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Mesenchymal stem cells protect endothelial cells from cytotoxic T lymphocyte induced lysis

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Abstract:

The integrity of the vasculature plays an important role in the success of allogeneic organ and haematopoietic stem cell transplantation. Endothelial cells (EC) have previously been shown to be the target of activated cytotoxic T lymphocytes (CTL) resulting in extensive cell lysis. Mesenchymal stromal or stem cells (MSC) are multipotent cells which can be isolated from multiple sites, each demonstrating immunomodulatory capabilities. They are explored herein for their potential to protect EC from CTL targeted lysis.

CD8⁺ T cells isolated from human PBMC were stimulated with mitotically inactive cells of a human microvascular endothelial cell line (CDC/EU.HMEC-1, further referred to as HMEC) for 7 days. Target HMEC were cultured in the presence or absence of MSC for 24 hours before exposure to activated allogeneic CTL for 4 hours. EC were then analysed for cytotoxic lysis by flow cytometry.

Culture of HMEC with MSC in the efferent immune phase (24 h before the assay) led to a decrease in HMEC lysis. This protection was lost when MSC were cultured in a transwell system, where contact between the MSC was withdrawn. Further analysis suggested that prostaglandin E2 (PGE2) has a role to play in MSC abrogation of lysis. The efficacy of multiple sources of MSC was also confirmed and the collaborative effect of MSC and the endothelium protective drug defibrotide was determined, with defibrotide enhancing the protection provided by MSC. These results support the use of MSC as an adjuvant cellular therapeutic in transplant medicine, be it alone or in conjunction with EC protective agents such as Defibrotide.

39 **Abbreviations**

40 EC: Endothelial cell

41 HMEC: CDC/EU.HMEC-1, an immortalized human microvascular endothelial cell line

42 IL-2: Interleukin 2

43 PBMC: Peripheral blood mononuclear cells

44 CTL: Cytotoxic T lymphocyte

45 BM-MSC: Bone marrow derived mesenchymal stem cell

46 PVSC: Perivascular (umbilical artery derived) MSC

47 UC-MSC: Umbilical cord matrix derived MSC

48 PGE2: Prostaglandin E2

49

50 **Highlights**

- 51 • MSC protect EC from CTL lysis
- 52 • Cell-to-cell contact and the release of PGE2 are involved in MSC protection
- 53 • MSC from different sources achieve similar results
- 54 • Defibrotide adds to the protection observed in MSC co-culture

55

Introduction

Allogeneic transplantation of organs or haematopoietic stem cells is often the only therapeutic option for end-stage organ disease or leukaemia, respectively. However, due to disparities in major and minor histocompatibility antigens between donor and recipient, severe inflammatory complications can occur, requiring immunosuppression. Unfortunately, immunosuppression seriously affects the quality of life of patients and is a major threat to the health economy. The vascular endothelium is the primary contact between foreign and self, and damage to vascular endothelial cells represents the primary adverse event finally resulting in loss of organ function. This central role and early appearance of endothelial damage makes it an ideal target for early therapeutic intervention. Endothelial damage to the graft from ischemic reperfusion injury in the case of solid organ transplantation (Boyle et al., 1997) or to the host from conditioning in allogeneic haematopoietic stem cell transplantation (Eissner et al., 1995, Paris et al., 2001), ultimately leads to loss of organ function and transplant failure. Damage to the mucosal barriers results in the expression of damage associated molecular patterns and the translocation of foreign antigen such as LPS across damaged membranes. This combined with the exposure to inflammatory mediators results in the development of an immunologically active state, rendering EC to become antigen-presenting cells. Previous work published by our group demonstrated the destructive repercussions of the invasion of cytotoxic T lymphocytes (CTL) which specifically target endothelial cells (EC) (Eissner et al., 2011). Importantly, these CTL show an unusual phenotype (CD28-negative) and are peculiarly enhanced in their cytotoxic activity by the co-culture with FoxP3⁺ regulatory T cells (Treg cells) (Eissner et al., 2011), suggesting that the currently available immunomodulatory mechanisms cannot protect EC against these effector cells.

