies may explain how MSCs are able to carry out long term immunosuppression given their transient presence. Thus, MSC/macrophage crosstalk may be essential and the therapeutic effects of MSCs can be party attributed to engulfment by monocytes/macrophages and their adoption of an acquired immunoregulatory phenotype. This needs to be investigated in other disease models, however, it is likely that the disease microenvironment may dictate the involvement of MSC phagocytosis in MSC therapeutic efficacy.

1.3.1.2 MSC influence on dendritic cells

Dendritic cells (DCs) are a specialised population of antigen presenting cells which play an important role in the regulation of the immune response. Maturation of DCs is critical for the presentation of antigens to T cells to initiate the adaptive immune response (Maqbool et al., 2020). MSCs suppress the migration and maturity of conventional dendritic cells (cDCs), making them less capable to facilitate $CD4^+$ T cell proliferation thus regulating the adaptive immune response (Jiang et al., 2005; Ramasamy et al., 2007). These actions are thought to be mediated through paracrine signalling as IL-6 secreted from MSCs and MSC EVs containing miRNAs have been shown to modulate DC maturation and function (Djouad et al., 2007; Reis et al., 2018). On the other hand, MSCs can promote the expansion of tolerogenic dendritic cells (DCregs) (Cahill et al., 2015; Lu et al., 2020, 2019). DCregs induce antigen-specific tolerance by suppressing immune cells such as effector T cells and inducing the differentiation of regulatory T cells (Tregs) (Ness et al., 2021). Studies have shown that the induction of DCregs by MSCs can alleviate acute lung injury in mice (Lu et al., 2020, 2019). Induction of DCregs by MSCs have been attributed to the production of HGF (Lu et al., 2019) and the activation of Notch signalling (Cahill et al., 2015; Lu et al., 2020).

1.3.1.3 MSC influence on type 2 innate lymphoid cells

As previously mentioned, ILCs are key mediators of type 2 immune responses and considered the innate counterpart of Th2 cells (Halim, 2016). Tissue resident MSCs have been shown to facilitate ILC2s in maintaining homeostasis. MSCs have been shown to act as a reservoir for IL-33 to sustain ILC2s and promote proliferation (Rana

et al., 2019). In disease settings, the administration of MSC EVs have been shown to alleviate allergic airway inflammation by inhibiting the function of ILC2s (Fang et al., 2020). Human PBMCs were isolated from allergic rhinitis patients and cocultured with MSC EVs. Flow cytometry analysis showed a reduction in the levels of IL-9$^+$ ILC2s and IL-13$^+$ ILC2s. MSCs suppression of ILC2s were partly attributed to miRNA-146a-5p (Fang et al., 2020). Conversely, a recent study showed that induced pluripotent stem cell-derived MSCs (iPSC-MSCs) were unable to directly suppress the frequency of IL-9$^+$ ILC2s and IL-13$^+$ ILC2s (Fan et al., 2021). Instead, they observed that iPSC-MSCs can indirectly suppress ILC2s through the activation of Tregs and the subsequent release of IL-10.

1.3.1.4 MSC influence on natural killer cells

Natural killer (NK) cells are cytotoxic innate immune cells capable of recognising and destroying virally, infected, allogeneic or abnormal host cells (Abel et al., 2018). NK cells provide a first line of defence targeting cells that escape cytotoxic T cell (CTL) recognition or display inadequate expression of self-MHC class I molecules. The immunomodulatory functions of NK cells are mediated in response to and through secretion of cytokines such as IFN-γ (Abel et al., 2018). Studies have shown that MSCs can suppress proliferation of resting NK cells, whereas they can only partially impair the proliferation of IL-2 activated NK cells (Najar et al., 2018; Spaggiari et al., 2006). Additionally they show that NK cells display impaired cytotoxicity and cytokine production following coculture with MSCs (Noone et al., 2013; Spaggiari et al., 2006). Spaggiari and colleagues also provide evidence that the inhibitory effects

of MSCs on NK cells is mediated by MSC secreted PGE2 and IDO (Spaggiari et al., 2008).

1.3.2 MSCs and the adaptive immune system

1.3.2.1 MSC influence on T cells

The modulation of innate immune cells by MSC, as described above, can have indirect suppressive effects on adaptive immunity. The ability to suppress T cell proliferation is a mainstay of MSC immunomodulation. The current consensus is that MSCs are able to suppress T cell proliferation by both paracrine and cell contact-dependent mechanisms in a dose dependent manner (Di Nicola et al., 2002; Krampera et al., 2006; Ren et al., 2008). MSCs can exert immunomodulatory effects on T cells through the secretion of soluble mediators IDO, PGE2, TGF-β, HGF, and galectins (Carty et al., 2018; Chen et al., 2020; Chinnadurai et al., 2014; English et al., 2007; Niu et al., 2017; Sioud et al., 2011). Studies have shown that MSCs incubated with T cells display significantly higher levels of suppression when in direct contact than under separating conditions (K. Zhou et al., 2018). Zhou et al. showed that direct cell-to-cell contact resulted in greater suppression of $CD4^+$ and $CD8^+$ T cells than indirect coculture (K. Zhou et al., 2018). Previous studies have shown that the adhesion molecules intracellular adhesion molecule-1 (ICAM-1) and VCAM-1 are important for MSC-mediated immunosuppression of T cells (Ren et al., 2010; Zheng et al., 2021). A recent study showed that MSCs licensed with GvHD-derived plasma enhanced MSC immunomodulation through the upregulation of ICAM-1 (Silva-Carvalho et al., 2020). In addition to the suppression of T cell proliferation, MSCs

have shown to suppress activation and induce apoptosis. MSCs have been shown to induce apoptosis in T cells through the FasL/Fas pathway (Akiyama et al., 2012; Zhao et al., 2012).

In addition to the suppression of $CD4^+$ and $CD8^+$ T cells, MSCs are known to induce the generation of Tregs (English et al., 2009). Tregs are $CD4^+$ T cells which highly express forkhead box P3 (FoxP3) and CD25 on their cell surface. Tregs exert immunomodulatory and anti-inflammatory effects to maintain homeostasis and peripheral tolerance through the secretion of inhibitory cytokines such as IL-10 and TGF-β (Vignali et al., 2008). The induction of Tregs by MSCs comes from the conversion of conventional $CD4^+$ T cells rather than the expansion of pre-existing natural Tregs (Azevedo et al., 2020). Azevedo et al. observed that coculture of natural Tregs with MSCs does not promote proliferation whereas conventional $CD4^+$ T cells adopt a Treg phenotype and proliferate in culture. This change in phenotype was related to epigenetic changes such as DNA methylation following interaction with MSCs (Azevedo et al., 2020; Engela et al., 2013). A number of MSC immunomodulatory factors have been shown to be involved in the induction of Tregs *in vitro* and *in vivo* including IDO, TGF-β1, HGF, and PGE2 secreted by MSCs have been shown to induce the generation of Tregs *in vitro* and *in vivo* (Azevedo et al., 2020; Bai et al., 2018; Chen et al., 2020; Ge et al., 2010; Melief et al., 2013). Conversion to Tregs through direct cell contact has also been observed. Luz-Crawford et al. reported that MSCs can impair IL-17 production from Th17 cells through the conversion to Tregs (Luz-Crawford et al., 2019). Treg conversion was facilitated through the transfer of mitochondria from MSCs to Th17 cells resulting in a change in bioenergetics and the conversion to a Treg phenotype. The ability to regulate the activity of T cells through the combination of direct and indirect mechanisms

demonstrates the dynamic nature of MSC immunomodulation. Additionally, MSCs ability to suppress immune cell proliferation whilst stimulating the generation of de-novo immunosuppressive cells accentuates their capabilities as a therapeutic.

1.3.2.2 MSC influence on B cells

While the effects of MSCs on T cells are well described with several mechanisms elucidated, the effects of MSCs on B cells in comparison are poorly detailed. MSCs have been shown to actually increase B cell survival and viability, however, inhibit their proliferation through the arrest of the cell cycle in the G_0/G_1 phase (Healy et al., 2015; Rosado et al., 2015; Tabera et al., 2008). Conversely, Traggiai et al. observed that a 1:1 coculture of MSCs and B cells promotes survival, proliferation and differentiation of B cells thereby enhancing immunoglobulin secretion (Traggiai et al., 2008). These discrepancies may be explained by the culture conditions or the activation state of either the MSCs or the B cells. Luk et al. shows that immunological conditions dictate MSC efficacy (Luk et al., 2017). They showed that under quiescent conditions, MSCs do not inhibit B cell proliferation or maturation. Interestingly, they did observe induction of regulatory B cells (Bregs). However, when stimulated with IFN-γ, MSCs inhibit B cell proliferation and maturation through the upregulation of IDO. Similar results were observed by Palomares Cabeza et al,; MSC incubation with IFN-γ prior to coculture impairs B cell proliferation, plasmoblast formation or IgG secretion (Palomares Cabeza et al., 2019). In addition to IDO playing a role in B cell regulation, TGF-β secretion and COX-2 signalling have been shown to mediate maturation of B cells (Park et al., 2020; Shin et al., 2017). Moreover, MSCs derived from IL-1 receptor antagonist (IL-1RA) knockout mice fail to impair B cell

differentiation into plasmablasts and the production of IgG antibodies (Luz-Crawford et al., 2016). Reports around the involvement of MSC-derived EVs in B cell modulation have also shown conflicting results. Administration of MSC-derived EVs was shown to block interaction between follicular T helper cells and germinal B cells in a mouse model of chronic graft versus host disease (GvHD). Additionally EVs were shown to reduce the ratio of B cell activation factor to B cells *in vivo* (Guo et al., 2020). On the contrary, Carreras-Planella et al. reports that MSC effects on B cells are a result of soluble mediators rather than EVs (Carreras-Planella et al., 2019).

In addition to MSCs supressing the differentiation of B cells into plasmablasts, an increasing amount of literature is being published on the generation of Bregs. Similar to the role of Tregs, Bregs can regulate immune responses through the production of IL-10 and interaction with pathogenic T cells (Mauri and Bosma, 2012). MSCs have been shown to ameliorate inflammation by upregulating IL-10 secreting Bregs (Chen et al., 2019; Cho et al., 2017; Garcia et al., 2022). Interestingly, analysis of a clinical trial involving the administration of allogenic MSCs into patients with severe progressive systemic sclerosis showed a role for Bregs in MSC efficacy (Loisel et al., 2023). They discovered an increase in circulating IL-10-producing Bregs in the responders compared to non-responders.

1.3.3 MSC therapeutic efficacy in preclinical models of allergic asthma

MSCs have been shown to ameliorate various aspects of allergic asthma pathophysiology as outlined in table 1.1. Generally, MSCs have been shown to abrogate allergen induced inflammation with MSC treated groups displaying reduced eosinophilia and reduced levels of Th2 cytokines in the BAL fluid (Abreu et al., 2018;

Choi et al., 2022; Cruz et al., 2015; de Castro et al., 2017; Duong et al., 2015; Hur et al., 2020; Kavanagh and Mahon, 2011; Kitoko et al., 2018; Lin et al., 2018; Mathias et al., 2013; Ou-Yang et al., 2011; Zhong et al., 2019). Conversely, other reports have shown MSCs are unable to dampen eosinophilia or reduce the levels of certain Th2 cytokines (Cruz et al., 2015; Kitoko et al., 2018; Lathrop et al., 2014). Such disparity of results in the literature is also seen in the ability for MSCs to affect airway remodelling. MSCs have been shown to attenuate airway remodelling in allergic asthma by reducing both collagen deposition and goblet cell hyperplasia (Castro et al., 2020; Choi et al., 2022; Zhong et al., 2019). On the other hand, some reports have observed no effect on goblet cell hyperplasia (Abreu et al., 2018; Kitoko et al., 2018; Royce et al., 2019) or subepithelial collagen deposition (Dai et al., 2018; Mariñas-Pardo et al., 2014) following MSC administration. MSCs effects on airway hyperresponsiveness is also a contentious issue with reports demonstrating their ability to restore lung function and decrease parameters associated with methacholine-mediated AHR in asthmatic mice (Abreu et al., 2013; Castro et al., 2020; Cruz et al., 2015; Dai et al., 2018; Hur et al., 2020; Lathrop et al., 2014; Ou-Yang et al., 2011; Song et al., 2015; Zeng et al., 2015). Whereas several reports have suggested MSCs have no effect (Choi et al., 2022; de Castro et al., 2017; Kitoko et al., 2018; Mariñas-Pardo et al., 2014). In order to consolidate the literature surrounding MSCs and asthma more focussed work into understanding the inflammatory microenvironment and use of clinically relevant methods is needed to consolidate the literature surrounding MSCs and asthma.

Table 1.1. MSC treatment in preclinical models of allergic asthma

Source	Route of administration	Allergen	Study Duration	Outcome	Ref
Human AD-MSC	i.v.	OVA	90 days	AHR→, inflammation↓, goblet cell hyperplasia↓, collagen deposition↓, RELM-β↓	(Choi et al., 2022)
Human BM-MSC	i.v.	OVA	29 days	AHR↓, eosinophilia↓, IL-4/5/13↓	(Hur et al., 2020)
Human AD-MSC	3 doses i.v.	HDM	28 days	IL-4/13↓, eotaxin↓, IL-10→, inflammation↓, $CD3^+CD4^+$↓, $SiglecF^+$↓, TGF-β↓, collagen deposition↓, AHR↓	(Castro et al., 2020)
Human iPSC derived MSC	3 doses i.v.	OVA	55 days	Inflammation↓, goblet cell hyperplasia↓, eosinophilia↓, IL-4/13↓, TGF-β↓, collagen deposition↓, pSmad2/3 expression↓	(Zhong et al., 2019)
Human iPSC-, and mesangioblast derived MSC	i.n.	OVA	77 days	Inflammation↓, goblet cell metaplasia→, collagen deposition↓, airway epithelial damage↓, TGF-β↓, AHR↓, airway myofibroblasts↓	(Royce et al., 2019)
Murine BM-MSC	i.t.	HDM	25 days	IL-4/IL-13↓, eotaxin↓, IL-10→, eosinophilia↓, collagen deposition↓, AHR↓,	(Abreu et al., 2019b)
Murine AD-MSC	i.t.	OVA	55 days	AHR↓, IL-1β/4/17↓, IL-10↑, $CD4^+CD25^+FoxP3^+$↑, goblet cell hyperplasia↓, collagen deposition→	(Dai et al., 2018)
Human ESC-MSC	i.v.	OVA	20 days	Inflammation↓, goblet cell hyperplasia↓, eosinophilia↓, lymphocytes↓, IL-4/5/13↓, OVA-specific IgE↓, $CD4^+CD25^+FoxP3^+$↑, *Ccl11/Ccl24/Il13/Il33/Ear11* expression↓	(Lin et al., 2018)
Murine BM-MSC	i.t.	HDM	22 days	AHR→, inflammation→, goblet cell hyperplasia→, eosinophils↓, B cells↓, CCL11/24→, IL-4/5→, TGF-β→, IL-10↑	(Kitoko et al., 2018)
Murine AD-MSC	i.t.	HDM	22 days	AHR→, inflammation→, goblet cell hyperplasia→, eosinophilia→, B cells→, CCL11/24→, IL-4/5→, TGF-β→, IL-10→	(Kitoko et al., 2018)

Murine BM-MSC	i.t.	HDM	22 days	IL-4/13↓, VEGF↓, IL-10→, eosinophilia↓, collagen deposition↓, goblet cell hyperplasia→, AHR↓	(Abreu et al., 2018)
Human AD-MSC	i.v.	OVA	54 days	AHR→, collagen deposition↓, TGF-β↓, eosinophilia↓, SiglecF^{+}↓, CD4^{+}CD25^{+}FoxP3^{+}↓, IL-4/5/13↓, eotaxin↓, IL-10→	(de Castro et al., 2017)
Murine BM-MSC	i.v.	HDM	18 days	Eosinophilia↓, mast cells↓, ILC2s↓, goblet cell hyperplasia↓, IgE↓, IL-5/13↓, AHR↓, CD11b^{+} DCs↓, IL-25↓	(Duong et al., 2015)
Human MSC-derived EVs	i.v.	AHE	19 days	AHR↓, inflammation↓, neutrophils↓, macrophages↓, eosinophils↓, lymphocytes↓, IL-4/5/17↓, IL-13/6→, IFN-γ↑, IL-10→, RANTES↓	(Cruz et al., 2015)
Murine BM-MSC	i.v.	OVA	20 days	Eosinophilia↓, lymphocytes↓, IL-4/5/13↓, IFN-γ↑, AHR↓, DCs↓,	(Zeng et al., 2015)
Human BM-MSC	i.v.	OVA	14 days	AHR↓, eosinophilia↓, macrophage M2 suppressive phenotype↑	(Song et al., 2015)
Murine AD-MSC	i.v.	HDM	22 days	AHR→, inflammation↓, Th1 cytokines↓, contractile tissue→	(Mariñas-Pardo et al., 2014)
Murine BM-MSC	i.v.	AHE	19 days	AHR↓, inflammation↓, IL-4/5/13→, IL-17↓, RANTES↓, IL-10→	(Lathrop et al., 2014)
Human AD-MSC	i.v.	OVA	12 days	Eosinophilia↓, IL-4/5/13↓, IFN-γ↑, Treg↑, IL-10 producing macrophages↑	(Mathias et al., 2013)
Murine BM-MSC	i.t.	OVA	54 days	AHR↓, alveolar collapse↓, inflammation↓, collagen deposition↓, IL-4/13↓, TGF-β↓, VEGF↓,	(Abreu et al., 2013)
Human iPSC derived MSC	i.v.	OVA	29 days	Inflammation↓, goblet cell hyperplasia↓, eosinophilia↓, neutrophils↓, macrophages↓, lymphocytes↓, IgE↓, IL-4/5/13↓, IFN-γ→	(Sun et al., 2012)
Murine BM-MSC	i.v.	OVA	18 days	Inflammation↓, eosinophilia↓, β-hexosaminidase↓, AHR↓, IL-4/5/9↓, IFN-γ↑	(Ou-Yang et al., 2011)

"↑" increase, "↓" decrease "→" no effect

1.3.4 Clinical trials

MSCs were first trialled as a therapeutic agent in human subjects in 1995 (Lazarus et al., 1995). Following this, MSCs have become one of the most experimentally studied cell therapies worldwide. Despite the vast number of promising preclinical studies using MSC therapy in a range of different diseases there has been limited success in replicating these in a clinical setting. Phase I and II trials have confirmed that intravenous (i.v.) administration of MSCs is safe. MSC therapy was granted approval only in Japan for use in the treatment of GvHD in 2015 (Muroi et al., 2016). However, a breakthrough was achieved in 2018 where the first allogenic stem cell therapy was approved in Europe. Adipose-derived mesenchymal stromal cells (darvadstrocel, previously Cx601) received central marketing authorisation approval by the European Medicines Agency (EMA) following a successful phase III clinical trial in Crohn's disease patients (Panés et al., 2016). The randomised, parallel group, placebo-controlled trial, NCT01541579, focused on the treatment of complex perianal fistulas with darvadstrocel. 120 million expanded allogenic adipose-derived MSCs of saline placebo were administered into the fistula lesions of the patients. Significant differences were observed in the darvadstrocel group compared to the control patients where 50% of the darvadstrocel group achieved combined remission versus 34% in the placebo. The darvadstrocel group also experienced less treatment-related adverse effects showing darvadstrocel as a safe and effective therapy in the treatment of complex perianal fistulas in patients with Crohn's disease. Following up one year later, MSCs were proven to be a safe and effective therapy long term with 56.3% achieving combined remission in the darvadstrocel group compared with 38.6% in the placebo control (Panés et al., 2018). Additionally, Mesoblast have conducted a phase III trial, NCT02336230, involving treatment of paediatric steroid-refractory acute GvHD using

allogenic bone marrow-derived MSCs (remestemcel-L) (Kurtzberg et al., 2020). Despite initial promising clinical outcomes Mesoblast have been unable to secure approval for remestemcel-L from regulatory bodies as they require additional efficacy studies.

To date, no clinical trials involving MSCs and asthma patients have been completed. However, a search of clinicaltrials.gov (search conducted March 2023) lists 5 MSC-related trials in human asthma patients (search terms: Asthma, MSC). 3 trials are actively recruiting (NCT05035862, NCT04883320, NCT05147688), one was terminated early by the sponsor (NCT03137199), and one status is now unknown (NCT02192736). Of the trials currently recruiting: NCT05147688 seeks to determine the safety and efficacy of allogeneic adult umbilical cord derived MSCs for the treatment of pulmonary diseases asthma and chronic obstructive pulmonary disease (COPD); NCT04883320 plans to compare the effects of MSC CM on airway epithelium cells of chronic asthma patients; NCT05035862 seeks to determine the safety and toxicity of IFN-γ primed allogeneic adult umbilical cord derived MSCs in moderate-severe persistent asthma.

1.3.5 Discrepancies between preclinical and clinical studies of MSC

Many factors have been hypothesised as to why outcomes of phase III clinical trials have fallen short of expectations from such successful preclinical studies. Namely, overall study design and fitness of MSCs infused are suggested to impact MSC product performance. In preclinical studies involving mice, MSCs are administered following adherent cell culture and harvest during the log phase of growth. Whereas clinical

allogenic MSCs are expanded until their exhaustive replicative limit before being cryopreserved. Cryopreserved cells are then thawed and immediately infused. MSCs that have reached their replicative limit are prone to becoming senescent where they experiences metabolic changes and become less efficacious (Samuelsson et al., 2009; Zhou et al., 2020).

Another reason for the discrepancies between successful preclinical models and previous clinical trials is the differences in dosing. Typically clinical trials involving MSCs use no more than 12 million cells/kg whilst preclinical models use vastly higher doses of around 50 million cells/kg (Galipeau and Sensébé, 2018). This vast difference in dosing means that the effects observed in preclinical models may not be indicative of the outcomes observed in the clinic. Therefore, we need to employ strategies to enhance MSC immunosuppression to allow them to be more efficacious at lower doses.

1.3.6 MSC Kinetics

Endogenous MSCs are present in the bone marrow and are thought to migrate through the bloodstream to sites of injury (Chan et al., 2022). MSCs can extravasate through capillaries and migrate to damaged tissues to mediate immunosuppression within the inflammatory niche. *In vitro* and *intra-vital* studies have revealed that MSC extravasation follows a similar paradigm to that of leukocytes and haematopoietic stem cells (Rüster et al., 2006; Teo et al., 2015). This includes a multistep process as follows: (1) tethering and rolling mediated by selectins, (2) activation of integrins, (3) integrin-dependent adhesion to endothelial cells, (4) transendothelial migration, (5) interstitial migration towards the target tissue (Nitzsche et al., 2017).

The initial step of endogenous MSC migration from the bone marrow is the priming of the endothelium by inflammatory mediators released from the site of injury. This priming is mediated through the endothelial adhesion molecules P- and E-selectin. P-selectin can be rapidly upregulated upon activation with histamine. E-selectin is upregulated by inflammatory stimuli such as TNF-α or IL-1β (Heemskerk et al., 2014). These selectins interact with CD44 expressed on MSCs to enable tethering and slow rolling (Katayama et al., 2005).

Pro-inflammatory cytokines such as TNF-α induce the next step of MSC systemic migration through the activation of VCAM-1 on the endothelium and $\alpha_4\beta_1$ integrin (very late antigen (VLA)-4) on the MSCs (Rüster et al., 2006). Finally, MSCs secrete matrix metalloproteinase (MMP)-2 and membrane type (MT)1-MMP to facilitate transendothelial migration (Ries et al., 2007; Steingen et al., 2008).

The most extensively studied MSC chemotactic axis is CXCR4/ stromal cell-derived factor (SDF)-1. SDF-1 [also referred to as C-X-C motif chemokine 12 (CXCL12)] is secreted at the sites of injury and facilitates the homing of cells expressing the CXCR4 receptor. SDF-1 has been shown to drive migration of MSCs in a dose dependent manner *in vitro* (Liu et al., 2012; Ponte et al., 2007; Wynn et al., 2004). In addition, animal studies and computational modelling have revealed the significance of the CXCR4 expression on the MSCs on *in vivo* homing (Jin et al., 2018; Kitaori et al., 2009). They observed that CXCR4 overexpression results in increased numbers at the site of injury and migration was inhibited by treatment with CXCR4 antagonist. In addition to CXCR4 being important to MSC homing, MSCs also express other chemokine receptors which have been shown to drive MSC migration. CXCR7 upregulation has been shown to promote MSC homing to the lungs and enhance repair

of lung injury in a phosphogene-induced acute lung injury model (Shao et al., 2019). In addition, the CCL27/CCR10 axis (Alexeev et al., 2013), and CCL5/CCR5 axis (Nishikawa et al., 2019) have also been shown to drive MSC migration *in vivo*. Despite these studies, there is little convincing *in vivo* evidence that endogenous MSCs find their way to the site of inflammation via the bloodstream. Hoogduijn et al. found no evidence for circulating MSCs in patients with organ injury (Hoogduijn et al., 2014). No endogenous MSCs were detected in the blood of patients with end-stage renal disease, end stage liver disease or heart transplant patients. Instead, it is thought that tissue resident MSCs may migrate within tissues to contribute to local immunomodulation. As for exogenous MSCs, Masterson et al. demonstrated that MSCs do not roll along the endothelium and fail to bind to the endothelium under laminar flow (Masterson et al., 2021). However, they did observe significantly higher rates of MSC retention in the pulmonary vasculature of mice with lung injury 24 hours after administration compared to healthy mice. This is in line with studies from our lab which show increased MSC retention in disease target organs (Carty et al., 2021, 2018). Higher retention is associated with enhanced therapeutic efficacy as they have a longer period to secrete soluble factors and interact with cells within the inflammatory niche (Carty et al., 2021). Carty et al. demonstrated that IFN-γ licensed MAPCs are more efficacious in a humanised model of GvHD compared to nontreated controls. Increased efficacy in the licensed MAPCs was associated with longer retention times at sites of injury suggesting persistence is important as there is a longer time for cells to modulate the microenvironment.

As a therapeutic, the most common route of administration of MSCs in preclinical models is i.v. due to it being the most feasible approach in human therapy. This has shown to be a safe method and it enables the administration of large numbers of MSCs.

MSCs administered i.v. localise to the lungs and become trapped in the lung vasculature (Sensebé and Fleury-Cappellesso, 2013). Pulmonary entrapment occurs due to their size, MSCs range from 15-30 μm in diameter after cell culture expansion whilst lymphocytes range from 4-12 μm (Zhuang et al., 2021). After the initial entrapment in the lungs a small number of the cells migrate to other tissues such as the liver and the spleen or to sites of injury (Carty et al., 2018). Until recently, the consensus was that viable functional MSCs were critical for efficacy through the secretion of immunomodulatory factors. However, we have previously outlined that in some conditions apoptotic MSCs are able to modulate the immune system following efferocytosis by macrophages (Pang et al., 2021; Witte et al., 2018). These studies may explain how MSCs are able to carry out long term immunosuppression given their transient presence. However, Galleu et al. showed that apoptotic MSCs were only efficacious in a mouse model of GvHD when infused intraperitoneally where i.v. infusion did not induce IDO production (Galleu et al., 2017). They also showed that the apoptotic MSCs exhibited a reduced efficacy compared to viable functional MSCs. Likewise, Giri and Galipeau showed that heat inactivated MSCs lose therapeutic efficacy in a mouse dextran sulfate sodium toxic colitis model (Giri and Galipeau, 2020). They attributed the loss in function to shortened persistence and accelerated clearance by phagocytosis. Together these data show that MSCs ability to modulate inflammation through a range of different mediators. Initially, trapped MSCs modulate inflammatory responses through their secretome where they secrete EVs and soluble mediators, however, they may then be taken up by macrophages and elicit phenotypic changes to further induce immunosuppression. These new advances into understanding MSC kinetics *in vivo* have brought welcome excitement to the MSC field and may help us to improve therapeutic efficacy in the future.

1.3.7 Strategies to Enhance MSC Potency

As previously mentioned, MSCs are able to interact with signals and soluble mediators in their surrounding microenvironment. Interaction between MSCs and the surrounding mediators within the niche is essential in MSCs activation of signalling pathways to potentiate their therapeutic effects. Ensuring MSCs receive the correct signals at the correct time is essential in their ability to modulate their microenvironment. Investigators have attempted to enhance MSCs therapeutic potential by licensing MSCs *ex vivo*. The term licensing refers to the process of activating MSCs leading to functional maturation (Krampera, 2011). Licensing aims to activate MSCs by mimicking conditions within a pro-inflammatory microenvironment or to induce phenotypic changes to improve their efficacy. Such strategies include alterations to physiological, biological, chemical, or genetic factors (Fig. 1.2).

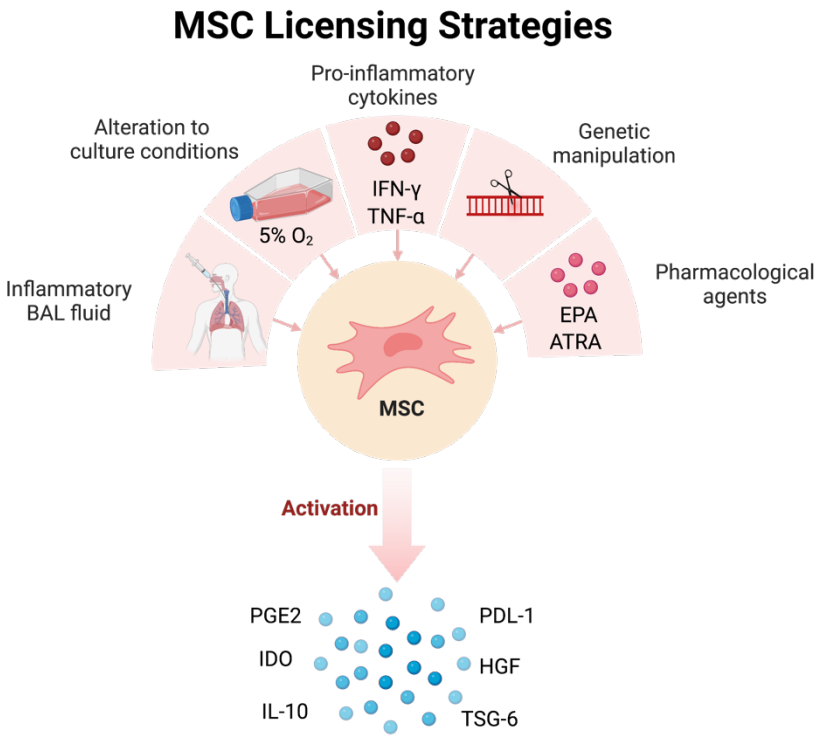

Figure 1.2. MSC licensing strategies. Licensing MSCs can lead to enhanced therapeutic efficacy through the amplification of various anti-inflammatory mediators.

1.3.7.1 MSC licensing with Hypoxia

Despite MSCs originating from niches with low oxygen availability, they are generally cultured and expanded in normoxic conditions. Respiratory diseases often impair gas exchange which leads to an ischemic environment *in vivo* (Noronha et al., 2021). Therefore, MSCs have been cultured in hypoxic conditions to precondition them and reduce cellular stress before administration. Hypoxic licensing alters cell metabolism, promotes MSC survival and enhances the angiogenic potential of MSCs (Liu et al., 2021; Mathew et al., 2017; Roemeling-van Rhijn et al., 2013). Hypoxic preconditioning of MSCs has been shown to increase their ability to reduce pro-inflammatory cytokines IL-6 and IL-1β, reduce reactive oxygen species and lung fibrosis in models of lung injury (Lan et al., 2015; Li et al., 2017). Cellular responses to hypoxia are controlled by hypoxia inducible factor (HIF)-1 which controls metabolism and maintains cell viability. HIF-1 also improved MSCs angiogenic potential through the upregulation of VEGF (Razban et al., 2012). Zhilai et al. showed that hypoxic preconditioning improved MSC efficacy in a rat model of spinal cord injury. They observed an increase in VEGF, HGF and brain-derived neutrotrophic factor. This upregulation was associated with increased axonal cell survival, reduced apoptosis, and attenuated inflammation (Zhilai et al., 2016).

1.3.7.2 Pharmacological Licensing and Genetic Manipulation

All-trans retinoic acid (ATRA) binds to retinoic acid receptors and regulates genes involved in apoptosis, differentiation and immune function. ATRA licensing of MSCs has been shown to increase MSC survival and wound healing capacity though the

increased expression of COX-2, CXCR4, CCR2, angiopoietin (Ang)-2, Ang-4, VEGF, and HIF-1α (Pourjafar et al., 2017). ATRA licensing MSCs demonstrated enhanced efficacy in elastase-induced emphysema resulting in lung tissue repair and improvement in lung function compared to non-treated controls. ATRA licensing was associated with activation of p70S6 kinase-1, a S6 kinase isoform involved in the mammalian target of rapamycin (mTOR) signalling pathway (Takeda et al., 2018).

Eicosapentaeoic acid (EPA) has been thought to be another possible licensing agent. EPA is an omega-3 polyunsaturated fatty acid which has been shown to inhibit inflammatory and remodelling responses in several inflammatory diseases (Calder, 2013). Studies have shown that EPA licensing improves MSC production of PGE2, TGF-β, IL-10, and resolvin D_1 compared to non-licensed MSCs (Abreu et al., 2018; Silva et al., 2019). EPA licensed MSCs were more efficacious than non-treated MSCs in a HDM model of allergic asthma (Abreu et al., 2018). Additionally, EPA licensing was shown to increase MSCs efficacy in a mouse model of sepsis yielding lower severity scores and improved survival rates (Silva et al., 2019).

Genetic strategies have been investigated to enhance MSC therapeutic efficacy by targeting MSC homing *in vivo*. Recently, Hervás-Salcedo et al. transfected human adipose tissue-derived MSCs with messenger RNAs containing CXCR4 and IL-10 (Hervás-Salcedo et al., 2021). Transfected MSCs or wild type MSCs were injected i.v. into an LPS-induced inflamed pad model. The transfected MSCs had significantly increased homing into inflamed pads and an enhanced anti-inflammatory effect compared to the wild type MSCs (Hervás-Salcedo et al., 2021). Other strategies include transducing MSCs with a lentiviral vector containing CXCR4. They found that injecting CXCR4 overexpressing MSCs in an inflammatory bowel disease model

resulted in reduced weight loss and longer colons compared to control MSCs (Zheng et al., 2019). Bioengineering MSCs through enzymatic treatment has also been investigated. Chou et al. enhanced migration of murine bone marrow derived MSCs by transfecting MSCs with fucosyltransferase VI (FTVI) (Chou et al., 2017). FTVI fucosylates N-glycans of CD44 to increase their affinity for E-selectin enhancing tethering and rolling on the endothelium to permit more efficient extravasation.

1.3.7.3 Licensing MSCs with Pro-inflammatory Cytokines

Several approaches have been utilised to prime MSCs including licensing with pro-inflammatory cytokines, with environmental stresses such as hypoxia or licensing with pharmacological agents. The most widely studied method of licensing is the exposure to pro-inflammatory cytokines most notably IFN-γ. Proteomic analysis revealed that IFN-γ leads to the differential expression of 210 proteins including IDO, PGE2, HGF, TGF-β, MHC-I, MHC-II, ICAM-1, and VCAM-1 (Guan et al., 2017). The extensively studied protein upregulated following IFN-γ exposure is the enzyme IDO. The IDO-mediated production of tryptophan metabolites such as kynurenine play a central role in MSC immunomodulation. Licensing of MSCs with IFN-γ increases immunosuppressive capacity through the regulation of IDO production (Boyt et al., 2020; English et al., 2007). IDO secreted from MSCs has been shown to suppress T cell proliferation and polarise macrophage into the M2 phenotype resulting in the production of IL-10 (English et al., 2007; François et al., 2012). Another immunoregulatory mediator upregulated in the presence of IFN-γ is the co-stimulatory modulate PDL-1. IFN-γ licensed MSCs were able to suppress T cell proliferation independent of IDO production through the expression of PDL-1 (Chinnadurai et al.,

2014). IFN-γ licensed MSCs and MSC-like cells [multipotent adult progenitor cells (MAPCs)] have been shown to be more efficacious than naïve MSCs in a preclinical models of sepsis (Baudry et al., 2019), GvHD (Carty et al., 2021; Corbett et al., 2021; Kim et al., 2018; Polchert et al., 2008), and colitis (Duijvestein et al., 2011).

Preconditioning MSCs with TNF-α has been shown to increase anti-inflammatory mediators PGE2, HGF, and TSG-6 thereby further enhancing their immunomodulatory potential. As previously mentioned, PGE2 plays a role in the polarisation of macrophages to the anti-inflammatory M2 type. In addition, PGE2 has shown to suppress lymphocyte proliferation (Carter et al., 2005), promote the induction of Tregs (Baratelli et al., 2005), and suppress the differentiation of dendritic cells and naïve T cells to Th17 cells (Spaggiari et al., 2009; Tumangelova-Yuzeir et al., 2019). TNF-α licensing has also been shown to limit IgE and histamine release, alleviating allergic symptoms (Su et al., 2015). TNF-α priming has also been shown to enhance MSCs neuroprotective effects on retinal ganglion cells through increasing pigment epithelium-derived factor and VEGF (Mead et al., 2020).

IL-1β, a key mediator of innate immune cell activation, has also been used to license MSCs to improve their therapeutic efficacy. Studies have indicated that IL-1β licensing can upregulate TSG-6 and PGE2 expression and activate the NF-κB signalling pathway (H. Liu et al., 2021). IL-1β licensed MSCs were shown to enhance repair following ischemia reperfusion injury through the upregulation COX-2 expression and PGE2 production (L. Liu et al., 2020).

In addition to single factor licensing, cocktails of pro-inflammatory cytokines have been shown to further augment the effects of MSCs. Preconditioning MSCs isolated

from menstrual blood with TNF-α and IFN-γ in combination resulted in increased release of extracellular vesicles and micro RNAs involved in immune response (de Pedro et al., 2021). They also observed enhanced suppression of $CD4^+$ and $CD8^+$ T cell proliferation showing that combination priming can work synergistically to create an additive effect. This was further supported where multifactorial priming of MSCs with IL-1β, TNF-α and IFN-γ further augmented immunoregulation of polymorphonuclear granulocytes (Hackel et al., 2021). Combination licensing aims to replicate the physiological conditions of the cytokine milleu within the inflammatory niche. This can be a powerful tool to enhance the therapeutic efficacy as it permits MSC activation with the same inflammatory mediators which the cells will encounter once they reach the site of injury. Studies have been performed to license MSCs with this milleu by collecting the BAL fluid or serum from disease models to prime MSCs *ex vivo*. Exposure of MSCs to the BAL fluid or serum from HDM challenged mice has been shown to increase therapeutic efficacy through the upregulation of IDO, TSG-6, TGF-β, and IL-1RA in a HDM model of allergic airway inflammation (Abreu et al., 2019b). This upregulation of MSC derived soluble mediators as a result of licensing led to decreased levels of IL-4, IL-13 and eotaxin in the BAL fluid of the treated mice and the polarisation of macrophages to a M2-like phenotype. Moreover, a study showed that licensing murine bone marrow-derived MSCs with serum from ARDS patients increased their IL-10 and IL-1RA production (Bustos et al., 2013). The ARDS serum licensed MSCs resulted in improved disease outcome with a reduction of TNF-α and IL-1β in the BAL fluid in a mouse model of ARDS compared to control MSCs. Moreover, a study observed that licensing MSCs with BAL fluid from ARDS patients for 24 hours led to greater ability to modulate macrophage phenotypes. They observed that ARDs licensed MSCs significantly increased expression of M2 markers

and phagocytic capacity of monocyte derived macrophages (Morrison et al., 2017). Conversely, a study showed that IL-6 was increased following MSC coculture with small airway epithelial cells in the presence of plasma from patients with ARDS (Islam et al., 2019). These results highlight the importance in understanding the microenvironment which MSCs are exposed to. Timing of exposure and environmental conditions are important determinants in whether MSCs will be beneficial or detrimental in inflammatory environments.

1.4 Aims and Objectives

This chapter has outlined the current literature surrounding asthma pathogenesis, the role of MIF in inflammatory diseases, and how MSCs can be utilised to act as a therapeutic to modulate the immune system. While preclinical studies have demonstrated efficacy for MSCs, their performance in a clinical setting has been underwhelming (Galipeau et al., 2021). Understanding the mediators at play within the site of inflammation and the modes of action of cell types is required to exploit the benefits of MSC therapy. Furthermore, identifying markers which may indicate a patient's likelihood of responding to MSC treatment is essential to harnessing their therapeutic potential. Thus, the aim of this thesis is to further develop our understanding into the role of MIF in asthma pathogenesis and provide data on the interactions between MIF and MSCs to further optimise their efficacy in the clinic. The goals of this thesis can be divided into two strands; investigating the role of MIF in asthma pathogenesis using novel transgenic mice expressing human MIF with differing polymorphisms in their promoter region in a HDM model of allergic asthma,

and identify optimal strategies for MSC therapy in allergic asthma in high and low MIF settings.

The aims of this thesis are outlined as follows:

(1) To investigate the role of MIF and the influence of the CATT promoter polymorphism in a HDM model of allergic airway inflammation
(2) To investigate the interactions of MIF and MSCs and elucidate the mechanisms by which these interactions occur *in vitro*
(3) To investigate the efficacy of MSCs in high vs low hMIF environments in a mouse model of allergic asthma
(4) To explore the efficacy of MIF licensing of MSCs and elucidate the mechanisms involved *in vivo*

Overall, this thesis aims to demonstrate the importance of understanding mediators governing the lung microenvironment and their effects on MSC efficacy. By understanding their interactions with MSCs we can utilise them to improve MSCs as a therapeutic. The data presented herein will contribute to a broader understanding of the influence that inflammatory mediators such as MIF have on MSC biology which can be applied to future studies and the use of MSCs in the clinic.

Chapter 2 Expression of the human MIF polymorphic allele $CATT_7$ exacerbates house dust mite driven allergic airway inflammation

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Based on Hazel Dunbar, Ian J. Hawthorne, Courteney Tunstead, Michelle E. Armstrong, Seamas C. Donnelly, Karen English. Blockade of MIF biological activity ameliorates house dust mite-induced allergic airway inflammation in humanised MIF mice. FASEB J. Accepted 2023. (Appendix I)

2.1 Abstract

Background – MIF plays a central role in the context of allergic asthma with high levels of MIF found in the serum of patients with severe asthma. The functional polymorphism -794 CATT which lies in the promoter region of the MIF gene affects disease outcome in a range of inflammatory disorders.

Methods – Humanised transgenic mice with either the high-expressing MIF promoter polymorphism ($CATT_7$) or the low-expressing MIF promoter polymorphism ($CATT_5$) were exposed to house dust mite (HDM) intranasally 3 times a week for 3 weeks. Cellular infiltration and Th2 cytokine levels within the bronchoalveolar lavage (BAL) fluid were assessed. Histological analysis of the lung tissue was performed to measure goblet cell hyperplasia, airway remodelling and immune cell infiltration. Airway hyperresponsiveness was determined through responsiveness to methacholine.

Results – $CATT_7$ mice challenged with HDM exhibit a more severe asthma phenotype compared to $CATT_5$ or WT C57BL/6 mice. Histologically, $CATT_7$ exhibit marked changes to lung morphology with significantly elevated goblet cell hyperplasia and collagen deposition. $CATT_7$ HDM mice also present with significantly higher numbers of eosinophils in the BAL fluid and significant increases in Th2 cytokines. Furthermore, $CATT_7$ mice exhibit hallmarks of chronic airway inflammation such as airway remodelling demonstrated by changes in airway hyperresponsiveness as early as 3 weeks in a HDM model.

