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Author: Jamal Mohammadi Ayenehdeh Bahare Niknam Shim Rasouli Seyed Mahmoud Hashemi Hossein Rahavi Nima Rezaei Masoud Soleimani Ali Liaeiha Mohammad Hossein Niknam Nader Tajik

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Immunomodulatory and Protective Effects of Adipose Tissue-derived Mesenchymal stem cells in an Allograft Islet Composite Transplantation for Experimental Autoimmune type 1 Diabetes

Jamal Mohammadi Ayenehdeh[1,2], Bahare Niknam[1,3], Shim Rasouli[4], Seyed Mahmoud Hashemi[4], Hossein Rahavi[1], Nima Rezaei[2,5], Masoud Soleimani[6], Ali Liaeiha[1], Mohammad Hossein Niknam[2], Nader Tajik[1]*

[1]*Immunology Research Center (IRC), Iran University of Medical Sciences, Tehran, Iran*

[2]*Department of Immunology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran*

[3]*Department of Immunology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran*

[4]*Department of Immunology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran*

[5]*Research Center for Immunodeficiencies, Children's Medical Center, Tehran University of Medical Sciences, Tehran, Iran*

[6]*Department of Stem Cell Biology, Stem Cell Technology Research Center, Tehran, Iran*

Correspondence should be addressed to: Nader Tajik, PhD; Division of Transplant Immunology and Immunogenetics, Immunology Research Center (IRC), Iran University of Medical Sciences, Shahid Hemmat Highway 14496, Tehran, Iran, Tel: +98-912-325-0344, Fax: +98-21-8862-2652, email: tajik.n@iums.ac.ir &nadertajik@yahoo.com

Running title: Immunomodulatory and protective effects of AT-MSCs in islets Tx

Highlights

- Syngenic AT-MSCs along with allograft islets embedded in hydrogelic composite and transplanted intraperitoneally in Streptozotocin (STZ) induced diabetic C57BL/6 mice.
- AT-MSCs co-transplanted with allograft decreased pro-inflammatory cytokines and increased regulatory cytokines and regulatory T cells (Treg) population.

- Our procedure could be an optimal islet transplantation method and applicable in clinical trials. Because in this combination and co- transplantation of allograft and AT-MSCs, AT-MSCs have a maximum capability to increase the Treg population via cell-cell contacts and soluble factors.

Abstract:

Background: Allogeneic islet transplantation could be an ideal alternative therapy for Type 1 Diabetes Mellitus (T1DM). Adipose Tissue-derived Mesenchymal Stem Cells (AT-MSCs) characterized by immunomodulatory and protective effects may have the potential to improve the outcome of this highly immunogenic transplant.
Methods: Syngenic AT-MSCs along with allograft islets embedded in hydrogelic composite and transplanted intraperitoneally in Streptozotocin (STZ) induced diabetic C57BL/6 mice.
Results: *In vitro* experiments of co-imbedded islets and AT-MSCs in a hydrogel revealed AT-MSCs are able to significantly increase insulin secretion. During a 32 days of post-transplant period, blood glucose monitoring showed a decrease from over 400mg/dl to less than 150mg/dl and at the end of 32 days, mice have been dissected and assessed. Graft histopathology demonstrated that hydrogel makes an artificial immune isolation site and AT-MSCs contribute greatly to the reduction of the immune cells infiltration. Analyses of mononuclear cells isolated from Mesenteric Lymph Nodes (MLNs) and spleen showed that AT-MSCs co-transplanted with allograft decreased pro-inflammatory cytokines and increased regulatory cytokines (for both MLNs and spleen) and regulatory T cells (Treg) population (only for MLNs). In addition, real time-PCR assays revealed that transcript levels of IDO, iNOS, and PDX1, significantly increased in allograft islets in the presence of AT-MSCs.
Conclusions: according to results, this investigation indicates that AT-MSCs can be regarded as promising complementary candidates for engineered-cell therapy using hydrogel composites in islet transplantation.

Abbreviations

AMDCC, Animal Models of Diabetic Complications Consortium; AT-MSCs, Adipose Tissue-derived Mesenchymal Stem Cells; CAC, Cell Activation Cocktail; H&E, Hematoxylin and Eosin; iNOS, inducible Nitric Oxide Synthase; IDO, Indoleamine 2,3-Dioxygenase; MLNs, Mesenteric Lymph Nodes; Pdx1,Pancreas and Duodenum homeobox1; STZ, Streptozotocin; T1DM, Type 1 Diabetes Mellitus; Treg, regulatory T cells

Key words

Mesenchymal stem cells; Type 1 diabetes mellitus; immunomodulatory and protective effects; allograft islet transplantation.

