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1 **The secretome of mesenchymal stem cells prevents islet beta cell apoptosis via**
2 **an IL-10-dependent mechanism**
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23
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ABSTRACT

Background: Type 1 diabetes mellitus (T1DM) is partly driven by autoimmune destruction of the pancreatic beta cell, facilitated by the release of inflammatory cytokines including IFNγ, TNF-α and IL-1β by cells of the innate immune system. Mesenchymal stem cells (MSCs) have been used to counteract autoimmunity in a range of therapeutic settings due to their secretion of trophic and immunomodulatory factors that ameliorate disease independently of the cells themselves.

Objective: The aim of this study was to assess the effect of the secretome of human bone-marrow derived MSCs on cytokine-driven beta cell apoptosis.

Methods: All experiments were conducted in two insulin-secreting islet cell lines (BRIN-BD11 and βTC1.6) with selected experiments confirmed in primary islets. MSC secretome was generated by conditioning serum-free media (MSC-CM) for 24 hours on sub-confluent MSC populations. The media was then removed and filtered in readiness for use.

Results: Exposure to IFNγ, TNF-α and IL-1β induced apoptosis in cell lines and primary islets. The addition of MSC-CM to cell lines and primary islets partially reversed cytokine-driven apoptosis. MSC-CM also restored glucose-stimulated insulin secretion in cytokine-treated cell lines, which was linked to improved cell viability following on from cytokine challenge. Characterization of MSC-CM revealed significant concentrations of IL-4, IL-10, PIGF and VEGF. Of these, IL-10 alone prevented cytokine-driven apoptosis. Furthermore, inhibition of IL-10 via the addition of blocking antibody reversed the anti-apoptotic effects of MSC-CM.

Conclusion: Overall, the protective effects of MSC-CM on islet beta cell survival appear to be largely IL-10-dependent.

50 **INTRODUCTION**

51

52 T1DM is a complex autoimmune disease in which several inflammatory cells inflict a
53 coordinated assault on the pancreatic islets of Langerhans and the insulin-producing
54 beta cells therein, contributing to an absolute insulin requirement. Autoimmune beta
55 cell destruction begins when autoantigens (i.e. GAD65) are released during
56 spontaneous turnover of beta cells. The antigens are processed by antigen presenting
57 cells and presented to CD4+ TH1 cells, which secrete cytokines including Interferon
58 (IFN)γ, Tumour Necrosis Factor (TNF)-α, TNF-β, and Interleukin (IL)-2. IFNγ causes
59 macrophages to become cytotoxic and release substantial quantities of cytokines
60 (including IFNγ, TNF-α, and IL-1β) leading to beta cell apoptosis [1]. It is believed that
61 beta cell mass is reduced by 70-80% at the time of diagnosis of T1DM. Due to the
62 absence of detectable beta cell necrosis and variable degrees of insulitis, it has been
63 suggested that beta cell loss occurs slowly over years [2]. This is supported by the
64 detection of insulin antibodies years before the appearance of clinical symptoms in the
65 susceptible individuals [3].

66

67 Mesenchymal stem/stromal cells / Medicinal Signaling Cells (MSCs) are multipotent
68 cells that can be found in almost all adult organs and tissues and are characterized by
69 their immunomodulatory abilities. In the context of diabetes research, MSCs have been
70 used to counteract autoimmunity and enhance islet engraftment and survival [4,5].
71 Despite the reported antidiabetogenic effects of MSCs [6], the mechanism of action
72 remains poorly understood. This is partly due to the diverse range of effects that MSCs
73 and their secreted products have on the surrounding environment. Emerging evidence
74 suggests that the therapeutic utility of MSCs could be based primarily on their
75 production of trophic and immunomodulatory factors. Indeed, the infusion of MSC
76 conditioned media (MSC-CM) every 3 days relieved hyperglycemia in a rodent model

77 of T2DM [7]. The animals showed enhanced concentrations of c-peptide and insulin
78 as well as improvements in glucose metabolism. The authors concluded that these
79 improvements largely stemmed from the secretion of cytokines and growth factors by
80 the MSCs. An earlier study revealed that trophic factors from MSCs aided islet survival
81 and function after transplantation [8]. This study reported high concentrations of IL-6,
82 vascular endothelial growth factor-A (VEGF-A), hepatocyte growth factor (HGF), and
83 transforming growth factor (TGF)-β found in MSC-CM. MSCs secrete soluble factors
84 that play multifactorial roles in the regulation of circulating inflammatory cells. For
85 example, MSCs secrete TGF-β and IL-10, which blocks T-cell proliferation [9,10], while
86 soluble factors secreted by MSCs are also believed to alter the secretion profile of
87 dendritic cells leading to increased production of anti-inflammatory cytokines including
88 IL-10 and decreased production of inflammatory cytokines including IFNγ [11].
89
90 It has been hypothesized that MSCs may offer protection against diabetes via
91 paracrine actions to include cytoprotective, anti-inflammatory, and anti-apoptotic
92 effects [12]. The current study sought to characterize the effect of the secretome of
93 human bone-marrow derived MSCs on cytokine-driven beta cell apoptosis. Here, we
94 report that the secretome of human MSCs protects beta cell lines and primary islets
95 from cytokine-driven apoptosis via an IL-10 dependent mechanism.