Over the last 10 years the clinical use of bone marrow derived mesenchymal stem cells (BM-MS) as a cellular therapeutic has risen dramatically, with over 350 clinical trials registered across a broad spectrum of diseases (Wei et al., 2013). Early work looked at MSC for their capacity to differentiate into multiple lineages (Friedenstein et al., 1966, Pittenger et al., 1999), testing their ability to replace damaged tissue by *in situ* differentiation. It is now, however, more commonly accepted that MSC home to the site of injury and influence the local environment through the release of soluble factors (Caplan and Dennis, 2006, Chen et al., 2008). The same can be observed in vascular biology where soluble mediators, such as VEGF and IGF, released by MSC in a model of myocardial infarction promote tube formation and protect cardiac cells against apoptosis (Sadat et al., 2007). Bader *et al.* also showed MSC can protect EC from apoptosis in hypoxic conditions associated with ischemic disease, improving viability and proliferation (Bader et al., 2014). These results support the use of MSC in transplant medicine, targeting protection or repair of the endothelium. Use of MSC in combination therapy has also become common practice, with publications into renal fibrosis (Huuskes et al., 2015) and clinical trials in GvHD (Le Blanc et al., 2008), siting increased attenuation of pathology.

Defibrotide is a polydisperse mixture of mostly single stranded oligonucleotides with anti-inflammatory, anti-ischemic, pro-fibrinolytic, and antithrombotic functions (Guglielmelli et al., 2012). As of 2013 it has been approved by the European Medicines Agency as a treatment for hepatic veno-occlusive disease (VOD), a severe endothelial complication following allogeneic haematopoietic stem cell transplantation. Treatment of EC with the chemotherapeutic drug fludarabine, also used in pre-transplant conditioning, results in apoptosis and allogeneic activation. The addition of defibrotide rescued EC from these adverse events without interfering with the desirable anti-leukemic and anti-T cell proliferative effects of the drug (Eissner et al.,

102 2002). These observations prompted investigation into not only the efficacy of MSC, but the
103 examination of defibrotide and MSC as a combination therapy in a cytotoxicity assay.

104

Materials and methods

Cell culture and reagents

The human dermal microvascular endothelial cell line CDC/EU.HMEC-1 (further referred to as HMEC) was kindly provided by the Centers for Disease Control and Prevention (Atlanta, Georgia, USA) (Ades et al., 1992). HMEC were cultured in MCDB131 medium, supplemented with 15% fetal calf serum (FCS), 1 µg/mL hydrocortisone (Sigma, Deisenhofen, Germany), 10 ng/mL epidermal growth factor (Collaborative Biochemical Products, Bedford, MA, USA) and antibiotics. Defibrotide was kindly provided by Dr. Terenzio Ignoni (Gentium, SpA., a Jazz Pharmaceuticals Company, Villa Guardia (CO), Italy). Bone marrow derived MSC (BM-MSC) were provided by Apceth GmbH & Co.KG, by the Department of Internal Medicine III, Regensburg University Medical Centre, Regensburg, Germany, or purchased from Pelobiotech (PELOBiotech GmbH, Planegg, Germany), Perivascular mesenchymal stem cells (PVSC) were isolated from the perivascular region of umbilical cord arteries (Department of Gynaecology, Wolfart-Klinik (Gräfelfing, Germany)) and Amniotic membrane-derived MSC (hAMSC) were kindly provided by Ornella Parolini, Centro di Ricerca E. Menni, Fomdazione Poliambulanza, Brescia, Italy. All MSC were cultured in Human Xeno-free Mesenchymal Stem Cell Medium (PELOBiotech GmbH, Planegg, Germany) containing supplement and 1% Penicillin/streptomycin. T lymphocytes were isolated from PBMC from either leukapheresis products by the Department of Clinical Chemistry and Transfusion Medicine (Regensburg University Medical Centre, Regensburg, Germany) or from buffy coats by the Irish Blood Transfusion Service and were cultured in RPMI containing 10% FCS, 1% L-glutamine, 1% Penicillin/streptomycin and 300U/ml of Interleukin 2 (IL-2);(Novartis, Basel, Switzerland). All reagents have been purchased from Life Technologies unless stated otherwise. All biological

samples were obtained with informed consent according to guidelines of the local Ethical review committees.

