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Extended survival versus accelerated rejection of nonhuman primate islet allografts: Effect of mesenchymal stem cell source and timing

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Mesenchymal stem cells (MSC) have been shown to be immunomodulatory, tissue regenerative, and graft promoting; however, several questions remain with regard to ideal MSC source and timing of administration. In this study, we utilized a rigorous preclinical model of allogeneic islet cell transplantation, incorporating reduced immune suppression and near to complete mismatch of major histocompatibility antigens between the diabetic cynomolgus monkey recipient and the islet donor, to evaluate both the graft promoting impact of MSC source, that is, derived from the islet recipient, the islet donor or an unrelated third party as well as the impact of timing. Co-transplant of MSC and islets on post-operative day 0, followed by additional IV MSC infusions in the first posttransplant month, resulted in prolongation of rejection free and overall islet survival and superior metabolic control for animals treated with recipient as compared to donor or third-party MSC. Immunological analyses demonstrated that infusion of MSC from either source did not prevent alloantibody formation to the islet or MSC donor; however, treatment with recipient MSC resulted in significant downregulation of memory T cells, decreased anti-donor T cell proliferation, and a trend toward increased Tregulatory:Tconventional ratios.

Abbreviations: CMV, cytomegalovirus; CP, c-peptide; D, donor; EIR, exogenous insulin requirements; FBG, fasting blood glucose; IEQ, islet equivalents; IH, intrahepatic; IS, immune suppression; MSC, mesenchymal stem cells; NHP, nonhuman primate; OIS, overall islet survival; MLR, mixed lymphocyte reaction; POD, post-operative day; PPG, post-prandial blood glucose; R, recipient; RFS, rejection free survival.

1 | INTRODUCTION

Several centers have reported extended islet cell transplant survival after intrahepatic infusion, with restoration of glycemic control, elimination of severe hypoglycemia, attainment of insulin independence, improved quality of life, and attenuation of complications in patients with type 1 diabetes.[1-9] The requirement for chronic immune suppression, with the associated clinical side effects[10] and adverse impact on islet cell revascularization and function,[11,12] limits islet cell transplantation to patients with poor quality of life and contributes to the need for multiple islet donors.

Results from studies of mesenchymal stem cell (MSC) infusion indicate significant local and systemic impacts on early inflammatory events and immune activation. Mediated through secretion of paracrine and endocrine mediators, as well as cell-to-cell contact,[13-15] the anti-inflammatory and immunomodulatory properties of MSC include tissue repair, enhanced angiogenesis, reduced oxidative stress,[16-20] suppressed proliferation, maturation, and/or differentiation of T and B cells, macrophages, dendritic cells, and natural killer cells and also generation of T regulatory cells and macrophages.[21-24]

Data from both rat and mouse studies demonstrated prolonged islet survival subsequent to co-transplantation of MSC with allogeneic islets in the omentum,[25] with syngeneic islets in the liver[26] or with allogeneic or syngeneic islets under the kidney capsule.[27] Bone marrow-derived MSC from nonhuman primates (NHP) are phenotypically and functionally similar to their human counterparts and are distinct from rodent MSC,[28-33] and intrahepatic co-transplantation of MSC and islets in a cynomolgus monkey islet/bone marrow transplant model significantly enhanced islet engraftment and survival.[28]

In this study, we assessed the graft-promoting effect of bone marrow-derived MSC on islet transplantation under the cover of reduced immune suppression. In addition, we compared the effect of MSC from different sources (obtained from the islet recipient, islet donor or an unrelated third party) as well as the impact of timing of MSC administration on islet transplant outcomes.

2 | MATERIALS AND METHODS

2.1 | Animals

Islet cell donor and recipient male cynomolgus monkeys (*Macaca fascicularis*; >4 and >2 years of age, respectively) were obtained from The Mannheimer Foundation, Inc. (Homestead, FL) and Charles River (Houston, TX) and were negative for TB, Herpes B, SRV, SIV, and STLV-1. Each donor–recipient pair was ABO compatible and was as mismatched as possible for major histocompatibility Class I and II alleles, identified via microsatellite analysis or deep sequencing[34-37] (Figure S1). All study transplant protocols were approved by The Institutional Animal Care and Use Committee of the University of Miami.

2.1.1 | Diabetes induction, management, and clinical monitoring

Diabetes was induced with 100 mg/kg IV streptozotocin (Zanosar, TEVA, Irvine, CA) as previously described[38] and was confirmed 2–4 weeks post-treatment and defined as fasting c-peptide <0.3 and stimulated <0.5 ng/ml. Details in Supporting Information.

2.2 | Immunologic monitoring

Immunophenotyping of whole blood, peripheral blood mononuclear cells from CFSE mixed lymphocyte reaction (MLR), and MSC, as well as alloantibody assessment, were undertaken via immunofluorescent staining and flow cytometric analysis as previously described.[38] All flow cytometry samples included a Live/Dead Fixable Near-IR Dead Cell Stain (Molecular Probes, Grand Island, NY) for viability assessment and were run on a Becton Dickinson LSR II cytometer and analyzed using Kaluza software (version 1.5a, Beckman Coulter). Full details are in Supporting Information.

2.3 | Islet isolation and transplantation

The donor pancreas was recovered and islets were isolated and purified as previously described.[39-41] After overnight culture at 37°C, islets were collected, washed, and counted to determine islet equivalents (IEQ)[40,41] and resuspended in 20-ml transplant media containing heparin (70 IU/kg). The final preparation was tested for bacterial contamination by Gram stain and endotoxin content and for in vitro functional capacity via perifusion. When transplanted together with MSC, islets plus MSC were incubated at room temperature for 19–37 min before intrahepatic (IH) infusion. Recipients underwent a mini-laparotomy and islets with or without MSC were infused via gravity drainage through a 24-gauge intravenous catheter.[41]

2.3.1 | Islet allograft outcomes: rejection free and overall islet survival

Following islet transplantation, insulin was administered as needed to target daily fasting blood glucose (FBG) in the 100–150 mg/dl range and post-prandial glucose (PPG) in the 100–200 mg/dl range. Daily exogenous insulin requirement (EIR), weekly fasting c-peptide (CP), and monthly hemoglobin A1C were assessed. The post-operative day (POD) prior to consistent destabilization of glycemic control, increased EIR, and decreased CP was considered the last day of rejection free graft survival (RFS). Overall islet survival (OIS) was the period during which fasting CP was >0.5 ng/ml.

2.4 | MSC isolation, expansion, and characterization

Bone marrow aspirates were harvested from the iliac crest of recipient, donor, or third-party monkeys and processed as previously described.[28] Passage 4 MSC were utilized for all transplant experiments. Full details for MSC expansion, characterization, and IV infusions are provided in Supporting Information.

2.5 | Transplant groups and immune suppression: experimental design

Figure 1 summarizes the research design. Reduced immune suppression (IS, Figure 1A) consisted of 10 mg/kg IV thymoglobulin (Genzyme Corp., Cambridge, MA) on POD −1, 0, 2, and 4; daily IM Tacrolimus (FK506, Astellas Pharma US, Inc., Northbrook, IL), from POD −2 or −1 through POD 27–30 and daily IM rapamycin from POD 28 on (LC Labs, Woburn, MA), with target trough levels of 8–10 and 8–12 ng/ml, respectively. Islets with or without MSC were transplanted into the liver on POD 0. Figure 1B summarizes the timing and route of administration of MSC: no MSC (islets alone on POD 0 [data from one control, H10C21, was included in a recent publication[38]]); POD 0 (islets + 1×10^6 MSC/kg on POD 0); POD 0 + IV (islets + 1×10^6 MSC/kg on POD 0 and 2×10^6 IV MSC/kg on POD 5, 11, 18, and 28); and delayed IV (islets alone on POD 0 and 2×10^6 IV MSC/kg on POD 1, 5, 11, 18, and 28). FK506 and rapamycin trough levels were monitored weekly. The end of study was 180 days.

2.6 | Histopathology

Explanted grafts were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned (5 um), and stained as described in Supporting Information.

2.7 | RNA isolation and RNA sequencing library preparation

The methods for RNA extraction from MSC and whole blood as well as details for RNAseq libraries are provided in Supporting Information.

