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REGENERATIVE MEDICINE

Interspecies Incompatibilities Limit the Immunomodulatory Effect of Human Mesenchymal Stromal Cells in the Rat

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ABSTRACT

Mesenchymal stem/stromal cells (MSC) are an immunomodulatory cell population which are under preclinical and clinical investigation for a number of inflammatory conditions including transplantation. In this study, a well-established rat corneal transplantation model was used to test the ability of human MSC to prolong corneal allograft rejection-free survival using a pre-transplant intravenous infusion protocol previously shown to be efficacious with allogeneic rat MSC. Surprisingly, pre-transplant administration of human MSC had no effect on corneal allograft survival. In vitro, human MSC failed to produce nitric oxide and upregulate IDO and, as a consequence, could not suppress rat T-cell proliferation. Furthermore, human MSC were not activated by rat pro-inflammatory cytokines. Thus, interspecies incompatibility in cytokine signaling leading to failure of MSC licensing may explain the lack of in vivo efficacy of human MSC in a rat tissue allotransplant model. Interspecies incompatibilities should be taken into consideration when interpreting preclinical data efficacy data in the context of translation to clinical trial. STEM CELLS *2018;36:1210–1215*

SIGNIFICANCE STATEMENT

Mesenchymal stromal cells (MSC) have great potential for use in a number of inflammatory diseases and in transplantation due to their immunomodulatory abilities. However, it has been shown in recent years that these cells require a pro-inflammatory stimulus to "license" their immunomodulatory capabilities. This article shows that a rat pro-inflammatory environment is insufficient to provide this license to human MSC and this is associated with a failure of the human cells to modulate T-cell activity in vitro and corneal allograft rejection in vivo. These findings highlight the potential that "false-negative" results could arise in pre-clinical experiments utilizing xenogeneic cells.

INTRODUCTION

Mesenchymal stem/stromal cells(MSC) possess immunomodulatory properties [1–4] which are mediated by both cell-cell contact [5] and soluble factors such as nitric oxide (NO) [6], prostaglandin-E_2 (PGE_2) [7], and indoleamine-2,3-dioxygenase (IDO) [8]. For translation of MSC research to the clinic, preclinical efficacy must be demonstrated and this is often considered to require proof of efficacy of the human cell product in an animal model. Indeed, such efficacy has been demonstrated in rodent models of experimental autoimmune encephalomyelitis [9], acute allergic rhinitis [10], type 2 diabetes [11], and myocardial injury [12]. However, failure to demonstrate efficacy of human cells in an appropriate animal model could lead to potentially beneficial human cell therapies being overlooked before entering the clinical trial process. In the case of MSC therapies, this xenogeneic barrier is of particular concern as activation by exposure to a pro-inflammatory environment (licensing) is reported to underlie the therapeutic effects of these cells [3, 6]. Whether or not a human cell therapy will be efficacious in a particular pre-clinical model is difficult to predict and relevant negative results may go unpublished [13]. In the case of corneal transplantation, systemically administered syngeneic [14] and allogeneic [3, 15] MSC have been shown to reduce rejection rates in rodent models but these findings have not yet been converted into a clinical therapy.