Separation and culture of CD8 T cells

CD8⁺ T cells were isolated using MagCelect CD8⁺ T cell isolation kit according to manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). Briefly, PBMC were negatively selected by incubation with biotin antibody cocktail followed by incubation with ferro-streptavidin and finally magnetic separation. Purity of isolation was determined by flow cytometric analysis.

Cytotoxicity Assay

CD8⁺ T cells were stimulated in a ratio of 1:1 with mitotically inactivated (50 µM Silibinin, Sigma-Aldrich, Deisenhofen, Germany) HMEC for 7 days in the presence of IL-2 (300 U/mL). Cytotoxicity was assessed according to a well established protocol (Piriou et al., 2000). Target HMEC were labelled with 30 µM 3,3'-dioctadecyloxacarbocyanine perchlorate (DIOC18₍₃₎) by incubating at 37°C for 15min. HMEC were then cultured in the presence or absence of MSC or drugs (day-1) for 24 hours followed by incubation with CD8⁺ T cells at descending effector to target ratios (20:1, 10:1 and 5:1) for another 4 hours. The percentage of specific lysis determined by counter staining the HMEC with propidium iodide (PI);(0.2 µg/mL, Sigma-Aldrich, Deisenhofen, Germany) and determining lysis by flow cytometry analysis (BD, Oxford, UK). Defibrotide was added to the culture system at day-1 at a concentration of 25µM and Indomethacin (Sigma-Aldrich, Deisenhofen, Germany) was used at a concentration of 40µM.

150 *Transwell Culture*

151 Transwell inserts for a 24 well plate with a 0.4µm pore size (Corning, Hazebrouck,
152 France) were used to separate the MSC from HMEC and CTL during the cytotoxicity assay.
153 5×10^4 DIOC18₍₃₎ labelled HMEC were seeded in the bottom of the 24 well plate, while 1×10^4
154 BM-MSC were plated either in contact with the HMEC or in the upper chamber of the transwell
155 insert. The cells were cultured for 24 hours in HMEC medium. The medium was removed and
156 re-placed with CTL medium before CTL were added to the bottom well of the plates and
157 cultured for a further 4 hours. The HMEC were collected and counter stained with PI and
158 examined by flow cytometry.

159 *PGE ELISA*

160 The amount of PGE-2 produced during a cytotoxicity assay was measured using a
161 commercially available ELISA Kit from Cayman Chemical (Ann Arbor, MI, USA). The PGE-2
162 assay is based on the competition between PGE-2 and PGE-2 acetylcholinesterase (AChE)
163 conjugate (PGE-2 tracer) for a limited amount of PGE-2 monoclonal antibody. ELISA were
164 carried out according to manufacturer's instructions. The microplate (96 well polystyrene) was
165 pre-coated with goat anti-mouse polyclonal antibody. The standards and samples in triplicate and
166 controls in duplicate (50 µl) were added to the plate, and read at an absorbance of 405 nm using
167 a multiscan plate reader (LabSystems, Thermo-Fisher, Waltham, MA, USA).

168 *Statistical Analysis*

169 Statistical analysis was performed using GraphPad Prism™ software (GraphPad, San
170 Diego, CA, USA). The students paired t test was used when statistical analysis was required
171 between two experimental groups. One way ANOVA was used to test for statistical significance

172 of differences when multiple experimental groups were compared. Data are presented as the $\pm$
173 standard error of the mean (SEM). P-values of $p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$ (***) were
174 considered statistically significant.