2.8 | Statistics and bioinformatics

Survival curves were analyzed by the Kaplan–Meier method and comparison between groups was performed using the log-rank test with $\alpha = 0.05$. Means of two groups were compared using paired or unpaired T tests and means of three or more groups were compared using ANOVA with post-hoc testing via Tukey's. Statistical analyses were performed using GraphPad Prism Software and SPSS Statistics version 25. Unless otherwise stated, data are represented as means ± SD, and p values < .05 were considered statistically significant. RNAseq bioinformatic and statistical analysis details are provided in Supporting Information.

3 | RESULTS

3.1 | Effect of MSC source and timing on islet allograft outcomes

Analyses of MSC phenotype, ability to differentiate into fat and bone and in vitro immunomodulatory capacity were undertaken to verify MSC identity (details in Supporting Information), and results are shown in Figure 2. To assess the in vivo immunomodulatory and graft promoting potential of MSC under the cover of reduced IS, we undertook studies to determine the impact of both timing of MSC infusion in relation to islet transplant (Figure 1) and source of MSC, that is, from the islet recipient, islet donor, or unrelated third party. Based on results from a previous islet/bone marrow transplant study, we tested the effect of IH co-transplant of MSC with islets on POD 0. As shown in Table S1, and regardless of MSC source, there was no prolongation of rejection free or overall islet survival as compared with the no MSC group.

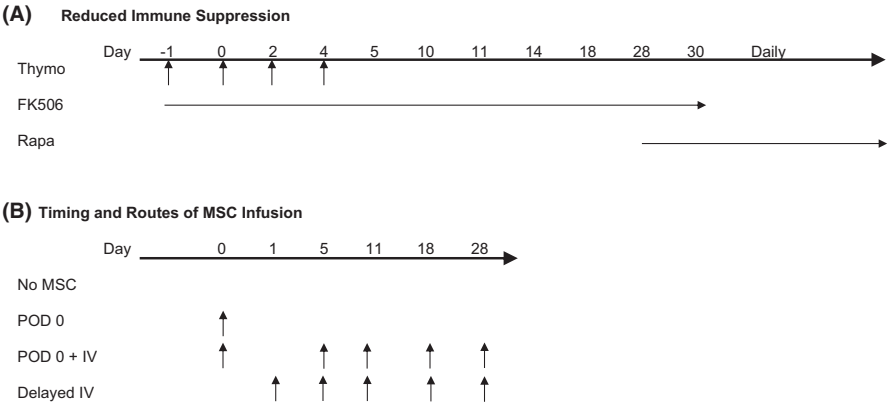


FIGURE 1 (A) Schematic of Reduced IS and (B) schematic of the design used to test the timing and route of MSC infusion. For all groups, islets were transplanted into the liver on POD 0, with or without MSC.

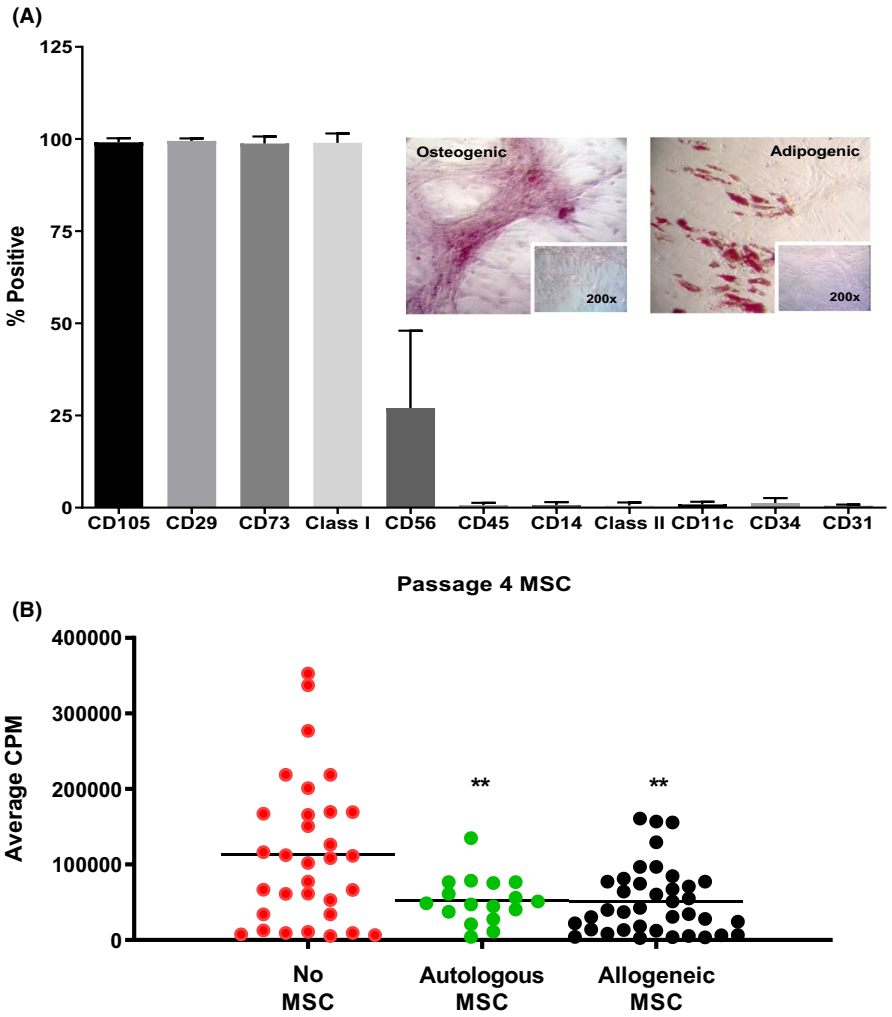


FIGURE 2 *In vitro* characterization of bone-marrow derived MSC from cynomolgus monkeys. (A) Phenotypic characterization of bone marrow-derived MSC (passage 4) from cynomolgus monkeys showing expression of MSC markers (CD105, CD29, CD73 and class I, with variable expression of CD56) and absence of markers for leukocytes (CD45, CD14, CD11c); endothelial cells (CD31, CD34) and HLA class II. Values represent mean ±SD; $n = 33$. Inset shows osteogenic and adipogenic differentiation. (B) Effect of MSC on mitogen induced (concanavalin A) proliferation of peripheral blood mononuclear cells, using self (autologous, $n = 17$) or MHC mismatched (allogeneic, $n = 38$) MSC. ** $p \leq .014$ vs no MSC ($n = 32$).

We explored the effect of additional IV infusions of MSC on POD 5, 11, 18, and 28, using recipient, donor, or third-party MSC. The results shown in Table 1 and Figure 3 demonstrate prolongation of rejection free and overall islet survival (RFS and OIS, respectively) for the recipient and donor MSC groups, with 3/5 animals in both groups maintaining graft function (OIS) through POD 180 versus 2/6 in the no MSC group and none in the third-party MSC group. In terms of both RFS and OIS, the recipient MSC group was significantly better as compared with third party at POD 60, 120, and 180 (Figure 3A,B), while the donor MSC group was only significantly different versus third party for RFS on POD 120. As compared with no MSC, the recipient MSC group experienced significant prolongation of RFS at POD 60 and OIS at POD 120 (Figure 3A,B). No significant difference was observed for recipient versus donor or donor versus no MSC groups at any time point; however, we consistently observed superior metabolic control in the recipient as compared with the donor MSC group. This metabolic distinction held true whether the animals received a marginal or full islet mass (less than or equal to 10,000 IEQ/kg, respectively) (Figure 4), with observation of more stable FBG and reduced EIR for the Recipient as compared with the donor MSC group, despite transplantation of a full mass in only 2/5 animals in the recipient versus 4/5 in the donor MSC groups; Table S3. Three of five animals in the recipient MSC group became insulin independent (42, 70, and 175 days) as compared with 1/5 in the donor MSC group (22 days).

TABLE 1 Effect of MSC source on islet allograft outcomes

MSC source group	Rejection free survival	Overall islet survival
No MSC	22, 30, 40, 46, 180, 180	28, 46, 61, 62, 180, 180
Third party	15, 24, 41, 53, 74	28, 39, 46, 67, 111
Donor	24, 72, 131, 146, 151	32, 95, 180, 180, 180
Recipient	60, 93, 105, 180, 180	123, 144, 180, 180, 180

Note: Shown are the POD for rejection free and overall islet survival. No MSC: intrahepatic islets alone on POD 0. The other three groups received 1×10^6 intrahepatic MSC/kg on POD 0, followed by IV infusion of 2×10^6 recipient, donor, or third party MSC/kg on POD 5, 11, 18, and 28. Source of MSC is as shown; islet recipient and donor were MHC mismatched, and third-party MSC were MHC mismatched (as much as possible) to the islet recipient and donor.