96 **METHODS**

97

98 *Beta cell models*

99 All experiments were conducted in two beta cell lines to ensure that data was not
100 skewed by the nuances of any individual cell line. BRIN-BD11 cells were purchased
101 from ECACC General Cell Collection (ECACC 10033003) and cultured as previously
102 described [13]. βTC1.6 cells were purchased from ATCC (ATCC CRL-11506, LGC
103 Standards, UK) and cultured according to the supplier's instructions. In brief, BRIN-
104 BD11 cells were cultured in RMPI and βTC1.6 cells cultured in DMEM (4.5g/L glucose).
105 Both culture media were supplemented with 10% fetal bovine serum (FBS; Lonza, UK)
106 and 1% Penicillin-Streptomycin (Lonza). Cells were routinely passaged with 1x
107 trypsin/EDTA (Lonza).

108

109 Where possible, experimental results were confirmed in primary islets isolated from
110 CD1 mice aged 12-16 weeks and bred in-house. All procedures were conducted in
111 accordance with the Animals Scientific Procedures Act 1986. Animals were euthanized
112 under Schedule 1 methods and the pancreas excised and transferred to Hank's
113 Balanced Salt Solution (HBSS) transport buffer comprising 0.14 M NaCl, 0.005 M KCl,
114 0.001 M $CaCl_2$, 0.0004 M $MgSO_4$, 0.0005 M $MgCl_2$, 0.0003 M Na_2HPO_4, 0.0004 M
115 KH_2PO_4, 0.006 M Glucose, 0.004 M $NaHCO_3$ and 10 mM Hepes. The pancreas was
116 chopped, placed in collagenase P (0.5 mg/ml collagenase clostridium histolyticum,
117 (Fisher, UK) in HBSS), and agitated at 37 °C for 10 minutes followed by the addition
118 of HBSS supplemented with 0.1% Bovine Serum Albumin (Sigma, UK) to neutralize
119 enzymatic action. Pancreatic tissue was then centrifuged for 5 mins at 1000 rpm, the
120 pellet washed three times in wash buffer (HBSS + 5% FBS), the homogenized tissue
121 passed through a fine mesh filter, and the filtrate centrifuged for 5 mins at 1000 rpm.
122 Pelleted islets were resuspended in RPMI media supplemented with 5% FBS and 1%

123 Penicillin-Streptomycin and hand-picked using a fine glass pipette. The islets were
124 maintained in an incubator at 37 °C and 5% CO_2 for 24 hours before experimentation.
125
126 *Cytokine stimulation of beta cell models*
127 Recombinant Tumor Necrosis Factor-α (TNF-α), Interferon Gamma (IFN-γ) and
128 Interleukin-1β (IL-1β) were purchased from PeproTech, UK. Cell lines were seeded at
129 1 x 10^5 cells/cm^2 and allowed to attach overnight. Islets were seeded at a density of 50
130 islets/cm^2 and maintained in culture overnight. Cell lines and islets were then exposed
131 to a range of IFNγ, TNF-α and IL-1β concentrations (0.1 ng/ml - 1000 ng/ml) for 24 h.
132 Determination of cytokine concentrations that induced an approximate reduction in cell
133 viability of 50% was established with MTT (Sigma, UK) in the first instance and
134 induction of apoptosis confirmed by TUNEL assay (TUNEL in situ direct DNA
135 fragmentation kit (Abcam, UK)).
136
137 *Human bone marrow-derived Mesenchymal Stem Cells (MSCs)*
138 Human Bone Marrow Mononuclear cells (hMNCs) were purchased from (Lonza, UK)
139 and hMSCs isolated according to a previously published methodology [14].
140 Mononuclear cells were seeded at a density of 1 x 10^5 MNC/cm^2. Culture vessels were
141 pre-coated with 10 ng/ml of fibronectin (Sigma, UK) in PBS for one hour at room
142 temperature. Seeded MNC cells were maintained in DMEM media supplemented with
143 5% FBS, 1% L-Glutamine (Lonza, UK), 1% Non-essential amino acid (Lonza, UK) and
144 1% Penicillin Streptomycin Amphotericin-B (Lonza, UK). After one week a 50% media
145 change was performed and cells incubated for a further week after which, a 100%
146 media change was performed. Routine media changes were performed twice weekly
147 thereafter. Cells were passaged at 80-90% confluency as described [14].
148

