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Mesenchymal stromal cell secretory factors induce sustained improvements in islet function pre- and post-transplantation

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Abstract

Background. Mesenchymal stromal cells (MSCs) enhance islet function both *in vitro* and *in vivo*, at least in part by secreting ligands which activate islet G-Protein Coupled Receptors (GPCRs). We assess whether pre-treatment with a defined "cocktail" of MSC-secreted GPCR ligands enhances islet functional survival *in vitro* and improves the outcomes of islet transplantation in an experimental model of diabetes. **Methods.** Isolated islets were cultured for 48h with ANXA1, or SDF-1, or C3a, alone or in combination. Glucose stimulated insulin secretion (GSIS) and cytokine-induced apoptosis were measured immediately after the 48h culture period, and at 24h or 72h following removal of the ligands from the culture media. Islets were syngeneically transplanted underneath the kidney capsule of streptozotocin-induced diabetic C57BL/6 mice and blood glucose levels monitored for 28 days. **Results.** Preculturing islets with a cocktail of ANXA1/SDF-1/C3a potentiated GSIS and protected islet cells from cytokine-induced apoptosis *in vitro*. These effects were maintained for up to 72h after the removal of the factors from the culture medium, suggesting a sustained protection of islet graft functional survival during the immediate post-transplantation period. Islets pre-treated with the cocktail of MSC secretory factors were more effective in reducing blood glucose in diabetic mice, consistent with their improved functional survival *in vivo*. **Discussion.** Preculturing islets with a cocktail of MSC secretory products offers a well-defined, cell-free approach to improve clinical islet transplantation outcomes while avoiding many of the safety, regulatory and logistical hurdles of incorporating MSCs into transplantation protocols.

Key Words: Diabetes, Islet Transplantation, Mesenchymal Stromal Cells, Secretome

Abbreviations

ANXA1	Annexin A1
C3a	Complement component C3a
GPCR	G-Protein Coupled Receptor
GSIS	Glucose Stimulated Insulin Secretion
MSC	Mesenchymal Stromal Cells
SDF-1	Stromal Cell Derived Factor 1
STZ	Streptozotocin
T1D	Type 1 Diabetes

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Introduction

Islet transplantation as a therapy for Type 1 Diabetes (T1D) is restricted by the limited availability of donor islets, loss of functional islets during pre-transplantation culture *in vitro* [1, 2] and further extensive loss during the immediate post-transplantation period when islet function and survival is compromised by the hypoxic, inflammatory host environment [3-5]. In experimental models a pre-transplant culture *in vitro* with mesenchymal stromal cells (MSCs) enhances β-cell secretory function *in vitro* [6-10], which correlates with superior graft function *in vivo* [6, 7]. The direct beneficial effects of MSCs on islet function can be attributed, at least in part, to paracrine effects of MSC-derived soluble mediators [11] and to MSC-generated extracellular matrix (ECM) [12], whilst MSC effects on the host niche may improve the survival of islet grafts by suppressing adverse immune responses [13-17] and enhancing revascularisation [18-20]. Identifying MSC secretory factors which improve islet function and transplantation outcomes raises the possibility of harnessing the therapeutic benefits of MSCs in a "cell-free" strategy [11, 21], thus avoiding the logistical, safety and regulatory concerns of including MSCs in clinical islet transplantation protocols.

G-protein coupled receptors (GPCRs) are the target for around 30% of known pharmaceutical therapeutics, and we have previously used a quantitative mRNA screening approach to identify over 250 different GPCRs expressed by islet cells [22] offering considerable scope for manipulating islet function. We have recently applied a similar approach to identify a panel of mouse and human MSC secretory products that are ligands for islet G-protein-coupled

