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Adipose tissue-derived mesenchymal stem cells rescue the function of islets transplanted in sub-therapeutic numbers via their angiogenic properties

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Abstract

A significant proportion of islets are lost following transplantation due to hypoxia and inflammation. We hypothesize that adipose tissue-derived mesenchymal stem cells (AD-MSCs) can rescue a sub-therapeutic number of transplanted islets by helping them establish a new blood supply and reducing inflammation. Diabetic mice received syngeneic transplantation with 75 (minimal), 150 (sub-therapeutic), or 225 (therapeutic) islets, with or without 1×10^6 mouse AD-MSCs. Fasting blood glucose (FBG) values were measured over 6 weeks with tissue samples collected for islet structure and morphology (H&E, insulin/glucagon staining). Histological and immunohistochemical analyses of islets were also performed at 2 weeks in animals transplanted with a sub-therapeutic number of islets, with and without AD-MSCs, to determine new blood vessel formation, the presence of pro-angiogenic factors facilitating revascularization, and the degree of inflammation. AD-MSCs had no beneficial effect on FBG values when co-transplanted with a minimal or therapeutic number of islets. However, AD-MSCs significantly reduced FBG values and restored glycemic control in diabetic animals transplanted with a sub-therapeutic number of islets. Islets co-transplanted with AD-MSCs preserved their native morphology and organization and exhibited less aggregation when compared to islets transplanted alone. In the sub-therapeutic group, AD-MSCs significantly increased islet revascularization and the expression of angiogenic factors including hepatocyte growth factor (HGF) and angiopoietin-1 (Ang-1) while also reducing inflammation. AD-MSCs can rescue the function of islets when transplanted in a sub-therapeutic number, for at least 6 weeks, via their ability to maintain islet architecture while concurrently facilitating islet revascularization and reducing inflammation.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Statement of animal welfare All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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Keywords

Islet transplantation; Islet number; Mesenchymal stem cells; Angiogenesis; Diabetes

Introduction

Compared to the whole pancreas transplantation, islet transplantation does not require the creation of a surgical vascular anastomosis. Hence, for islets to survive following their engraftment, they need to rebuild a complex network of blood vessels between each islet and the host-derived microvascular bed to ensure they receive an adequate supply of nutrients and oxygen to meet the needs of the islet graft (Carlsson et al. 2001; Pepper et al. 2013). However, this process can take up to 3 weeks, during which time the islets must survive by relying on the diffusion of oxygen and nutrients into cells (Komatsu et al. 2017); this is not ideal given the high metabolic requirement of the cells within the islets, especially the insulin-producing beta cells (Sato et al. 2011). As a result, up to 60% of the islet graft is lost within 2 weeks following transplantation from islet hypoxia due to an underdeveloped vascular supply, as well as the instant blood-mediated inflammatory reaction (IBMIR) towards the islets (Biarnes et al. 2002; Kanak et al. 2014; Lau et al. 2009; Nilsson et al. 2011). This situation is made worse by the fact that islet loss actually begins before transplantation as a result of islets suffering hypoxic stress from the isolation process (Linn et al. 2006). If not enough islets survive the transplantation procedure, the remaining islets ultimately get “overworked” resulting in them eventually failing from exhaustion, which further jeopardizes the overall success of the transplant (Boland et al. 2017). To account for these issues, a large number of islets are therefore transplanted to ensure that enough survive. Hence, patients receiving an allogenic islet transplant will typically receive at least 5000 islet equivalents/kg (i.e., 300,000–400,000 islets) per transplantation (McCall and Shapiro 2012). Sometimes, this requires procurement of islets from at least two donors or patients requiring two islet transplantations, which is problematic given the limited donor pool (Bruni et al. 2014).

One strategy to improve islet engraftment and survival following transplantation is to co-transplant mesenchymal stem cells (MSCs) with islets. MSCs can be procured from different tissues (i.e., adipose tissue (AD-MSC), bone marrow (BM-MSC), peripheral blood, placenta, and umbilical cord (UC-MSC)) with studies showing that they can act as a “mobile drug store” to protect and regenerate damaged cells through the release of angiogenic, anti-inflammatory, anti-apoptotic, immunomodulatory, and anti-fibrotic factors (Caplan 2016, Caplan and Correa 2011, Le Le Blanc and Davies 2018, Tao et al. 2016, Watt et al. 2013). BM-MSCs have been shown to improve the survival and function of transplanted islets through their paracrine function, with studies demonstrating that they can accelerate islet revascularization (Figliuzzi et al. 2014; Ito et al. 2010; Sakata et al. 2010; Yoshimatsu et al. 2015). While BM-MSCs are the most frequently investigated MSC, and often designated as the gold standard, they have to be procured from bone marrow aspirates which requires a painful invasive procedure. AD-MSCs have emerged as a new and readily available source of MSCs that can be isolated with high yield from liposuction or lipoplasty procedures without the use of enzymes or the need for ex vivo expansion (Bianchi et al. 2013;

Tremolada et al. 2016). Compared to BM-MSCs, AD-MSCs have also demonstrated an improved proliferative capability and enhanced paracrine actions (Ikegame et al. 2011; Im 2017; Omar and Aboulkhair 2017; Strioga et al. 2012). Indeed, recent studies are now also showing that AD-MSCs can also increase islet survival and function, both in vitro as well as in vivo following transplantation (Cavallari et al. 2012; Ohmura et al. 2010; Schive et al. 2017; Yamada et al. 2014).

In previous studies using ischemic disease models, AD-MSCs have been shown to have potent pro-angiogenic properties through the secretion of a variety of trophic factors (Kim et al. 2007; Moon et al. 2006; Rehman et al. 2004). These pro-angiogenic properties of AD-MSCs are largely attributed to the release of vascular endothelial growth factor (VEGF) (Song et al. 2010), as well as other factors which include hepatocyte growth factor (HGF), and basic fibroblast growth factor (bFGF) (De Francesco et al. 2009; Gómez-Mauricio et al. 2016). Although Ohmura et al. (Ohmura et al. 2010) confirmed an increase of vWF-positive cells following co-transplantation of AD-MSCs and islets, what still remains unknown is the mechanism by which AD-MSCs can facilitate islet revascularization. Furthermore, in this study, they investigated using different ratios of AD-MSCs and islets and found that normoglycemia could only be achieved when a high number of islets alone (i.e., 400) were transplanted (Ohmura et al. 2010). When a lower number of islets were transplanted, AD-MSCs were needed to ensure initial graft survival and achieve normoglycemia; this effect was dependent on the dose of AD-MSCs, with a lower number of AD-MSCs having no effect (1×10^5) and a higher number of AD-MSCs being able to temporarily restore normoglycemia ($2-4 \times 10^5$). However, in either case (i.e., with or without AD-MSCs), normoglycemia could only be maintained for 13 days.