149 hMSC multipotency determination was established via differentiation into osteogenic,
150 adipogenic and chondrogenic cells using chemical induction with differentiation media
151 as outlined in the Supplement and as shown in Figure S1 (Supplement).
152
153 *Preparation of MSC-conditioned media (CM)*
154 MSC conditioned media (MSC-CM) was prepared by medium-cell contact with 70%
155 confluent hMSCs for 24 hours. MSCs were washed once with 10 ml PBS and twice
156 with 10 ml of serum free DMEM. Either 15 ml RPMI-1640 or DMEM media (Lonza, UK)
157 was then left in contact with the MSCs for 24 hours after which the conditioned media
158 was collected, centrifuged to remove any cell debris, filtered through 0.2 μm filter then
159 stored at -80 °C until required for experimental use.
160
161 *Measurements of cellular viability and apoptosis*
162 MTT reagent 5 mg/ml (Sigma, UK) was mixed with RPMI1640 media, added to cells
163 and incubated for 2 hours at 37 °C. MTT solution was removed and DMSO added to
164 each well before incubation at 37 °C for a further 45 minutes. The absorption was
165 measured with a micro-plate reader (Dynatech, MR5000 version 3.7) at a wavelength
166 of 570 nm with a reference wavelength reading at 650 nm.
167
168 Following optimization of cytokine concentration, TUNEL (terminal deoxynucleotidyl
169 transferase mediated deoxyuridine triphosphate nick end labelling) assay (TUNEL in
170 situ direct DNA fragmentation kit (Abcam, UK)) was used to determine if reductions in
171 cell viability observed with the MTT assay resulted from apoptosis. The TUNEL assay
172 was performed following a modified version of the manufacturer's protocol. Media was
173 first removed from cells and islets (islets were gently centrifuged at 900 rpm prior to
174 each step in the following protocol), washed once with PBS, and fixed with 95%
175 methanol for 10 mins. Methanol was removed and the cells washed twice with washing
176 buffer, and then re-suspended in staining solution comprising reaction buffer, TDT

177 enzyme, FITC_dUTP and ddH_2O. The cells were incubated at 37 °C for one hour and
178 staining solution removed. The cells were washed with rinse buffer twice after which
179 DAPI (4,6-Diamidino-2-phenylindole) (Sigma, UK) was added for 30 mins at room
180 temperature. Images of cell lines were acquired by fluorescent microscope (Olympus
181 Fluoview, Nikon Eclipse, Japan) while islets were visualised using a laser scanning
182 confocal microscope (Olympus, Japan).
183
184 *Glucose-stimulated insulin secretion*
185 To determine the effect of MSC-CM on insulin secretion from pancreatic beta cells, cell
186 lines were seeded at a density of 1 x 10^5 cells/cm^2 and allowed to attach overnight.
187 Following this step the cells were treated with pro-inflammatory cytokines (IFN-γ. TNF-
188 α, IL-1β) with and without MSC-CM. Glucose solutions were prepared in 1x Hepes
189 buffered saline (HBS comprising 10 mM Hepes, 145 mM NaCl, 5 mM KCl and 1 mM
190 $MgSO_4$) at three different concentrations (1.1 mM, 5.6 mM and 16.7 mM D-Glucose).
191 After exposing the cells to cytokines for 24 hours, the media was removed and the
192 cells were washed twice with 1 ml HBS followed by addition of 1.1 mM glucose solution
193 for 40 mins. This was then removed and 1.1, 5.6, or 16.7 mM glucose solution added
194 for a further 20 mins after which the supernatant was removed and stored at -20 °C
195 until further analysis. Cells were lysed using 200 µl/well/24well plate RIPA buffer
196 (Sigma) and transferred to fresh tubes, which were maintained on ice with regular
197 vortexing for 20 mins. Lysates were centrifuged at full speed and 4 °C for 20 mins. The
198 total protein present in the resulting supernatants was quantified using the Pierce BCA
199 Protein Assay Kit (Thermo Scientific, UK) according to the manufacturer's instructions.
200 Insulin secretion into the supernatants was quantified using ALPCO ELISA kits
201 (ALPCO, USA) according to the manufacturer's instructions.
202
203 *Characterization of conditioned media*

204 Following identification of candidates from a secretome screen of MSC-CM (Marwan
205 and Forsyth, under review) ELISA was used to quantify the concentration of
206 interleukin-4 (IL-4), interleukin-10 (IL-10), vascular endothelial growth factor (VEGF)
207 and placental growth factor (PlGF) in our MSC-CM. ELISA development kits were
208 purchased from PeproTech (UK) and assays were developed for each cytokine or
209 growth factor according to the manufacturer's instructions.
210
211 *Statistical Analysis*
212 Data are presented as mean ± minus standard deviation (SD) for a given number of
213 observations (n) as indicated in the Figure legends. Groups of data were compared
214 using two-tailed unpaired Student t-tests, or One-way ANOVA with post-hoc test
215 (Graphpad, PRISM software, USA), with significance being accepted if $P<0.05$.

