TNF-α/IL-1β licensed mesenchymal stromal cells promote corneal allograft survival via myeloid cell mediated induction of Foxp3+ regulatory T cells in the lung

Nick Murphy[1], Oliver Treacy[1,2], Kevin Lynch[1,2], Maurice Morcos[1], Paul Lohan[1], Gerry Fahy[3], Matthew D. Griffin[1,4], Aideen E. Ryan[1,2,4], Thomas Ritter[1,4*]

[1]Regenerative Medicine Institute (REMEDI), School of Medicine, College of Medicine, Nursing and Health Sciences National University of Ireland, Galway, Ireland; [2]Discipline of Pharmacology and Therapeutics, School of Medicine, College of Medicine, Nursing and Health Sciences National University of Ireland, Galway, Ireland; [3]University Hospital Galway, Department of Ophthalmology, National University of Ireland, Galway, Ireland; [4]CÚRAM Centre for Research in Medical Devices, School of Medicine, College of Medicine, Nursing and Health Sciences, National University of Ireland Galway, Galway, Ireland

***Corresponding author:**

Thomas Ritter PhD,
Regenerative Medicine Institute, School of Medicine, College of Medicine, Nursing and Health Sciences,
Biomedical Sciences
National University of Ireland, Galway
Galway, Ireland
Phone: 00353-91-495329
Email: thomas.ritter@nuigalway.ie

Running title: TNF-α/IL-1β licensed MSCs promote corneal allograft survival

List of abbreviations

7-AAD – 7-Aminoactinomycin D
ACK – Ammonium-chloride-potassium
Allo – Allogeneic
Auto – Autologous
BSS – Balanced salt solution
CD – Cluster of differentiation
CFSE – Carboxyfluorescein succinimidyl ester
COX-2 – Cyclooxygenase-2
DA – Dark agouti
dLN – Draining lymph nodes
DTH – Delayed-type hypersensitivity
FBS – Foetal bovine serum
Foxp3 – Forkhead box P3
GAPDH – Glyceraldehyde 3-phosphate dehydrogenase
GvHD – Graft versus host disease
HBSS – Hank's balanced salt solution
HLA – Human leukocyte antigen
IDO – Indoleamine 2,3-dioxygenase
IFN-γ – Interferon –gamma
IL-1β – Interleukin-1 beta
iNOS – inducible nitric oxide synthase
Lew – Lewis
MACS – Magnetic-activated cell sorting
MHC – Major histocompatibility complex
MSCs – Mesenchymal stromal cells
NO – Nitric oxide
PBMC – Peripheral blood mononuclear cells
PGE2 – Prostaglandin E2
POD – Post-operative day
PTGS2 – Prostaglandin-endoperoxide synthase 2
RPMI – Roswell Park Memorial Institute
SMT – S-methylisothiourea
TCR – T cell receptor
TGF-β1 – Transforming growth factor-beta 1
TNF-α – Tumor necrosis factor-alpha
TOR – Time of rejection
T-regs – Regulatory T cells
TSG-6 – Tumor necrosis factor-inducible gene 6
UTR – Untreated
αMEM – Minimum essential medium – Alpha modification

Abstract

Mesenchymal stromal cells (MSCs) have shown promise as a therapy for immune mediated disorders including transplant rejection. Our group previously demonstrated the efficacy of pre-transplant, systemic administration of allogeneic but not syngeneic MSCs in a rat model of cornea transplantation. The aim of this study was to enhance the immunomodulatory capacity of syngeneic MSCs. *In vitro*, MSCs licensed with TNF-α/IL-1β ($MSC^{TNF\text{-}\alpha/IL\text{-}1\beta}$) potently suppressed syngeneic lymphocyte proliferation. *In vivo*, when administered post-transplantation, non-licensed syngeneic MSCs improved graft survival from 0 to 50% while $MSC^{TNF\text{-}\alpha/IL\text{-}1\beta}$ improved survival to 70%. Improved survival was associated with increased $CD4^+CD25^+Foxp3^+$ regulatory T-cells (T-regs) and decreased pro-inflammatory cytokine expression in the draining lymph node. $MSC^{TNF\text{-}\alpha/IL\text{-}1\beta}$ demonstrated a more potent immunomodulatory capacity compared to non-licensed MSCs, promoting an immune regulatory lung $CD11b/c^+$ myeloid cell population and significantly expanding T-regs in the lung and spleen. *Ex vivo*, we observed that lung-derived myeloid cells act as an intermediary of MSC's immunomodulatory function. MSC-conditioned myeloid cells suppressed stimulated $CD4^+$ lymphocyte proliferation and promoted the expansion of T-regs from naïve lymphocytes. This work illustrates how syngeneic MSC therapy can be enhanced by licensing and optimisation of timing strategies and further highlights the important role of myeloid cells in mediating MSC's immunomodulatory capacity.

Key words: pro-inflammatory cytokine licensing, immunomodulation, cornea transplantation, immune suppression, autologous MSC therapy

Introduction

Mesenchymal stromal cells (MSCs), a population of non-hematopoietic stromal cells with potent immunosuppressive and immunomodulatory properties, are being investigated for their ability to inhibit immune-mediated rejection in animal models of allograft transplantation (1-6). The immune regulatory capacity of MSCs is well documented, and demonstrates that they possess the potential to suppress pro-inflammatory responses of both the adaptive and innate arms of the immune system as well as to enrich populations of immune regulatory cells (7-10). In response to the adaptive immune response, MSCs directly inhibit the proliferation of activated T cells, induce and expand $Foxp3^+$ regulatory T cells (T-regs) and may also modulate the B cell response (7, 10-14). More recently, a growing body of literature has described the ability of MSCs to modulate the innate immune response and to promote the generation of innate regulatory cells (9, 15, 16). Although the mechanism of MSC-mediated immunomodulation following intravenous administration (i.v.) remains to be fully elucidated, it is evident that the lung (where the majority of MSCs become trapped and are subsequently cleared within 24 hours) plays an important role (15-18). Our group and others have reported that MSCs enrich innate immune regulatory cells in the lung following i.v. administration (9, 15, 16, 19-21).

Currently our group and others are investigating the immunomodulatory properties of MSCs for the prevention of immune mediated rejection in cornea transplantation (keratoplasty) (4, 15, 16, 19). Owing to the immune privileged nature of the eye, keratoplasty is typically performed without human leukocyte antigen (HLA) tissue matching or the indefinite use of immunosuppressive therapy and yet the survival rate within the first year is over 90% (22). However, the ten year prognosis is much poorer due to increased rates of rejection in "high risk" patients. The risk of graft failure in these patients is heightened due to an increased risk of immune-mediated rejection (23-25). For these patients systemic immunosuppressants are potentially efficacious in prolonging high risk corneal allograft survival but with the caveat that they trigger considerable side effects including nephrotoxicity, hepatoxicity, leucopenia, gastrointestinal disorders and increased risk of malignancies and infections (26-29). As corneal blindness is not a life-threatening disorder, the long-term side effects of immunosuppressant therapies may outweigh the benefits of prolonged graft survival in some high risk patients. Therefore, there is a significant need to develop novel, safe immunomodulatory therapies to alleviate immune-mediated rejection episodes in corneal allograft patients.

Previous results from our laboratory have demonstrated the efficacy of pre-transplant infusion of allogeneic (allo) but not autologous (auto) MSC in a fully allogeneic, MHC-mismatched rat model of cornea transplantation (4). MSCs acquire their immunomodulatory properties upon exposure to inflammatory stimuli such as IFN-γ, TNF-α and IL-1β (8, 30). Ren et al, demonstrated that blocking the inflammatory stimulus by utilising IFN-γ receptor deficient MSCs prevented MSC mediated T cell suppression and abolished MSCs ability to prevent graft versus host disease (GvHD) in mice (8). Similarly, in another model of GvHD, recipients of IFN-$\gamma^{-/-}$ donor T cells did not respond to MSC therapy however the immunomodulatory function of the MSC could be restored by pre-treatment with IFN-γ (31). This suggests that in our previous work the expression of allo-antigen by allo-MSCs provides a priming stimulus that enhances their immune modulatory effects while auto-MSCs administered to an immunologically compatible non-inflamed (pre-transplantation) host did not receive the required priming stimulus (4). As increasingly, evidence suggests that allo-MSCs trigger an allo-specific immune mediated cellular and humoral response which may be deleterious to the long term outcome of the graft this study investigated whether auto-MSC therapy could be enhanced to promote rejection free graft survival. (32-34).

We hypothesised that pre-emptive priming ("licensing") of auto-MSC could overcome the lack of efficacy in preventing cornea transplantation rejection by better promoting regulatory innate and adaptive immune cells. We demonstrate that auto-MSCs administered post-operatively promote rejection free graft survival and licensing with a combination of TNF-α and IL-1β ($MSC^{TNF\text{-}\alpha/IL\text{-}1\beta}$) further enhances their immunomodulatory capacity. Auto-MSC therapy is associated with increased $Foxp3^{+}$ T-regs in the lungs post-infusion and in the draining lymph node at the estimated time of rejection and this immunomodulatory effect can be enhanced by TNF-α/IL-1β licensing. Finally, we demonstrate that MSC-mediated induction of Foxp3+ regulatory T cells (Tregs) was dependent upon a lung-derived myeloid cell intermediary. This work contributes to the growing body of literature highlighting the importance of lung myeloid cells in mediating MSCs immunomodulatory function.