receptors (GPCRs), and so have the potential to influence islet functional survival [11]. We have now screened these mouse [11] and human [12] MSC "secretomes" based on (i) high expression levels in MSC populations known to enhance islet functional survival; (ii) expression of their cognate receptors in mouse and human islets [22]; (iii) published evidence of their ability to influence islet functional survival [23-27]. These screens identified collagen-3A1 (COL3A1); annexin A1 (ANXA1); stromal cell-derived factor-1 (SDF-1)/chemokine (C-X-C motif) ligand (CXCL) 12; and complement component C3 as the most highly expressed islet GPCR ligands in mouse and human MSC populations. The main function of COL3A is likely as a component of ECM, and we have recently reported that decellularized, MSC-derived ECM has beneficial effects on mouse and human islet function [12]. Similarly, we have previously reported beneficial effects of ANXA1 on mouse [11] and human [12] islet function, with ECM acting as a reservoir for ANXA1 [12]. In the current study we have therefore focused on the potential of SDF-1 and C3a to influence islet functional survival in the context of a pre-implantation cocktail therapy. We suggest that this MSC-based, non-cellular approach will enable simple and defined modifications to transplantation protocols to improve the efficiency of clinical islet transplantation.

Methods

Islet isolation and culture

Islets were isolated by collagenase digestion (1mg/ml; type XI; Sigma-Aldrich, Poole, UK) followed by density gradient separation (Histopaque-1077; Sigma-Aldrich). Briefly, 2-3ml of cold (4^{o}C) collagenase solution (1mg/ml) was injected into the pancreas via the common bile duct following clamping at the ampulla of vater. The distended pancreas was removed and incubated in a water bath at 37°C for 10min. After washing with MEM supplemented with 10% newborn calf serum ,100U/ml penicillin and 0.1mg/ml streptomycin the pancreatic tissue

was vortexed and passed through a sieve to discard contaminating exocrine tissue. A purification gradient was generated by adding Histopaque® to the pancreatic tissue. The pancreatic tissue in histopaque was vortexed briefly and 10ml of MEM plus supplements added before centrifugation at 1900 x g, 10°C for 24min. Islets were removed from the interphase of the histopaque and MEM and washed three times in RPMI-1640 medium. Islets were hand-picked into groups of 100 for preculture in RPMI supplemented with 10% Foetal Bovine Serum (FBS) and 100U/ml penicillin plus 0.1mg/ml streptomycin either alone, with recombinant human ANXA1-alone, recombinant mouse SDF-1-alone, recombinant mouse C3a-alone, or with combinations of these factors (R & D Systems, Abingdon, UK). Human ANXA1 has 88% sequence homology with mouse and rat ANXA1 [28] and we have demonstrated previously that human recombinant ANXA1 has similar functional effects on both human and mouse islets [11, 12].

C3a and SDF-1 ELISA

To confirm the expression of C3a and SDF-1 protein in MSC lysates and to determine whether C3a and SDF-1 are released into the MSC culture media (DMEM supplemented with 10% Foetal Bovine Serum (FBS) and 100U/ml penicillin + 0.1mg/ml streptomycin), MSCs were seeded into Nunclon™ 35mm petri dishes, to mimic the experimental set-up utilised for our direct contact islet-MSC co-culture system [6, 7]. After 2 days, MSCs from each petri dish were trypsinised and resuspended in ice-cold PBS supplemented with cOmplete ULTRA mini protease inhibitors (Roche Diagnostics, Burgess Hill, UK), then sonicated. The MSC-conditioned media (CM) from each Petri dish was also collected and concentrated x24 using 3,000NMWL Amicon® Ultra 2ml centrifugal filters (Merck Millipore, Middlesex, UK). Control samples were MSC culture media alone, which was concentrated x24. C3a and SDF-1 were measured in MSC lysates and CM using ELISAs (Cloud-Clone Corp, Houston, USA and R&D Life Sciences, Abingdon, UK).

Islet secretory function in vitro

Insulin secretion *in vitro* was assessed using static incubations of isolated islets. Islets were pre-incubated for 2h in RPMI containing 2mmol/L glucose. Groups of three islets were transferred into 1.5ml Eppendorf tubes and incubated at 37°C in a bicarbonate-buffered physiological salt solution containing 2mmol/L $CaCl_2$ and 0.5mg/ml BSA [29] and either 2 or 20mmol/L glucose. After 1h, samples of the incubation medium were taken and stored at -20°C until assayed for insulin content using in-house RIA [30, 31].