1. INTRODUCTION

Decades of research on diabetes have shown that Type 1 Diabetes Mellitus (T1DM) is an autoimmune related disease, in which specific T cells irreversibly destroy the pancreatic β cells, which consequently leads to hyperglycemia and diabetes (1-4). Exogenous insulin administration is not efficiently capable of controlling the blood glucose levels and somehow increases the risk of secondary complications (3). Transplantation could be considered as a therapeutic option for T1DM (5-7). Islet transplantation due to a lack of additional cells and induction of optimized transplantation as a consequence, have enough capacity to decrease blood glucose levels and is less invasive than the whole pancreas transplantation, which makes it an ideal alternative therapy (1, 8, 9). However, several problems need to be solved before islets transplantation including the need for systemic immunosuppressive drugs, which are highly toxic (10). Mesenchymal Stem Cells (MSCs), exert natural immunosuppressive and protective effects through cell-cell contacts and by secreting soluble factors (11, 12). MSCs originate from different tissues, which amongst them Adipose Tissue derived MSCs (AT-MSCs) benefit from being easily accessed abundantly from adult tissues which makes them more ideal for cell based therapy applications (13, 14).

An important application of these cells in cell therapy is the utilization of immunomodulatory properties of AT-MSCs in the immune response to alloantigens (2). Systemic administration of MSCs results in complications, such as improper homing and tumor induction (15). One solution avoiding MSC-Therapy's complications is using these cells locally and close to the transplants. It was hard to assure these cells remain in considered position until tissue engineering technology has come to aid. One of the most common scaffolds in tissue engineering are hydrogel compositions.

Hydrogels are water swollen substances and they are considered as a good three dimensional (3-D) cell culture system, for any type of mammalian cells including islets as ideal candidates for transplantation in T1DM patients. Furthermore, the physiology of the cells cultured in this type of medium, is much more better than those conventionally done on flat surfaces of tissue culture plates, as reflected in differential gene expression (16), also in cell behavior and differentiation experiments (17).Thus, a 3-D cell culture is the better choice for investigating cell function (6).

. Immunoisolation of the islets by hydrogel in Maleimide-Dextran polymer offers the prospect of transplanting allograft, without the need for immunosuppression. Small pore size of the hydrogel prevents the passage of immune cells, which protects the islets from immune rejection and any

direct graft contact with the host. Moreover, in the present study, it is capable of improving the outcomes of islet transplantation by facilitating the exchange of oxygen, nutrients, insulin and so on.

Among various transplantation sites in the experimental animal models of diabetes (18), we selected peritoneum cavity because of our large quantity of hydrogelic composite, containing both MSCs and allograft islets. On the other hand, it is easy and more relevant in human application than in the other sites, such as the renal capsule.

In current study, syngeneic AT-MSCs selected because of their less allogenecity and easy access for clinical applications via liposuction and imbedded them along with the allograft transplant in hydrogel, which consequently prevented their spreading into the body. Thus, AT-MSCs protective and immune dampening factors were in close contact with the transplant. We evaluated AT-MSCs therapeutic effects on T1DM mice model transplanted with islets used in hydrogel composites in comparison with groups without AT-MSCs. This evaluation took place investigating the presence of Treg cells and its function by flowcytometric, Real-Time PCR, ELISA, histopathological analyses and their compliance with blood glucose levels.

2. MATERIALS AND METHODS

2.1. Experimental animals

Two female mice 6–8-weeks-old; C57BL/6 and BALB/c, were obtained from the Pasteur Institute, Tehran, Iran. Pancreatic islets and AT-MSCs were isolated from BALB/c and C57BL/6, respectively.

All animal experiments were undertaken in accordance with the Animal Care guidelines of Tehran University of Medical Sciences, Tehran, Iran. The animals adapted into their new environment within one week of arrival.

2.2. Low-dose streptozotocin diabetic mouse model

The C57BL/6 mice, as subjects for transplantation, were rendered diabetic by AMDCC (Animal Models of Diabetic Complications Consortium) Protocols (19). Multiple low-doses (50 mg/kg, for 5 consecutive days) of Streptozotocin (STZ) (Sigma, USA), solubilized in the sodium citrate buffer, with pH 4.5, injected intraperitoneally within 10 minutes of preparation. The mice were tested for sufficient levels of hyperglycemia at 4 weeks post-injection.