Conclusion – We have developed a reproducible model of severe asthma which encapsulates features of chronic disease such as airway remodelling when challenged with a clinically relevant allergen for 3 weeks driven by a clinically relevant human

cytokine. Our model displays the dominant role of MIF allelic variants in the inflammatory lung manifestations and demonstrates a novel role for MIF in driving airway remodelling.

2.2 Introduction

Macrophage migration inhibitory factor (MIF) is a pleiotropic cytokine implicated in the pathogenesis of many inflammatory disorders (Bernhagen et al., 1993; Foote et al., 2004; Leech et al., 1999; Mikulowska et al., 1997). MIF is stored preformed in intracellular pools of immune cells to aid rapid release into the extracellular milieu (Calandra et al., 1994). The rapid induction of MIF release allows it to amplify production of other proinflammatory cytokines and override the effects of anti-inflammatory glucocorticoids (Bucala, 1996; Calandra et al., 1995). The MIF gene is highly conserved among species and is located on 22q11.2 (Budarf et al., 1997). Two functional polymorphisms within the promoter region have been identified, at positions -173, single nucleotide polymorphism (G to C), and at -794, tetranucleotide repeat sequence CATT (5-8) (Baugh et al., 2002; Donn et al., 2002).

The importance of MIF in inflammatory disease was first highlighted in the 1990's where it was found to potentiate lethal endotoxemia (Bernhagen et al., 1993). Following this, MIF has been extensively studied and has been shown to play a role in many diseases such as rheumatoid arthritis (Leech et al., 1999), systemic lupus erythematosus (Foote et al., 2004), multiple sclerosis (Benedek et al., 2017), severe acute pancreatitis (Sakai et al., 2003), and autoimmune kidney disease (Lan et al., 1997). These studies highlight the importance of MIF as higher levels are associated with more severe forms of each of these diseases. The CATT MIF polymorphism in the promoter region comprises of 5-8 repeats of the CATT sequence and has been found to be important in a range of inflammatory diseases (Awandare et al., 2009; Baugh et al., 2002; Benedek et al., 2017; Das et al., 2016; Liu et al., 2018; Savva et al., 2016; Sreih et al., 2011). Functionally, the CATT polymorphism affects the

activity of the MIF promoter where a shorter number of CATT repeats correlates with lower transcriptional activity, whilst longer numbers of CATT repeats within promoter region of the MIF gene correlates with more severe disease, particularly among the 7/7 and 8/8 haplotypes.

Asthma is a complex multifactorial disease affecting over 300 million people worldwide (*Global Initiative for Asthma. Global Strategy for Asthma management and Prevention, 2020. Available from: www.ginasthma.org.*, n.d.). Allergic asthma is characterised by sensitisation to specific and/or non-specific stimuli resulting in airway hyperresponsiveness (AHR), airway inflammation, and goblet cell metaplasia (Djukanović et al., 1990; Holgate et al., 2015). Repeated exacerbations lead to remodelling and narrowing of the airways (Al-Muhsen et al., 2011). Common environmental stimuli include house dust mite (HDM), mould, cigarette smoke, and pet dander (Custovic, 2015; Kim et al., 2010; Robays et al., 2009). HDM, a trigger in up to 85% of asthmatic patients (Nelson et al., 1996), has proteolytic activity to cleave epithelial tight junctions after inhalation to permit uptake by submucosal antigen-presenting cells surrounding the upper airways (Gregory and Lloyd, 2011). Lung inflammation is orchestrated by the release of prototypical Th2 cytokines, IL-4, IL-5, and IL-13 which drive the release of inflammatory mediators into the surrounding microenvironment (Robinson et al., 1992; Walker et al., 1992).

MIF has been shown to be one of these inflammatory mediators and plays a central role in the context of asthma. The link between MIF and asthma was first identified when elevated levels of MIF were detected in the bronchoalveolar lavage fluid of asthma patients compared to healthy controls (Rossi et al., 1998). Higher levels of MIF was also detected in the sputum and serum of asthma patients with the highest

levels of MIF present in severe steroid resistant cases (Yamaguchi et al., 2000). Current mouse models have knocked out or blocked MIF which have shown to reduced disease pathogenesis (Amano et al., 2007; Das et al., 2011; Kobayashi et al., 2006; Li et al., 2021; Mizue et al., 2005; Wang et al., 2006). Instead we have taken the novel approach of exposing humanised mice with the high-expressing $CATT_7$ allele or the low expressing $CATT_5$ allele to HDM to establish the role of MIF on disease severity. Previous investigations have shown that human and murine CD74 share 77% homology whilst human and murine MIF share 85% homology. This high conservation permits the binding of human MIF to murine CD74 to initiate MIF signalling cascades (Chen et al., 2023, 2021; Pantouris et al., 2015).

We hypothesise that elevated levels of MIF as a result of $CATT_7$ allele will further potentiate HDM driven asthma pathophysiology leading to more severe disease compared to the low expressing $CATT_5$ variant.

The objectives of this chapter are outlined as follows:

(1) Characterise the effect of MIF allelic variants on human *Mif* gene expression and protein production under basal and stimulated conditions

(2) Investigate the role of MIF and the influence of the CATT polymorphism using humanised $CATT_5$ and $CATT_7$ mice in a HDM model of allergic airway inflammation and remodelling

2.3 Materials and Methods

2.3.1 Ethics Statement

All procedures involving the use of animals or human materials were carried out by licensed personnel. Ethical approval for all work was granted by the biological research ethics committee of Maynooth University (BRESC-2018-013). Project authorization was received from the scientific animal protection unit of the health products regulatory agency (HPRA) under AE19124/P022 whereby the terms of the animal experiments within this project were outlined and adhered to.

2.3.2 Humanised MIF mice

Two C57BL/6N mouse strains expressing the human high- or low-expression *MIF* alleles (*MIF*^{CATT7} [(C57BL/6NTac-Miftm3884.1(MIF)Tac-Tg(CAG-Flpe)2Arte] and *MIF*^{CATT5} [C57BL/6NTac-Miftm3883.1(MIF)Tac-Tg(CAG-Flpe)2Arte] mice) were created using vector-based recombinant replacement of murine *Mif* by Taconic Biosciences (Rensselaer, NY) (Fig. 2.1). Validation of the expression of human and not murine *MIF* mRNA was verified by qPCR, and -794 CATT-length dependent stimulated MIF production was confirmed *in vivo* (Shin et al., 2022).

2.3.3 Preparation of cell suspensions

A single cell suspension of splenocytes from the spleens of wildtype, $CATT_5$, and $CATT_7$ mice were prepared by mashing spleens through a 40 μm pore cell strainer. Splenocytes were centrifuged at 300 g and red blood cells were lysed with RBC lysis

buffer for 2 minutes. Cells were then quenched with warm Roswell Park Memorial Institute (RPMI) 1640 medium GlutaMAX™ (Gibco, Paisley, UK) supplemented with 10% (v/v) foetal bovine serum (FBS) (BioSera), 1% (v/v) Penicillin/Streptomycin (Sigma-Aldrich), and 0.1% mercaptoethanol. $1x10^6$ splenocytes were seeded into wells of a 6 well plate.

Femurs and tibias were flushed with warm RPMI 1640 medium GlutaMAX™ supplemented with 10% (v/v) FBS (BioSera) and 1% (v/v) Penicillin/Streptomycin (Sigma-Aldrich). Cells were collected and seeded into T175 flasks in cRPMI supplemented with 10% L929 conditioned medium. L929 cell line produces high amounts of macrophage colony stimulating factor (M-CSF) and other proteins stimulating macrophage differentiation (Heap et al., 2021). $1x10^6$ bone marrow-derived macrophages (BMDMs) were seeded into wells of a 6 well plate.

2.3.4 HDM-induced airway inflammation model

MIF^{CATT7}, *MIF*^{CATT5} or littermate control wildtype (WT) mice were challenged with 25 µg HDM extract (*Dermatophagoides pteronyssinus,* Greer Laboratories, Lenoir, NC, USA) in 25 µl phosphate buffered saline (PBS) intranasally 3 days weekly for 3 weeks under light isoflurane anaesthesia. Control mice were challenged with 25 µl PBS under the same conditions. Vials of whole body Der p HDM extract contained a protein content between 12-43 mg with endotoxin levels ranging from 400-20000 EU/vial. Batches of HDM were validated by analysing haematoxylin and eosin staining of lung sections in WT mice. This to ensure comparable levels of immune cell infiltration and disease severity were achieved in each batch.

2.3.5 Quantitative Real-time PCR

Total RNA was isolated from lung tissue homogenised in TRIzol reagent (Thermo Fisher Scientific, Carlsbad, CA, USA) as per manufacturer's instructions. cDNA was generated using qScript cDNA synthesis kit (Quantabio, Beverly, MA, USA) and quantitative real-time PCR was performed using PerfeCTa SYBR green fastmix (Quantabio) according to manufacturer's instructions. The following primers were used: murine HPRT: [5'-AGG GATT TGA ATC ACG TTT G-3' (forward) and 5'-TTT ACT GGC AAC ATC AAC AG-3' (reverse)]; human MIF: [5'-CAG GGT CTA CAT CAA CTA TTA C-3' (forward) and 5'-TTA TTT CTC CCC ACC AGA AG-3' (reverse)].

2.3.6 Collection of BALF

Bronchoalveolar lavage (BAL) fluid was obtained through 3 aspirations of PBS. After centrifugation, protease inhibitor (Roche Diagnostics, Mannheim, Germany) was added to the supernatants before Th2 cytokine analysis. Total numbers of viable BAL cells were counted using ethidium bromide/ acridine orange staining on haemocytometer slides. $1x10^5$ cells were cytospun onto microscope slides and stained with Kwik Diff kit stain (Richard-Allan Scientific, Kalamazoo, MI, USA). Differential cells counts were derived by counting a minimum of 300 leukocytes on randomly selected fields under a light microscope at 20X magnification.

2.3.7 Enzyme-linked immunosorbent assay (ELISA)

Levels of mIL-4 (Biolegend, San Diego, CA, USA), mIL-5 (Biolegend), mIL-13 (eBioscience, San Diego, CA, USA), and hMIF (R&D Systems, Minneapolis, MN, USA) were determined using commercial ELISA kits, according to manufacturer's instructions.

2.3.8 Lung Histology

Lungs were removed and fixed in 10% neutral buffered formalin, paraffin embedded, and 5 μm slices were mounted onto slides for histological analysis. Lung tissue was stained with haematoxylin and eosin (H&E), periodic acid-Schiff (PAS) or Masson's Trichrome to analyse immune cell infiltration, goblet cell hyperplasia or extracellular matrix deposition respectively. H&E analysis was carried out as previously described (Ehrentraut et al., 2013). Briefly, blinded scoring was performed as follows: infiltration or aggregation of inflammatory cells in air space or vessel wall: 1 only wall, 2 1–5 cells in air space, 3 intermediate, 4 severe (air space congested); interstitial congestion and hyaline membrane formation: 1 normal lung, 2 moderate, 3 intermediate, 4 severe; haemorrhage: 0 absent, 1 present. Goblet cell hyperplasia was determined by the % of PAS positive cells in airways relative to airway diameter. Extracellular matrix deposition was calculated by analysing the % of positive staining using the trainable Weka segmentation plugin on Fiji open-source software.

2.3.9 Lung mechanics

Analysis of airway responsiveness to methacholine (Sigma-Aldrich, Laramie, WY, USA) 24 hours post final HDM challenge was performed using FlexiVent system (Scireq, Montreal, QC, Canada) as previously described (Cyphert-Daly et al., 2019). Briefly, mice were anaesthetized with a cocktail of ketamine and medetomidine before being cannulated and mechanically ventilated at respiratory rate of 150 breaths/min, a tidal volume of 10 ml/kg and a positive-end respiratory pressure of 3 cmH_2O. To ensure passive breathing, 0.5 mg/kg atracurium besilate (Kalcex, Riga, Latvia) was administered before commencement of the dose response script. Increasing concentrations of methacholine (0, 3.125, 12.5, 25 mg/ml) were aerosolised using the FlexiVent FX aeroneb nebuliser attachment and readings of airway resistance (R_N), tissue damping (G) and tissue elastance (H) were recorded.

2.3.10 Statistical analysis

All data are presented as mean ± SEM. Results of two or more groups were compared by analysis of variance (ANOVA) followed by the *post-hoc* Tukey's multiple comparison test. Response to different concentrations of methacholine were analysed by 2way 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.

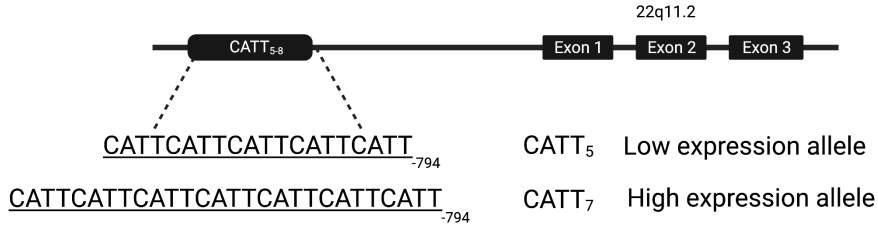


Figure 2.1. Schematic of the human MIF gene and the repeat polymorphism CATT in the MIF promoter region (rs5844572). The human MIF gene located on chromosome 22 (22q11.2) is composed of 3 short exons made up of 107, 172, and 66 base pairs interspersed by 2 introns. The functional polymorphism CATT which is repeated 5 to 8 times lies at position -794.

2.4 Results

2.4.1 Functional -794CATT polymorphisms lead to different expression levels of hMIF under basal and disease conditions.

To fully characterise the effect of the CATT microsatellite repeat in the promoter region of the MIF gene we analysed hMIF production under basal and disease conditions. BAL fluid obtained from naïve $CATT_7$ mice displayed markedly higher levels of human MIF compared to $CATT_5$ mice (Fig 2.2A). BMDMs and splenocytes were also isolated from naïve $CATT_7$, $CATT_5$, and WT C57BL/6 mice. BMDMs and splenocytes isolated from $CATT_7$ mice secrete significantly higher levels of hMIF than $CATT_5$ mice (Fig. 2.2B, 2.2C). Upon stimulation with HDM intranasally 3 times a week for 3 consecutive weeks (Fig. 2.2D), BAL fluid was obtained, and lungs were snap frozen for RNA isolation and qPCR analysis. In a disease environment, $CATT_7$ mice had significantly higher levels of hMIF in the BAL fluid relative to $CATT_5$ (Fig. 2.2E). In addition, there was a significant increase in the mRNA expression of human *Mif* in the $CATT_7$ mice compared to the $CATT_5$ (Fig. 2.2F).

These data comprehensively show that the CATT polymorphism is responsible for differential production of hMIF under basal and disease conditions. This provides a valuable tool to investigate the impact of hMIF polymorphisms in the pathophysiology of a mouse model of house dust mite induced allergic airway inflammation.

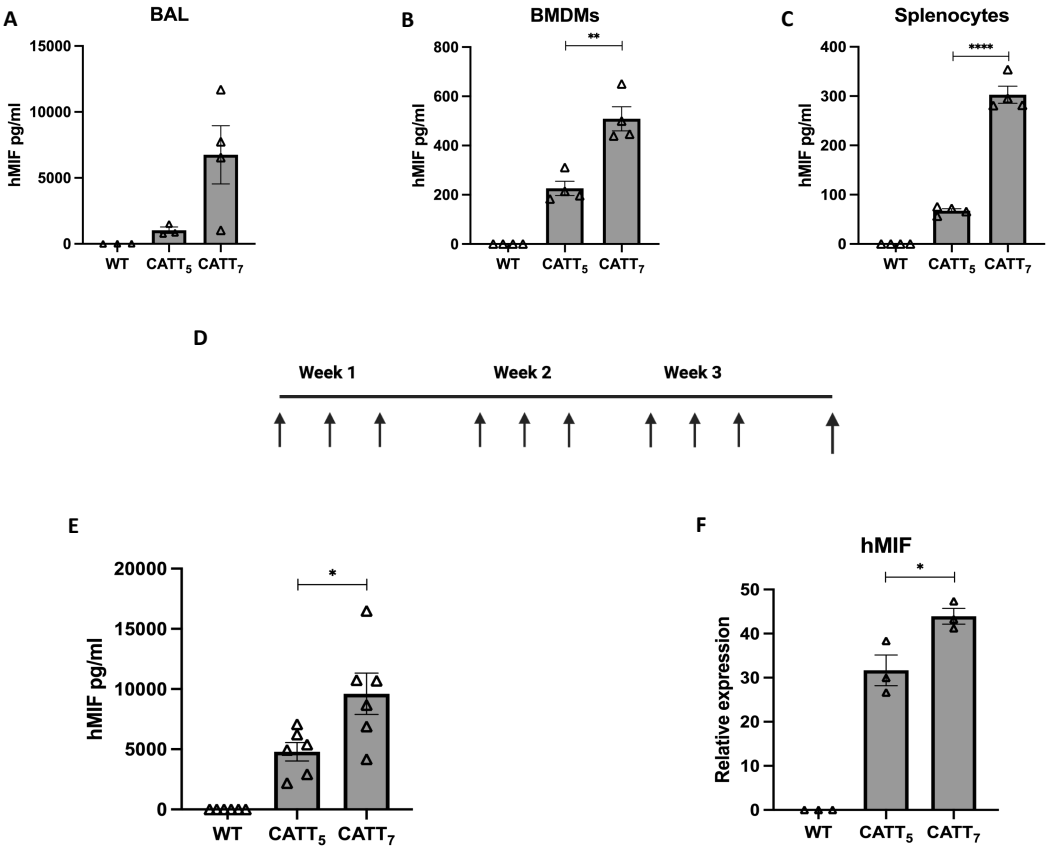


Figure 2.2. Functional -794CATT polymorphisms lead to different expression levels of hMIF under basal and disease conditions. A hMIF levels in BAL fluid of naïve WT, $CATT_5$ and $CATT_7$ mice. **B** hMIF production from BMDMs of WT, $CATT_5$ and $CATT_7$ mice. **C** hMIF production from splenocytes of WT, $CATT_5$ and $CATT_7$ mice. **D** Timeline for HDM exposure. Mice were intranasally challenged with HDM 3 times a week for 3 consecutive weeks. **E** Levels of hMIF detected in the BAL fluid of WT, $CATT_5$ and $CATT_7$ mice challenged with HDM. **F** Relative expression of hMIF detected in lung homogenates of WT, $CATT_5$ and $CATT_7$ mice challenged with HDM detected by real-time PCR. Data are presented as mean ± SEM; N=3-6 per group. Statistical analysis was carried out using one-way ANOVA followed by the *post-hoc* Tukey's multiple comparison test where *p<0.05, **p<0.01, ****p<0.0001.

2.4.2 Human $CATT_7$ allele significantly increases the Th2 signature in a HDM model of allergic airway inflammation

Absence of MIF in models of allergic inflammation has been shown to reduce the levels of Th2 cytokines in the BAL fluid (Das et al., 2011; Mizue et al., 2005). To study the effect of different levels of endogenous MIF on asthma severity, $CATT_7$, $CATT_5$, and WT C57BL/6 mice were challenged with HDM. 4 hr post final challenge the mice were sacrificed, and the BAL fluid was obtained. $CATT_7$ mice challenged with HDM have significantly higher numbers of immune cells in the bronchoalveolar lavage fluid compared to WT mice (Fig. 2.3A). $CATT_7$ mice also exhibit a marked increase in total BAL cell number compared to $CATT_5$ although not significant (Fig. 2.3A). Differential cell counts demonstrated that the predominant cell type in the BAL fluid are eosinophils (Fig. 2.3B, 2.3C, 2.3D).

To further characterise the influence of hMIF on asthma pathophysiology we explored its effects on the prototypical Th2 signature in the BAL fluid. IL-4, IL-5, and IL-13 have been shown to be critical in the development of airway hyperresponsiveness, eosinophilic responses, and goblet cell hyperplasia, and responsible for the overall asthma phenotype. Low levels of Th2 cytokines were detected in the BAL fluid of $CATT_5$ and WT HDM mice (Fig. 2.3E, 2.3F, 2.3G). However, we observed significantly elevated levels of IL-4, IL-5, and IL-13 in $CATT_7$ HDM compared to $CATT_7$ PBS group (Fig. 2.3E, 2.3F, 2.3G). Furthermore, significantly higher levels of IL-4 and IL-13 were detected in $CATT_7$ HDM compared to $CATT_5$ and WT HDM (Fig. 2.3E, 2.3G). A marked increase in IL-5 was also detected although not significant (Fig. 2.3F). These data show that the $CATT_7$ polymorphism contributes to significant

eosinophil infiltration into the bronchoalveolar space and generates a prominent Th2 cytokine profile which may contribute to a more severe asthma phenotype.

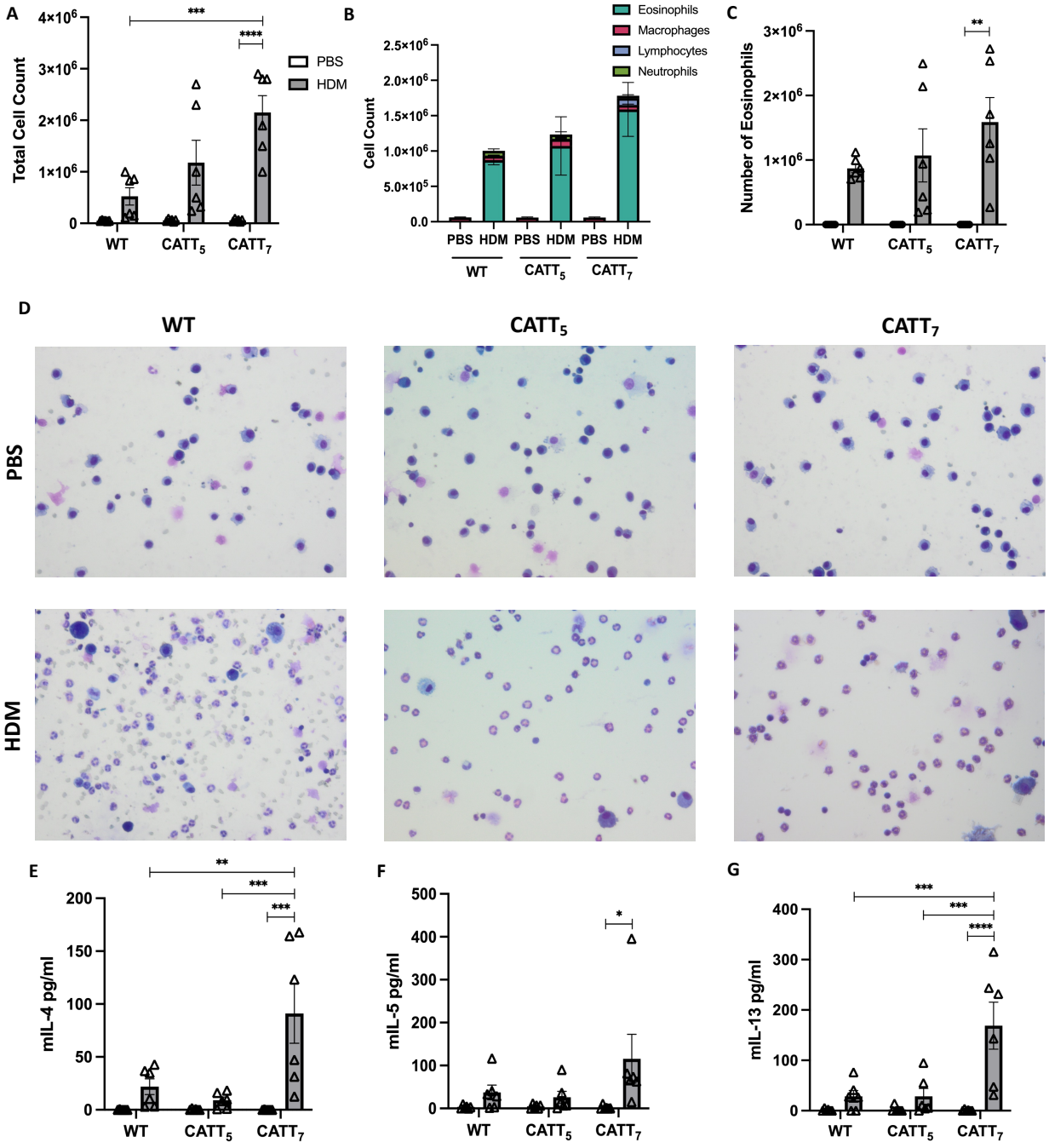


Figure 2.3. Human $CATT_7$ allele significantly increases the Th2 signature in a HDM model of allergic airway inflammation. **A** Total cell count recovered from the BAL. **B** Number of macrophages, eosinophils (**C**), lymphocytes, and neutrophils were determined by differential staining of cytospins. **D** Representative images of cells obtained from the BAL fluid at 20X magnification. Cytokine levels of **E** IL-4, **F** IL-5 and **G** IL-13 in the BAL fluid determined by ELISA. Data are presented as mean ± SEM; N=6 per group. Statistical analysis was carried out using one-way ANOVA followed by the *post-hoc* Tukey's multiple comparison test where *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Part of these data were generated in collaboration and appear in the thesis of Hazel Dunbar.

2.4.3 Human $CATT_7$ allele exacerbates hallmarks of asthma pathophysiology

To further investigate the effect of elevated Th2 cytokine production in the BAL fluid of the $CATT_7$ mice we examined the lung histology. Lung tissue from $CATT_7$, $CATT_5$, and WT mice exposed to either HDM or PBS control were fixed in formalin and sectioned onto slides.

Excessive production of mucus and the associated pathophysiological changes are hallmarks in a range of respiratory diseases including asthma (Boucherat et al., 2013). We investigated the effect of the CATT polymorphism on goblet cell hyperplasia by staining lung tissue with PAS (Fig. 2.4A). PBS groups exhibited very low levels of PAS positive staining, whilst $CATT_5$ HDM exhibit slightly higher numbers of PAS positive cells relative to the control compared to the WT (Fig. 2.4A, 2.4B). Strikingly, we found that $CATT_7$ mice challenged with HDM have significantly higher levels of goblet cell hyperplasia compared to $CATT_5$ and WT HDM (Fig. 2.4B).

In addition to investigating goblet cell hyperplasia, we examined the effect of the -794CATT polymorphism on airway remodelling. Sections were stained with Masson's trichrome stain to evaluate subepithelial collagen deposition (Sheehan and Hrapchak, 1980). $CATT_5$, and WT mice displayed similar levels of disease pathology, exhibiting small but significant increases in collagen deposition compared to the PBS controls (Fig. 2.5A, 2.5B). Interestingly, $CATT_7$ HDM display significantly higher levels of subepithelial fibrosis compared to the lower MIF expressing $CATT_5$ allele and WT groups (Fig. 2.5B); suggesting that high levels of hMIF contributes to airway remodelling in a HDM model of allergic airway inflammation.

H&E staining revealed that HDM significantly induces cellular infiltration surrounding the airways in all groups compared to the PBS control (Fig. 2.6A, 2.6B).

However, $CATT_7$ HDM exhibited significantly higher airway immune cell infiltration compared to both $CATT_5$ and WT HDM (Fig. 2.6A, 2.6B). Analysis of H&E score revealed no significant difference in immune cell infiltration between the $CATT_5$ and WT HDM groups (Fig. 2.6A).

These data suggest that high levels of human MIF exacerbate asthma pathophysiology by increasing mucin production through the induction of goblet cell hyperplasia, increasing deposition of subepithelial collagen thereby contributing the airway remodelling, and increasing the infiltration of immune cells surrounding the airways.

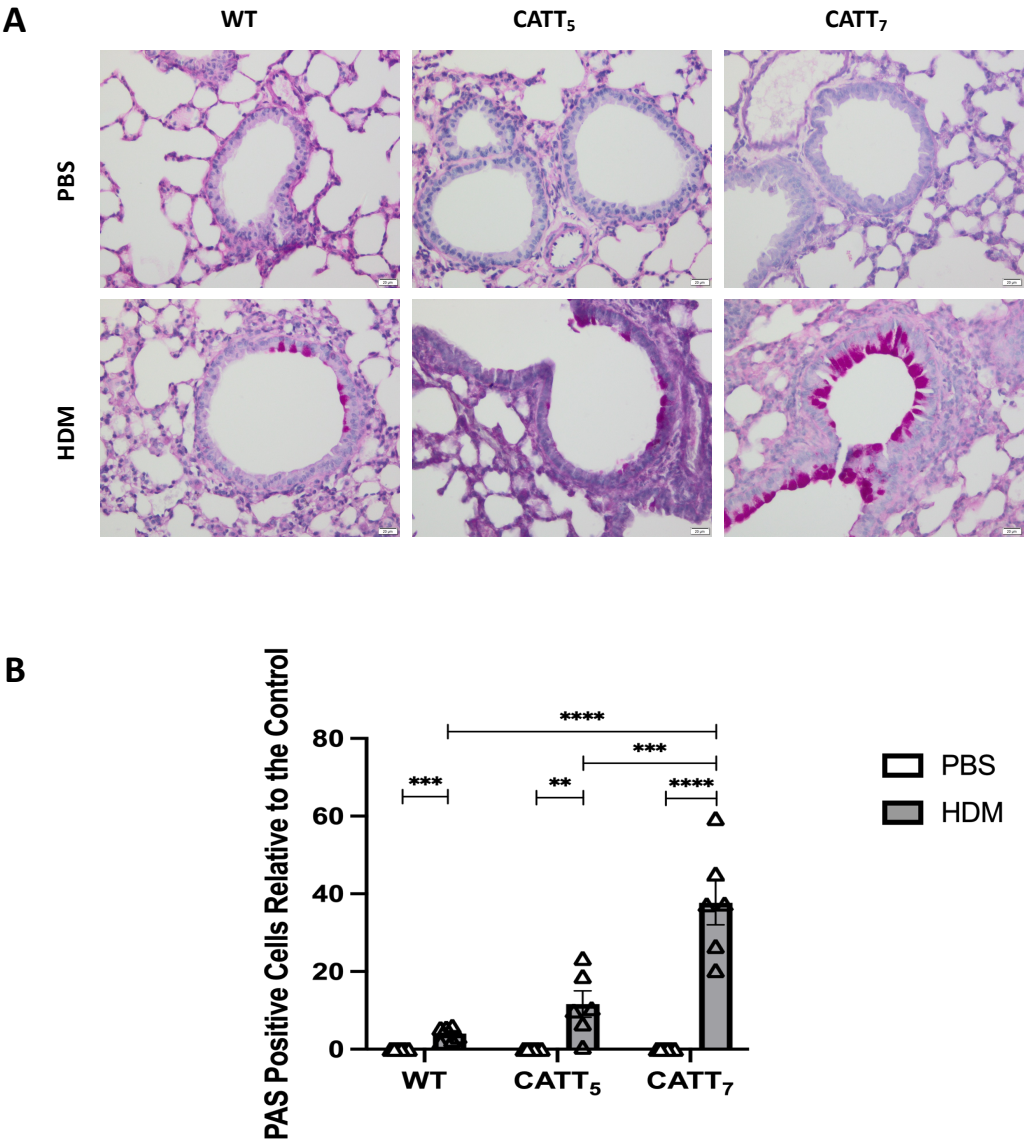


Figure 2.4. Human $CATT_7$ allele exacerbates goblet cell hyperplasia in a house dust mite model of allergic asthma. . Periodic acid Schiff (PAS) staining of lung tissue from WT, $CATT_5$ and $CATT_7$ mice. PBS and HDM groups received PBS or HDM i.n. 3 times a week for 3 consecutive weeks. Mice were sacrificed on day 21. **A** Representative images of lung tissue stained with periodic acid Schiff at 20X magnification, scale bar = 20 µm. **B** Goblet cell metaplasia was investigated through the quantitation of PAS positive cells. Data are presented as mean ± SEM; N=6 per group. Statistical analysis was carried out using one-way ANOVA followed by the *post-hoc* Tukey's multiple comparison test where **p<0.01, ***p<0.001, ****p<0.0001. Part of these data were generated in collaboration and appear in the thesis of Hazel Dunbar.

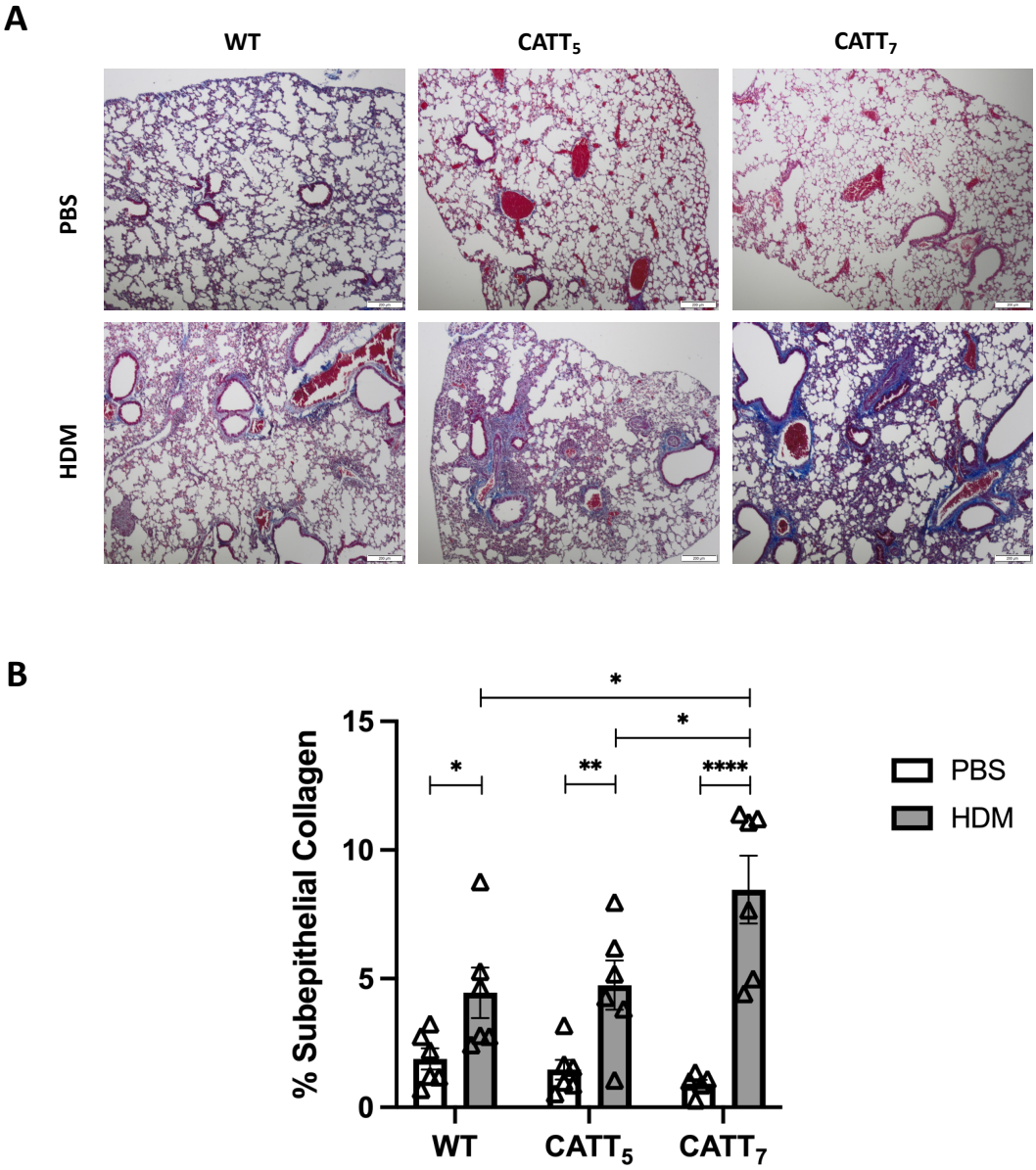


Figure 2.5. Human $CATT_7$ allele significantly increases collagen deposition in a house dust mite model of allergic asthma. Masson's trichrome staining of lung tissue from WT, $CATT_5$ and $CATT_7$ mice. PBS and HDM groups received PBS or HDM i.n. 3 times a week for 3 consecutive weeks. Mice were sacrificed on day 21. **A** Representative images of lung tissue stained with Masson's trichome at 4X magnification, scale bar = 200 µm. **B** Quantitation of % subepithelial collagen. Data are presented as mean ± SEM; N=6 per group. Statistical analysis was carried out using one-way ANOVA followed by the *post-hoc* Tukey's multiple comparison test where *p<0.05, **p<0.01, ****p<0.0001. Part of these data were generated in collaboration and appear in the thesis of Hazel Dunbar.

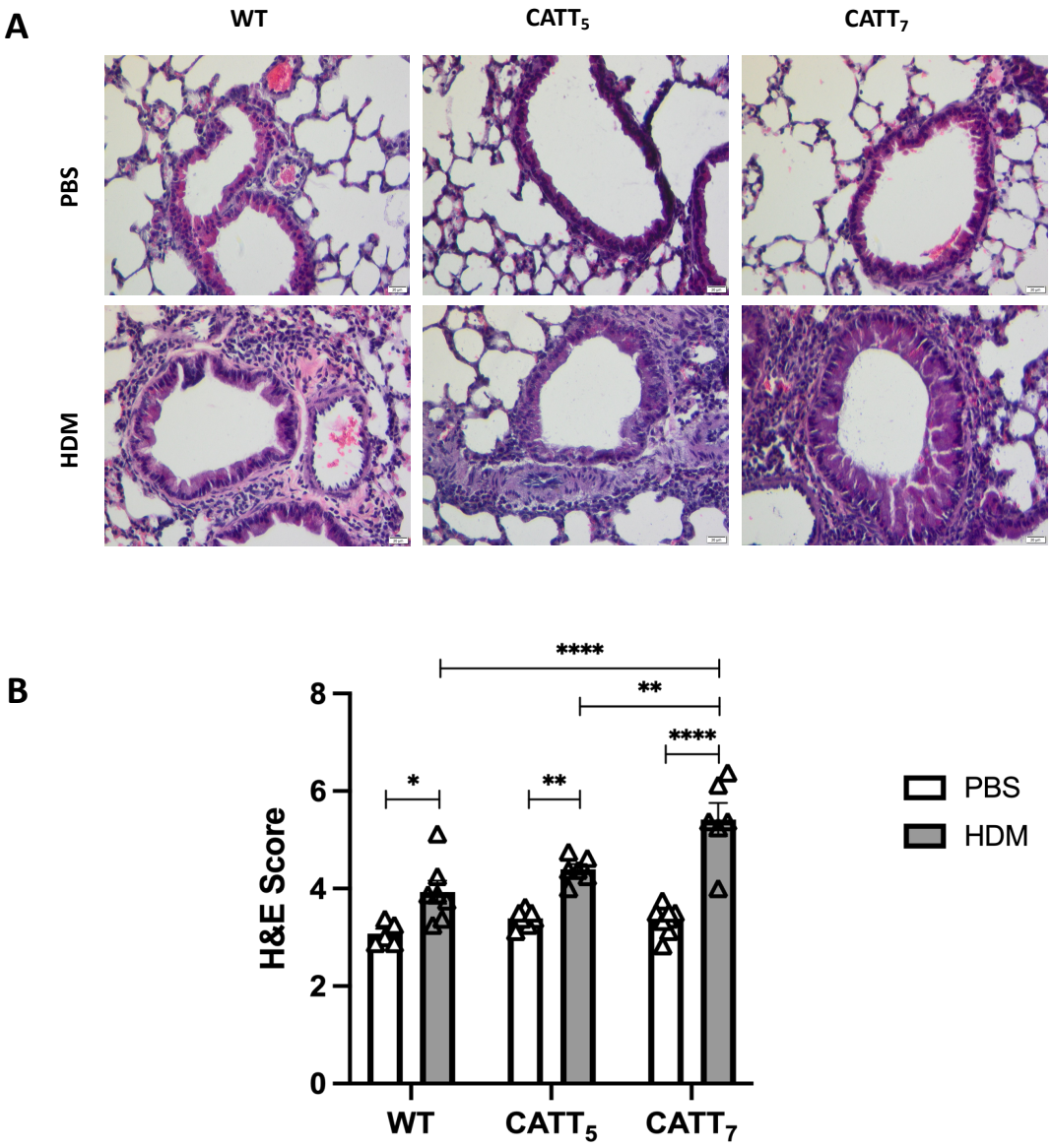


Figure 2.6. Human $CATT_7$ allele exacerbates airway inflammation in a house dust mite model of allergic asthma. Haematoxylin and eosin (H&E) staining of lung tissue from WT, $CATT_5$ and $CATT_7$ mice. PBS and HDM groups received PBS or HDM i.n. 3 times a week for 3 consecutive weeks. Mice were sacrificed on day 21. Representative images of lung tissue stained with H&E at 20X magnification, scale bar = 20 µm. . **B** Quantitation of airway inflammation in H&E-stained lung tissue. Data are presented as mean ± SEM; N=6 per group. . Statistical analysis was carried out using one-way ANOVA followed by the *post-hoc* Tukey's multiple comparison test where *p<0.05, **p<0.01, ****p<0.0001. Part of these data were generated in collaboration and appear in the thesis of Hazel Dunbar.

2.4.4 Changes in lung mechanics in response to increasing concentrations of methacholine in HDM challenged $CATT_7$, $CATT_5$ and WT mice

Airway hyperresponsiveness and remodelling are major hallmarks of asthma, and thus it is important for preclinical models to reflect this. It has been well documented that due to genetic differences, C57BL/6 mice have a higher resistance to airway hyperresponsiveness compared to the more sensitive BALB/c mice in acute airway inflammatory models (Atochina et al., 2003; Hove et al., 2009). We examined HDM-induced AHR in response to aerosolised methacholine challenge using the FlexiVent system. $CATT_7$ HDM mice exhibited a marked increase in airway resistance (R_N) at 12.5 mg/ml and 25 mg/ml doses compared to the rest of the groups (Fig. 2.7A). Analysis of the area under curve (AUC) revealed that R_N in response to methacholine challenge was significantly higher in $CATT_7$ HDM mice compared to PBS control (Fig. 2.7D). A trend of increased tissue (G) and tissue elastance (H) was also observed in the $CATT_7$ mice at the 25 mg/ml dose (Fig. 2.7B, 2.7C, 2.7E, 2.7F). No difference was observed between the other groups.

These data show that $CATT_7$ mice challenged with HDM exhibit higher levels of airway remodelling and AHR compared to WT littermate control C57BL/6 mice.

