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

4
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26 **Running title:** TNF- α /IL-1 β licensed MSCs promote corneal allograft survival
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34 **List of abbreviations**

- 35 7-AAD – 7-Aminoactinomycin D
36 ACK – Ammonium-chloride-potassium
37 Allo – Allogeneic
38 Auto – Autologous
39 BSS – Balanced salt solution
40 CD – Cluster of differentiation
41 CFSE – Carboxyfluorescein succinimidyl ester
42 COX-2 – Cyclooxygenase-2
43 DA – Dark agouti
44 dLN – Draining lymph nodes
45 DTH – Delayed-type hypersensitivity
46 FBS – Foetal bovine serum
47 Foxp3 – Forkhead box P3
48 GAPDH – Glyceraldehyde 3-phosphate dehydrogenase
49 GvHD – Graft versus host disease
50 HBSS – Hank’s balanced salt solution
51 HLA – Human leukocyte antigen
52 IDO – Indoleamine 2,3-dioxygenase
53 IFN- γ – Interferon –gamma
54 IL-1 β – Interleukin-1 beta
55 iNOS – inducible nitric oxide synthase
56 Lew – Lewis
57 MACS – Magnetic-activated cell sorting
58 MHC – Major histocompatibility complex
59 MSCs – Mesenchymal stromal cells
60 NO – Nitric oxide
61 PBMC – Peripheral blood mononuclear cells
62 PGE2 – Prostaglandin E2
63 POD – Post-operative day
64 PTGS2 – Prostaglandin-endoperoxide synthase 2
65 RPMI – Roswell Park Memorial Institute
66 SMT – S-methylisothiourea
67 TCR – T cell receptor
68 TGF- β 1 – Transforming growth factor-beta 1
69 TNF- α – Tumor necrosis factor-alpha
70 TOR – Time of rejection
71 T-regs – Regulatory T cells
72 TSG-6 – Tumor necrosis factor-inducible gene 6
73 UTR – Untreated
74 α MEM – Minimum essential medium – Alpha modification
75

76 **Abstract**

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

95

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

98

99

100 **Introduction**

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

117 Currently our group and others are investigating the immunomodulatory properties of MSCs
118 for the prevention of immune mediated rejection in cornea transplantation (keratoplasty) (4,
119 15, 16, 19). Owing to the immune privileged nature of the eye, keratoplasty is typically
120 performed without human leukocyte antigen (HLA) tissue matching or the indefinite use of
121 immunosuppressive therapy and yet the survival rate within the first year is over 90% (22).
122 However, the ten year prognosis is much poorer due to increased rates of rejection in “high
123 risk” patients. The risk of graft failure in these patients is heightened due to an increased risk
124 of immune-mediated rejection (23-25). For these patients systemic immunosuppressants are
125 potentially efficacious in prolonging high risk corneal allograft survival but with the caveat
126 that they trigger considerable side effects including nephrotoxicity, hepatotoxicity, leucopenia,
127 gastrointestinal disorders and increased risk of malignancies and infections (26-29). As
128 corneal blindness is not a life-threatening disorder, the long-term side effects of
129 immunosuppressant therapies may outweigh the benefits of prolonged graft survival in some
130 high risk patients. Therefore, there is a significant need to develop novel, safe
131 immunomodulatory therapies to alleviate immune-mediated rejection episodes in corneal
132 allograft patients.

133 Previous results from our laboratory have demonstrated the efficacy of pre-transplant
134 infusion of allogeneic (allo) but not autologous (auto) MSC in a fully allogeneic, MHC-
135 mismatched rat model of cornea transplantation (4). MSCs acquire their immunomodulatory
136 properties upon exposure to inflammatory stimuli such as IFN- γ , TNF- α and IL-1 β (8, 30).
137 Ren et al, demonstrated that blocking the inflammatory stimulus by utilising IFN- γ receptor
138 deficient MSCs prevented MSC mediated T cell suppression and abolished MSCs ability to
139 prevent graft versus host disease (GvHD) in mice (8). Similarly, in another model of GvHD,
140 recipients of IFN- γ ^{-/-} donor T cells did not respond to MSC therapy however the
141 immunomodulatory function of the MSC could be restored by pre-treatment with IFN- γ (31).
142 This suggests that in our previous work the expression of allo-antigen by allo-MSCs provides
143 a priming stimulus that enhances their immune modulatory effects while auto-MSCs
144 administered to an immunologically compatible non-inflamed (pre-transplantation) host did
145 not receive the required priming stimulus (4). As increasingly, evidence suggests that allo-
146 MSCs trigger an allo-specific immune mediated cellular and humoral response which may be
147 deleterious to the long term outcome of the graft this study investigated whether auto-MSC
148 therapy could be enhanced to promote rejection free graft survival. (32-34).
149 We hypothesised that pre-emptive priming (“licensing”) of auto-MSC could overcome the
150 lack of efficacy in preventing cornea transplantation rejection by better promoting regulatory
151 innate and adaptive immune cells. We demonstrate that auto-MSCs administered post-
152 operatively promote rejection free graft survival and licensing with a combination of TNF- α
153 and IL-1 β (MSC^{TNF- α /IL-1 β}) further enhances their immunomodulatory capacity. Auto-MSC
154 therapy is associated with increased Foxp3⁺ T-regs in the lungs post-infusion and in the
155 draining lymph node at the estimated time of rejection and this immunomodulatory effect can
156 be enhanced by TNF- α /IL-1 β licensing. Finally, we demonstrate that MSC-mediated
157 induction of Foxp3⁺ regulatory T cells (Tregs) was dependent upon a lung-derived myeloid
158 cell intermediary. This work contributes to the growing body of literature highlighting the
159 importance of lung myeloid cells in mediating MSCs immunomodulatory function.
160