Results

MSC protect endothelial cells against allogeneic CD8⁺ CTL

Previous data published by our group demonstrated the existence of a strictly EC-specific CD8⁺ T cell subpopulation that is peculiarly enhanced by the co-administration of Treg cells (Eissner et al., 2011). Following up on this work, the present study looked to investigate the role of MSC in protecting EC from targeted killing. Untouched CD8⁺ T cells were isolated and co-cultured with allogenic EC (HMEC) for 7 days. 24 hours before the cytotoxicity assay, HMEC were stained with DIOC and cultured in the presence or absence of BM-MSC at a ratio of 5:1, respectively. The cytotoxicity assay involves culturing the primed CTL with the treated or untreated HMEC for 4h, staining with PI and analysing the expression of PI versus DIOC by flow cytometry. When cultured in the presence of BM-MSC there was a significant decrease in the extent of cell lysis elicited by the CTL. The CTL were titrated resulting in a titrated rate of cell death, in each case the MSC reduced the extent of EC killing (Fig. 1A), with significance at 20:1 graphed as a bar chart (Fig. 1B), with the 10:1 and 5:1 ratios also reaching significance (data not shown).

Following on from this work, efforts were made to determine the signalling mechanisms involved in MSC protection of EC. However, before this could be achieved more needed to be understood about the physical interactions between the cells in culture. The lysis of EC by CTL was herein shown to be fully MHC Class I restricted, as pre-treatment of HMEC with w6/32, an MAH Class I-restricted monoclonal antibody, prior to their encounter with CTL, completely abrogated the CTL activity. The extent of lysis was reduced to that observed with MSC co-culture (Supplementary Fig. 1). Lastly, to eliminate the possibility that MSC were simply

masking the contact sites on EC from the CTL by physically covering them, human heart explant cells (HH7) as size-matched negative controls were used in the place of MSC in a cytotoxicity assay. These heart explant cells failed to protect the EC from cell death thus negating the possibility of MSC simply shrouding the EC (Supplementary Fig. 2).

MSC protection requires contact

MSC signalling has provoked a long running debate as to the importance of cell-cell contact over the release of soluble factors. Results from transwell cytotoxicity experiments performed herein showed that in the absence of cell contact MSC failed to protect HMEC from CTL induced lysis (Fig 2A). The extent of HMEC lysis did not increase to levels observed in untreated HMEC however, suggesting that MSC may release some soluble factor that infers some modicum of protection but not to the extent of MSC in contact conditions.

The multitude of cytokines and stimulants released by MSC posed a problem in narrowing down the potential candidate's eliciting the protective effects on EC. A cytokine array on the supernatant from transwell experiments was performed, also including the supernatant from MSC in contact with CD8⁺ T cells but in the absence of HMEC. There was little difference between the supernatant from MSC in contact and those in transwell to suggest a candidate for signalling. However, when PGE2 was examined by competitive ELISA there was a drop in the level of PGE in the supernatant of cells from the transwell group (data not shown). Following on from this work a COX inhibitor that prevents the synthesis of prostaglandins, indomethacin, was added to the HMEC and MSC co-culture during the 24 hours prior to the cytotoxicity assay and resulted in an increase in EC lysis despite the presence of MSC (Fig 2B).

Protection evident in MSC from multiple sources

BM-MSC are one of the most commonly used and studied groups of stem cells predominantly due to the ease of isolation and their initial beneficial use in autologous MSC therapy. However, MSC isolated from the umbilical cord and placenta have been shown to be less immunogenic than BM-MSC while maintaining the same modulatory abilities (Pianta et al., 2014). In order to determine whether MSC from different sources would work as well in this system, MSC from the perivascular region of umbilical cord arteries (PVSC) and MSC of the amniotic membrane (hAMSC) were tested in a cytotoxicity assay with various CTL donors. Though BM-MSC provide the greatest protection on HMEC with only 24% of the cell population undergoing lysis, PVSC (38%) and hAMSC (37%) still significantly reduce the level of CTL lysis (Fig 3).

Defibrotide enhances the protective effect of MSC

Combination therapy, particularly in the case of cellular therapy has received a lot of attention in the last number of years. Defibrotide as described above has been tested successful in the treatment of VOD, its known interaction with endothelial cells led to a study of the drug in the stabilisation of the endothelial compartment (Koehl et al., 2007). Previously published work from our group defined a role for defibrotide in protecting the endothelium from CTL targeted lysis without interfering with the regular growth and function of the cells (Eissner et al., 2002). This study sought to determine whether Defibrotide, given in combination with MSC, would increase the protection achieved by the MSC alone. In line with previous findings MSC decreased the lysis of EC, notably however, the addition of defibrotide increased the significance of inhibition, adding to the abrogation of lysis achieved by MSC alone (Fig. 4).