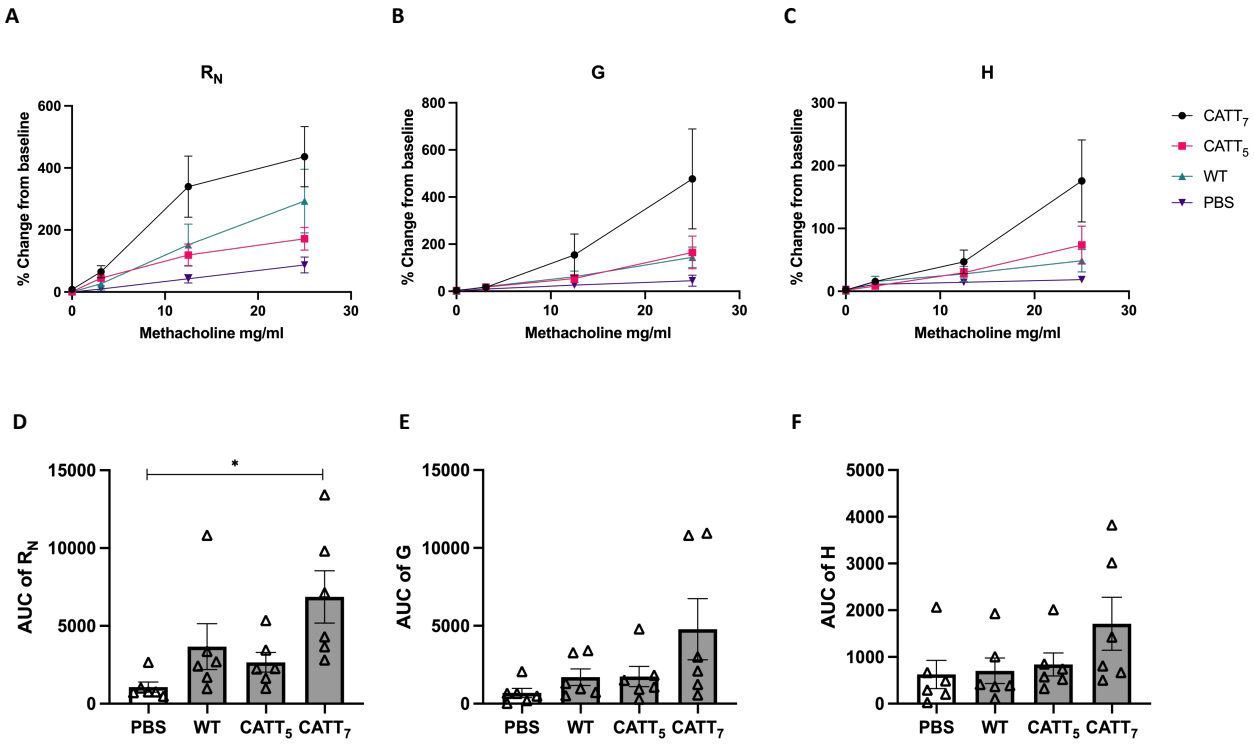


Figure 2.7. Changes in lung mechanics in response to increasing concentrations of methacholine in HDM challenged $CATT_7$, $CATT_5$ and WT mice. Airway hyperresponsiveness determined by **A** airway resistance, **B** tissue damping, and **C** tissue elastance (R_N, G, and H respectively). Data are presented as peak response normalized to the baseline and expressed as % increase over baseline ± SEM. Mean area under curve (AUC) of **D** R_N, **E** G, and **F** H against increasing concentrations of methacholine was determined; N=6 per group. Statistical analysis was carried out using one-way ANOVA followed by the *post-hoc* Tukey's multiple comparison test where *p<0.05.

2.5 Discussion

In this study, we have shown that increased expression of human MIF leads to elevated allergic airway inflammation in a clinically relevant model of allergic asthma. We have demonstrated that differences in human MIF production from $CATT_5$ and $CATT_7$ humanised transgenic mice result in different pathophysiological phenotypes. $CATT_7$ mice challenged with HDM present with pulmonary eosinophilia and elevated levels of IL-4, IL-5, and IL-13 in the BAL fluid. This exacerbated Th2 cytokine profile in the lungs of $CATT_7$ results in increased immune cell infiltration, goblet cell hyperplasia and subepithelial fibrosis deposition.

Previous models have demonstrated the importance of MIF in the pathogenesis of asthma through knockdown (Li et al., 2021; Magalhães et al., 2009, 2007), inhibition (Amano et al., 2007; Chen et al., 2010; Kobayashi et al., 2006; Lan et al., 2020; Li et al., 2020) or blocking of a MIF binding site (Das et al., 2011). We have taken the novel approach of using humanised mouse models with low- and high-expressing human MIF alleles. The -794 CATT MIF polymorphism has been found to be important in a range of diseases (Awandare et al., 2009; Baugh et al., 2002; Benedek et al., 2017; Das et al., 2016; Liu et al., 2018; Savva et al., 2016; Sreih et al., 2011), where high-expressing MIF alleles are associated with higher levels of inflammation and a more severe disease phenotype. The results of this study have established a dominant role of MIF allelic variants in the inflammatory lung manifestations of a relevant mouse model of asthma.

Mizue et al. first demonstrated the association between the CATT polymorphism and asthma whereby asthma patients with the low-expressing $CATT_5$ MIF allele presented with less severe clinical disease, higher FEV1 and fewer hospital admissions (Mizue

et al., 2005). Our present findings align with the existing literature as $CATT_5$ mice challenged with HDM had a milder phenotype with less immune cell infiltration, lower Th2 signature, leading to lower goblet cell metaplasia, airway remodelling and a more subdued response to methacholine challenge compared to the high-expressing $CATT_7$ allele. These data confirm the human genetic data and other supportive clinical studies of the MIF cytokine.

In addition to highlighting the role of MIF in asthma we have generated a scale of inflammatory disease which may allow more accurate investigation of potential new drugs to treat severe asthma. The use of a clinically relevant allergen in a disease driven by a human cytokine provides opportunity to determine mechanistic actions and therapeutic efficacy of human cell therapies such as mesenchymal stromal cells and regulatory T cells in the treatment of severe inflammatory disorders. This model also allows for examination of features which are more typically observed in chronic asthma models. The presentation of airway hyperresponsiveness and airway remodelling requires allergen exposure beyond 5 weeks (Johnson et al., 2004; Penton et al., 2013; Salehi et al., 2017; Vroman et al., 2017), however, $CATT_7$ mice challenged with HDM 3 times a week for 3 consecutive weeks exhibit a marked response to methacholine challenge and significant subepithelial collagen deposition compared to WT C57BL/6 mice. We have provided a reproducible model which exhibits features of airway remodelling and suggests the development of airway remodelling is MIF-dependent.

In summary, we have generated a model of severe asthma exhibiting features of chronic disease when challenged with a clinically relevant allergen, driven by a clinically relevant human cytokine. Furthermore, the validation of the humanised MIF

mouse will enable the utility of this model in downstream pharmaceutical development, both in asthma and other MIF-dependent diseases in the realm of airway disease, oncology, infection, and autoimmunity.

Chapter 3 Macrophage migration inhibitory factor (MIF) enhances Mesenchymal stromal cell (MSC) functions *in vitro*

3.1 Abstract

Macrophage migration inhibitory factor (MIF) is a pleiotrophic cytokine involved in driving innate and adaptive immune responses. MIF possesses a complex immunobiology through its ability to bind to multiple receptors making it a master regulator of host immune responses. MIF has been shown to be highly expressed in some diseases and its dysregulation has been implicated in the pathogenesis of a range of inflammatory and proliferative disorders. Mesenchymal stromal cells (MSCs) rely on the influence of extrinsic factors within the surrounding microenvironment to activate them to potentiate their therapeutic effects. Therefore, understanding disease microenvironments in which MSCs are exposed to is important in improving their potential as a future therapy. We sought to investigate the interactions between MSCs and hMIF *in vitro* to understand its influence on MSC biology. Endogenous human MIF secreted from bone marrow-derived macrophages (BMDMs) of transgenic mice expressing high or low levels of human MIF were used in the assays. The effect of hMIF on MSC chemotaxis, survival, expansion, and immunomodulation were analysed. hMIF was shown to drive migration of MSCs through binding to CXCR4, not CXCR2. MIF mediated chemotaxis occurred in a dose dependent manner. Moreover, MSC expansion was enhanced in the presence of high levels of hMIF. Interestingly, high levels of hMIF induced COX-2 expression alone and significantly amplified expression in combination with TNF-α. Functionally, we observed that pre-treatment of MSCs with high levels of MIF improved their ability to suppress T cell proliferation in a co-culture assay.

In conclusion, our data reveal that MIF can improve MSCs functions *in vitro*. This demonstrates that MSCs may thrive in environments where MIF production is

dysregulated. Furthermore, these data provide evidence for the use of MIF in MSC licensing which may have broad implications for the effective translation of MSC directed therapies.

3.2 Introduction

Mesenchymal stromal cells (MSCs) are a heterogenous population of non-haematopoietic multipotent progenitor cells that display reparative properties. MSCs have also been shown to possess a range of immunoregulatory abilities and are capable of affecting the functionality of both innate and adaptive immune cells (Le Blanc and Mougiakakos, 2012; Prockop and Oh, 2012). MSCs are plastic in their function and display both pro-inflammatory and anti-inflammatory effects based on stimuli from the surrounding microenvironment requiring threshold levels of inflammation to polarise them into an anti-inflammatory phenotype (English, 2013; English et al., 2014; C. H. Masterson et al., 2021; Weiss et al., 2019). Despite MSCs being one of the most experimentally studied cell therapies worldwide, translation of promising preclinical studies into a clinical setting has proved difficult. Further optimization of MSC-based therapy is urgently needed; a deeper understanding of the interactions of MSCs and pro-inflammatory cytokines within diseased microenvironments is needed to increase their therapeutic potential. Priming of MSCs with cytokines present in the inflammatory microenvironment has been found to facilitate MSC anti-inflammatory function. Licensing human BM-MSCs with tumour necrosis factor alpha (TNF-α) or interferon gamma (IFN-γ) have been shown to produce immunomodulatory secreted factors such as prostaglandin E2 (PGE2) (English et al., 2007), indolamine-2,3-dioxygenase (IDO) (Boyt et al., 2020; Prasanna et al., 2010; Roemeling-van Rhijn et al., 2013), and TSG6 (Wang et al., 2018). In addition to the production of immunomodulatory factors, licensing has been shown to enhance MSC function by upregulating pro-survival pathways and increasing persistence *in vivo* (Baldari et al., 2017; Carty et al., 2021).

Macrophage migration inhibitory factor (MIF) is a pleiotropic cytokine with chemokine-like characteristics. MIF is an upstream regulator of the innate and adaptive immune system and its dysregulation can lead to a range of inflammatory diseases including asthma (Rossi et al., 1998), rheumatoid arthritis (Leech et al., 2003, 1999), systemic lupus erythematosus (Foote et al., 2004, 2004), multiple sclerosis (Benedek et al., 2017) and diabetes (Herder et al., 2006). In humans, a functional polymorphism has been identified within the MIF promoter region at position -794 where a tetranucleotide repeat sequence CATT can affect the transcriptional activity (Baugh et al., 2002). Functionally, the CATT polymorphism affects the activity of the MIF promoter where the shorter number of CATT repeats correlates with lower transcriptional activity, whilst longer numbers of CATT repeats within promoter region of the MIF gene correlate with higher transcriptional activity. The CATT MIF polymorphism in the promoter region comprises of 5-8 repeats of the CATT sequence and has been found to be important in a range of inflammatory diseases. Typically, patients with the low-expressing $CATT_5$ allele are associated with less severe disease compared to the other higher expressing haplotypes such as $CATT_7$ and $CATT_8$ which tend to experience much more severe disease (Baugh et al., 2002; Hizawa et al., 2004; Mizue et al., 2005; Shin et al., 2022).

In addition to being an important mediator of inflammation, MIF has shown to promote survival of cells by inhibiting p53 (Fukaya et al., 2016; Hudson et al., 1999; Leech et al., 2003; Mitchell et al., 2002). MIF has been shown to act in the resolution phase of tissue repair to promote survival and proliferation of endothelial cells, fibroblasts, epithelial cells, placental cells, neural crest derived cells (Djudjaj et al., 2017; Fallica et al., 2014; Hofmann et al., 2021; Ietta et al., 2018; Li et al., 2019; Ohta et al., 2012; Soppert et al., 2018). Additionally MIF has been shown to be a driver of

cell migration to sites of inflammation through its ability to act like a chemokine. MIF promotes the chemotaxis of dendritic cells, neutrophils, B cells, eosinophils, and myeloid derived suppressor cells through interaction with one or several of its receptors (Alampour-Rajabi et al., 2015; de Souza et al., 2015; Ives et al., 2021; Klasen et al., 2014; Santos et al., 2011; Zhang et al., 2017).

These properties make MIF an interesting candidate, and warrants further study of the interactions between MIF and MSCs. We hypothesise that MIF may act as a priming agent leading to improved MSC efficacy and immunomodulatory function in relevant inflammatory disease contexts. The few studies that have investigated the influence of MIF on MSCs have focussed on the pro-survival nature of MIF either by the modulation of cellular senescence (Palumbo et al., 2014; Xia et al., 2015b) or rescue from apoptosis (Xia and Hou, 2018). Furthermore, studies exploring the effect of MIF on MSC migration and homing have presented opposing conclusions (Lourenco et al., 2015; Xiong et al., 2012). We seek to consolidate the literature surrounding the interactions of MIF and MSCs in order to provide greater clarity to the field.

The objectives of this chapter are outlined as follows:

(1) Study the effect of human MIF on MSC migration *in vitro* and elucidate the mechanisms involved
(2) Investigate the effect of human MIF on MSC survival and expansion *in vitro*
(3) Determine the effect of human MIF on MSC immunomodulation by analysing key immunomodulatory mediators and MSCs ability to suppress human T cell proliferation in a co-culture assay

3.3 Materials and Methods

3.3.1 Ethical Approval

As stated in 2.3.1.

3.3.2 Animal Strains

Two C57BL/6N mouse strains expressing the human high- or low-expression *MIF* alleles (*MIF*^{CATT7} [(C57BL/6NTac-Miftm3884.1(MIF)Tac-Tg(CAG-Flpe)2Arte] and *MIF*^{CATT5} [C57BL/6NTac-Miftm3883.1(MIF)Tac-Tg(CAG-Flpe)2Arte] mice) were created using vector-based recombinant replacement of murine *Mif* by Taconic Biosciences (Rensselaer, NY). Validation of the expression of human and not murine *MIF* mRNA was verified by qPCR, and -794 CATT-length dependent stimulated MIF production was confirmed *in vivo* (Shin et al., 2022).

3.3.3 Human bone marrow derived MSC culture

Three different human bone marrow-derived MSC (BM-MSC) donors were obtained from RoosterBio Inc. (Frederick, MD, USA). MSC donors were validated based on their expression of CD73, CD90 and CD105, the lack of expression of the haematopoietic and endothelial markers CD11b, CD14, CD19, CD34, CD45, CD79a and HLA-DR. Furthermore, the functionality of MSCs were validated through their ability to secrete IDO following IFN-γ stimulation and ability to suppress human T cell proliferation in coculture. MSCs were first expanded in RoosterBio proprietary expansion medium (RoosterBasal and RoosterBooster) for the first two passages

according to manufacturer's instructions. Following this MSCs were cultured and maintained in Dulbecco's Modified Eagles Media Low Glucose (cDMEM, Sigma-Aldrich, Arklow, Wicklow, Ireland) supplemented with 10% (v/v) foetal bovine serum (FBS) (BioSera) and 1% (v/v) Penicillin/Streptomycin (Sigma-Aldrich). Human MSCs were seeded at $1x10^6$ cells per T175 flask and cultured at 37 °c in 5% CO_2. Media was replenished every 2-3 days, and cells split once they achieved 80% confluency. All experiments were carried out between passages 2-5.

3.3.4 Cryopreservation and recovery from liquid nitrogen

At passage 1 and 2, MSCs were frozen in CryoStore CS5 freezing medium at $1x10^6$ cells/ml (Sigma Aldrich). From passage 3-5, MSCs were suspended at $1x10^6$ cells/ml in freezing media (DMEM, 70% (v/v); FBS, 20% (v/v); dimethyl sulfoxide (DMSO), 10% (v/v)) and transferred to cryo-tubes (ThermoFisher Scientific, Surrey, UK). Cells were cooled at a rate of 1°c per minute to -80°c then transferred to liquid nitrogen for long term storage.

3.3.5 Generation of Endogenous Human MIF

Supernatants containing endogenous human MIF was generated from bone marrow-derived macrophages (BMDMs) of two C57BL/6 mouse strains expressing the high- or low expressing MIF alleles ($CATT_7$ and $CATT_5$, respectively) (Fig. 1). $CATT_7$, $CATT_5$ and littermate control wildtype (WT) mice were challenged with 25 µg house dust mite (HDM) extract 25 µg HDM extract (*Dermatophagoides pteronyssinus,*

Greer Laboratories, Lenoir, NC, USA) in 25 µl phosphate buffered saline (PBS) intranasally 3 days weekly for 3 weeks under light isoflurane anaesthesia. Four hours post final challenge, femurs and tibias were flushed with warm Roswell Park Memorial Institute (RPMI) 1640 medium GlutaMAX^{TM} (Gibco, Paisley, UK) supplemented with 10% (v/v) foetal bovine serum (FBS) (BioSera) and 1% (v/v) Penicillin/Streptomycin (Sigma-Aldrich). Cells were collected and seeded into T175 flasks in cRPMI supplemented with 10% L929 conditioned medium. L929 cell line produces high amounts of macrophage colony stimulating factor (M-CSF) and other proteins stimulating macrophage differentiation (Heap et al., 2021). After 96 hours, supernatants were collected, sterile filtered (0.22 µM pore size) and stored at -20°c. The conditioned media generated in this manner will be referred to as $CATT_7$ CM, $CATT_5$ CM, and WT CM hereafter (Fig.1A).

3.3.6 Chemotaxis Migration Assay

Cell migration was assessed using Sarstedt transwell polyethylene terephthalate (PET) inserts with 8 µM pore size (Nümbrecht, Germany). $1x10^4$ MSCs in serum free media were seeded into the apical compartment of the transwell chamber. 600 µl of chemotactic solution containing either recombinant human MIF in serum free media or endogenous human MIF from $CATT_5$ or $CATT_7$ CM was added to the basal compartment of the transwell chamber and incubated at 37°c overnight. A negative control of serum free media in the basal compartment was used. Where indicated, MIF inhibitors [(S,R)-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester, known as ISO-1, 100 µM] and [3-(29-methylphenyl)-isocoumarin, known as SCD-19, 100 µM] were used. In such cases, inhibitors of MIF were pre-incubated with

the chemotactic solution 1 hr before the migration assay. Furthermore, CXCR2 and CXCR4 chemokine receptor inhibitors [reparixin (40 μM) and AMD3100 (50 μg/ml), respectively, both from Sigma-Aldrich] were added to the assay. In those instances, MSCs were pre-incubated with the chemokine receptor inhibitor 1 hr before the assay. Migration was assessed in a similar manner as previously described (Lourenco et al., 2015). Briefly, MSCs that had migrated onto the underside of the insert were fixed with ice cold methanol and stained with 0.5% crystal violet then counterstained with haematoxylin. The inserts were cut out and mounted on microscope slides. Images were taken of 10 fields of view using a brightfield microscope (Olympus Life Science, Waltham, MA, USA) and number of migrated cells were counted.

3.3.7 Flow Cytometry

For the analysis of chemokine receptor expression, MSCs were seeded at $1x10^5$ cells per well in 6 well plates. MSCs were stimulated with either recombinant human MIF (rhMIF) (100 ng/ml) (a kind donation from R. Bucala, Yale School of Medicine, Yale University, New Haven, CT, USA) or $CATT_7$ CM (containing high levels of endogenous MIF) for 6 hr. To ensure there was no variation in receptor expression over the course of the migration assay, a timecourse of 1, 6 and 24 hr was also performed on cells treated with $CATT_7$ CM. The cells were detached from the plates using ethylenediaminetetraacetic acid (EDTA) solution (0.5mM) with agitation. Cells were incubated with the following flow cytometry antibodies: CD74 [PE (phycoerythrin); clone 5-329], CD182 (CXCR2) [PerCP (Peridinin Chlorophyll Protein Complex)-eFluor™710; clone 5E8-C7-F10], CD184 (CXCR4) [APC (allophycocyanin); clone 12G5]. All antibodies were purchased from ThermoFisher

Scientific. Cells were acquired and analysed on the Attune NxT Flow Cytometer (ThermoFisher Scientific).

3.3.8 Intracellular Staining of Immunomodulatory Factors

MSCs were seeded at $1x10^5$ cells per well in 6 well plates. MSCs were stimulated with $CATT_7$ BMDM conditioned media and pro-inflammatory cytokines [IL-4 (20 ng/ml), TNF-α (1 or 5 ng/ml), IFN-γ (1 or 5ng/ml)] for 24 hr. All cytokines were purchased from Peprotech (Cranbury, NJ, USA). Cells were incubated with 1X Brefeldin A (ThermoFisher Scientific) for 4 hours before harvest. Cells were prepared for intracellular staining using the Intracellular FoxP3 kit as per manufacturer's instructions. Cells were stained with COX-2 (PE; clone AS67) and IDO (eFluor660; clone eyedio) for 45 min. Cells were then washed in flow cytometry staining buffer and acquired using the Attune Nxt Flow Cytometer. COX-2 and IDO antibodies were purchased from BD Pharmingen (San Diego, CA, USA) and ThermoFisher Scientific, respectively.

3.3.9 Annexin V PI Apoptosis Assay

To induce apoptosis, MSCs in the presence or absence of $CATT_7$ CM were treated with staurosporine (STS, 500 nM) (Enzo Life Sciences, Lausen, Switzerland) for 6 hr at 37°c, 5% CO_2. Apoptotic cells were detected using Annexin V PI apoptosis detection kit (ThermoFisher Scientific) according to manufacturer's instructions. Briefly, both adherent and detached cells were washed in PBS following trypinisation. MSCs were resuspended in 1X Annexin binding buffer at a density of $1x10^6$ cells/ml and stained with 1 µl Annexin V APC at room temperature for 15 min. Cells were

washed and resuspended in Annexin binding buffer. 1 µl PI was added just before acquisition on the Attune Nxt Flow Cytometer.

3.3.10 MSC Expansion Assay

$1.4x10^5$ MSCs were seeded out into T25 flasks in cDMEM or 50:50 cDMEM and WT CM or $CATT_7$ CM for 72 hr. Cells were trypsinised and stained with ethidium bromide/ acridine orange and counted on a haemocytometer. MIF inhibitors ISO-1 (100 µM) and SCD-19 (100 µM) were used to determine MIF specificity. In such cases, inhibitors of MIF were pre-incubated with the conditioned media 1 hr before the expansion assay.

3.3.11 Western Blot

Cells were washed in ice cold PBS and lysed with radioimmunoprecipitation assay (RIPA) lysis buffer supplemented with $NaVO_3$ (100 µM), phenylmethylsulfonyl fluoride (PMSF, 100 µM), DTT (50 mM) and protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) on ice. Protein concentrations in the lysates were normalised using the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Protein lysates were resolved using 8% sodium dodecyl sulfate-poly-acrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Merck Millipore, Wicklow, Ireland). The membranes were blocked with 5% skimmed milk in tris-buffered saline containing 0.1% Tween 20 (TBST) for 30 min. Membranes were washed and incubated with primary

antibodies overnight at 4°C. The membranes were subsequently incubated with secondary antibodies at room temperature for 1 hr. Membranes were incubated with ECL substrate (Merck Millipore), exposed, and developed according to manufacturer's instructions. Antibodies used: rabbit anti-Akt [(1:1000) clone C67E7], rabbit anti-Phospho-Akt [(Ser473) (1:1000) clone D9E], mouse anti-alpha tubulin [(1:1000) clone DM1A]. Secondary antibodies used: anti-rabbit horseradish peroxidase (HRP) (1:2000), anti-mouse HRP (1:2000). All antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA).

3.3.12 T Cell Suppression assay

Human PBMCs were isolated from buffy packs (Irish Blood Transfusion Service), by Ficoll density gradient centrifugation. $5x10^4$ Carboxyfluorescein succinimidyl ester (CFSE) labelled PBMC were co-cultured (Fisher Scientific) with BM-MSC in a 1:20 ratio (2.5×10^3 cells/well). 24 hours prior to co-culture, BM-MSCs were incubated with $CATT_7$ CM or $CATT_7$ CM + SCD-19 (100 μM). After the 24 hours, BM-MSCs were washed with PBS before adding the PBMCs. Activation and expansion of human T cells was carried out using ImmunoCult™ human CD3/CD28 T cell activator antibody mix (STEMCELL Technologies, Cambridge, UK). After 4 days, PBMCs were harvested and the % and number of proliferating $CD3^+$ cells were analysed by flow cytometry (Attune Nxt Flow Cytometer).

3.3.13 Statistical Analysis

All data are presented as mean ± SEM. Results of two or more groups were compared by 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.

3.4 Results

3.4.1 Human BM-MSCs constitutively express canonical and non-canonical MIF receptors

CD74 is the classical receptor for MIF however, contrary to its name, MIF displays chemokine-like properties and is a non-cognate ligand of the chemokine receptors CXCR2 and CXCR4 (Bernhagen et al., 2007). To explore whether MIF and MSCs interact, we sought to determine whether MSCs express the classical and non-classical receptors of MIF. We showed that MSCs constitutively express CD74, CXCR2 and CXCR4 (Fig. 3.1A, 3.1B, 3.1C). CXCR4 had the highest expression on the surface of MSCs compared to CXCR2 and the classical MIF receptor CD74. Once we had elucidated that MSCs express MIF receptors we tested whether MIF binding influences receptor expression. We exposed MSCs to different concentrations of recombinant human MIF (1, 10 and 100 ng/ml) for 6 hr and measured receptor expression by flow cytometry. We found that recombinant hMIF had no effect on the expression levels of MIF receptors CD74 (Fig. 3.1A), CXCR2 (Fig. 3.1B), and CXCR4 (Fig. 3.1C) in MSCs. In addition, we measured MIF receptor expression on MSCs following exposure to high levels of endogenous human MIF from BMDM supernatants of $CATT_7$ mice ($CATT_7$ CM). Endogenous human MIF was obtained by collecting BMDM supernatants from humanised MIF mice challenged with HDM 3 times a week for 3 weeks (Fig. 3.2A). As expected, the $CATT_7$ mice with the high expressing MIF allele produce significantly higher levels of human MIF than $CATT_5$ mice with the low expressing MIF allele (Fig. 3.2B). Similar to the observations with the recombinant hMIF, exposure to high levels of human endogenous MIF from $CATT_7$ CM had no effect on % CD74, CXCR2, and CXCR4 on the cell surface (Fig.

3.2C, 3.2D, 3.2E). To further validate the results, we exposed MSCs to endogenous MIF for several different timepoints (1, 6 and 24 hrs). We observed no changes in MIF receptor expression 1 Hr, 6 Hr or 24Hr following exposure (Fig. 3.2F, 3.2G, 3.2H). These results suggest that MSCs possess the machinery to respond to MIF stimulation.

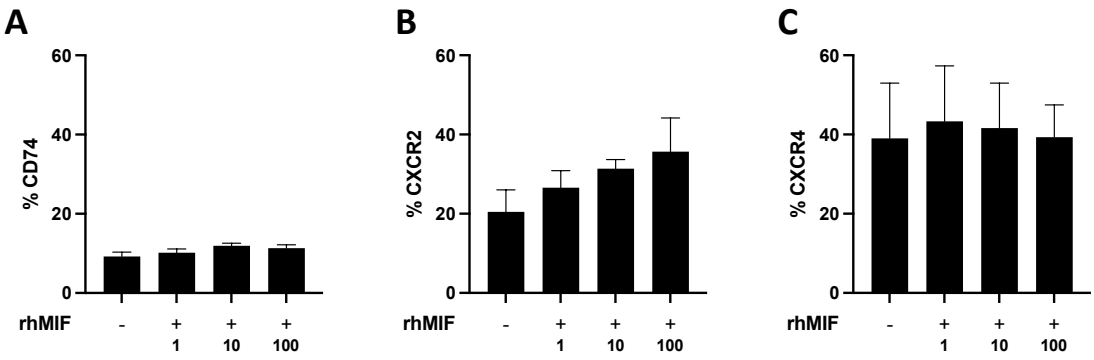
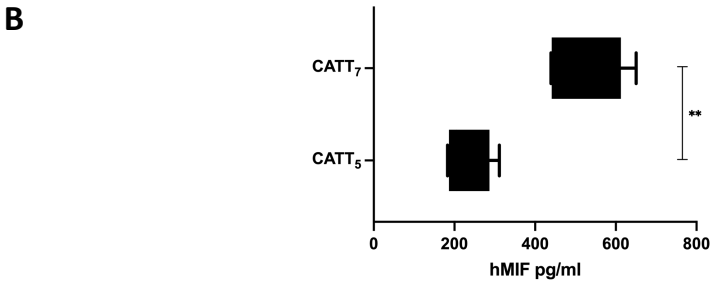
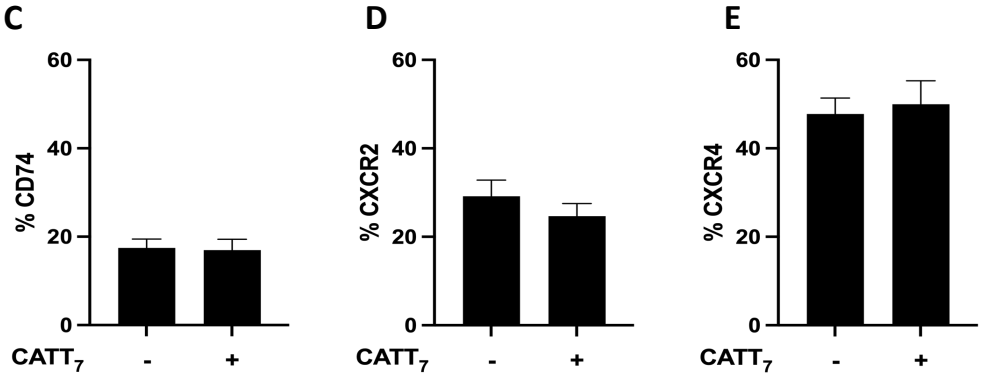


Figure 3.1. Human BM-MSCs constitutively express canonical and non-canonical MIF receptors. Human BM-MSCs were treated with different concentrations of rhMIF (1, 10 or 100 ng/ml) for 6 Hr. Expression of MIF receptors CD74 (**A**), CXCR2 (**B**), and CXCR4 (**C**) on the surface of BM-MSC were assessed by flow cytometry. Data are presented as mean ± SEM and are representative of 3 independent experiments performed in triplicate using 3 MSC donors. Statistical analysis was carried out using one-way ANOVA followed by the *post-hoc* Tukey's multiple comparison test. No statistical significance was found between the groups. Instances hereafter the statistical test used will not be stated in the figure legend.


A
CATT7
CATT5
WT
HDM i.n
3/week x 3
weeks
BMDMs
96 Hr
cRPMI
10% L929 CM
Harvest CM


B
CATT7
CATT5
**
0
200
400
600
800
hMIF pg/ml


C
% CD74
60
40
20
0
CATT7 - +
D
% CXCR2
60
40
20
0
CATT7 - +
E
% CXCR4
60
40
20
0
CATT7 - +

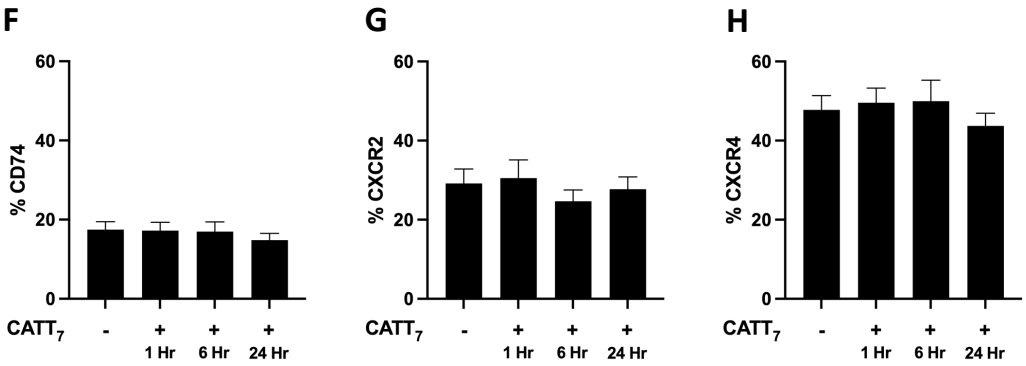

F
% CD74
60
40
20
0
CATT7 - + + +
1 Hr 6 Hr 24 Hr
G
% CXCR2
60
40
20
0
CATT7 - + + +
1 Hr 6 Hr 24 Hr
H
% CXCR4
60
40
20
0
CATT7 - + + +
1 Hr 6 Hr 24 Hr

Figure 3.2. Effects of endogenous hMIF on canonical and non-canonical MIF receptors. A Schematic detailing the generation $CATT_7$, $CATT_5$, and WT CM from mice challenged with 25 μg HDM 3 times a week for 3 weeks. **B** Levels of human MIF in $CATT_7$ and $CATT_5$ CM detected by hMIF ELISA; N=4 mice per group. PBS controls not shown. BM-MSCs were exposed to $CATT_7$ CM containing high levels of endogenous hMIF and the % CD74 (**C**), CXCR2 (**D**), and CXCR4 (**E**) was assessed. CD74 (**F**), CXCR2 (**G**), and CXCR4 (**H**) MIF receptor expression on BM-MSCs was measured after endogenous hMIF stimulation at 6 Hr, 12 Hr and 24 Hr timepoints. Data are presented as mean ± SEM and are representative of 3 independent experiments performed in triplicate. Statistical analysis was carried out using unpaired t test where **$p<0.01$

3.4.2 Recombinant and endogenous MIF drive human BM-MSC chemotaxis *in vitro*

Chemotaxis is described as the being the movement of cells toward a chemoattractant gradient (Jin et al., 2008). Despite MIF lacking the conserved structural properties of a classical chemokine, MIF has shown chemokine-like functions by modulating the migration of leukocytes to inflamed tissues (Bernhagen et al., 2007). Its propensity to display chemokine-like properties is achieved through interaction with CXCR2 and CXCR4 as well as CXCR7 in some cases (Alampour-Rajabi et al., 2015; Klasen et al., 2014; Lourenco et al., 2015; Schwartz et al., 2009). MSCs have been shown to home to sites of tissue injury, transmigrating across the endothelium to ameliorate inflammation (Eseonu and De Bari, 2015; Leibacher and Henschler, 2016). Despite extensive studies into MSC homing, the sheer number of important factors involved means mechanisms are still poorly understood (Ponte et al., 2007). In order to investigate the effects of MIF on MSC migration, MSCs were seeded into transwell chambers containing different concentrations of recombinant human MIF (1 and 100 ng/ml) or human platelet derived growth factor (PDGF) as a positive control. We observe significant MSC-driven migration towards rhMIF at 1 and 100 ng/ml (Fig. 3.3). MIF has dose-dependent chemotactic effects on MSCs, with 100 ng/ml having a significantly higher chemotactic index than 1 ng/ml of rhMIF. To verify the observations with the rhMIF we repeated the transwell migration assays using endogenous MIF from the low-expressing $CATT_5$ mice, high expressing $CATT_7$ mice or littermate WT mice (as a negative control) in the form of BMDM CM. We observe significantly higher numbers of migrating MSCs toward the $CATT_7$ CM compared to the WT CM control, suggesting MIF drives MSC chemotaxis (Fig. 3.4A). The high MIF expressing $CATT_7$ CM also displayed a higher chemotactic index compared to

the low expressing $CATT_5$ although not significant. To further validate the specificity of the MIF-mediated effect observed in the $CATT_7$ CM, we blocked MIF using two different MIF inhibitors, ISO-1 and SCD-19. ISO-1 is a small molecule MIF inhibitor which binds within the hydrophobic pocket that contains highly conserved amino acids known to be essential for MIF pro-inflammatory activity (Al-Abed et al., 2005; Al-Abed and VanPatten, 2011). SCD-19 is an inhibitor of the isocoumarin class which reports 100% inhibition of MIF biological activity at a concentration of 100 μM (Kok et al., 2018). $CATT_7$ supernatants were incubated with either ISO-1 or SCD-19 (both 100 μM) for 1 hr at 37°c in the lower chamber of the transwell. Subsequently, MSCs were added into the upper chamber and were subjected to the migration assay. The chemotactic response was abrogated when MIF biological activity was blocked with both ISO-1 and SCD-19 (Fig. 3.4B). Blocking with SCD-19 resulted in less directed migration than ISO-1, suggesting greater inhibition of MIF biological activity.

Taken together, these findings show MIF strongly promotes MSC chemotaxis *in vitro* and may suggest that endogenous MIF may enhance MSC migration *in vivo*.

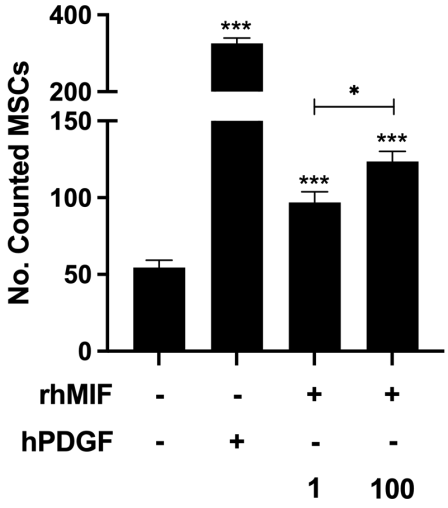


Figure 3.3. Recombinant human MIF triggers human BM-MSC chemotaxis in a dose dependent manner. *In vitro* chemotaxis assay was performed using transwell chambers containing different concentrations of rhMIF (1 and 100 ng/ml). $1x10^4$ human BM-MSCs in 100 µl serum free DMEM were added to the upper chamber of the transwell and incubated overnight. The lower chambers were added 30 min prior to cell seeding to allow the transwell to equilibrate. Serum free DMEM and hPDGF (50 ng/ml) were used as negative and positive controls respectively. Data are presented as mean ± SEM and are representative of 3 independent experiments performed in triplicate. Statistical analysis was carried out using one-way ANOVA followed by the *post-hoc* Tukey's multiple comparison test where *$p<0.05$, ***$p<0.001$.

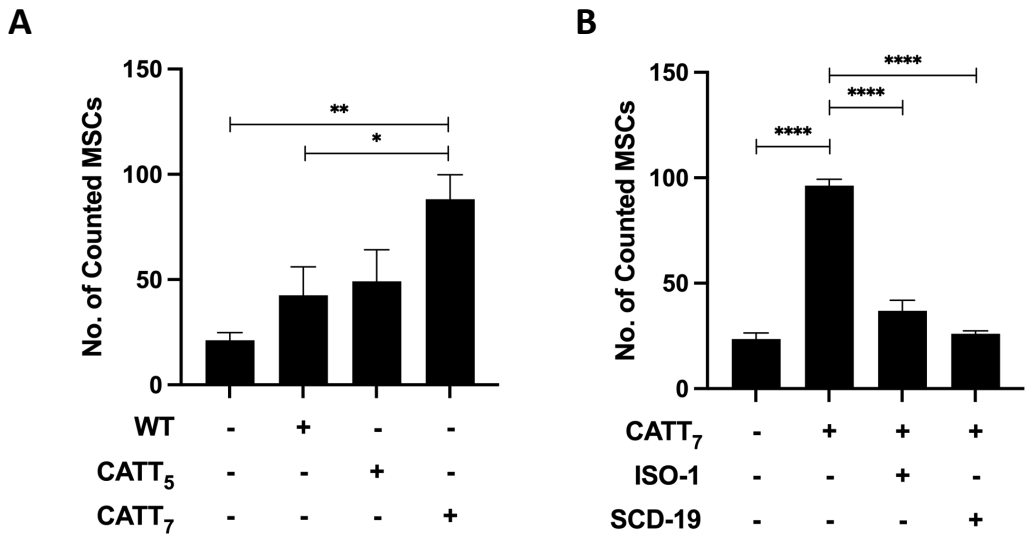


Figure 3.4. Endogenous human MIF in BMDM conditioned media from $CATT_7$ mice drives human BM-MSC chemotaxis *in vitro*. **A** *In vitro* chemotaxis assay was performed using transwell chambers containing RPMI or WT CM, $CATT_5$ CM or $CATT_7$ CM. $1x10^4$ human BM-MSCs in 100 µl serum free RPMI were added to the upper chamber of the transwell and incubated overnight. The lower chambers were added 30 min prior to cell seeding to allow the transwell to equilibrate. **B** MIF inhibitors ISO-1 and SCD-19 were added to $CATT_7$ CM 1 Hr before to block MIF biological activity. Data are presented as mean ± SEM and are representative of 3 independent experiments performed in triplicate. Statistical analysis was carried out using one-way ANOVA followed by the *post-hoc* Tukey's multiple comparison test where *$p<0.05$, **$p<0.01$, ****$p<0.0001$.

3.4.3 MIF-driven BM-MSC chemotaxis is mediated by the MIF-CXCR4 axis, not CXCR2.

Based on our previous results showing MSCs express chemokine receptors CXCR2 and CXCR4, of which MIF has a strong binding affinity to, we sought to determine the receptors involved in MIF-driven migration. To determine the receptor involved, we incubated cells with reparixin or AMD3100 for 1 hr at 37°c before seeding into the assay. We found that incubating MSCs with reparixin, a non-competitive allosteric inhibitor of CXCR1 and CXCR2 (Bertini et al., 2004), had no effect on MIF-driven migration (Fig. 3.5A). However, when we subjected MSCs to AMD3100, a highly potent and selective CXCR4 antagonist (Schols et al., 1997), MIF-driven migration was abrogated (Fig. 3.5A). These data suggest that MIF-driven migration is mediated through binding to CXCR4 and not CXCR2. Upon establishing the involvement of the MIF-CXCR4 axis we confirmed our finding by showing that AMD3100 remained bound to the CXCR4 receptor rather than mediating any off-target effects over the duration of the migration assay (Fig. 3.5B).

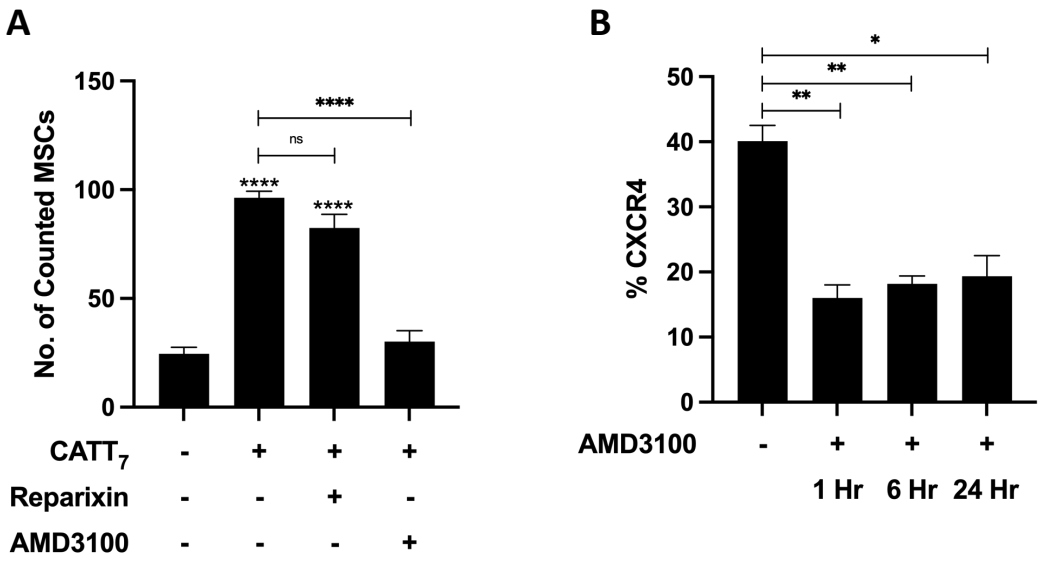


Figure 3.5. Inhibition of CXCR4 abrogates MIF-driven BM-MSC chemotaxis. A *In vitro* chemotaxis assay was performed using transwell chambers containing RPMI or $CATT_7$ CM. Human BM-MSCs were incubated with reparixin (40 μM) or AMD3100 (50 μg/ml) 1 Hr before seeding to block CXCR2 or CXCR4, respectively. **B** To demonstrate the long-lasting inhibitory effect of AMD3100 on the CXCR4 receptor, a time course the length of the transwell assay was performed. BM-MSCs were incubated with AMD3100 then stained with anti-CXCR4 antibody. % CXCR4 expression was determined by flow cytometry. Data are presented as mean ± SEM and are representative of 3 independent experiments performed in triplicate. Statistical analysis was carried out using one-way ANOVA followed by the *post-hoc* Tukey's multiple comparison test where *$p<0.05$, **$p<0.01$, ****$p<0.0001$, ns not significant.