Hence, in order to fully test the ability of AD-MSCs to rescue islets, our study used a higher number of AD-MSCs (1×10^6), tested a much lower number of islets (75, 150, and 225 islets), and examined the effects of AD-MSCs over a much longer duration (up to 6 weeks). In addition, given that AD-MSCs are able to stimulate angiogenesis, we evaluated the mechanisms by which they can help a sub-optimal number of islets survive transplantation by facilitating their revascularization and engraftment.

Materials and methods

Animals

All experiments were approved by the Administrative Panel on Laboratory Animal Care (APLAC) at Stanford University. Male C57BL/6 mice, at 6–8 weeks of age, were purchased from Charles River Laboratories (Wilmington, MA) and used for either pancreatic islet isolation or islet transplantation. All animals were maintained in pathogen-free conditions at the Research Animal Facility at Stanford University and had free access to food and water with a 12 h:12 h light/dark cycle. Fasting blood glucose (FBG) levels were measured using a Bayer Contour blood glucose monitoring system following tail vein clipping and each animal was fasted for 6 h before any measurement was taken. Animals were randomly allocated either into six groups (75, 150, and 225 islets alone and 75, 150, and 225 islets + AD-MSCs) for 6 weeks or into two groups (150 islets alone and 150 islets + AD-MSCs) for 2 weeks. All groups contained 4–6 animals.

Induction of diabetes

To induce diabetes, each animal received 180 mg/kg Streptozotocin (STZ; Sigma-Aldrich) via intraperitoneal (*i.p.*) injection. For any animal which did not demonstrate a rise in blood glucose after 72 h following *i.p.* injection of STZ, a second dose was administered. Mice which did not develop hyperglycemia following a second dose of STZ were excluded from the study. Mice were considered diabetic once they demonstrated two consecutive FBG values >19.4 mmol/l, at which point they were randomly allocated into an experimental group for islet transplantation.

AD-MSCs isolation, culture, and characterization

Mouse adipose tissue was obtained from the lower abdomen in male C57BL/6 mice at 6–8 weeks of age, as previously described (Sung et al. 2008). In brief, procured adipose tissue was washed with sterile phosphate buffered saline (PBS), minced with scissors, and then digested with 1 mg/ml type I collagenase (Sigma-Aldrich) in serum-free medium at 37 °C for 3 h. The digestion was then inactivated with an equal volume of DMEM (Gibco) supplemented with 10% fetal bovine serum (FBS; Invitrogen). All samples were then filtered through a 100- μ m mesh filter to remove any debris. The cellular pellets were collected and then re-suspended in DMEM containing 10% FBS in a humidified incubator at 37 °C with 5% carbon dioxide. Spindle-shaped cells appeared on day 3, with cell reaching 70–90% confluence within 4–5 days. Cells were then split and sub-cultured.

AD-MSCs from passage number 3–5 were used for all transplantation studies with cells examined using a Zeiss LSM710 Confocal Microscope. For cell surface marker expression, adherent AD-MSCs were detached, disaggregated into single cells, and then stained with the following antibodies for 40 min at 4 °C: phycoerythrin (PE)-conjugated mouse monoclonal antibodies against CD34, CD90, and CD105 and allophycocyanin (APC)-conjugated mouse monoclonal antibody against CD45 (Biolegend). Following incubation, AD-MSCs were washed twice with PBS before being re-suspended with 0.5 ml PBS at which point their surface marker expression compared to unstained AD-MSCs (as control) was determined using the Guava® easyCyte system (Millipore, Darmstadt, Germany).

Islet isolation

Pancreatic islets were isolated from C57BL/6 mice by means of collagenase digestion and histopaque gradients, as previously described (Neuman et al. 2014). In brief, the pancreas was surgically exposed in mice and the pancreatic duct isolated and cannulated. The pancreas was then distended using an infusion of 2–3 ml of Hank's balanced salt solution (HBSS, Sigma-Aldrich) supplemented with 0.1% bovine serum albumin (BSA; Sigma-Aldrich) containing 1 mg/ml of collagenase VI (Sigma-Aldrich). Following distension, the pancreas was carefully dissected and incubated for 10 min in a 37 °C water bath. Islets were purified by gradient centrifugation on Histopaque-1119 and 1077 (Sigma-Aldrich), and then individually handpicked and cultured in p60 culture dish containing RPMI 1640 medium supplemented with 10% FBS. Islets were visually inspected, manually counted, and their purity determined using dithizone (DTZ) staining (Sigma-Aldrich). Islets were also stained with fluorescein diacetate (FDA) and propidium iodide (PI) (Sigma-Aldrich) and then

examined under both rhodamine and FITC filters equipped on a Leica fluorescence microscope (Leica microsystem, Wetzlar, Germany) to determine viability.

Islet transplantation

Experimental groups were group 1: 75 islets alone; group 2: 150 islets alone; group 3: 225 islets alone; group 4: 75 islets + 1×10^6 AD-MSCs; group 5: 150 islets + 1×10^6 AD-MSCs; and group 6: 225 islets + 1×10^6 . After islets were removed from culture, they were washed once with PBS and then either re-suspended alone, or with AD-MSCs, in 1:1 mixture of PBS and Matrigel (BD Bioscience). This mixture was then injected, using a micropipette, beneath the right kidney capsule. Animals were then followed with FBG measurements taken twice a week. All animals were humanly sacrificed at either 2 or 6 weeks following islet transplantation depending on the experimental group. The kidney containing the transplanted islet graft was then carefully removed for histological and/or immunohistochemical evaluation.

Histology and immunohistochemistry (IHC)

Graft-bearing kidneys were fixed in 10% (vol/vol) neutral buffered formalin (NBF) and embedded in paraffin at 2 or 6 weeks post-transplantation, sectioned (5 μ m thick) using a HM 355S automatic microtome (ThermoFisher Scientific). Samples were either stained with hematoxylin and eosin (H&E) or different primary antibodies (AbCam) including guinea pig polyclonal antibodies to insulin (1:200), mouse monoclonal antibodies to glucagon (1:1000), tumor necrosis factor- α (TNF- α ; 1:100), rabbit monoclonal antibodies to vWF (1:400), rabbit polyclonal antibodies to cluster of differentiation 31 (CD31; 1:100), VEGF (1:100), HGF (1:100), or angiopoietin-1 (Ang-1; 1:100). All stained sections were scanned for analyses using a NanoZoomer (Hamamatsu Photonics, Hamamatsu, Japan).