Utilizing recipient MSC, we undertook further analysis of the impact of MSC timing on graft outcomes. Results are shown in Figure 5A,B. Groups included POD 0 MSC given into the liver with islets alone (POD 0, $n = 4$), POD 0 MSC plus IV MSC on POD 5, 11, 18, and 28 (POD 0 + IV, $n = 5$), and POD 0 islets alone plus delayed IV MSC on POD 1, 5, 11, 18, and 28 (delayed IV,

n = 4). The POD 0 + IV group yielded significant prolongation of RFS and OIS at all time points as compared to POD 0 MSC alone (POD 0, *n* = 4) and of OIS on POD 120 and 180 as compared with the delayed IV group. One animal transplanted with islets alone on POD 0 and given IV recipient MSC on POD 5, 11, 18, and 28 experienced RFS of 57 days and OIS of 88 days (omit POD 0 MSC group, Table S3), consistent with the delayed IV group.

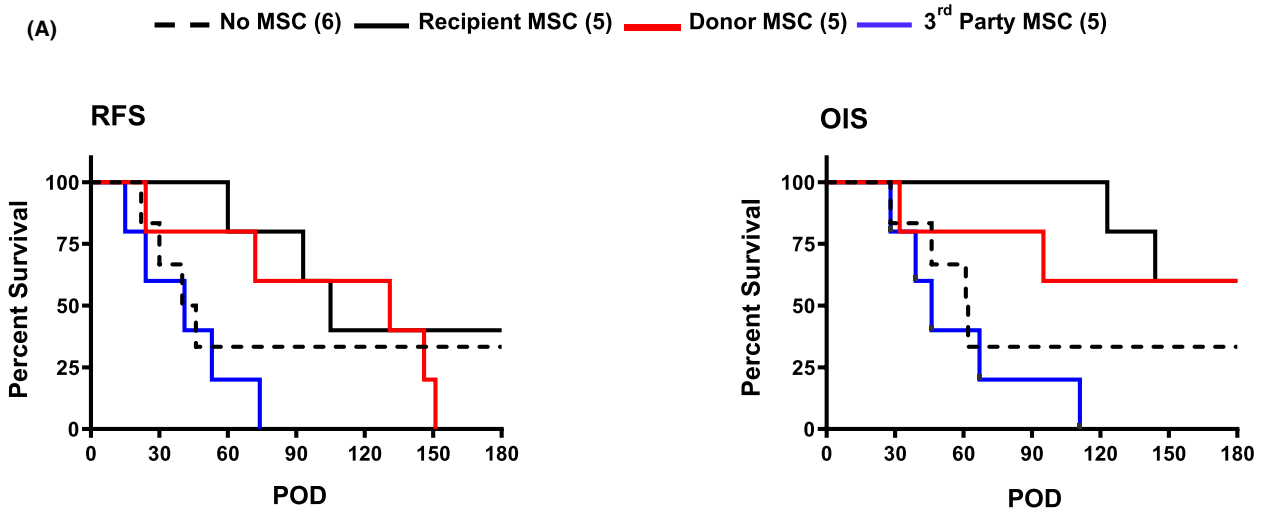
Blocking TNF-α activity via treatment with Enbrel resulted in limited RFS and OIS for two of three POD 0 + IV animals treated with recipient MSC (Table S2).

3.2 | Immunologic analyses

Results from immunophenotypic analyses of peripheral blood samples, obtained prior to and posttransplant for the no MSC, recipient, donor, and third-party MSC groups, are shown for central and effector memory T cells in Figure 6. Data are represented as the % change from baseline of the % for each subset. Both CD3/4 (Figure 6A) and CD3/8 (Figure 6B) central memory T cells were significantly lower in the recipient MSC group as compared with all other groups on POD 28 ($p < .05$). Significant differences were also observed for the recipient MSC group versus no MSC and donor MSC on POD 14 for the CD3/4 central memory and on POD 28 for the CD3/8 effector memory T cells; similar data were obtained for % change from baseline of absolute cell counts (not shown). Comparison of the ratio of Tregulatory cells ×100/T conventional cells (Treg/Tconv ratio) between the no MSC, donor MSC, and recipient MSC groups revealed a trend ($p = .07$) for increased ratios in the Recipient MSC group as compared with the others (Figure S2).

The anti-donor proliferative responsiveness of CD3/4 and CD3/8 T cells was assessed in CFSE MLR at 2 months posttransplant. Representative dot plots for each group are shown in Figure 7. The % change for CD3/4 T cells posttransplant was comparable for the recipient and no MSC animals (−45% and −53%, respectively); in contrast, increased proliferation was observed for the donor (20%) and a very high increase for the third-party animal (382%). The recipient MSC animal had the lowest % change in proliferation of CD3/8 T cells posttransplant (−39%), with stepwise increases in the no MSC (27%), donor MSC (48%), and third-party MSC (150%) groups. At the 2-month time point, all animals were being treated with rapamycin monotherapy but graft status varied. Exogenous

(A)



(B)

P Values for Rejection Free (RFS) and Overall Islet Survival (OIS)

	RFS 60 p ≤	RFS 120 p ≤	RFS 180 p ≤	OIS 60 p ≤	OIS 120 p ≤	OIS 180 p ≤
R vs No MSC	0.031				0.031	
R vs 3rd Party	0.013	0.006	0.006	0.049	0.002	0.002
Donor vs 3rd Party		0.049				

FIGURE 3 (A) Kaplan Meier survival curves for rejection free (RFS) and overall islet survival (OIS) in recipients of islet transplants with no MSC (*n* = 6) or together with intrahepatic MSC on POD 0 and IV MSC on POD 5, 11, 18 and 28. No MSC, dashed black line; recipient MSC, black line (*n* = 5); donor MSC, red line (*n* = 5); and third-party MSC, blue line (*n* = 5). (B) Statistical analysis of Kaplan Meier survival curves performed using the log-rank test with α = 0.05. R, recipient MSC; 3rd Party, third-party MSC; Donor, donor MSC.

insulin requirement, rejection free survival and overall islet survival are detailed in the Figure 7 legend. The "No MSC" animal had recently rejected and lost all graft function, with slightly reduced CD3/4 and increased CD3/8 proliferation observed; the "third party" recipient had lost all function several weeks prior, which is in line with the sensitization observed in the MLR. The "donor" had partial graft function, was clinically rejection free but began rejecting on POD 72 and lost all function on POD 95, which may explain the increased CD3/8 proliferation. In contrast, the "recipient" was insulin independent and rejection free with decreased proliferation in both the CD3/4 and CD3/8 populations. Anti-donor hypo-responsiveness persisted through the end study (POD 180) for the recipient MSC animal, with −57% and −77% proliferation for CD3/4 and CD3/8 cells, respectively. Results for all animals at 2 months posttransplant are shown in Figure S3A and demonstrate significantly greater anti-islet donor CD3/4 T cell proliferation for the third-party MSC group as compared with all other groups, as well as significantly greater B cell proliferation versus the recipient MSC group. At approximately 4 months posttransplant, the recipient MSC group had significantly lower anti-islet donor proliferation in both the CD3/4 and 3/8 T cell compartments as compared with the donor MSC group (Figure S3A), with the exception of one animal that was undergoing rejection at the time of sampling and analysis.

Shown in Figure 8 are the data for Class I and II alloantibody, analyzed in samples obtained pre-transplant and prior to the onset of rejection (time point prior to the end of RFS, shown in Table 1). Minimal changes in Class I alloantibody levels were observed for most groups, with the highest changes from baseline occurring in 2/4 animals in the delayed IV group and notable increases against both the islet and the MSC donor in one animal in the third party (POD 0 + IV) MSC group. For anti-Class II, minimal changes were observed for the third-party group, possibly due to early rejection and end of study, but the same 2/5 animals in the delayed IV group experienced high increases. Three of five animals in the recipient and 5/5 in the donor MSC group showed increases, with the degree of change from baseline higher in the donor MSC as compared with all but the delayed IV group.