3.4.4 Effect of MIF on staurosporine-induced apoptosis of BM-MSCs.

MIF is a pleiotropic cytokine implicated in the pathogenesis of inflammation and cancer. Elevated concentrations of MIF is associated with tumorigenesis and cancer cell survival (Fukaya et al., 2016; Lue et al., 2007; Wilson et al., 2005). Moreover, MIF has been shown to play a cytoprotective role in inflammation and injury. In kidney diseases MIF has been identified as a protective factor of tubular cells by counteracting cell cycle arrest (Djudjaj et al., 2017). MIF has also been shown to act as a pro-survival factor for many different cell types in conditions of oxidative stress (Ietta et al., 2018; Li et al., 2019; Nguyen et al., 2003; Ohta et al., 2012; Soppert et al., 2018). In order to determine the ability of MIF to prolong survival in MSCs, we treated MSCs with staurosporine (STS) (500 nM) for 6 hr. STS is a highly potent inducer of apoptosis through caspase-dependent and caspase-independent mechanism making it a useful tool in cancer therapy (Belmokhtar et al., 2001; Zhang et al., 2004). MSCs were trypsinised, washed and stained with Annexin V PI for analysis by flow cytometry. After 6 hr, roughly 40% of MSCs were Annexin V^+PI^-, indicating they are in the early stages of apoptosis (Fig. 3.6A, 3.6C). MSCs that were incubated with $CATT_7$ CM in addition to STS exhibited lower levels of apoptosis than those treated with STS alone yet not statistically significant (Fig. 3.6A). The same trends were observed when we looked at the % total apoptosis by measuring the percentage of Annexin V^+PI^+ cells (Fig. 3.6B). These data show that MIF may provide some degree of cytoprotection to MSCs undergoing apoptosis.

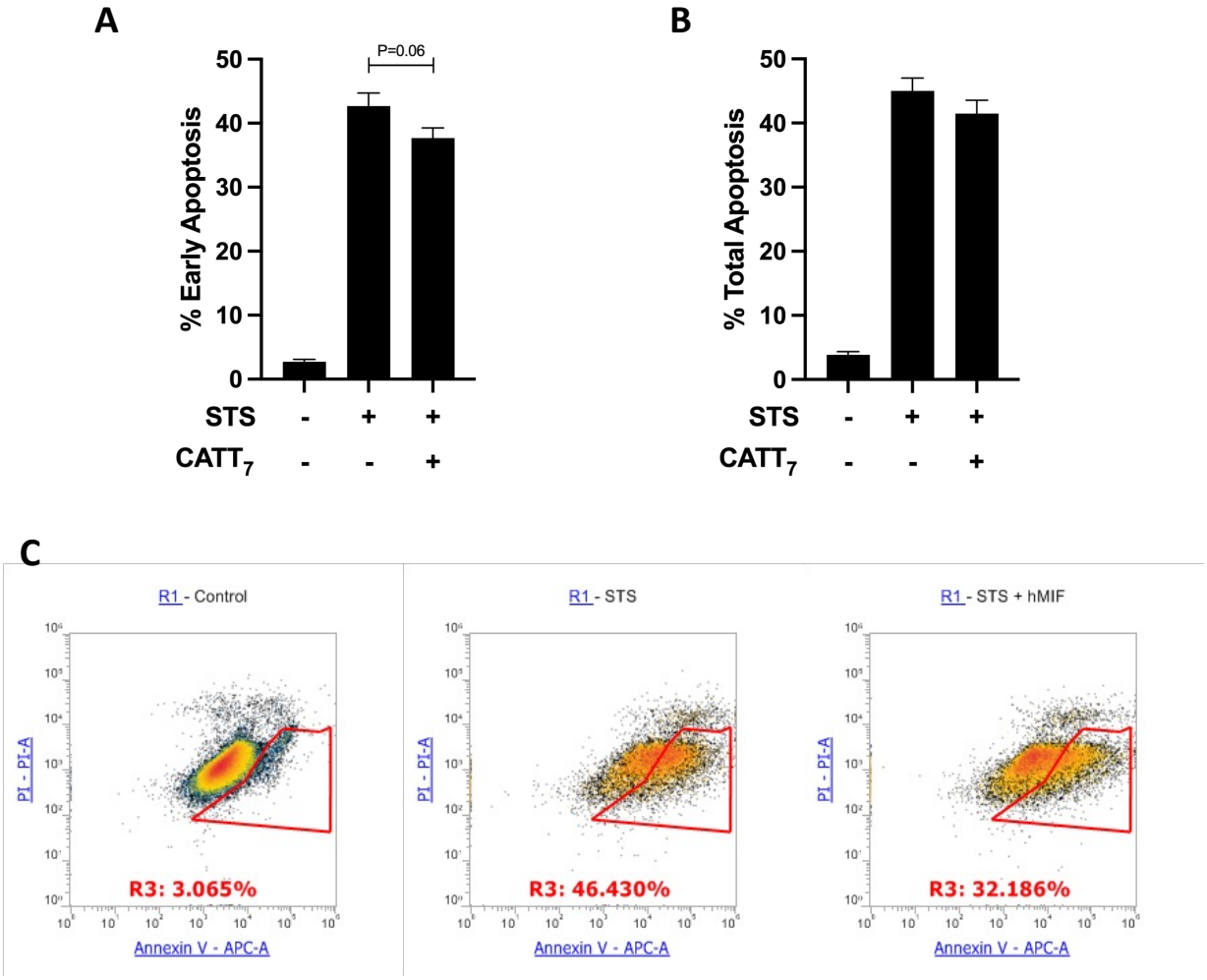


Figure 3.6. Effect of MIF on staurosporine-induced apoptosis of BM-MSCs. BM-MSCs were seeded out in 6 well plates and left to adhere overnight. Annexin V PI staining for apoptosis by flow cytometry was performed on MSCs incubated in complete medium or $CATT_7$ CM and treated with staurosporine (500 nM) for 6 Hr. **A** % early apoptotic cells and **B** % total apoptosis was determined. **C** Early apoptotic cells are identified by Annexin V^+ PI^- gating. Data are presented as mean ± SEM and are representative of 3 independent experiments performed in triplicate.

3.4.5 Endogenous hMIF enhances BM-MSC expansion *in vitro*

Next, we investigated the effects of MIF on MSC expansion *in vitro*. Previous studies have shown that MIF has the ability to support cell proliferation *in vitro* (Lan et al., 2018; Ohta et al., 2012; Utispan and Koontongkaew, 2021). Increasing the number of MSCs within the inflammatory niche could prove to be important in enhancing MSC immunoregulatory effects. MSCs were seeded out into T25 flasks in complete medium or 50:50 complete medium and WT CM or $CATT_7$ CM for 72 hr. Cells were trypsinised and cell counts were performed. We observed that high levels of MIF significantly enhanced MSC expansion *in vitro* compared to the complete medium and WT control groups (Fig. 3.7A). We observed more than a 1.5-fold increase in the number of MSCs present after incubation with $CATT_7$ CM. We confirmed the role of MIF using ISO-1 and SCD-19 which were added to the $CATT_7$ CM 1 hr before the assay. The presence of MIF inhibitors significantly reduced the ability of the $CATT_7$ CM to drive MSC expansion further affirming the result (Fig. 3.7B).

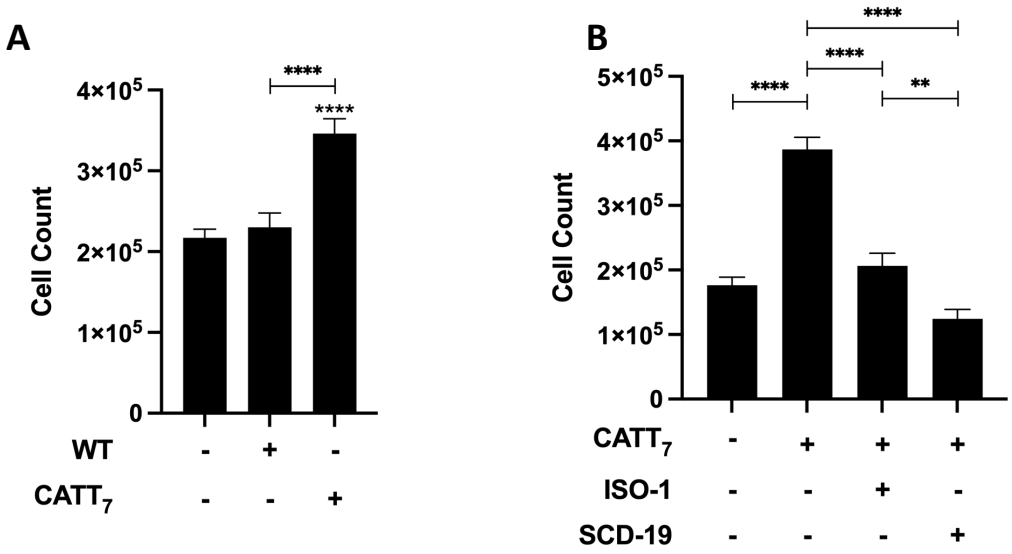


Figure 3.7. Endogenous hMIF enhances BM-MSC expansion in vitro. BM-MSCs were seeded out into T25 flasks at a density of $5.7x10^3$ cells/cm^2 in complete medium or 50:50 complete medium and WT CM or $CATT_7$ CM for 72 Hr. Cells were trypsinised and **A** cell counts were performed using ethidium bromide/ acridine orange staining. **B** MIF inhibitors ISO-1 and SCD-19 were added to $CATT_7$ BMDM supernatants 1 Hr before BM-MSC incubation to block MIF biological activity. Data are presented as mean ± SEM and are representative of 3 independent experiments performed in triplicate. Statistical analysis was carried out using one-way ANOVA followed by the *post-hoc* Tukey's multiple comparison test where **$p<0.01$, ****$p<0.0001$.

3.4.6 Western Blot analysis of Akt and p-Akt in hMIF stimulated BM-MSCs

On establishing the involvement of MIF in MSC survival and expansion we examined possible mechanisms involved. MIF-induced signaling acts through relatively few downstream pathways, which converge on the MAPK, PI3K, NF-kB, and AMPK signalling pathways (Leng et al., 2003; Mitchell et al., 1999; Shi et al., 2006; Starlets et al., 2006). In other cellular contexts, MIF can directly promote cell survival and cell growth through activation of the PI3K-Akt pathway (Bloom et al., 2016; Gore et al., 2008; Kleemann et al., 2000; Lue et al., 2007). To delineate if the PI3K-Akt pathway plays a role in MSC expansion and survival we measured the expression levels of Akt and phosphorylated Akt (p-Akt) by western blot. We observed that incubation with $CATT_7$ CM induces Akt phosphorylation after 10 min (Fig 3.8A, 3.8B). Interestingly, inhibition of MIF with SCD-19 had little to no effect on the phosphorylation of Akt. These results indicate that other factors within the $CATT_7$-derived supernatants may also trigger cell signalling.

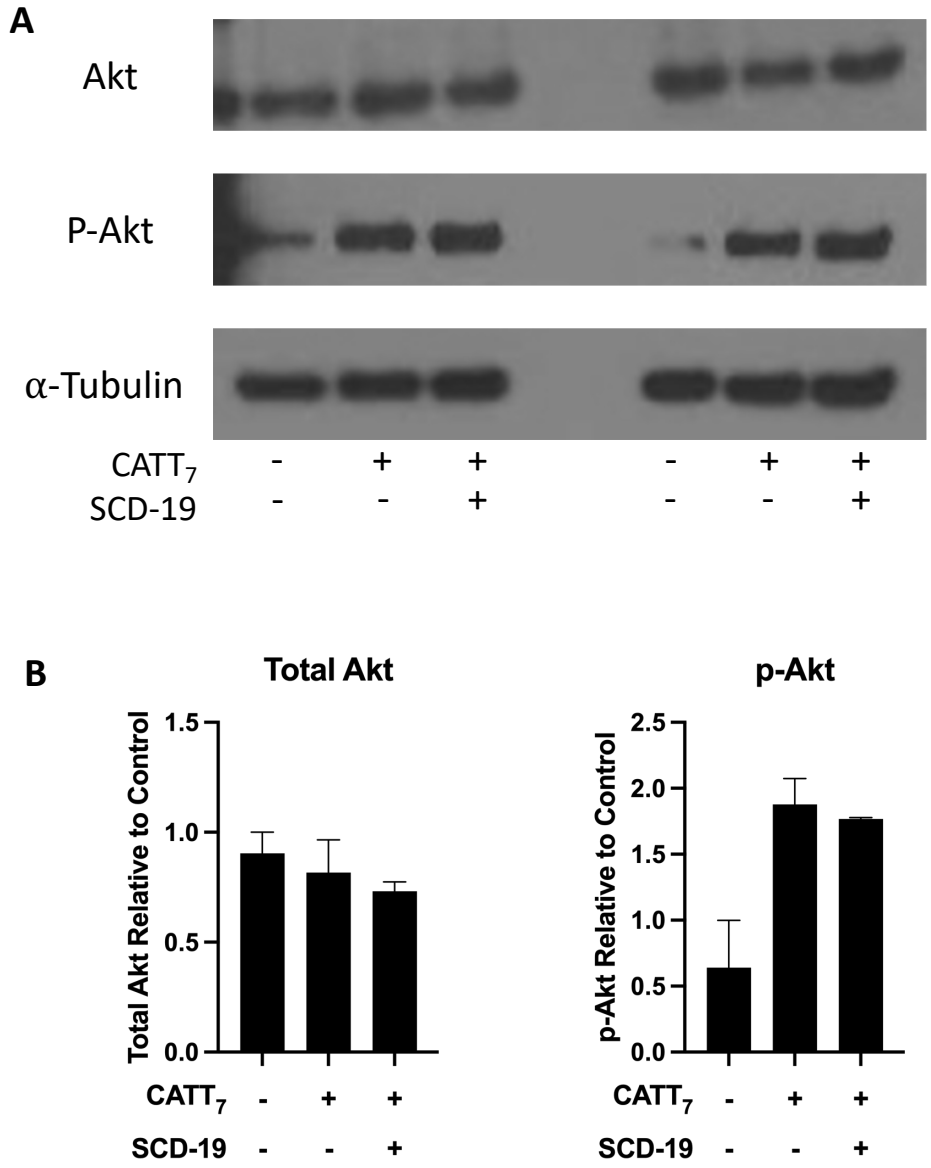


Figure 3.8. Western Blot analysis of Akt and p-Akt in hMIF stimulated BM-MSCs. A BM-MSCs were stimulated for 15 min with $CATT_7$ CM or $CATT_7$ CM preincubated with SCD-19 1 Hr prior to stimulation. After 15 min, lysates were generated and examined for protein levels of Akt and p-Akt by western blot. α-tubulin was used as a loading control. **B** Densitometric analysis was performed to determine the ratio of proteins of interest to the loading control. Data are presented as mean ± SEM and are representative of 2 independent experiments performed in triplicate.

3.4.7 hMIF upregulates COX-2 in human BM-MSC

MIF has been shown to activate a number of pathways. In addition to PI3K-Akt activation, MIF has been shown to activate the MAP kinase pathway (MAPK) (Hofmann et al., 2021; Mitchell et al., 1999; Zhang et al., 2016). MIF has an established role as an upstream regulator of cyclooxygenase 2 (COX-2) through the activation of the MAPK signalling pathway (Carli et al., 2009; Mitchell et al., 2002). COX-2 is the rate limiting enzyme that catalyses the conversion of arachidonic acid to prostaglandins such as prostaglandin E2 (PGE2) (Sampey et al., 2001). COX-2 and PGE2 are widely reported mediators of MSC immunosuppression (Carty et al., 2021; Li et al., 2015). COX-2 expression by MSCs plays an important role in PGE2 biosynthesis, mediating PGE2 immunosuppression. We analysed MSC expression of COX-2 in the presence or absence of human MIF by flow cytometry. MSCs constitutively express COX-2 however we observed a significant upregulation of the % of COX-2 expressing MSCs following MIF stimulation (Fig. 3.9A) We also observed an increase in the COX-2 MFI in MSCs stimulated with MIF (Fig. 3.9B) These data show that MIF is capable of increasing the immunomodulatory capacity of MSCs through the upregulation of COX-2 expression.

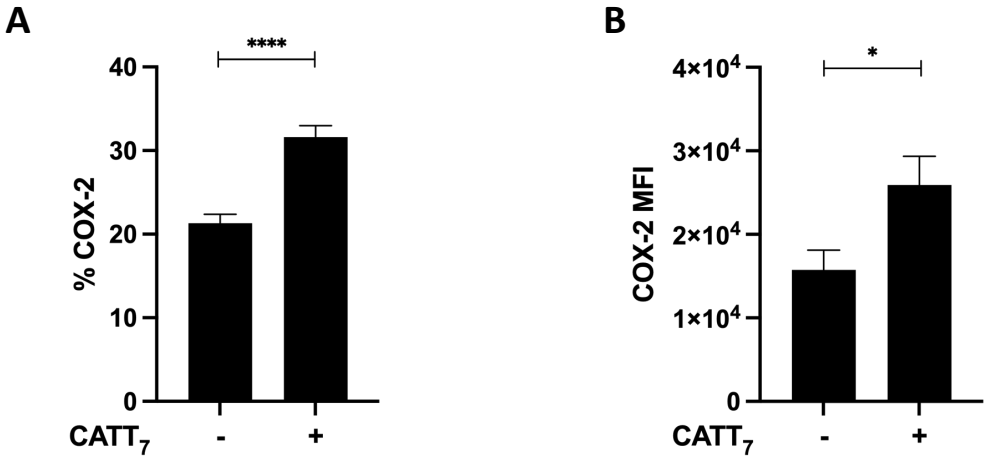


Figure 3.9. hMIF upregulates COX-2 in human BM-MSC. Cells were stimulated with $CATT_7$ CM for 24 Hr. Cells were typsinised then surface stained before staining intracellularly for COX-2. **A** % COX-2 and **B** mean fluorescence intensity (MFI) of COX-2 was measured. Data are presented as mean ± SEM and are representative of 3 independent experiments performed in triplicate. Statistical analysis was carried out using unpaired t test where **p<0.01, ****p<0.0001.

3.4.8 hMIF enhances TNF-α stimulation of COX-2 in BM-MSCs

A suggested approach to enhance MSC efficacy in clinical trials is to prime MSCs with proinflammatory cytokines within the inflammatory niche or prior to infusion. Priming of MSCs by biological means using pro-inflammatory cytokines (Baudry et al., 2019; Boyt et al., 2020; English et al., 2007; Kim et al., 2018; Klinker et al., 2017; Prasanna et al., 2010; Ragni et al., 2020) or biochemically through the exposure to hypoxia (Lavrentieva et al., 2010; J. Liu et al., 2021; Mathew et al., 2017; Roemeling-van Rhijn et al., 2013) have been shown to enhance their therapeutic potential. It is recognised that MSCs may behave differently to priming with different combinations of pro-inflammatory cytokines. Cytokines such as TNF-α and IFN-γ further enhance MSCs immunoregulatory ability and this effect is amplified when used in tandem (Boland et al., 2018; de Pedro et al., 2021). Upon establishment that MIF may increase MSCs therapeutic potential through the upregulation of COX-2, we stimulated MSCs with MIF and different cytokines (IL-4, TNF-α and IFN-γ) and measured COX-2 and IDO expression by flow cytometry. Stimulation of MSCs with IL-4 had no effect on COX-2 (Fig. 10A-C) or IDO production (Fig. 3.11A, 3.11B) suggesting that IL-4 is unable to precondition MSCs like other pro-inflammatory cytokines. Presence of IL-4 does not abrogate elevated COX-2 levels mediated by MIF stimulation. As other authors have previously shown, TNF-α is able to drive COX-2 upregulation in a dose dependent manner (English et al., 2007). Strikingly, the presence of MIF alongside TNF-α is not redundant but in fact amplifies COX-2 production with significant increases in the percentage of COX-2 expressing MSCs and COX-2 MFI (Fig. 3.10A, 3.10B). The percentage of COX-2 and COX-2 MFI were significantly elevated when MSCs were incubated with $CATT_7$ supernatants and both concentrations of TNF-α

suggesting that maximal stimulation was not achieved with TNF-α alone and MSCs immunomodulatory potential can be amplified. Stimulating MSCs with TNF-α had no effect on IDO production (Fig. 3.11A, 3.11B). Stimulation of MSCs with IFN-γ had no influence on COX-2 production (Fig. 3.12), however, IFN-γ stimulation significantly increases % IDO expressing cells and IDO MFI in a dose dependent manner as predicted (Fig. 3.13A, 3.13B). Interestingly, amplification in MSCs immunomodulatory capacity is not observed when MSCs are primed with both MIF and IFN-γ. In fact, MIF appears to downregulate IDO production in the presence of IFN-γ (Fig. 3.13A). These data suggest that priming MSCs with a combination of MIF and TNF-α can increase their ability to modulate the immune system, however, this amplified effect is not achieved with IFN-γ.

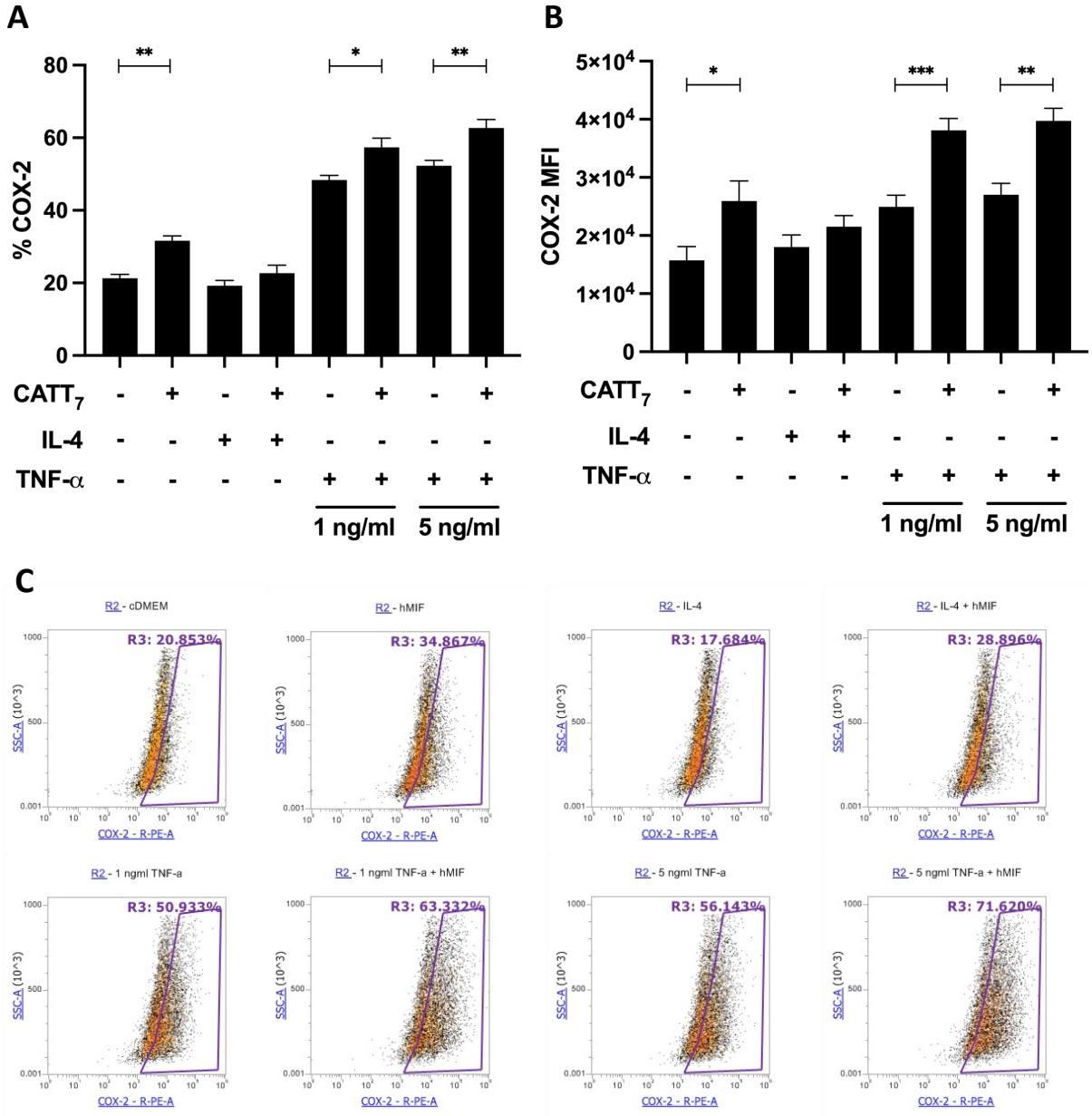


Figure 3.10. hMIF enhances TNF-α stimulation of COX-2 in BM-MSCs. $CATT_7$ CM treated BM-MSCs were treated with IL-4 (20 ng/ml) or TNF- α (1 or 5 ng/ml) for 24 Hr. Cells were first gated on forward scatter (FSC) and side scatter (SSC) to exclude debris. CD73 + cells were selected and **A** % COX-2 and **B** mean fluorescence intensity (MFI) of COX-2 was measured. **C** Representative images of COX-2 density plots and gating. Data are presented as mean ± SEM and are representative of 3 independent experiments performed in triplicate. Statistical analysis was carried out using one-way ANOVA followed by the *post-hoc* Tukey's multiple comparison test where *p<0.05, **p<0.01, ***p<0.001

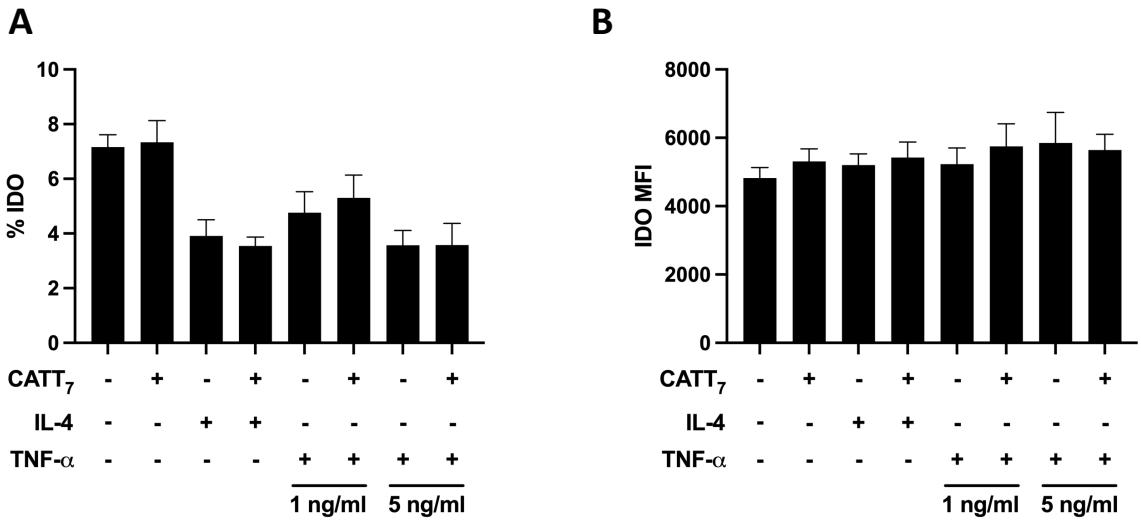


Figure 3.11. Stimulation of BM-MSCs with IL-4 or TNF-α has no effect on IDO production in BM-MSCs. $CATT_7$ CM treated BM-MSCs were treated with IL-4 (20 ng/ml) or TNF- α (1 or 5 ng/ml) for 24 Hr. Cells were typsinised then surface stained before staining intracellularly for IDO. **A** % IDO and **B** mean fluorescence intensity (MFI) of IDO were measured. Data are presented as mean ± SEM and are representative of 3 independent experiments performed in triplicate. Statistical analysis was carried out using one-way ANOVA followed by the *post-hoc* Tukey's multiple comparison test.

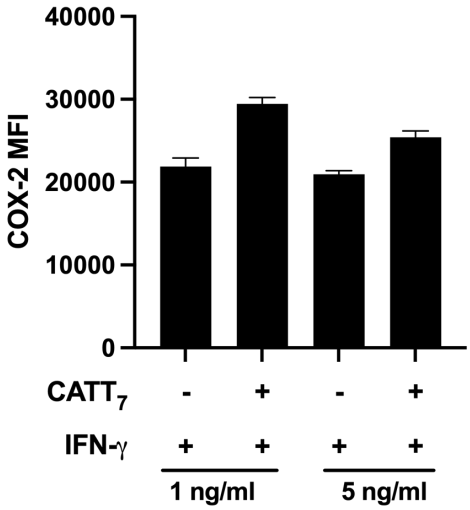


Figure 3.12. Stimulation of BM-MSC with IFN-γ had no effect on COX-2 production. $CATT_7$ CM treated BM-MSCs were treated with IFN-γ (1 or 5 ng/ml) for 24 Hr. Cells were typsinised then surface stained before staining intracellularly for COX-2. Mean fluorescence intensity (MFI) of IDO was measured. Data are presented as mean $\pm$ SEM and are representative of 3 independent experiments performed in triplicate. Statistical analysis was carried out using one-way ANOVA followed by the *post-hoc* Tukey's multiple comparison test.

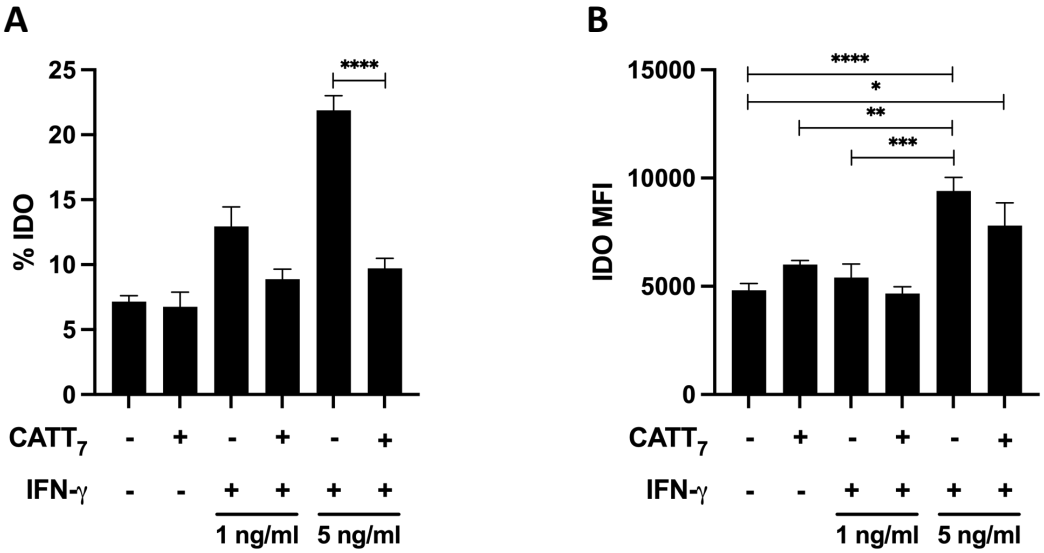


Figure 3.13. hMIF has no effect on IDO production in BM-MSCs. $CATT_7$ treated BM-MSCs were treated with IFN-γ (1 or 5 ng/ml) for 24 Hr. Cells were typsinised then surface stained before staining intracellularly for IDO. **A** % IDO and **B** mean fluorescence intensity (MFI) of IDO was measured. Data are presented as mean $\pm$ SEM and are representative of 3 independent experiments performed in triplicate. Statistical analysis was carried out using one-way ANOVA followed by the *post-hoc* Tukey's multiple comparison test where *p<0.05, **p<0.01, ***p<0.001 ****p<0.0001.

3.4.9 hMIF enhances BM-MSC suppression of T cell proliferation

After discovering that MIF licensing drives COX-2 production in MSCs we decided to probe this further. To ascertain whether COX-2 upregulation resulted in any functional changes to MSCs ability to modulate the immune system we tested their ability to suppress T cell proliferation. PBMCs were activated with human CD3/CD28 T cell activator mix (aPBMC) and cocultured with MSCs at a 1:20 ratio in a 96-well round bottomed plate for 4 days. As a positive control aPBMCs were incubated in the absence of BM-MSCs and exhibited the highest % $CD3^+$ T cell proliferation (Fig. 3.14A) and total number of proliferating $CD3^+$ T cells (Fig. 3.14B). The presence of MSCs significantly inhibited $CD3^+$ T cell proliferation. Interestingly, MSCs licensed with high levels of hMIF from $CATT_7$ CM displayed enhanced suppression of T cell proliferation compared to the untreated MSCs. To delineate whether hMIF was responsible for this enhanced suppression of T cell proliferation $CATT_7$ CM was incubated with SCD-19 (100 μM) 1 hr before adding to MSCs. Presence of SCD-19 abrogated the enhanced suppression observed in the hMIF licensed group. The SCD-19 group had significantly higher numbers of proliferating $CD3^+$ T cells than the $CATT_7$ group. Similar results were observed with recombinant human MIF (Fig. 3.15).

These data show that licensing MSCs with hMIF increases MSCs immunomodulatory capacity. This is shown through an enhanced ability to suppress T cell proliferation *in vitro* and is likely mediated through the upregulation of COX-2.

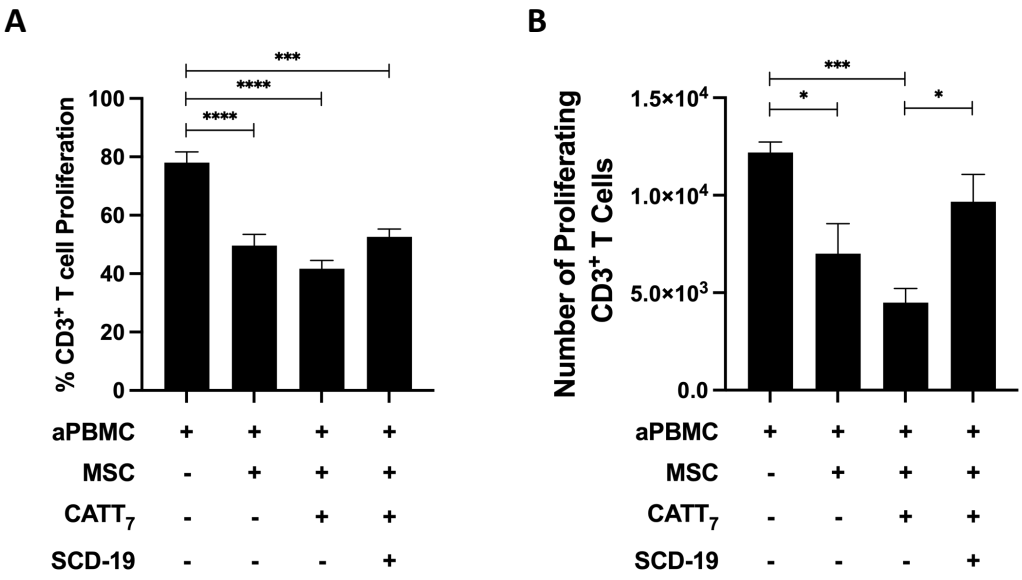


Figure 3.14. hMIF enhances BM-MSC suppression of T cell proliferation. MSCs were seeded (2.5×10^3 per well) into a 96 well round bottom plate followed by the addition of human CD3/CD28 T cell activator antibody activated CFSE labelled PBMCs (aPBMC) (5×10^4 per well). $CATT_7$ groups were pre-stimulated with $CATT_7$ CM 24 Hr prior to coculture. As a control SCD-19 (100 μM) was added to the $CATT_7$ CM 1 Hr before BM-MSC stimulation to block MIF biological activity. On day four, cells were harvested and stained with anti-CD3 and 7AAD to analyse $CD3^+$ T cell proliferation by flow cytometry. **A** % $CD3^+$ T cells undergoing proliferation and **B** number of proliferating $CD3^+$ T cells were measured. Data are presented as mean ± SEM and are representative of 2 independent experiments performed in triplicate. Statistical analysis was carried out using one-way ANOVA followed by the *post-hoc* Tukey's multiple comparison test where *p<0.05, ***p<0.001, ****p<0.0001.

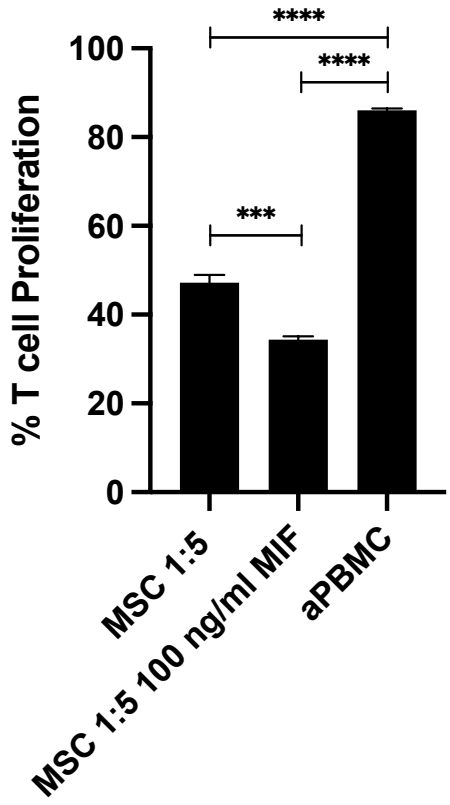


Figure 3.15. rhMIF enhances BM-MSC suppression of T cell proliferation. MSCs were seeded (1×10^4 per well) into a 96 well round bottom plate followed by the addition of human CD3/CD28 T cell activator antibody activated CFSE labelled PBMCs (aPBMC) (5×10^4 per well). BM-MSCs were stimulated with rhMIF (100 ng/ml) 24 Hr prior to coculture. On day four, cells were harvested and stained with anti-CD3 and 7AAD to analyse $CD3^+$ T cell proliferation by flow cytometry. **A** % proliferating $CD3^+$ T cells were measured. Data are presented as mean ± SEM and are representative of one experiment. Statistical analysis was carried out using one-way ANOVA followed by the *post-hoc* Tukey's multiple comparison test where ***p<0.001, ****p<0.0001.

3.5 Discussion

Understanding the mechanisms by which MSCs mediate their anti-inflammatory and pro-reparative effects is essential to improving their therapeutic efficacy in patients. To do this we need to investigate the pro-inflammatory cytokines within the targeted inflammatory niche. A cytokine which warrants such study is MIF, an upstream regulator of inflammation found to be differentially expressed in a range of inflammatory diseases (Baugh et al., 2002; Mizue et al., 2005). Stark differences in inflammatory microenvironments in separate cohorts of patients may be a reason for MSCs limited success in phase III clinical trials. In this study we demonstrate the effects of human MIF on human bone marrow derived MSC function *in vitro*.

Our initial investigations involved using recombinant human MIF to study its influence on MSCs. However, the use of recombinant human MIF has previously been criticised in the literature due to issues with protein degradation and concerns over purity. The synthesis of recombinant MIF through bacterial expression introduces the possibility of endotoxin contamination which can bring its own biological influence and can lead to non-specific effects (Kudrin et al., 2006). The conclusions of historical studies with high levels of bacterial contamination have been thrown into question and opposing observations as a result of possible LPS contamination has muddied the current available literature (Bloom et al., 2016). To overcome the problems associated with recombinant human MIF we harvested BMDMs from humanised transgenic $CATT_7$ and $CATT_5$ mice. In these mice, murine *Mif* has been replaced by human high- or low expression alleles, $CATT_7$ and $CATT_5$ respectively. The BMDMs harvested from these mice express differential levels of endogenous human MIF allowing us to investigate the effect of high and low levels of human MIF on MSCs. BMDMs from

littermate wildtype mice were used as controls. The CM retrieved from the BMDMs of the $CATT_7$, $CATT_5$, and WT mice was used neat rather than being purified, therefore, controls were needed to ensure results were not affected by confounders within the supernatant. After observing similar results using both recombinant and endogenous, we switched to using fresh endogenous MIF to navigate through the concerns surrounding protein degradation and endotoxin contamination as well as being able to implement relevant controls to ensure our results were MIF specific. Firstly, we looked at the expression of MIF receptors CD74, CXCR2 and CXCR4 on the surface of MSCs in the presence or absence of human MIF. Unlike B cells or human embryonic kidney 293 (HEK 293) cells, MSCs binding of MIF did not elicit receptor internalisation (Alampour-Rajabi et al., 2015; Bernhagen et al., 2007) suggesting that internalisation is not essential for signal transduction. These differences in results could be associated with the cell type used or the timepoints at which receptor expression was measured. Alampour-Rajabi et al. used murine pre-B-lymphoma cells transfected with human CXCR4 and measured 30 min after exposure to recombinant human MIF instead of the 6 hr timepoint in our studies. However, our data align with existing literature on MSC receptor interactions where binding of the classical ligands to MIF receptors does not elicit a receptor internalisation and recycle loop (Marquez-Curtis and Janowska-Wieczorek, 2013; Park et al., 2017; Zhang et al., 2007).

Moreover, we have shown that MIF can promote MSC migration *in vitro*. MIF-driven migration was abrogated in the presence of AMD3100 but not reparixin suggesting that migration was mediated through MIF binding to CXCR4 and not CXCR2 (Fig 3.16). To prove the observations were MIF specific we used two different MIF inhibitors, ISO-1 and SCD-19. ISO-1 belongs to the isoxazline class of inhibitors

which binds to the tautomerase active site on MIF to inhibit MIF biological activity (Lubetsky et al., 2002). Following its discovery, the use of ISO-1 was found to attenuate the growth of prostate cancer cells, inhibit colon tumour growth and metastasis, reduce melanoma cell survival, alleviate pancreatic and lung injury in rats with acute pancreatitis in pregnancy, and attenuate inflammation and airway hyperresponsiveness in a preclinical model of COPD by successfully blocking MIF activity (He et al., 2009; Meyer-Siegler et al., 2006; Russell et al., 2016; Tanese et al., 2015; Y. Zhou et al., 2018). However, a concern over reported results involving ISO-1 has been growing over the lack of reproducibility and consistency. Values of ISO-1 half-maximal inhibitory concentration (IC_{50}) have been reported to range from 7 μM to over 100 μM (Trivedi-Parmar and Jorgensen, 2018). Furthermore, a study exploring the role of MIF in an animal model of interstitial cystitis/bladder pain syndrome (IC/BPS) showed that ISO-1 blocked bladder inflammatory and micturition changes in the absence of MIF (Ma et al., 2019). This suggests that ISO-1 can induce non-specific effects independent of MIF. Therefore, to ensure our results were MIF specific we also used a highly potent inhibitor of MIF, SCD-19, which completely inhibits MIF at 100 μM and boasts a more favourable inhibitor constant (Ki) value compared to ISO-1 (Mawhinney et al., 2015; Tynan et al., 2017).

Previous studies have shown that MIF driven recruitment of leukocytes such as monocytes and neutrophils is CXCR2 dependent (Bernhagen et al., 2007; Cho et al., 2010; Xu et al., 2015). However, our results align with a previous study by Lourenco et al. which demonstrates a role for the MIF-CXCR4 axis in the recruitment of MSCs to tumours (Lourenco et al., 2015). It is not surprising that MIF mediates MSCs homing through binding to CXCR4 as the SDF-1α-CXCR4 has been highlighted as

one of the main mechanisms of MSC chemotaxis (Bhakta et al., 2006; Marquez-Curtis and Janowska-Wieczorek, 2013; Won et al., 2014).