On H&E staining, transplanted islets were assessed for their overall individual morphology as well as for any infiltration of inflammatory cells (i.e., number of inflammatory cells per mm^2). For each animal, three histological sections from different areas of the islet graft were evaluated for individual endocrine aggregates (i.e., areas of positive staining separated from each other by $> 50 \mu\text{m}$ of non-endocrine tissue). The endocrine area refers to the sum of the area of all endocrine aggregates within a histological section. As previously described (Rackham et al. 2013), the vascular density (i.e., positive staining for vWF and CD31), and presence of pro-angiogenic factors (i.e., positive staining for VEGF, HGF, and Ang-1) or an inflammatory factor (i.e., positive staining for TNF- α) were determined using islets from five to nine different sections from at least four animals within experimental groups. All positive staining within islets was then quantified using FIJI ImageJ software (Jensen 2013).

Statistical analysis

FBG values following transplantation were assessed using two-way ANOVA with repeated measures comparing the effect of time, group, and interactions between time and group. A Student's unpaired *t* test was also used to determine differences in the staining results between the transplantation groups. For all comparisons, statistical significance was accepted when $P < 0.05$.

Results

AD-MSC characterization

AD-MSCs had a long and thin morphology with widely dispersed filopodia and flattened polygonal extensions. Guava analysis demonstrated AD-MSCs to express CD105 (90.53 ± 5.45), and CD90 (92.41 ± 3.62) (positive) surface antigen markers, with no expression of hematopoietic markers such as CD34 (1.92 ± 0.43) and CD45 (2.43 ± 0.72) (negative).

Fasting blood glucose response following islet transplantation

Following STZ treatment, all animals became hyperglycemic with their FBG values increasing from an overall average of 6.6 ± 0.4 mmol/l (baseline) to 25.6 ± 1.5 mmol/l (post-STZ treatment) (Fig. 1a–c). Following transplantation of a therapeutic number of islets alone (i.e., 225 islets), there was a significant reduction in FBG values in all animals by 6 weeks (21.3 ± 1.5 vs. 6.5 ± 0.3 mmol/l; $P < 0.05$) with re-establishment of glycemic control seen from as early as 1 week following islet transplantation. This effect was similar when a therapeutic number of islets were co-transplanted with AD-MSCs with no overall difference observed between groups (Fig. 1c). When a sub-therapeutic number of islets alone (i.e., 150 islets) were transplanted, there was an initial trend for the FBG values to decrease by 3 weeks (19.1 ± 3.3 mmol/l), but this was reversed by 6 weeks so animals remained hyperglycemic (27.5 ± 2.7 vs. 26.1 ± 2.5 mmol/l; $P > 0.05$; Fig. 1b). However, when a sub-therapeutic number of islets were co-transplanted with AD-MSCs, there was a reduction in FBG values over 6 weeks (22.2 ± 1.0 vs. 9.4 ± 0.8 mmol/l; $P < 0.05$) with animals demonstrating re-establishment of glycemic control from 2.5 weeks following islet transplantation (Fig. 1b). When a minimal number of islets alone (i.e., 75 islets) were transplanted into diabetic animals, there was no change in the FBG values by 6 weeks (24.9 ± 0.8 vs. 28.1 ± 1.9 mmol/l; $P > 0.05$). Furthermore, no additional beneficial effect was seen when a minimal number of islets were co-transplanted with AD-MSCs with animals remaining hyperglycemic at 6 weeks following transplantation (29.3 ± 0.8 vs. 30.8 ± 1.5 mmol/l; $P > 0.05$; Fig. 1a).

Pancreatic islet morphology following transplantation

At 6 weeks post-transplantation, islets could only be consistently identified in the experimental groups which had been transplanted with 150 or 225 islets (Fig. 2a–f). In the experimental groups which received 75 islets, we could not reliably identify islets within tissue samples. In all groups, when islets were co-transplanted with AD-MSCs, they retained their native size and spherical morphology, clustered less, and maintained their intrinsic architecture with beta cells (positive insulin staining) located in the center and alpha cells (positive glucagon staining) located in the periphery of the islet (Figs. 2e–f and 3a–h). In contrast, when islets were transplanted without AD-MSCs, they lost their spherical morphology and tended to cluster more, thereby making it difficult to readily identify single islets (Fig. 2b, c). In addition, many of these islets had a more disorganized architecture as demonstrated by loss of their spatial organization of beta and alpha cells, as seen by positive glucagon staining located throughout the islets rather than being specifically localized in the periphery (Fig. 3a–h).

The endocrine index of islets following transplantation

At 6 weeks post-transplantation, the total endocrine area was greater when more islets were transplanted; this was evident by there being more staining for insulin and glucagon in groups which had 225 islets transplanted compared to those which only received 150 islets. When 225 islets were transplanted, the total endocrine area was similar without or with co-transplantation of AD-MSCs ($78,449 \pm 9379$ vs. $84,867 \pm 8835 \mu\text{m}^2$ of positive staining per section; $P > 0.05$; Fig. 3i). However, when 150 islets were transplanted, there was a higher total endocrine area in the group which was co-transplanted with AD-MSCs compared to the group in which islets were transplanted alone ($29,772 \pm 5160$ vs. $11,377 \pm 2958 \mu\text{m}^2$ of positive staining per section; $P < 0.05$; Fig. 3i).

The vascular index of islets following transplantation

At 2 weeks post-transplantation, islets demonstrated no significant change in CD31 when they were transplanted either alone or with AD-MSCs (94 ± 20 vs. 94 ± 3 positive cells per mm^2 ; $P > 0.05$; Fig. 4a–e). However, islets co-transplanted with AD-MSCs demonstrated a significantly higher expression of vWF compared to those transplanted alone (1193 ± 208 vs. 444 ± 73 positive cells per mm^2 ; $P < 0.05$; Fig. 4c–f). In keeping with the increase in microvascular density seen in islets co-transplanted with AD-MSCs, islets also demonstrated an increase in HGF (1081 ± 234 vs. 126 ± 20 positive cells per mm^2 ; $P < 0.05$; Fig. 5a–g) and Ang-1 (925 ± 45 vs. 81 ± 15 positive cells per mm^2 ; $P < 0.05$; Fig. 5c–g) staining compared to the control group in which 150 islets were transplanted alone. There was no significant difference in VEGF staining within the islets, regardless of whether or not they were co-transplanted with AD-MSCs (530 ± 73 vs. 468 ± 60 positive cells per mm^2 ; $P > 0.05$; Fig. 5b–g).