161 **Materials and Methods**

162 **Animals and corneal transplantation**

163 All procedures performed on animals were approved by the Animals Care Research Ethics
164 Committee of the National University of Ireland (Galway, Ireland) and conducted under
165 license from the Health Product Regulatory Authority (HPRA) of Ireland. All animals were
166 housed and cared for under Standard Operating Procedures of the Animal Facility at the
167 National Centre for Biomedical Engineering Science, NUI Galway. A well established, fully
168 allogeneic major histocompatibility complex (MHC) class I/II disparate cornea transplant
169 model was used for these studies. Male Dark Agouti (DA, RT-1^{av1}) rats served as graft donors
170 and Lewis (Lew, RT-1^l) rats served as recipients. All animals were aged between 8-14 weeks
171 old and obtained from Harlan Laboratories (Bicester, UK) and housed with food and water ad
172 lib. Orthotopic corneal transplantation was performed as reported previously [1-4]. Briefly,
173 isoflurane was systemically administered at a concentration of 2-2.5% in medical oxygen
174 (BOC, Galway, Ireland) with a flow rate of 2l/minute. 1% Tetracaine (Chauvin
175 Pharmaceuticals, Kingston upon Thames, UK) was administered as a local anesthesia and
176 atropine 1%, tropicamide 1% and phenylephrine 1% (all Chauvin Pharmaceuticals) were
177 administered for iris dilation. A 2.5mm graft bed was prepared and a 3mm donor graft was
178 sutured in place with 8-10 interrupted 10-0 Ethilon sutures (Ethicon, Livingston, Scotland).
179 Antibiotic ointment containing chloramphenicol was applied to the graft. To irrigate the
180 corneal tissue Alcon BSS (Alcon, Hemel Hempstead, UK) was applied to the graft. Graft
181 transparency as an indicator of rejection was scored every 2-3 days using an operating
182 microscope at 25x magnification. The grading score was as follows: 0 – completely
183 transparent cornea; 0.5 – slight corneal opacity; 1 – slight corneal opacity; 1.5 – modest
184 corneal opacity, vessels still visible; 2.0 – moderate opacity, few iris details visible; 2.5 –
185 high corneal opacity; only pupil margin visible; 3.0 – complete corneal opacity; anterior
186 chamber not visible. A graft was considered to be rejected when an opacity score of 2.5 was
187 recorded on two consecutive days or one score of 3.0. Animals with surgical complications
188 were excluded.

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

190 MSCs were isolated from the bone marrow of Lewis Rats (8-12 weeks old). Animals were
191 euthanized by CO₂ inhalation and the femurs and tibias removed. The bone marrow was
192 flushed from the bones in rat MSC medium (10 % fetal bovine serum (FBS) (Sigma-Aldrich,
193 Missouri, US) in equal parts F-12 nutrient mixture and α MEM (both Gibco/Thermo Fisher

194 Scientific)) to isolate bone marrow progenitors. Cells were pelleted by centrifugation at 400 x
195 g for 5 mins and washed with Dulbecco's Phosphate Buffered Saline (DPBS) (Gibco/Thermo
196 Fisher Scientific, Massachusetts, US). Cells were re-suspended in rat MSC medium and
197 seeded at a density of 9×10^5 cells/cm² in T-175 flasks (NUNC, Thermo Fisher Scientific)
198 with rat MSC medium. Cells were incubated at 37°C, 5% CO₂ and 90% humidity. Medium
199 was changed every 3-4 days and cells were passaged at 85% confluency. MSCs were cultured
200 and used in subsequent experiments up to passage 6 (P6). The cells consistently differentiated
201 to osteogenic and adipogenic lineages in culture. In addition, the cells positively expressed
202 characteristic MSC markers CD29, CD73 and CD90, expressed low levels of MHC I and
203 were negative for CD45, CD80, CD86 and MHC II (Supplementary Figure S1).

204 **Pro-inflammatory cytokine licensing of LEW MSCs**

205 LEW MSCs were seeded at a density of 7.5×10^3 cells/cm³ in a T175 flask or 6 well plate
206 (Sarstedt, Germany) in rat MSC medium and incubated at 37°C, 5% CO₂ and 90% humidity
207 for 12 hours. Medium was removed and replaced with rat MSC medium containing
208 recombinant rat pro-inflammatory cytokines IFN- γ (50ng/ml), TNF- α (25ng/ml) and/or IL-1 β
209 (50ng/ml) (Peprotech, UK) and cells were placed in a humidified tissue culture incubator at
210 37°C, 5% CO₂ for 72 hours. Medium was removed, cells were washed with DPBS, 5ml/T175
211 of 0.25% trypsin (Sigma-Aldrich) was added and cells were incubated for 3 minutes at 37°C,
212 5% CO₂. Trypsin was neutralised by adding twice the volume of serum-containing media.
213 Cells were then centrifuged at 400 x g, washed with DPBS (x2) and counted using a
214 haemocytometer.

215 **Intravenous administration of MSCs**

216 MSCs, cultured as described above in the presence or absence of pro-inflammatory rat
217 cytokines, were washed in DPBS (x3) and filtered through a 40 μ M filter before
218 administration. Rats were anaesthetized by brief inhalation of isoflurane and MSCs (1×10^6
219 cells in 1ml PBS) were injected i.v. through the lateral tail vein using a 25G needle.