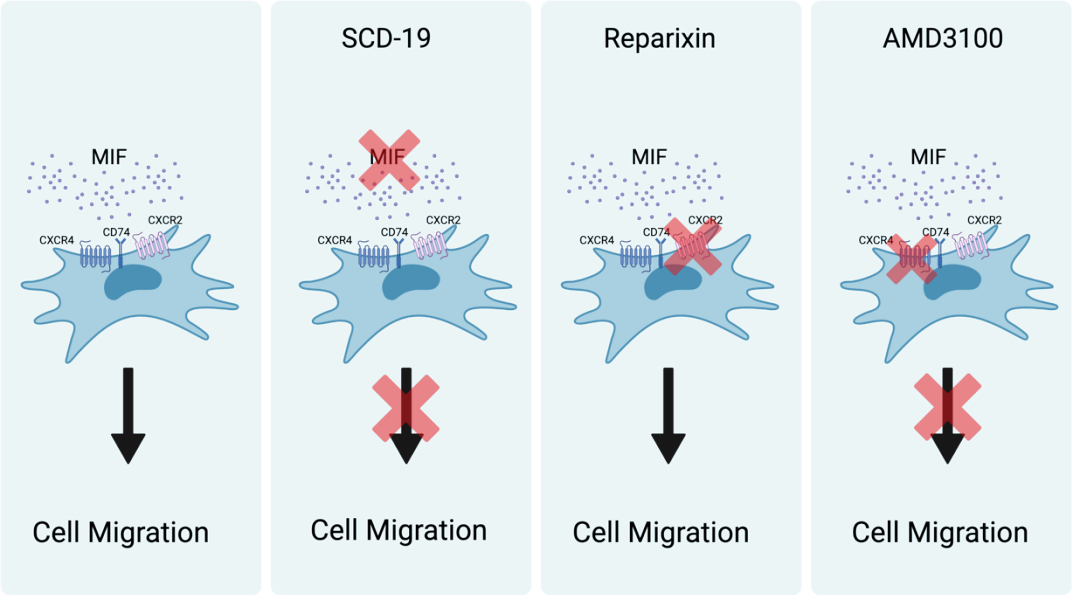


Fig. 3.16. hMIF drives MSC migration through CXCR4. Blockade of CXCR4 receptor abrogates MIF driven migration of MSCs.

Furthermore, studies have shown how MIF sustains cell survival and limits apoptosis by inhibiting P53 (Hudson et al., 1999; Leech et al., 2003; Lue et al., 2007; Mitchell et al., 2002). However, published studies describing the pro-survival nature of MIF *in vitro* have often been performed in cells undergoing stress such as accumulation of reactive oxygen species in serum deprived cells or those exposed to hypoxia (Ietta et al., 2018; Soppert et al., 2018; Xia et al., 2015a). MSCs ability to cope and maintain viability in conditions of severe environmental stresses make it difficult to study (Deschepper et al., 2011; Russo et al., 2020). Additionally, other prominent publications describing the anti-apoptotic nature of MIF have used agents which alter the mitochondrial membrane permeability such as sodium nitroprusside and the

topoisomerase inhibitor camptothecin. (Hudson et al., 1999; Leech et al., 2003; Nguyen et al., 2003; Shi et al., 2006). MSCs are resistant to both agents and are unable to induce apoptosis, therefore, another approach was required (Babajani et al., 2020; Nicolay et al., 2016). We opted to use STS to induce apoptosis in MSCs. Despite showing that MIF has a small protective effect on MSCs, we did not observe the same levels of protection observed in other papers (Gore et al., 2008; Li et al., 2019; Nguyen et al., 2003). This is likely to be explained by the mechanism of action of STS. STS induces apoptosis through caspase-dependent and caspase independent mechanisms and making it a highly potent inducer of apoptosis. Such strong signals to elicit the induction of apoptosis make it very difficult to show protection.

Furthermore, our results show that MIF enhances MSC expansion which may improve MSC efficacy *in vivo*. Cytokine mediators often function as growth factors. These findings are consistent with other reports showing that MIF can enhance the proliferation and survival of other cell types such as macrophages (Shi et al., 2006), kidney cells (Djudjaj et al., 2017), and neural stem/progenitor cells (Ohta et al., 2012). Despite evidence that shows MSCs are only present for a transient period after infusion (Witte et al., 2018), the MSC secretome is still an important facet of MSC efficacy, therefore, retention time and MSC number at the site of inflammation remains to be important. Reports showing MIF as a regulator of proliferation and survival have suggested it is mediated through the activation of the PI3K-Akt pathway (Costa et al., 2016; Gore et al., 2008; Soppert et al., 2018; Xia and Hou, 2018). We observed an upregulation of p-Akt in MSCs after incubation with the $CATT_7$ CM, however interestingly, this was not abrogated in the presence of the MIF inhibitor SCD-19. This finding highlights the disadvantage of using BMDM supernatants to study the role of endogenous MIF. Despite using WT CM as a baseline control to show our findings

are a result of high levels of MIF, we are unable to prevent other factors in the supernatant from also acting on the MSCs. The BMDM supernatants could contain a plethora of different cytokines and growth factors that could initiate signal transduction. However, we can show that the activation of signalling pathways by these other possible factors do not have an impact functionally through the use of the highly specific MIF inhibitor SCD-19.

We also showed that MIF can enhance MSC immunomodulation through the upregulation of COX-2, the catalyst for the production of PGE2 (Sampey et al., 2001). PGE2 facilitates MSC immunomodulation through the suppression of immune cells such as dendritic cells (Spaggiari et al., 2009), natural killer cells (Spaggiari et al., 2008) and T cells (Duffy et al., 2011) and the induction of $CD4^+CD25^+FoxP3^+$ regulatory T cells (English et al., 2009). Our findings align with other studies showing MIF as an upstream regulator of COX-2 through the activation of the MAPK signalling pathway (Carli et al., 2009; Mitchell et al., 2002). Notably, we found that MIF upregulated COX-2 production but not IDO production in MSCs. This finding suggests that the ability of MIF to enhance MSC immunomodulation is specific to the MAPK signalling pathway and does not activate the Janus kinase (JAK)- signal transducer and activator of transcription 1 (STAT1) pathway (Kim et al., 2018). In addition, we found that MIF can amplify TNF-α mediated upregulation of COX-2. This additive effect suggests that MIF and TNF-α can work synergistically to further enhance MSC function.

Taken together, we show that MIF has the ability to enhance MSC function through driving MSC migration, enhancing MSC ability to survive and expand, and increase MSC immunomodulatory potential through the upregulation of COX-2. These data

provide a greater understanding of interactions between MIF and MSCs. As mentioned previously, differences in MIF polymorphisms can result in contrasting disease severities due to the difference in inflammatory microenvironments. A patient with the 5/5 haplotype will have lower levels of MIF in basal or stimulated conditions (Baugh et al., 2002). The 5/5 haplotype is more likely to have a less severe disease, whereas a 7/7 population tend to experience more severe disease conditions due to the higher levels of circulating MIF (Awandare et al., 2009; Das et al., 2016; Mizue et al., 2005; Savva et al., 2016). We have shown that high levels of hMIF from BMDM supernatants of $CATT_7$ mice are able to enhance various properties of MSCs which may increase their potency. This information may allow us to stratify patient populations with different expression levels of MIF for MSC therapy as MSCs appear more efficacious in the presence of MIF. Patients with high expressing haplotypes of the CATT polymorphism such as those with 7/7 or 8/8 may respond better to MSC therapy compared to patients with the low expressing 5/5 haplotype. However, more interestingly, this could reveal a new opportunity of MSC optimisation as a cell therapy through licensing. Licensing has already been shown to be a useful tool to bolster MSCs function with cytokines such as TNF-α and IFN-γ. Notably we observed that licensing with MIF alone or in combination with other cytokines provide enhanced immunomodulatory ability. Our findings again present the unusual dichotomy when it comes to MSC therapy where a proinflammatory cytokine like MIF which is responsible for the pathogenesis of inflammatory diseases can be beneficial for MSC therapy in its ability to activate and license them to enhance their function.

Chapter 4 High levels of human Macrophage migration inhibitory factor (MIF) potentiate Mesenchymal stromal cell (MSC) efficacy in a murine model of allergic asthma

4.1 Abstract

Asthma is a large contributor to morbidity and mortality worldwide. Current asthma therapies focus on reducing symptoms but fail to restore existing damage. Mesenchymal stromal cell (MSC) therapy has emerged as an attractive candidate by its ability to ameliorate airway inflammation and reverse airway remodelling. However, success of MSCs in clinical trials have been limited as not all patients respond to such therapy. A possible reason for this is differences in patient disease microenvironments as MSCs require a threshold level of inflammation to carry out their therapeutic effects. A greater understanding of the interactions between MSCs and mediators within the disease microenvironment, and strategies to address the discrepancies between MSC efficacy *in vivo* and in clinical trials are needed. In this study we sought to investigate the efficacy of MSCs in high vs low hMIF environments using humanised mice in a HDM model of allergic asthma. We also sought to determine the effect of MIF licensing on MSC efficacy and elucidate the mechanisms involved. Intravenously infused human bone marrow-derived MSCs (BM-MSCs) significantly attenuated airway inflammation in high MIF expressing $CATT_7$ mice by reducing the number of eosinophils and levels of IL-4, IL-5, and IL-13 in the bronchoalveolar lavage (BAL) fluid. MSCs also had a significant effect on airway remodelling in the $CATT_7$ mice with reduced subepithelial collagen deposition and goblet cell hyperplasia. Little to no effects of MSC administration were observed in the low MIF expressing mice or wildtype controls. Differences in efficacy correlated with retention as MSCs appear to be retained longer in the lungs of $CATT_7$ mice compared to $CATT_5$ or wildtypes. Furthermore, we demonstrate that MIF can be used to license MSCs prior to administration to enhance their therapeutic efficacy. MIF licensing potentiated MSCs anti-inflammatory effects at a previously ineffective

dose. Mechanistically, MIF binding to CD74 expressed by MSCs lead to upregulation of COX-2 expression. Blockade of CD74 or COX-2 function in MSCs prior to administration attenuated the efficacy of MIF-licensed MSCs in vivo.

In conclusion, these data demonstrate the importance of understanding the disease microenvironment prior to MSC administration. Additionally, we also provide a novel MIF-licensing strategy to further potentiate MSCs therapeutic potential *in vivo*.

4.2 Introduction

In the preceding chapters we have investigated the influence of MIF in asthma. Using humanised high-expressing and low-expressing MIF mice in a HDM model of allergic airway inflammation we demonstrated the pivotal role MIF plays in asthma pathogenesis. High levels of human MIF resulted in a significant increase in airway inflammation as a result of elevated levels of Th2 cytokines promoting infiltration of eosinophils into the airways. Furthermore, high levels of MIF were associated with airway remodelling with significant mucus hyperplasia, subepithelial collagen deposition, and airway hyperresponsiveness generating a more severe asthma phenotype. Currently, there is a heavy reliance on inhaled corticosteroids and long acting β2-adenoceptor agonists in the treatment of type 2-high asthma. The introduction of novel biologics such as benralizumab and dupilumab targeting Th2 cytokine receptors and tezepelumab targeting the alarmin TSLP have been shown to significantly reduce exacerbations and improve FEV1 values (Bleecker et al., 2016; Corren et al., 2020; Ortega et al., 2016; Sverrild et al., 2021). However, not all patients are responders, and they fail to reverse any existing airway remodelling in patients. In order to maintain efficacy, treatments like dupilumab require administration via injection every 2 weeks. New long-lasting therapeutics able to attenuate inflammation and reverse remodelling are needed to address the pitfalls in the current treatment and management of allergic asthma.

Mesenchymal stromal cells (MSCs) have emerged as a possible alternative therapy. MSCs immunomodulatory and anti-fibrotic properties have driven interest given asthma's pathology. Administration of MSCs intratracheally or intravenously has been shown to be effective in reducing airway inflammation and airway

hyperresponsiveness in OVA (Abreu et al., 2013; Choi et al., 2022; Dai et al., 2018; de Castro et al., 2017; Hur et al., 2020; Mathias et al., 2013; Ou-Yang et al., 2011; Royce et al., 2019, 2017; Song et al., 2015; Zhong et al., 2019), HDM (Abreu et al., 2018, 2019b; Braza et al., 2016; Castro et al., 2020; Duong et al., 2015; Kitoko et al., 2018; Shin et al., 2021), and aspergillus hyphal extract (Cruz et al., 2015; Lathrop et al., 2014) models. However, to understand the mechanisms involved and to make MSCs a viable therapeutic in the clinic more focussed translational work is needed.

Under basal conditions MSCs lie in a homeostatic state. MSCs only become polarised to an anti-inflammatory phenotype in the presence of extrinsic factors. Once polarised, MSCs modulate their surrounding microenvironment (Liu et al., 2022). Licensing has been shown to improve MSC therapeutic efficacy by polarising MSCs prior to administration. Licensing through exposure to hypoxia (J. Liu et al., 2021; Mathew et al., 2017; Roemeling-van Rhijn et al., 2013; Zhilai et al., 2016), inflammatory cytokines (Carty et al., 2021; Corbett et al., 2021), and pharmacological factors (Silva et al., 2019; Takeda et al., 2018) have all been shown to improve MSC efficacy in a range of inflammatory diseases.

Therefore, this chapter seeks to investigate the relationship between MIF and MSCs *in vivo* and to define conditions for optimal MSC therapeutic efficacy. Following the discovery that high levels of hMIF can enhance the immunomodulatory capacity and migration of MSCs *in vitro* we hypothesised that administration of MSCs into the high expressing $CATT_7$ mice may result in improved therapeutic outcomes compared to administration into $CATT_5$ or wildtype mice. We hope that the data in this chapter will allow us to make conclusions relevant to asthma disease in humans through the use of

human cell therapy to treat mice expressing a human cytokine in the presence of a clinically relevant allergen.

The objectives of this chapter are outlined as follows:

(1) Investigate the efficacy of MSCs in high vs low hMIF environments in a mouse model of allergic asthma
(2) Determine the biodistribution of MSCs in high and low hMIF environments following systemic administration
(3) Investigate the effect of MIF licensing on MSC efficacy in a mouse model of allergic asthma
(4) Elucidate the mechanisms involved in MIF-licensed MSC efficacy *in vivo*

4.3 Materials and Methods

4.3.1 Ethical Approval

As stated in 2.3.1.

4.3.2 Human bone marrow derived MSC culture

Carried out as stated in 3.3.3.

4.3.3 Animal Strains

Two C57BL/6N mouse strains expressing the human high- or low-expression *MIF* alleles (*MIF*^{CATT7} [(C57BL/6NTac-Miftm3884.1(MIF)Tac-Tg(CAG-Flpe)2Arte] and *MIF*^{CATT5} [C57BL/6NTac-Miftm3883.1(MIF)Tac-Tg(CAG-Flpe)2Arte] mice) were created using vector-based recombinant replacement of murine *Mif* by Taconic Biosciences (Rensselaer, NY). Validation of the expression of human and not murine *MIF* mRNA was verified by qPCR, and -794 CATT-length dependent stimulated MIF production was confirmed *in vivo* (Shin et al., 2022). Littermate wildtype (WT) and *Mif*$^{-/-}$ (MIF KO) (a kind donation from R. Bucala, Yale School of Medicine, Yale University, New Haven, CT, USA) mice were used as controls.

4.3.4 HDM-induced Airway Inflammation Model and Therapeutic Protocol

MIF^{CATT7}, *MIF*^{CATT5} or WT mice were challenged with 25 µg HDM extract (*Dermatophagoides pteronyssinus,* Greer Laboratories, Lenoir, NC, USA) in 25 µl phosphate buffered saline (PBS) intranasally (i.n.) 3 days weekly for 3 weeks under light isoflurane anaesthesia. Control mice were challenged with 25 µl PBS under the same conditions. On day 14, after HDM challenge, mice received an intravenous (i.v.) injection of $1x10^6$ MSCs in 300 µl into the tail vein. For the dose curve $1x10^6$, $5x10^5$, $1x10^5$, and $5x10^4$ were administered i.v. into HDM challenged $CATT_7$ mice on day 14.

4.3.5 Licensing of MSCs with Endogenous Human MIF

CM from BMDMs of $CATT_7$ mice was generated as described in chapter 3. Additionally, KO CM was generated from MIF KO mice as a control. Licensing MSCs was performed by removing existing media, washing with PBS, and incubating cells with $CATT_7$ CM (^{CATT7}MSC) or MIF KO CM (^{KO}MSC) for 24 Hr. $5x10^4$ or $1x10^6$ licensed MSCs were administered i.v. into HDM challenged $CATT_7$ or WT mice on day 14, respectively. Where indicated, MSCs were treated with COX-2 inhibitor indomethacin (10 µM) for 30 min. Following pre-treatment, MSCs were licensed with $CATT_7$ CM for 24 hr as described above. Moreover, mouse anti-CD74 neutralising antibody and isotype control were added to the assay. MSCs were pre-treated with anti-CD74 neutralising antibody (clone LN2) (10 µg/ml) or IgG1 κ isotype control (clone T8E5) (10 µg/ml) for 30 min. MSCs were then licensed with $CATT_7$ CM for 24 hr before administration.

4.3.6 Collection of Bronchoalveolar Lavage (BAL) Fluid

BAL fluid collected and analysed as stated in 2.3.6.

4.3.7 Enzyme-linked immunosorbent assay (ELISA)

Levels of mIL-4 (Biolegend, San Diego, CA, USA), mIL-5 (Biolegend) and, mIL-13 (eBioscience, San Diego, CA, USA) were determined using commercial ELISA kits, according to manufacturer's instructions.

4.3.8 Lung Histology

On day 21, mice were sacrificed for histological analysis. Lungs were processed and stained as stated in 2.3.8.

4.3.9 Cryo-Imaging

$1x10^6$ MSCs were labelled with the Qtracker® 625 labelling kit (ThermoFisher Scientific) according to manufacturer's instructions before being administered i.v. on day 14. On day 15, mice were humanely euthanised, and the lungs were embedded in mounting medium for cryotomy (O.C.T compound, VWR Chemicals, Leuven, Switzerland), frozen in liquid nitrogen and stored at -80°c. Sectioning and imaging were carried out using the automated CryoViz™ imaging system (BioInvision Inc., Cleveland, OH, USA). Images were then processed to generate 3D images using

CryoViz™ processing, and the number of detected cells was quantified using cell detection software (BioInvision) (Carty et al., 2021).

4.3.10 Statistical Analysis

All data are presented as mean ± SEM. Results of two or more groups were compared by 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.

4.4 Results

4.4.1 Human BM-MSCs significantly reduce goblet cell hyperplasia in $CATT_7$ mice challenged with HDM

Firstly, to investigate the impact of high- and low-expressing MIF alleles on MSC treatment of allergic airway inflammation we examined the lung histology. $CATT_7$, $CATT_5$, and WT mice exposed to HDM intranasally 3 times a week for 3 weeks. $1x10^6$ human BM-MSCs were administered via tail vein injection on day 14 before being sacrificed on day 21. Lung tissue was removed, formalin fixed and sectioned onto slides. Slides were stained with PAS to highlight mucin production to assess the level of goblet cell hyperplasia. As observed in chapter 2, $CATT_7$ mice exhibit significantly higher level of goblet cell hyperplasia compared to their WT and $CATT_5$ low expression counterparts. Interestingly, we observed that administration of BM-MSCs into HDM challenged $CATT_7$ mice significantly reduced the number of mucin

secreting cells in the airways (Fig. 4.1A, 4.1B). We observed no change in goblet cell hyperplasia in WT or $CATT_5$ mice when treated with BM-MSC (Fig. 4.1A, 4.1B). These data suggest that MSCs are more efficacious at reducing goblet cell hyperplasia in more severe inflammatory environments such as in high MIF expressing $CATT_7$ mice.

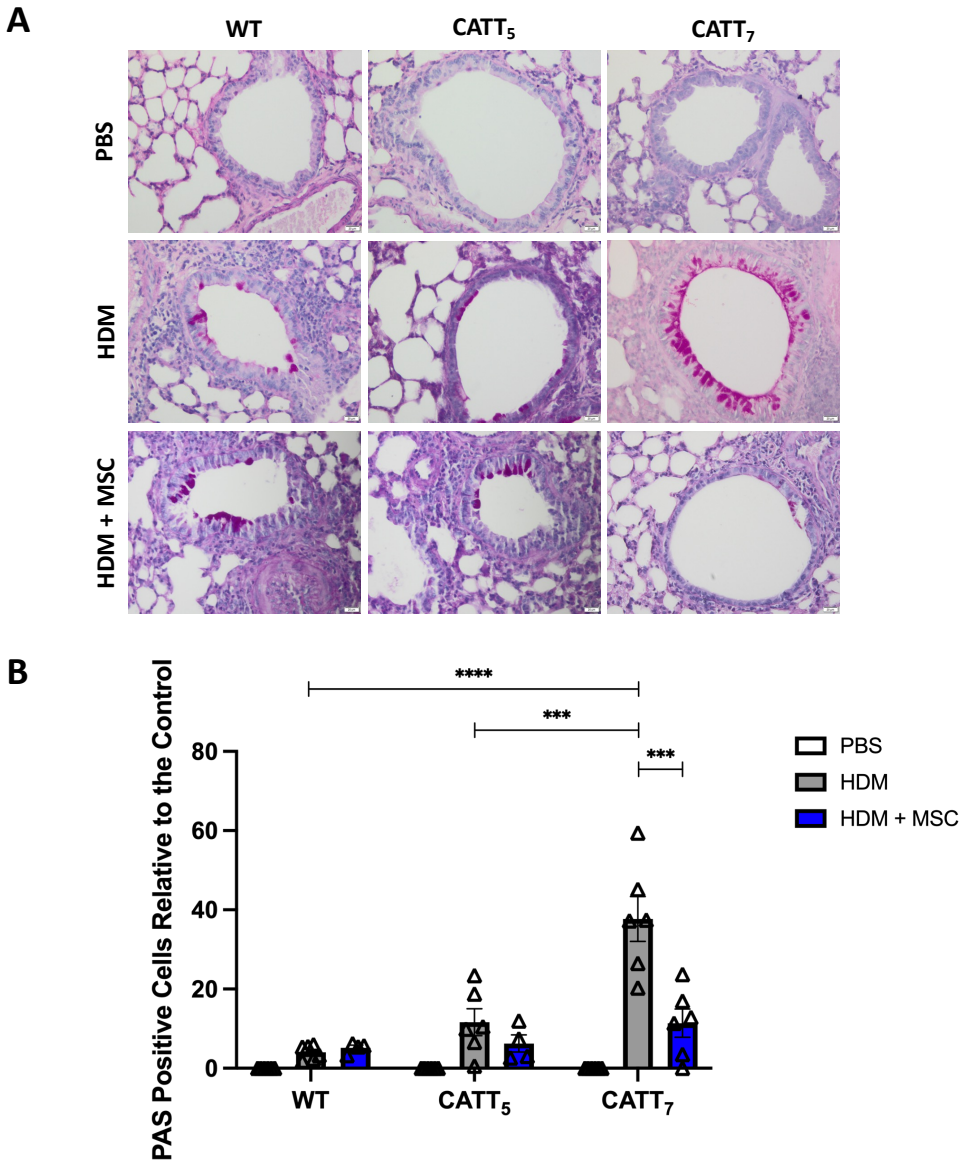


Figure 4.1 Human BM-MSCs significantly reduce goblet cell hyperplasia in $CATT_7$ mice challenged with HDM. Periodic acid Schiff (PAS) staining of lung tissue from WT, $CATT_5$ and $CATT_7$ mice. PBS and HDM groups received PBS or HDM i.n. 3 times a week for 3 consecutive weeks. $1x10^6$ human BM-MSCs were administered i.v. to the HDM+MSC groups on day 14. Mice were sacrificed on day 21. **A** Representative images of lung tissue stained with PAS at 20X magnification, scale bar = 20 µm. **B** Goblet cell hyperplasia was investigated through the quantitation of PAS positive cells. Data are presented as mean ± SEM; N=6 per group. Statistical analysis was carried out using one-way ANOVA followed by the *post-hoc* Tukey's multiple comparison test where ***p<0.001, ****p<0.0001.

4.4.2 Human BM-MSCs significantly reduce collagen deposition in $CATT_7$ mice challenged with HDM

In addition to studying airway remodelling through the assessment of goblet cell hyperplasia we also assessed the level of subepithelial fibrosis. Slides were stained with Masson's trichrome to determine the amount of collagen deposition in the airways. As shown in chapter 2, $CATT_7$ HDM mice display significantly higher levels of subepithelial fibrosis compared to the lower MIF expressing $CATT_5$ and WT groups. Quantification of the % collagen in the lung tissue showed that administration of BM-MSCs ameliorates subepithelial collagen deposition in HDM challenged $CATT_7$ mice (Fig. 4.2A, 4.2B). Furthermore, no statistical significance was observed between the $CATT_7$ PBS group and the BM-MSC treated group suggesting BM-MSC treatment had alleviated the inflammation induced fibrosis. No statistical difference was found between HDM and HDM + MSC groups in the WT or $CATT_5$ mice.

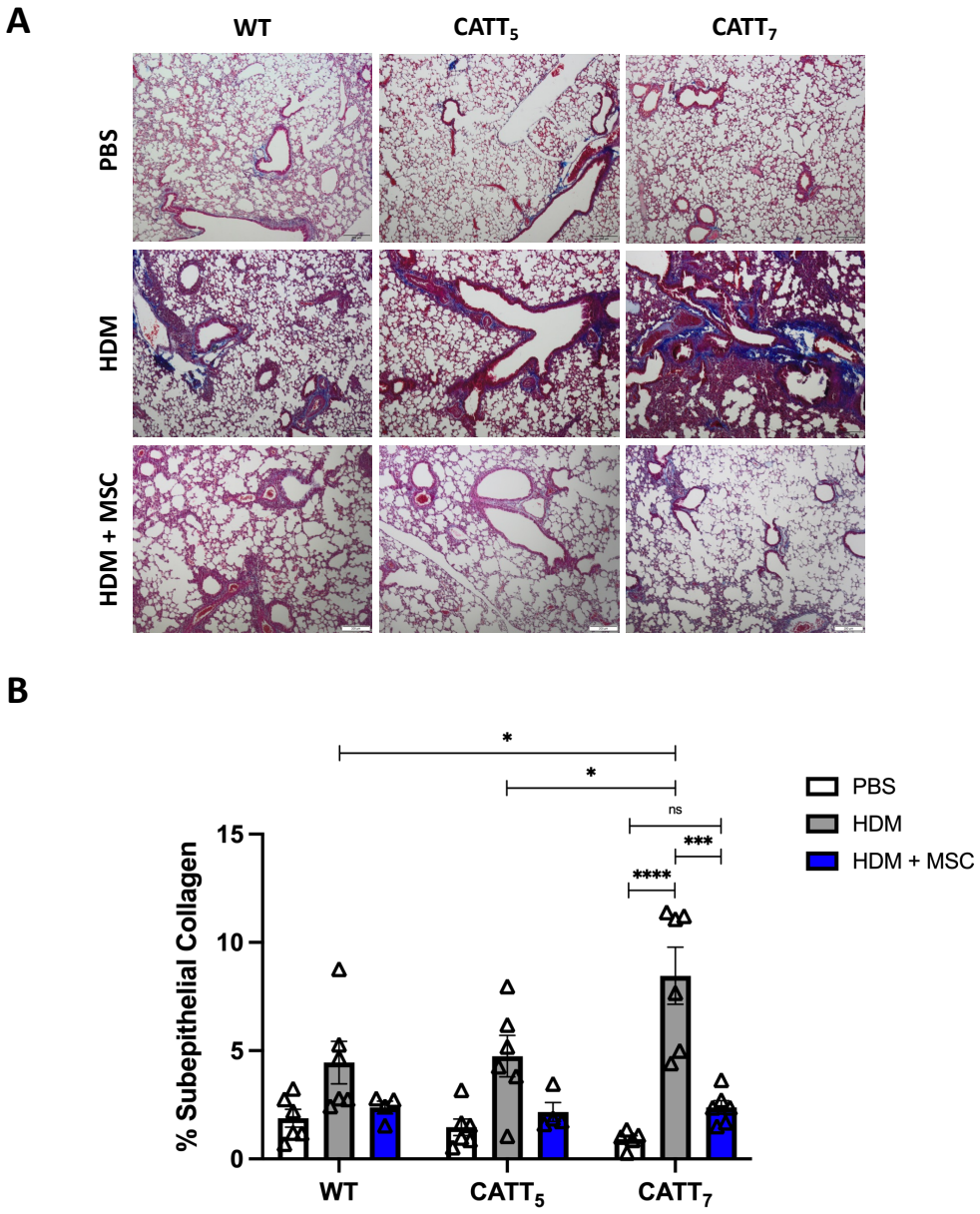


Figure 4.2. Human BM-MSCs significantly reduce collagen deposition in $CATT_7$ mice challenged with HDM. Masson's trichrome staining of lung tissue from WT, $CATT_5$ and $CATT_7$ mice. PBS and HDM groups received PBS or HDM i.n. 3 times a week for 3 consecutive weeks. $1x10^6$ human BM-MSCs were administered i.v. to the HDM+MSC groups on day 14. Mice were sacrificed on day 21. **A** Representative images of lung tissue stained with Masson's trichome at 4X magnification, scale bar = 200 µm. **B** Quantitation of % subepithelial collagen. Data are presented as mean ± SEM; N=4-6 per group. Statistical analysis was carried out using one-way ANOVA followed by the *post-hoc* Tukey's multiple comparison test where *p<0.05, ***p<0.001, ****p<0.0001, ns non significant.

4.4.3 Human BM-MSCs significantly reduce inflammatory infiltrate surrounding airways in $CATT_7$ mice challenged with HDM

H&E staining highlights the level of immune cell infiltration surrounding the airways. As detailed in chapter 2, $CATT_7$ HDM mice exhibit significantly higher airway immune cell infiltration compared to both $CATT_5$ and WT HDM. BM-MSCs markedly reduced H&E score in all groups, although significant difference was only observed in the $CATT_7$ group (Fig. 4.3A, 4.3B). Next, we examined inflammatory infiltrate into the lungs following BM-MSC treatment of HDM challenged mice by harvesting the BAL fluid. BAL fluid was centrifuged to isolate cells. Following cell counting, $1x10^5$ cells were spun onto slides using a cytocentrifuge. Slides were stained with Kwik Diff stain to differentiate between eosinophil, lymphocyte, macrophage, and neutrophil populations. We observed no difference in total cell counts of WT HDM and WT HDM MSC or between $CATT_5$ HDM and $CATT_5$ HDM MSC groups (Fig. 4.4A). However, we observed a significant reduction in the number of infiltrating cells in the BAL fluid of $CATT_7$ mice following BM-MSC treatment (Fig. 4.4B). Following analysis of the differential cell counts we identified eosinophils as the main cell infiltrating into the lung tissue (Fig. 4.4A). As a result of this, the trends observed in the total number of eosinophils mirrored that of the total cell count (Fig. 4.4C). Whereby, MSC treatment significantly reduced the number of infiltrating eosinophils in $CATT_7$ mice yet had no effect in the $CATT_5$ and WT groups (Fig. 4.4C).

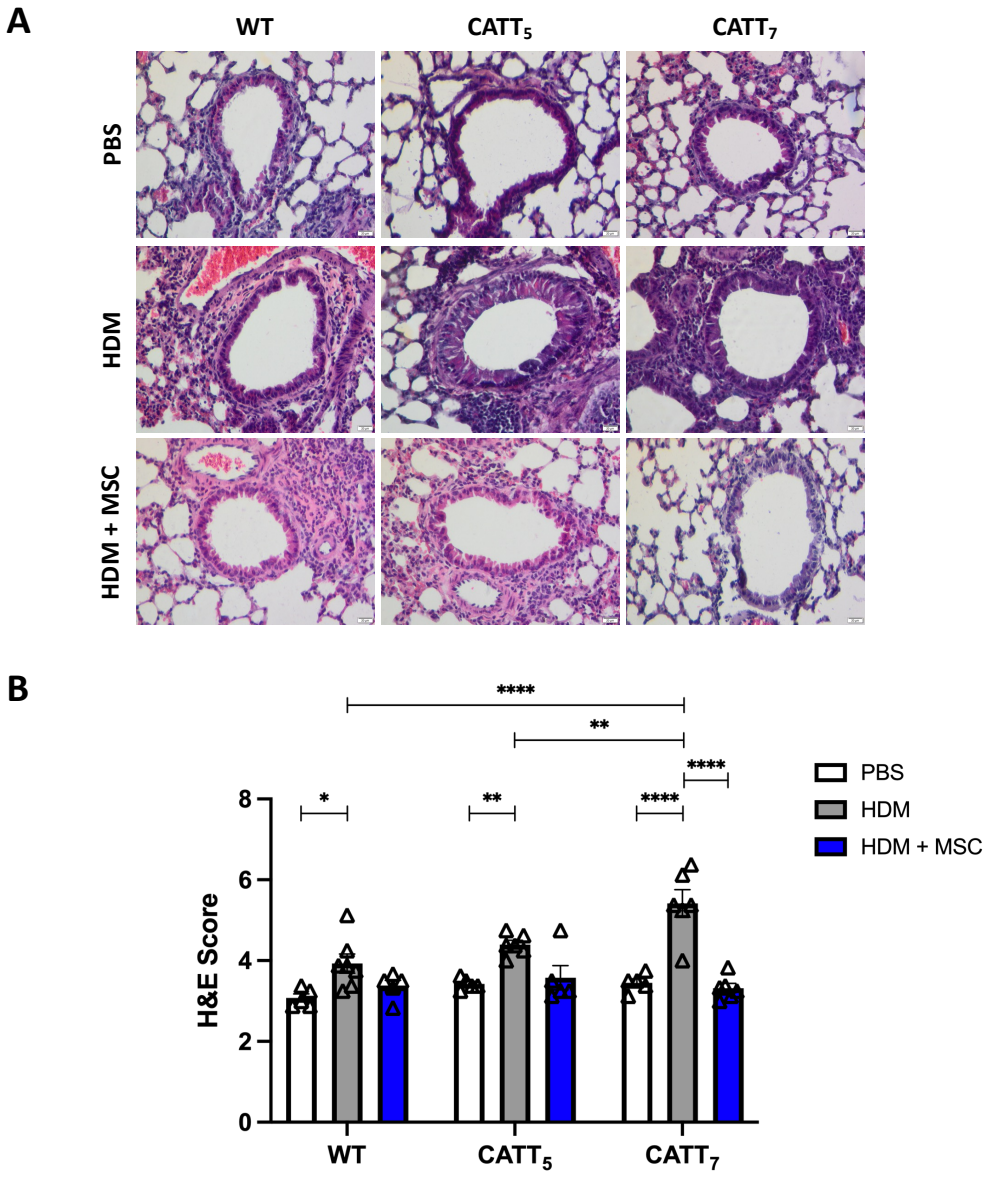


Figure 4.3. Human BM-MSCs significantly reduce inflammatory infiltrate surrounding airways in CATT$_7$ mice challenged with HDM. Haematoxylin and eosin (H&E) staining of lung tissue from WT, CATT$_5$ and CATT$_7$ mice. PBS and HDM groups received PBS or HDM i.n. 3 times a week for 3 consecutive weeks. 1x10^6 human BM-MSCs were administered i.v. to the HDM+MSC groups on day 14. Mice were sacrificed on day 21. **A** Representative images of lung tissue stained with H&E at 20X magnification, scale bar = 20 µm. **B** Quantitation of airway inflammation in H&E-stained lung tissue. Data are presented as mean ± SEM; N=4-6 per group. Statistical analysis was carried out using one-way ANOVA followed by the *post-hoc* Tukey's multiple comparison test where *p<0.05, **p<0.01, ****p<0.0001.

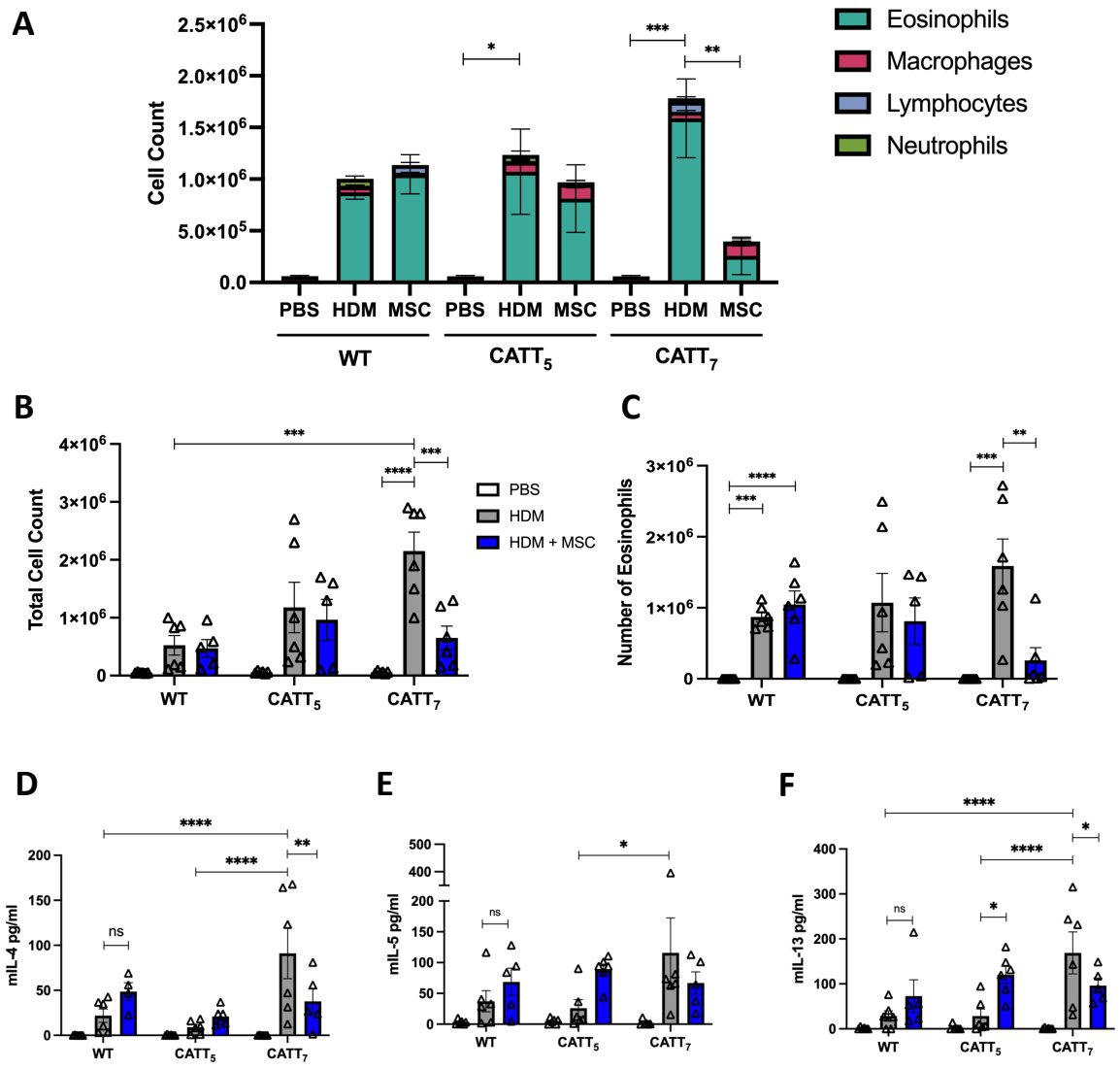


Figure 4.4. Human BM-MSCs significantly reduce levels of Th2 cytokines in the BAL fluid of CATT$_7$ mice challenged with HDM. PBS and HDM groups received PBS or HDM i.n. 3 times a week for 3 consecutive weeks. 1x10^6 human BM-MSCs were administered i.v. to the HDM+MSC groups on day 14. BAL was performed 4 Hr post final HDM challenge on day 18. **A** Cells recovered from the BAL fluid were stained with Kwik Diff stain to identify the number of macrophages, eosinophils, lymphocytes, and neutrophils. **B** Total cell counts and numbers of eosinophils (**C**) recovered from the BAL were enumerated. Cytokine levels of **D** IL-4, **E** IL-5 and **F** IL-13 in the BAL fluid determined by ELISA. Data are presented as mean ± SEM; N=4-6 per group. Statistical analysis was carried out using one-way ANOVA followed by the *post-hoc* Tukey's multiple comparison test where *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns non significant.

4.4.4 Human BM-MSCs significantly reduce levels of Th2 cytokines in the BAL fluid of $CATT_7$ mice challenged with HDM

Next, we investigated the Th2 cytokines in the BAL fluid by ELISA. IL-4, IL-5 and IL-13 produced by Th2 cells and ILC2s in the inflammatory microenvironment co-ordinate the inflammatory immune response. BM-MSC treatment appeared to be ineffective at alleviating Th2 cytokines in the BAL fluid of $CATT_5$ and WT mice (Fig. 4.4D, 4.4E, 4.4F). Interestingly we observed a trend of increased levels of Th2 cytokines following BM-MSC treatment in WT and $CATT_5$ mice. However, only IL-13 was significantly higher in the $CATT_5$ HDM MSC group compared to $CATT_5$ HDM. Whereas, BM-MSCs significantly reduced HDM-driven production of IL-4 and IL-13 in $CATT_7$ mice (Fig. 4.4D, 4.4E, 4.4F). Additionally, BM-MSCs slightly reduced IL-5 levels in the $CATT_7$ mice, however, this was not statistically significant. These data show that BM-MSCs are effective at alleviating eosinophil infiltration and reducing Th2 cytokines in a high MIF expressing model of allergic asthma.

4.4.5 High levels of hMIF significantly enhance BM-MSC retention in a HDM model of allergic asthma

Next, we analysed the biodistribution of BM-MSCs following administration into HDM challenged WT, $CATT_5$ and $CATT_7$ mice. $1x10^6$ fluorescently labelled BM-MSCs were administered via tail vein injection on day 14. On day 15 mice were sacrificed and the lungs prepared for CryoViz imaging (Fig. 4.5A, 4.5B, 4.5C). Significantly higher numbers of labelled BM-MSCs were detected in the lung of high MIF expressing $CATT_7$ mice compared to the low expressing $CATT_5$ or WT littermate control (Fig. 4.5C, 4.5D). However, the number of clusters of labelled BM-MSCs

within the lungs remained unchanged among the groups (Fig. 4.5E). These data suggest that high levels of MIF enhance MSC retention at the site of injury. This is important as increasing the time at the site of injury allows provides a longer window for BM-MSCs to carry out their therapeutic effects before clearance.