216 **RESULTS**

217

218 *MSC-CM ameliorates cytokine-driven apoptosis in beta cell models*

219 Cytokine concentration was optimized by MTT assay. Following 24h exposure to 1
220 μg/ml IFNγ, 100 ng/ml TNF-α and 100 ng/ml IL-1β, both BRIN-BD11 and βTC1.6 cells
221 displayed significant reductions in cellular viability of up to 50% (Figure 1 and Table
222 S1, Supplement). These concentrations were used for subsequent experiments. In
223 primary islets, the equivalent concentrations were 100 ng/ml IFNγ, 100 ng/ml TNF-α
224 and 100 ng/ml IL-1β (Figure 1 and Table S1, Supplement). In both the BRIN-BD11 and
225 βTC1.6 cell lines, MSC-CM was able to ameliorate (P<0.05 – 0.001) these reductions
226 in cellular viability (Figure 1). Modest improvements (P<0.05) in viability were observed
227 in primary islets in response to both IFNγ and IL-1β in the presence of MSC-CM.
228 However, MSC-CM had little effect on the viability of primary islets following exposure
229 to TNF-α (Figure 1).

230

231 To confirm that observed reductions in cellular viability resulted from apoptosis rather
232 than necrosis, the percentage of TUNEL positive cells was assessed following
233 exposure to optimal concentrations of cytokines, in the presence and absence of MSC-
234 CM (Figure 2A). Exposure to 1 μg/ml IFNγ caused an 8-fold increase in apoptosis in
235 BRIN-BD11 cells (P<0.001) and a 28-fold increase in βTC1.6 cells (P<0.001).
236 Treatment of BRIN-BD11 and βTC1.6 cells with 100 ng/ml TNF-α or 100 ng/ml IL-1β
237 also elicited significant increases in the percentage of TUNEL positive cells (TNF-α: 7-
238 fold increase in BRIN-BD11 cells and 26-fold increase in βTC1.6 cells (P<0.001); IL-
239 1β: 6-fold increase in BRIN-BD11 cells and 21-fold increase in βTC1.6 cells (P<0.001)
240 (Figure 2B). Positive control (1% H_2O_2) resulted in 12- and 39-fold increases in
241 apoptosis (P<0.001) in BRIN-BD11 and βTC1.6 cells respectively (Figure 2B). In all
242 instances, increases in apoptotic frequency in response to cytokine challenge were
243 largely reversed by MSC-CM (Figure 2). Representative images suggest that MSC-

244 CM is also protective against cytokine-driven apoptosis in primary islets (Figure S2,
245 Supplement).
246
247 *MSC-CM restores glucose stimulated insulin secretion by enhancing islet cell viability,*
248 *but not function*
249 Examination of insulin secretion in response to 1.1, 5.6 and 16.7 mM D-glucose before
250 and after the addition of MCS-CM revealed that in all instances, glucose-stimulated
251 insulin secretion from BRIN-BD11 (Figure 3) and βTC1.6 (Figure 4) cells was
252 significantly higher (P<0.01-P<0.001) in the presence of MSC-CM. At stimulatory
253 concentrations of glucose (16.7 mM), a 1.2-fold increase ($P<0.05$) in insulin release
254 was observed in untreated BRIN-BD11 cells cultured in the presence of MSC-CM
255 (Figure 3). A significant impact of MSC-CM on insulin release was not observed in
256 untreated βTC1.6 cells (Figure 4). However, MSC-CM resulted in significant
257 enhancements in insulin release in cells treated with 1 µg/ml IFNγ, 100 ng/ml TNF-α
258 or 100 ng/ml IL-1β for 24h prior to acute exposure to glucose. This observation was
259 true of all glucose concentrations tested and consistent for both the BRIN-BD11
260 (Figure 3) and βTC1.6 (Figure 4) cell lines. Culture of BRIN-BD11 cells in the presence
261 of MSC-CM and 1 µg/ml IFNγ, 100 ng/ml TNF-α or 100 ng/ml IL-1β for 24h prior to
262 exposure to 16.7 mM glucose resulted in 1.5-fold increases in insulin release in all
263 instances ($P<0.001$; Figure 3). In the βTC1.6 cell line, MSC-CM resulted in 1.5-fold
264 increases in insulin release in response to 16.7 mM glucose in cells treated with IFNγ
265 or IL-1β ($P<0.001$) and a 1.7-fold increase in cells treated with TNF-α ($P<0.001$, Figure
266 4).
267
268 With the demonstration that MSC-CM protects against beta cell apoptosis, we wished
269 to determine if the observed increase in insulin secretion was a direct consequence of
270 enhanced beta cell survival. Therefore, data was standardized according to protein
271 concentration, which acted as a surrogate for cell number in this instance. Following