220 **Cell isolation and flow cytometry**

221 The lung was digested by mincing lung tissue into small pieces, followed by digestion by
222 incubating in Hank's Balanced Salt Solution (HBSS) (Gibco/Thermo Fisher Scientific)
223 containing collagenase IV (200U/ml) (Gibco/Thermo Fisher Scientific) and DNase I
224 (200U/ml) (Sigma-Aldrich) at 37°C with shaking (150 rpm) for 2 hours. Single-cell
225 suspensions of the lymph node, spleen and digested lung were prepared by gentle mashing of

226 the organs through a 40 μ M cell strainer (Thermo Fisher Scientific) in a petri dish containing
227 10mls DPBS. The homogenates were then centrifuged at 800 x g for 5 minutes. The lymph
228 node cells were washed in DPBS and counted using a haemocytometer. The spleen and lung
229 cells were re-suspended in ACK lysis buffer (distilled water, 0.15M NH₄CL, 10mM KHCO₃,
230 Sodium EDTA 0.1mM) and incubated on ice for 5 minutes. The reaction was stopped with
231 serum-containing medium. Cells were centrifuged at 800 x g for 5 minutes, washed and re-
232 suspended in DPBS and counted.

233 For flow cytometry, 1x10⁵ cells/sample were stained with the following anti-rat antibodies:
234 CD4, CD8, CD25, CD45, CD11b/c, MHC II (all Biolegend, California, USA) and with the
235 dead-cell exclusion dye 7-AAD (Thermo Fisher Scientific). For Foxp3 staining, cells were
236 fixed and permeabilised with Foxp3/Transcription Factor Staining Buffer Kit
237 (eBioscience/Thermo Fisher Scientific) as per the manufacturer's instructions and then
238 stained with anti-rat Foxp3 (Thermo Fisher Scientific). Samples were analysed using a BD
239 FACSCanto II Flow Cytometer (BD BioSciences, California, USA). Flow cytometry data
240 was analysed using FlowJo analysis software version 10 (Tree Star Inc., Ashland, OR, USA).

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

246 **RNA isolation and RT-PCR**

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

257 ***In vitro* co-culture assays**

258 For lymphocyte proliferation assays, lymph nodes were isolated from LEW rats and single
259 cell suspensions of lymphocytes were prepared as described above. Cells were re-suspended
260 in T cell medium (RPMI-1640 (Gibco/Thermo Fisher Scientific) supplemented with 10%
261 heat-inactivated FBS (Sigma-Aldrich), 2mmol/l L-glutamine, 100U/ml penicillin, 100µg/ml
262 streptomycin (all Life Technologies/Thermo Fisher Scientific), 0.1 mmol/l non-essential
263 amino acids, 1 mmol/l sodium pyruvate and 55µmol/l 2β-mercaptoethanol (all Sigma-
264 Aldrich)). The cells were labelled with CellTrace Violet Cell Proliferation Kit
265 (Invitrogen/Thermo Fisher Scientific) according to the manufacturer's instructions and
266 seeded in a 96 well plate at a concentration of 2×10^5 cells/100µl of T cell medium with or
267 without anti-rat anti-CD3/CD28 Dynabeads (Thermo Fisher Scientific) at a ratio of 1:1. In
268 various experiments, untreated or cytokine-stimulated MSCs were added to wells of
269 lymphocytes at a concentration of 2×10^4 cells/50µl (ratio of 1:10 lymphocyte:MSC) of rat
270 MSC medium. To inhibit nitric oxide (NO) production, S-methylisothiourea (SMT) was
271 added to the cultures at a final concentration of 100 µM. Co-cultures were incubated at 37°C,
272 5% CO₂ and 90% humidity for 96 hours followed by antibody staining and flow cytometric
273 analysis.

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

288 **Quantification of cytokines, prostaglandin E2 (PGE2) and NO**

289 Culture supernatants were analysed for the presence of PGE2 and transforming growth factor
290 beta 1 (TGF- β 1) by ELISA (R&D Systems,UK) following the manufacturer's guidelines.
291 IFN- γ , TNF- α and IL-2 concentrations were quantified as part of the Th Complete 14-Plex
292 Rat ProcartaPlex Panel (Thermo Fisher Scientific) and quantified by Bio-Plex 200 system
293 (Bio-Rad, California, USA). NO concentration in culture supernatants was quantified by
294 Griess assay. 100 μ l of supernatant were combined with an equal volume of Griess reagent
295 (1% sulphanilamide and 0.1% N-1-(naphthyl)-ethylenediamine-diHCl in 2.5% H3PO4) in a
296 96-well flat bottom plate and absorbance measured immediately at 540nm on a plate reader
297 (Perkin Elmer, Ireland). A sodium nitrite standard curve was used to calculate NO
298 concentration.