The inflammatory response in islets following transplantation

At 2 weeks post-transplantation, islets that were cotransplanted with AD-MSCs demonstrated a lesser degree of inflammation as demonstrated by a reduction in the amount of inflammatory cell infiltrate when compared to islets that were transplanted alone (146 ± 31 vs. 278 ± 52 positive cells per mm^2 ; $P < 0.05$; Fig. 6a–e). In addition, there was also a corresponding reduction in the presence of TNF- α within the islets that were co-transplanted with AD-MSCs compared to those that were transplanted alone (74 ± 14 vs. 449 ± 52 positive cells per mm^2 ; $P < 0.05$; Fig. 6c–e). Of note, islets which were transplanted alone also subjectively demonstrated areas of hemorrhage within islets (Fig. 6).

Discussion

In this study, we used an STZ-induced diabetic mouse model to investigate the effects of pancreatic islet co-transplantation with AD-MSCs. The kidney capsule was chosen as our site of transplantation given that it is a well-established site of islet transplantation in small animals which is easily accessible. Our results show when pancreatic islets were transplanted alone, only a therapeutic (225 islets) number of islets resulted in restoration of glucose homeostasis over 6 weeks, with neither a sub-therapeutic (150 islets) nor minimal (75 islets) number of islets alone having the capacity to reverse hyperglycemia in diabetic animals. Although there was no additional biological benefit when AD-MSC were co-

transplanted with a therapeutic number of islets (i.e., the speed of hyperglycemia reversal and the maintenance of this response over 6 weeks), AD-MSCs reversed hyperglycemia and restored glucose homeostasis when co-transplanted with a sub-therapeutic number of islets for up to 6 weeks. Furthermore, we demonstrated that this effect was due to AD-MSCs promoting angiogenesis to facilitate islet revascularization and hence their engraftment and survival. As previously noted, we also found that AD-MSCs were able to reduce the inflammatory response in the transplanted islets.

In contrast to previous studies which have shown that AD-MSCs co-transplanted with islets can only maintain normoglycemia for 13 days (Ohmura et al. 2010), we have shown that with a higher amount of AD-MSCs, we can maintain normoglycemia in both sub-therapeutic and therapeutic number of transplanted islets for up to 6 weeks. One possible explanation for this could be due to the release of more trophic factors from AD-MSCs which can further support islet engraftment. Also, while there is no information available on passage number of AD-MSCs used in previous studies, we used AD-MSCs from early passage numbers (i.e., 2–4) which have been shown to have a greater regenerative effect compared to those isolated from a higher passage number (Bogdanova et al. 2014). Future research will therefore aim to study the effect of AD-MSC passage number on transplanted islet survival and function.

Although islet transplantation can maintain glycated hemoglobin levels $\leq 7\%$ while offering freedom from life-threatening severe hyperglycemia in T1DM patients with problematic hypoglycemia (Hering et al. 2016), there is a substantial loss of islets following transplantation. Our results show that when AD-MSCs are co-transplanted with islets, they improve their function and viability especially when given with a sub-therapeutic number of islets; here, they are able to help restore glycemic control in diabetic animals by promoting islet revascularization and reducing the inflammatory response towards islets. These results would suggest that AD-MSCs could potentially rescue sub-optimal transplants thereby reducing the number or additional transplants required for any one individual. Although no difference was seen in the biological response (i.e., return to normoglycemia) when a therapeutic number of islets was transplanted, the islets cotransplanted with AD-MSCs demonstrated a healthier morphology and preserved alpha and beta spatial organization with reduced aggregation/clustering; combined, this will enable these islets to have a better long-term outcome and improved revascularization. In keeping with this, studies have shown that the architecture, organization, and morphology of islets play an important role in their function and outcome following transplantation and that these can be maintained by co-transplanting with MSCs (Rackham et al. 2011). This can be attributed to the fact that when islets aggregate, the diffusion of oxygen and nutrients to cells within the center of larger aggregates will be limited compared to smaller aggregates or separated islets, thereby affecting their function and ultimately their survival (Carlsson et al. 1998; Dionne et al. 1989). Furthermore, the revascularization of smaller islets (and hence aggregates) has also been shown to be more efficient when compared to larger islets (Kampf et al. 2006). Also, the presence of a collagen matrix between islets, which is eliminated when islets aggregate together, has been shown to be beneficial to islet graft function following transplantation (Jalili et al. 2011). In our study, we used Matrigel® as an extracellular matrix (ECM) to provide a physical scaffold for transplanted islets (Stendahl et al. 2009). The presence of an ECM can help support transplanted islets via its ability to bind, store, and protect the

degradation of growth factors, cytokines, and other soluble signaling molecules (Flaumenhaft and Rifkin 1992; Folkman et al. 1988), especially those that are released from AD-MSCs that have been co-transplanted with islets. These effects are likely to be further enhanced given that the ECM can help regulate islet survival, insulin secretion, and proliferation, in addition to preserving and restoring the spherical morphology of islets (Beattie et al. 2002; Bosco et al. 2000; Hayek et al. 1995; Lucas-Clerc et al. 1993; Nagata et al. 2002; Navarro-Alvarez et al. 2008; Wang and Rosenberg 1999). Taken together, these beneficial effects of Matrigel will contribute to the improved responses seen when AD-MSCs are co-transplanted with a sub-therapeutic number of islets.