Liver tissues harvested from an insulin independent animal in the recipient MSC group were sectioned and dual stained for insulin plus CD3, CD20, CD68, or FoxP3. Results are shown in Figure 9. Several insulin positive islets were observed with peri-islet lymphocyte cuffs, ranging from barely detectable to extensive. Occasional intra-islet lymphocytes were also observed. FoxP3+ cells were detected in several of the sections that contained peri-islet lymphocyte cuffs; this was also observed for tissue taken from an insulin independent no MSC animal. FoxP3+ cells appear to be associated with stable graft function, as they were not observed in infiltrated islets. Peri-islet lymphocytes were predominantly CD3+, with some CD20+ cells and rare CD68+ cells.

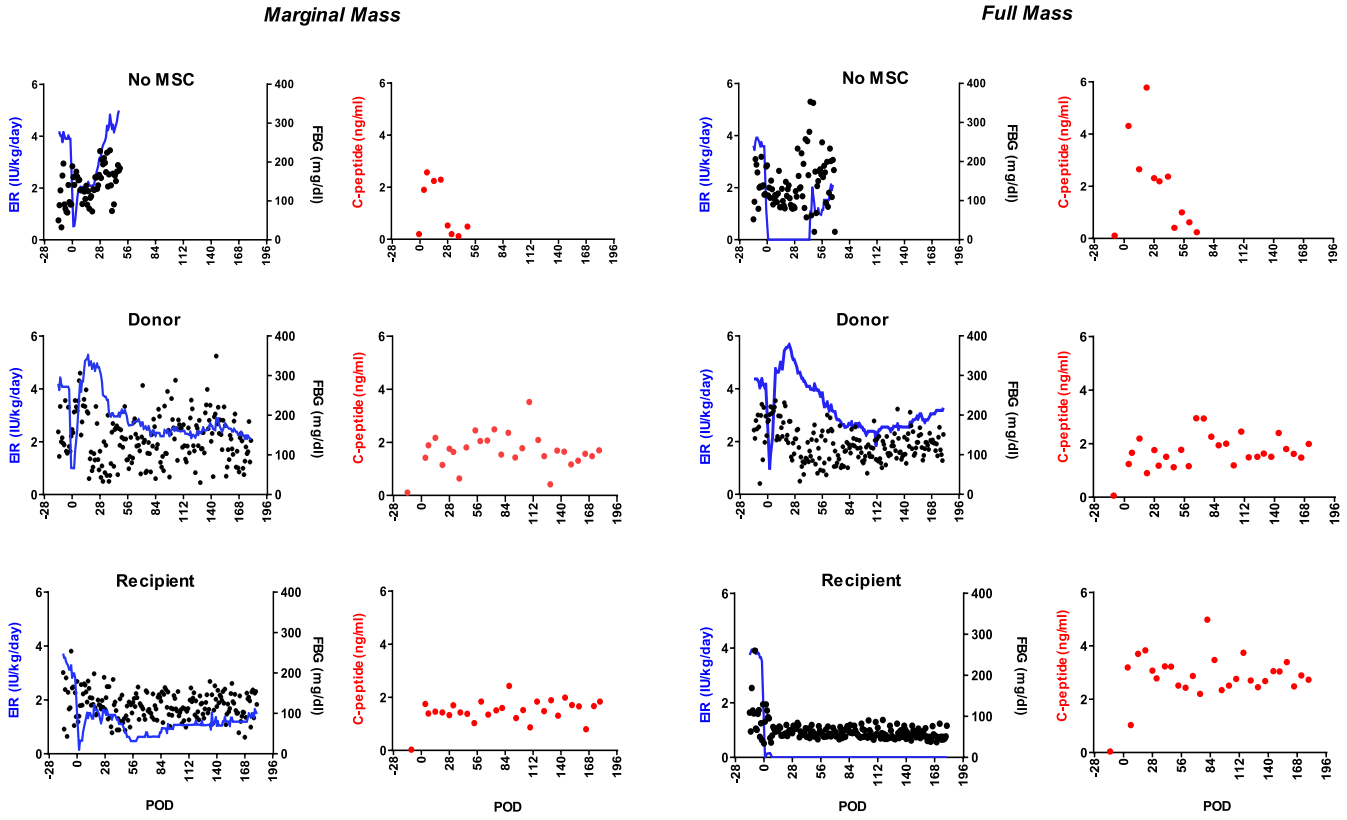
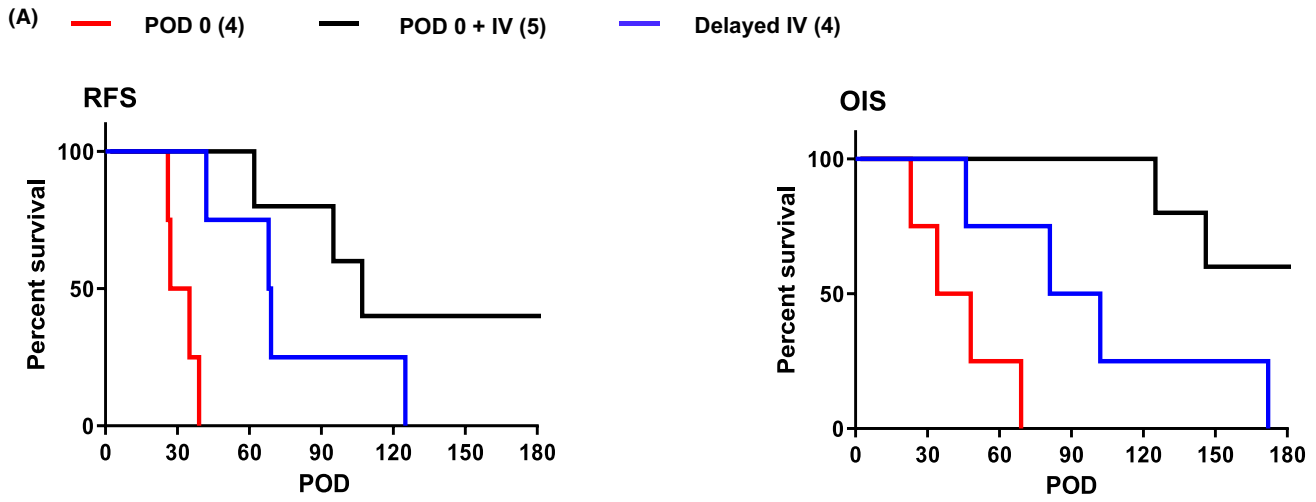


FIGURE 4 Metabolic outcomes in representative animals transplanted with a marginal (left panel) or a full mass (right panel) of allogeneic islets alone (no MSC) or co-transplanted with donor or recipient derived MSC. Exogenous insulin requirements (EIR, blue line), fasting blood glucose (FBG, black dots), and fasting C-peptide (red dots).



(B) **P Values for Rejection Free (RFS) and Overall Islet Survival (OIS)**

	RFS 60 P ≤	RFS 120 P ≤	RFS 180 P ≤	OIS 60 P ≤	OIS 120 P ≤	OIS 180 P ≤
POD 0 vs POD 0 + IV	0.003	0.003	0.003	0.022	0.003	0.003
POD 0 vs Delayed IV	0.007	0.007	0.007		0.034	0.034
POD 0 + IV vs Delayed IV					0.022	0.035

FIGURE 5 (A) Kaplan Meier survival curves for rejection free survival (RFS) and overall islet survival (OIS) using recipient MSC and varied timing of administration: intrahepatic MSC/islet co-transplant on POD 0 (POD 0, red line, $n = 4$); intrahepatic co-transplant on POD 0 plus IV MSC on POD 5, 11, 18 and 28 (POD 0 + IV, black line, $n = 5$); intrahepatic islets alone on POD 0 plus MSC on POD 1, 5, 11, 18 and 28 (Delayed IV, blue line, $n = 4$). (B) Statistical analysis of Kaplan Meier survival curves performed using the log-rank test with $\alpha = 0.05$.