Materials and Methods

Animals and corneal transplantation

All procedures performed on animals were approved by the Animals Care Research Ethics Committee of the National University of Ireland (Galway, Ireland) and conducted under license from the Health Product Regulatory Authority (HPRA) of Ireland. All animals were housed and cared for under Standard Operating Procedures of the Animal Facility at the National Centre for Biomedical Engineering Science, NUI Galway. A well established, fully allogeneic major histocompatibility complex (MHC) class I/II disparate cornea transplant model was used for these studies. Male Dark Agouti (DA, RT-1^{avl}) rats served as graft donors and Lewis (Lew, RT-1^{l}) rats served as recipients. All animals were aged between 8-14 weeks old and obtained from Harlan Laboratories (Bicester, UK) and housed with food and water ad lib. Orthotopic corneal transplantation was performed as reported previously [1-4]. Briefly, isoflurane was systemically administered at a concentration of 2-2.5% in medical oxygen (BOC, Galway, Ireland) with a flow rate of 2l/minute. 1% Tetracaine (Chauvin Pharmaceuticals, Kingston upon Thames, UK) was administered as a local anesthesia and atropine 1%, tropicamide 1% and phenylephrine 1% (all Chauvin Pharmaceuticals) were administered for iris dilation. A 2.5mm graft bed was prepared and a 3mm donor graft was sutured in place with 8-10 interrupted 10-0 Ethilon sutures (Ethicon, Livingston, Scotland). Antibiotic ointment containing chloramphenicol was applied to the graft. To irrigate the corneal tissue Alcon BSS (Alcon, Hemel Hempstead, UK) was applied to the graft. Graft transparency as an indicator of rejection was scored every 2-3 days using an operating microscope at 25x magnification. The grading score was as follows: 0 – completely transparent cornea; 0.5 – slight corneal opacity; 1 – slight corneal opacity; 1.5 – modest corneal opacity, vessels still visible; 2.0 – moderate opacity, few iris details visible; 2.5 – high corneal opacity; only pupil margin visible; 3.0 – complete corneal opacity; anterior chamber not visible. A graft was considered to be rejected when an opacity score of 2.5 was recorded on two consecutive days or one score of 3.0. Animals with surgical complications were excluded.

Bone marrow (BM) derived rat MSC isolation, culture and expansion

MSCs were isolated from the bone marrow of Lewis Rats (8-12 weeks old). Animals were euthanized by CO_2 inhalation and the femurs and tibias removed. The bone marrow was flushed from the bones in rat MSC medium (10 % fetal bovine serum (FBS) (Sigma-Aldrich, Missouri, US) in equal parts F-12 nutrient mixture and αMEM (both Gibco/Thermo Fisher

Scientific)) to isolate bone marrow progenitors. Cells were pelleted by centrifugation at 400 x g for 5 mins and washed with Dulbecco's Phosphate Buffered Saline (DPBS) (Gibco/Thermo Fisher Scientific, Massachusetts, US). Cells were re-suspended in rat MSC medium and seeded at a density of $9x10^5$ cells/cm^2 in T-175 flasks (NUNC, Thermo Fisher Scientific) with rat MSC medium. Cells were incubated at 37°C, 5% CO_2 and 90% humidity. Medium was changed every 3-4 days and cells were passaged at 85% confluency. MSCs were cultured and used in subsequent experiments up to passage 6 (P6). The cells consistently differentiated to osteogenic and adipogenic lineages in culture. In addition, the cells positively expressed characteristic MSC markers CD29, CD73 and CD90, expressed low levels of MHC I and were negative for CD45, CD80, CD86 and MHC II (Supplementary Figure S1).

Pro-inflammatory cytokine licensing of LEW MSCs

LEW MSCs were seeded at a density of $7.5x10^3$ cells/cm^3 in a T175 flask or 6 well plate (Sarstedt, Germany) in rat MSC medium and incubated at 37°C, 5% CO_2 and 90% humidity for 12 hours. Medium was removed and replaced with rat MSC medium containing recombinant rat pro-inflammatory cytokines IFN-γ (50ng/ml), TNF-α (25ng/ml) and/or IL-1β (50ng/ml) (Peprotech, UK) and cells were placed in a humidified tissue culture incubator at 37°C, 5% CO_2 for 72 hours. Medium was removed, cells were washed with DPBS, 5ml/T175 of 0.25% trypsin (Sigma-Aldrich) was added and cells were incubated for 3 minutes at 37°C, 5% CO_2. Trypsin was neutralised by adding twice the volume of serum-containing media. Cells were then centrifuged at 400 x g, washed with DPBS (x2) and counted using a haemocytometer.

Intravenous administration of MSCs

MSCs, cultured as described above in the presence or absence of pro-inflammatory rat cytokines, were washed in DPBS (x3) and filtered through a 40μM filter before administration. Rats were anaesthetized by brief inhalation of isofluorane and MSCs ($1x10^6$ cells in 1ml PBS) were injected i.v. through the lateral tail vein using a 25G needle.

Cell isolation and flow cytometry

The lung was digested by mincing lung tissue into small pieces, followed by digestion by incubating in Hank's Balanced Salt Solution (HBSS) (Gibco/Thermo Fisher Scientific) containing collagenase IV (200U/ml) (Gibco/Thermo Fisher Scientific) and DNAse I (200U/ml) (Sigma-Aldrich) at 37°C with shaking (150 rpm) for 2 hours. Single-cell suspensions of the lymph node, spleen and digested lung were prepared by gentle mashing of

the organs through a 40μM cell strainer (Thermo Fisher Scientific) in a petri dish containing 10mls DPBS. The homogenates were then centrifuged at 800 x g for 5 minutes. The lymph node cells were washed in DPBS and counted using a haemocytometer. The spleen and lung cells were re-suspended in ACK lysis buffer (distilled water, 0.15M NH_4CL, 10mM $KHCO_3$, Sodium EDTA 0.1mM) and incubated on ice for 5 minutes. The reaction was stopped with serum-containing medium. Cells were centrifuged at 800 x g for 5 minutes, washed and re-suspended in DPBS and counted.

For flow cytometry, $1x10^5$ cells/sample were stained with the following anti-rat antibodies: CD4, CD8, CD25, CD45, CD11b/c, MHC II (all Biolegend, California, USA) and with the dead-cell exclusion dye 7-AAD (Thermo Fisher Scientific). For Foxp3 staining, cells were fixed and permeabilised with Foxp3/Transcription Factor Staining Buffer Kit (eBioscience/Thermo Fisher Scientific) as per the manufacturer's instructions and then stained with anti-rat Foxp3 (Thermo Fisher Scientific). Samples were analysed using a BD FACSCanto II Flow Cytometer (BD BioSciences, California, USA). Flow cytometry data was analysed using FlowJo analysis software version 10 (Tree Star Inc., Ashland, OR, USA).

To isolate lung derived $CD11b/c^+$ myeloid cells, a single cell suspension was prepared from the lungs of LEW rats as described above and $CD11b/c^+$ cells were enriched by magnetic column separation using anti-rat CD11b/c microbeads (Miltenyi Biotec, Germany) according to the manufacturer's guidelines. Cells were washed in DPBS and counted, followed by downstream analysis by flow cytometry or RT-PCR or seeded in co-culture assays.

RNA isolation and RT-PCR

Single cell suspensions were centrifuged at 800 x g, supernatants were removed and RNA was extracted with the Isolate II RNA MiniKit (Bioline) following manufacturer's guidelines. cDNA was synthesized using RevertAid H Minus Reverse Transcriptase (Thermo Fisher Scientific) with oligo (dT)12-18 Primers (Thermo Fisher Scientific). Two-step, quantitative real-time polymerase chain reaction (qRT-PCR) was performed to quantify relative mRNA expression. For primer sequences of *Ifn-γ*, *Il-1β*, *Il-10*, *Ido*, *Tnf-α*, *Tgfβ1*, *Ptgs2* and *Gapdh* see Table 1. Samples were normalised to expression of *Gapdh* (housekeeping gene) and expression relative to untreated allogeneic controls was calculated. All qRT-PCR was performed on the ABI Step-one machine (Applied Biosystems, UK) according to standard program settings.

In vitro co-culture assays

For lymphocyte proliferation assays, lymph nodes were isolated from LEW rats and single cell suspensions of lymphocytes were prepared as described above. Cells were re-suspended in T cell medium (RPMI-1640 (Gibco/Thermo Fisher Scientific) supplemented with 10% heat-inactivated FBS (Sigma-Aldrich), 2mmol/l L-glutamine, 100U/ml penicillin, 100µg/ml streptomycin (all Life Technologies/Thermo Fisher Scientific), 0.1 mmol/l non-essential amino acids, 1 mmol/l sodium pyruvate and 55µmol/l 2β-mercaptoethanol (all Sigma-Aldrich)). The cells were labelled with CellTrace Violet Cell Proliferation Kit (Invitrogen/Thermo Fisher Scientific) according to the manufacturer's instructions and seeded in a 96 well plate at a concentration of $2x10^5$ cells/100µl of T cell medium with or without anti-rat anti-CD3/CD28 Dynabeads (Thermo Fisher Scientific) at a ratio of 1:1. In various experiments, untreated or cytokine-stimulated MSCs were added to wells of lymphocytes at a concentration of $2x10^4$ cells/50µl (ratio of 1:10 lymphocyte:MSC) of rat MSC medium. To inhibit nitric oxide (NO) production, S-methylisothiourea (SMT) was added to the cultures at a final concentration of 100 µM. Co-cultures were incubated at 37°C, 5% CO_2 and 90% humidity for 96 hours followed by antibody staining and flow cytometric analysis.