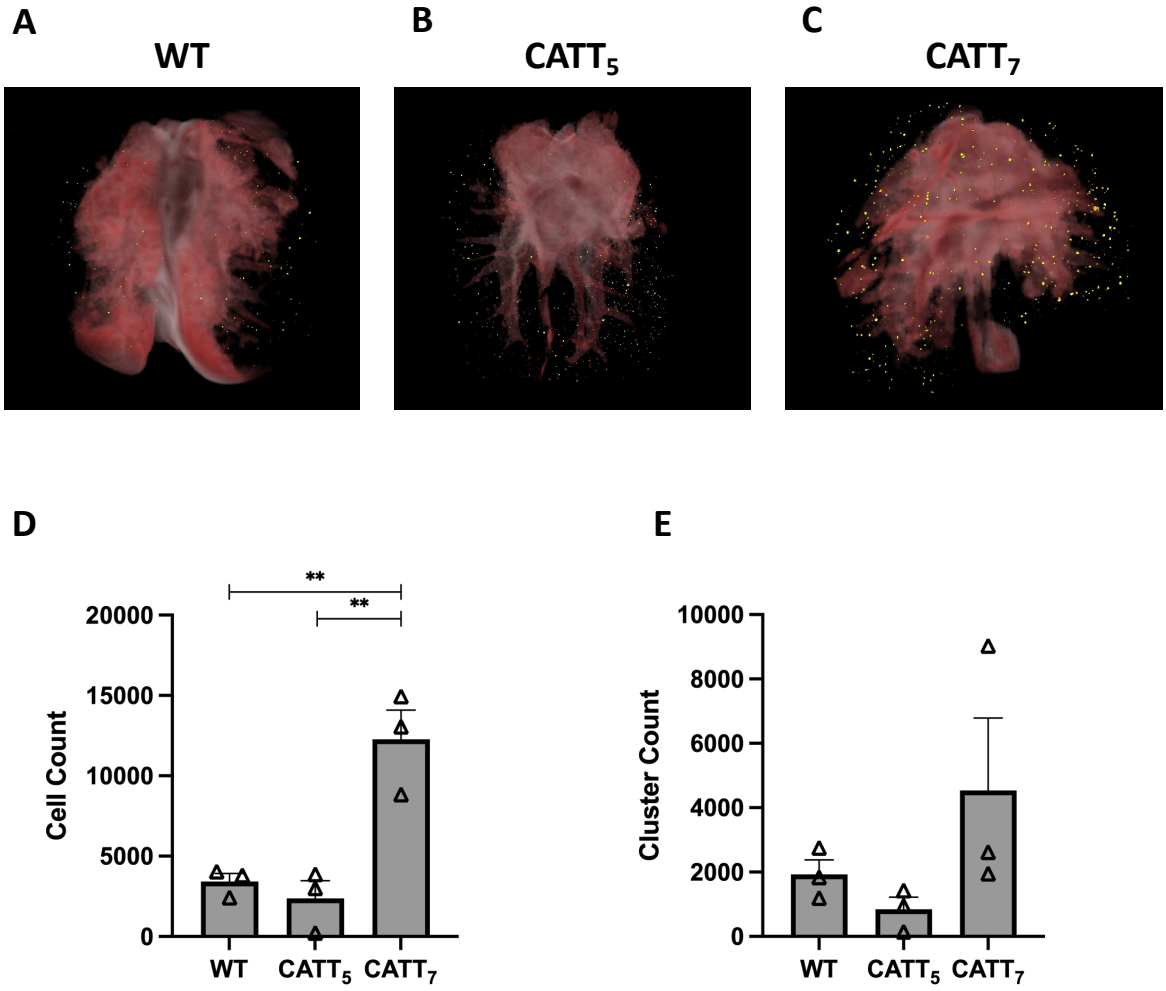


Figure 4.5. High levels of hMIF significantly enhance BM-MSC retention in a HDM model of allergic airway inflammation. HDM was administered i.n. 3 times a week for 2 weeks. On day 14, $1x10^6$ Qtracker 625- labelled hMSCs were administered i.v. in WT, $CATT_5$ or $CATT_7$ mice. 24 hr later the lungs were harvested, embedded in OCT compound and frozen at -80. Tissue blocks were sectioned and imaged using the CryoVizTM (Bioinvision, Cleveland, OH, USA) imaging system. 3D images show representative lung images from **A** WT, **B** $CATT_5$ and **C** $CATT_7$ mice with detected MSCs shown in yellow. **D** Total number of MSCs detected in the lung and **E** number of clusters were quantified using CryoVizTM Quantification software. Data are presented as mean ± SEM; N=3 per group. Statistical analysis was carried out using one-way ANOVA followed by the *post-hoc* Tukey's multiple comparison test where **$p<0.01$.

4.4.6 MIF licensed MSCs are ineffective at reducing immune cell infiltration in the lungs of HDM challenged WT mice

Following the observation that BM-MSCs were more efficacious in a high MIF environment we attempted to mimic the physiological conditions *ex vivo* to improve BM-MSC efficacy in low MIF environments. $1x10^6$ BM-MSCs were stimulated with BMDM conditioned media from $CATT_7$ mice for 24 hours prior to administration. BMDM conditioned media from MIF KO mice was used as a control. On day 14 ^{CATT7}MSC or ^{KO}MSC were injected into the tail vein of HDM challenged WT mice. BAL fluid was collected 4 hours post final challenge on day 18 and cell counts were performed. We observed no difference in total cell count and eosinophil number between the HDM control group and the MIF licensed MSC group (Fig. 4.6A, 4.6B), suggesting that licensing MSCs with MIF is ineffective at ameliorating immune cell infiltration in a low inflammatory microenvironment.

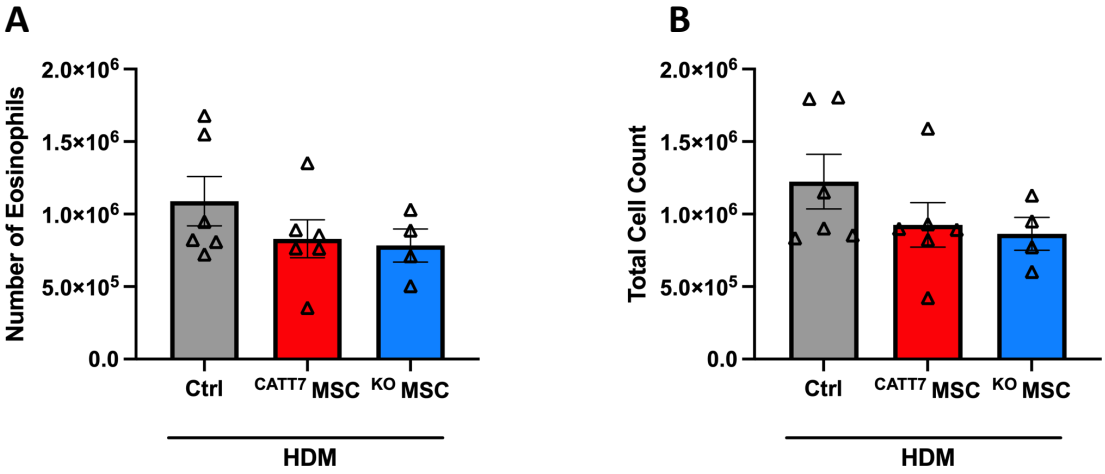


Figure 4.6. MIF licensed MSCs have no effect on immune cell infiltration in the BAL fluid of HDM challenged WT mice. MSCs were licensed with BMDM supernatant from $CATT_7$ or MIF KO mice for 24 Hr before administration into WT mice on day 14. BAL was performed 4 Hr post final HDM challenge on day 18. **A** Total cell count recovered from the BAL. **B** BAL fluid eosinophil count determined by differential staining of cytospins. Data are presented as mean ± SEM; N=4-6 per group.

4.4.7 Titration of BM-MSC doses in $CATT_7$ mice challenged with HDM

Next, we investigated if MIF licensing could improve MSC efficacy in the high MIF expressing $CATT_7$ mice. To do this we first had to perform a pilot study determine the dose at which MSCs lose efficacy. BM-MSC at doses of $1x10^6$, $5x10^5$, $1x10^5$, and $5x10^4$ were administered i.v. into HDM challenged $CATT_7$ on day 14. On day 18, BAL fluid was collected, cell counts were performed and Th2 cytokines were measured. We discovered that BM-MSCs maintained efficacy as low as $1x10^5$ cells displaying a significant reduction in immune cell infiltration (4.7A, 4.7B). Furthermore, we observed lower levels of Th2 cytokines IL-4 (Fig. 4.7C), IL-5 (Fig. 4.7D), and IL-13 (Fig. 4.7E) although not significant. This is likely due to it being a small pilot and only using 2-3 mice per group. We observed that BM-MSCs were no longer able to carry out their immunosuppressive effects at a dose of $5x10^4$. At $5x10^4$ BM-MSCs were unable to reduce the number of eosinophils infiltrating into the lungs (Fig. 4.7A, 4.7B) or regulate Th2 cytokine production (Fig. 4.7C, 4.7D, 4.7E).

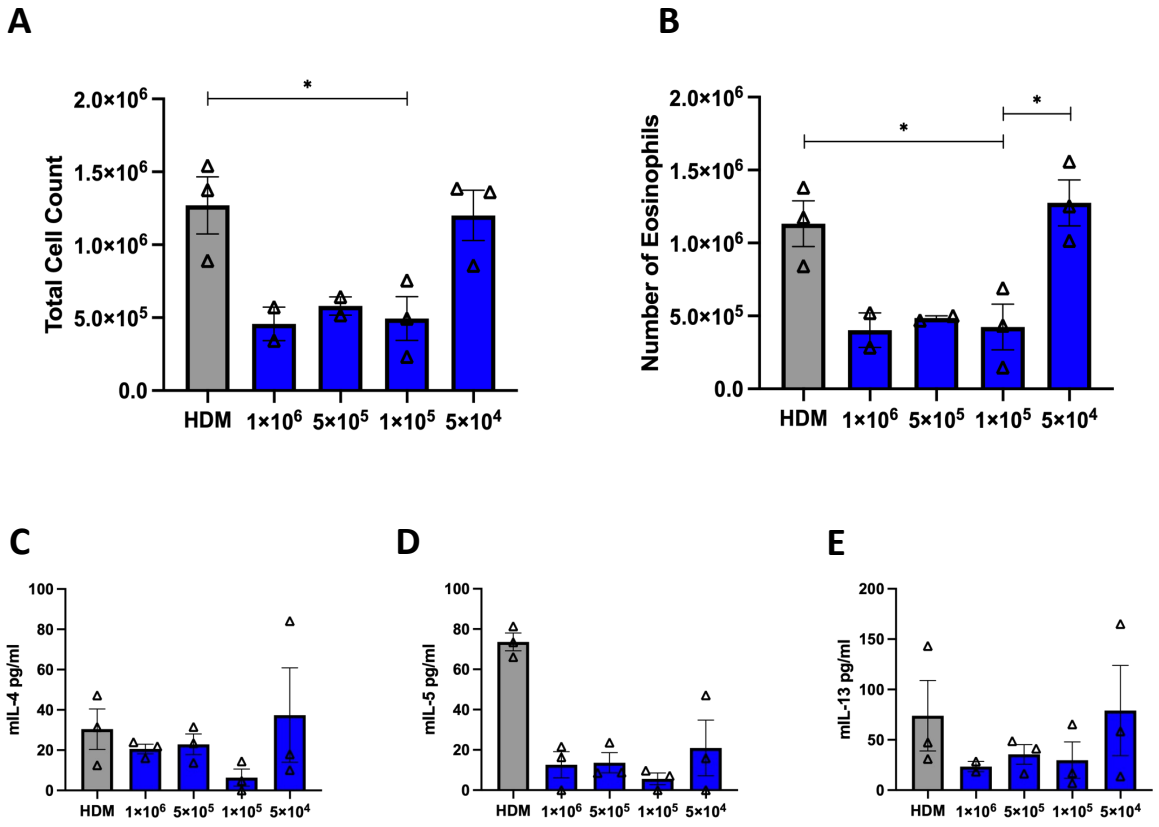


Figure 4.7. Titration of BM-MSC doses in CATT$_7$ mice challenged with HDM. To determine the point where MSCs lose efficacy in CATT$_7$ mice, a range of doses were administered on day 14. BAL was performed 4 Hr post final HDM challenge on day 18. **A** Total cell count recovered from the BAL. **B** Number of eosinophils obtained from the BAL fluid. Cytokine levels of **C** IL-4, **D** IL-5 and **E** IL-13 in the BAL fluid determined by ELISA. Data are presented as mean ± SEM; N=2-3 per group. Statistical analysis was carried out using one-way ANOVA followed by the *post-hoc* Tukey's multiple comparison test where *p<0.05.

4.4.8 MIF licensing restores MSC efficacy at low doses in $CATT_7$ mice

The previous data provided information on the dose at which BM-MSCs lose their effectiveness in our model. We then used this dose to investigate the effect of MIF licensing on MSC therapeutic efficacy. BM-MSCs were first licensed by stimulation with BMDM conditioned media from $CATT_7$ or KO mice for 24 hours. $5x10^4$ MSCs, MIF licensed MSCs (^{CATT7}MSC), or MIF KO licensed MSCs (^{KO}MSC) were administered into $CATT_7$ mice via tail vein injection on day 14 in HDM challenged mice. On day 18, BAL fluid was collected, cell counts were performed and Th2 cytokines were measured. We observed a significant reduction in total cell counts and number of eosinophils in the ^{CATT7}MSC group compared to the non-licensed MSC and KO control (Fig. 4.8A, 4.8B). We observed no difference in macrophage number between the MSC and ^{CATT7}MSC groups (Fig. 4.8C).

Furthermore, analysis of the Th2 cytokines in the BAL fluid showed similar trends to the cellular infiltration with the ^{CATT7}MSC group displaying slightly lower levels of IL-4, IL-5, and IL-13 compared to control group although not significant (Fig. 4.8D, 4.8E, 4.8F). The control MSC group and the ^{KO}MSC group displayed similar levels of immune cell infiltration and Th2 cytokine production suggesting the effects observed in the ^{CATT7}MSC group are MIF specific. Unexpectedly, however, the number of macrophages were significantly elevated in the ^{KO}MSC group. These data show that MIF licensing can restore MSC immunosuppressive function at doses that would normally be ineffective.

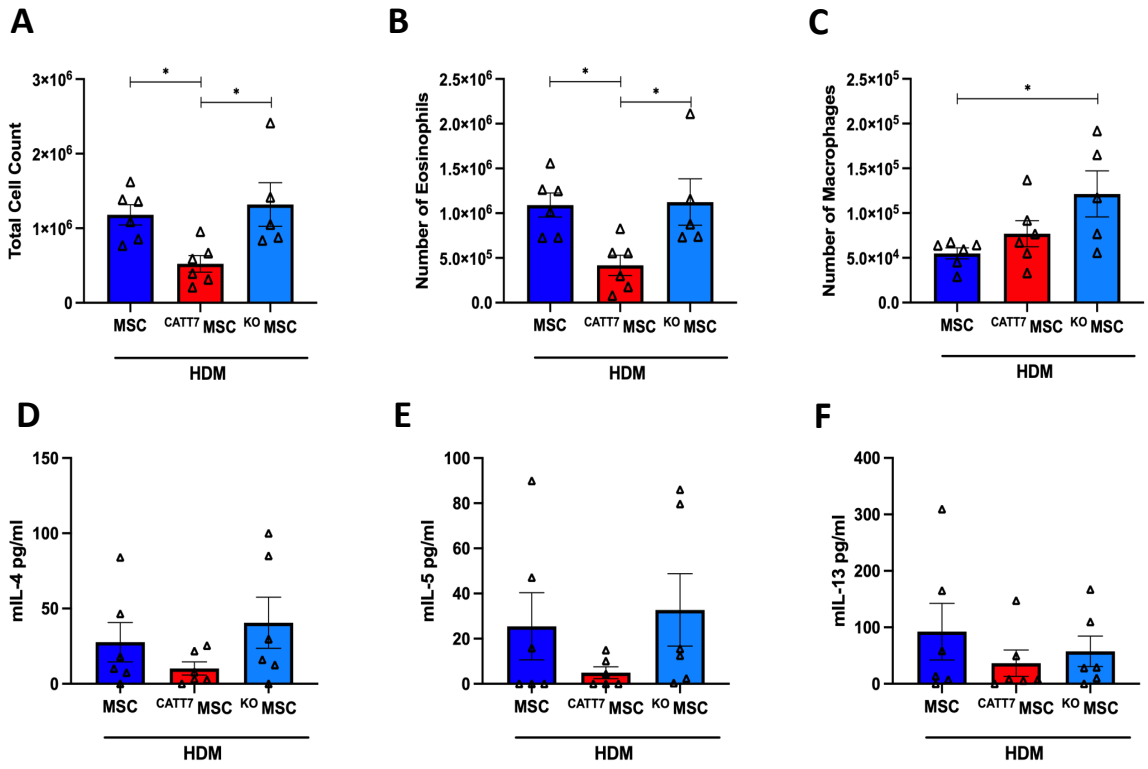


Figure 4.8. MIF licensing restores MSC efficacy at low doses in CATT$_7$ mice. Once it was determined that MSCs lose their efficacy in the HDM model at 5x10^4, we used that dose as a benchmark for licensing. 5x10^4 MSCs were administered to HDM challenged CATT$_7$ mice on day 14. ^{CATT7}MSCs were licensed with CATT$_7$ BMDM supernatant for 24 Hr prior to i.v. administration. The control group ^{KO}MSCs were generated by licensing MSCs with BMDM supernatant from MIF KO mice 24 Hr prior to i.v. administration. BAL was performed 4 Hr post final HDM challenge on day 18. **A** Total number of cells in the BAL was determined and differential cell counts were performed on the collected cells to determine the numbers of **B** eosinophils and **C** macrophages. Cytokine levels of **D** IL-4, **E** IL-5 and **F** IL-13 in the BAL fluid determined by ELISA. Data are presented as mean ± SEM; N=5-6 per group. Statistical analysis was carried out using one-way ANOVA followed by the *post-hoc* Tukey's multiple comparison test where *p<0.05.

4.4.9 Blocking COX-2 abrogates therapeutic efficacy of MIF licensed BM-MSCs

COX-2 is the rate-limiting enzyme involved in the synthesis of arachidonic acid to PGE2, a key mediator in the immunomodulatory effects of MSCs. To assess the role of COX-2 on MIF licensed MSCs we inhibited COX-2 with indomethacin. BM-MSCs were treated with indomethacin (10 μM) for 30 min. Following the 30 min pre-treatment, cells were incubated with $CATT_7$ CM for 24 hours. To further validate the involvement of MIF in the improvement of MSC efficacy, we blocked CD74 signalling using anti-CD74 neutralising antibody. BM-MSCs were treated with anti-CD74 neutralising antibody (10 μg/ml) or IgG1 isotype control (10 μg/ml) for 30 min. Cells were then incubated with $CATT_7$ CM for 24 hours. Analysis of the BAL fluid cell counts showed that pre-treating BM-MSCs with indomethacin significantly reduces MIF licensed BM-MSCs ability to suppress immune cell infiltration. The indomethacin group experienced significantly higher numbers of immune cells, particularly eosinophils, in the BAL fluid compared to the control group (Fig. 4.9A, 4.9B). Additionally, the analysis of the Th2 cytokines in the BAL fluid showed a significant increase in the levels of IL-13 (Fig. 4.9E) in the indomethacin group compared to the control. However, no difference was observed in IL-4 or IL-5 levels (Fig. 4.9C, 4.9D). Taken together, these results show that COX-2 is an important mediator in the enhancement of therapeutic efficacy associated with MIF licensing. Furthermore, blocking of CD74 abrogates MIF licensed BM-MSC immunosuppression. The anti-CD74 group displayed significantly higher numbers of eosinophils in the BAL fluid than the non-treated control (Fig. 4.9A, 4.B). Blocking CD74 abolished MIF licensed MSCs ability to reduce the levels of IL-13 in the BAL fluid (Fig. 4.9E). Similar trends were observed with in the levels of IL-4 and IL-5 yet not significant (Fig. 4.9C, 4.9D). Interestingly, we observed a significant difference in

eosinophil number between the indomethacin group and the anti-CD74 group (Fig. 9B). These data indicate that MIF enhances MSCs immunomodulatory capacity mainly through CD74 signalling to upregulate COX-2 production.

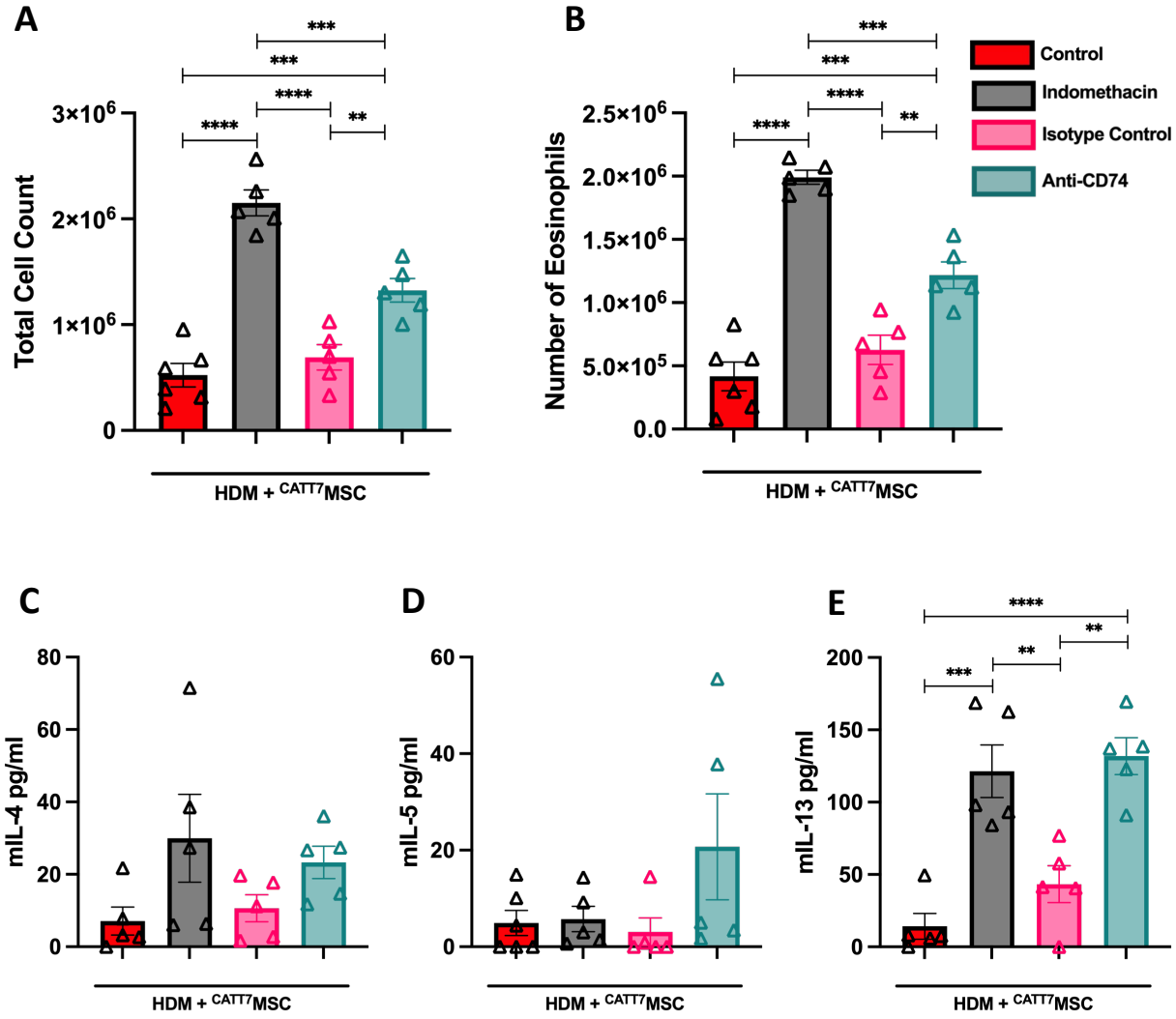


Figure 4.9. Blocking COX-2 abrogates ^{CATT7}MSC therapeutic efficacy.

BM-MSCs were pre-treated with indomethacin (10 µM), anti-CD74 neutralising antibody (10 µg/ml) or IgG1 isotype control (10 µg/ml) 30 min before $CATT_7$ CM licensing. Licensed BM-MSCs were injected into $CATT_7$ mice on day 14 of the HDM-mediated allergic asthma model. BAL fluid was collected 4 hours post final challenge on day 18. **A** Total number of cells in the BAL was determined and differential cell counts were performed on the collected cells to determine the numbers of **B** eosinophils. Cytokine levels of **C** IL-4, **D** IL-5 and **E** IL-13 in the BAL fluid determined by ELISA. Data are presented as mean ± SEM; N=5-6 per group. Statistical analysis was carried out using one-way ANOVA followed by the *post-hoc* Tukey's multiple comparison test where **p<0.01, ***p<0.001, ****p<0.0001.

4.5 Discussion

Despite MSCs promising results in preclinical models of disease, MSC therapy has often yielded disappointing results in the clinical setting. A reason for this is the heterogeneity of humans and the complexity of severe disease. Patients with similar clinical presentations will respond differently due to factors such as the composition of their inflammatory disease microenvironment. Recent studies have shown that not all inflammatory microenvironments are equal and will dictate whether MSCs will be beneficial or further exacerbate inflammation (Islam et al., 2019). Abreu et al. exposed MSCs to BAL fluid from ARDS patients or other lung diseases such as cystic fibrosis (non-ARDS) (Abreu et al., 2019a). They found that exposure to non-ARDS BAL fluid was more effective at inducing anti-inflammatory phenotype in monocytes compared to exposure to ARDS BALF. The importance of the microenvironment was exemplified in a clinical trial which sought to determine the effect of allogenic bone marrow-derived MSCs in patients with moderate to severe COPD (Weiss et al., 2013). Analysis of all 62 patients who received 4 monthly infusions showed that MSC treatment had no significant effect on frequency of exacerbations or pulmonary function tests. However, post-hoc analysis revealed that MSC treatment was effective in a subset of patients (Weiss et al., 2021). Stratification into subgroups of high (≥ 4 mg/L) and low (≤4 mg/L) baseline CRP levels showed that MSC treatment resulted in significant improvements among patients with high baseline CRP levels. Patients with baseline CRP levels over 4 mg/L responded to MSC treatment with significant improvements in pulmonary function and exercise tolerance compared to placebo controls. To improve MSCs viability as a therapeutic there must be more efforts into the identification of subsets of patients more likely to respond to cell therapy i.e. those with more pronounced inflammatory profiles.

Firstly, we explored the effect of high and low levels of endogenous human MIF on MSC efficacy in a HDM model of allergic asthma.

Previous studies demonstrating the positive effects of MSC treatment in asthma models have mostly been performed on OVA sensitised mice (Abreu et al., 2013; Choi et al., 2022; Dai et al., 2018; de Castro et al., 2017; Hur et al., 2020; Lin et al., 2018; Malaquias et al., 2018; Mathias et al., 2013; Ou-Yang et al., 2011; Royce et al., 2019, 2017; Song et al., 2015; Yu and Chiang, 2018; Zhong et al., 2019). These studies have outlined MSCs ability to alleviate hallmark features of asthma, however, OVA models require sensitisation via the peritoneum in combination with the adjuvant alum to prime the immune response. Despite eliciting a strong Th2 immune response, systemic sensitisation with OVA occurs in the absence of pulmonary antigen presenting cells (Aun et al., 2017). This makes it difficult to elucidate the interactions between MSCs and stages of the innate immune response or their crosstalk with the airway epithelium. The use of HDM such as that used in our study or other clinically relevant allergens such as cockroach extract or the fungus *Alternaria alternata* elicit an immune response intranasally without the presence of an adjuvant (Maltby et al., 2017). The protease activity possessed by these allergens can directly damage the lung epithelium while the endotoxin presence in the allergen engages toll-like receptor (TLR) activity creating a clinically relevant disease microenvironment (Gregory and Lloyd, 2011). These processes trigger the release of alarmins such as IL-25, IL33 and TSLP from the epithelial cells to modulate the activation of DCs and Th2 cells. In turn driving the Th2 immune response mediated by Th2 cells and ILC2s (Drake et al., 2014; Hammad and Lambrecht, 2021; Lei et al., 2020). Therefore, the use of HDM permits the study of these innate immune processes that are otherwise not activated when using OVA making it a more suitable method to investigate the effect of MSCs on allergic asthma.

Furthermore, MSCs were injected intravenously via tail vein injection on day 14 of a 3-week HDM model in order to reproduce a clinically relevant situation, as lung inflammation and remodelling were established. Other studies exploring the effect of MSCs in allergic asthma models have used i.t. (Abreu et al., 2019b, 2018, 2013; Kim et al., 2022; Kitoko et al., 2018; Shin et al., 2021) or i.n. (Royce et al., 2019, 2017) routes of administration. These routes of administration have been shown to be more effective as they provide direct exposure to the site of injury. Shin et al. observed that administration of MSCs intratracheally, but not intravenously, significantly reduced IL-13 production in the lungs of Alternaria alternata challenged mice (Shin et al., 2021). The aforementioned studies have provided valuable information regarding the ability of MSCs to suppress Th2 inflammation, however, their route of administration means they are less informative with regards to the mechanism of action. Administration of cells i.v. is the most relevant route of delivery in the clinic therefore it is important to study the effects of systemic MSC treatment. MSCs administered i.v. have previously shown to be effective in some allergic asthma models (Cruz et al., 2015; Hur et al., 2020; Lathrop et al., 2014; Mathias et al., 2013; Zhong et al., 2019).

Our results show that BM-MSC treatment was highly effective in ameliorating airway inflammation in the high MIF expressing $CATT_7$ mice challenged with HDM. We observed that MSCs markedly reduced IL-4, IL-5, and IL-13 in the BAL fluid and in turn abrogated pulmonary eosinophilia in $CATT_7$ group. Our data aligns with several papers in the literature demonstrating the ability of human MSCs to ameliorate eosinophil infiltration by reducing the levels of Th2 cytokines (Choi et al., 2022; Cruz et al., 2015; de Castro et al., 2017; Hur et al., 2020; Lin et al., 2018; Mathias et al., 2013).

In addition to ameliorating inflammation, we observed that BM-MSCs also improves features of airway remodelling in the $CATT_7$ mice. We observed a significant reduction in collagen deposition within the subepithelial space and a significant decrease in goblet cell hyperplasia. Interestingly, we did not see the same changes in the WT and the low MIF expressing $CATT_5$ mice suggesting that high levels of MIF were responsible for improving MSCs efficacy. The reduced efficacy of MSCs in the WT and $CATT_5$ mice is likely attributed to lack of inflammation present. A small therapeutic effect was observed with attenuated subepithelial collagen deposition and goblet cell hyperplasia, however, we detected little to no change in the number of infiltrating cells or Th2 cytokines following MSC administration into $CATT_5$ or WT mice. A reason for the lack of inflammation present could have been due to the strain of mice used. C57BL/6 mice such as the ones in our study are almost always used in the generation of transgenic and knockout mouse lines. C57BL/6 mice present more of a bias towards Th1 immunity compared to the more Th2 BALB/c strain which can result in a more subdued allergic reaction in response to environmental stimuli such as HDM (Fallon and Schwartz, 2020). There have been several instances where researchers also observed poor responses to MSC treatment of allergic airway inflammation in C57BL/6 mice. Kitoko et al. challenged C57BL/6 mice with HDM under the same protocol of 25 μg of HDM 3 times a week for 3 weeks. $1x10^5$ BM-MSCs or adipose tissue-derived MSCs (AD-MSCs) were administered intratracheally one day after the final challenge and the mice were sacrificed either 3 or 7 days later. They observed no improvements in goblet cell hyperplasia or levels of the Th2 cytokines IL-4 and IL-5 with only marginal changes observed in immune cell infiltration following MSC administration (Kitoko et al., 2018). Furthermore, Abreu et al. only observed a small level of suppression following MSC treatment with no

changes in airway remodelling in OVA challenged C57BL/6 mice (Abreu et al., 2013). More recently, Castro et al. presented their findings on the requirement of at least 2 doses of human AD-MSCs to reverse airway remodelling and alleviate inflammation in HDM challenged C57BL/6 mice (Castro et al., 2020).

Current treatments of asthma include conventional treatments (inhaled corticosteroids and long acting β2-adenoceptor agonists) and novel biologics (mepolizumab, benralizumab, and dupilumab) (Imanirad and Tabatabaian, 2022). These therapies aim to treat symptoms of asthma by targeting Th2 inflammation, however, do little in the way of reversing existing damage. We show that human MSC treatment is capable of ameliorating airway remodelling in $CATT_7$ mice by reducing subepithelial collagen deposition and goblet cell hyperplasia. This suggests that high levels of MIF may facilitate activation of MSCs improving their therapeutic efficacy and leading to reversal of airway remodelling. The literature surrounding MSCs effect on airway remodelling is conflicting. Overall, our data aligns with the majority of the current literature demonstrating that MSCs are able to attenuate airway remodelling by reducing collagen deposition and/ or goblet cell hyperplasia (Abreu et al., 2019b; Choi et al., 2022; de Castro et al., 2017; Mariñas-Pardo et al., 2014; Royce et al., 2019, 2017; Zhong et al., 2019). However, it has also been reported in the literature that MSCs are ineffective at ameliorating goblet cell hyperplasia (Abreu et al., 2018; Kitoko et al., 2018; Royce et al., 2019) or subepithelial collagen deposition (Dai et al., 2018; Mariñas-Pardo et al., 2014). A reason for these discrepancies could be down to source of MSCs. It is well documented that MSCs from different sources can behave in different ways and possess different immunomodulatory properties depending on the extracellular milleu (Hass et al., 2011). Kitoko et al. demonstrated the differences in bone marrow derived and adipose tissue derived murine MSC efficacy in a HDM

model of allergic asthma (Kitoko et al., 2018). They determined that bone marrow derived MSCs were more efficacious through their ability to reduce eosinophilia in the lung and increase IL-10 levels. Choi et al. came to similar conclusions with human MSCs where bone marrow derived MSCs were able to more effectively attenuate features of airway inflammation, remodelling and AHR in a chronic model of allergic asthma compared to adipose tissue derived MSCs (Choi et al., 2022). Other factors such as severity of model, time of infusion, MSC fitness, dosing, and route of administration may also be reasons for the contradictory results (Abreu et al., 2017).

In order for newly licensed biologics such as dupilumab to maintain efficacy and suppression of inflammation it requires administration every 2 weeks (Faverio et al., 2023). Although long term efficacy was not our focus, previous studies have demonstrated the long-term effects of MSC treatment. Mariñas-Pardo et al. demonstrated that the therapeutic effects of murine MSCs were sustained for at least 2 weeks after administration (Mariñas-Pardo et al., 2014). HDM was administered 3 times a week for 4 weeks prior to MSC administration and continued for the 2 weeks following. At the 6-week cut-off point the MSC treated mice had significantly lower numbers of eosinophils in the BAL fluid and an improvement in airway hyperresponsiveness compared to the non-treated group.

Therefore, to determine whether the increased efficacy observed in the $CATT_7$ mice was due to the MSCs being preferentially activated in the high MIF environment or if there was simply just a larger scale of disease to achieve statistical significance, we analysed their biodistribution. If the biodistribution was the same among the groups it would provide an indication that there was no preferential activation. However, we detected significantly higher numbers of MSCs in the lungs of $CATT_7$ compared to

$CATT_5$ or littermate control 24 hours following administration. It is suggested that short-term effects of MSCs are mediated by their diverse secretome and the longer-term effects of MSC therapy are a result of direct interaction with other cell types (de Witte et al., 2017). Their biodistribution and retention time dictates which cells they interact with through paracrine or direct means. Previous studies conducted by our lab have shown that pre-licensing multipotent adult progenitor cells (MAPCs) with IFN-γ enhanced their retention in acute GvHD target organs in a humanised model of aGvHD (Carty et al., 2021). MAPCs are a population of postnatal cells isolated from the bone marrow which similar to MSCs have pro-reparative and immunomodulatory capacities. Enhanced MAPC retention was associated with improved overall therapeutic efficacy with improvement to survival rates and disease pathology. We suggest high levels of MIF activated MSCs increasing retention which contributed to their enhanced therapeutic efficacy. Increased longevity at the site of injury means they have a longer period to secrete soluble factors and interact with cells in the inflammatory microenvironment.

Interestingly, we observed no difference in cluster count within the lungs between the groups. This shows that there is no difference in dissemination of cells throughout lungs of the $CATT_7$ mice compared to the other groups. Suggesting that there is no preferential migration to different sites within the lung of the $CATT_7$ mice compared to the $CATT_5$ and WT mice. A recent study supports these data where MSC retention is increased in pulmonary vasculature following injury (Claire H. Masterson et al., 2021). They hypothesised that upregulation in adhesion molecules do not facilitate rolling but do play a role in retention following pulmonary entrapment. Unfortunately, we did not examine the effect of high levels of MIF on expression of ligands involved in adhesion.

To track the location of the MSCs *in vivo* the cells were labelled with a Qtracker labelling kit. In order for the CryoViz imaging system to quantify the number of labelled cells the fluorescent qDots are required to be tightly packed together within the cell to be detected (Luk et al., 2016). Only intact cells are picked up by the detection software and thus cells that have broken apart or phagocytosed go undetected. Low levels of autofluorescence in the lung meant we were able to accurately detect the number of labelled cells retained. However, the detection of the MSCs do not provide any information on their viability.

Taken together these data suggest that MSCs are more efficacious in the high MIF environment of $CATT_7$ mice. By investigating the effects of different concentrations of a human cytokine on the efficacy of human MSCs in a model of allergic asthma using a clinically relevant allergen we have identified a specific disease microenvironment which supports and enhances MSC efficacy. The use of our humanised model aims to provide a more accurate depiction of how human MSCs would interact in subsets of patients compared to conventional murine models. Despite exploring the effect of a human cytokine on human MSCs there are still limitations as we are unable to fully mimic clinical severe allergic asthma. However, these results may have implications in tailoring MSC treatment in cases of severe asthma. Our results have demonstrated that MSCs are less efficacious in low MIF environments. Patients with 5/5 or 6/6 haplotypes tend to have lower levels of circulating MIF and therefore may not respond as well to MSC treatment. Whereas patients with 7/7 or 8/8 haplotypes are more likely to have high levels of circulating MIF which may lead to greater MSC activation and enhanced therapeutic efficacy.

Following on from the discovery that MSC administration into $CATT_7$ mice led to improved MSC efficacy we investigated strategies to use the high MIF microenvironment to potentiate the effects of MSCs. Past work in our lab has focussed on different licensing strategies of MSCs to enhance MSC efficacy. Previously, we have demonstrated how IFN-γ licensing can improve MSC efficacy in a humanised model of acute GvHD and how they can be affected by endogenous factors such as peroxisome proliferator-activated receptor (PPAR)δ ligands or treatments like cyclosporine A (Carty et al., 2021; Corbett et al., 2021). Previous studies have shown how licensing with pharmacological agents or endogenous factors can further enhance the effects of MSC therapy in preclinical models of asthma (Abreu et al., 2019b, 2018; Kim et al., 2022). We sought to investigate whether licensing MSCs with CM derived from the high MIF expressing $CATT_7$ mice could potentiate their immunomodulatory effects. Using a low MSC dose ($5x10^4$ cells) which failed to protect against HDM-driven allergic airway inflammation, we showed that licensing low dose MSCs with $CATT_7$ CM enhanced low dose MSC therapeutic efficacy. Our findings agree with the current literature as doses reported for the systemic administration of MSCs in preclinical models of asthma range from $1x10^6$ to $1x10^5$ cells per mouse (Shin et al., 2021; Zhong et al., 2019). $CATT_7$ licensed MSCs at a previously ineffective dose were able to significantly reduce the numbers of eosinophils in the bronchoalveolar space. Furthermore, we detected markedly lower levels of Th2 cytokines in the BAL fluid. Licensing with BMDM CM from MIF knockout mice failed to restore MSCs immunosuppressive function suggesting that our findings were MIF specific.

One of the main criticisms of preclinical research is the use of doses which far exceed what would be reasonable in the clinic. Analysis of clinical trials using i.v. injection of MSCs reveals that the minimal effective dose used ranges from 1-2 million cells/kg

(Kabat et al., 2019). Studies which have investigated the i.v. administration of MSCs in preclinical models of allergic asthma administer doses which equate to 4-40 million cells/kg with the majority at the higher end of the scale (Table 4.1). The efficacy observed with MIF licensed MSCs using $5x10^5$ cells per mouse results in an effective dose of 2 million cells/kg. This shows that through MIF licensing we are able to restore MSC efficacy at a dose akin to what is used in clinical trials.

Table 4.1. Systemic administration of MSCs in preclinical models of allergic asthma

Model	Source	Number administered	Estimated Dose[a] Cells/kg	Ref
OVA	Human AD-MSC	$6.25x10^5$	$25x10^6$	(Choi et al., 2022)
OVA	Human BM-MSC	$6.25x10^5$	$25x10^6$	(Choi et al., 2022)
HDM	Human AD-MSC	$1x10^5$ (2 doses)	$4x10^6$	(Castro et al., 2020)
OVA	Human BM-MSC	$1x10^6$	$40x10^6$	(Malaquias et al., 2018)
OVA	Human ESC-MSC	$1x10^6$	$40x10^6$	(Lin et al., 2018)
OVA Poly I:C	Human UC-MSC	$1x10^5$	$4x10^6$	(Kwak et al., 2018)
OVA	Human AD-MSC	$1x10^5$	$4x10^6$	(de Castro et al., 2017)
OVA Poly I:C	Human UC-MSC	$6x10^5$	$24x10^6$	(Hong et al., 2017)
AHE	Murine BM-MSC	$1x10^6$	$40x10^6$	(Cruz et al., 2016)
HDM	Murine BM-MSC	$5x10^5$	$20x10^6$	(Braza et al., 2016)
HDM	Murine BM-MSC	$1x10^6$	$40x10^6$	(Duong et al., 2015)
AHE	Human BM-MSC	$1x10^6$	$40x10^6$	(Cruz et al., 2015)
OVA	Human BM-MSC	$1x10^6$	$40x10^6$	(Song et al., 2015)
HDM	Murine AD-MSC	$3x10^5$	$12x10^6$	(Mariñas-Pardo et al., 2014)
AHE	Murine BM-MSC	$1x10^6$	$40x10^6$	(Lathrop et al., 2014)
OVA	Human BM-MSC	$1x10^6$	$40x10^6$	(Mathias et al., 2013)

Footnote: [a]Under the assumption that the mice used had an average weight of 25g

We then sought to elucidate the mechanisms involved. MIF mediated signal transduction is primarily initiated by binding to the classical receptor CD74 (Kang and Bucala, 2019). We showed that blocking CD74 on the surface of MSCs ultimately abolished their immunosuppressive abilities. These results not only reaffirmed that the licensing with $CATT_7$ CM was MIF mediated but also showed that these effects were through binding to CD74. MIF signal transduction through CD74 binding has been shown to initiate a range of signalling pathways which induce cell proliferation, resistance to apoptosis, and the promotion of repair (Bucala and Shachar, 2014; Farr et al., 2020; Imaoka et al., 2019; Klasen et al., 2018; Soppert et al., 2018). Furthermore, MIF binding to CD74 has been shown to activate cytosolic phospholipase A2 ($cPLA_2$). $cPLA_2$ is a critical component of the pro-inflammatory cascade and is shown to play a role in the suppression of glucocorticoid action (Mitchell et al., 1999). Moreover, $cPLA_2$ activation results in the mobilisation of arachidonic acid from membrane phospholipids which is a precursor to the synthesis of prostaglandins (Flaster et al., 2007). Interestingly MIF has also shown to upregulate COX-2 expression, a rate limiting step in the synthesis of prostaglandins such as PGE2 (Carli et al., 2009; Mitchell et al., 2002; Sampey et al., 2001). MIF has been shown to have no effect on the expression of COX-1 (Carli et al., 2009).

The COX-2/PGE2 pathway has been extensively documented as being one of the key mediators driving MSC immunomodulation. We hypothesised that the COX-2/PGE2 pathway could be involved in the restoration of MSCs immunomodulatory capacity following $CATT_7$ licensing. To investigate, we pre-treated MSCs with indomethacin prior to licensing. Indomethacin is a potent non-selective inhibitor of COX-1 and COX-2 (Sun et al., 2018). We showed that blocking COX-2 abrogated therapeutic efficacy of $CATT_7$ licensed MSCs. Interestingly we observed that blocking of COX-

2 via indomethacin had more pronounced effect than blocking CD74. COX-2 is constitutively expressed in human MSCs, therefore, inhibition with indomethacin also blocks basal COX-2 expression which will contribute to the therapeutic effects observed.

Taken together these data show that MIF licensing can improve MSC therapeutic efficacy through the upregulation of COX-2 which likely drives PGE2 production. Our data agrees with several studies in the literature which also reveal the ability of MIF to improve MSC efficacy *in vivo*. The Zhang group demonstrated the ability of MIF to improve MSC therapeutic efficacy by tranducing MSCs with a lentiviral vector containing *Mif* cDNA thus promoting MIF overexpression (Liu et al., 2020; Yuelin Zhang et al., 2019; Zhu et al., 2021). They demonstrate that MIF overexpression in MSCs can improve their therapeutic effects resulting in improved cardiac function in a myocardial infarct model. However, we have further demonstrated the effects of *ex vivo* MIF licensing on MSC therapeutic efficacy. We have also explored how exogenous MIF can prime MSCs by binding to CD74 and upregulating COX-2 expression thereby enhancing MSCs immunomodulatory abilities.