299 **Statistical Analysis**

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

307

308 **Results**

309 **IL-1 β in combination with TNF- α or IFN- γ stimulates a potent immunosuppressive** 310 **MSC phenotype**

311 In this study we aimed to enhance the immune modulatory properties of auto-MSCs by
312 “licensing” with pro-inflammatory cytokines. Based on the literature, IFN- γ , TNF- α and IL-
313 1 β were selected to license the MSCs for 72 hours (35-37). To test their immunomodulatory
314 function, cytokine-licensed LEW MSCs were cultured with anti-CD3/CD28 bead-stimulated
315 LEW lymphocytes for a period of 96 hours. We observed significant suppression of overall
316 lymphocyte proliferation when MSCs were licensed with IL-1 β in combination with either
317 IFN- γ (MSC^{IFN- γ /IL-1 β}) or TNF- α (MSC^{TNF- α /IL-1 β}) (Figure 1A and B) compared to lymphocytes
318 stimulated in the absence of MSC. Analysis of lymphocyte proliferation beyond 3
319 generations demonstrated that MSCs licensed with IL-1 β alone (MSC^{IL-1 β}) suppressed
320 lymphocyte proliferation (Figure 1 A and C) in addition to MSC^{IFN- γ /IL-1 β} and MSC^{TNF- α /IL-1 β}
321 (Figure 1A and C). Untreated MSCs (MSC^{UTR}) or MSCs licensed with IFN- γ alone (MSC^{IFN- γ}
322 γ), TNF- α alone (MSC^{TNF- α}) or IFN- γ and TNF- α combined (MSC^{IFN- γ /TNF- α}) did not
323 significantly suppress lymphocyte proliferation (Figure 1 A-C). In the context of corneal
324 allograft transplantation, rejection is mediated primarily by CD4⁺ T cells and associated
325 delayed type hypersensitivity (DTH) responses (38-43). To test whether licensed MSCs
326 preferentially inhibited CD4⁺ T cells, the proliferation of the CD4⁺ and CD8⁺ T cell subsets
327 was separately assessed in the same experiments. As shown in Figures 1D and 1E, both
328 MSC^{IFN- γ /IL-1 β} and MSC^{TNF- α /IL-1 β} significantly inhibited proliferation of CD4⁺ and CD8⁺ T
329 cells, demonstrating that the licensed MSCs similarly suppress both subsets. In summary,
330 these results demonstrate LEW MSCs acquire a potent capacity to inhibit syngeneic
331 lymphocyte proliferation when licensed with the pro-inflammatory cytokine IL-1 β in
332 combination with IFN- γ or TNF- α .

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

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

341 lymphocyte co-cultures, detectable levels of NO were observed in all wells in which MSCs
342 were present, indicating that the interaction of MSCs and lymphocytes stimulates the
343 production of NO (Figure 2B). Compared to stimulated lymphocytes alone, significant
344 increases in the concentration of NO was observed in co-culture wells containing MSC^{IL-1 β}
345 (12.68 \pm 7 μ M, p \leq 0.05), MSC^{IFN- γ /IL-1 β} (15.58 \pm 6.37 μ M, p \leq 0.01) or MSC^{TNF- α /IL-1 β}
346 (18.82 \pm 4.58 μ M, p \leq 0.01), while MSC^{TNF- α /IL-1 β} also increased the NO concentration compared
347 to MSC^{UTR} (5.04 \pm 2.26 μ M, p \leq 0.05) (Figure 2B).

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

352 Phenotypic comparison of MSC^{TNF- α /IL-1 β} and MSC^{UTR} showed that they expressed
353 comparable surface levels of characteristic MSC markers CD90 and CD29 while MSC<sup>TNF- α /IL-
354 1 β</sup> had higher surface expression of CD73 and CD44 as well as low-level MHC I, not present
355 on MSC^{UTR}. Neither MSC type expressed MHC II, CD45 or CD86 (Supplementary Figure
356 S1).

357 **MSC^{TNF- α /IL-1 β} suppression of syngeneic lymphocytes is primarily mediated by nitric** 358 **oxide**

359 To confirm the role of NO as the primary mediator of licensed MSCs' immunosuppressive
360 capacity, MSCs were co-cultured with stimulated lymphocytes in the presence of S-
361 methylisothiourrea (SMT), a preferential iNOS inhibitor. As predicted, total lymphocyte
362 proliferation was not inhibited by MSC^{UTR} either in the absence or presence of SMT (Figure
363 3A and B). In contrast, the ability of MSC^{TNF- α /IL-1 β} to inhibit lymphocyte proliferation
364 (1.29% \pm 1.011, p \leq 0.001) compared to stimulated controls (92.57% \pm 3.5) was abolished by the
365 addition of SMT (88.55% \pm 0.78) (Figure 3A and B). Analysis of the co-culture supernatants
366 confirmed that NO was successfully inhibited by addition of SMT (Figure 3C). NO is
367 reported to increase the enzymatic activity of cyclooxygenase-2 (COX-2) resulting in
368 increased production of the immune-regulatory factor PGE₂ (46). In keeping with this, PGE₂
369 was detected in co-cultures in the presence of MSC^{UTR} (61.92 \pm 20.76ng/ml), further increased
370 in the presence of MSC^{TNF- α /IL-1 β} (126.81 \pm 9.94ng/ml, p \leq 0.001) and was reduced to
371 concentrations comparable to MSC^{UTR} in the presence of SMT (55.78 \pm 19.76ng/ml, p \leq 0.001)
372 (Figure 3D).

373 While both MSC^{UTR} and MSC^{TNF- α /IL-1 β} inhibited the production of IFN- γ and TNF- α in co-

374 culture with syngeneic lymphocytes, the addition of SMT did not abolish this effect -
375 indicating that, in keeping with previous reports, the ability of MSCs to inhibit lymphocyte
376 secretion of pro-inflammatory cytokines is independent of NO (Figure 3E, F) (47). Increased
377 levels of the T cell growth factor IL-2 were detected in the presence of MSC^{TNF- α /IL-1 β}
378 compared to stimulated lymphocytes alone and this was reversed by the addition of SMT
379 (Figure 3C). This result is in keeping with a mechanism of action of NO by T cells
380 undergoing the normal initial activation sequence in response to stimulation (calcium flux, T
381 cell receptor (TCR) modulation and secretion of IL-2) but subsequently fail to respond to IL-
382 2 resulting in its accumulation in the culture medium (48, 49).
383 In summary, our findings indicate that rat MSC^{TNF- α /IL-1 β} suppression of syngeneic T-cell
384 proliferation and IL-2 consumption as well as increased PGE₂ production are dependent on
385 NO, while MSC-mediated lymphocyte polarisation is NO-independent.