272 standardization, many of the apparent increases in insulin secretion were abolished
273 indicating that improvements in glucose-stimulated insulin secretion likely results from
274 enhancements in the viability of cells, rather than augmentation of beta cell function
275 (Figure 3, BRIN-BD11 cells and Figure 4, βTC1.6).
276
277 *MSCs secrete high concentrations of anti-inflammatory proteins*
278 To explore the content of our MSC-CM, possible candidates were chosen based on
279 data obtained from a secretome cytokine array (Data not shown). MSC-CM was
280 analyzed for IL-4, IL-10, VEGF and PIGF content by commercially available ELISA
281 assays. IL-10 was the most abundant of the candidates and measured at respective
282 concentrations of 3270 ± 378 pg/ml and 3039 ± 122 pg/ml in RMPI1640 and DMEM
283 MSC-CM, respectively (Figure 5). Significant concentrations of VEGF (RPMI1640:
284 2315 ± 61 pg/ml; DMEM: 1423 ± 382 pg/ml), PIGF (RPMI1640: 153 ± 20 pg/ml; DMEM:
285 109± 46 pg/ml) and IL-4 (RPMI1640: 93 ± 7 pg/ml; DMEM: 107 ± 6 pg/ml) were also
286 detected (Figure 5). For comparison, the expression of each candidate protein was
287 also assessed in RMPI1640 or DMEM medium that had not undergone conditioning
288 by MSCs. These proteins were not detected in either medium indicating that they are
289 secreted products of hMSCs (Figure 5).
290
291 *IL-10 confers protection against IFNγ or TNF-α-induced apoptosis.*
292 We next assessed the impact of each of these candidates on cellular viability and
293 apoptosis. BRIN-BD11 and βTC1.6 cells were exposed to rising concentrations (0.01
294 – 100 ng/ml) of recombinant IL-4, IL-10, VEGF and PIGF in the presence or absence
295 of pro-apoptotic IFNγ, TNF-α, and IL-1β. A significant increase in cell viability was
296 noted between cells treated with IFN-γ or TNF-α alone and those treated with IFNγ or
297 TNF-α in the presence of recombinant IL-10. At 1 ng/ml IL-10, BRIN-BD11 cells
298 showed a 46% improvement (P<0.001) in IFNγ-driven reductions in cellular viability
299 and a 25% improvement (P<0.01) in response to TNF-α treatment. Similar findings

300 were observed in βTC1.6 cell lines (Figure 6). IL-10 appeared to have no effect on IL-
301 1β mediated reductions in cellular viability. Furthermore, addition of IL-4, VEGF or
302 PIGF conferred little protection against cytokine-driven reductions in cellular viability
303 (Data not shown).
304
305 We next confirmed that improvements in cellular viability in response to IL-10 resulted
306 from reductions in apoptosis. BRIN-BD11 and βTC1.6 cells were exposed to IFN-γ or
307 TNF-α in the presence or absence of 1 ng/ml IL-10 and induction of apoptosis
308 investigated by TUNEL assay. As shown in Figure 7, significant (P<0.001) reductions
309 in the number of TUNEL positive cells were observed in the presence of IL-10. In the
310 BRIN-BD11 cell line, 95% reductions in the number of TUNEL positive cells were
311 observed in cells treated with IFN-γ and TNF-α in the presence of IL-10, compared
312 with those treated with IFN-γ or TNF-α alone (Figure 7B, P<0.001). In the βTC1.6, the
313 number of TUNEL positive cells observed in response to IFN-γ and TNF-α was
314 reduced by 88% and 84% respectively when IL-10 was present (Figure 7B, P<0.001).
315
316 *The anti-apoptotic action of MSC-CM is largely driven by IL-10.*
317 We evaluated the expression of IL-10 receptors in BRIN-BD11 and βTC1.6 cells at the
318 transcriptional level by RT-PCR. IL-10RA and IL-10RB mRNA transcription was
319 confirmed in both BRIN-BD11 and βTC1.6 cells as shown in Figure S4 (Supplement).
320 To determine if the anti-apoptotic actions of MSC-CM was partly facilitated by the anti-
321 inflammatory action of IL-10, MSC-CM was depleted of IL-10 through the addition of
322 100 ng/ml of anti-IL-10 antibody (PeproTech). IL-10 depleted MSC-CM was applied to
323 BRIN-BD11 and βTC1.6 cells along with 1 µg/ml IFNγ or 100 ng/ml TNF-α. Cell viability
324 was significantly reduced in cells treated with IL-10 depleted MSC-CM (Figure 8). In
325 comparison with cells treated with IFNγ and MSC-CM, BRIN-BD11 cells displayed a
326 31 ± 1.4% reduction in cell viability when cells were treated with IFNγ plus IL-10-
327 depleted MSC-CM. Under the same conditions, βTC1.6 cells displayed a 32 ± 3.7%

328 reduction in viability (Figure 8). Treatment of BRIN-BD11 and βTC1.6 cells with TNF-
329 α plus IL-10 depleted MSC-CM resulted in respective 33 ± 3.2% and 30 ± 4.1%
330 reductions in cell viability when compared with cells cultured in the presence of TNF-α
331 plus complete MSC-CM (Figure 8). Furthermore, an almost complete reversal of the
332 anti-apoptotic effect of MSC-CM was observed in the presence of the anti-IL-10
333 antibody (Figure 9).