Islet Apoptosis in vitro

To assess caspase 3/7 activity, luciferase activity dependent on caspase 3/7 – mediated generation of luciferase substrate was measured, according to the manufacturer's instructions (Promega, Southampton, UK). Islets were precultured alone or with recombinant factors as described for individual experiments. For the final 20h of the culture period, half of the islets in each culture dish were exposed to mixed cytokines (50U/ml IL-1β, 1000U/ml IFN-γ and 1000U/ml TNF-α (PeproTech, London, UK) and half of the islets served as controls without cytokines. All islets were incubated in serum-free media for this final 20h. Islets were picked into groups of 5 islets/well and Caspase-Glo 3/7 reagent was added. After 1 hr, light emission was detected using a Turner Biosystems Veritas microplate luminometer (Promega).

Experimental animals

Male CD1 mice (Charles River, Margate, Kent) aged 8-12 weeks were used as islet donors for *in vitro* investigations. Male C57Bl/6 mice (Envigo, Huntingdon, UK) aged 8-12 weeks were used as donors and recipients for syngeneic islet transplantations. Mice were made diabetic by intraperitoneal streptozotocin (STZ) injection (180mg/kg; Sigma-Aldrich) one week prior to transplantation and those with a non-fasting blood glucose concentration of ≥20mmol/l for

more than three consecutive days were used as recipients. Blood glucose concentrations were determined using a blood glucose meter and strips (Accu-Chek; Roche, Burgess Hill, UK) with blood obtained from a pin prick to the tail. Where blood glucose levels exceeded the upper limit of the Accu-Chek meter, a stat strip express meter and strips was used (upper limit 50mmol/L). All animal procedures were approved by our institution's Ethics Committee and carried out under license, in accordance with the UK Home Office Animals (Scientific Procedures) Act 1986.

Renal subcapsular islet transplantation

Mice were transplanted with 150 islets, precultured either alone, with 5nmol/L recombinant ANXA1-alone, or with a cocktail of 5nmol/L ANXA1, 10nmol/L SDF-1 and 10nmol/L C3a, for 2 days prior to transplantation. Mice were anaesthetised with 1–5% isoflurane and 95% oxygen (1l/min). Carprofen (Caprieve, 4mg/k.g; Norbrook, UK) was administered subcutaneously prior to the surgical procedure. A lumbar incision was made, the kidney exposed and an incision made in the capsule. Islets that had been centrifuged into pellets in PE50 polyethylene tubing (Becton Dickinson, Sparks, MD, USA) were placed underneath the kidney capsule using a Hamilton syringe (Fisher, Pittsburg, PA, USA). Bupivacaine (Marcaine 0.5% solution, 2mg/kg, Aspen Medical, UK) was administered subcutaneously at the transplantation site for post-operative analgesia. Body weight and blood glucose concentrations of recipient mice were monitored every day for the first week post-transplantation and every 3 to 4 days thereafter. At the end of the 28-day monitoring period the graft-bearing kidney and STZ-pancreas were removed for analysis of insulin content.

Statistical analysis

Statistical analysis used Student's *t* test or ANOVA, as appropriate. Two-way repeated-measurement ANOVA was used with Bonferroni's post hoc test to analyse repeated

measurements in the same animal at different time points. A p value of $p < 0.05$ was considered significant. All data are expressed as means ± SEM.

Results

Effects of SDF-1 and C3a on islet function in vitro

Mouse adipose MSCs contained and secreted substantial amounts of both SDF-1 and C3a confirming that the high level of their mRNAs previously reported [11] is translated into secreted proteins. MSC extracts contained 7.7±1.8ng SDF-1 per 200,000 cells (n=6), and analysis of MSC-conditioned media showed that approximately 20% of this SDF-1 was released into the medium over 48h (1.7ng±0.1ng/well, n=6). MSC extracts contained 14.1±2.3pg C3a per 200,000 cells (n=5), and over 60% of this was released into the medium over 48h (12.0±0.9pg/well (n=5)). Control samples of MSC culture media-alone (supplemented with 10% FBS and 100U/ml penicillin plus 0.1mg/ml streptomycin) contained comparatively low amounts of SDF-1 and C3a immunoreactivity (<0.1% of MSC- conditioned media).