Lung-derived CD11b/c^+ cells, enriched by magnetic column separation as described above, were re-suspended in macrophage medium (RPMI-1640 (Thermo Fisher Scientific), 15% L929 cell conditioned medium, 10% FBS, 2mmol/l L-glutamine, 1% non-essential amino acids, 1mmol/l sodium pyruvate, 1% penicillin/streptomycin, 55µmol/l 2β-mercaptoethanol) at a concentration of $1x10^5$ cells/100µl and were cultured at a ratio of 1:1 with MSCs ($1x10^5$ cells/100µl in rat MSC medium) in a 96 well round bottomed plate for 48 hours. Cells were then lifted by trypsinisation and the magnetic column separation protocol was used to re-enrich the CD11b/c^+ cells. CD11b/c positivity and the viability of re-enriched CD11b/c^+ cells was assessed by flow cytometry (Supplementary Figure S2).These MSC-conditioned CD11b/c^+ cells were then co-cultured with freshly-prepared LEW lymphocytes prepared from whole lymph node homogenates at a ratio 1:1 ($1x10^5$ cells/100µl each)) with or without anti-rat CD3/CD28 (both BD Biosciences) bound Dynabeads (Thermo Fisher Scientific) at a ratio of 1:1 bead:lymphocyte for 96 hours followed by antibody staining and flow cytometric analysis.

Quantification of cytokines, prostaglandin E2 (PGE2) and NO

Culture supernatants were analysed for the presence of PGE2 and transforming growth factor beta 1 (TGF-β1) by ELISA (R&D Systems,UK) following the manufacturer's guidelines. IFN-γ, TNF-α and IL-2 concentrations were quantified as part of the Th Complete 14-Plex Rat ProcartaPlex Panel (Thermo Fisher Scientific) and quantified by Bio-Plex 200 system (Bio-Rad, California, USA). NO concentration in culture supernatants was quantified by Griess assay. 100µl of supernatant were combined with an equal volume of Griess reagent (1% sulphanilamide and 0.1% N-1-(naphthyl)-ethylenediamine-diHCl in 2.5% H3PO4) in a 96-well flat bottom plate and absorbance measured immediately at 540nm on a plate reader (Perkin Elmer, Ireland). A sodium nitrite standard curve was used to calculate NO concentration.

Statistical Analysis

All statistical analysis was performed using GraphPad Prism software Version 5 (La Jolla, USA). Data were assessed for normal distribution using the Kolmogrov-Smirnov test for normality. One-way ANOVA was used for multiple comparison tests for results from *in vitro* and *in vivo* experiments followed by Tukey's multiple comparison post-test. Data are presented as mean ± SEM. Kaplan-Meier survival curves with log-rank (Mantel Cox) test were used for analysis of allograft survival. Significance was denoted as: *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$.

Results

IL-1β in combination with TNF-α or IFN-γ stimulates a potent immunosuppressive MSC phenotype

In this study we aimed to enhance the immune modulatory properties of auto-MSCs by "licensing" with pro-inflammatory cytokines. Based on the literature, IFN-γ, TNF-α and IL-1β were selected to license the MSCs for 72 hours (35-37). To test their immunomodulatory function, cytokine-licensed LEW MSCs were cultured with anti-CD3/CD28 bead-stimulated LEW lymphocytes for a period of 96 hours. We observed significant suppression of overall lymphocyte proliferation when MSCs were licensed with IL-1β in combination with either IFN-γ ($MSC^{IFN\text{-}\gamma/IL\text{-}1\beta}$) or TNF-α ($MSC^{TNF\text{-}\alpha/IL\text{-}1\beta}$) (Figure 1A and B) compared to lymphocytes stimulated in the absence of MSC. Analysis of lymphocyte proliferation beyond 3 generations demonstrated that MSCs licenced with IL-1β alone ($MSC^{IL\text{-}1\beta}$) suppressed lymphocyte proliferation (Figure 1 A and C) in addition to $MSC^{IFN\text{-}\gamma/IL\text{-}1\beta}$ and $MSC^{TNF\text{-}\alpha/IL\text{-}1\beta}$ (Figure 1A and C). Untreated MSCs (MSC^{UTR}) or MSCs licensed with IFN-γ alone ($MSC^{IFN\text{-}\gamma}$), TNF-α alone ($MSC^{TNF\text{-}\alpha}$) or IFN-γ and TNF-α combined ($MSC^{IFN\text{-}\gamma/TNF\text{-}\alpha}$) did not significantly suppress lymphocyte proliferation (Figure 1 A-C). In the context of corneal allograft transplantation, rejection is mediated primarily by $CD4^+$ T cells and associated delayed type hypersensitivity (DTH) responses (38-43). To test whether licensed MSCs preferentially inhibited $CD4^+$ T cells, the proliferation of the $CD4^+$ and $CD8^+$ T cell subsets was separately assessed in the same experiments. As shown in Figures 1D and 1E, both $MSC^{IFN\text{-}\gamma/IL\text{-}1\beta}$ and $MSC^{TNF\text{-}\alpha/IL\text{-}1\beta}$ significantly inhibited proliferation of $CD4^+$ and $CD8^+$ T cells, demonstrating that the licensed MSCs similarly suppress both subsets. In summary, these results demonstrate LEW MSCs acquire a potent capacity to inhibit syngeneic lymphocyte proliferation when licensed with the pro-inflammatory cytokine IL-1β in combination with IFN-γ or TNF-α.

TNF-α/ IL-1β licensing significantly increases NO production by MSCs

We next investigated the mechanism of licensed MSC-mediated suppression of T-cell proliferation. As previous studies have reported nitric oxide (NO) to be the key mediator of the T-cell suppressive effects of rodent MSCs, we assayed the NO levels in licensed MSC cultures and in licensed MSC-lymphocyte co-cultures (44, 45). Interestingly, following 72-hour licensing, only $MSC^{TNF\text{-}\alpha/IL\text{-}1\beta}$ produced increased levels of NO (6.61±0.92 μM, $p \leq 0.001$) in the supernatant compared to MSC^{UTR} (0.2±0.25μM), while the concentrations in all other groups were low or undetectable (Figure 2A). In the supernatants of licensed MSC-

lymphocyte co-cultures, detectable levels of NO were observed in all wells in which MSCs were present, indicating that the interaction of MSCs and lymphocytes stimulates the production of NO (Figure 2B). Compared to stimulated lymphocytes alone, significant increases in the concentration of NO was observed in co-culture wells containing $MSC^{IL\text{-}1\beta}$ (12.68±7μM, $p \leq 0.05$), $MSC^{IFN\text{-}\gamma/IL\text{-}1\beta}$ (15.58±6.37μM, $p \leq 0.01$) or $MSC^{TNF\text{-}\alpha/IL\text{-}1\beta}$ (18.82±4.58μM, $p \leq 0.01$), while $MSC^{TNF\text{-}\alpha/IL\text{-}1\beta}$ also increased the NO concentration compared to MSC^{UTR} (5.04±2.26μM, $p \leq 0.05$) (Figure 2B).

These results suggest that NO production is directly linked to the potency of licensed MSCs' ability to suppress syngeneic lymphocyte proliferation. As $MSC^{TNF\text{-}\alpha IL\text{-}1\beta}$ demonstrated the most potent suppressive capacity, subsequent experiments focused on comparing MSC^{UTR} with $MSC^{TNF\text{-}\alpha/IL\text{-}1\beta}$.

Phenotypic comparison of $MSC^{TNF\text{-}\alpha/IL\text{-}1\beta}$ and MSC^{UTR} showed that they expressed comparable surface levels of characteristic MSC markers CD90 and CD29 while $MSC^{TNF\text{-}\alpha/IL\text{-}1\beta}$ had higher surface expression of CD73 and CD44 as well as low-level MHC I, not present on MSC^{UTR}. Neither MSC type expressed MHC II, CD45 or CD86 (Supplementary Figure S1).

$MSC^{TNF\text{-}\alpha/IL\text{-}1\beta}$ suppression of syngeneic lymphocytes is primarily mediated by nitric oxide

To confirm the role of NO as the primary mediator of licensed MSCs' immunosuppressive capacity, MSCs were co-cultured with stimulated lymphocytes in the presence of S-methylisothiourea (SMT), a preferential iNOS inhibitor. As predicted, total lymphocyte proliferation was not inhibited by MSC^{UTR} either in the absence or presence of SMT (Figure 3A and B). In contrast, the ability of $MSC^{TNF\text{-}\alpha/IL\text{-}1\beta}$ to inhibit lymphocyte proliferation (1.29%±1.011, $p \leq 0.001$) compared to stimulated controls (92.57%±3.5) was abolished by the addition of SMT (88.55%±0.78) (Figure 3A and B). Analysis of the co-culture supernatants confirmed that NO was successfully inhibited by addition of SMT (Figure 3C). NO is reported to increase the enzymatic activity of cyclooxygenase-2 (COX-2) resulting in increased production of the immune-regulatory factor PGE_2 (46). In keeping with this, PGE_2 was detected in co-cultures in the presence of MSC^{UTR} (61.92±20.76ng/ml), further increased in the presence of $MSC^{TNF\text{-}\alpha/IL\text{-}1\beta}$ (126.81±9.94ng/ml, $p \leq 0.001$) and was reduced to concentrations comparable to MSC^{UTR} in the presence of SMT (55.78±19.76ng/ml, $p \leq 0.001$) (Figure 3D).

While both MSC^{UTR} and $MSC^{TNF\text{-}\alpha/IL\text{-}1\beta}$ inhibited the production of IFN-γ and TNF-α in co-

culture with syngeneic lymphocytes, the addition of SMT did not abolish this effect - indicating that, in keeping with previous reports, the ability of MSCs to inhibit lymphocyte secretion of pro-inflammatory cytokines is independent of NO (Figure 3E, F) (47). Increased levels of the T cell growth factor IL-2 were detected in the presence of $MSC^{TNF\text{-}\alpha/IL\text{-}1\beta}$ compared to stimulated lymphocytes alone and this was reversed by the addition of SMT (Figure 3C). This result is in keeping with a mechanism of action of NO by T cells undergoing the normal initial activation sequence in response to stimulation (calcium flux, T cell receptor (TCR) modulation and secretion of IL-2) but subsequently fail to respond to IL-2 resulting in its accumulation in the culture medium (48, 49).