334 **DISCUSSION**

335

336 The immunomodulatory effects of MSCs are well established. Although the
337 mechanism remains unclear, several studies have shown that MSC transplantation
338 can improve the metabolic profile of diabetic animal models [15]. Some studies have
339 suggested a cardinal role for the secretome and the paracrine signals it exerts (rather
340 than stem cell differentiation) in the regenerative effects observed following therapeutic
341 stem cell administration [16]. It is thought that the secretome consists of a complex set
342 of proteins, growth factors, cytokines, angiogenic factors, hormones and extracellular
343 matrix proteins [17], which have important biological roles including replication, cell
344 growth, differentiation, and apoptosis [18]. In addition to the direct secretion of soluble
345 factors there is also increasing attention focused on the release of extracellular
346 vesicles (exosomes, microvesicles) carrying potentially therapeutic, bioactive cargo
347 [20–22]. Several studies have investigated the manner by which these soluble factors
348 act and it is generally thought that they may either act directly, by mediating
349 intracellular pathways in injured cells, or indirectly, by inducing the secretion of
350 functionally active products from adjacent tissues [19].

351

352 In the present study, we sought to evaluate the anti-apoptotic effect of the MSC
353 secretome. Conditioned media from human bone marrow derived MSCs (MSC-CM)
354 was added to insulin-secreting cell lines and primary islets in the presence or absence
355 of cytokines known to promote beta cell apoptosis, namely IFNγ, TNF-α, and IL-1β a
356 synergistic effect of these cytokines in the demise of the beta cell [23]. However, the
357 concentrations and interaction of pro-inflammatory cytokines are thought to vary
358 significantly during the development of T1DM. This may explain the different levels of
359 protection achieved through blocking the action of these cytokines in rat models of
360 autoimmune diabetes [24]. The current study sought to understand the individual
361 contribution of each cytokine in beta cell apoptosis and to identify specific mechanisms

362 by which MSC-CM may confer protection against this process. Therefore, cells were
363 exposed to individual cytokines and their independent effects studied.
364
365 Unsurprisingly, IFNγ TNF-α and IL-1β each induced apoptosis in beta cell models and
366 primary islets. Addition of MSC-CM to cell lines and primary islets largely reversed
367 cytokine-driven apoptosis. This is consistent with prior observations that MSCs and
368 their secretome confer protection against cytokine-driven islet loss and that islet
369 function is enhanced islet function via secreted products [25]. Here, MSC-CM restored
370 glucose-stimulated insulin secretion in cytokine-treated cell lines. However, it has been
371 shown that enhancements in insulin secretion in response to MSC co-culture likely
372 result from improvements in cell viability rather than enhancements in the secretory
373 function of the cells [26]. Our insulin secretory data was therefore standardized
374 according to the protein content of the cells. The enhancements in glucose stimulated
375 insulin secretion observed in the presence of MSC-CM were lost upon standardization
376 of the data, suggesting that this effect was indeed linked to improvements in cellular
377 viability rather than any direct enhancement in functionality of the cells.
378
379 Soluble factors secreted by MSCs are believed to alter the secretion profile of dendritic
380 cells leading to increased production of anti-inflammatory cytokines like IL-10 and
381 decreased production of inflammatory cytokines like IFN-γ and TNF-α [11]. MSCs can
382 reduce T cell infiltration into pancreatic islets and the progression to diabetes via the
383 induction of IL10-secreting FOXP3(+) T cells [27]. MSCs produce anti-apoptotic effects
384 not only through their ability to restore the local microenvironment, but also by
385 specifically producing anti-inflammatory or anti-apoptotic proteins including IL-10 [28].
386 Tang and colleagues also report that MSC-treated cardiac cells displayed lower levels
387 of pro-apoptotic factors including Bax and cleaved caspase 3 while the levels of pro-
388 angiogenic factors including VEGF and FGF were increased [29]. Consistently, the

389 restoration of cardiac function in response to MSC therapy has been linked to the
390 secretion of paracrine protective factors rather than myocardial regeneration [30].
391
392 In the current study, characterization of MSC-CM revealed significant concentrations
393 of IL-4, IL-10, PIGF and VEGF. Prior work has shown that IL-4 and IL-10 can directly
394 impact beta cell function and promote beta cell viability. Furthermore, circulating levels
395 of both cytokines are reduced in T1D [31–33]. The potential cytoprotective
396 mechanisms of IL-4 and IL-10 in beta cells are complex. As summarized by Russell
397 and Morgan [34], both cytokines are thought to reduce oxidative stress and to inhibit
398 various inflammatory pathways including NF-κB, likely via stabilization of IκB [34]. The
399 potential roles of PIGF and VEGF in beta cell survival are less well studied. PIGF is a
400 member of the VEGF sub-family with confirmed roles in angiogenesis and vascular
401 regeneration. PIGF-overexpressing transgenic mice displayed inflammation and
402 evidence of metabolic disease when receiving a high fat diet [35]. It has been
403 suggested that VEGF governs the formation of intra-islet capillaries during
404 embryogenesis [36] and improves graft revascularization when islets are implanted
405 [37]. However, of the four abundant candidates identified in our MSC-CM only IL-10
406 was found to prevent cytokine-driven apoptosis. Importantly, the anti-apoptotic effect
407 of IL-10 was only observed in response to IFNγ, or TNF-α challenge and not in
408 response to IL-1β suggesting that the pathways involved are highly specific. IL-10
409 signals via unique receptor complexes that do not share homology with IL-4, PIGF, or
410 VEGF signaling. Expression of both isoforms of the IL-10 receptor (IL-10RA and IL-
411 10RB) was confirmed in the two cell lines used in this study (Supplement, Figure S3),
412 which is consistent with findings in human islets [34]. Indeed, inhibition of IL-10 action
413 by addition of blocking antibody reversed the anti-apoptotic effects of MSC-CM.
414 Overall, the protective effects of MSC-CM on islet cell survival appear to be largely IL-
415 10-dependent.