386 **MSC^{TNF- α /IL-1 β} significantly prolong corneal allograft survival in a fully allogeneic rat** 387 **model**

388 Our group has previously demonstrated that syngeneic MSCs failed to prolong allograft
389 survival when administered pre-transplantation (4). Based on this finding, in this study, we
390 used a post-transplant administration strategy consisting of i.v. administration of 1×10^6
391 syngeneic MSC^{UTR} or MSC^{TNF- α /IL-1 β} on post-operative day (POD) 1 and POD 7 in a fully
392 MHC-mismatched rat corneal transplant model. Increased rejection-free corneal allograft
393 survival was observed in LEW rats that received syngeneic MSC^{UTR} (50%, $p \leq 0.05$) as well as
394 MSC^{TNF- α /IL-1 β} (70%, $p \leq 0.001$) compared to allograft controls (0%) (Figure 4A). Although not
395 statistically significant, a trend toward superior rejection-free survival was observed in the
396 MSC^{TNF- α /IL-1 β} -treated compared to MSC^{UTR}-treated group (Figure 4A). Trends toward
397 decreased graft opacity were observed in both MSC^{UTR}-treated and MSC^{TNF- α /IL-1 β} -treated
398 animals compared to untreated controls (Figure 4B and E), while there was also a trend
399 toward increased neovascularisation observed in MSC^{TNF- α /IL-1 β} -treated animals compared to
400 the other two groups (Figure 4C). The maximum opacity score recorded for each animal was
401 significantly reduced in both the MSC^{UTR}-treated (2.38 ± 0.23 , $p \leq 0.01$) and MSC^{TNF- α /IL-1 β} -
402 treated (2.35 ± 0.41 , $p \leq 0.01$) groups compared to untreated controls (2.9 ± 0.21) (Figure 4D).
403 In summary, these results demonstrate that the post-transplant administration of syngeneic
404 MSCs prolongs fully MHC-mismatched corneal allograft survival with a trend toward
405 superior rejection-free survival following administration of MSC^{TNF- α /IL-1 β} .

406 **MSC^{TNF- α /IL-1 β} therapy enriches myeloid cells in the lung and Foxp3⁺ T-reg proportions**
407 **in the lung and spleen**

408 It has become increasingly clear that the majority of MSCs administered i.v. become trapped
409 in the lung and are cleared within 24 hours (17, 18, 50, 51). Therefore, it is likely that MSCs
410 exert their immunomodulatory effect at this site before being cleared. In flow cytometry
411 analyses carried out on tissues dissected at POD 9, we observed proportionate increases in
412 CD11b/c⁺MHCII⁺ expressing CD45⁺ cells (Figure 5A for gating strategy) in the lungs of
413 MSC^{UTR}-treated allograft recipients (7.67% \pm 1.43, $p\leq 0.05$) compared to untreated controls
414 (3.79% \pm 1.25) with a further increase in the MSC^{TNF- α /IL-1 β} -treated group in the lung
415 (11.29% \pm 3.57, $p\leq 0.001$ and $p\leq 0.05$ compared to untreated and MSC^{UTR}-treated respectively)
416 (Figure 5B) and in the spleen (Supplementary Figure S3). To investigate their
417 immunomodulatory phenotype, lung CD11b/c⁺ cells were enriched from the lungs of all three
418 groups at POD 9 and were analysed by RT-PCR. In this analysis, trends toward increased
419 mRNA expression for the immunomodulatory genes *Ptgs2*, *Il-10* and indoleamine 2,3
420 dioxygenase (*Ido*) as well as a significant decrease in *Tnf- α* mRNA were observed in lung
421 CD11b/c⁺ cells from MSC^{TNF- α /IL-1 β} -treated animals (Figure 5C).

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

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

437 **MSC^{TNF- α /IL-1 β} increase Foxp3⁺ Tregs and decrease pro-inflammatory cytokines in the**
438 **lung, spleen and draining lymph node at the expected time of corneal allograft rejection**

439 To analyse the immune response at the expected time of rejection (TOR), the proportions of
440 Foxp3⁺ T-regs and the mRNA expression of pro-inflammatory cytokines *Ifn- γ* , *Tnf- α* and *Il-*
441 *1 β* were analysed in lungs, spleens and dLNs of control and MSC-treated corneal allograft
442 recipients at POD 17-19.