In summary, our findings indicate that rat $MSC^{TNF\text{-}\alpha/IL\text{-}1\beta}$ suppression of syngeneic T-cell proliferation and IL-2 consumption as well as increased PGE_2 production are dependent on NO, while MSC-mediated lymphocyte polarisation is NO-independent.

$MSC^{TNF\alpha/IL1\beta}$ significantly prolong corneal allograft survival in a fully allogeneic rat model

Our group has previously demonstrated that syngeneic MSCs failed to prolong allograft survival when administered pre-transplantation (4). Based on this finding, in this study, we used a post-transplant administration strategy consisting of i.v. administration of $1x10^6$ syngeneic MSC^{UTR} or $MSC^{TNF\text{-}\alpha/IL\text{-}1\beta}$ on post-operative day (POD) 1 and POD 7 in a fully MHC-mismatched rat corneal transplant model. Increased rejection-free corneal allograft survival was observed in LEW rats that received syngeneic MSC^{UTR} (50%, $p \leq 0.05$) as well as $MSC^{TNF\text{-}\alpha/IL\text{-}1\beta}$ (70%, $p \leq 0.001$) compared to allograft controls (0%) (Figure 4A). Although not statistically significant, a trend toward superior rejection-free survival was observed in the $MSC^{TNF\text{-}\alpha/IL\text{-}1\beta}$-treated compared to MSC^{UTR}-treated group (Figure 4A). Trends toward decreased graft opacity were observed in both MSC^{UTR}-treated and $MSC^{TNF\text{-}\alpha/IL\text{-}1\beta}$-treated animals compared to untreated controls (Figure 4B and E), while there was also a trend toward increased neovascularisation observed in $MSC^{TNF\text{-}\alpha/IL\text{-}1\beta}$-treated animals compared to the other two groups (Figure 4C). The maximum opacity score recorded for each animal was significantly reduced in both the MSC^{UTR}-treated (2.38 ± 0.23, $p \leq 0.01$) and $MSC^{TNF\text{-}\alpha/IL\text{-}1\beta}$-treated ($2.35 \pm 0.41$, $p \leq 0.01$) groups compared to untreated controls (2.9 ± 0.21) (Figure 4D). In summary, these results demonstrate that the post-transplant administration of syngeneic MSCs prolongs fully MHC-mismatched corneal allograft survival with a trend toward superior rejection-free survival following administration of $MSC^{TNF\text{-}\alpha/IL\text{-}1\beta}$.

MSC$^{TNF\text{-}\alpha/IL\text{-}1\beta}$ therapy enriches myeloid cells in the lung and Foxp3$^+$ T-reg proportions in the lung and spleen

It has become increasingly clear that the majority of MSCs administered i.v. become trapped in the lung and are cleared within 24 hours (17, 18, 50, 51). Therefore, it is likely that MSCs exert their immunomodulatory effect at this site before being cleared. In flow cytometry analyses carried out on tissues dissected at POD 9, we observed proportionate increases in CD11b/c$^+$MHCII$^+$ expressing CD45$^+$ cells (Figure 5A for gating strategy) in the lungs of MSC^{UTR}-treated allograft recipients (7.67%±1.43, $p \leq 0.05$) compared to untreated controls (3.79%±1.25) with a further increase in the MSC$^{TNF\text{-}\alpha/IL\text{-}1\beta}$-treated group in the lung (11.29%±3.57, $p \leq 0.001$ and $p \leq 0.05$ compared to untreated and MSC^{UTR}-treated respectively) (Figure 5B) and in the spleen (Supplementary Figure S3). To investigate their immunomodulatory phenotype, lung CD11b/c$^+$ cells were enriched from the lungs of all three groups at POD 9 and were analysed by RT-PCR. In this analysis, trends toward increased mRNA expression for the immunomodulatory genes *Ptgs2*, *Il-10* and indoleamine 2,3 dioxygenase (*Ido*) as well as a significant decrease in *Tnf-α* mRNA were observed in lung CD11b/c$^+$ cells from MSC$^{TNF\text{-}\alpha/IL\text{-}1\beta}$-treated animals (Figure 5C).

In the same flow cytometry analyses, a proportionate increase in lung CD4$^+$CD25$^+$Foxp3$^+$ (Figure 5D for gating strategy) T-regs at POD 9 was demonstrated in the MSC^{UTR}-treated group (7.66%±0.65, $p \leq 0.01$) compared to untreated controls (3.73%±0.3) with a further increase in the lungs of the MSC$^{TNF\text{-}\alpha/IL\text{-}1\beta}$-treated group (10.5%±1.51, $p \leq 0.05$). In the latter group, there was also an increase in the proportion of T-regs in the spleen (5.6%±0.58) compared to both untreated (4.33%±0.29, $p \leq 0.01$) and MSC^{UTR}-treated (4.86%±0.37, $p \leq 0.05$) groups (Figure 5E and F). At this early time-point, no differences were observed among the groups in the proportions of T-regs in the corneal allograft-draining lymph nodes (dLN) with the exception that the T-reg proportion was lower for the MSC$^{TNF\text{-}\alpha/IL\text{-}1\beta}$-treated compared to the MSC^{UTR}-treated group (Figure 5G) (52).

Taken together, these results demonstrate increased accumulation at POD 9 of potential regulatory myeloid cells and T-regs in the lungs, as well as proportionate increases in splenic T-regs but not dLNs of corneal allograft recipients treated with MSC$^{TNF\text{-}\alpha/IL\text{-}1\beta}$ on PODs 1 and 7. Similar, but less potent, localized immunomodulatory effects were also present in allograft recipients treated with MSC^{UTR}.

MSC$^{TNF\text{-}\alpha/IL\text{-}1\beta}$ increase Foxp3$^+$ Tregs and decrease pro-inflammatory cytokines in the lung, spleen and draining lymph node at the expected time of corneal allograft rejection

To analyse the immune response at the expected time of rejection (TOR), the proportions of Foxp3$^+$ T-regs and the mRNA expression of pro-inflammatory cytokines *Ifn-γ*, *Tnf- α* and *Il-1β* were analysed in lungs, spleens and dLNs of control and MSC-treated corneal allograft recipients at POD 17-19.

As shown in Figure 6A, increased proportions of T-regs remained present in the lungs of MSC$^{TNF\text{-}\alpha/IL\text{-}1\beta}$-treated animals at the TOR (5.22% ± 0.2 compared to 3.43% ± 0.24 for untreated controls, $p \leq 0.001$) but not in MSC^{UTR}-treated animals (3.23% ± 0.37). *Ifn-γ* mRNA expression was not detected in the lungs of control or MSC treated animals. MSC$^{TNF\text{-}\alpha/IL\text{-}1\beta}$ but not MSC^{UTR} were associated with reduced *Il-1β* mRNA expression in the lungs while lung *Tnf-α* mRNA expression was reduced in both MSC-treated groups compared to allograft controls (Figure 6B). Similarly, in the spleen, increased proportions of T-regs were observed in the MSC$^{TNF\text{-}\alpha/IL\text{-}1\beta}$-treated animals (5.85% ± 0.86) compared to untreated controls (4.42 ± 0.56, $p \leq 0.01$) and MSC^{UTR}-treated animals (4.58% ± 0.76, $p \leq 0.01$) (Figure 6C). Both MSC^{UTR} and MSC$^{TNF\text{-}\alpha/IL\text{-}1\beta}$ treatments were associated with reduced *Ifn-γ*, *Il-1β* and *Tnf-α* expression in the spleen compared to untreated controls with spleen *Ifn-γ* expression lower in MSC$^{TNF\text{-}\alpha/IL\text{-}1\beta}$-treated compared to the MSC^{UTR}-treated group (Figure 6D). In contrast to findings at POD 9, analyses of cells from the dLNs indicated increased proportions of T-regs in both MSC^{UTR}-treated (4.05% ± 0.79, $p \leq 0.05$) and MSC$^{TNF\text{-}\alpha/IL\text{-}1\beta}$-treated (4.55% ± 0.33, $p \leq 0.01$) control animals (2.79% ± 0.57) (Figure 6E). This finding was accompanied by lower mRNA expression of *Ifn-γ* and *Tnf-α* in both MSC treatment groups (Figure 6F).

Overall, these results demonstrate that both MSC^{UTR} and MSC$^{TNF\text{-}\alpha/IL\text{-}1\beta}$ therapies significantly increase the proportions of T-regs and lower expression of DTH-associated cytokines in the critical organ in determining the fate of the graft – the dLN – at the expected TOR. Notably, however, only MSC$^{TNF\text{-}\alpha/IL\text{-}1\beta}$ therapy was associated with a persistent T-reg presence in distant organs such as the lung and spleen, suggesting a more potent systemic immune modulation.

MSCs indirectly promote Foxp3$^+$ regulatory T cells via a myeloid cell intermediary

To investigate the mechanism underlying MSC-mediated promotion of increased Foxp3$^+$ T-regs in corneal allograft recipients, the ability of MSCs to directly enrich Foxp3$^+$Tregs *in vitro* in direct co-culture with syngeneic unstimulated lymphocytes was determined (Figure 7A and B). MSC^{UTR} did not alter the percentage of CD25$^+$Foxp3$^+$ cells among CD4$^+$ T cells

compared to unstimulated lymphocyte controls while $MSC^{TNF\text{-}\alpha/IL\text{-}1\beta}$ significantly reduced the percentage of $CD25^+Foxp3^+$ cells - indicating that syngeneic MSCs do not directly induce $Foxp3^+$ T-regs from unstimulated lymphocytes ($p \leq 0.05$) (Figure 7A and B).