We have previously reported the expression of the GPCRs for SDF-1 and C3a on both mouse and human islets, these being CXCR4/CXCR7 and C3aR, respectively [27, 32]. To determine whether exogenous SDF-1 or C3a influence islet functional survival we measured cytokine-induced apoptosis and glucose-stimulated insulin secretion (GSIS) in islets that had been precultured for 48h with nmol/L concentrations of recombinant proteins. Initial experiments assessed the effects of SDF-1 and C3a alone over a range of concentrations to determine which were effective in our *in vitro* screens of islet insulin secretory function and cytokine-induced apoptosis. The optimal concentrations were standardised for subsequent experiments investigating dual and triple combinations of SDF-1 and/or C3a with previously investigated

ANXA1. Preculturing islets for 48h with SDF-1 protected them from inflammatory cytokines in a dose-dependent manner, with a statistically significant effect observed at 10nmol/L SDF-1 (Figure 1A). The protective effect of this concentration of SDF-1 was reproducible between different islet preparations and increasing the concentration to 20nmol/L conferred no further protection (Figure 1B). Preculturing islets with SDF-1 for 48h had no influence on GSIS (Figure 1C), in contrast to the stimulatory effects of preculture with ANXA-1, which were consistently observed (Figure 1D [11]). Preculture with a dual combination of ANXA1 and SDF-1 did not further potentiate GSIS over effects seen with ANXA1-alone (Figure 1D). Thus, SDF-1 preculture protected islets from cytokine-induced apoptosis but did not influence GSIS.

In contrast, preculturing islets for 48h with 10nmol/L C3a potentiated GSIS, with no further potentiation observed at 100nmol/L C3a (Figure 2A). Concentrations of C3a lower than 10nmol/L had no reproducible effect on GSIS (data not shown). Figure 2B shows that preculture with 10nmol/L C3a was equally effective as preculture with ANXA1 at enhancing GSIS [11]. However, the effects of C3a and ANXA1 on GSIS were not additive, (Figure 2C). Similarly, in separate experiments, we observed no significant additive effects on GSIS of islet preculture with a cocktail of ANXA1/C3a/SDF-1 (islets precultured alone, 2.6±0.3ng/islet/h; ANXA1, 3.2±0.5ng/islet/h; ANXA1/C3a, 2.9±0.4ng/islet/h; ANXA1/C3a/SDF-1, 3.4±0.2ng/islet/h, n=10, $p > 0.05$). Preculturing islets for 48h with C3a-alone had a similar protective influence on cytokine-induced apoptosis to that observed with ANXA1-alone (Figure 2D) or SDF-1-alone (Figure 1A, B), but the anti-apoptotic effects of C3a and ANXA1 were not additive (Figure 2D). Thus, C3a preculture had similar effects to ANXA1 to enhance GSIS and to protect islets from cytokine-induced apoptosis but there was no additive influence to enhance the functional survival of islets at a time point reflecting that at which islets would typically be transplanted, immediately after the preculture period.

Sustained improvement in islet function following preculture with ANXA1, SDF-1 and/or C3a

Having demonstrated that 48h preculture with ANXA1, SDF-1 and/or C3a exerted effects on islets which are likely to be beneficial during the immediate post-transplantation period, we next assessed whether the beneficial effects persisted after removal of the islet-GPCR ligands from the culture medium for 24-72h, since in the transplantation setting these MSC-biotherapeutics would be present during the *in vitro* preculture period, but absent post-transplantation *in vivo*.