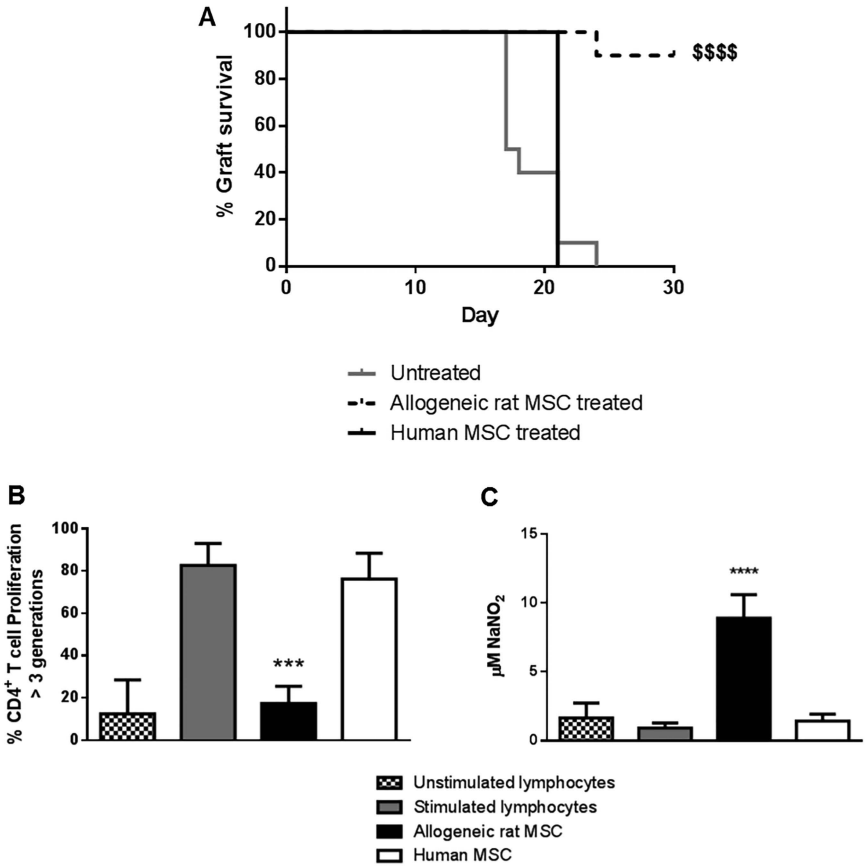

Figure 1. Human MSC are incapable of prolonging rat corneal allograft survival. **(A):** Kaplan-Meier survival curves depicting corneal allograft survival in rats treated with allogeneic rat MSC ($n = 10$) (dashed black line) administered IV 7 days and 1 day prior to transplantation, human MSC ($n = 6$) (solid black line), delivered at the same time-points and untreated control animals ($n = 10$) (gray line). $^{\$\$\$\$}$, $p < .0001$ Log rank (Mantel-Cox) test versus untreated animals. **(B):** $CD4^+$ T cell proliferation after coculture of CFSE stained anti CD3/anti CD28 stimulated Lewis rat lymphocytes with allogeneic rat (black bar) or human (white bar) MSC. **(C):** Nitric oxide levels in the supernatants of coculture experiment after 4 days. Error bars represent mean ± SEM for 3 experimental replicates. Statistical analysis performed using one-way ANOVA with Tukey's post-test, ***, $p < .001$; ****, $p < .0001$ versus stimulated lymphocytes. Abbreviation: MSC, mesenchymal stem cells.

In this study, human MSC (hMSC) were administered intravenously prior to corneal allotransplantation in rats [3, 16, 17]. In contrast to our reported results for allogeneic rat MSC [3], the human MSC did not prolong rejection-free corneal allograft survival. In an effort to understand the reasons for this lack of efficacy, we performed a series of in vitro experiments which demonstrated that, in contrast to rat MSC, hMSC failed to respond to rat pro-inflammatory cytokines and did not suppress proliferation of rat T-lymphocytes. These results reveal a potential preclinical barrier to clinical translation of hMSC for tissue transplantation.

Materials and Methods

Animal Procedures

Male Lewis (RT-1^l) and Dark Agouti (DA) (RT-1^{avl}) rats aged 8–14 weeks were purchased from Envigo (Oxon, U.K.) and housed in a fully accredited bio-resource. All procedures were approved by the NUI Galway Animal Care Research Ethics Committee and authorized by the Health Product Regulatory Authority (HPRA). Corneal transplantation was performed on Lewis rats using DA donor corneas with rejection determined based on graft opacification as previously described [3, 16]. Tail vein injections of 1×10^6 human or DA MSC in sterile saline were carried out 7 and 1 days prior to transplantation.

Culture Procedures

hMSC were isolated by plastic adherence from bone marrow of healthy donors and expanded in culture as previously described after ethical approval from Galway University Hospital. [3, 18, 19]. Rat MSC were isolated from 8-week-old male DA rats [18]. MSC were between passage 3 and 5 for all experiments.

For proliferation assays, lymph node cell suspensions were prepared from 6- to 12-week-old Lewis rats as previously described [5]. MSC were cocultured at 1:50 ratio with CFSE-labeled, polyclonally stimulated lymph node cells and proliferation was quantified by flow cytometry based on CFSE dilution [5, 18]. Concentrations of NO and PGE_2 were measured in supernatants using the Griess assay and ELISA respectively [3, 5].