416 **CONCLUSION**

417

418 In this study we show that (1) factors secreted from MSCs are sufficient to promote
419 islet beta cell survival in response to cytokine challenge, (2) that this increase in
420 survival is able to sustain glucose-stimulated insulin secretion in the face of
421 inflammatory challenge and (3) that IL-10 plays a significant part in the anti-apoptotic
422 effects of MSC-CM, which may indicate some of the mechanisms by which MSCs
423 confer protection on pancreatic islet beta cells.

424 **DECLARATION OF INTERESTS**

425 The authors have no conflicts of interest to declare

426

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431 (HSC R&D) & Ulster University

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559 1.

560 **FIGURE LEGENDS**

561

562 **Figure 1: MSC-CM protection against cytokine-driven reductions in islet cell**
563 **viability.**

564 Following exposure to increasing concentrations of IFNγ, TNF-α or IL-1β for 24 h, the
565 cellular viability of BRIN-BD11 cells, βTC1.6 cells and primary islets grown in standard
566 non-conditioned medium (NCM) or MSC-conditioned medium (CM) was assessed by
567 colorimetric MTT assay. Data are normalized to untreated controls and presented as
568 mean ± standard deviation (SD). n=4 with all experiments assayed in duplicate.
569 **P*<0.05, ***P*<0.01, ****P*<0.001 and *****P*<0.0001 compared with non-conditioned
570 media. MSC-CM, Mesenchymal Stem Cell Conditioned Medium; IFNγ, Interferon
571 gamma; TNF-α, tumour necrosis factor alpha; IL-1β, Interleukin 1 beta.

572

573 **Figure 2: MSC-CM confers protection against cytokine-driven apoptosis in islet**
574 **cell models.**

575 BRIN-BD11 and βTC1.6 cells were exposed to 1 μg/ml IFN-γ, 100 ng/ml TNFα, and
576 100 ng/ml IL-1β for 24h in the presence or absence of MSC-CM and the induction of
577 apoptosis assessed by TUNEL assay. **(A)** Fluorescent images showing the ability of
578 MSC-CM to reduce the % positive TUNEL cells after cytokine challenge. 1% H_2O_2
579 acted as a positive control in these experiments. Blue staining represents DAPI
580 staining of the nuclei while green staining indicates TUNEL positive cells **(B)** The %
581 positive TUNEL cells was measured by calculating the number of TUNEL positive cells
582 divided by the total number of cells. Data are presented as mean ± standard deviation
583 (SD) with n=3. ****P*<0.001 compared with untreated controls. The scale bar in all
584 images equals 100 μm. MSC-CM, Mesenchymal Stem Cell Condition Media; IFNγ,
585 Interferon gamma; TNF-α, tumour necrosis factor alpha; IL-1β, Interleukin 1 beta.

586

587

588 **Figure 3: MSC-CM restores glucose-stimulated insulin secretion in BRIN-BD11**
589 **cells following cytokine challenge.**
590 BRIN-BD11 cells were exposed to 1 µg/ml IFN-γ, 100 ng/ml TNFα, and 100 ng/ml IL-
591 1β for 24h prior to exposure to rising concentrations of D-Glucose (1.1, 5.6, 16.7 mM)
592 in the presence or absence of MSC-CM. Insulin secretion was measured by ELISA
593 and data presented according to insulin concentration or insulin concentration as a
594 function of protein content. Data are presented as mean ± standard deviation (SD).
595 n=4 with all experiments assayed in duplicate. $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$
596 compared with corresponding treatments in the absence of MSC-CM. MSC-CM,
597 Mesenchymal Stem Cell Conditioned Media; IFN-γ, Interferon gamma; TNFα, Tumour
598 Necrosis Factor alpha; IL-1β, Interleukin-1 beta.
599
600 **Figure 4: MSC-CM restores glucose-stimulated insulin secretion βTC1.6 cells**
601 **following cytokine challenge.**
602 βTC1.6 cells were exposed to 1 µg/ml IFN-γ, 100 ng/ml TNFα, and 100 ng/ml IL-1β for
603 24h prior to exposure to rising concentrations of D-Glucose (1.1, 5.6, 16.7 mM) in the
604 presence or absence of MSC-CM. Insulin secretion was measured by ELISA and data
605 presented according to insulin concentration or insulin concentration as a function of
606 protein content. Data are presented as mean ± standard deviation (SD). n=4 with all
607 experiments assayed in duplicate. $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$ compared with
608 corresponding treatments in the absence of MSC-CM. MSC-CM, Mesenchymal Stem
609 Cell Conditioned Media; IFN-γ, Interferon gamma; TNFα, Tumour Necrosis Factor
610 alpha; IL-1β, Interleukin-1 beta.
611
612 **Figure 5: Quantification of candidate anti-inflammatory and anti-apoptotic**
613 **proteins in MSC-CM**
614 The concentration of candidate anti-inflammatory or anti-apoptotic proteins in MSC-
615 CM was quantified by ELISA assays. The results showed a high concentration of IL-