443 As shown in Figure 6A, increased proportions of T-regs remained present in the lungs of
444 MSC^{TNF- α /IL-1 β} -treated animals at the TOR (5.22% \pm 0.2 compared to 3.43% \pm 0.24 for
445 untreated controls, $p \leq 0.001$) but not in MSC^{UTR}-treated animals (3.23% \pm 0.37). *Ifn- γ* mRNA
446 expression was not detected in the lungs of control or MSC treated animals. MSC^{TNF- α /IL-1 β}
447 but not MSC^{UTR} were associated with reduced *Il-1 β* mRNA expression in the lungs while
448 lung *Tnf- α* mRNA expression was reduced in both MSC-treated groups compared to allograft
449 controls (Figure 6B). Similarly, in the spleen, increased proportions of T-regs were observed
450 in the MSC^{TNF- α /IL-1 β} -treated animals (5.85% \pm 0.86) compared to untreated controls (4.42 \pm
451 0.56, $p \leq 0.01$) and MSC^{UTR}-treated animals (4.58% \pm 0.76, $p \leq 0.01$) (Figure 6C). Both
452 MSC^{UTR} and MSC^{TNF- α /IL-1 β} treatments were associated with reduced *Ifn- γ* , *Il-1 β* and *Tnf- α*
453 expression in the spleen compared to untreated controls with spleen *Ifn- γ* expression lower in
454 MSC^{TNF- α /IL-1 β} -treated compared to the MSC^{UTR}-treated group (Figure 6D). In contrast to
455 findings at POD 9, analyses of cells from the dLNs indicated increased proportions of T-regs
456 in both MSC^{UTR}-treated (4.05% \pm 0.79, $p \leq 0.05$) and MSC^{TNF- α /IL-1 β} -treated (4.55% \pm 0.33,
457 $p \leq 0.01$) control animals (2.79% \pm 0.57) (Figure 6E). This finding was accompanied by lower
458 mRNA expression of *Ifn- γ* and *Tnf- α* in both MSC treatment groups (Figure 6F).

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

465 **MSCs indirectly promote Foxp3⁺ regulatory T cells via a myeloid cell intermediary**

466 To investigate the mechanism underlying MSC-mediated promotion of increased Foxp3⁺ T-
467 regs in corneal allograft recipients, the ability of MSCs to directly enrich Foxp3⁺ Tregs *in*
468 *vitro* in direct co-culture with syngeneic unstimulated lymphocytes was determined (Figure
469 7A and B). MSC^{UTR} did not alter the percentage of CD25⁺Foxp3⁺ cells among CD4⁺ T cells

470 compared to unstimulated lymphocyte controls while $MSC^{TNF-\alpha/IL-1\beta}$ significantly reduced the
471 percentage of $CD25^+Foxp3^+$ cells - indicating that syngeneic MSCs do not directly induce
472 $Foxp3^+$ T-regs from unstimulated lymphocytes ($p \leq 0.05$) (Figure 7A and B).
473 As we had observed increased proportions of $CD11b/c^+MHCII^+$ myeloid cells and T-regs in
474 the lungs at the same time-point *in vivo*, we next investigated whether MSCs induced T-regs
475 via a myeloid cell intermediary. As outlined in Figure 7C, this was tested by culturing
476 MSC^{UTR} and $MSC^{TNF-\alpha/IL-1\beta}$ with syngeneic $CD11b/c^+$ myeloid cells sorted from the lungs of
477 naïve LEW rats, then subsequently culturing the MSC-conditioned myeloid cells with either:
478 a) stimulated LEW lymphocytes to investigate the myeloid cell's suppressive capacity
479 (Figure 7F and G); or b) unstimulated LEW lymphocytes to investigate their capacity to
480 enrich $Foxp3^+$ T-regs (Figure 7H and I). In culture supernatants from the primary myeloid
481 cell-MSc co cultures, concentrations of PGE_2 and $TGF-\beta 1$ were higher in the presence of
482 either MSC^{UTR} or $MSC^{TNF-\alpha/IL-1\beta}$ compared to untreated myeloid cells, while increased
483 concentrations of NO were observed in the presence of $MSC^{TNF-\alpha/IL-1\beta}$ only (Figure 7D and E,
484 Supplementary Figure S4). In the subsequent myeloid cell-lymphocyte co-cultures, we
485 observed potent inhibition of $CD4^+$ lymphocyte proliferation in the presence of myeloid cells
486 conditioned with MSC^{UTR} or $MSC^{TNF-\alpha/IL-1\beta}$ but not in the presence of unconditioned myeloid
487 cells (Figure 7F and G). In addition to suppressing T cell proliferation, myeloid cells
488 conditioned with MSC^{UTR} or $MSC^{TNF-\alpha/IL-1\beta}$ were also associated with increased proportions
489 of $CD4^+CD25^+Foxp3^+$ T-regs compared to unconditioned $CD11b/c^+$ myeloid cells when co-
490 cultured with unstimulated syngeneic lymphocytes ($p \leq 0.05$ and $p \leq 0.01$, respectively) (Figure
491 7H and I).
492 No increase in the concentration of NO was observed in the supernatants from co-cultures of
493 $CD11b/c^+$ myeloid cells and lymphocytes (Supplementary Figure S4). In the unstimulated co-
494 cultures of MSC-conditioned myeloid cells and lymphocytes, we observed that
495 concentrations of PGE_2 and $TGF-\beta 1$ were differentially affected by the licensing status of the
496 MSCs. Specifically, $MSC^{TNF-\alpha/IL-1\beta}$ -conditioned myeloid cells were associated with higher
497 PGE_2 and lower $TGF-\beta 1$ compared to MSC^{UTR} -conditioned myeloid cells (Figure 7J and K).
498 A similar trend was observed in co-cultures with stimulated lymphocytes (Supplementary
499 Figure S5).
500 In summary, we demonstrate that both MSC^{UTR} and $MSC^{TNF-\alpha/IL-1\beta}$ condition lung-derived
501 myeloid cells towards an immune regulatory phenotype capable of suppressing syngeneic
502 lymphocyte proliferation and inducing $CD4^+CD25^+Foxp3^+$ T-regs from naïve unstimulated
503 lymphocytes. Potent induction of PGE_2 and $TGF-\beta 1$ may represent an important feature of

504 MSC-reprogrammed lung myeloid cells and these known immunomodulatory mediators may
505 also be differentially induced by resting and cytokine-licensed MSC.
506

507 **Discussion**

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

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

531 Therefore, while licensing may prove to be a beneficial strategy to enhance auto-MSC
532 therapy it may have an adverse effect in an allogeneic setting.