Discussion

The current study sought to examine the role of MSC in the protection of EC from CTL induced lysis. Herein we have shown that MSC from multiple sources, not only bone marrow, can abrogate EC cytotoxicity and work cooperatively with defibrotide to prevent EC death. PGE₂ was demonstrated to play a partial role in MSC protection with global inhibition of COX signalling increasing EC lysis to levels similar to that of EC and CTL alone. To our knowledge these findings are the first of their kind, establishing a role for MSC protection of EC from CTL killing.

MSC influence over vascular cells has been studied in depth, in most cases their efficacy is demonstrated in models of vascular pathophysiological diseases (Togel et al., 2007, Bader et al., 2014, Sadat et al., 2007). Though much is yet unknown about their mechanism of action, many such studies agree that the health of the endothelium is a major factor in the pathogenesis of vascular diseases (Aird, 2003) and organ failure (Paris et al., 2001). Previous work by our group into the role of CTL in EC cell lysis identified a sub population of CD8⁺CD28⁻ T lymphocytes, that once activated target allogenic EC and induce cell lysis. The same study also challenged the established dogma of Treg cells suppressing lymphocyte activity, showing that the presence of Treg in this system enhanced the lytic activity of CTL (Eissner et al., 2011). This was an important discovery as Treg are commonly studied as potential cellular therapeutic in the treatment of GvHD (Edinger et al., 2003). Following on from this work, MSC were examined herein for their ability to abrogate the MHC Class I restricted lysis of EC by CTL. There are two possible routes for MSC to function in this system; through interaction with the HMEC 24 hours prior to the cytotoxicity assay, or through their interaction with the CTL during the cytotoxicity assay. Size exclusion assays using heart explant cells, similar in size to MSC, eliminated the

possibility that the MSC were simply masking the HMEC and thus interaction sites, while animal studies, such as that by Togel *et al.*, demonstrate how the release of trophic mediators by MSC have a protective and regenerative effect on EC (Togel *et al.*, 2007). In the context of MSC inhibition of CTL, *in vitro* studies have concluded that MSC inhibit the proliferation and cytotoxicity of CTL (Uccelli *et al.*, 2008) without themselves being susceptible to CTL killing (Rasmusson *et al.*, 2007). Given the short exposure time between the MSC and CTL, it stands to reason that the MSC directly affect the EC. It is interesting to note that cell-to-cell contact between EC and MSC is required for their full protective efficacy, which is in contrast to studies with mixed lymphocyte cultures where MSC could inhibit T cell proliferation even in a transwell system (Weiss *et al.*, 2008, Hartmann *et al.*, 2010). However, evidence from inhibitor studies herein suggest a role for PGE2 in EC protection (just like it can be seen in T cell inhibition (Rossi *et al.*, 2012, Hartmann *et al.*, 2010)), yet levels of the prostaglandin increase where MSC and CTL are in contact (data not shown). One hypothesis to explain these results is that of licencing. MSC are known to react to the local milieu, a characteristic elegantly described by Tobin *et al* where in the absence of prior licencing, MSC failed to improve the outcome of *in vivo* GvHD following early administration (Tobin *et al.*, 2013). In this instance, the interaction between MSC and CTL may trigger an increase in PGE2 production resulting in protection of EC. This hypothesis is further supported by the fact that when MSC in transwell experiments failed to achieve the same level of inhibition. The clear abrogation of EC lysis by MSC demonstrated in this study suggests that future adjuvant therapies may look to MSC as an additional treatment option for solid organ rejection and GvHD, particularly in cases where the endothelium is the therapeutic target and Treg cell treatment would be unsuitable.

