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Function of Cryopreserved MSCs with and without IFN- γ pre-licensing is Context Dependent

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Success of mesenchymal stromal cell (MSC) therapies in disease models has sparked hundreds of clinical trials in humans[1]. While the first MSC clinical trials, for pediatric osteogenesis imperfecta[2] and GvHD[3], utilized fresh cultured MSC, the majority of clinical trials and companies developing commercially available MSC-based products today utilize cryopreserved MSCs. Cryopreservation simplifies logistics of cell therapy, but is not without potential drawbacks and the full impact of cryopreservation on MSC function in specific disease contexts is not well understood. In recent years, several groups have examined the impact of cryopreservation on MSC function with mixed results[4–8]. A particular focus has been around whether or not MSC's diverse immunomodulatory functions remain intact following cryopreservation. Several groups have reported reduced potency of MSCs following cryopreservation[6, 8] while others have seen no significant decline in immunosuppressive function[5, 9, 10]. Some discrepancy can be attributed to poor viability following cryopreservation and differences in PBMC suppression assay protocols that may not be sensitive enough to detect differences in potency.

The excellent paper by Chinnadurai *et al.* adds to the evaluation of the fitness of cryopreserved MSCs by demonstrating that thawed MSCs are susceptible to lysis by cytotoxic T lymphocytes (CTL) in co-culture assays [11]. Cryopreserved MSCs co-cultured with PBMCs in direct contact failed to suppress PBMC proliferation and in some cases mildly enhanced PBMC proliferation while cryopreserved MSCs co-cultured with PBMCs in transwells remained suppressive. As could be expected, allogeneic thawed MSCs were far more sensitive to T-cell mediated lysis than autologous MSC after co-culture. Together these results suggest increased allo-reactivity to recently thawed MSCs. To investigate the cause of MSC's failure to suppress PBMCs and avoid lysis by CTLs the authors determined the rate at which MSCs are capable of suppressing degranulation of CD8+ T cells and found cryopreserved MSCs failed to suppress degranulation at all time points while fresh MSCs suppressed degranulation by day 2 of the co-culture. To restore the immunosuppressive potency of cryopreserved MSCs, cells were pre-licensed with IFN- γ for 48 hours before cryopreservation so that upon thawing, MSCs would already have high levels of IDO, a potent immunosuppressive enzyme. Indeed, pre-licensed cryo-MSCs had high levels of IDO mRNA and protein and avoided CTL mediated lysis while suppressing PBMC proliferation

Author Contributions

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equally as well as fresh MSCs. IDO activity is known to suppress CD8⁺ CTL[12] and is likely responsible for the improved survival and potency of pre-licensed cryo-MSCs. Thus they suggest pre-licensing MSCs with IFN- γ may inform strategies to overcome the detrimental effects of cryopreservation on MSC.

We recently reported that cryopreserved MSCs maintain viability, responsiveness to inflammatory stimuli, and growth factor secretion and showed a small and not-significant decline in their ability to suppress PBMCs in co-cultures *in vitro*[9]. In addition, cryopreserved MSCs performed equally as well as fresh MSCs when used to rescue retinal ganglion cells (RGC) following an ischemia reperfusion injury to the eye [9]. We then sought to improve the therapy further by pre-licensing MSCs with IFN- γ prior to freezing. Using a similar strategy to Chinnadurai, we pretreated MSCs with IFN- γ for 24 or 48 hours and froze the MSCs as reported[9]. We then examined the level of IDO expression after thawing and plating MSC in IFN- γ containing media. Both batches of primed MSCs were evaluated for IDO protein content 8, 24, and 48 hours after thaw or until they had been exposed to IFN- γ for a total of 72 hours (Fig. 1A). At 8 and 24 hours after thawing, the 24-hour pre-licensed group had less IDO content compared to fresh MSC but the discrepancy in IDO content was no longer noticeable at 48 hours after thawing (72 hours of total IFN- γ exposure). The 48 hour pre-licensed group performed considerably better in this assay, displaying comparable levels of IDO content at both 8 and 24 hours after thawing. We then took the 48 hour pre-licensed cryo-MSC and tested to see if they would outperform fresh MSCs in an ischemia/reperfusion model *in vivo*. To our surprise, the IFN- γ pre-licensed cryo-MSCs lost effectiveness *in vivo*, rescuing fewer RGC than either fresh or unlicensed cryopreserved MSC (Fig. 1B,C).

While MSCs licensed with IFN- γ are known to increase expression of immunosuppressive factors, treatment also dramatically increases surface expression of MHC-I and MHC-II molecules[13] which may accelerate the detection and clearance of MSC via xeno-recognition. Notably, in our ischemia/reperfusion model all human MSC were cleared from the mouse eyes by day 7[9]. Further analysis is needed to determine if the reduced effect of pre-licensed MSC was due to hastened immune detection and clearance of MSC or changes in secreted factors that support RGC survival. While a syngeneic or autologous transplant model would allow for analysis of the fate of pre-licensed cryo-MSC independent of rejection mechanisms, it would not be without significant drawbacks. Notably, MSC biology diverges significantly between human and mice, with documented differences in chemokine receptors[14] as well as their use of central immunomodulatory mechanisms, with murine MSC utilizing iNOS while human MSC employ IDO[15]. In addition, since the goal of our work was to move toward an off-the-shelf therapy for I/R injury that can be administered within hours of the onset of an ischemic event, the analogous human application would likely employ allogeneic MSC, and thus MSC would likely suffer from enhanced allo-recognition in a pre-licensed state. Our current data is insufficient to fully conclude that pre-licensed cryo-MSC have no place in the world of cell-therapy, but highlight the need for future work in this area to proceed cautiously with careful attention paid toward *in vivo* immune detection and clearance.