533 Recent evidence has highlighted the importance of MSC-mediated immunomodulation in the
534 lung for their subsequent systemic anti-inflammatory effects following i.v. administration (9,
535 15, 19). Despite the majority of i.v. administered MSCs being cleared from the lung within
536 the first 24 hours, our group and others have demonstrated that their immunological imprint
537 persists for longer due to either direct or indirect modulation of lung myeloid cells to an
538 immune regulatory phenotype (9, 15-17, 19).

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

541 in the promotion of an immune regulatory myeloid cell phenotype has been described (20, 21,
542 60). Braza and colleagues demonstrated that lung macrophages that had phagocytosed
543 PKH26 labelled MSCs displayed an M2 phenotype not observed in PKH26⁻ macrophages
544 (60). Galleu et al, reported that cytotoxic T cell-induced MSC apoptosis in the lung was
545 required to induce a regulatory phenotype in the engulfing phagocyte (21). De Witte and
546 colleagues recently reported that the majority of i.v.-delivered MSCs were not viable by 24
547 hours post infusion and were detected as phagocytosed particles of monocytes (20). Our
548 results further highlight the potential importance of lung myeloid cells in the transfer of
549 MSCs immunomodulatory effect. We also demonstrate that licensing of auto-MSCs enhances
550 their capacity to modulate the myeloid cells. This effect is likely mediated through cross-talk
551 via the release of soluble mediators such as NO, PGE₂, and TGF- β which create a local anti-
552 inflammatory environment and, potentially, pre-condition the myeloid cell before further
553 reprogramming occurs upon phagocytosis (61).

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

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

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

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

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

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603 **Conflict of Interest Statement**

604 The authors declare no conflict of interest

606 **Authors and Contributors**

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

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627 **Figure Legends**

628 **Figure 1. IL-1 β in combination with TNF α or IFN γ induces a potently**

629 **immunosuppressive phenotype in Lewis rat MSCs.** Lewis rat MSCs were stimulated with
630 IFN- γ , TNF- α and/or IL-1 β (500U/ml) for 72 hours in monolayer culture. Licensed Lewis rat
631 MSCs were then co-cultured with anti-CD3/CD28 bead stimulated Lewis rat lymphocytes for
632 96 hours. (A) Representative examples of CFSE profiles of lymphocytes (grey) following co-
633 culture with licensed MSCs compared to control stimulated lymphocytes alone (black dashed
634 line). (B) Quantification of percentage of total lymphocyte proliferation following co-culture
635 with licensed MSCs. (C) Quantification of percentage of lymphocyte proliferation greater
636 than three generations. (D) Percentage proliferation of CD4⁺ lymphocytes. (E) Percent
637 proliferation of CD8⁺ lymphocytes. (Representative of three independent experiments one-
638 way ANOVA n=3, mean \pm SEM, *p=0.05, **p=0.01, ***p=0.001).

639

640 **Figure 2. TNF- α /IL-1 β licensing significantly increases NO production in Lewis rat**

641 **MSCs.** (A) Griess assay results of the supernatants of Lewis rat MSCs cultured in monolayer
642 for 72 hours with rat MSC media supplemented IFN- γ , TNF- α and/or IL-1 β (500U/ml) (One-
643 way ANOVA, Tukey's multiple comparison post test, n=3, mean \pm SEM, ***p \leq 0.001). (B)
644 Griess assay results of the supernatants from MSC:Lymphocyte co culture assays described
645 in Figure 1 (One-way ANOVA, Tukey's multiple comparison post test, n=3, mean \pm SEM,
646 *p \leq 0.05, **p \leq 0.01).

647

648 **Figure 3 Inhibition of NO production abrogates the ability of MSC^{TNF- α /IL-1 β} to suppress**

649 **lymphocyte proliferation but MSC mediated lymphocyte polarisation is NO**
650 **independent.** Anti-CD3/CD28 bead stimulated Lewis rat lymphocytes were co-cultured with
651 Lewis rat MSC^{UTR} or MSC^{TNF- α /IL-1 β} in the presence or absence of NO inhibitor SMT
652 (500 μ M) for 96 hours. (A) Representative lymphocyte CFSE profiles and (B) the percentage
653 proliferation of the lymphocytes quantified by CFSE dye dilution measured by flow
654 cytometry following coculture with syngeneic MSC^{UTR} or MSC^{TNF- α /IL-1 β} in the presence or
655 absence of SMT (One-way ANOVA n=3, Tukey's multiple comparison post-test
656 ***p \leq 0.001). The supernatants were analysed by: (C) Griess assay to detect NO
657 concentration, (D) ELISA for the concentration of PGE₂ and (E,F,G) multiplex assay to
658 detect the concentrations of (E) IFN- γ , (F) TNF- α and (G) IL-2 (All assays were analysed by
659 One-way ANOVA n=3, Tukey's multiple comparison post-test, mean \pm -SEM, *p \leq 0.05,
660 **p \leq 0.01, ***p \leq 0.001).