As we had observed increased proportions of $CD11b/c^+MHCII^+$ myeloid cells and T-regs in the lungs at the same time-point *in vivo*, we next investigated whether MSCs induced T-regs via a myeloid cell intermediary. As outlined in Figure 7C, this was tested by culturing MSC^{UTR} and $MSC^{TNF\text{-}\alpha/IL\text{-}1\beta}$ with syngeneic $CD11b/c^+$ myeloid cells sorted from the lungs of naïve LEW rats, then subsequently culturing the MSC-conditioned myeloid cells with either: a) stimulated LEW lymphocytes to investigate the myeloid cell's suppressive capacity (Figure 7F and G); or b) unstimulated LEW lymphocytes to investigate their capacity to enrich $Foxp3^+$ T-regs (Figure 7H and I). In culture supernatants from the primary myeloid cell-MSC co cultures, concentrations of PGE_2 and TGF-β1 were higher in the presence of either MSC^{UTR} or $MSC^{TNF\text{-}\alpha/IL\text{-}1\beta}$ compared to untreated myeloid cells, while increased concentrations of NO were observed in the presence of $MSC^{TNF\text{-}\alpha/IL\text{-}1\beta}$ only (Figure 7D and E, Supplementary Figure S4). In the subsequent myeloid cell-lymphocyte co-cultures, we observed potent inhibition of $CD4^+$ lymphocyte proliferation in the presence of myeloid cells conditioned with MSC^{UTR} or $MSC^{TNF\text{-}\alpha/IL\text{-}1\beta}$ but not in the presence of unconditioned myeloid cells (Figure 7F and G). In addition to suppressing T cell proliferation, myeloid cells conditioned with MSC^{UTR} or $MSC^{TNF\text{-}\alpha/IL\text{-}1\beta}$ were also associated with increased proportions of $CD4^+CD25^+Foxp3^+$ T-regs compared to unconditioned $CD11b/c^+$ myeloid cells when co-cultured with unstimulated syngeneic lymphocytes ($p \leq 0.05$ and $p \leq 0.01$, respectively) (Figure 7H and I).

No increase in the concentration of NO was observed in the supernatants from co-cultures of $CD11b/c^+$ myeloid cells and lymphocytes (Supplementary Figure S4). In the unstimulated co-cultures of MSC-conditioned myeloid cells and lymphocytes, we observed that concentrations of PGE_2 and TGF-β1 were differentially affected by the licensing status of the MSCs. Specifically, $MSC^{TNF\text{-}\alpha/IL\text{-}1\beta}$-conditioned myeloid cells were associated with higher PGE_2 and lower TGF-β1 compared to MSC^{UTR}-conditioned myeloid cells (Figure 7J and K). A similar trend was observed in co-cultures with stimulated lymphocytes (Supplementary Figure S5).

In summary, we demonstrate that both MSC^{UTR} and $MSC^{TNF\text{-}\alpha/IL\text{-}1\beta}$ condition lung-derived myeloid cells towards an immune regulatory phenotype capable of suppressing syngeneic lymphocyte proliferation and inducing $CD4^+CD25^+Foxp3^+$ T-regs from naïve unstimulated lymphocytes. Potent induction of PGE_2 and TGF-β1 may represent an important feature of

MSC-reprogrammed lung myeloid cells and these known immunomodulatory mediators may also be differentially induced by resting and cytokine-licensed MSC.

Discussion

Cell therapies, in particular MSC-based therapies, have shown promising results in clinical trials for the treatment of inflammatory disorders. Despite this, the *in vivo* mechanism of action of i.v. administered MSCs remains to be fully understood. Our work using either licensed or non-licensed (naïve) MSCs expand our knowledge by demonstrating that systemic delivery of autologous MSC in the days following corneal transplantation was associated with expansion of T-regs – likely through a regulatory myeloid cell intermediate. Previously we demonstrated that allo-MSCs but not auto-MSCs prolonged corneal allograft survival when administered pre-transplantation in our DA rat cornea to LEW rat recipient transplant model (4). In the previous study, auto-MSCs administered to a non-inflamed, immunologically compatible host may not have received a sufficient activation stimulus to trigger their immunomodulatory properties.

Here we describe two modifications which work synergistically to enhance the efficacy of auto-MSC therapy. Firstly, pro-inflammatory cytokine licensing mimics the inflammatory milieu, stimulating the immune modulatory capacity of the MSCs and secondly, the efficacy is further enhanced by post-transplantation administration where the primed MSC encounters an inflamed host. As the immune privileged status of MSCs has come into question with the accumulating evidence that anti-donor immune responses are mounted against allo-MSCs, our results describe how optimising auto-MSC therapy can significantly enhance their immune modulatory capacity are of potential clinical importance (33, 53-55). Notably, as demonstrated here and as previously reported, licensing of MSCs may increase their immunogenicity by up-regulating expression of MHC molecules I and II (31, 54, 56). Indeed, it has previously been reported that licensed allo-MSCs are associated with increased T cell responses and humoral responses compared to their untreated counterparts (33, 57, 58). Therefore, while licensing may prove to be a beneficial strategy to enhance auto-MSC therapy it may have an adverse effect in an allogeneic setting.

Recent evidence has highlighted the importance of MSC-mediated immunomodulation in the lung for their subsequent systemic anti-inflammatory effects following i.v. administration (9, 15, 19). Despite the majority of i.v. administered MSCs being cleared from the lung within the first 24 hours, our group and others have demonstrated that their immunological imprint persists for longer due to either direct or indirect modulation of lung myeloid cells to an immune regulatory phenotype (9, 15-17, 19).

Directly, MSCs are reported to modulate lung myeloid cells via PGE2, TSG-6 and TGF-β dependent mechanisms (9, 15, 19, 59). More recently, the importance of MSC phagocytosis

in the promotion of an immune regulatory myeloid cell phenotype has been described (20, 21, 60). Braza and colleagues demonstrated that lung macrophages that had phagocytosed PKH26 labelled MSCs displayed an M2 phenotype not observed in PKH26$^-$ macrophages (60). Galleu et al, reported that cytotoxic T cell-induced MSC apoptosis in the lung was required to induce a regulatory phenotype in the engulfing phagocyte (21). De Witte and colleagues recently reported that the majority of i.v.-delivered MSCs were not viable by 24 hours post infusion and were detected as phagocytosed particles of monocytes (20). Our results further highlight the potential importance of lung myeloid cells in the transfer of MSCs immunomodulatory effect. We also demonstrate that licensing of auto-MSCs enhances their capacity to modulate the myeloid cells. This effect is likely mediated through cross-talk via the release of soluble mediators such as NO, PGE_2, and TGF-β which create a local anti-inflammatory environment and, potentially, pre-condition the myeloid cell before further reprogramming occurs upon phagocytosis (61).

MSC-educated myeloid cells are reported to directly suppress the inflammatory response or mediate their disease-modifying effects by enriching other immune regulatory cells such as T-regs (15, 20). Our results indicate that MSC-educated lung-derived myeloid cells have the potential to both directly suppress the inflammatory response by inhibiting lymphocyte proliferation and to expand populations of T-regs. The MSC-mediated expansion of T-regs appears to be dependent upon the myeloid cell intermediary as MSCs do not directly enrich T-regs from naïve lymphocytes. In keeping with this, de Witte and colleagues demonstrated that MSC-conditioned monocytes induced T-regs in mixed lymphocyte reactions and Melief et al, demonstrated that T-reg expansion was dependent upon induction of an anti-inflammatory macrophage phenotype and that MSCs failed to induce T-regs in macrophage depleted PBMC cultures (10, 20).

Proportionate increases in T-regs were observed in the dLN at the average TOR in both MSC^{UTR} and $MSC^{TNF\text{-}\alpha/IL\text{-}1\beta}$ treated animals. Importantly, immunological activity in the dLN has been shown to be critical in determining the fate of allogeneic corneal transplants. Yamagami and colleagues highlighted the importance of the dLN by showing that its removal prior to transplantation resulted in 100% rejection-free survival (52). In this study, the expanded T-reg proportions are associated with *Ifn-γ* and *Tnf-α* mRNA expression, indicating that MSC-educated myeloid cell-mediated induction of T-regs inhibits the DTH responses in the dLN resulting in prolongation of graft survival (62).

A further important finding in the current study is the enhanced immunomodulatory effect of licensed MSCs in the lung and spleen. Evidence of lung myeloid cell reprogramming and the

persistence of expanded T-regs proportions in the lung and spleen at the TOR was only observed in licensed MSC-treated animals. Licensed MSCs more potently modulated the lung, the first organ encountered upon infusion, therefore, local administration such as subconjunctival injection in the case of cornea transplantation could further enhance the immunomodulatory capacity of licensed MSCs. Furthermore, potent immunomodulation of the lung and spleen upon i.v. infusion indicates that licensed auto-MSCs may have therapeutic efficacy for disease processes that directly affect these organs such as acute lung injury or in systemic disorders such as graft versus host disease (GvHD).

In summary, our findings demonstrate that the immunomodulatory capacity of auto-MSC therapy can be enhanced by a pro-inflammatory cytokine licensing pre-infusion regimen and by optimisation of the timing of administration. Our results indicate that autologous i.v. infused MSCs potently modulate the immune cell repertoire in the lung, conditioning myeloid cells to an immune regulatory phenotype which is subsequently associated with increased $Foxp3^+$ T-regs. Expansion of $Foxp3^+$ T-regs in MSC treated animals are observed in the dLN at the TOR where they suppress the DTH response that is known to be a critical element of corneal transplant rejection.