286 The cooperative relationship between MSC and the endothelial stabilising drug
287 defibrotide demonstrated herein, speaks to their capacity as a potential prophylactic treatment in
288 host conditioning or organ transplantation. This study has also shown that the source of MSC
289 does not alter the protection provided. MSC from placental sources are known to be less
290 immunogenic than those isolated from the bone marrow (Rossi et al., 2012) and thus offer a
291 greater degree of immune privilege for 3rd party transplantation. They also show an increased
292 rate of proliferation allowing for a more efficient isolation (Pianta et al., 2014). Prevention is
293 always better than cure; we have shown that MSC therapy protects the endothelium from
294 targeted lysis, and if used in conjunction with Defibrotide can improve endothelial health,
295 reducing the risk of graft rejection. The health of the endothelium is key to the success of
296 transplant medicine and MSC are demonstrated herein to offer a tangible means of preventing
297 EC lysis from allogenic immune cells.

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Figure Legends

Figure 1. Allogeneic bone marrow MSC protect HMEC from CTL induced lysis. CD8⁺ T cells (CTL) were stimulated with HMEC, treated with 50 μ M Silibinin, for 7 days in the presence of IL-2. Cytotoxicity assays were performed with HMEC cultured 24h previous in the presence (green squares) or absence (red circles) of BM-MSC in the ratio of 5:1 HMEC to MSC. Effector cells (CTL) were cultured with target cells (HMEC) in descending ratios of 20:1, 10:1 and 5:1 for 4 hours. EC lysis was determined by counter staining with propidium iodide (PI) and analysed by flow cytometry (A). Results from multiple experiments were graphed using data from the 20:1 ratio groups (B). n=12, ***P $\leq$ 0.001.

Figure 2. Cell contact is required for MSC protection of EC in cytotoxicity assay. CTL were cultured with HMEC for 7 days. At day -1 5×10^4 HMEC were seeded in a 24 well plate, 1×10^4 BM-MSC were added to the culture either in contact with the HMEC or in a transwell chamber. The cells were cultured for 24h before addition of CTL to the 24 well plate, always in the lower chamber, in contact with the HMEC. As before cells were cultured for 4 hours then counterstained with PI and analysed by flow cytometry (A). PGE2 production was inhibited by the addition of Indomethacin (40 μ M) to the HMEC and BM-MSC co-culture at day-1 (B). Results from multiple experiments were graphed using data from the 20:1 ratio groups. n=3, *P $\leq$ 0.05, ***P $\leq$ 0.001.

Figure 3. Co-culture of HMEC with multiple sources of MSC result in protection from CTL lysis. CTL were cultured with HMEC for 7 days. At day -1 HMEC were co-cultured in the presence or absence of BM-MSC, PVSC or hAMSC at a ratio of 5:1 respectively for 24h.

Targets were analysed for lysis as described in figure legend 1. Data from 4 independent experiments with 4 different PBMC donors. * $P \leq 0.05$.

Figure 4. Defibrotide adds to the protective effect of MSC. CTL were cultured with HMEC for 7 days. At day -1 HMEC were co-cultured in the presence or absence of BM-MSC at a ratio of 5:1, with or without the addition of Defibrotide (25 μ M) for 24h. Targets were analysed for lysis as described in figure legend 1. Results from multiple experiments were graphed using data from the 20:1 ratio groups. n=3, * $P \leq 0.05$, ** $P \leq 0.01$.

Supplementary Figure 1. CTL lysis of HMEC is MHC Class I dependent. CTL were cultured with HMEC for 7 days. At day -1 HMEC were co-cultured in the presence or absence of BM-MSC at a ratio of 5:1, with or without the addition of w6/32 for 24h. Targets were analysed for lysis as described in figure legend 1. Results from multiple experiments were graphed using data from the 20:1 ratio groups. n=3, * $P \leq 0.05$, ** $P \leq 0.01$.

Supplementary Figure 2. MSC size matched cells have no effect on EC lysis by CTL. CTL were cultured with HMEC for 7 days. At day -1 HMEC were co-cultured in the presence or absence of BM-MSC or HH7 cells at a ratio of 5:1 for 24h. Targets were analysed for lysis as described in figure legend 1. Results from multiple experiments were graphed using data from the 20:1 ratio groups. n=3, * $P \leq 0.05$, ** $P \leq 0.01$.

Figure 1

A.

B.

Figure 2

Figure 3

Figure 4

Supplementary
Figure 1

Supplementary
Figure 2