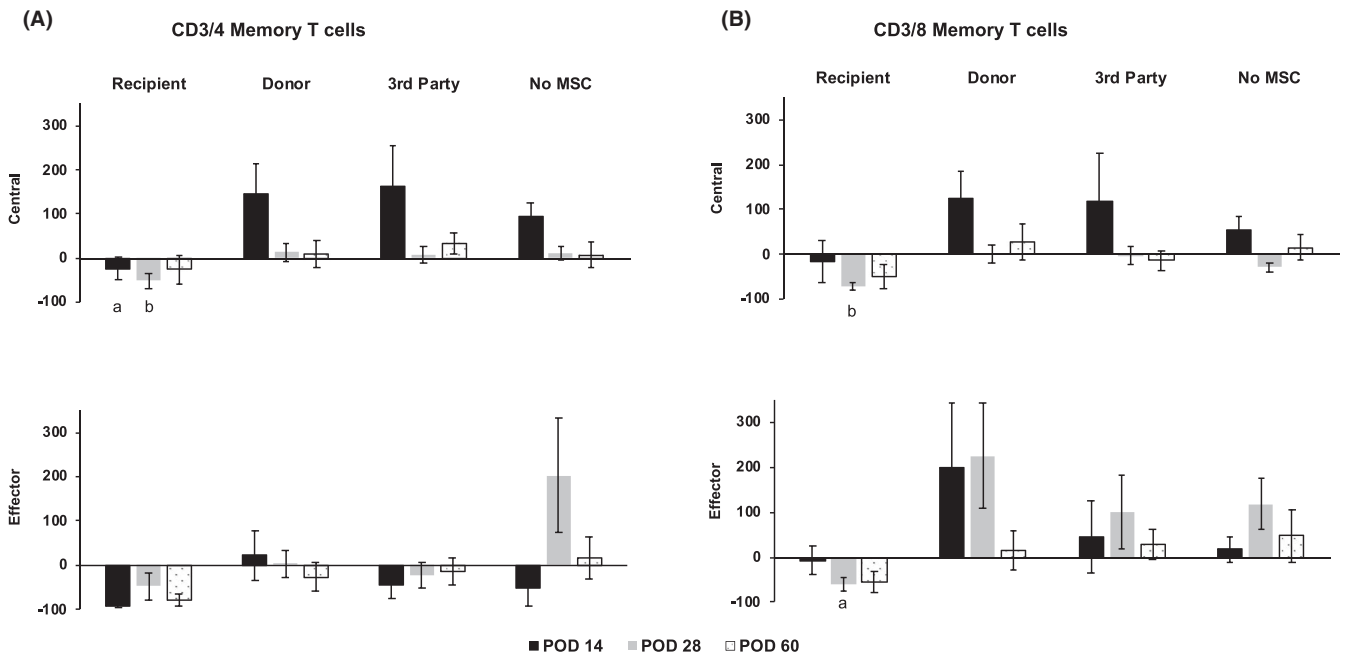


FIGURE 6 (A) Mean values ± SE for CD3/4 (left panels) and (B) CD3/8 (right panels) central and effector memory T cells on POD 14 (black bars), 28 (gray) and 60 (white with dots). Data are represented as the % change from baseline of the % for each subset. (a) $p < .05$ for recipient MSC vs no MSC and donor MSC; (b) $p < .05$ for recipient MSC vs all groups.

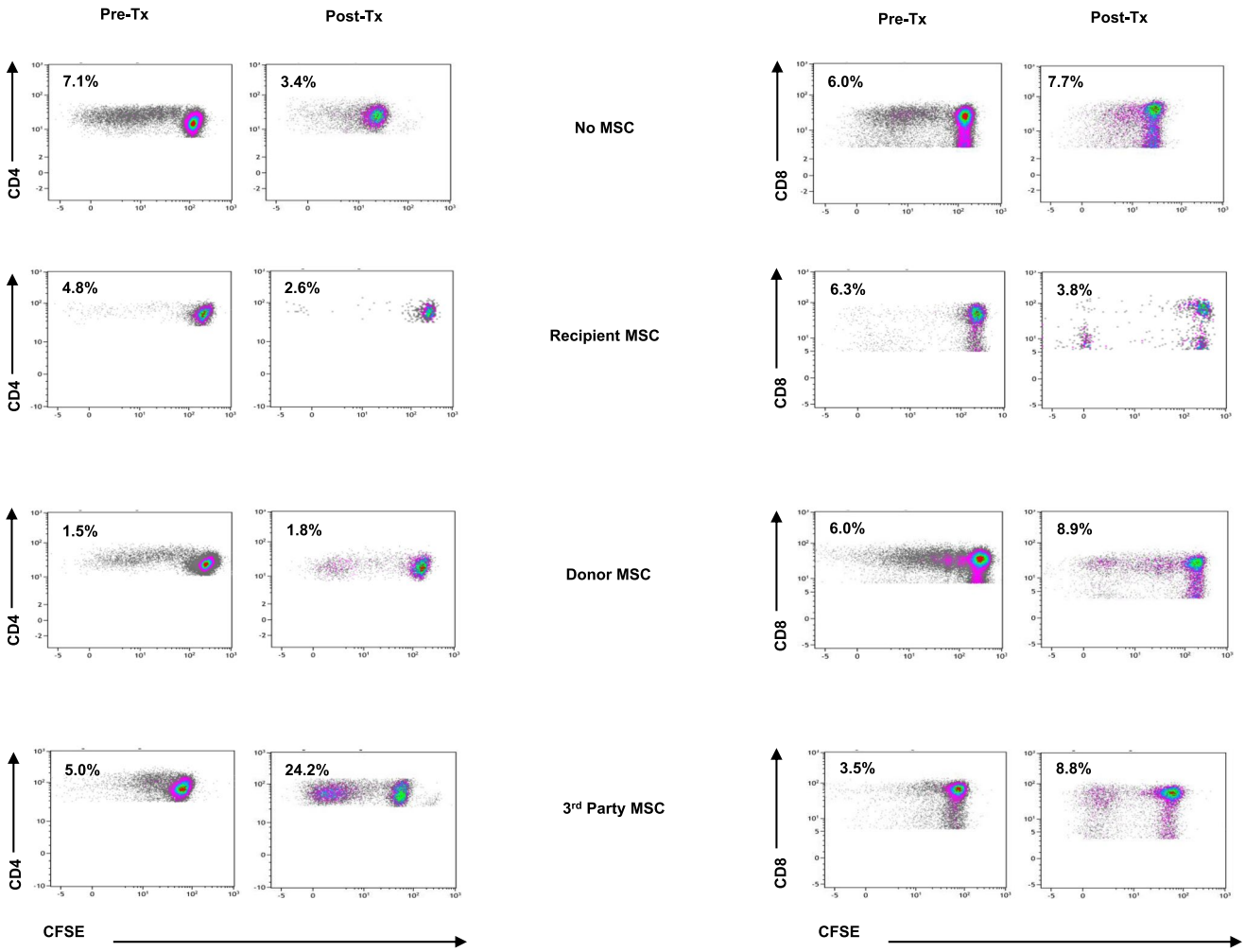


FIGURE 7 CFSE MLR. At two months post-transplant (between POD 55–62), recipient peripheral blood mononuclear cells were isolated, labeled with CFSE and cultured with autologous (not shown) or allogeneic (from the islet donor) cells for 6 days, followed by flow cytometric analysis of proliferation in viable lymphocytes. Shown are representative animals for each MSC group for CD3/4 (left side) and 3/8 (right side) positive lymphocytes. Control cultures revealed minimal proliferation (not shown). Percentage of proliferating CD3/4 and 3/8 cells is shown on each dot plot. The % change in proliferation of T cells for each time point was calculated as follows: the % of proliferating cells for control cultures (recipient vs self) was subtracted from values for anti-donor cultures; the % change in proliferating cells for post as compared to pre-transplant values was then calculated (Post – Pre/Pre*100). The % change for CD3/4 T cells post-transplant was comparable for the recipient and no MSC animals (−45% and −53%, respectively); in contrast, increased proliferation was observed for the donor (20%) and a very high increase for the third-party animal (382%). The recipient MSC animal had the lowest % change in proliferation of CD3/8 T cells post-transplant (−39%), with stepwise increases in the no MSC (27%), donor MSC (48%), and third-party MSC (150%) groups. The ID, POD at collection of sample, status, RFS and OIS for the representative animals are: 'No MSC': H10C21, POD 62, receiving exogenous insulin (3.0 IU/kg/day), 46 and 62; 'Recipient MSC'; H12C18, POD 62, insulin-free for 56 days, 180, 180; 'Donor MSC': H10C62, POD 62, receiving exogenous insulin (3.2 IU/kg/day); 72, 95 and '3rd party MSC': H12C29, POD 55, receiving exogenous insulin (3.3 IU/kg/day), 24 and 39 (see also Table S3).