Acknowledgments

This study was supported by a Science Foundation Ireland Investigator Award (grant number 12/IA/1624; NM, OT, KL, MM, PL, MDG, TR). This work was also supported by grants from the Health Research Board of Ireland (grant number HRA_POR/2013/341) (NM, MDG, TR), a Science Foundation Ireland Starting Investigator Grant (grant number 15/SIRG/3456; AR) and the European Regional Development Fund. The authors wish to acknowledge the advice, assistance and technical expertise of Ms. Georgina Shaw and Dr. Linda Howard, NUI Galway. All flow cytometry experiments were performed in the NUI Galway Flow Cytometry Core Facility which is supported by funds from NUI Galway, Science Foundation Ireland, the Irish Government's Programme for Research in Third Level Institutions, Cycle 5 and the European Regional Development Fund.

Conflict of Interest Statement

The authors declare no conflict of interest

Authors and Contributors

- Substantial contributions to the conception or design of the work (**NM, OT, MDG, AR, TR)**; or the acquisition (**NM, OT, KL, PL, MM)**, analysis (**NM, OT, KL, PL, MM, GF)**, or interpretation of data for the work (**NM, OT, PL, GF, AR, MDG, TR)**; AND
- Drafting the work or revising it critically for important intellectual content (**NM, AR, MDG, TR)**; AND
- Final approval of the version to be published (**NM, OT, KL, MM, PL, GF, AR, MDG, TR)**; AND
- Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved **(NM, OT, KL, MM, PL, GF, AR, MDG, TR)**.

Figure Legends

Figure 1. IL-1β in combination with TNFα or IFNγ induces a potently immunosuppressive phenotype in Lewis rat MSCs. Lewis rat MSCs were stimulated with IFN-γ, TNF-α and/or IL-1β (500U/ml) for 72 hours in monolayer culture. Licensed Lewis rat MSCs were then co-cultured with anti-CD3/CD28 bead stimulated Lewis rat lymphocytes for 96 hours. (A) Representative examples of CFSE profiles of lymphocytes (grey) following co-culture with licensed MSCs compared to control stimulated lymphocytes alone (black dashed line). (B) Quantification of percentage of total lymphocyte proliferation following co-culture with licensed MSCs. (C) Quantification of percentage of lymphocyte proliferation greater than three generations. (D) Percentage proliferation of $CD4^+$ lymphocytes. (E) Percent proliferation of $CD8^+$ lymphocytes. (Representative of three independent experiments one-way ANOVA n=3, mean ± SEM, *p=0.05, **p=0.01, ***p=0.001).

Figure 2. TNF-α/IL-1β licensing significantly increases NO production in Lewis rat MSCs. (A) Griess assay results of the supernatants of Lewis rat MSCs cultured in monolayer for 72 hours with rat MSC media supplemented IFN-γ, TNF-α and/or IL-1β (500U/ml) (One-way ANOVA, Tukey's multiple comparison post test, n=3, mean ± SEM, ***p≤0.001). (B) Griess assay results of the supernatants from MSC:Lymphocyte co culture assays described in Figure 1 (One-way ANOVA, Tukey's multiple comparison post test, n=3, mean ± SEM, *p≤0.05, **p≤0.01).

Figure 3 Inhibition of NO production abrogates the ability of $MSC^{TNF\text{-}\alpha/IL\text{-}1\beta}$ to suppress lymphocyte proliferation but MSC mediated lymphocyte polarisation is NO independent. Anti-CD3/CD28 bead stimulated Lewis rat lymphocytes were co-cultured with Lewis rat MSC^{UTR} or $MSC^{TNF\text{-}\alpha/IL\text{-}1\beta}$ in the presence or absence of NO inhibitor SMT (500μM) for 96 hours. (A) Representative lymphocyte CFSE profiles and (B) the percentage proliferation of the lymphocytes quantified by CFSE dye dilution measured by flow cytometry following coculture with syngeneic MSC^{UTR} or $MSC^{TNF\text{-}\alpha/IL\text{-}1\beta}$ in the presence or absence of SMT (One-way ANOVA n=3, Tukey's multiple comparison post-test ***p≤0.001). The supernatants were analysed by: (C) Griess assay to detect NO concentration, (D) ELISA for the concentration of PGE_2 and (E,F,G) multiplex assay to detect the concentrations of (E) IFN-γ, (F) TNF-α and (G) IL-2 (All assays were analysed by One-way ANOVA n=3, Tukey's multiple comparison post-test, mean+-SEM, *p≤0.05, **p≤0.01, ***p≤0.001).

Figure 4. Syngeneic MSC^{UTR} and $MSC^{TNF\text{-}\alpha/IL\text{-}1\beta}$ prolong corneal allograft survival when administered post operatively. Lewis rat MSC^{UTR} or $MSC^{TNF\text{-}\alpha/IL\text{-}1\beta}$ were administered intravenously on post-operative day (POD) 1 and POD 7 to a Lewis rat receiving an allogeneic Dark Agouti (DA) corneal allograft. (A) Kaplan-Meier survival curve analysis of allogeneic transplant controls (black line) (n=10), corneal allograft + MSC^{UTR} (blue line) (n=8) and corneal allograft + $MSC^{TNF\text{-}\alpha/IL\text{-}1\beta}$ (orange line) (n=10). (Log-rank (Mantel-Cox) test, *p=0.05, ***p=0.001, NS = not significant). (B) Opacity and (C) neovascularization scores up to POD 30. (D) Maximum opacity score recorded per animal, one score of opacity 3 or two consecutive scores of 2.5 classed as graft rejection (One-way ANOVA, Tukey's multiple comparison post-test, mean±SEM, **p≤0.01)). (E) Representative light microscope images of the corneal allograft at POD 10 to POD 30.

Figure 5. $MSC^{TNF\text{-}\alpha/IL\text{-}1\beta}$ therapy potently enriches $Foxp3^+$ Tregs and modulates $CD11b/c^+$ cells in transplanted animals at POD 9. Lewis rats receiving MSC^{UTR} or $MSC^{TNF\text{-}\alpha/IL\text{-}1\beta}$ on POD 1 and 7 were sacrificed on POD 9 and their lungs, dLN and

spleen analysed by flow cytometry. (A) Gating strategy to identify CD11b/c+MHCII$^+$ cells in the CD45$^+$ fraction of cells isolated from the lung and (B) Graphs showing results of CD45$^+$ cells expressing CD11b/c and MHCII. (C) RT-PCR analysis of CD11b/c$^+$ cells MACS sorted from the lung at POD 9 assessing mRNA expression of *Tgf-β1, Ptgs2, Il-10, Ido, Tnf-α* and *Il-1β*. (D) Gating strategy to gate CD25$^+$Foxp3$^+$ cells in tissues of corneal graft recipients. Flow cytometry analysis of the proportion of CD4$^+$ T cells expressing CD25 and Foxp3 in the (E) lung, (F) spleen and (G) dLN. (One-way ANOVA, Tukey's multiple comparison post-test, mean±SEM, *p≤0.05 **p≤0.01, ***p≤0.001)

Figure 6. At the time of rejection Foxp3$^+$ Tregs are observed in the periphery of MSC$^{TNF\text{-}α/IL\text{-}1β}$ and in the draining lymph node of both MSC^{UTR} and MSC$^{TNF\text{-}α/IL\text{-}1β}$ treated animals. Lewis rats receiving MSC^{UTR} or MSC$^{TNF\text{-}α/IL\text{-}1β}$ on POD 1 and 7 were sacrificed at the average time of rejection (TOR) and their lungs, dLN and spleen analysed by flow cytometry and RT-PCR. (A) Graph showing flow cytometry results of CD4$^+$ T cells co-expressing CD25 and Foxp3 in the lungs of transplanted animals. (B) RT-PCR results for the mRNA expression of IFN-γ, IL-1β and TNF-α from cells isolated from the lungs of transplanted animals. (C) Graph of flow cytometry analysis CD4$^+$CD25$^+$Foxp3$^+$ cells and (D) RT-PCR results for the mRNA expression of IFN-γ, IL-1β and TNF-α from cells isolated from the spleen of transplanted animals. (E) Flow cytometry analysis of CD4$^+$CD25$^+$Foxp3$^+$ cells and (F) mRNA expression IFN-γ, IL-1β and TNF-α from cells isolated from the dLN (One-way ANOVA, Tukey's multiple comparison post-test, mean±SEM, *p≤0.05 **p≤0.01, ***p≤0.001)

Figure 7. MSC^{UTR} and MSC$^{TNF\text{-}α/IL\text{-}1β}$ induce Foxp3$^+$ Tregs via promotion of an immune regulatory phenotype in lung derived CD11b/c$^+$ myeloid cells associated with increases in PGE$_2$ and TGF-β1. MSC^{UTR} or MSC$^{TNF\text{-}α/IL\text{-}1β}$ were cultured with unstimulated syngeneic lymphocytes for 96 hours. The percentage of CD25$^+$Foxp3$^+$ cells of the CD4$^+$ T cells was assessed by flow cytometry. (A) Representative dot plots and (B) graphed results of the percentages CD25$^+$Foxp3$^+$ cells in unstimulated lymphocytes alone or in the presence of MSC^{UTR} or MSC$^{TNF\text{-}α/IL\text{-}1β}$.
(C) Schematic of experimental design, CD11b/c$^+$ cells MACS sorted from the lung were cultured with MSC^{UTR} or MSC$^{TNF\text{-}α/IL\text{-}1β}$ for 48 hours, the MSC conditioned CD11b/c$^+$ cells were MACS sorted again and cultured with stimulated or unstimulated lymphocytes for 96 hours. (D, E) Concentration of PGE$_2$ and TGF-β1 in the supernatants of MSC-CD11b/c$^+$ cell co-cultures detected by ELISA (F) Quantification of lymphocyte proliferation following co culture of anti-CD3/CD28 bead stimulated lymphocytes with MSC conditioned lung CD11b/c$^+$ cells and (G) representative plots of lymphocyte CFSE profiles. (H) Quantification of the percentage of CD25+Foxp3+ cells of the CD4+ lymphocytes following co culture of unstimulated lymphocytes with lung sorted, MSC conditioned, CD11b/c$^+$ cells and (I) representative plots of the proportions of CD25$^+$Foxp3$^+$ cells of the CD4$^+$ lymphocytes (One-way ANOVA n=4, Tukey's multiple comparison post-test, mean±SEM, *p≤0.05, **p≤0.01, ***p≤0.001). (J, K) The supernatants from stimulated lymphocyte-CD11b/c$^+$ cell co-cultures detected by ELISA for PGE$_2$ and TGF-β1. For results from supernatants unstimulated lymphocyte-CD11b/c$^+$ cell co cultures see Supplementary Figure 4. (One-way ANOVA n=3, Tukey's multiple comparison post-test, mean±SEM, *p≤0.05, **p≤0.01, ***p≤0.001).