Following islet transplantation, islets need to rapidly re-establish their vascular network to ensure they receive an adequate supply of nutrients and oxygen for their survival. Within 3–5 days following their transplantation, islets start to form new blood vessels and by 7–14 days, blood flow to the transplanted islets is established with further vascular development, organization, and functional maturation continuing over several months (Jansson and Carlsson 2002; Patan 2000; Vajkoczy et al. 1995). For these reasons, we chose 14 days as our time point to examine the differences in vascular development between our experimental groups when a sub-therapeutic number of islets were transplanted. In our study, transplanted islets demonstrated positive staining for both CD31 as well as vWF at 14 days post-transplantation. While CD31 is thought to be one of the earliest markers of endothelial cell differentiation and hence specific for early angiogenesis (Baldwin et al. 1994; Rongish et al. 1996), vWF acts as a regulator of angiogenesis, controlling vessel proliferation and maturation following initial formation through multiple pathways (Randi and Laffan 2017). Our data is supported by previous studies which have also shown that over the first few weeks following islet transplantation, vWF immunoreactivity becomes more homogenous and less speckled reflecting an increase in microvasculature density (Watanabe et al. 2000). When AD-MSCs were co-transplanted with islets, we observed no significant difference in CD31 expression and this can be attributed to the fact that majority of the endothelial cell differentiation required to form the new blood vessels supplying the transplanted islets has already happened by 14 days. However, there was a significant increase in vWF expression within islets suggesting that AD-MSCs are helping to promote vessel proliferation and maturation, in order to form a more functional microcirculation. To explain the increased vascularity observed within islets co-transplanted with AD-MSCs at 14 days, we investigated three pro-angiogenic factors (i.e., VEGF, HGF, Ang-1); these factors have not only been shown to play a role in islet revascularization following transplantation, but have also been shown to be secreted by AD-MSCs (Kalinina et al. 2015).

During islet development, VEGF-A is secreted by beta cells and directly stimulates the proliferation of vascular endothelial cells as well as their release of vWF, which in turn promotes endothelial cell proliferation, migration, and sprouting of tip cells in the early stages of angiogenesis (Gerhardt 2008; Johansson et al. 2006; Konstantinova and Lammert 2004; Leung et al. 1989). Following islet transplantation, VEGF-A is expressed in islets from as early as 1 day with expression still seen up to 28 days, albeit much reduced (Watanabe et al. 2003). The driving force behind VEGF-A expression is likely due to the increased expression of hypoxia-inducible factor-1 α (HIF-1 α) which is strongly expressed in islets at day 1 following transplantation, peaking around day 3, and then gradually

diminishing by day 14 (Miao et al. 2006). Although our results show that VEGF-A is expressed within transplanted islets at day 14, there was only slight increase in its expression when islets were cotransplanted with AD-MSCs which failed to reach statistical significance. This is somewhat interesting given that AD-MSCs have been shown to upregulate VEGF in porcine islets when they were co-cultured with AD-MSCs. However, our result could be explained by either a difference in the species being tested (i.e., porcine vs. mouse), experimental conditions (i.e., in vitro vs. in vivo), or timing. In the latter case, while we may have expected islets co-transplanted with AD-MSCs to exhibit an increased expression of VEGF via its release from either AD-MSCs (as a result of inflammatory signals and/or hypoxia) or beta cells themselves, this may have occurred at earlier time points with any difference at 14 days now less evident. Our results also showed no significant difference in CD31 between groups in keeping with previous studies which have shown a temporal correlation between VEGF-A and CD31 expression (Watanabe et al. 2003). Other important pro-angiogenic factors which have been shown to be involved in the neovascularization of transplanted islets include HGF and Ang-1 (Golocheikine et al. 2010; Su et al. 2007). In contrast to VEGF, we observed that both of these factors were significantly increased in islets that were co-transplanted with AD-MSCs. Although HGF and Ang-1 can both stimulate angiogenesis and islet microvascular density, they both have additional roles after blood vessels have been formed—HGF increasing endothelial permeability and inhibiting endothelial cell apoptosis (Book et al. 1999; Bussolino et al. 1992; Morishita et al. 1997), while Ang-1 binds to the receptor tyrosine kinase Tie-2 to promote the survival and integrity of blood vessels (Brindle et al. 2006). Hence, given that islets cotransplanted with AD-MSCs have more blood vessels at day 14, it is plausible that AD-MSCs could either be working directly (via releasing these angiogenic factors) or indirectly (via their paracrine action on beta cells which then release these angiogenic factors) to help the maturation of blood vessels which have already been formed. In turn, this results in improved glucose profiles and increased glucose-stimulated insulin secretion in islets co-transplanted with AD-MSCs. Furthermore, while both VEGF-A and HGF act synergistically to enhance angiogenesis after islet transplantation (Golocheikine et al. 2010), previous studies have shown that the expression of HGF actually becomes stronger in the transplanted islets over time (Watanabe et al. 2003) and hence this effect is likely amplified in the presence of AD-MSCs based on our data. Given that Ang-1 can also confer cytoprotective effects on islets, as well enhance their engraftment and preserve their functional mass (Su et al. 2007), it is plausible that the transplantation conditions favor the release of this cytokine directly from AD-MSCs.

Our results also showed that when AD-MSCs are cotransplanted with islets, they can protect them by reducing the inflammatory response within the graft (Angaswamy et al. 2012). This is supported by data from our study which showed that AD-MSCs could reduce the degree of inflammatory cells and TNF- α (i.e., potent pro-inflammatory cytokine) present within transplanted islets. One potential mechanism responsible for this finding is the ability of MSCs to adopting an immunosuppressive phenotype where they then release indoleamine 2,3-dioxygenase (IDO), prostaglandin-E2 (PGE2), nitric oxide, transforming growth factor β (TGF- β), hepatocyte growth factor (HGF), and interleukins 6 and 10; all of which can reduce the inflammatory response to transplanted islets (Yeung et al. 2012).

In summary, AD-MSCs can rescue the function of transplanted islets for up to 6 weeks, especially when they are transplanted in sub-therapeutic numbers, via their ability to maintain islet architecture while concurrently facilitating islet revascularization through their pro-angiogenic properties and reducing inflammation.