We examined the effects on GSIS and apoptosis of a 48h preculture with ANXA1, SDF-1 and C3a, alone or in combination, at 24h and 72h after their removal from the culture medium, to determine whether the beneficial effects would persist throughout a time period reflecting the immediate post-transplantation period (Figure 3). Preculture with ANXA1+SDF1 had beneficial effects on GSIS which persisted for 24h after removal of the ligands from the medium (Figure 3A), but this was not maintained to 72h (Figure 3B). ANXA1+C3a preculture did not have sustained effects on GSIS at 24h (Figure 3A) or 72h (Figure 3B) after their removal from the medium. Preculturing islets with a cocktail of ANXA1+SDF-1+C3a had no additional effects to that of dual ANXA1+SDF-1 preculture at 24h (Figure 3A), but by 72h after removal of the MSC-biotherapeutics, only the cocktail of all three islet-GPCR ligands (ANXA1+SDF-1+C3a) caused a persistent potentiation of GSIS (Figure 3B). Figure 3 (C, D) shows the sustained effects of preculture with ANXA1, SDF-1 and/or C3a on the apoptotic responses of islets to inflammatory cytokines. When assessed 24h after removal of the ligands from the medium (Figure 3C), apoptosis was reduced in islets precultured with ANXA1-alone, or ANXA1 in any combination with the other ligands. C3a-alone also caused a reduction in apoptosis but this did not reach statistical significance (Figure 3C). However, by 72h after removal of the ligands, only the cocktail of ANXA1+SDF-1+C3a preculture induced a

sustained and significant reduction of apoptosis when the islets were exposed to inflammatory cytokines (Figure 3D). Thus, preculture of islets with a cocktail of MSC secretory factors influences islet insulin secretory and apoptotic responses for up to 72h after their exposure.

Effects of preculture on in vivo function of transplanted islets

Preculturing islets for 48h with a cocktail of ANXA1, SDF-1 and C3a immediately prior to their transplantation into severely hyperglycaemic mice had modest effects on their ability to regulate blood glucose *in vivo*, as shown in Figure 4. Thus, the average blood glucose concentrations of mice transplanted with ANXA1/SDF-1/C3a precultured islets were consistently lower than those of mice transplanted with islets precultured alone (Figure 4A), with a significant decrease in AUC (Figure 4B). We have previously reported a modest effect of preculture with ANXA1-alone on islet graft function [11] and we observed a similar effect in the current study, where mice receiving islets precultured with ANXA1-alone had lower blood glucose than mice transplanted with islets precultured alone at all time points, although this did not achieve statistical significance (Figure 4A). Notably, the average blood glucose concentrations of mice transplanted with ANXA1+SDF-1+C3a precultured islets were consistently lower than those of mice transplanted with ANXA1-alone precultured islets (Figure 4A, B). In terms of reversing hyperglycaemia (i.e. non-fasting blood glucose concentrations ≤ 11.1 mmol/L for at least two consecutive readings), by the end of the 28-day study this was achieved in 2/8 mice transplanted with ANXA1+SDF-1+C3a precultured islets, 1/9 mice transplanted with ANXA1-alone precultured islets, and 0/7 of mice transplanted with islets precultured alone.

At the end of the 28-day study there was considerable variation in the graft insulin content, ranging from 1.0-2.2µg, 0.7-6.9µg and 0.9-5.7µg in mice transplanted with control islets, ANXA1-pre-treated islets, and ANXA1/SDF-1/C3a-pre-treated islets, respectively. The mean

insulin content was approximately 30% higher in grafts consisting of ANXA1-alone precultured islets and 50% higher in grafts consisting of ANXA1/SDF-1/C3a precultured islets, compared to those consisting of islets precultured alone, although this was not statistically significant (1.51+0.17µg/graft vs. 1.98+0.63µg/graft vs. 2.27+0.77µg/graft, islet-alone vs. ANXA1-alone vs. ANXA1/SDF-1/C3a precultured islets, respectively). The insulin content of the pancreas in all STZ-treated mice was approximately ten times lower than that of the subcapsular islet grafts. There was no difference in the STZ-pancreas insulin content for mice transplanted with islets precultured alone, with ANXA1-alone, or with the cocktail of MSC-derived biotherapeutics (0.22±0.02µg vs. 0.21±0.03µg vs. 0.21±0.03µg, $p > 0.05$ n=8, One-Way ANOVA with Dunnet's post hoc test).