References

1. Ge, W., Jiang, J., Arp, J., Liu, W., Garcia, B., and Wang, H. (2010) Regulatory T-cell generation and kidney allograft tolerance induced by mesenchymal stem cells associated with indoleamine 2,3-dioxygenase expression. *Transplantation* **90**, 1312-1320
2. Casiraghi, F., Azzollini, N., Todeschini, M., Cavinato, R. A., Cassis, P., Solini, S., Rota, C., Morigi, M., Introna, M., Maranta, R., Perico, N., Remuzzi, G., and Noris, M. (2012) Localization of Mesenchymal Stromal Cells Dictates Their Immune or Proinflammatory Effects in Kidney Transplantation. *American Journal of Transplantation* **12**, 2373-2383
3. Zhou, H. P., Yi, D. H., Yu, S. Q., Sun, G. C., Cui, Q., Zhu, H. L., Liu, J. C., Zhang, J. Z., and Wu, T. J. (2006) Administration of donor-derived mesenchymal stem cells can prolong the survival of rat cardiac allograft. *Transplantation proceedings* **38**, 3046-3051
4. Treacy, O., O'Flynn, L., Ryan, A. E., Morcos, M., Lohan, P., Schu, S., Wilk, M., Fahy, G., Griffin, M. D., Nosov, M., and Ritter, T. (2014) Mesenchymal Stem Cell Therapy Promotes Corneal Allograft Survival in Rats by Local and Systemic Immunomodulation. *American Journal of Transplantation* **14**, 2023-2036
5. Xia, X., Chen, W., Ma, T., Xu, G., Liu, H., Liang, C., Bai, X., Zhang, Y., He, Y., and Liang, T. (2012) Mesenchymal stem cells administered after liver transplantation prevent acute graft-versus-host disease in rats. *Liver transplantation : official publication of the American Association for the Study of Liver Diseases and the International Liver Transplantation Society* **18**, 696-706
6. Tian, W., Liu, Y., Zhang, B., Dai, X., Li, G., Li, X., Zhang, Z., Du, C., and Wang, H. (2015) Infusion of mesenchymal stem cells protects lung transplants from cold ischemia-reperfusion injury in mice. *Lung* **193**, 85-95
7. Murphy, N., Lynch, K., Lohan, P., Treacy, O., and Ritter, T. (2016) Mesenchymal stem cell therapy to promote corneal allograft survival: current status and pathway to clinical translation. *Current opinion in organ transplantation* **21**, 559-567
8. Ren, G., Zhang, L., Zhao, X., Xu, G., Zhang, Y., and Roberts, A. I. (2008) Mesenchymal stem cell-mediated immunosuppression occurs via concerted action of chemokines and nitric oxide. *Cell stem cell* **2**
9. Nemeth, K., Leelahavanichkul, A., Yuen, P. S. T., Mayer, B., Parmelee, A., Doi, K., Robey, P. G., Leelahavanichkul, K., Koller, B. H., Brown, J. M., Hu, X., Jelinek, I., Star, R. A., and Mezey, E. (2009) Bone marrow stromal cells attenuate sepsis via prostaglandin E2-dependent reprogramming of host macrophages to increase their interleukin-10 production. *Nature medicine* **15**, 42-49
10. Melief, S. M., Schrama, E., Brugman, M. H., Tiemessen, M. M., Hoogduijn, M. J., Fibbe, W. E., and Roelofs, H. (2013) Multipotent stromal cells induce human regulatory T cells through a novel pathway involving skewing of monocytes toward anti-inflammatory macrophages. *Stem cells (Dayton, Ohio)* **31**, 1980-1991
11. English, K., Ryan, J. M., Tobin, L., Murphy, M. J., Barry, F. P., and Mahon, B. P. (2009) Cell contact, prostaglandin E(2) and transforming growth factor beta 1 play non-redundant roles in human mesenchymal stem cell induction of CD4+CD25(High) forkhead box P3+ regulatory T cells. *Clinical and experimental immunology* **156**, 149-160
12. Prevosto, C., Zancolli, M., Canevali, P., Zocchi, M. R., and Poggi, A. (2007) Generation of CD4+ or CD8+ regulatory T cells upon mesenchymal stem cell-lymphocyte interaction. *Haematologica* **92**, 881-888

13. Luz-Crawford, P., Kurte, M., Bravo-Alegria, J., Contreras, R., Nova-Lamperti, E., Tejedor, G., Noel, D., Jorgensen, C., Figueroa, F., Djouad, F., and Carrion, F. (2013) Mesenchymal stem cells generate a CD4+CD25+Foxp3+ regulatory T cell population during the differentiation process of Th1 and Th17 cells. *Stem cell research & therapy* **4**, 65
14. Fan, L., Hu, C., Chen, J., Cen, P., Wang, J., and Li, L. (2016) Interaction between Mesenchymal Stem Cells and B-Cells. *International journal of molecular sciences* **17**, 650
15. Ko, J. H., Lee, H. J., Jeong, H. J., Kim, M. K., Wee, W. R., Yoon, S.-o., Choi, H., Prockop, D. J., and Oh, J. Y. (2016) Mesenchymal stem/stromal cells precondition lung monocytes/macrophages to produce tolerance against allo- and autoimmunity in the eye. *Proceedings of the National Academy of Sciences* **113**, 158-163
16. Oh, J. Y., Lee, R. H., Yu, J. M., Ko, J. H., Lee, H. J., Ko, A. Y., Roddy, G. W., and Prockop, D. J. (2012) Intravenous mesenchymal stem cells prevented rejection of allogeneic corneal transplants by aborting the early inflammatory response. *Molecular therapy : the journal of the American Society of Gene Therapy* **20**, 2143-2152
17. Eggenhofer, E., Benseler, V., Kroemer, A., Popp, F., Geissler, E., Schlitt, H., Baan, C., Dahlke, M., and Hoogduijn, M. (2012) Mesenchymal stem cells are short-lived and do not migrate beyond the lungs after intravenous infusion. *Frontiers in Immunology* **3**
18. Fischer, U. M., Harting, M. T., Jimenez, F., Monzon-Posadas, W. O., Xue, H., and Savitz, S. I. (2009) Pulmonary passage is a major obstacle for intravenous stem cell delivery: the pulmonary first-pass effect. *Stem Cells Dev* **18**
19. Lohan, P., Murphy, N., Treacy, O., Lynch, K., Morcos, M., Chen, B., Ryan, A. E., Griffin, M. D., and Ritter, T. (2018) Third-Party Allogeneic Mesenchymal Stromal Cells Prevent Rejection in a Pre-sensitized High-Risk Model of Corneal Transplantation. *Frontiers in Immunology* **9**
20. de Witte, S. F. H., Luk, F., Sierra Parraga, J. M., Gargesha, M., Merino, A., Korevaar, S. S., Shankar, A. S., O'Flynn, L., Elliman, S. J., Roy, D., Betjes, M. G. H., Newsome, P. N., Baan, C. C., and Hoogduijn, M. J. (2018) Immunomodulation By Therapeutic Mesenchymal Stromal Cells (MSC) Is Triggered Through Phagocytosis of MSC By Monocytic Cells. *Stem cells (Dayton, Ohio)* **36**, 602-615
21. Galleu, A., Riffo-Vasquez, Y., Trento, C., Lomas, C., Dolcetti, L., Cheung, T. S., von Bonin, M., Barbieri, L., Halai, K., Ward, S., Weng, L., Chakraverty, R., Lombardi, G., Watt, F. M., Orchard, K., Marks, D. I., Apperley, J., Bornhauser, M., Walczak, H., Bennett, C., and Dazzi, F. (2017) Apoptosis in mesenchymal stromal cells induces in vivo recipient-mediated immunomodulation. *Science translational medicine* **9**
22. Niederkorn, J. Y. (2013) Corneal Transplantation and Immune Privilege. *International reviews of immunology* **32**, 10.3109/08830185.08832012.08737877
23. Sangwan, V. S., Ramamurthy, B., Shah, U., Garg, P., Sridhar, M. S., and Rao, G. N. (2005) Outcome of corneal transplant rejection: a 10-year study. *Clinical & experimental ophthalmology* **33**, 623-627
24. Maguire, M. G., Stark, W. J., Gottsch, J. D., Stulting, R. D., Sugar, A., Fink, N. E., and Schwartz, A. (1994) Risk factors for corneal graft failure and rejection in the collaborative corneal transplantation studies. Collaborative Corneal Transplantation Studies Research Group. *Ophthalmology* **101**, 1536-1547
25. Waldock, A., and Cook, S. (2000) Corneal transplantation: how successful are we? *The British Journal of Ophthalmology* **84**, 813-815