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Abbreviations

AD	Adipose tissue
Ang-1	Angiopoietin-1
APLAC	Administrative Panel on Laboratory Animal Care
bFGF	Basic fibroblast growth factor
BM	Bone marrow
DTZ	Dithizone
FBG	Fasting blood glucose
FDA	Fluorescein diacetate
HBSS	Hank's balanced salt solution
HGF	Hepatocyte growth factor
HIF-1α	Hypoxia-inducible factor-1 α
IBMIR	Instant blood-mediated inflammatory reaction
IDO	Indoleamine 2,3-dioxygenase
ITX	Islet transplantation
MSC	Mesenchymal stem cell
NBF	Neutral buffered formalin
PGE2	Prostaglandin E2
PI	Propidium iodide
STZ	Streptozotocin
TNF-α	Tumor necrosis factor- α
T1DM	Diabetes mellitus type 1
UC	Umbilical cord

VEGF	Vascular endothelial growth factor
vWF	Von Willebrand factor

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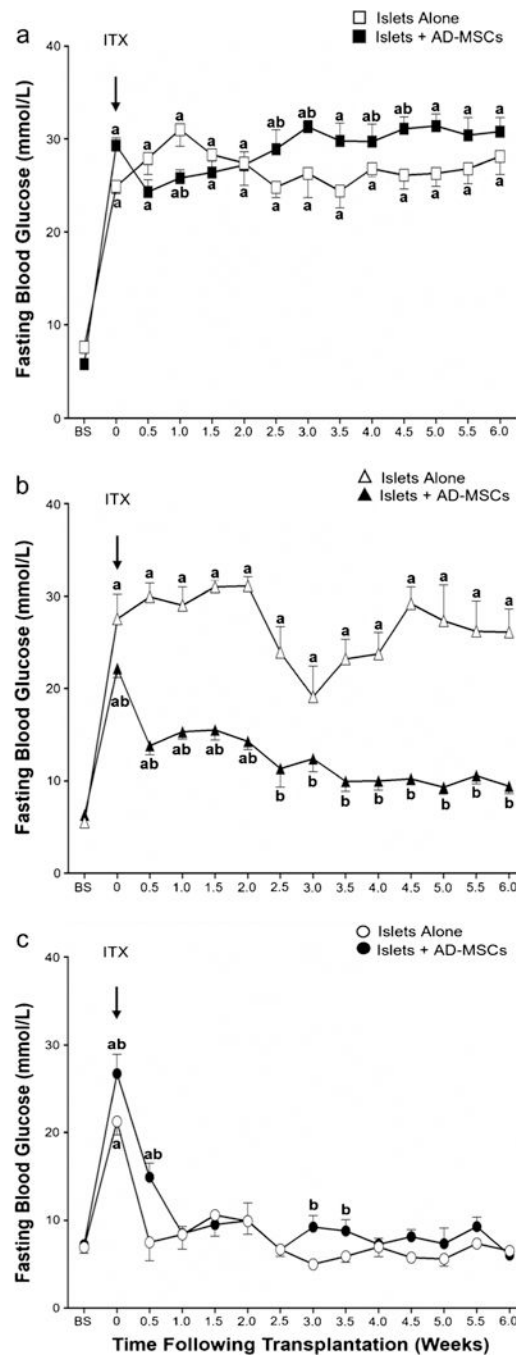


Fig. 1. Fasting blood glucose levels in mice following islet transplantation. Values represent the mean \pm S.E.M. at baseline (BS), islet transplantation (ITX arrow), and at week (W) 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, and 6.0 following islet transplantation. Animals received either 75 islets alone (white square), 75 islets with AD-MSCs (black square), 150 islets alone (white up-pointing triangle), 150 islets with AD-MSCs (black up-pointing triangle), 225 islets alone (white circle), and 225 islets with AD-MSCs (black circle).

Significant differences: ^a $P < 0.05$, baseline vs. all other time points; ^b $P < 0.05$, islets alone vs. islets + AD-MSCs (two-way RM ANOVA with post hoc Tukey test)

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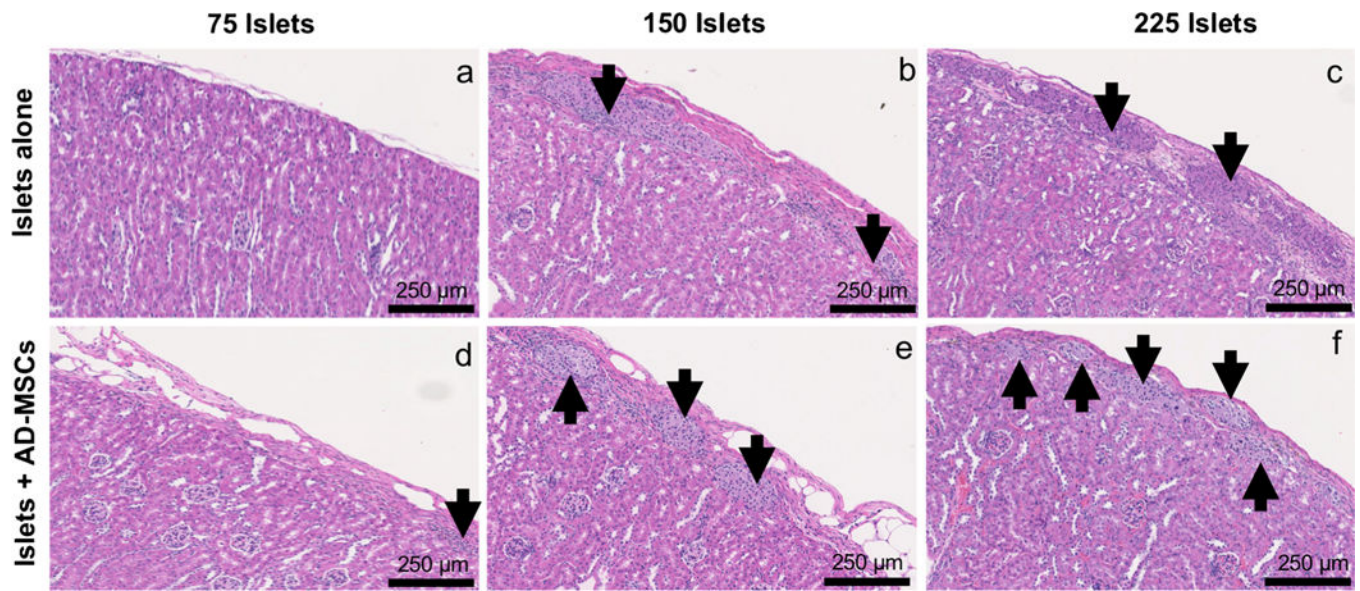


Fig. 2. The morphology of transplanted islets. Representative H&E images of 75, 150, or 225 islets that were transplanted under the kidney capsule, either alone (**a–c**) or with AD-MSCs (**d–f**), at 6 weeks following transplantation. Arrows indicate islets present within the representative images