661

662 **Figure 4. Syngeneic MSC^{UTR} and MSC^{TNF- α /IL-1 β} prolong corneal allograft survival when**

663 **administered post operatively.** Lewis rat MSC^{UTR} or MSC^{TNF- α /IL-1 β} were administered
664 intravenously on post-operative day (POD) 1 and POD 7 to a Lewis rat receiving an
665 allogeneic Dark Agouti (DA) corneal allograft. (A) Kaplan-Meier survival curve analysis of
666 allogeneic transplant controls (black line) (n=10), corneal allograft + MSC^{UTR} (blue line)
667 (n=8) and corneal allograft + MSC^{TNF- α /IL-1 β} (orange line) (n=10). (Log-rank (Mantel-Cox)
668 test, *p=0.05, ***p=0.001, NS = not significant). (B) Opacity and (C) neovascularization
669 scores up to POD 30. (D) Maximum opacity score recorded per animal, one score of opacity
670 3 or two consecutive scores of 2.5 classed as graft rejection (One-way ANOVA, Tukey's
671 multiple comparison post-test, mean \pm SEM, **p \leq 0.01)). (E) Representative light microscope
672 images of the corneal allograft at POD 10 to POD 30.

673

674 **Figure 5. MSC^{TNF- α /IL-1 β} therapy potently enriches Foxp3⁺ Tregs and modulates**

675 **CD11b/c⁺ cells in transplanted animals at POD 9.** Lewis rats receiving MSC^{UTR} or
676 **MSC^{TNF- α /IL-1 β} on POD 1 and 7 were sacrificed on POD 9 and their lungs, dLN and**

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

685
686 **Figure 6. At the time of rejection Foxp3⁺ Tregs are observed in the periphery of**
687 **MSC^{TNF-α/IL-1β} and in the draining lymph node of both MSC^{UTR} and MSC^{TNF-α/IL-1β}**
688 **treated animals.** Lewis rats receiving MSC^{UTR} or MSC^{TNF-α/IL-1β} on POD 1 and 7 were
689 sacrificed at the average time of rejection (TOR) and their lungs, dLN and spleen analysed by
690 flow cytometry and RT-PCR. (A) Graph showing flow cytometry results of CD4⁺ T cells co-
691 expressing CD25 and Foxp3 in the lungs of transplanted animals. (B) RT-PCR results for the
692 mRNA expression of IFN-γ, IL-1β and TNF-α from cells isolated from the lungs of
693 transplanted animals. (C) Graph of flow cytometry analysis CD4⁺CD25⁺Foxp3⁺ cells and (D)
694 RT-PCR results for the mRNA expression of IFN-γ, IL-1β and TNF-α from cells isolated
695 from the spleen of transplanted animals. (E) Flow cytometry analysis of CD4⁺CD25⁺Foxp3⁺
696 cells and (F) mRNA expression IFN-γ, IL-1β and TNF-α from cells isolated from the dLN
697 (One-way ANOVA, Tukey's multiple comparison post-test, mean±SEM, *p≤0.05 **p≤0.01,
698 ***p≤0.001)

699
700 **Figure 7. MSC^{UTR} and MSC^{TNF-α/IL-1β} induce Foxp3⁺ Tregs via promotion of an immune**
701 **regulatory phenotype in lung derived CD11b/c⁺ myeloid cells associated with increases**
702 **in PGE₂ and TGF-β1.** MSC^{UTR} or MSC^{TNF-α/IL-1β} were cultured with unstimulated syngeneic
703 lymphocytes for 96 hours. The percentage of CD25⁺Foxp3⁺ cells of the CD4⁺ T cells was
704 assessed by flow cytometry. (A) Representative dot plots and (B) graphed results of the
705 percentages CD25⁺Foxp3⁺ cells in unstimulated lymphocytes alone or in the presence of
706 MSC^{UTR} or MSC^{TNF-α/IL-1β}.

707 (C) Schematic of experimental design, CD11b/c⁺ cells MACS sorted from the lung were
708 cultured with MSC^{UTR} or MSC^{TNF-α/IL-1β} for 48 hours, the MSC conditioned CD11b/c⁺ cells
709 were MACS sorted again and cultured with stimulated or unstimulated lymphocytes for 96
710 hours. (D, E) Concentration of PGE₂ and TGF-β1 in the supernatants of MSC-CD11b/c⁺ cell
711 co-cultures detected by ELISA (F) Quantification of lymphocyte proliferation following co
712 culture of anti-CD3/CD28 bead stimulated lymphocytes with MSC conditioned lung
713 CD11b/c⁺ cells and (G) representative plots of lymphocyte CFSE profiles. (H) Quantification
714 of the percentage of CD25⁺Foxp3⁺ cells of the CD4⁺ lymphocytes following co culture of
715 unstimulated lymphocytes with lung sorted, MSC conditioned, CD11b/c⁺ cells and (I)
716 representative plots of the proportions of CD25⁺Foxp3⁺ cells of the CD4⁺ lymphocytes (One-
717 way ANOVA n=4, Tukey's multiple comparison post-test, mean±SEM, *p≤0.05, **p≤0.01,
718 ***p≤0.001). (J, K) The supernatants from stimulated lymphocyte-CD11b/c⁺ cell co-cultures
719 detected by ELISA for PGE₂ and TGF-β1. For results from supernatants unstimulated
720 lymphocyte-CD11b/c⁺ cell co cultures see Supplementary Figure 4. (One-way ANOVA n=3,
721 Tukey's multiple comparison post-test, mean±SEM, *p≤0.05, **p≤0.01, ***p≤0.001).

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723

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