2.3. *Isolation of islets and AT-MSCs*

Pancreatic islets were isolated from BALB/c mice, as previously described in our previous study (20). In summary, after anesthetizing the mice with IP injection of ketamin (10 mg/kg) and xylazin (100 mg/kg) mixture, a V shaped incision was made at the middle of abdomen and the pancreas was pulled away from the body. Then, the mice were euthanized by cervical dislocation. Pancreas was inflated with 1 ml of cold collagenase type XI (1 mg/ml; Sigma), for at least 10 times in several sites, and incubated at a temperature of 37^{o}C for 10 minutes. After filtering the digested material with a tea strainer, the tube is refilled with 10-15 ml cold PBS to dilute the present components, thus terminating enzymatic digestion. The islets could be hand-picked under a stereomicroscope, ready for applying in hydrogel scaffolds.

AT-MSCs isolated from abdominal adipose tissue of C57BL/6 mice as discussed in our previous study. Briefly, isolated tissue washed 3 times with PBS and extracellular matrix digested with collagenase type 1. After centrifugation, pellet have been transferred to Dulbecco's modified Eagle's medium (DMEM, GIBCO) containing 10% fetal bovine serum (FBS, GIBCO) and 2 mM L-glutamine, penicillin, and streptomycin (all from Invitrogen) as MSCs culture media and incubated (37∘C in 5% CO_2). At adherent cells optimum confluency, these cells harvested and at third passage assessment of AT-MSCs done with immunophenotyping (21).

2.4. Hydrogel preparation

For preparation of the 100 µl of hydrogel (3D-Life Dextran-PEG Hydrogel kit, Cellendes, Germany), following the manufacturer's instructions, combination of water, 10x CB (Concentrated Buffer) pH 5.5, and Maleimide-Dextran were placed in a reaction tube. After 5 minutes, the cell suspensions (2×10^5AT-MSCs with or without the 100 islets) were added. The content of the reaction tube were transferred into a pipette tip (to form proper shape) and mixed with the Polyethylene Glycols (PEG-Link) by pipetting three times up and down. After, at least, one minute for gel formation, culture medium was carefully added to cover the gel, until transplantation.

2.5. In vitro function assay (insulin secretion) of islets co-embedded with AT-MSCs into the hydrogel

For assessment of the islet adaptation in the hydrogel and the *in vitro* effects of the AT-MSCs on islet function, AT-MSCs were co-embedded with islets, at a density of 2×10^5 cells/100 µl hydrogel, to determine its impact on insulin secretion. Triplicate batches of 10 islets in equal numbers and similar sizes, were washed and divided into three groups, including free islets without Gel (Islet), islets imbedded into hydrogel (Gel+Islet), hydrogel containing composites of islets and MSCs (Gel+MSC+Islet), cultured in mediums with different concentrations of glucose. These mediums consist of T0 or a control medium (DMEM low Glucose (Sigma) + 10% FBS + 1% glutamine + 1% Penicillin/Streptomycin) with no extra glucose, T1 (control medium + 150 mg/dl glucose) and T2 (control medium + 300 mg/dl glucose). ٠.2 ml of control medium were added to microplates and incubated for 30 minutes, at 37℃, pH 7.4, with 5% CO_2. Glucose solutions were added into the T1 and T2 medium wells after incubation to get glucose concentrations about 150 mg/dl and 300 mg/dl, respectively. All wells were incubated for 60 minutes under the same conditions, similar to those of first incubation (22-24). Thereafter, the supernatants were collected after gentle centrifugation and insulin assay were conducted by ELISA method, using mice standard insulin.

2.6. Hydrogelic composite transplantation

The diabetic recipient mice with blood glucose concentration >400 mg/dl were used for transplantation. Surgery were performed with ketamine (10 mg/kg) and xylazin (100 mg/kg) mixture in the surgery room. Prepared composite in four groups were transplanted, intraperitoneally, by making a small incision. A minimal mass of 200 islets in two composites of 100 µl were transplanted into every candidate mice. Five groups consist of Control (without any transplantation), Hydrogel alone (Gel), AT-MSCs imbedded hydrogel (Gel+MSC), Islet imbedded hydrogel (Gel+Islet) and AT-MSCs along with Islets imbedded hydrogel (Gel+MSC+Islet), delivered into the peritoneal cavity using sterile devices. Blood glucose levels were monitored within 4 day intervals by Glucometer (Accu-chek, Performa).

2.7. Hydrogel composite recovery

The mice were dissected by cervical dislocation, 32 days after transplantation. Composites were collected from the peritoneal cavity and washed in saline buffer solution.

2.8. Hematoxylin and Eosin (H&E) staining

For an *in vivo* assessment of transplanted islets, isolated grafts at three time points (10, 20 and 32 days) were subjected to histopathological examinations. hydrogelic grafts were recovered, fixed in 10% formaldehyde, embedded in paraffin, and cut into 5-mm sections. Prepared sections were stained using Hematoxylin-Eosin staining due to instructions of standard protocol (13). All grafts were examined by an independent pathologist.