3.3 | Genomic studies

Principal component analysis of the expression data acquired by sequencing of RNA isolated from 33 MSC preparations used in the transplant studies revealed no clustering of preps, indicating no underlying significant differences between them. RNAseq data for peripheral blood samples obtained from transplanted animals at 2 time points, pretransplant (POD −1 or −2) and post-transplant (around POD 55), were analyzed. Considering the relatively small number of animals per group for this kind of analysis, we pooled animals into two groups based on disparate RFS: Group 1 consisted of animals with RFS >140 days (n = 6; including two controls [no MSC], two D, and two R); and Group 2 animals had RFS ≤30 days (n = 7; including one control, one D, two third party, and three POD 0). Analysis of the white blood cell count and lymphocyte count for the pre-transplant and 2 month posttransplant time points revealed no significant difference between the two groups for either parameter (data not shown). No consistent differential gene expression was observed between pre-transplant samples from the two groups. Paired differential

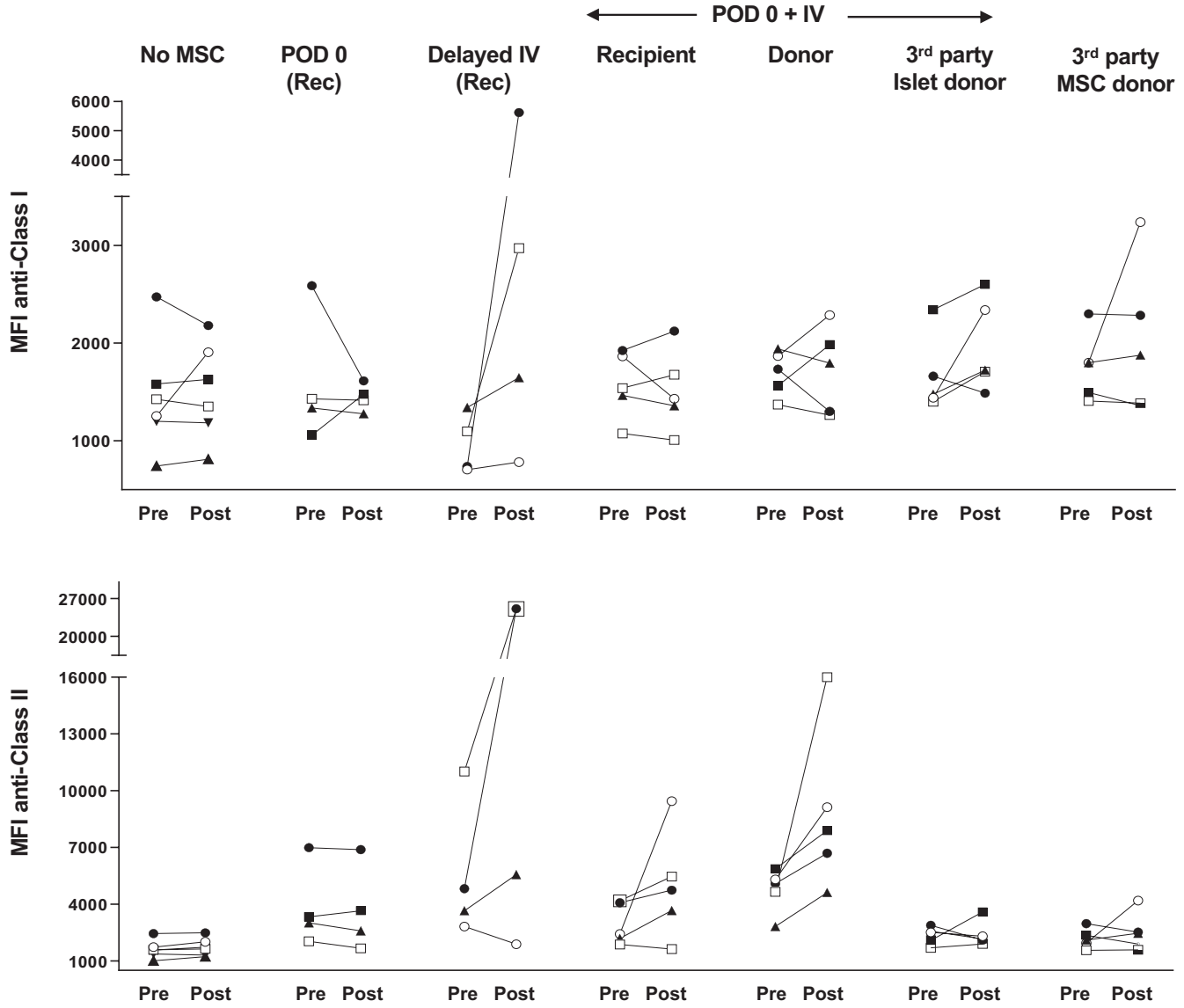


FIGURE 8 Recipient alloantibodies specific for the islet or third-party MSC donor. Leukocytes from the islet donor (for all but the data for the third-party MSC donor) or from the third-party MSC donor were incubated with recipient serum collected pre-transplant (Pre) and at a time point just after the end of rejection free survival (Post). Donor T (class I specific, top panel) and B (class II specific, bottom panel) cells were analyzed for binding of recipient serum. Data are represented as mean fluorescence intensity (MFI). No MSC: intrahepatic islets alone on POD 0; POD 0 (Rec): intrahepatic islets plus recipient MSC on POD 0; Delayed IV (Rec): intrahepatic islets alone on POD 0 + IV recipient MSC on POD 1, 5, 11, 18, and 28. POD 0 +IV: intrahepatic islets +MSC on POD 0 and IV MSC on POD 5, 11, 18, and 28, from either the recipient, donor, or third-party islet donor. Third-party MSC donor: intrahepatic islets +third-party MSC on POD 0 and IV third-party MSC on POD 5, 11, 18, and 28.

expression analysis of pretransplant vs posttransplant samples within each group revealed genes upregulated (186 and 58, Groups 1 and 2, respectively) and downregulated (163 and 27, Groups 1 and 2, respectively) with FDR <0.05 and fold change ±1.5 in either direction in both groups (Figure 10).

There were no genes anti-correlated (i.e., significantly down in one group and up in the other and vice versa). Pathway enrichment analyses of the differentially expressed genes showed upregulation of genes involved in the innate immune response to viral infection for the group with longer duration RFS (Group 1); genes involved in regulation of inflammatory responses were upregulated in the groups with short term RFS (Group 2, Table 2). Critical pathways involved in graft rejection were downregulated in Group 1 (Table 3), including gene transcripts involved in T cell proliferation (ITK), signal 1 (CD3G, LCK), and signal 2 (CD40L, ICOS). There were no significant pathways downregulated in Group 2.

4 | DISCUSSION

Utilization of banked, allogeneic MSC would facilitate therapeutic application; however, optimization of cell source in terms of