26. Birnbaum, F., Bohringer, D., Sokolovska, Y., Sundmacher, R., and Reinhard, T. (2005) Immunosuppression with cyclosporine A and mycophenolate mofetil after penetrating high-risk keratoplasty: a retrospective study. *Transplantation* **79**, 964-968
27. Birnbaum, F., Mayweg, S., Reis, A., Bohringer, D., Seitz, B., Engelmann, K., Messmer, E. M., and Reinhard, T. (2009) Mycophenolate mofetil (MMF) following penetrating high-risk keratoplasty: long-term results of a prospective, randomised, multicentre study. *Eye (London, England)* **23**, 2063-2070
28. Sommerer, C., Schnitzler, P., Meuer, S., Zeier, M., and Giese, T. (2011) Pharmacodynamic monitoring of cyclosporin A reveals risk of opportunistic infections and malignancies in renal transplant recipients 65 years and older. *Therapeutic drug monitoring* **33**, 694-698
29. Durnian, J. M., Stewart, R. M. K., Tatham, R., Batterbury, M., and Kaye, S. B. (2007) Cyclosporin-A associated malignancy. *Clinical ophthalmology (Auckland, N.Z.)* **1**, 421-430
30. Krampera, M., Cosmi, L., Angeli, R., Pasini, A., Liotta, F., Andreini, A., Santarlasci, V., Mazzinghi, B., Pizzolo, G., Vinante, F., Romagnani, P., Maggi, E., Romagnani, S., and Annunziato, F. (2006) Role for interferon-gamma in the immunomodulatory activity of human bone marrow mesenchymal stem cells. *Stem cells (Dayton, Ohio)* **24**, 386-398
31. Polchert, D., Sobinsky, J., Douglas, G., Kidd, M., Moadsiri, A., Reina, E., Genrich, K., Mehrotra, S., Setty, S., Smith, B., and Bartholomew, A. (2008) IFN-gamma activation of mesenchymal stem cells for treatment and prevention of graft versus host disease. *Eur J Immunol* **38**, 1745-1755
32. Griffin, M. D., Ryan, A. E., Alagesan, S., Lohan, P., Treacy, O., and Ritter, T. (2013) Anti-donor immune responses elicited by allogeneic mesenchymal stem cells: what have we learned so far? *Immunology and cell biology* **91**, 40-51
33. Schu, S., Nosov, M., O'Flynn, L., Shaw, G., Treacy, O., Barry, F., Murphy, M., O'Brien, T., and Ritter, T. (2012) Immunogenicity of allogeneic mesenchymal stem cells. *Journal of cellular and molecular medicine* **16**, 2094-2103
34. Griffin, M. D., Ritter, T., and Mahon, B. P. (2010) Immunological aspects of allogeneic mesenchymal stem cell therapies. *Human gene therapy* **21**, 1641-1655
35. Ren, G., Zhang, L., Zhao, X., Xu, G., Zhang, Y., Roberts, A. I., Zhao, R. C., and Shi, Y. (2008) Mesenchymal Stem Cell-Mediated Immunosuppression Occurs via Concerted Action of Chemokines and Nitric Oxide. *Cell Stem Cell* **2**, 141-150
36. English, K., Barry, F. P., Field-Corbett, C. P., and Mahon, B. P. (2007) IFN-γ and TNF-α differentially regulate immunomodulation by murine mesenchymal stem cells. *Immunology letters* **110**, 91-100
37. Chen, H., Min, X. H., Wang, Q. Y., Leung, F. W., Shi, L., Zhou, Y., Yu, T., Wang, C. M., An, G., Sha, W. H., and Chen, Q. K. (2015) Pre-activation of mesenchymal stem cells with TNF-alpha, IL-1beta and nitric oxide enhances its paracrine effects on radiation-induced intestinal injury. *Scientific reports* **5**, 8718
38. Hegde, S., Beauregard, C., Mayhew, E., and Niederkorn, J. Y. (2005) CD4+ T-Cell–Mediated Mechanisms of Corneal Allograft Rejection: Role of Fas-Induced Apoptosis. *Transplantation* **79**, 23-31
39. Hegde, S., and Niederkorn, J. Y. (2000) The role of cytotoxic T lymphocytes in corneal allograft rejection. *Investigative ophthalmology & visual science* **41**, 3341-3347
40. Yamada, J., Kurimoto, I., and Streilein, J. W. (1999) Role of CD4+ T cells in immunobiology of orthotopic corneal transplants in mice. *Investigative ophthalmology & visual science* **40**, 2614-2621

41. He, Y. G., Ross, J., and Niederkorn, J. Y. (1991) Promotion of murine orthotopic corneal allograft survival by systemic administration of anti-CD4 monoclonal antibody. *Investigative ophthalmology & visual science* **32**, 2723-2728
42. Ayliffe, W., Alam, Y., Bell, E. B., McLeod, D., and Hutchinson, I. V. (1992) Prolongation of rat corneal graft survival by treatment with anti-CD4 monoclonal antibody. *The British Journal of Ophthalmology* **76**, 602-606
43. Niederkorn, J. Y., Stevens, C., Mellon, J., and Mayhew, E. (2006) CD4+ T-cell-independent rejection of corneal allografts. *Transplantation* **81**, 1171-1178
44. Sato, K., Ozaki, K., Oh, I., Meguro, A., Hatanaka, K., Nagai, T., Muroi, K., and Ozawa, K. (2007) Nitric oxide plays a critical role in suppression of T-cell proliferation by mesenchymal stem cells. *Blood* **109**, 228-234
45. Ren, G., Zhang, L., Zhao, X., Xu, G., Zhang, Y., Roberts, A. I., Zhao, R. C., and Shi, Y. (2008) Mesenchymal stem cell-mediated immunosuppression occurs via concerted action of chemokines and nitric oxide. *Cell stem cell* **2**, 141-150
46. Salvemini, D., Misko, T. P., Masferrer, J. L., Seibert, K., Currie, M. G., and Needleman, P. (1993) Nitric oxide activates cyclooxygenase enzymes. *Proceedings of the National Academy of Sciences of the United States of America* **90**, 7240-7244
47. Zinöcker, S., and Vaage, J. T. (2012) Rat Mesenchymal Stromal Cells Inhibit T Cell Proliferation but Not Cytokine Production Through Inducible Nitric Oxide Synthase. *Frontiers in Immunology* **3**, 62
48. Strickland, D., Kees, U., and Holt, P. (1994) Suppression of T-cell activation by pulmonary alveolar macrophages: dissociation of effects on TcR, IL-2R expression, and proliferation. *European Respiratory Journal* **7**, 2124-2130
49. Strickland, D., Kees, U. R., and Holt, P. G. (1996) Regulation of T-cell activation in the lung: alveolar macrophages induce reversible T-cell anergy in vitro associated with inhibition of interleukin-2 receptor signal transduction. *Immunology* **87**, 250-258
50. Agrawal, H., Shang, H., Sattah, A. P., Yang, N., Peirce, S. M., and Katz, A. J. (2014) Human adipose-derived stromal/stem cells demonstrate short-lived persistence after implantation in both an immunocompetent and an immunocompromised murine model. *Stem cell research & therapy* **5**, 142
51. Leibacher, J., Dauber, K., Ehser, S., Brixner, V., Kollar, K., Vogel, A., Spohn, G., Schafer, R., Seifried, E., and Henschler, R. (2017) Human mesenchymal stromal cells undergo apoptosis and fragmentation after intravenous application in immune-competent mice. *Cytotherapy* **19**, 61-74
52. Yamagami, S., and Dana, M. R. (2001) The critical role of lymph nodes in corneal alloimmunization and graft rejection. *Investigative ophthalmology & visual science* **42**, 1293-1298
53. Zangi, L., Margalit, R., Reich-Zeliger, S., Bachar-Lustig, E., Beilhack, A., Negrin, R., and Reisner, Y. (2009) Direct imaging of immune rejection and memory induction by allogeneic mesenchymal stromal cells. *Stem cells (Dayton, Ohio)* **27**, 2865-2874
54. Badillo, A. T., Beggs, K. J., Javazon, E. H., Tebbets, J. C., and Flake, A. W. (2007) Murine bone marrow stromal progenitor cells elicit an in vivo cellular and humoral alloimmune response. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation* **13**, 412-422
55. Nauta, A. J., Westerhuis, G., Kruisselbrink, A. B., Lurvink, E. G., Willemze, R., and Fibbe, W. E. (2006) Donor-derived mesenchymal stem cells are immunogenic in an allogeneic host and stimulate donor graft rejection in a nonmyeloablative setting. *Blood* **108**, 2114-2120

56. Le Blanc, K., Tammik, C., Rosendahl, K., Zetterberg, E., and Ringden, O. (2003) HLA expression and immunologic properties of differentiated and undifferentiated mesenchymal stem cells. *Experimental hematology* **31**, 890-896
57. Cho, P. S., Messina, D. J., Hirsh, E. L., Chi, N., Goldman, S. N., and Lo, D. P. (2008) Immunogenicity of umbilical cord tissue derived cells. *Blood* **111**
58. Rafei, M., Birman, E., Forner, K., and Galipeau, J. (2009) Allogeneic mesenchymal stem cells for treatment of experimental autoimmune encephalomyelitis. *Molecular therapy : the journal of the American Society of Gene Therapy* **17**, 1799-1803
59. Eggenhofer, E., and Hoogduijn, M. J. (2012) Mesenchymal stem cell-educated macrophages. *Transplantation Research* **1**, 12-12
60. Braza, F., Dirou, S., Forest, V., Sauzeau, V., Hassoun, D., Chesne, J., Cheminant-Muller, M. A., Sagan, C., Magnan, A., and Lemarchand, P. (2016) Mesenchymal Stem Cells Induce Suppressive Macrophages Through Phagocytosis in a Mouse Model of Asthma. *Stem cells (Dayton, Ohio)* **34**, 1836-1845
61. Cheung, T. S., and Dazzi, F. (2018) Mesenchymal-myeloid interaction in the regulation of immunity. *Seminars in immunology* **35**, 59-68
62. Niederkorn, J. Y. (2007) Immune Mechanisms of Corneal Allograft Rejection. *Current eye research* **32**, 1005-1016