Discussion

Recent experimental studies have demonstrated the beneficial effects that MSCs exert directly on islet function and on the outcomes of islet transplantation [6-9], but these observations have yet to translate into improvements in human islet function for clinical islet transplantation in T1D. One reason for this is that currently human islets are almost exclusively implanted via the hepatic portal vein [33], which does not facilitate co-engraftment of islets and MSCs because their different sizes influence where they will lodge in the circulation. Thus, after intraportal delivery the islets (100-200µm diameter) lodge in the hepatic microvessels, while the much smaller MSCs will most likely pass through the microvessels to lodge in capillary beds of the liver or other organs [34]. An alternative strategy to co-transplanting islets and MSCs is a pre-transplantation co-culture period *in vitro*, and studies in rodent models have reported enhanced islet function [8, 9] which correlates with improved transplantation outcomes [6, 7]. However, there are also impediments to applying these observations to clinical islet transplantation protocols. For example, scaling up co-culture systems to accommodate the large numbers of human islets and MSCs required for clinical transplantation is technically

challenging. In addition, the inherent heterogeneity in different MSC populations [35] presents problems in standardisation. However, the recent demonstration that some of the beneficial effects of MSCs on islet function are mediated through MSC secretory products [11, 12] offers the potential of using these molecules in defined protocols to improve islet functional survival, thus avoiding the logistical, safety and regulatory problems of including MSCs in clinical islet transplantation protocols. The current study therefore focused on defining a cocktail of MSC-derived secretory molecules with the potential to improve the functional survival of islet grafts. We have previously reported that the most highly expressed of these molecules, Annexin A1 (ANXA1), mimicked the beneficial effects of MSC preculture on β-cell insulin secretory function *in vitro*, but had only modest effects on the capacity of an islet graft to regulate blood glucose in hyperglycaemic mice, suggesting that additional MSC secretory products factors are needed to fully mimic the beneficial effect of MSCs *in vivo* [11]. In the present study we have therefore investigated the effects of other MSC-secreted islet GPCR ligands, alone or in combination with ANXA1, on the function and survival of islets during an *in vitro* preculture period, and on subsequent transplantation outcomes.

SDF-1 is a peptide chemokine initially identified in bone marrow stromal cells, but now known to be expressed in stromal tissues of many organs [36], including the microvascular endothelium of pancreatic islets [25]. We here demonstrate that MSC populations which have beneficial effects on islet functional survival synthesise and secrete substantial amounts of SDF-1. Our observation that pre-incubation with SDF-1 protected isolated islets from subsequent cytokine-induced apoptosis is consistent with previous studies demonstrating that SDF-1 has an important role in cell survival responses. SDF-1 is upregulated in stromal tissues and β-cells in response to injury [24] suggesting an endogenous pro-survival function within islets. Similarly, exogenous SDF-1 reduced apoptosis in the INS-1 β-cell line [37], while experimental overexpression of SDF-1 in β-cells protected them from STZ- and thapsigargin-

induced apoptosis [24]. However, exposure to SDF-1 in our pre-treatment protocol had no detectable effect on GSIS, in direct contrast to the beneficial effects of ANXA1 on GSIS in this study, and in our previous studies [11]. These observations suggest that SDF-1 and ANXA1 influence β-cell functional survival via distinct mechanisms, and thus validate the inclusion of both molecules in an islet pre-treatment regimen.

C3, a key protein in the complement system, is converted by proteolytic cleavage to biologically active products, including C3a. The adipokine adipsin (complement factor D) improves islet β-cell function by increasing the conversion of circulating C3 into C3a, which has acute effects to increase islet oxygen consumption rate [26], and is a potent stimulator of insulin secretion in mouse [26] and human islets [27]. We here demonstrate that MSCs secrete C3a into the culture media, and that preculture with exogenous C3a has persistent effects on islet function, consistent with C3a being a soluble mediator through which MSCs influence islet function. The effects of C3a pre-treatment on islet functional survival were similar to those of ANXA1, both enhancing GSIS and reducing cytokine-induced apoptosis, and the effects of C3a and ANXA1 were not additive on either functional end-point. The lack of additive effects may suggest similar mechanism(s) through which both molecules influence islet cell function, or it may be a consequence of the endpoints for assessing islet function *in vitro*. Thus, preculture with either molecule essentially suppressed cytokine-induced apoptosis, such that additive effects would not be readily detected in this *in vitro* model. Similarly, if preculture with either molecule maintains optimal GSIS at the 48h time-point immediately following the preculture period, it is unlikely that additive effects would be detected. Nonetheless, the demonstrable effects of C3a preculture on islet secretory function and survival validate its inclusion in an islet pre-treatment regimen.