Taken together, these data have highlighted the importance of understanding the disease microenvironment prior to MSC administration. We have demonstrated that MSCs have propensity to be more effective in high MIF environments. MSCs were able to significantly attenuate inflammation in HDM challenged $CATT_7$ mice but not $CATT_5$ or WTs. We observed a significant reduction in airway eosinophilia as well as reduced levels of IL-4, IL-5, and IL-13 in the BAL fluid. Furthermore, MSCs were able to significantly improve airway remodelling through the ability to ameliorate

subepithelial collagen deposition and goblet cell hyperplasia. We discovered that MSCs were retained for longer in the high MIF environment meaning they had a longer time to release their secretome and influence the cells within the lung tissue. We also revealed that MIF can bind to CD74 and act as a licensing mediator to improve MSC therapeutic efficacy by upregulating COX-2 expression. The knowledge gained from this chapter can be used to further optimise MSCs as a therapy and provide a basis for future studies regarding the effects of MSCs on the immune response in high MIF environments such as in asthma patients exhibiting the $CATT_7$ polymorphism.

Chapter 5 Discussion

Following its discovery in the 1960s, macrophage migration inhibitory factor (MIF) has shown to play a pivotal role in a range of inflammatory diseases. As a cytokine, its non-classical shape and lack of receptor redundancy allows MIF to drive inflammation by influencing its environment in a multitude of ways (Bloom et al., 2016). Initial studies on MIF focussed on its ability to regulate host inflammatory responses through the suppression of glucocorticoid action and amplify the inflammatory response by promoting expression of inflammatory mediators such as of TNF, IFN-γ, IL-1β, IL-2, IL-6, and IL-8 (Bernhagen et al., 1993; Calandra et al., 1995; Donnelly et al., 1997). MIF also possess chemokine-like characteristics through its ability to bind to chemokine receptors CXCR2, CXCR4, and CXCR7 to drive recruitment of leukocytes to sites of injury (Alampour-Rajabi et al., 2015; Klasen et al., 2014; Schwartz et al., 2009; Xu et al., 2015). Furthermore, MIF can also play a cytoprotective role by enhancing cell survival and promoting cellular proliferation (Ietta et al., 2018; Ohta et al., 2012; Soppert et al., 2018). Whilst MIF dysregulation can contribute to the pathogenesis of inflammatory disorders, we identified that the characteristics it possesses may actually enhance MSC fitness. Therefore, the aim of this thesis was to develop a better understanding of MIF's role in the regulation of inflammatory disorders and how MIF influences MSC efficacy within the disease microenvironment *in vivo*.

Firstly, this thesis has demonstrated the dominant role of MIF in both the pathogenesis of airway inflammation and airway remodelling in a HDM model of allergic asthma. We observed significantly higher levels of airway eosinophilia and Th2 cytokines in the BAL fluid of $CATT_7$ mice compared to the $CATT_5$ group. Interestingly, we also observed signs of airway remodelling in the $CATT_7$ mice with excess ECM production in the form of subepithelial collagen deposition and significant goblet cell hyperplasia.

Additionally, $CATT_7$ mice displayed airway hyperresponsiveness following methacholine challenge. The importance of this work is twofold. Firstly, for the first time we have shown that 7/7 transgenic mice with high levels of MIF develop an aggressive asthma phenotype with severe airway inflammation and remodelling. Current links between airway inflammation and airway remodelling are still ambiguous. However, our data suggest that airway remodelling may also be MIF dependent. Secondly, from a practical standpoint, we have generated a reproducible model capable of displaying hallmarks of airway remodelling with 9 instillations of HDM over 3 weeks. Typically, after 3 weeks of allergen exposure mice will present with signs of inflammation and airway hyperresponsiveness but rarely remodelling as seen in our WT control mice. Normally, to observe features of airway remodelling such as subepithelial collagen deposition mice need to be subjected to allergen exposure over the course of 5-12 weeks (Woo et al., 2018). We have established a reproducible model emphasising the role of human MIF in the development of allergic asthma (Dunbar, Hawthorne et al, Accepted, FASEB J). Using high and low MIF expressing transgenic mice we have generated a MIF-dependent scale of inflammation which can be used to investigate the differences of disease microenvironment on MSC therapy. Previous studies investigating the role of MIF in asthma have focussed on blocking or knocking out MIF (Amano et al., 2007; Das et al., 2011; Kobayashi et al., 2006; Li et al., 2021; Mizue et al., 2005; Wang et al., 2006). Instead, we have introduced the novel method of using humanised mice expressing different clinically relevant haplotypes of the MIF CATT promoter polymorphism. Not only have we demonstrated that MIF drives the clinical features and pathology of asthma we have established the dominant role of MIF allelic variants in determining disease severity. Showing for the first time that the $CATT_7$ haplotype results in a more severe form of

asthma. This data adds to the human genetic data and other supportive clinical studies of the MIF cytokine which associate the $CATT_5$ haplotype with milder disease (Baugh et al., 2002; Hizawa et al., 2004; Mizue et al., 2005), and the $CATT_7$ haplotype with more severe disease (Awandare et al., 2009; Benedek et al., 2017; Liu et al., 2018; Savva et al., 2016; Shin et al., 2022).

One of the primary aims of this thesis was to investigate the effect of high versus low MIF environments on MSC efficacy in a HDM model of allergic asthma. At the start of this project the effects of MIF on MSCs were largely unknown. Firstly, we demonstrated that MSCs possess the machinery to respond to MIF showing they constitutively express the main MIF receptors CD74, CXCR2, and CXCR4. We showed for the first time that MSCs are more efficacious in the severe high MIF expressing $CATT_7$ mice compared to the mild $CATT_5$ or WT. We demonstrated that MSCs were able to ameliorate Th2 inflammation by reducing the numbers of eosinophils and the levels of Th2 cytokines in the BAL fluid in the $CATT_7$ mice. Furthermore, MSCs were able to more effectively ameliorate aspects of airway remodelling in a high MIF environment with significant reductions in subepithelial collagen deposition and goblet cell hyperplasia in the $CATT_7$ mice. We investigated several factors which could explain the enhanced efficacy of MSCs in the $CATT_7$ mice. Firstly, we showed how MIF can promote MSC migration *in vitro* through the engagement of the CXCR4 receptor. And whilst it is generally accepted that MSCs do not preferentially migrate to the lung but are instead trapped in the lung vasculature, previous studies have shown that strategies to increase interaction with the CXCR4 receptor on MSCs can increase therapeutic efficacy. Genetic manipulation of MSCs to overexpress CXCR4 has been shown to augment MSCs potential in myocardial infarct, acute lung injury, and local inflammation models (Hervás-Salcedo et al., 2021;

Yang et al., 2015; Zhang et al., 2008). These reports indicate that the enhanced MSC efficacy observed in the $CATT_7$ may result from the interaction of MIF with the CXCR4 receptor.

Secondly, we investigated the effect of MIF on MSC survival. Most studies involving MSCs and MIF focussed on the effect of MIF on MSC survival (Ohta et al., 2012; Palumbo et al., 2014; Xia et al., 2018, 2015b, 2015a; Xia and Hou, 2018). Among these studies they found that MIF was able to rescue rat MSCs from hypoxia/serum deprivation induced hypoxia (Xia et al., 2018, 2015a, 2015b). Unfortunately, we were unable to reproduce these results as hypoxia/serum deprivation was an insufficient method of inducing apoptosis in human MSCs (data not shown). Reports have shown human and rat MSCs to behave differently e.g., their ability to differentiate along osteogenic and chondrogenic lineages so it is feasible that there may also be differences in their susceptibility to apoptosis (Scuteri et al., 2014). Instead, we used staurosporine to induce apoptosis. STS is a highly potent inducer of apoptosis as it induces apoptosis through caspase-dependent and caspase independent mechanisms. MIF is thought to mediate apoptosis resistance through the regulation of P53 therefore using STS elicit the induction of apoptosis make it very difficult to show protection (Fukaya et al., 2016; Hudson et al., 1999; Leech et al., 2003; Mitchell et al., 2002). We showed that MIF may have a small protective effect on MSCs, we did not observe the same levels of protection observed in other papers (Gore et al., 2008; Li et al., 2019; Nguyen et al., 2003). Our data indicate that resistance to apoptosis is unlikely to be responsible for the enhanced efficacy observed in the $CATT_7$ mice.

Overexpression of MIF has been reported in several cancer types where it has been shown to drive cancer progression (Kindt et al., 2016). Modulating cellular

proliferation has been reported to be one of the mechanisms behind MIF driven carcinogenesis. MIF has been shown to drive the proliferation of cells in hepatocellular carcinomas, non-small-cell lung carcinomas, human melanoma, and head and neck squamous cell carcinomas (Cao et al., 2021; Guo et al., 2013; Huang et al., 2014; Oliveira et al., 2014; Utispan and Koontongkaew, 2021; Wirtz et al., 2021). The ability of MIF to promote proliferation has also been shown to be beneficial by inducing proliferation of neural stem/progenitor cells to stimulate neurogenesis (Ohta et al., 2012; Zhang et al., 2013). We showed for the first time that MIF can enhance MSC expansion *in vitro.* We also investigated how MIF might be promoting the expansion of MSCs. We observed that exposure of MSCs to $CATT_7$ CM induced the expression of pAkt. Unfortunately we were not able to show that this was MIF specific as incubation with SCD-19 prior to exposure did not abrogate pAkt expression. This appears to be due to the slew of other factors likely present within the $CATT_7$ CM leading to changes in protein expression, however, seem to not lead to any functional changes. Despite this lack of specificity, our data aligns with the literature which suggests that activation of P13K/Akt signalling pathway is responsible for MIF driven proliferation (Cao et al., 2021; Cui et al., 2016; Oliveira et al., 2014). Cui et al. demonstrates that pre-treatment with MK-2206, an Akt inhibitor, or LY294002, a PI3K inhibitor, can block MIF induced cardiac stem cell proliferation (Cui et al., 2016). The PI3K/Akt signalling pathway does not appear to be the sole driver of MIF induced proliferation as Cui et al. also showed that incubation with compound c, an AMPK inhibitor, also abrogated MIF's proliferative effects. Nevertheless, the ability of MIF to enhance MSC expansion may have partly contributed to the increased numbers of MSCs detected in the lungs of $CATT_7$ mice 24 Hr post administration. It is possible that exposure of MSCs to MIF within the inflammatory microenvironment

drove MSC expansion leading to the increased retention and number of MSCs in the lungs $CATT_7$ mice compared to the $CATT_5$ and wildtype mice.

This is important as the more MSCs present for longer at the site of inflammation means there is a greater release of the MSC secretome to modulate the surroundings, thus making MSCs more efficacious in the $CATT_7$ microenvironment. Our results highlighted the importance of understanding the disease microenvironment and for the first time showed how MSCs are more efficacious in niches with high MIF.

Using the *in vitro* data generated which demonstrated how MIF can increase MSC fitness and based on our lab's expertise in licensing we decided to assess the effect of MIF exposure on MSCs prior to administration *in vivo*. We first attempted to license MSCs with $CATT_7$ CM prior to administration into WT mice. We found that MIF licensing was unable to enhance MSC efficacy in this model. Instead, using a low MSC dose ($5x10^4$ cells) which failed to protect against HDM-driven allergic airway inflammation, we showed that pre-licensing low dose MSCs with $CATT_7$ CM could restore their anti-inflammatory ability, revealing a novel licensing strategy. We demonstrated the ability for MIF licensing to enhance MSC efficacy within the dose range commonly used in clinical trials (Kabat et al., 2019). To ensure these effects were MIF specific we pre-treated MSCs with an anti-CD74 antibody which nullified the licensing effects.

COX-2 is a key mediator of MSC directed immunosuppression due to its central role in the synthesis of PGE2. PGE2 can elicit immunosuppression in a range of ways modulate the maturation of dendritic cells, suppress proliferation of activated T cells, and drive the differentiation of macrophages into an immunosuppressive phenotype (English et al., 2009; Kulesza et al., 2023). However, the upregulation of COX-2 has

been shown to induce a pro-inflammatory phenotype in other cellular contexts promoting inflammatory oedema, nervous injury and endometriotic lesions (Carli et al., 2009; Wang et al., 2011). Previous reports showed MIF alone can activate synoviocytes in rheumatoid arthritis, ectopic endometrial cells, spinal microglia, and astrocytes to upregulate the expression of COX-2 and PGE2 production (Carli et al., 2009; Sampey et al., 2001; Wang et al., 2011; Yuxin Zhang et al., 2019). We found that human MIF could also evoke COX-2 expression in human MSCs. To ascertain whether the upregulation of COX-2 resulted in enhanced immunosuppression we co-cultured $CATT_7$ simulated MSCs with PBMCs activated with CD3/CD28 T cell activator mix. We discovered that MIF pre-treatment enhanced MSCs ability to suppress T cells which was abrogated in the presence of MIF inhibitor SCD-19. For the first time we demonstrate the ability for MIF to upregulate COX-2 expression in MSCs leading to enhanced immunomodulatory properties. These results appear to be specific to the MIF mediated upregulation of COX-2 expression which likely induced PGE2 production as we detected no changes in IDO production following exposure. Therefore, to elucidate whether COX-2 was responsible for mediating the therapeutic effects following MIF licensing we blocked COX-2 with indomethacin prior to administration. We found that indomethacin abrogated the therapeutic effects driven by MIF licensing providing mechanistic evidence that MIF licensing enhances therapeutic efficacy through the modulation of COX-2. These data align with the current literature which state that MIF is able to facilitate COX-2 and PGE2 production and highlight the role of the COX-2/PGE2 axis in the regulation of MSC immunomodulation (Abreu et al., 2018; Kulesza et al., 2023; Yuxin Zhang et al., 2019).

The discrepancies observed between the efficacy of MIF licensed MSCs in $CATT_7$ and WT mice may be explained by the immunosuppression results obtained previously. In chapter 3 we observed that MIF exposure can increase COX-2 expression in MSCs, however, maximal stimulation of COX-2 expression is not achieved. This was observed when we exposed MSCs to $CATT_7$ CM and TNF-α. This dual stimulation was not redundant and in fact amplified the percentage of COX-2 expressing MSCs and COX-2 MFI. We believe that in our $CATT_7$ mice MIF licensing initially enhances COX-2 expression but the MSCs then receive a second hit of cytokines following administration which further amplifies COX-2 production resulting in immunosuppression. Conversely, we believe that there is insufficient inflammation present in the lungs of WT mice to amplify COX-2 expression resulting in a lack of immunosuppression.

In this study we have found BM-MSCs to be effective at alleviating hallmark features of asthma in a model of severe allergic asthma through the upregulation of COX-2. However, we did not directly examine how MSCs carried out their effects on a cellular level. The most obvious effect of MSC administration into a HDM induced model of allergic asthma was the capacity to ameliorate pulmonary eosinophilia. To date, there have been no studies that have shown the ability for MSCs to directly interact with and suppress eosinophils. Instead, MSCs have been shown to interact with other cells within the inflammatory microenvironment leading to the depletion of pro-inflammatory signals which drive eosinophil recruitment, activation, and survival. Dendritic cells play an essential role in the development of allergic asthma. DCs are specialised antigen-presenting cells which migrate to draining lymph nodes to initiate the Th2 response (Sakurai et al., 2021). MSCs have been shown to attenuate immune responses in asthma by directly influencing dendritic cells (Cahill et al., 2015; Duong

et al., 2015; Zeng et al., 2015). MSCs can alter the function of lung DCs by suppressing their ability to migrate to mediastinal lymph nodes. Additionally, they have been shown to inhibit DC activation and maturation during the effector phase of allergic airway inflammation (Duong et al., 2015; Zeng et al., 2015). Furthermore, MSCs have been shown to dampen the inflammatory response by inducing differentiation of DCs into a tolerogenic phenotype. DCregs have been shown to induce Treg differentiation and upregulate IL-10 (Cahill et al., 2015). It is reported that PGE2 produced by MSCs is responsible for these immunomodulatory effects as DC differentiation and function is restored in the presence of PGE2 inhibitor NS-398 (Spaggiari et al., 2009; Spaggiari and Moretta, 2013). We hypothesise that the anti-inflammatory effects observed when MSCs are subjected to high levels of MIF may be partly attributed to the modulation of dendritic cells.

Our work has focussed on administration of MSCs when there is persistent airway inflammation and features of airway remodelling has been established. We administered MSCs at day 14 of our acute severe asthma model rather than other studies which administer at day 0 (Ou-Yang et al., 2011; Shin et al., 2021; Sun et al., 2012). This approach provides greater insight into the effectiveness of MSC therapy in asthma patients as treatment would most likely only be used in severe persistent cases. Studies have shown that the cells involved in orchestrating airway inflammation changes as the asthmatic phenotype develops. Instead of Th2 cells being the chief producers of IL-4, IL-5, and IL-13 within the disease microenvironment it has been shown that ILC2s contribute to the development of experimental asthma (Christianson et al., 2015; Halim et al., 2012). The production of Th2 cytokines makes ILC2s a critical mediator in not only airway inflammation through the maturation of DCs and the initiation of the adaptive immune response but also to lung remodelling as IL-4

and IL-13 can induce airway hyperresponsiveness and goblet cell hyperplasia (Halim et al., 2016, 2014; Winkler et al., 2019). The ability for MIF licensed MSCs to markedly reduce the levels of IL-4 and IL-13 in the BALF fluid could be attributed to the suppression of ILC2 activity. This hypothesis would agree with the existing literature on MSCs and ILC2s where EVs derived from MSCs can impair ILC2 function (Fang et al., 2020; Shin et al., 2021). Where, exposure to MSC derived EVs results in a reduction in IL-13+ ILC2s in PBMCs from allergic rhinitis patients. Interestingly, this could be mediated by PGE2 as ILC2s constitutively express PGE2 receptors EP2 and EP4. The binding of PGE2 to these receptors has been shown to suppress human ILC2 cytokine production and proliferation.

Furthermore, one of the most documented mechanisms of MSC immunomodulation is the ability to polarise macrophages into an anti-inflammatory M2 like phenotype. However, under the previous M1 M2 framework macrophages in asthma were considered M2 as they are associated with Th2 inflammation and thought to play a role in would healing. Using polarisation markers that are indicative of macrophage functions we now know that macrophages in asthma patients experience impaired efferocytosis and fewer macrophages express IL-10 (Draijer et al., 2017; Huynh et al., 2005).

Upon treatment with MSCs we did not observe a change in the number of alveolar macrophages obtained from the BAL fluid compared to HDM only. The current literature on the effect of MSCs on macrophage number appears to be inconsistent. Some studies observe an increase in the number of alveolar macrophages (Kim et al., 2022; Mathias et al., 2013; Ren et al., 2021), whilst others agree with our findings that there is no change (Braza et al., 2016; Song et al., 2015). What does appear consistent

is the ability of MSCs to polarise macrophages towards an immunosuppressive phenotype. Studies have shown that MSC administration in mouse models of asthma is dependent on macrophage-mediated induction of IL-10 production from macrophages and other cell sources (Abreu et al., 2019b, 2018; Braza et al., 2016; Kim et al., 2022; Mathias et al., 2013). By doing this it is hypothesised that MSCs generate a tolerogenic environment to counterbalance the antigen-mediated airway inflammation. It has been shown that the COX-2/PGE2 axis is also involved in MSC mediated polarisation of macrophages through the binding to EP4 (Cao et al., 2020; Ortiz-Virumbrales et al., 2020; Vasandan et al., 2016; Wang et al., 2021). These studies indicate that the upregulation of COX-2 because of MIF licensing may have improved therapeutic efficacy through the polarisation of alveolar macrophages towards an immunosuppressive phenotype.

However, it is important to note that MSCs could have induced the immunosuppressive effects following phagocytosis/efferocytosis by macrophages. As stated previously, it is believed that MSCs initially modulate the inflammatory microenvironment through the secretion of immunomodulatory factors such as PGE2. They are then thought to modulate cellular metabolism of professional phagocytes following phagocytosis/efferocytosis resulting in longer-term immunosuppression (Braza et al., 2016; Pang et al., 2021; Witte et al., 2018). Due to the small numbers of MIF licensed MSCs administered in our model it is unlikely that the MIF-induced upregulation of COX-2 was wholly responsible for the therapeutic effects observed. And whilst the interaction between MSCs and macrophages was beyond the scope of this study it is very possible that MIF licensing may have enhanced macrophage reprogramming following uptake.

In the wider context this thesis has provided insight into the complex role of MIF in allergic asthma. We demonstrated how the CATT polymorphism can influence MIF production and for the first time how it can influence the severity of asthma. Furthermore, we identified that MSCs are preferentially activated in high MIF environments leading to enhanced therapeutic efficacy. The identification of MSCs to have enhanced functions in HDM challenged $CATT_7$ mice has wider implications than just the treatment of asthma. The $CATT_7$ subvariant in humans has been shown to increase disease severity in a range of inflammatory disorders including rheumatoid arthritis (Baugh et al., 2002; Radstake et al., 2005). To date clinical trials involving MSC treatment of rheumatoid arthritis have shown promising results however the response rates are quite low (20-45%) (Álvaro-Gracia et al., 2017; Sarsenova et al., 2021). Identification of patients expressing high CATT polymorphisms may indicate which patients will respond and improve response rates. Finally, we have presented a novel licensing strategy for MSCs which improves their therapeutic efficacy through the upregulation of COX-2 expression.

However, this thesis has come with some limitations which should be addressed. The main criticism of the research presented is the low numbers of readouts used to depict the pathophysiology observed in the HDM model of allergic airway inflammation. Due to lack of resources and availability of mice we could only focus on the core aspects of allergic airway inflammation such as differential cell counts from the BAL fluid, prototypical Th2 cytokines, and analysis of lung mechanics. Ideally, we would have also analysed other cytokines elevated in Th2 disease such as IL-9 and eotaxin or those involved in the sensitisation phase including IL-25, IL-33 and TSLP.

Obtaining these data would have provided insight on whether different MIF allelic variants are important during the early phases of asthma pathophysiology or whether the presence of high levels of MIF exacerbates the disease during the effector phase. The BAL fluid was the primary focus of this research due to how easy it is to process. Alternatively, we could have taken lung homogenates instead. This would have allowed us to build a larger picture of the state of the lung as we could have analysed alveolar cells, bronchial cells and interstitial macrophages. Furthermore, lysates for western blot could have been generated from homogenates to analyse eosinophilic proteins or cell death pathways. We did homogenise single lobes to extract RNA for qPCR analysis, however, we were unable to generate any consistent data. Additional readouts which may have improved our data would be the study of total IgE and HDM-specific IgE in the serum. IgE plays an integral role in antigen presentation and degranulation of basophils and mast cells which may have improved our understanding of how high levels of MIF results in severe allergic airway inflammation and mechanisms by which MSCs can reduce this.

To investigate the influence of MIF on MSCs we used CM from BMDMs of $CATT_7$ mice. The supernatants used were analysed for MIF levels prior to being used, however, we did not analyse other factors which might have also been present. It is likely that the CM contained a range of cytokines and growth factors which will have also had an impact on MSC responses. We attempted to mitigate any effects and ensure the results we observed were MIF specific by using SCD-19 or ISO-1 to block MIF activity *in vitro.* Moreover, *in vivo* we licensed MSCs with CM from MIF KO mice and used an anti-CD74 neutralising antibody to block MIF binding to the CD74 receptor. The use of ultrafiltration spin filters could have been used to filter out some of the confounders present in the supernatants. Despite showing that MIF can enhance

COX-2 expression *in vitro* leading to enhanced suppression of T cell proliferation and that blocking COX-2 with indomethacin abrogates the therapeutic effects of MIF licensed MSCs *in vivo* we did not measure PGE2 production. Lastly, this study would have benefitted from the use of asthma patient samples. MIF has been shown to be upregulated in the BAL fluid, serum and sputum of asthma patients. Genotyping of such samples to determine the number of CATT repeats and exposure to MSCs would allow us to further probe the effects of human MIF on MSC therapeutic efficacy.

In the future we plan to look more closely at the interactions between MSCs and immune cells in a high microenvironment. This includes the use of chlodronate liposomes to deplete alveolar macrophages and to examine the numbers of IL-4+IL-13+ ILC2s following administration of MIF licensed MSCs.

However, despite the limitations of this thesis we have further developed our understanding on how MIF can affect MSC efficacy. These could have implications particularly in the design of future clinical trials involving MSCs and asthma. We have identified that MSCs are more efficacious in $CATT_7$ mice where the levels of MIF are higher. We speculate that stratification of patients based on the number of CATT repeats prior to MSC treatment may lower the risk of failure and bring MSC-based therapies a step closer to regulatory approval.

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Chapter 7 Appendix I

Blockade of MIF biological activity ameliorates house dust mite-induced allergic airway inflammation in humanised MIF mice

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Running head: High MIF allele expression drives HDM-induced allergic airway inflammation and remodelling

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Abstract

Macrophage migration inhibitory factor (MIF) expression is controlled by a functional promoter polymorphism, where the number of the tetranucleotide repeats ($CATT_n$) corresponds to level of MIF expression. To examine the role of this polymorphism in a preclinical model of allergic asthma, novel humanised MIF mice with increasing CATT repeats ($CATT_5$ and $CATT_7$) were used to generate a physiologically relevant scale of airway inflammation following house dust mite (HDM) challenge. $CATT_7$ mice expressing high levels of human MIF developed an aggressive asthma phenotype following HDM challenge with significantly elevated levels of immune cell infiltration, production of inflammatory mediators, goblet cell hyperplasia, subepithelial collagen deposition and airway resistance compared to wildtype controls. Importantly the potent MIF inhibitor SCD-19 significantly mitigated the pathophysiology observed in $CATT_7$ mice after HDM-challenge, demonstrating the fundamental role of endogenous human MIF expression in the severity of airway inflammation *in vivo*. Up to now, there are limited reproducible in vivo models of asthma airway remodelling. Current asthma medications are focused on reducing the acute inflammatory response but have limited effects on airway remodelling. Here we present a reproducible preclinical model that capitulates asthma airway remodelling and suggests that in addition to having pro-inflammatory effects MIF may play a role in driving airway remodelling.

Keywords: Macrophage Migration Inhibitory Factor, MIF, House Dust Mite, Allergic Asthma, Severe Asthma, Airway Inflammation, Airway Remodelling, MIF Inhibitors

Introduction

Asthma is a complex multifactorial disease affecting over 300 million people worldwide (1). Allergic asthma is characterised by sensitisation to specific and/or non-specific stimuli resulting in airway hyperresponsiveness (AHR), airway inflammation, and goblet cell hyperplasia (2, 3). Common environmental stimuli include house dust mite (HDM), mould, cigarette smoke, and pet dander (4, 5). HDM, a trigger in up to 85% of asthmatic patients (6) has proteolytic activity to cleave epithelial tight junctions after inhalation to permit uptake by submucosal antigen-presenting cells surrounding the upper airways (7). Lung inflammation is orchestrated by the release of prototypical Th2 cytokines, IL-4, IL-5, and IL-13 which drive the release of inflammatory mediators into the surrounding microenvironment (8). Preclinical models of allergic asthma have provided significant contributions for the understanding of allergic airway inflammation; however, we have limited access to reproducible models of asthma airway remodelling (9).

Macrophage migration inhibitory factor (MIF) is detected at high levels in the bronchoalveolar lavage fluid (BALF) and serum of asthmatic patients (10). The level of MIF expression can vary in humans due to a functional repeat polymorphism implicating a tetranucleotide sequence 'CATT', found at position -794 in the promoter region of the MIF gene (11). Four types of allelic variations were found, classified as 5-CATT, 6-CATT, 7-CATT and 8-CATT, with the 5-CATT repeat allele presenting the lowest promoter activity (12). Interestingly low MIF 5-CATT allele correlates with lower levels of inflammation and thus milder forms of asthma. Studies have established a role for MIF in asthma, with use of MIF deficient mice ($MIF^{-/-}$), anti-MIF antibodies and small molecular weight inhibitors. In a mouse model of OVA-induced allergic airway inflammation, $MIF^{-/-}$ mice had lower levels of pulmonary inflammation, Th2 cytokines and airway hyperresponsiveness (AHR) compared to wildtype controls (10). Administration of a MIF neutralising antibody mitigated the MIF-related induction of AHR in an OVA model, but notably had no effect on the production of Th2 cytokines or IgE (13). The prototypical MIF antagonist ISO-1 could abrogate AHR and airway inflammation in mice challenged with HDM, along with illustrating MIF's role in epithelial barrier dysfunction in vitro (14). A polyclonal anti-MIF antibody decreased the number of cellular infiltration in BALF from OVA-

induced allergic mice but failed to decrease Th2 cytokines or IgE (15). The link between MIF and airway remodelling has also been investigated in OVA challenged mice. ISO-1 decreased autophagy in smooth muscle cells, thus reducing the incidence of airway remodelling after OVA sensitisation (16).

Current treatments for asthma focus on the management of symptoms and consist of utilisation of bronchodilators and glucocorticoid steroids to control the intensity and number of allergic exacerbations. The more recently developed biologics target Th2 driven inflammation, however, these medications have limited effects on airway remodelling (9). MIF is known to suppress the action of glucocorticoids (17, 18) and therefore the levels of MIF expressed by asthma patients may have a major impact on their responsiveness to therapeutic strategies.

To better understand the potential impact of high allele human MIF expression on the severity of allergic airway inflammation and remodelling, we have generated novel humanised mice expressing high (CATT7) or low (CATT5) levels of human MIF. Using a specific MIF inhibitor, we have examined the specificity of human MIF expression in driving HDM-induced allergic airway inflammation.

Materials and Methods

Ethical Approval

All procedures involving the use of animals were carried out by licensed personnel. 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.

Humanised MIF mice

Two mouse strains expressing the human high- or low-expression *MIF* alleles (*e.g.,* *MIF*^{CATT7} [(C57BL/6NTac-Miftm3884.1(MIF)Tac-Tg(CAG-Flpe)2Arte] and *MIF*^{CATT5} [C57BL/6NTac-Miftm3883.1(MIF)Tac-Tg(CAG-Flpe)2Arte] mice) were created using vector-based recombinant replacement of murine *Mif* by Taconic Biosciences (Rensselaer, NY, US). Validation of the expression of human and not murine *MIF* mRNA was verified by qPCR, and -794 CATT-length dependent stimulated MIF production was confirmed *in vivo* (data not shown) *(19).*

Mouse model of house dust mite induced acute allergic airway inflammation

WT, CATT5 and CATT7 mice (6-18 weeks old) were challenged with 25µg of house dust mite (HDM) allergen, *Dermatophagoides pteronyssinus* (Greer Labs, Lenoir, NC, US) or PBS control intranasally (I.N.) 3 days weekly for 3 weeks under light isoflurane anaesthesia.

MIF Inhibitors

SCD-19 (3-(2-methylphenyl)-1H-isochromen-1-one) (Specs.net), or ISO-1 ((S,R)-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester) (Tocris) small

molecular weight inhibitors of macrophage migration inhibitory factor (MIF) enzymatic activity was used in a house dust mite model of acute allergic airway inflammation. 35mg/kg of SCD-19 was administered intraperitoneally (I.P.) twice weekly for three weeks; day 0, 4, 7, 11, 14, and 18.

Histology

On day 21 of the model, lungs were harvested. Tissue was fixed in 10% (v/v) neutral buffered formalin (Sigma-Aldrich) for 24 hours. Tissue was then processed using an automated processor (Shandon Pathcentre, Runcorn, UK) and embedded in paraffin wax using a Shannon Histocentre 2 (Shandon). Once sectioned with a Shandon Finesse 325 microtome (Thermo-Shandon, Waltham, MA, USA), tissue sections (5µm) were stained for Masson's Trichrome (Sigma-Aldrich), Periodic Acid Schiff (Abcam) and Haemotoxylin and Eosin-Y (Richard Allan Scientific). Samples were air dried and a coverslip was mounted with DPX mounting media (BDH). 4X and 20X images were taken using an Olympus BX51 light microscope.

Histological Scoring

Following staining, slides were coded without reference to prior treatment and examined in a blind manner. For H&E, images were scored using a composite scale (1–9); comprising of infiltration or aggregation of inflammatory cells in air space or vessel wall [1 = only wall, 2 = few cells (1–5 cells) in air space, 3 = intermediate, 4 = severe (air space congested)]; interstitial congestion and hyaline membrane [formation: 1 = normal lung, 2 = moderate (<25% of lung section), 3 = intermediate (25–50% of lung section), 4 = severe (>50% of lung section)]; haemorrhage: (0 = absent, 1 = present) (20). 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. Collagen deposition was calculated by analysing the % of positive staining following Masson's Trichrome staining using the trainable Weka segmentation plugin on Fiji open-source software.

Bronchoalveolar lavage fluid (BALF) Retrieval

Mice were sacrificed by lethal overdose of sodium pentobarbital via I.P. injection on day 18 of the model, 4 hours after last challenge. A tracheostomy and cannulation was performed, where a 27 gauge cannula was secured in place with sutures. 1ml of cold endotoxin free PBS was infused into the lungs through the cannula using a 1ml syringe for 3 gentle instillations. BALF was placed into an eppendorf and kept on ice before being centrifuged at 300g for 5 minutes at 4°C. The supernatant was collected, aliquoted and 10X protease inhibitor solution (Roche) was added to prevent protein degradation.

BALF Cell Analysis

Cells were isolated and resuspended in 100µl of endotoxin-free PBS for counting. Cyto-spin funnels were pre-wet by spinning with 300µl of PBS onto glass slides at 600rpm for 5 minutes. $1x10^5$ cells in a volume of 300µl of PBS were spun onto fresh labelled glass slides at 600rpm for 10 minutes using a RotoFix 32 cytocentrifuge (Hettich Zentrifugen). Slides were airdried before being stained with Kwik-Diff™ Stain (Shandon, ThermoScientific); 25 seconds in fixative, 15 seconds in solution I and 15 seconds in solution II. Slides were imaged on an Olympus BX51 light microscope until 300 cells could be counted. Cells were identified as being neutrophils, eosinophils, macrophages or lymphocytes.

Cytokine Analysis

BALF supernatants were analysed for Th2 cytokines IL-4 (Biolegend), IL-5 (Biolegend), IL-13 (eBioscience) and human MIF (R&D Systems) by ELISA following the manufacturer's instructions.

FlexiVent® Lung Function

Mice were anesthetised with 150mg/kg ketamine and 2mg/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. The mouse 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 minutes, alfaxan and 0.5mg/kg Tracium, a neuromuscular blocking agent (NMBA), was administered through the tail vail catheter. The measurement of lung function was initiated and approximately 100µl of PBS or increasing concentrations of the bronchoconstrictor methacholine (MCh) (3.125, 12.5 and 25mg/ml) was loaded into the nebulizer. Upon completion of lung function measurements at baseline and following increasing aerosolized methacholine challenges, the ventilator was stopped and the mouse was euthanised using either I.P. injection of sodium pentobarbital or via cervical dislocation.

Statistical Methods

All data are presented as mean ± SEM. Results of two or more groups were compared by analysis of variance (ANOVA) followed by the post-hoc Tukey's multiple comparison test. were analyzed using a statistical software package (GraphPad Prism, San Diego, CA). Response to different concentrations of methacholine were analysed by 2way 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.

Results

Functional -794CATT polymorphic mouse tissues express different levels of hMIF under basal and disease conditions.

Novel humanised MIF mice were generated to capitulate the varying levels of MIF expression under the functional promoter polymorphism within the human population. C57BL/6 mice were humanised by replacing the murine MIF gene with the human counterpart. Within this human MIF gene, 794 downstream of the promoter region, where the number of tetranucleotide repeats correlates with MIF allele expression (11), 5 repeats of this tetranucleotide sequence 'CATT' generated $CATT_5$ mice, containing the low expressing MIF allele. 7 repeats of this tetranucleotide sequence 'CATT' generated $CATT_7$ mice, containing the high expressing MIF allele (Figure 1A). To characterise the effect of the CATT microsatellite repeat we analysed hMIF production under basal and disease conditions. Bronchoalveolar lavage fluid (BALF) (Figure 1B), bone marrow-derived macrophages (BMDMs) (Figure 1C) and splenocytes (Figure 1D) isolated from $CATT_7$ mice secrete significantly higher levels of hMIF than $CATT_5$ or wildtype (WT) mice (Figure 1B-D).

To investigate the role of the human MIF polymorphism in a disease setting, a model of acute allergic airway inflammation was generated. The clinically relevant house dust mite (HDM) allergen was administered intranasally three times a week for three weeks to induce airway inflammation (Figure 1E). BALF was obtained, and lungs were snap frozen for RNA isolation and qPCR analysis on day 21. significantly higher levels of hMIF were detected in $CATT_7$ BALF compared to BALF from $CATT_5$ or WT mice (Figure 1F). Similarly, the relative expression of human *Mif* was significantly increased in the $CATT_7$ compared to the $CATT_5$ mouse lung tissue (Figure 1G).

These data comprehensively show that the CATT polymorphism is responsible for differential production of hMIF under basal and disease conditions. This model allows us to investigate the role of high versus low hMIF in the pathophysiology of acute airway inflammation in a relevant preclinical model.

Human $CATT_7$ allele significantly increases the Th2 signature in a HDM model of allergic airway inflammation.

Absence of MIF in models of allergic inflammation has been shown to reduce the levels of Th2 cytokines in the BALF (10, 21). To study the effect of different levels

of endogenous MIF on asthma severity, $CATT_7$, $CATT_5$, and WT C57BL/6 mice were challenged with HDM intranasally 3 times a week for 3 consecutive weeks. 4 hr post final challenge the mice were sacrificed, and the BALF was obtained. The BALF total leukocyte counts show that $CATT_7$ mice challenged with HDM have significantly higher numbers of immune cells in the bronchoalveolar space compared to WT mice (Figure 2A). $CATT_7$ mice also exhibit a marked increase in cell number compared to $CATT_5$ although not significant (Figure 2A). Differential cell counts demonstrated that the predominant cell type in the BALF are eosinophils (Figure 2B).

To further characterise the influence of hMIF on asthma pathophysiology we explored its effects on the prototypical Th2 signature. IL-4, IL-5, and IL-13 have been shown to be critical in the development of airway hyperresponsiveness, eosinophilic responses, and goblet cell hyperplasia, and responsible for the overall asthma phenotype. Th2 cytokines were detected in the BALF of $CATT_5$ and WT HDM mice (Figure 2C, 2D, 2E). However, we observed significantly elevated levels of IL-4, IL-5, ad IL-13 in $CATT_7$ HDM compared to $CATT_7$ PBS group (Figure 2C, 2D, 2E). Furthermore, significantly higher levels of IL-4 and IL-13 were detected in $CATT_7$ HDM compared to $CATT_5$ and WT HDM (Figure 2C, 2E). A marked increase in IL-5 was also detected although not significant (Figure 2D). These data show that the $CATT_7$ polymorphism generates a prominent Th2 cytokine profile which may contribute to a more severe asthma phenotype.

The Human $CATT_7$ allele exacerbates hallmarks of asthma pathophysiology.

Excessive production of mucus and the associated pathophysiological changes are hallmarks in a range of respiratory diseases including asthma (22). We investigated the effect of the CATT polymorphism on goblet cell hyperplasia by staining lung tissue with PAS (Figure 3A). PBS control groups exhibited very low levels of PAS positive staining, whilst $CATT_5$ HDM exhibit slightly higher numbers of PAS positive cells relative to the control compared to the WT (Figure 3A, 3B). Strikingly, $CATT_7$ mice have significantly higher levels of goblet cell hyperplasia compared to $CATT_5$ and WT mice following HDM challenge (Figure 3B).

In addition to investigating goblet cell hyperplasia, we examined the effect of the -794CATT polymorphism on airway remodelling. To determine the extent of remodelling, we stained lung tissue with Masson's trichrome to highlight subepithelial collagen. The trends in the Masson's trichrome staining mirrored that of the PAS, with $CATT_5$, and WT mice displaying similar levels of disease pathology. Both groups exhibit a significant yet small increase in collagen deposition compared to the PBS controls (Figure 3C, 3D). $CATT_7$ mice challenged with HDM display significantly higher levels of subepithelial fibrosis compared to the lower MIF expressing $CATT_5$ allele and WT groups (Figure 3D); suggesting that high levels of hMIF contributes to airway remodelling in a HDM model of allergic airway inflammation.

H&E staining revealed that HDM significantly induces cellular infiltration surrounding the airways in all groups compared to the PBS control (Figure 3E, 3F). However, $CATT_7$ HDM exhibited significantly higher airway immune cell infiltration compared to both $CATT_5$ and WT HDM (Figure 3E, 3F). There was no significant difference in immune cell infiltration between the $CATT_5$ and WT HDM groups according to the H&E score (Figure 3F).

These data suggest that high levels of human MIF exacerbate allergic airway pathophysiology by increasing mucin production through the induction of goblet cell hyperplasia, increasing deposition of subepithelial collagen thereby contributing the airway remodelling, and increasing the infiltration of immune cells surrounding the airways.

High levels of human MIF alters HDM-induced lung mechanics in response to increasing concentrations of methacholine.

Airway hyperresponsiveness (AHR) and remodelling are a major hallmark of asthma and allergic airway inflammation, and as such it is important for models to represent this. We examined HDM-induced AHR in response to aerosolised methacholine challenge using the FlexiVent system. $CATT_7$ HDM mice exhibited a marked increase in airway resistance (R_N) at 12.5 mg/ml and 25 mg/ml doses compared to the rest of the groups (Figure 4). A trend of increased tissue (G) and tissue elastance (H) was demonstrated in the $CATT_7$ mice at the 25 mg/ml dose (Figure 4). It has been well

documented that due to genetic differences, C57BL/6 have a higher resistance to airway hyperresponsiveness compared to the more sensitive BALB/c mice in acute airway inflammatory models (23) (24), and this is reflected here in our humanised MIF mice. These data show that $CATT_7$ mice challenged with HDM exhibit higher levels of airway remodelling and AHR compared to WT C57BL/6 mice.

The MIF Inhibitor SCD-19 decreases HDM-induced total cell counts and Th2 cytokines in BALF from $CATT_7$ mice.

High human MIF expression has been demonstrated to play a role in driving the physiological hallmarks of allergic airway inflammation such as eosinophil infiltration (Figure 2A, B), increased Th2 cytokines (Figure 2C-E), goblet cell hyperplasia (Figure 3A, B), subepithelial collagen deposition (Figure 3C, D) and decreased lung function (Figure 4).

MIF is known to initiate its biological effects through its active site (25). Therefore, to fully elucidate MIF's role in our model, the MIF antagonist SCD-19 was utilized to block this active site, as this alters the conformation of MIF and impairs its interaction with other molecules. Throughout the previous data, no significant difference was noted between the low MIF expressing $CATT_5$ mice and WT mice. Thus, the remainder of this study focused on comparing the high MIF expressing $CATT_7$ mice and WT mice. In addition to receiving HDM challenge, SCD-19 was administered intraperitoneally twice a week for three weeks.

Administration of SCD-19 to $CATT_7$ mice challenged with HDM significantly decreased human MIF production in BMDMs derived from $CATT_7$ mice (Supp. Figure 1). The total BALF cell count was decreased in $CATT_7$ HDM challenged mice that received SCD-19 compared to HDM challenged $CATT_7$ mice that received the vehicle control (Figure 5A). There was no significant difference between WT groups. BALF from SCD-19 treated $CATT_7$ mice had decreased numbers of eosinophils compared to $CATT_7$ mice that received the vehicle control (Figure 5B). There was no significant difference between WT groups. Although not statistically significant, there is a visible trend of decreased total cellular infiltration in the BALF of SCD-19 treated $CATT_7$ mice compared to vehicle control mice.