616 10 in MSC-CM Irrespective of whether RMPI1640 (**A**) or DMEM (**B**) medium was
617 conditioned. Candidates were not detected in non-conditioned media. Data are
618 presented as mean ± standard deviation (SD). N=3 with samples assayed in duplicate.
619
620 **Figure 6: Recombinant IL-10 protects against IFN-γ and TNF-α-driven reductions**
621 **in cellular viability.**
622 The viability of BRIN-BD11 and βTC1.6 cells was assessed by calorimetric MTT assay
623 after exposure to IFNγ, TNF-α, or IL-1β ± rising concentrations of IL-10 as indicated in
624 the Figure. Data are normalized to untreated controls and presented as mean ±
625 standard deviation (SD). N=4 with all experiments assayed in duplicate. ***P*<0.01 and
626 ****P*<0.001. IFNγ, Interferon gamma; TNF-α, tumour necrosis factor alpha; IL-1β,
627 Interleukin 1 beta; IL-10, Interleukin 10.
628
629 **Figure 7: Recombinant IL-10 protects against IFN-γ and TNF-α-driven apoptosis.**
630 **(A)** Fluorescent images showing the ability of IL-10 (1 ng/ml) to reduce the % positive
631 TUNEL cells after treatment with a combination of IL-10 and 1 μg/ml of IFN-γ or 1 μg/ml
632 of TNF-α. 1% H_2O_2 acted as a positive control in these experiments. Blue staining
633 represents DAPI staining of the nuclei while green staining indicates TUNEL positive
634 cells **(B)** The % positive TUNEL cells were measured by calculating the number of
635 positive TUNEL cells divided by the total number of cells. Data is presented as mean
636 ± standard deviation (SD) with n=3. ****P*<0.001 and *****P*<0.0001 compared with
637 untreated controls. The scale bars in all images equal 100 μm. IFNγ, Interferon
638 gamma; TNF-α, tumour necrosis factor alpha; IL-10, Interleukin 10.
639
640 **Figure 8: MSC-CM protection against beta cell susceptibility to IFN-γ or TNF-α**
641 **challenge is IL-10-dependent**
642 The viability of BRIN-BD11 (A) and βTC1.6 cells (B) was assessed by colorimetric MTT
643 assay after the addition of 100 ng/ml of anti-IL-10 ± IFN-γ or TNF-α. Data are

644 normalized to untreated controls and presented as mean ± standard deviation (SD).
645 n=3 with all experiments assayed in duplicate. $^{**}P<0.01$ and $^{***}P<0.001$ compared
646 with corresponding non-conditioned media control (i.e. RMPI or DMEM). MSC-CM,
647 Mesenchymal Stem Cell Conditioned Media; IFN-γ interferon gamma, TNF-α tumour
648 necrosis factor alpha.

649

650 **Figure 9: Blockage of IL-10 prevents the anti-apoptotic activities of MSC-CM.**
651 BRIN-BD11 and βTC1.6 cells were exposed to 1 μg/ml IFN-γ or 100 ng/ml TNFα, for
652 24h in the presence or absence of IL-10 depleted MSC-CM (100 ng/ml anti-IL-10
653 antibody) and the induction of apoptosis assessed by TUNEL assay. **(A)** Fluorescent
654 images showing the inability of IL-10 depleted MSC-CM to reduce the % positive
655 TUNEL cells after cytokine challenge. 1% H_2O_2 acted as a positive control in these
656 experiments. Blue staining represents DAPI staining of the nuclei while green staining
657 indicates TUNEL positive cells **(B)** The % positive TUNEL cells was measured by
658 calculating the number of TUNEL positive cells divided by the total number of cells.
659 Data are presented as mean ± standard deviation (SD) with n=3. $^{***}P<0.001$
660 compared with untreated controls. The scale bar in all images equals 100 μm. MSC-
661 CM, Mesenchymal Stem Cell Condition Media; IFNγ, Interferon gamma; TNF-α,
662 tumour necrosis factor alpha; IL-10, Interleukin 10.