For cytokine response and licensing assays, 1×10^5 human or 2×10^5 rat MSC were cultured per well of a 6 well plate in the presence or absence of optimized concentrations of human (50 ng/ml IFN-γ, 10 ng/ml TNF-α, 10 ng/ml

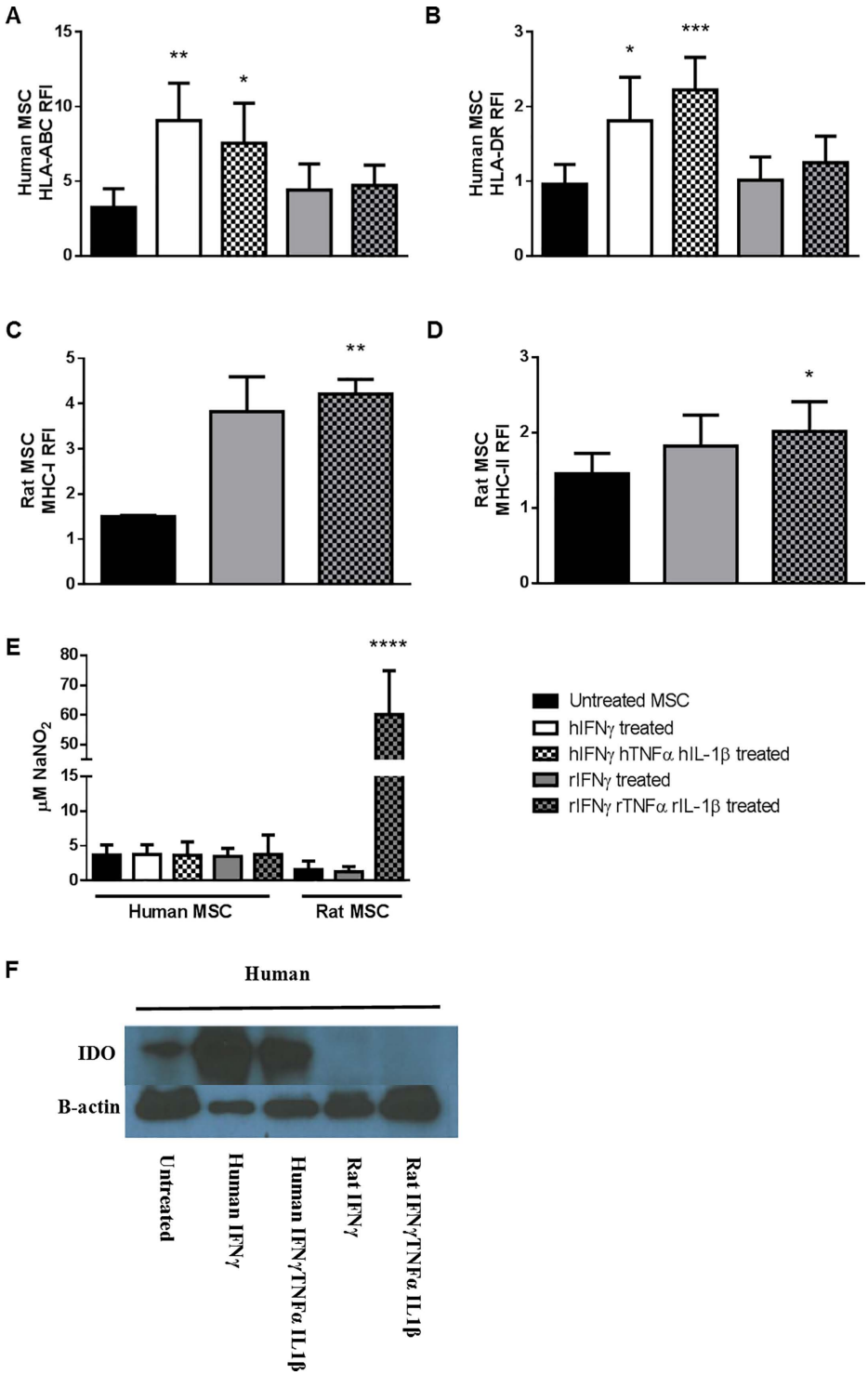

Figure 2. Human MSC cell surface profile is unaffected by rat cytokine treatment. **(A):** HLA-ABC RFI on human MSC 48 hours after no stimulation (black bar) or stimulation with hIFNγ alone (white bar), hIFNγ, hTNFα, hIL-1β (black and white checkered bar), rIFNγ alone (gray bar) or rIFNγ, rTNFα, rIL-1β (gray and black checkered bar). **(B):** HLA-DR RFI on human MSC following 48 hours under the same five conditions. **(C):** MHC-I RFI on rat MSC 48 hours after no stimulation (black bar) or stimulation with rIFNγ (gray bar) or rIFNγ, rTNFα, rIL-1β (gray and black checkered bar). **(D):** MHC-II RFI on rat MSC following 48 hours under the same three conditions. **(E):** Nitric oxide levels in culture supernatants of human and rat MSC 48 hours after no stimulation or stimulation with human or rat cytokines. **(F):** IDO and β-actin protein levels in human MSC 48 hours after no stimulation or stimulation with human or rat cytokines. Error bars represent mean ±SEM for 3 experimental replicates. All statistical analyses performed using One way ANOVA with Tukey's post-test, *, $p < .05$; **, $p < .01$; ***, $p < .001$; ****, $p < .0001$ versus untreated cells. Abbreviations: hIFNγ, human interferon gamma; hIFNγ, human interferon gamma; hIL-1β, human interleukin 1β; hTNFα, human tumor necrosis factor alpha; MSC, mesenchymal stem cells; RFI, relative fluorescent intensity; rIFNγ, rat interferon gamma; rIL-1β, rat interleukin 1β; rTNFα, rat tumor necrosis factor alpha.

IL-1β) or rat (500 U/ml IFN-γ, TNF-α, or IL-1β) cytokines (PeproTech, London, U.K.) prior to use in subsequent assays.

Immunoblotting

For STAT1 and pSTAT1 immunoblot, MSC were stimulated with cytokines for 15 minutes, for IDO immunoblot, MSC were stimulated with cytokines for 48 hours. Cells were lysed and 15 μg of protein loaded per lane of a 10% SDS-PAGE gel before transfer to a nitrocellulose membrane. Chemiluminescence was detected using a Pierce ECL Western Blotting substrate kit (Thermo-Fisher, Dublin, Ireland). Densitometry of bands was determined using ImageJ software (version 1.51, NIH).

Statistical Analysis

Statistical analyses were performed using Log-rank (Mantel-Cox) for graft survival or one-way ANOVA with Tukey's post-test for other experiments. Statistical analyses were performed using GraphPad Prism software (Version 6) (La Jolla, CA).