No SCD-19-specific differences were observed in BALF IL-4 protein levels (Figure 5D). However, lower levels of IL-5 were detected in the BALF of SCD-19 $CATT_7$ mice compared to vehicle control $CATT_7$ mice (Figure 5E). There was no significant difference between SCD-19 and vehicle control WT groups, which was expected as we have previously noted that WT mice already have low levels of MIF production. Importantly, levels of BALF IL-13 were significantly decreased in SCD-19 $CATT_7$ mice compared to vehicle control $CATT_7$ mice (Figure 5F).

SCD-19 significantly decreases HDM-induced lung pathology in $CATT_7$ mice.

High levels of human MIF in the $CATT_7$ mice drives airway remodelling and inflammation following repeated exposure to the clinically relevant allergen HDM. Here we examined the capacity for SCD-19 to prevent this exacerbation-related pathology.

SCD-19 significantly abrogated the number of PAS positive cells present compared to $CATT_7$ mice that received the vehicle control (Figure 6A,B). As expected, there was no significant difference between WT mice that received SCD-19 and the vehicle control (Figure 6A,B). In high human MIF expressing $CATT_7$ mice, SCD-19 significantly reduced the percentage of subepithelial collagen present after three weeks of intervention, compared to the vehicle control (Figure 6C,D). $CATT_7$ vehicle mouse lung sections had statistically significant higher levels of collagen deposition compared to WT vehicle mice. Moreover, SCD-19 had no effect in WT mice (Figure 6C,D). Lastly, lung sections were analysed for cellular infiltration using H&E staining. High expression of human MIF amplified cellular infiltration, as $CATT_7$ mice that received SCD-19 had a significantly lower H&E score compared to those that received the vehicle control (Figure 6E,F). $CATT_7$ vehicle mice had significantly higher H&E scores compared to WT vehicle mice. Moreover, similarly to our previous histological findings, SCD-19 had no effect in WT mice (Figure 6E,F). In a similar manner, the MIF inhibitor; ISO-1 significantly reduced goblet cell hyperplasia, subepithelial collagen deposition and airway inflammation in CATT7 mice challenged with HDM (Supp. Figure 2). In WT mice, ISO-1 had no effect (Supp. Figure 2).

These data support our hypothesis that MIF is a key factor in driving acute airway inflammation in our house dust mite model, as blocking the biological activity of MIF reduced lung inflammation.

SCD-19 improves lung function by decreasing airway resistance in $CATT_7$ mice in response to increasing concentrations of methacholine .

We have previously shown that mice possessing the high human MIF expression allele ($CATT_7$) had increased airway resistance (Figure 4A), tissue damping (Figure 4B) and tissue elasticity (Figure 4C) after inhaling increasing concentrations of methacholine compared to mice possessing the low human MIF expression allele ($CATT_5$) and WT mice.

To further investigate our hypothesis that human MIF is driving the development of preclinical signs of allergic airway inflammation following HDM challenge in the $CATT_7$ mice, we measured the respiratory mechanics of mice that received SCD-19. In $CATT_7$ mice, SCD-19 had the ability to decrease HDM-induced inflammation at a mechanical level, by reducing the percentage increase of airway resistance (Figure 7A), tissue damping (Figure 7B) and tissue elasticity (Figure 7C) from baseline, compared to $CATT_7$ vehicle mice.

Discussion

The biological role of macrophage migration inhibitory factor (MIF) has been previously documented in a plethora of inflammatory lung conditions (19, 26-31), including asthma (32) (10, 13, 16, 33). The low human MIF expressing $CATT_5$ promoter polymorphism correlates with a milder manifestation of asthma symptoms (10). We hypothesised that the $CATT_7$ promoter polymorphism which expresses high levels of human MIF could be linked to increased severity of allergic asthma. Using novel humanised MIF mice to create a physiological scale of allergic airway inflammation in response to the clinically relevant aeroallergen house dust mite, this

study set out to investigate the biological role of this pro-inflammatory cytokine on key hallmarks associated with this atopic condition.

We demonstrate that intranasal challenge of HDM can drive enhanced MIF production in the lung of $CATT_7$ and $CATT_5$ mice, perhaps illustrating a positive feedback loop resulting in the exacerbations of physiological asthmatic characteristics. In a similar fashion, MIF is known to act in an autocrine and paracrine fashion to promote downstream cytokine production (18, 21, 34, 35).

MIF's crucial contribution in this model of house dust mite-induced allergic airway inflammation was particularly clear at a histological level with significantly increased mucin-producing goblet cell hyperplasia and subepithelial collagen deposition in the presence of high levels of human MIF in $CATT_7$ mice. Airway remodelling occurs in uncontrolled cases of asthma, as repeated lung injury by inhaled insults and over-production of fibrotic tissue result in goblet cell hyperplasia and increased subepithelial collagen. This alteration in tissue architecture has consequences in the mechanical functioning of the lung, resulting in an increase in airway hyperresponsiveness, resistance, tissue damping and elasticity. Our findings further clarified the physiological role of MIF in HDM-induced airway inflammation, as humanised high MIF expressing $CATT_7$ mice had increased airway resistance, tissue damping (energy dissipation into alveoli) and elastance (energy conservation in the alveoli) in response to increasing concentrations of the chemical bronchoconstrictor methacholine. This study provides new insights on the role of MIF in driving airway remodelling.

Blockade of MIF using the small molecule antagonist (S,R)3-(4-hy-droxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester (ISO-1) has been shown to significantly reduce the pathology associated with OVA-induced (36), HDM-induced (14) airway inflammation and remodelling and in a neutrophilic experimental severe asthma model (37). The MIF small molecule inhibitor SCD-19 has been tested in a range of disease systems including lung cancer (25) and infectious disease (38) studies. SCD-19 potently inhibits MIF activity but its therapeutic efficacy had not been tested in an

allergic airway inflammation model. Although previous studies have utilised anti-MIF antibodies and the small molecule inhibitor ISO-1 in mouse models of inflammatory conditions (14, 15, 36, 39), this is the first study to do so in humanised MIF mice expressing human relevant MIF polymorphic alleles.

In this study, SCD-19 significantly mitigated the MIF-associated increase in inflammatory histopathology, in a dose-dependent manner, reiterating the importance of this cytokine in the induction and maintenance of experimental asthma, as described previously (36). SCD-19 decreased the total cells present in bronchoalveolar lavage in $CATT_7$ mice. SCD-19 had no effect on IL-4 production, but decreased IL-5 and significantly decreased IL-13 production in the BALF from $CATT_7$ mice compared to vehicle control. In line with our data, administration of an anti-MIF antibody in an OVA model also did not affect IL-4 levels in BALF, but anti-MIF treatment during OVA sensitisation significantly decreased eosinophil infiltration in BALB/c mice (13). Conversely in C57BL/6 mice, MIF inhibition with SCD-19 did not decrease eosinophil infiltration in the BALF of HDM-challenged $CATT_7$. Transgenic mice are routinely generated on a C57BL/6 background, which may be a limitation of this study, as Th2 atopic allergy models are usually performed in BALB/c. As a result, readouts may have a lower baseline than those performed in BALB/c mice. Similarly, levels of cellular and eosinophil infiltration may be lower than suspected in our model, due to the mice used being generated of the C57BL/6 genetic background. Furthermore, SCD-19 may not decrease eosinophil infiltration as efficiently as seen with an anti-MIF antibody in an OVA model (13), as our Th2 response was not high enough at baseline levels to be further suppressed by SCD-19. The timing of MIF inhibitor administration is fundamental, as the use of ISO-1 in a model of severe neutrophilic asthma could only abrogate HDM-induced airway inflammation when administered both 30 mins prior and 6 hours after allergen challenge (37). Taking into account the SCD-19 dosing regimen, along with the suboptimal genetic background of the mice used, this may help to explain the incomplete effects of SCD-19 in this model. As previously mentioned, collagen deposition and goblet cell hyperplasia were seen to be significantly increased at this acute timepoint, but more HDM challenges might be required to see the full effects of chronic inflammation in our model. SCD-

19 blocks MIF's conformationally sensitive tautomerase active site that overlaps functionally with MIF (CD74) receptor binding. Here we show that SCD-19 extensively diminishes HDM-induced histopathology in $CATT_7$ mice, along with having a subtle anti-inflammatory impact in the BALF of these mice when administered starting at first allergen challenge. Moreover, SCD-19 abrogated the airway resistance (R_N), tissue damping (G) and tissue elasticity (H) in $CATT_7$ mice compared to the vehicle control, showing a clear association between lower levels of tissue remodelling at a histological level and overall lung function.

In terms of asthma therapies, MIF is known to counter-regulate the effects of glucocorticoids, a steroidal treatment to manage severe asthmatic symptoms. In the future, small molecular weight MIF inhibitors may not only be used as a monotherapy for asthma patients with high MIF expression 7-7 genotypes (those genetically pre-dispositioned to secrete higher levels of this pro-inflammatory cytokine), but also as part of a synergistic regimen where they could initially work to inhibit MIF function, but also to enhance or restore the efficacy of glucocorticoids in the clinic.

This study demonstrated the ability of the MIF antagonist SCD-19 to abrogate HDM-induced cellular infiltration, goblet cell hyperplasia and subepithelial fibrosis. Furthermore, SCD-19 decreased airway hypersensitivity, but did not affect cell populations within the BALF retrieved from these novel humanised MIF mice.

Here we demonstrate that high MIF allele expression leads to enhanced severity of allergic airway inflammation driven by the clinically relevant allergen HDM. This study is the first to use novel humanised MIF mice to investigate the role of endogenous MIF expression on house dust mite-induced allergic asthma by utilising small molecular weight inhibitors in vivo.

To conclude, this study demonstrates the important role of MIF in further driving allergic airway inflammation and potentially airway remodelling and provides a novel, clinically relevant and reproducible model of allergic airway remodelling. Further experiments are required however, perhaps using therapeutic as opposed to prophylactic administration of SCD-19 to determine the ability of SCD-19 to reduce airway remodelling. These data pave the way for a new therapeutic avenue for the

utilisation of small molecule anti-MIF strategies that are both ant-inflammatory and that can potentially reduce airway remodelling in allergic asthma. This study is of high scientific and translational relevance given the obvious superiority of small molecules over biologic approaches (e.g., antibodies) to treating asthma. The validation of the humanised MIF mouse model is an additional advance, as it will enable the utility of this model in downstream pharmaceutical development, both in asthma and other MIF-dependent diseases in the sphere of airway disease, oncology, infection, and autoimmunity.

Data Availability Statement

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

Conflict of Interest Statement

The authors declare no conflict of interest.

Author's Contributions

HD performed research, data analysis, study design, and wrote the manuscript; IJH performed research, data analysis, study design and wrote the manuscript. HD and IJH should be conjoint first authors. CT performed research and data analysis; 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.

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Figure Legends:

Figure 1. Functional -794CATT polymorphisms lead to different expression levels of hMIF under basal and disease conditions. A Human MIF gene containing a functional polymorphism in position 794 downstream of the promoter region, where the number of tetranucleotide repeats correlates with promoter activity and MIF allele expression; 5 repeats = $CATT_5$ mice, 7 repeats = $CATT_7$ mice. **B** hMIF levels in BAL fluid of naïve WT, $CATT_5$ and $CATT_7$ mice. **C** hMIF production from BMDMs of WT, $CATT_5$ and $CATT_7$ mice. **D** hMIF production from splenocytes of WT, $CATT_5$ and $CATT_7$ mice. **E** Timeline for HDM exposure. Mice were intranasally challenged with HDM 3 times a week for 3 consecutive weeks. **F** Levels of hMIF detected in the BAL fluid of WT, $CATT_5$ and $CATT_7$ mice challenged with HDM. **G** Relative expression of hMIF detected in lung homogenates of WT, $CATT_5$ and $CATT_7$ mice challenged with HDM detected by real-time PCR. Data are presented as mean ± SEM; N=3-6 per group. *p<0.05, **p<0.01, ****p<0.0001.

Figure 2. Human $CATT_7$ allele significantly increases the Th2 signature in a HDM model of allergic airway inflammation. **A** Total cell count recovered from the BAL. **B** BAL fluid eosinophil count determined by differential staining of cytospins. Cytokine levels of **C** IL-4, **D** IL-5 and **E** IL-13 in the BAL fluid determined by ELISA.

Data are presented as mean ± SEM; N=6 per group. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Figure 3. Human $CATT_7$ allele exacerbates airway inflammation in a house dust mite model of allergic asthma. A Representative images of lung tissue stained with periodic acid Schiff at 20X magnification, scale bar = 20 µm. **B** Goblet cell hyperplasia was investigated through the quantitation of PAS positive cells. **C** Representative images of lung tissue stained with Masson's trichrome at 4X magnification, scale bar = 200 µm. **D** Quantitation of % subepithelial collagen. **E** Representative images of lung tissue stained with H&E from WT, 5CATT and 7CATT mice challenged with HDM or PBS control at 20X magnification, scale bar = 20 µm. **F** Quantitation of airway inflammation in H&E-stained lung tissue. Data are presented as mean ± SEM; N=6 per group. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Figure 4. Changes in lung mechanics in response to increasing concentrations of methacholine in HDM challenged $CATT_7$, $CATT_5$ and WT mice. Airway hyperresponsiveness determined by airway resistance, tissue damping, and tissue elastance (R_N, G, and H respectively). Data are presented as peak response normalized to the baseline and expressed as % increase over baseline ± SEM; N=6 per group.

Figure 5. MIF inhibitor SCD-19 decreases total cell counts and Th2 cytokines in BALF from $CATT_7$ mice. Mice were challenged with 25µg of HDM I.N. three times a week for three weeks, in addition to receiving 35mg/kg of SCD-19 or vehicle control I.P. twice weekly for three weeks. **A** Total cell count recovered from BAL fluid. **B** Number of Eosinophils from differential cell counts of BAL fluid from $CATT_7$ and WT mice . **C** 300 cells were counted and identified based on morphology. Cytokine levels of **D** IL-4, **E** IL-5 and **F** IL-13 in the BAL fluid determined by ELISA. Data are presented as mean ± SEM; N=6 per group. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Figure 6. MIF antagonist SCD-19 significantly decreases HDM-induced allergic airway inflammation in $CATT_7$ mice. A Representative images of lung tissue stained with Periodic Acid Schiff at 20X magnification, scale bar = 20 μm. **B** Goblet cell hyperplasia was investigated through the quantitation of PAS positive cells relative to the control. **C** Representative images of lung tissue stained with Masson's trichrome at 4X magnification, scale bar = 200 μm. **D** Quantitation of % subepithelial collagen. **E** Representative images of lung tissue stained with H&E from WT and $CATT_7$ mice challenged with HDM or PBS control and received SCD-19 or vehicle control at 20X magnification, scale bar = 20 μm. **F** Quantitation of airway inflammation in H&E-stained lung tissue. Data are presented as mean ± SEM; N=6 per group. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Figure 7. SCD-19 can decrease HDM-induced airway resistance (R_N), tissue damping (G) and tissue elasticity (H) in $CATT_7$ mice compared to vehicle control. $CATT_7$ mice were challenged with 25μg of HDM or PBS control I.N. three times a week for three weeks, in addition to receiving 35mg/kg of SCD-19 or vehicle control I.P. twice weekly for three weeks. 24hr after last challenge, a tracheostomy was performed and lung function was measured using a FlexiVent® instrument (SCIREQ) in response to PBS or increasing concentrations of methacholine (3.125, 12.5 and 25mg/ml). **A** Airway Resistance. **B** Tissue Damping. **C** Tissue Elasticity. Data are presented as peak response normalized to the baseline and expressed as % increase over baseline N=4-6 per group.

Supplementary Figure 1. MIF antagonist SCD-19 significantly decreases hMIF levels in $CATT_7$ mice. A hMIF production from BMDMs of $CATT_7$ mice that received 35mg/kg of SCD-19 or vehicle control I.P. twice weekly for three weeks. Data are presented as mean ± SEM; N=3 per group. *p<0.05.

Supplementary Figure 2. MIF antagonist ISO-1 ameliorates HDM-induced allergic airway inflammation in $CATT_7$ mice. Mice were challenged with 25μg of HDM I.N. three times a week for three weeks, in addition to receiving 35mg/kg of ISO-1 or vehicle control I.P. twice weekly for three weeks. **A** Representative images of lung tissue stained with Periodic Acid Schiff at 20X magnification, scale bar = 20 μm. **B** Goblet cell hyperplasia was investigated through the quantitation of PAS positive cells relative to the control. **C** Representative images of lung tissue stained with Masson's trichrome at 4X magnification, scale bar = 200 μm. **D** Quantitation of % subepithelial collagen. **E** Representative images of lung tissue stained with H&E from WT, $CATT_5$ and $CATT_7$ mice challenged with HDM or PBS control at 20X magnification, scale bar = 20 μm. **F** Quantitation of airway inflammation in H&E-stained lung tissue. Data are presented as mean ± SEM; N=6 per group. ***$p<0.001$, ****$p<0.0001$.

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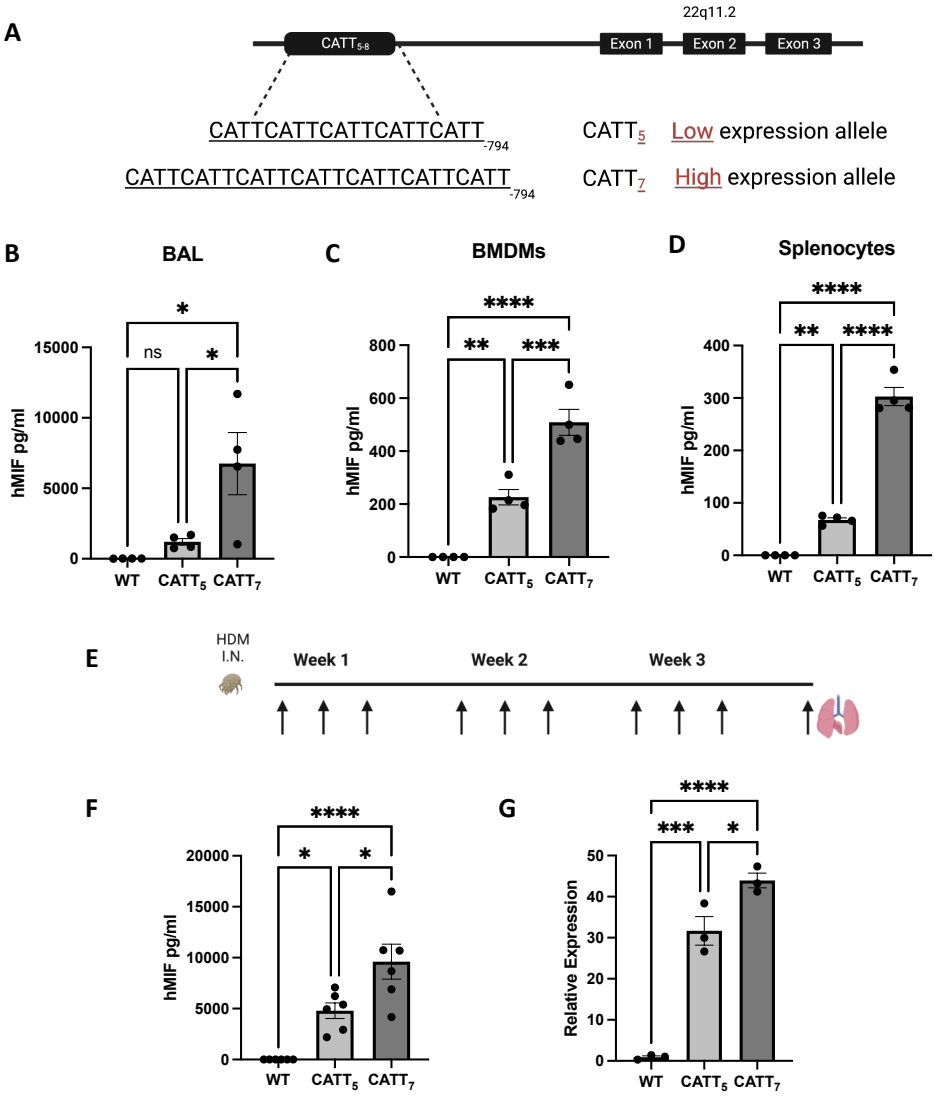

A
22q11.2
CATT5-8
Exon 1
Exon 2
Exon 3
CATTCATTCATTCATTCATT-794
CATT5 Low expression allele
CATTCATTCATTCATTCATTCATTCATT-794
CATT7 High expression allele
B
BAL
C
BMDMs
D
Splenocytes
hMIF pg/ml
WT
CATT5
CATT7
E
HDM
I.N.
Week 1
Week 2
Week 3
F
G
Relative Expression

Figure 1.

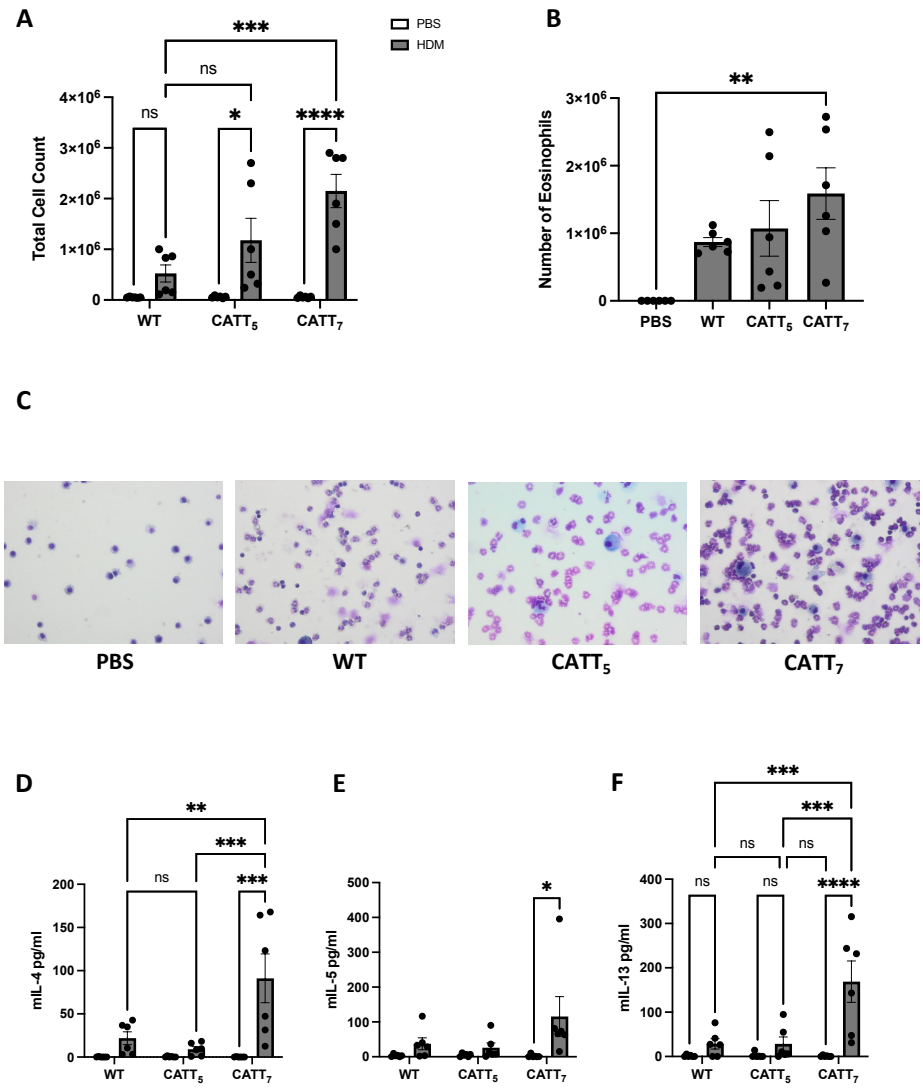

A
PBS
HDM
***
ns
ns
*
****
Total Cell Count
4×10^6
3×10^6
2×10^6
1×10^6
0
WT
CATT5
CATT7
B
**
Number of Eosinophils
3×10^6
2×10^6
1×10^6
0
PBS
WT
CATT5
CATT7
C
PBS
WT
CATT5
CATT7
D
**
***
ns
***
mIL-4 pg/ml
200
150
100
50
0
WT
CATT5
CATT7
E
*
mIL-5 pg/ml
500
400
300
200
100
0
WT
CATT5
CATT7
F
***
***
ns
ns
ns
ns
****
mIL-13 pg/ml
400
300
200
100
0
WT
CATT5
CATT7

Figure 2.

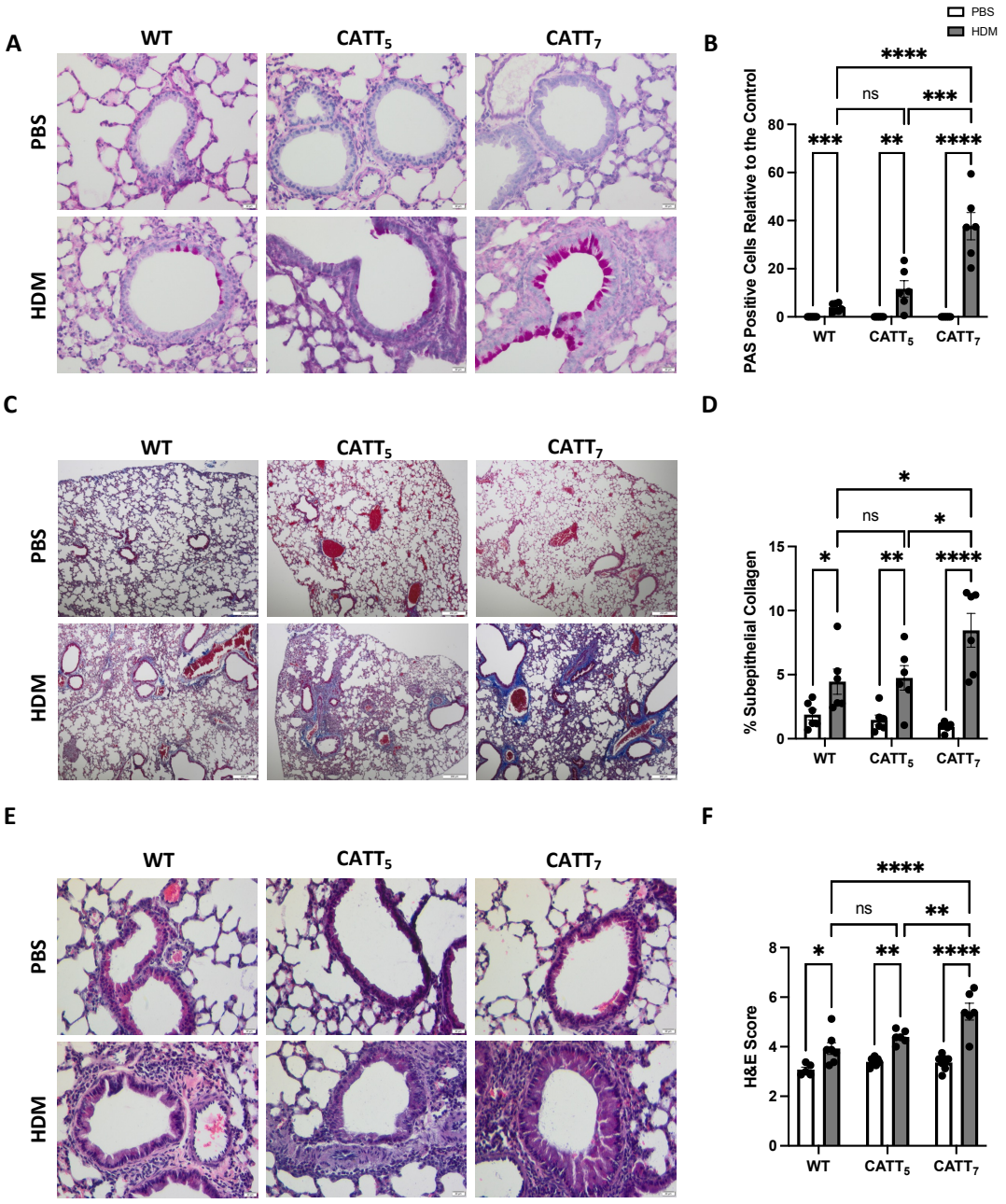

A
WT
CATT5
CATT7
PBS
HDM
B
PBS
HDM
****
ns
***
***
**
****
PAS Positive Cells Relative to the Control
80
60
40
20
0
WT
CATT5
CATT7
C
WT
CATT5
CATT7
PBS
HDM
D
*
ns
*
*
**
****
% Subepithelial Collagen
15
10
5
0
WT
CATT5
CATT7
E
WT
CATT5
CATT7
PBS
HDM
F
****
ns
**
*
**
****
H&E Score
8
6
4
2
0
WT
CATT5
CATT7

Figure 3.

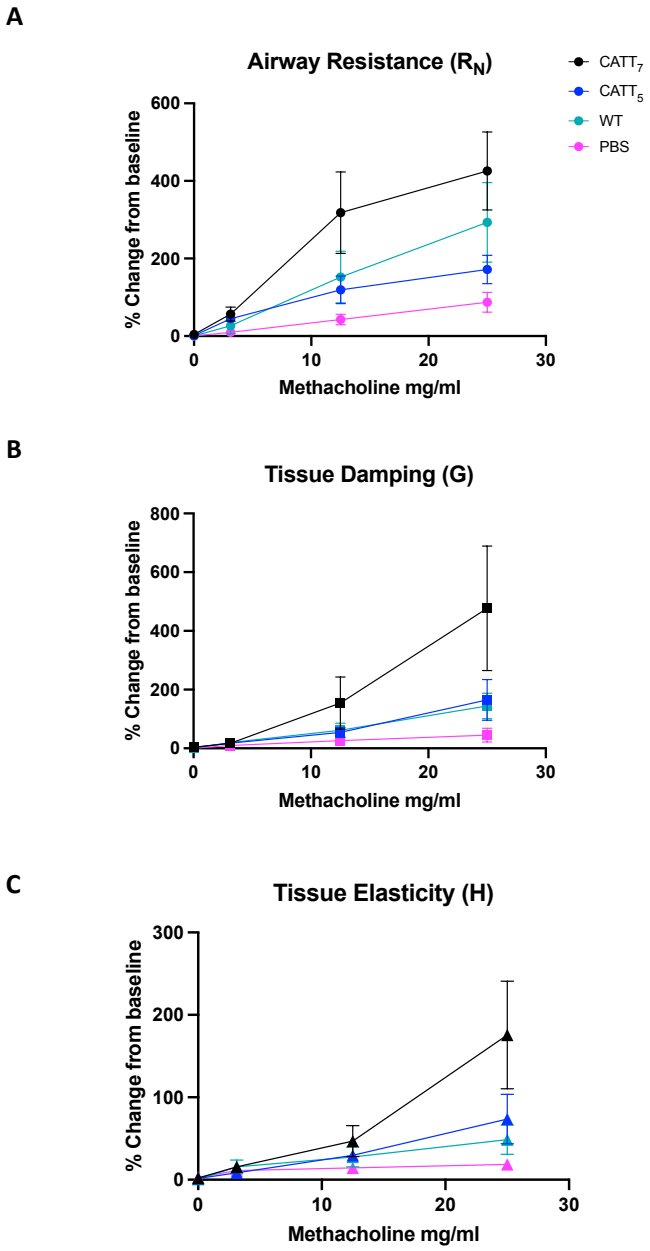

A
Airway Resistance (R_N)
CATT$_7$
CATT$_5$
WT
PBS
% Change from baseline
600
400
200
0
0
10
20
30
Methacholine mg/ml
B
Tissue Damping (G)
% Change from baseline
800
600
400
200
0
0
10
20
30
Methacholine mg/ml
C
Tissue Elasticity (H)
% Change from baseline
300
200
100
0
0
10
20
30
Methacholine mg/ml

Figure 4.

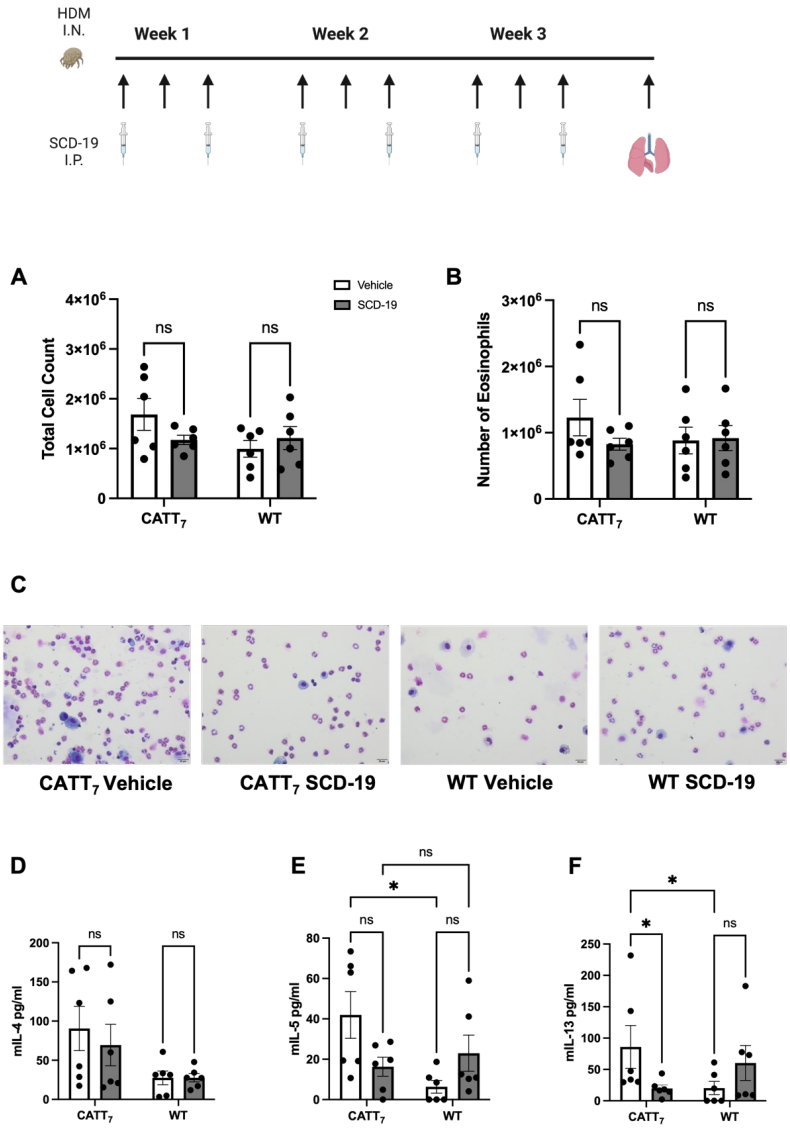

HDM
I.N.
Week 1
Week 2
Week 3
SCD-19
I.P.
A
Vehicle
SCD-19
Total Cell Count
4×10^6
3×10^6
2×10^6
1×10^6
0
ns
ns
CATT7
WT
B
Number of Eosinophils
3×10^6
2×10^6
1×10^6
0
ns
ns
CATT7
WT
C
CATT7 Vehicle
CATT7 SCD-19
WT Vehicle
WT SCD-19
D
mIL-4 pg/ml
200
150
100
50
0
ns
ns
CATT7
WT
E
mIL-5 pg/ml
80
60
40
20
0
ns
*
ns
ns
CATT7
WT
F
mIL-13 pg/ml
250
200
150
100
50
0
*
*
ns
CATT7
WT

Figure 5.

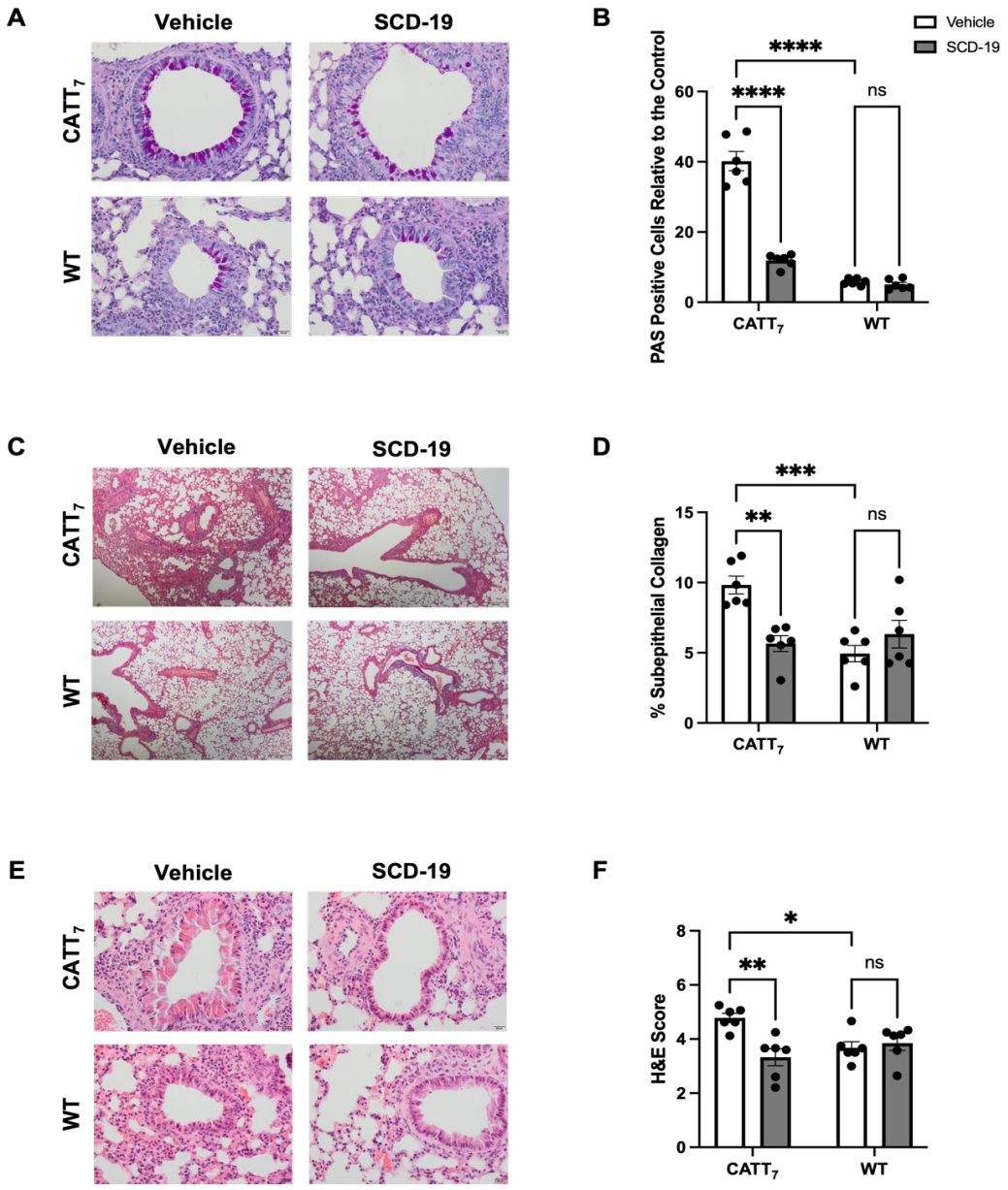
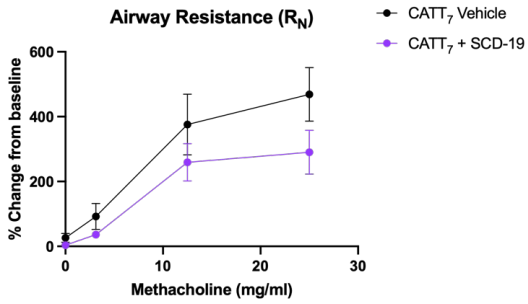
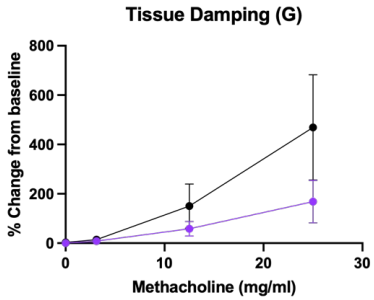

A
Vehicle
SCD-19
CATT7
WT
B
Vehicle
SCD-19
PAS Positive Cells Relative to the Control
****
****
ns
60
40
20
0
CATT7
WT
C
Vehicle
SCD-19
CATT7
WT
D
% Subepithelial Collagen
***
**
ns
15
10
5
0
CATT7
WT
E
Vehicle
SCD-19
CATT7
WT
F
H&E Score
*
**
ns
8
6
4
2
0
CATT7
WT

Figure 6.

A


Airway Resistance (R_N)
$CATT_7$ Vehicle
$CATT_7$ + SCD-19
% Change from baseline
600
400
200
0
0
10
20
30
Methacholine (mg/ml)

B


Tissue Damping (G)
% Change from baseline
800
600
400
200
0
0
10
20
30
Methacholine (mg/ml)

C

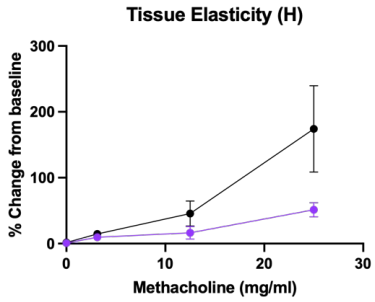
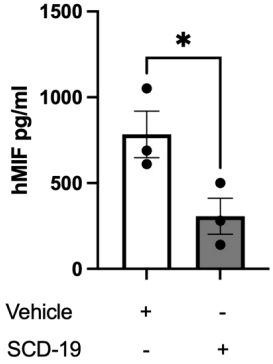

Tissue Elasticity (H)
% Change from baseline
300
200
100
0
0
10
20
30
Methacholine (mg/ml)

Figure 7.



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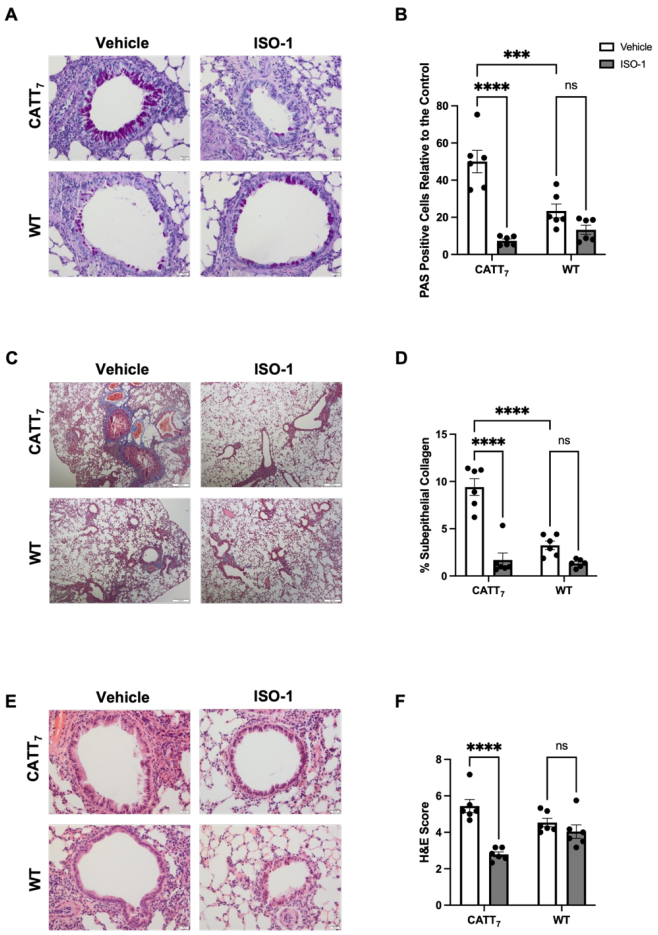

Supplementary Figure 2.