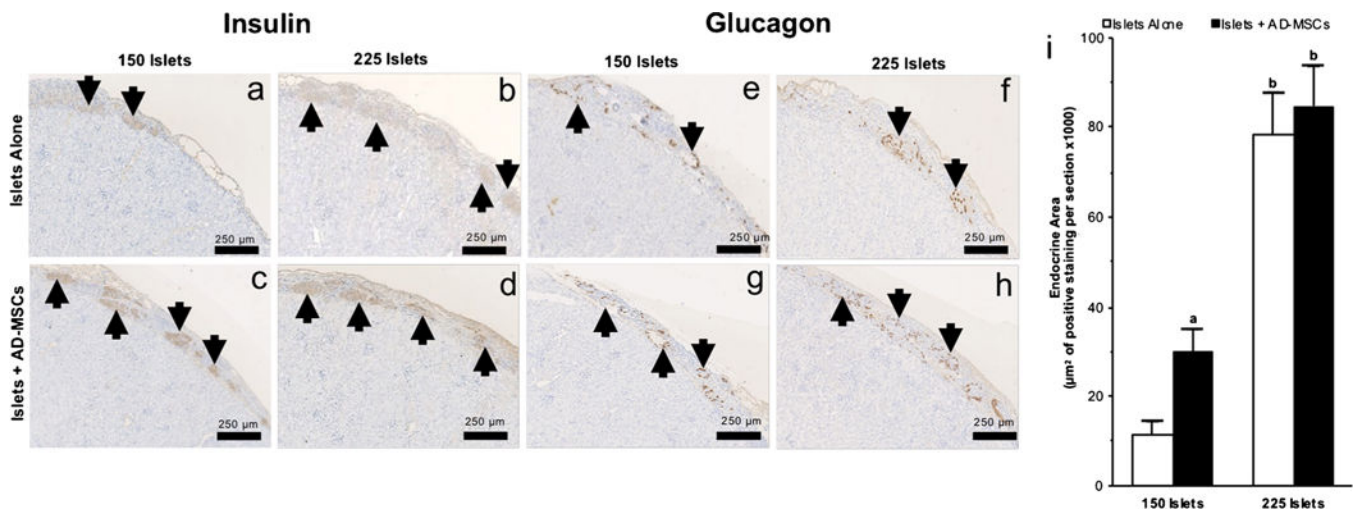


Fig. 3. The endocrine index of transplanted islets. **a–h** Representative images following insulin (**a–d**) and glucagon (**e–h**) immunohistochemical staining of 150 or 225 islets that were transplanted under the kidney capsule, either alone or with AD-MSCs, at 6 weeks following transplantation. Arrows indicate islets present within the representative images. **i** Quantification of the positive staining endocrine area in samples where 150 or 225 islets were either transplanted alone (white square) or with AD-MSCs (black square). Significant differences: ^a $P < 0.05$ for islets alone vs. islets + AD-MSCs; ^b $P < 0.05$, 150 vs. 250 islets (Student's unpaired t test)

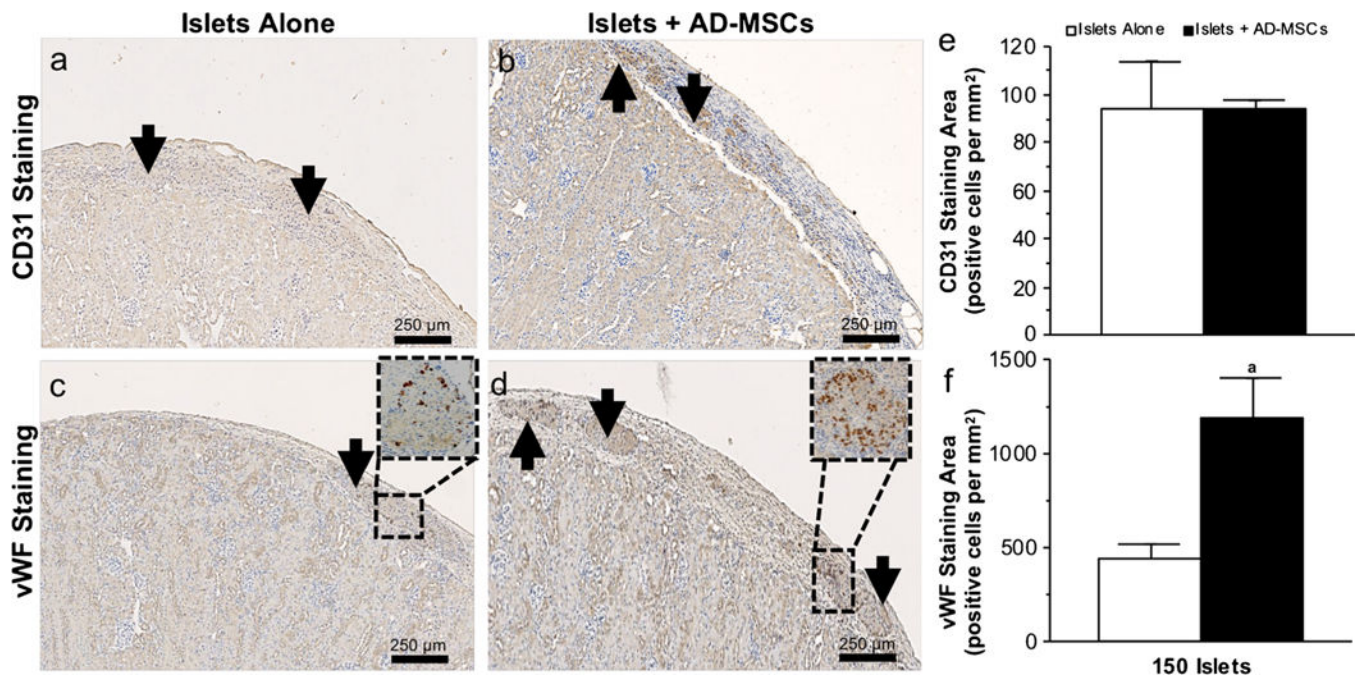


Fig. 4.