Chinnadurai et al.'s report[11] that cryopreserved MSCs can be killed via CTL mediated lysis is further evidence that MSCs are immune evasive in nature, but only evade destruction if their immunosuppressive facilities are intact[1]. Both the Chinnadurai et al. report[11] and our observed negative impact of pre-licensing on MSCs *in vivo* (Fig. 1B) highlight the need to understand in greater detail the multiple mechanisms by which MSCs are cleared *in vivo* and how cryopreservation and other preconditioning regimens extend or shorten their persistence *in vivo*. In addition, our observation that pre-licensed cryopreserved MSCs performed worse in the retinal ischemia/reperfusion model demonstrates the need to evaluate the suitability of cryopreserved or otherwise manipulated MSCs in a disease specific context. MSC exert multiple mechanisms including immune suppression, secretion of growth factors[16], and even donation of mitochondria[17]. However, each can be differentially impacted by cryopreservation and preconditioning strategies and the appropriateness of such strategies can not be determined outside of the context of a specific pathology.

Studies in animal models to date have shown that cryopreserved MSC are effective in treating disease models of colitis[18], allergic airway inflammation[10], and ischemia/reperfusion injury to the eye[9]. In contrast, cryo-MSC failed to induce a chondrogenic response in a mouse-based chondrocyte-responsive bioassay suggesting cryo-MSC may be unsuitable for treatment of osteogenesis imperfecta[19]. In humans, cryopreserved MSC have elicited positive responses in clinical trials for critical limb ischemia[20] while retrospective analysis of GvHD patients receiving fresh versus thawed MSC suggest fresh MSC are more efficacious[8]. Thus cryopreserved MSCs may be suboptimal and inappropriate for the treatment of some conditions, while being adequate and necessary for others. As our understanding of the disease specific therapeutic mechanisms employed by MSCs grows, so to will our ability to identify culture conditions and cryopreservation techniques that maintain or enhance rather than hinder MSC potency.

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Figure 1. IFN- γ priming enhances IDO expression of cryopreserved MSCs *in vitro* but is detrimental to MSC performance in an ischemia/reperfusion injury *in vivo*

(A): Representative Western blot of IDO protein in human MSCs pretreated with 100 ng/mL rhIFN- γ for 24 hr or 48 hr prior to cryopreservation (Pre-cryo IFN- γ), followed by re-stimulation for an additional 8, 24, or 48 hr (Post-cryo IFN- γ), compared to fresh MSC cultures grown in 100 ng/mL IFN- γ . β -Actin served as a loading control. Western blots were performed as previously described[9] using primary antibodies for IDO and β -Actin (1:500 rabbit anti-IDO (12006S, Cell Signaling, Danvers, MA), 1:20,000 mouse anti- β -actin (1406030, Ambion, Thermo Scientific, Waltham, MA)). Densitometry measurements made with LI-COR ImageStudio software.

(B): Quantitative analysis of RGC survival in eyes after retinal I/R injury revealed a significant rescue effect after transplantation of fresh MSC and cryo-MSC, while cryo-MSC preconditioned in 100ng/mL IFN- γ for 48 hrs show diminished rescue of RGCs (mean $\pm$ SD, One-way ANOVA with Tukey honest significant difference post hoc test to correct for multiple comparisons, $p < 0.05$ considered significant). All animal experiments were carried out in accordance with the ARVO Statement for the Use of Animals in Ophthalmology and Vision Research and were approved by the IACUC committee of the University of Iowa.

Unilateral retinal damage was induced by Ischemia/Reperfusion (I/R) injury as described earlier[9]. Two-month old C57BL6/J (The Jackson Laboratory, Bar Harbor, ME) were anaesthetized by intraperitoneal injection of Xylazin/Ketamine (10 mg/kg and 100 mg/kg, respectively). Eyes received 0.5% proparacaine eye drops for topical analgesia, pupils were dilated with 0.5% tropicamide (both Akorn, Lake Forest, IL), and corneas were kept moist until animals had fully recovered (GenTeal, Alcon, Fort Worth, TX). Intraocular pressure was elevated to 80 mmHg for 60 minutes by cannulating the anterior chamber with a sterile 30-gauge needle connected to an elevated saline reservoir. Animals were allowed to rest for 2 hours after IOP elevation to simulate a reperfusion injury after which they were sedated and received intraocular injection of PBS or 30,000 MSC suspended in 3 μ l of PBS. The right eyes of all mice received no manipulation and served as controls. After 7 days, animals were euthanized and RGC survival was quantified by staining for γ -synuclein, a marker for retinal ganglion cells (RGC), and the number of surviving RGC was determined. Briefly, retinas were incubated overnight with mouse anti- γ -synuclein primary antibody solution (1:400, Abnova Corporation, Walnut, CA, USA), followed by several rinses in PBS and incubation with an Alexa Fluor 488 donkey anti-mouse secondary antibody (1:300, Life technologies, Grand Island, NY). After another PBS wash, retinas were whole-mounted, cover slipped and imaged. Twelve images ($318 \times 318 \mu\text{m}$, 40X magnifications) were taken at predetermined mid-peripheral locations using a Nikon Eclipse i80 confocal microscope (Nikon Instruments Inc, Melville, NY). γ -synuclein positive RGC were counted in a masked fashion by an independent observer using the cell counter plugin in ImageJ software (NIH).