Results

Intravenous donor-specific and third-party allogeneic rat MSC, administered in two preoperative doses, prolong rejection-free survival of rat corneal allografts [3]. However, when hMSC (which were shown to be effective at modulating human T cell proliferation in vitro (data not shown)) were administered by the same protocol, all grafts rejected before day 30 and rejection-free survival was no different to that of untreated recipients (Fig. 1A).

As $CD4^+$ T-cells are important mediators of corneal allograft rejection [20], rat (DA) and hMSC were cocultured with antibody-stimulated Lewis rat lymph node cells and $CD4^+$ T-cell proliferation was quantified and compared with cultures lacking MSC. As shown in Figure 1B, DA MSC suppressed Lewis $CD4^+$ T-cell proliferation to the level of unstimulated cultures while hMSC had no suppressive effect at a ratio of 1:50, even though they do have some mild suppressive ability at higher ratios (Supporting Information Fig. S1). Rodent MSC are reported to suppress T-cell proliferation via NO production [6] and supernatants from cocultures containing DA MSC had increased NO compared with stimulated and unstimulated cultures without MSC. In contrast, supernatants from hMSC-containing cocultures had no increase in NO (Fig. 1C).

Exposure of MSC to pro-inflammatory cytokines results in their upregulation of surface activation markers such as MHC-I, MHC-II, and toll-like receptors [19, 21–26]. Additionally, pro-inflammatory cytokines increase MSC production of soluble immunomodulatory mediators [22, 27]. hMSC were exposed to rat or human IFN-γ or IFN-γ, TNF-α, and IL-1β in combination. hMSC demonstrated increased surface HLA-ABC (Fig. 2A) and HLA-DR expression (Fig. 2B) in response to human but not to rat cytokines. In contrast, DA MSC responded to rat cytokines with increased surface MHC-I (Fig. 2C) and MHC-II (Fig. 2D), demonstrating bioactivity of the rat cytokine preparations used. hMSC could not be induced to produce NO by either human or rat cytokines while DA MSC, as previously reported [6], strongly produced NO in response to rat IFN-γ, TNF-α, and IL-1β (Fig. 2E). Furthermore, IDO expression was strikingly increased in hMSC after exposure to human, but not rat, cytokines (Fig. 2F). In fact, the rat cytokines seemed to suppress the modest amount of endogenous IDO expressed by hMSC, suggesting the possibility of an antagonistic or modulatory effect.