It is perhaps not surprising that exposure to biologically active molecules secreted by MSCs influenced the ability of islet β-cells to maintain insulin secretion and resist inflammatory

cytokines, since the molecules were selected on the basis of their receptors being expressed by islet cells [22]. However, these effects were consistently observed in the subsequent absence of the exogenous molecules of interest after the pre-incubation period, and the current study has also demonstrated that some of these potentially beneficial effects persisted for at least 72h after the pre-incubation period. Thus, pre-treatment of isolated islets with ANXA1-alone, or in combination with SDF-1 was effective for up 24h after exposure, although only the cocktail of all three molecules endowed persistent beneficial effects on GSIS and apoptosis up to 72h after withdrawal of the ligands. These *in vitro* observations therefore suggest that a 48h pre-incubation with the cocktail of molecules can confer on islets improved GSIS and resistance to inflammatory cytokines over the immediate post-transplantation period when they are most at risk of functional failure. In accordance with this, our *in vivo* studies demonstrated that islets precultured with the cocktail of ANXA1, SDF-1 and C3a were more effective than untreated islets or those precultured with ANXA1-alone at reducing hyperglycaemia in STZ-treated graft recipients. The lower blood glucose levels observed in recipients of cocktail-treated islets most likely reflects a combination of their enhanced GSIS and their resistance to inflammatory stresses in the host transplantation niche, as indicated by the maintained graft insulin content at 28-days post-transplantation in cocktail-treated islets. These observations are important because a large proportion of a clinical islet graft (50-80%) is functionally compromised in the immediate (24-72h) post-transplantation period [4], most likely because of deleterious responses of transplanted islet cells to an inflammatory and hypoxic host environment [3-5]. Strategies which limit the post-transplantation loss of islets are therefore likely to improve the outcome of individual grafts, and also enable the limited pool of donor islets to treat more people with T1DM by avoiding the current clinical practice of administering multiple grafts to achieve normoglycemia [38]. The current study focused on ligands for islet cell GPCRs but MSCs also secrete a range of ligands which act through non-GPCRs, raising the real possibility

that extending our screening to all β-cell surface receptors may further enhance the efficacy of this therapeutic approach to islet transplantation.

In summary, we have identified a cocktail of MSC secretory products which has the potential to maintain functional β-cell mass during the *in vitro* pre-culture period and to support islet functional survival after transplantation. We suggest that pre-treatment with this cocktail of ANXA1, SDF-1 and C3a offers a well-defined, cell-free approach [21] to improve clinical islet transplantation outcomes while avoiding many of the safety, regulatory and logistical hurdles of incorporating MSCs into transplantation protocols.

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Conflicts of Interest

The authors declare that there are no conflicts of interest associated with this manuscript.

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Figure Legends

***Figure 1: In vitro* function of islets precultured with exogenous Stromal Cell Derived Factor-1. A.** Dose-dependent protection from cytokine-induced apoptosis following 48h preculture with SDF-1 and the subsequent presence of SDF-1 during the final 20h cytokine incubation. 8-12 replicates of 5 islets per well in each culture group assayed, $*p < 0.01$ vs. islets precultured alone in the presence of cytokines for the final 20h of the 3-day culture period **B.** Dose-dependent protection from cytokine-induced apoptosis following preculture with SDF-1, as in (A); an effect that plateaus at 10nmol/L SDF-1. **C, D.** Insulin release at 2mmol/L and 20mmol/L glucose of 30 replicates of 3 mouse islets per Eppendorf tube; **(C)** precultured either alone, with 10nmol/L SDF-1, or with 20nmol/L SDF-1 for 48h followed by subsequent GSIS assays in the absence of exogenous SDF-1 and; **(D)** precultured either alone, with 5nmol/L ANXA1-alone, with 10nmol/L SDF-1-alone, or with a dual combination of 5nmol/L ANXA1 and 10nmol/L SDF-1, for 48h followed by subsequent GSIS assays in the absence of exogenous ANXA1 and/or SDF-1 $*p < 0.01$ vs. islets precultured alone at the same glucose concentration. The *p* values (A-D) were calculated using two-way ANOVA Bonferonni's post hoc test. Data were consistent between three separate islet preparations.