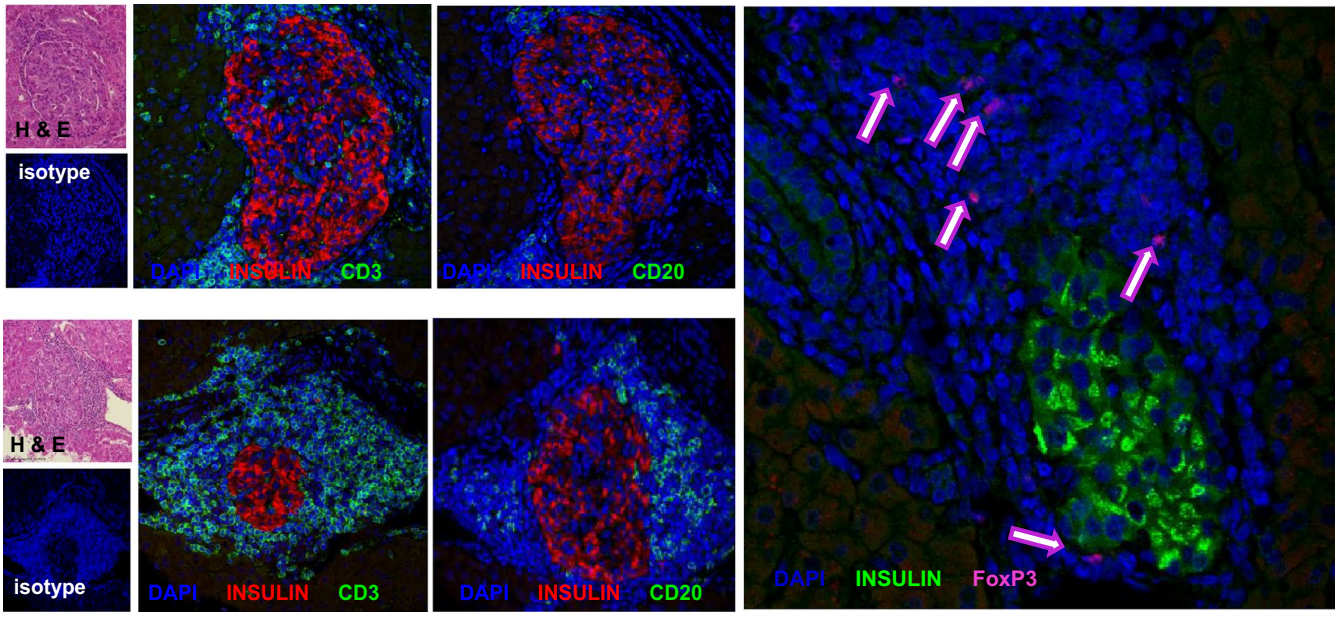


FIGURE 9 Liver biopsies from a stable, insulin independent islet transplant recipient in the Recipient MSC group. Insulin positive islets with peri-islet lymphocyte cuffs of varying degrees were present: minimal (top) and extensive (bottom), with both CD3 and CD20 positive cells identified. Foxp3+ cells were present in the lymphocyte cuffs (far right panel).

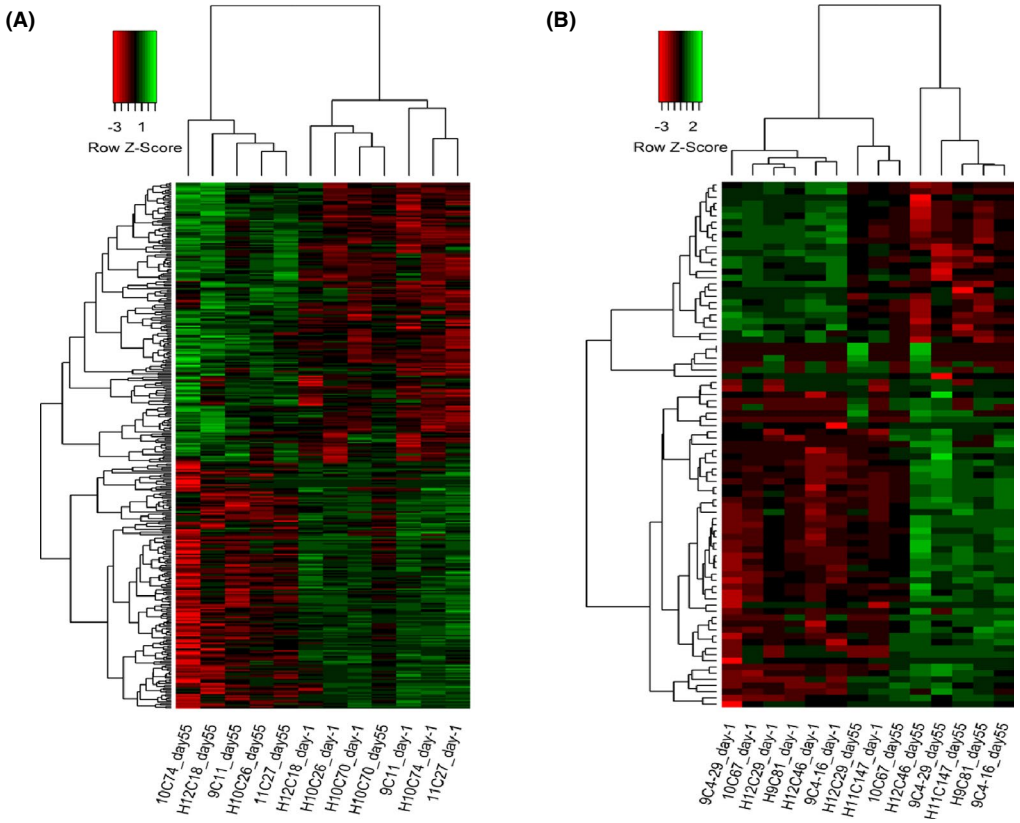
both MHC match and timing of infusion for maximal efficacy has not been established. The exceptionally restricted MHC diversity of Mauritian cynomolgus monkeys, with only seven distinct haplotypes,[36] allowed for a rigorous examination of the effect of MHC disparity between MSC and islet transplant recipients. Analysis of pancreas sections obtained at necropsy revealed an absence of insulin positive tissue for all recipients (data not shown); therefore, observed metabolic function was due to intrahepatic islets.

Under reduced IS, enhanced graft survival and function were achieved using MSC derived from the islet recipient, co-transplanted with islets on POD 0 and followed by additional IV infusions on POD 5, 11, 18, and 28. Animals treated with third-party MSC had poorer outcomes than those treated with donor MSC or with reduced IS alone. Using recipient MSC and the POD 5, 11, 18, and 28 timing of IV MSC administration, our preliminary data support the importance of intrahepatic MSC on POD 0, with significantly prolonged overall islet survival observed for this group as compared to those in which MSC were first administered IV on POD 1. The higher dosage of MSC administered to the POD 0 plus IV group, as compared with POD 0 alone, may also have contributed to the prolongation of islet function and survival.

Significant reduction of CD3/4 and 3/8 memory T cells and of anti-donor T cell responsiveness in CFSE MLR, as well as a trend toward higher T reg/T conv ratios, were observed for the POD 0 plus IV recipient MSC group. With regard to alloantibody, the data suggest that intrahepatic administration of MSC together with islets does not stimulate robust alloantibody formation against the islet donor. Recipients of delayed IV MSC alone yielded the greatest increases in anti-islet donor alloantibody responses, which suggests that intraportal administration of MSC may limit alloantibody formation. Additional study is needed to support these preliminary observations. The lack of class II alloantibody for the third-party MSC group may be attributable to the shorter rejection free survival time and early termination of the experiments.

Our results add to the growing body of evidence disproving the "immune-privileged" status of MSC[42,43] and support the hypothesis that some degree of matching with either donor or recipient MHC contributes to islet allograft protection in the context of reduced IS. Administration of autologous MSC proved to be safe in recent clinical trials, including for patients with recent onset [44] or established type 1 diabetes.[45] Moreover, in vitro studies comparing MSC from healthy versus type 1 diabetic donors suggest that despite differential gene expression, MSC from diabetic patients may be suitable for autologous therapy.[46]

FIGURE 10 Comparison of transcript expression for samples obtained pre-transplant (day −1) vs samples from POD 55 (day 55) for animals in Group 1 (A, C) and Group 2 (B, D). Inset indicates color keys. Group 1 consisted of animals with RFS >140 days (n = 6; including two controls [no MSC: 9C11 and H10C26], two POD 0 + IV MSC donor [D; H10C70 and H10C74], and two POD 0 + IV MSC recipient [R; 11C27 and H12C18]); and Group 2 animals had RFS ≤30 days (n = 7; including one control [no MSC; 9C4-29], one POD 0 + IV MSC D [H11C147], two POD 0 + IV MSC third-party [3rd; H12C29 and H12C46], and three POD 0 third-party MSC [10C67, H9C81, and 9C4-16]). C and D show the MHC typing for islet allograft donors (D) and recipients (R) used for Group 1 and Group 2, respectively. No consistent differential gene expression was observed between pre-transplant samples from the 2 groups.