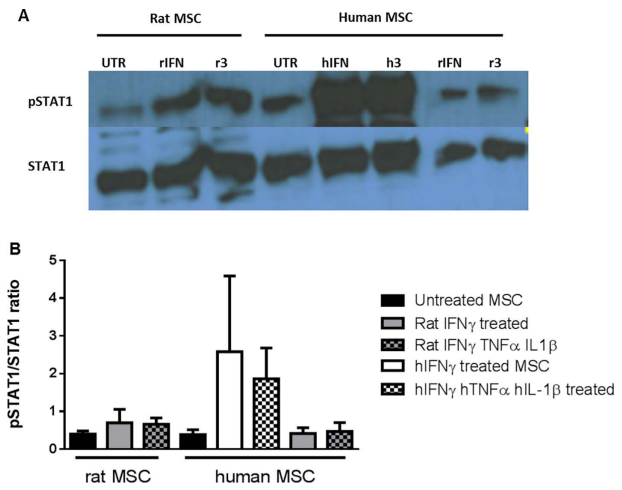

Figure 3. Rat pro-inflammatory cytokines do not provide an activation stimulus to human MSC. **(A):** pSTAT1 and STAT1 protein levels by Western blot (representative blot) on cell lysates from rat or human MSC 15 minutes after no stimulation (UTR) or stimulation with rat interferon gamma alone (rIFN), rat interferon gamma, rat interleukin 1β and rat tumor necrosis factor alpha (r3), human interferon gamma alone (hIFN), human interferon gamma, human interleukin 1β, and human tumor necrosis factor alpha (h3). **(B):** Graph of the ratio of pSTAT1 to total STAT1 in cells 15 minutes after cytokine treatment from densitometry of the bands in three independent blots. Error bars represent mean ± SEM for 3 experimental replicates. Statistical analyses performed using one way ANOVA with Tukey's post-test. Abbreviations: hIFN, human interferon gamma; MSC, mesenchymal stromal cells; rIFN, rat interferon gamma.

Phosphorylation of STAT1 has been reported as one of the earliest events in the activation of MSC, and we found it was increased in DA MSC treated with rat cytokines and in hMSC treated with human cytokines but not in hMSC treated with rat cytokines (Fig. 3A, 3B).

Finally, we compared the potency of rat cytokine-licensed DA and hMSC to suppress rat T-cell proliferation. While DA MSC had potent anti-proliferative effects on both $CD4^+$ and $CD8^+$ Lewis T-cells, hMSC had no suppressive effect regardless of cytokine conditioning (Fig. 4A, 4B) or providing increased concentrations of rat cytokines (**Supporting Information Fig. S2**). Quantification of NO and PGE_2 in the coculture supernatants indicated that the presence of rat MSC was associated with high concentrations of these molecules, while the presence of hMSC was associated with minimal NO and low concentrations of PGE_2 (Fig. 4C, 4D).

Discussion

These data show, in a specific rat model (corneal allo-transplantation), that hMSC are ineffective as an immunomodulatory intervention at a dose and timing regimen that proved highly effective for allogeneic rat MSC. Upon further investigation, we observed that hMSC, unlike rat MSC, are incapable of exerting immunomodulatory effects on rat T-cells, which are major effectors in corneal transplant rejection [20]. This