***Figure 2: In vitro* function of islets precultured with exogenous complement component C3a A, B.** Insulin release at 2mmol/L and 20mmol/L glucose of 10-20 replicates of 3 mouse islets per Eppendorf tube; **(A)** precultured either alone, with 10nmol/L C3a-alone or with 100nmol/L C3a-alone, for 48h followed by subsequent GSIS assays in the absence of exogenous C3a; (**B)** precultured either alone, with 5nmol/L ANXA1-alone or with 10nmol/L C3a-alone for 48h followed by subsequent GSIS assays in the absence of exogenous C3a or ANXA1; **(C)** precultured either alone, with 5nmol/L ANXA1-alone or with a dual combination of 5nmol/L ANXA1 and 10nmol/L C3a for 48h followed by subsequent GSIS assays in the absence of exogenous C3a and/or ANXA1, $p < 0.05$ vs. islets precultured alone at the same glucose concentration. **D.** Protection from cytokine-induced apoptosis following a 48h

preculture with 5nmol/L ANXA1-alone, 10nmol/L C3a-alone or a dual combination of 5nmol/L ANXA1 and 10nmol/L C3a, and the subsequent presence of specified MSC-biotherapeutics during the final 20h cytokine incubation. 8-12 replicates of 5 islets per well in each culture group assayed, *$p < 0.05$ vs. islets precultured alone in the presence of cytokines for the final 20h of the 3-day culture period. The *p* values (A-D) were calculated using two-way ANOVA Bonferonni's post hoc test.

Figure 3: Preculturing islets with a cocktail of MSC secretory factors ensures sustained improvements to islet insulin secretory function and protection from cytokine-induced apoptosis. A, B: Insulin release at 2mmol/L and 20mmol/L glucose of 30 replicates of 3 mouse islets per Eppendorf tube, precultured alone, with 5nmol/L ANXA1-alone, with 5nmol/L ANXA1 and 10nmol/L SDF-1, with 5nmol/L ANXA1 and 10nmol/L C3a, or with a cocktail of 5nmol/L ANXA1, 10nmol/L SDF-1 and 10nmol/L C3a, for 48h, before removal of the MSC-derived biotherapeutics for 1 day **(A)** or 3 days **(B),** * $p < 0.05$ and ** $p < 0.01$ vs. islets cultured alone at the same glucose concentration. **C, D:** Protection of islets from cytokine-induced apoptosis after preculture with MSC-derived biotherapeutics either alone, in dual combination, or a cocktail of all three factors (as of legend) for 48h, before removal of the MSC-derived biotherapeutics for 1 day **(C)** or 3 days **(D),** 8-12 replicates of 5 islets per well were assayed, * $p < 0.05$ and ** $p < 0.01$ vs. islets cultured alone with cytokines, + $p < 0.05$ vs. islets cultured alone without cytokines. The *p* values **(A-D)** were calculated using two-way ANOVA with Bonferroni post hoc test.

***Figure 4:* *In vivo* function of islets precultured alone, with ANXA1-alone, or with a cocktail of MSC secretory factors. A.** Average blood glucose concentrations of STZ-diabetic mice transplanted with 150 islets precultured for 48h either alone, with ANXA1-alone, or with a cocktail of ANXA1/SDF-1/C3a, *$p < 0.05$ vs. mice transplanted with islets precultured alone (repeated-measurements ANOVA with Bonferroni post hoc test, n=7-9). **B.** Area under the

curve (AUC) of STZ-diabetic mice transplanted with 150 islets precultured for 48h either alone, with ANXA1-alone, or with a cocktail of ANXA1/SDF-1/C3a, *$p < 0.05$ vs. mice transplanted with islets precultured alone (One-way ANOVA with Dunn's post hoc test, n=7-9).