The vascular index of transplanted islets. **a–d** Representative images following CD31 (**a, b**) and vWF (**c, d**) immunohistochemical staining of 150 islets that were transplanted under the kidney capsule, either alone or with AD-MSCs, at 2 weeks following transplantation. Arrows indicate islets present within the representative images. Magnified views of the box region were shown for vWF. **e, f** Quantification of the positive CD31 and vWF staining in samples where 150 islets were either transplanted alone (white square) or with AD-MSCs (black square). Significant differences: ^a $P < 0.05$ for islets alone vs. islets + AD-MSCs (Student's unpaired *t* test)

(Student's unpaired *t* test)

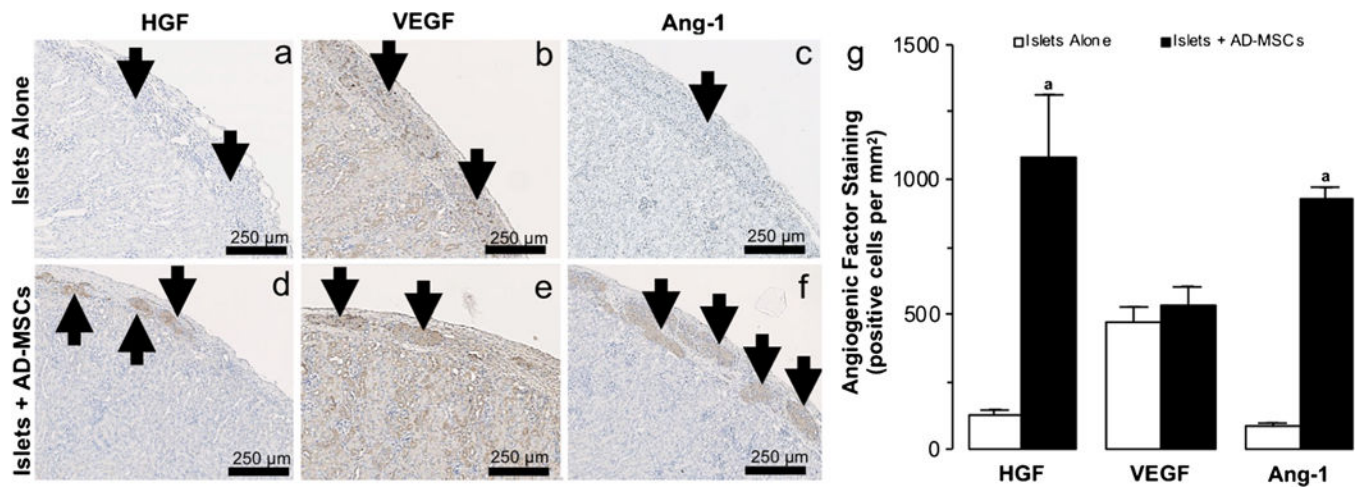


Fig. 5. Angiogenic factors within transplanted islets. **a–f** Representative images following hepatocyte growth factor: HGF (**a, d**), vascular endothelial growth factor: VEGF (**b, e**), and angiopoietin-1: Ang-1 (**c, f**) immunohistochemical staining of 150 islets that were transplanted under the kidney capsule, either alone or with AD-MSCs, at 2 weeks following transplantation. Arrows indicate islets present within the representative images. **g** Quantification of the positive HGF, VEGF, and Ang-1 staining in samples where 150 islets were either transplanted alone (white square) or with AD-MSCs (black square). Significant differences: ^a $P < 0.05$ for islets alone vs. islets + AD-MSCs (Student's unpaired t test)

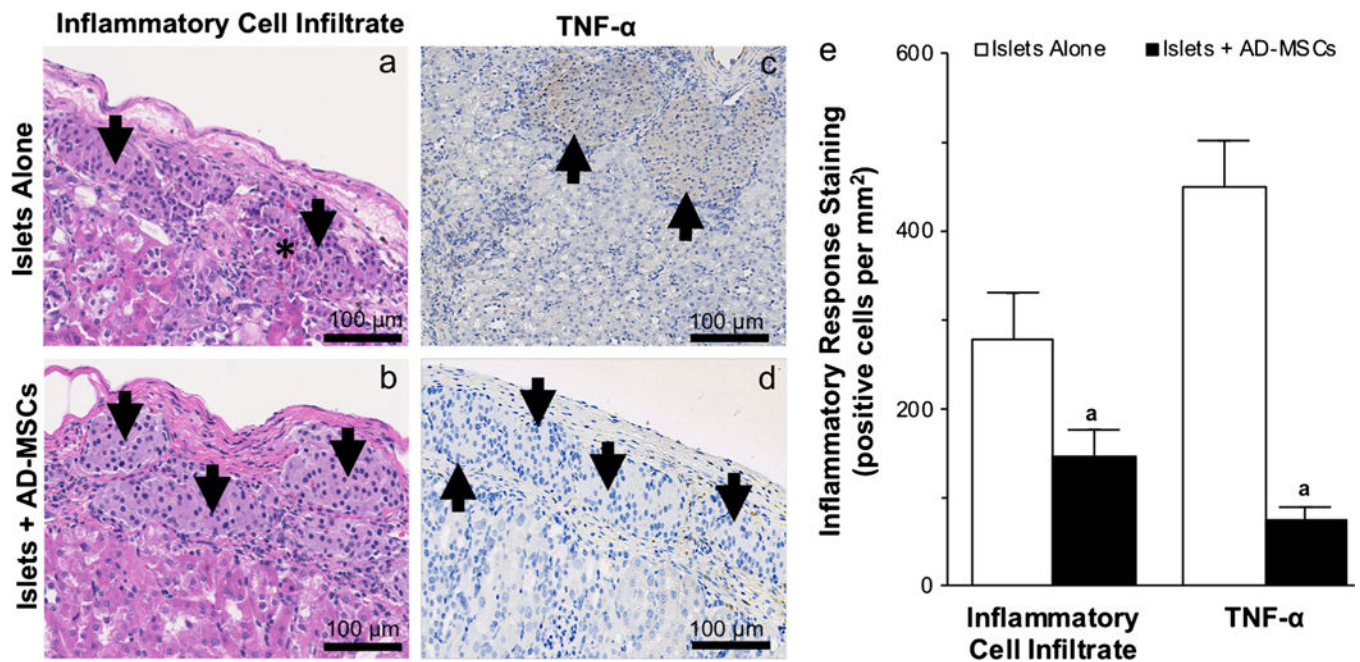


Fig. 6. The inflammatory response within transplanted islets. **a–d** Representative images of the inflammatory cell infiltrate on H&E staining (**a, b**) and following tumor necrosis factor- α : TNF- α immunohistochemical staining (**c, d**) of 150 islets that were transplanted under the kidney capsule, either alone or with AD-MSCs, at 2 weeks following transplantation. Arrows indicate islets present within the representative images. Asterisk (*) indicates area of hemorrhage within the representative images. **e** Quantification of the inflammatory cell infiltrate and TNF- α staining in samples where 150 islets were either transplanted alone (white square) or with AD-MSCs (black square). Significant differences: ^a $P < 0.05$ for islets alone vs. islets + AD-MSCs (Student's unpaired t test)