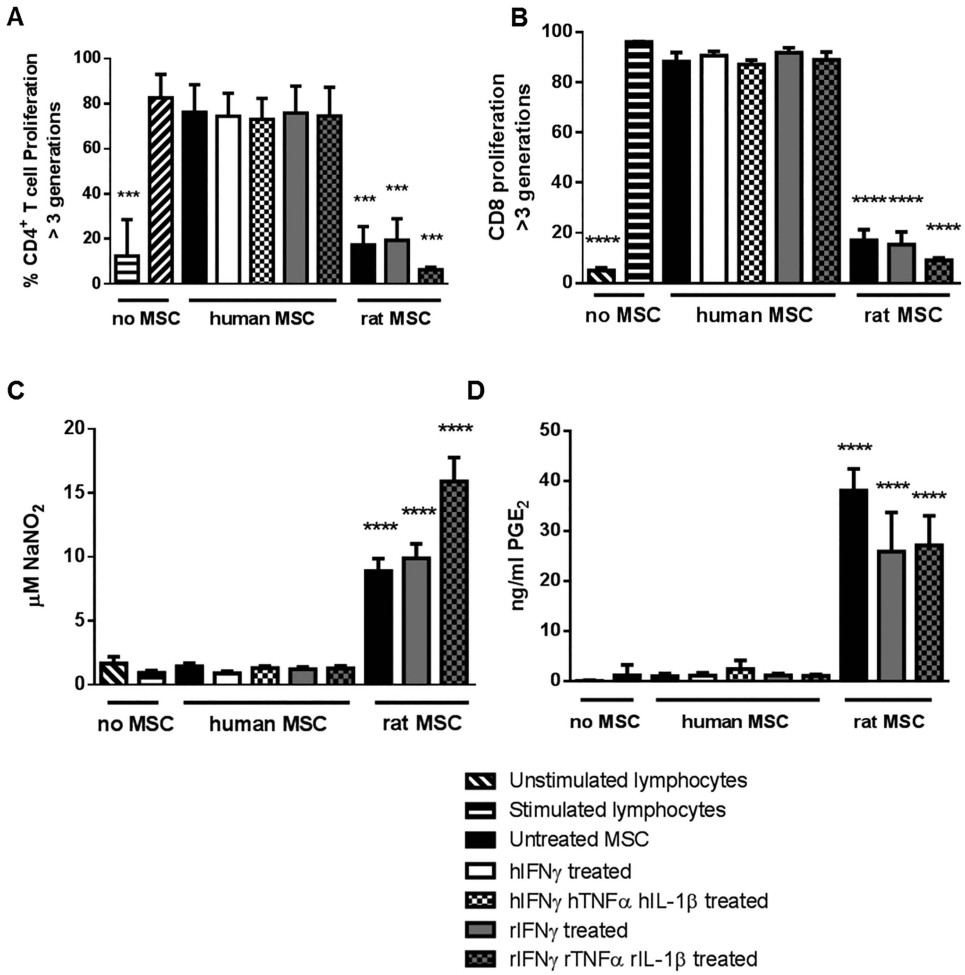

Figure 4. Human MSC do not produce the immunomodulatory molecules necessary to modulate rat T cell proliferation. **(A):** $CD4^+$ T cell proliferation after 4 days coculture of CFSE stained anti CD3/anti CD28 stimulated Lewis rat lymphocytes with pretreated rat or human MSC. **(B):** $CD8^+$ T cell proliferation from the same coculture experiments. **(C):** Nitric oxide levels in coculture supernatants after 4 days. **(D):** PGE_2 levels in coculture supernatants after 4 days. Error bars represent mean ±SEM for 3 experimental replicates. All statistical analyses performed using One way ANOVA with Tukey's post-test, ***, $p < .001$; ****, $p < .0001$ versus stimulated lymphocytes. Abbreviation: MSC, mesenchymal stem/stromal cell.

lack of modulation is likely due to the fact that, unlike rat MSC, hMSC cannot be induced to produce NO (a fact that is unsurprising, as it is well accepted that hMSC primarily rely on other immunomodulatory factors [28]) or upregulate IDO in response to rat pro-inflammatory cytokines or upon coculture with lymphocytes [5]. Recently, STAT signaling has been shown to have an important role in the early stages of MSC activation [29]. Lack of STAT1 phosphorylation in hMSC following exposure to rat IFN-γ, with or without rat TNF-α and IL-1β, further indicates that a rat pro-inflammatory micro-environment is insufficient to activate hMSC. Importantly, the rat cytokine preparations used in these experiments demonstrated appropriate activities when added to rat MSC cultures. Our findings may be specific to rat as others have shown that hMSC can be effective in a mouse model [30].

In conclusion, these data indicate that, for corneal allotransplantation, preclinical efficacy testing of hMSC in a well-established rat model provided a false negative result, likely due to interspecies incompatibilities in the cytokine signals required for in vivo activation of immune modulatory mechanisms. Our results are of high relevance to the process of translation of MSC therapies from the preclinical to clinical arenas as demonstration of in vivo efficacy is often pursued through the use of "human-to-rodent" in vivo models.

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Author Contributions

P.L.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; O.T.: collection and/or assembly of data, data analysis and interpretation, final approval of manuscript; M.M., E.D., and Y. O'D.: collection and/or assembly of data, final approval of manuscript; A.E.R.: conception and design, final approval of manuscript; S.J.E.: conception and design, financial support, final approval of manuscript; T.R. and M.D.G.: conception and design, financial support, data analysis and interpretation, manuscript writing, final approval of manuscript.

Disclosure of Potential Conflicts of Interest

Y.O'D. and S.J.E. are employees of Orbsen Therapeutics Ltd. The other authors indicated no potential conflicts of interest.

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