(C)

Group 1	Status	ID	Mafa-A Haplotype 1	Mafa-B Haplotype 1	Mafa-DRB Haplotype 1	Mafa-DQA/DQB Haplotype 1	Mafa-DPA/DPB Haplotype 1	Mafa-A Haplotype 2	Mafa-B Haplotype 2	Mafa-DRB Haplotype 2	Mafa-DQA/DQB Haplotype 2	Mafa-DPA/DPB Haplotype 2
No MSC	R	9C11	M3A	M3B	M3DR	M3DQ	M3DP	M1A	M1B	M6DR	M4M7DQ	M4M7DP
	D	7C1	M4A	M4B	M4DR	M4DQ	M4DP	M2A	M2B	M2DR	M2DQ	M2DP
	R	H10C26	M5A	M5B	M5DR	M5DQ	M5DP	M3A	M3B	M1DR	M1DQ	M1DP
	D	H8C62	M4A	M2B	M4DR	M4DQ	M3DP	M7A	M7B	M7DR	M7DQ	M7DP
POD 0 + IV MSC: D	R	H10C70	M6A	M6B	M6DR	M6DQ	M6DP	M1A	M4B	M4DR	M4DQ	M4DP
	D	8C4-46	M3A	M3B	M3DR	M3DQ	M3DP	M2A	M2B	M2DR	M2DQ	M2DP
	R	H10C74	M3A	M3B	M2DR	M2DQ	M2	M6A	M6B	M6DR	M6DQ	M6DP
	D	6C67	M1A	M1B	M1DR	M1DQ	M1DP	M4A	M4B	M4DR	M4DQ	M4DP
POD 0 + IV MSC: R	R	11C27	M4A	M4B	M4DR	M4DQ	M4DP	M6A	M6B	M6DR	M6DQ	M6DP
	D	6C156	M1A	M1B	M1DR	M1DQ	M1DP	M2A	M2B	M2DR	M2DQ	M2DP
	R	H12C18	M3A	M3B	M2DR	M2DQ	M2DP	M1A	M5B	M5DR	M5DQ	M5DP
	D	H8C58	M3A	M3B	M3DR	M3DQ	M3DP	M6A	M6B	M1DR	M1DQ	M1DP

(D)

Group 2	Status	ID	Mafa-A Haplotype 1	Mafa-B Haplotype 1	Mafa-DRB Haplotype 1	Mafa-DQA/DQB Haplotype 1	Mafa-DPA/DPB Haplotype 1	Mafa-A Haplotype 2	Mafa-B Haplotype 2	Mafa-DRB Haplotype 2	Mafa-DQA/DQB Haplotype 2	Mafa-DPA/DPB Haplotype 2
No MSC	R	9C4-29	M4A	M4B	M1DR	M1DQ	M1DP	M2A	M2B	M2DR	M2DQ	M2DP
	D	6C163	M4A	M5B	M5DR	M5DQ	M5DP	M3A	M3B	M3DR	M3DQ	M3DP
POD 0 + IV MSC: D	R	H11C147	M2A	M2B	M2DR	M2DQ	M2DP	M3A	M3B	M3DR	M3DQ	M3DP
	D	6C33	M1A	M1B	M1DR	M1DQ	M1DP	M4A	M4B	M4DR	M4DQ	M4DP
POD 0 + IV MSC: 3rd	R	H12C29	M1A	M2B	M4DR	M4DQ	M4DP	M7A	M7B	M7DR	M7DQ	M7DP
	D	6C76	M1A	M1B	M1DR	M1DQ	M1DP	M3A	M3B	M3DR	M3DQ	M3DP
	R	H12C46	M2A	M2B	M2DR	M2DQ	M2DP	M6A	M6B	M6DR	M6DQ	M6DP
	D	8C42	M1A	M1B	M1DR	M1DQ	M1DP	M4A	M4B	M4DR	M4DQ	M4DP
POD 0 MSC: 3rd	R	H9C81	M1A	M2B	M1DR	M1DQ	M1DP	M6A	M6B	M6DR	M6DQ	M6DP
	D	7C143	M2A	M2B	M2DR	M2DQ	M2DP	M3A	M3B	M3DR	M3DQ	M3DP
	R	9C4-16	M3A	M3B	M3DR	M3DQ	M3DP	M4A	M4B	M4DR	M4DQ	M4DP
	D	6C171	M1A	M2B	M1DR	M1DQ	M1DP	M2A	M2B	M2DR	M2DQ	M2DP
	R	10C67	M1A	M2B	M1DR	M1DQ	M1DP	M6A	M6B	M6DR	M6DQ	M6DP
	D	H8C19	M3A	M3B	M3DR	M3DQ	M3DP	M4A	M4B	M4DR	M4DQ	M4DP

TABLE 2 Signaling pathways for genes upregulated after islet transplantation in Groups 1 and 2

Group	Pathway	Count	%	*p*	Corrected *p*	FE	Genes
1	Defense response to virus	13	6.99	3.10 E−10	2.59E−07	12.83	*AZU1*, *IFIT3*, *IFIT2*, *OASL*, *IFIT1*, *ISG15*, *DDX60*, *BNIP3L*, *CXCL9*, *RSAD2*, *OAS2*, *MX1*, *MX2*
2	Positive regulation of inflammatory response	5	8.62	2.06E−05	0.0061	29.85	*S100A8*, *TLR1*, *S100A9*, *TLR2*, *FABP4*

Note: Group 1 consisted of animals with RFS >140 days ($n = 6$; including two controls [no MSC], two D, and two R); and Group 2 animals had RFS ≤30 days ($n = 7$; including one control, one D, two third party, and three POD 0).

TABLE 3 Signaling pathways for genes downregulated after islet transplantation in Group 1

Pathway	Count	%	*p*	Corrected *p*	FE	Genes
T cell receptor signaling pathway	8	5.37	2.08E−05	0.002307	9.14	*TK*, *CD3G*, *CD40LG*, *FYN*, *ICOS*, *RASGRP1*, *LCK*, *CD4*
Primary immunodeficiency	5	3.36	1.89E−04	0.010425	16.8	*CD40LG*, *ICOS*, *LCK*, *CD4*, *IL7R*
Cell adhesion molecules (CAMs)	8	5.37	1.89E−04	0.006971	6.48	*SDC1*, *ITGA6*, *CD40LG*, *ICOS*, *ITGB7*, *CD2*, *CD4*, *CD6*
Hematopoietic cell lineage	6	4.03	4.97E−04	0.013707	8.87	*CD3G*, *ITGA6*, *ITGA1*, *CD2*, *CD4*, *IL7R*

Utilization of anti-inflammatory therapy is considered critical in islet transplantation; however, it has also been demonstrated that MSC are activated with inflammatory cytokines, including tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ).[47,48] We hypothesized that treatment with a TNF-α blocker might obviate or attenuate the impact of MSC infusion; inclusion of Enbrel resulted in 2/3 animals having very limited rejection free and overall islet survival; however, the numbers are small and further studies will be required to elucidate the impact of blocking TNF-α on MSC efficacy.

Differences in MSC product may be attributable to different tissue or individual donor source, as well as to inter-lab variation in methods for isolation and culture. Phenotypic, functional, and RNAseq analyses of our MSC preparations revealed consistency of product, which lends strength to our ability to assess differences between transplant groups. Downregulation of transcripts from genes involved in T cell activation and proliferation was observed at 2 months posttransplant for animals with long-term rejection free survival as compared with those with RFS ≤30 days; this finding was not attributable to differences in white blood cell or lymphocyte count. For the two animals in the No MSC group that experienced extended graft survival, the RNA seq data suggests that immunologic changes may have occurred that enabled graft survival in the absence of adjunct cell therapy. We recognize that these findings do not directly elucidate the impact or mechanism of action of MSC infusion on graft outcomes, but the results suggest potential molecular pathways to target for the enhancement of rejection free survival.

In conclusion, infusion of Recipient derived MSC, together with islets on POD 0 and in subsequent IV infusions over the first posttransplant month, resulted in enhanced metabolic outcomes and significantly better rejection free and overall islet transplant outcomes. These outcomes were associated with reduction in memory T cells and reduced anti-donor immune reactivity. Incorporation of agents that limit alloantibody formation, such as anti-CD154, may further enhance rejection free and overall graft survival and could potentially be achieved via substitution of a standard IS agent with costimulatory blockade. Further study is merited to assess the impact of anti-inflammatory agents in MSC based protocols, but the outcomes obtained in a fully MHC mismatched and rigorous preclinical model—under the cover of reduced immune suppression—support the potential of MSC for attainment of enhanced engraftment and survival of islets.

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DISCLOSURE

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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