2.9. Cytokine assays

Isolated mononuclear cells from Spleen and Mesenteric Lymph Nodes (MLNs) of the transplanted mice were separately adjusted to 1.5×10^6/ml. Cell Activation Cocktail (CAC) without Brefeldin A (BioLegend) was added into each well and incubated at 37°C, with 5% CO_2 (according to manufacturer's instructions). After 6 hours of incubation, supernatants of the cells were collected after gentle centrifugation and kept frozen at -20^0C, until cytokine assay. The representative cytokine levels of each T cell subpopulations (Th1, Th2, Th17 and Treg), including interferon-γ (IFN-γ), interleukin 4 (IL-4), interleukin 17 (IL-17) and transforming growth factor-β1 (TGF-β1) were assessed by Single-AnalyteELISArray Kits (Qiagen).

2.10. Flow cytometry

Mononuclear cells from the MLNs and spleen of each group were adjusted to 1.5×10^6 cells/well and stimulated with a CAC with Brefeldin A (BioLegend), for 6 hours. activated cells were harvested and washed with Cell Staining Buffer (BioLegend). For Treg analysis, we used Mouse TregFlow™ Kit (BioLegend). The cells distributed 0.3×10^6 cells/100µl. Following the manufacturer's instructions, the activated cells were stained with Mouse Treg Flow Kit (FOXP3 Alexa Fluor® 488 /CD_4 APC/ CD_{25} PE from BioLegend). Analysis of stained cells were performed using flow cytometer (BD FACSCalibur™), row data analysis were performed using FlowJO.

2.11. Real time -PCR

Collected allograft composites were cut into pieces and degraded by dextranase prepared in the kit. Total RNA were isolated from hydrogel composite, using an RNeasy®PlusMini Kit (Qiagen). cDNA was prepared with RevertAid Reverse Transcriptase-Fermentase (Thermo Fisher Scientific Inc.) and synthetized cDNA were subjected to conventional PCR (35 cycles)and

electrophoresis on a 2% agarose gel. Bands were visualized under UV (Ultra Violet) light and cDNA have been confirmed. Real-time PCR were performed with specific primers for β2M, IDO, iNOS, PDX1 genes (Table1) and the Green-High Rox PCR Master Mix reagents (Ampliqon), using a StepOnePlus™ Real-Time PCR System. Selected genes expression were assessed independently, and β2M (β2 micro globulin) expression were used as a control for normalization.

2.12. Statistical Analysis

Statistical analysis were performed using Statistical Package Graph Pad Prism 6. Data were presented as mean±SD and compared using a one way ANOVA (Analysis of Variance). *Tukey's Multiple Comparison Test* was used to assess the effect of changes in differences among the categorical variables. *P<0.05, **P<0.01 and ***P<0.001 were considered to be statistically significant.

3. RESULTS

3.1. Insulin secretion

Co-embedded islets with MSCs increased glucose-stimulated insulin secretion, as compared to the group only embedded with islets in three different glucose concentration mediums, including control (no extra glucose T0), 150 mg/dl (T1) and 300 mg/dl (T2). There were no significant alterations between free islets and Gel+Islet groups; whereas, there were a significant difference in Gel+MSC+Islet group in both T1 and T2 (Gel+MSC+Islet vs. Islet and Gel+Islet; $p<0.01$; 10.8±2.1 vs. 5.26±1.6 or 4.45±0.9; 16.2±1.8 vs. 8.43±2.2 or 7.33±2.3) (Figure 1).

3.2. Effect of AT-MSC's presence on leukocyte infiltration

. Histopathology (Figure 2.) of normal pancreas showed normal islets of Langerhans in-between normal pancreatic acini (arrow) and normal pancreatic duct (A), and in the diabetic pancreas, it showed ruptured and destructed islets of Langerhans with damages in β-cells (B). Isolated grafts containing islets (10 days (C) and 20 days (D)), after transplantation also were subjected to histopathological examinations which showed intact islets. Finally, all grafts in the transplanted groups were isolated after 32 days. Results indicated that the immune cells (*esp.* leukocytes)

could not infiltrate into the grafts (E-H) in AT-MSC transplanted groups (F and H) in comparison with corresponding groups (E and G), respectively.

3.3. Graft function

Blood-glucose levels (tail vein blood samples) of all mice were measured regularly using Glucometer (Accu-check, Performa) for about one month every day, before 9:00 a.m.. Blood glucose levels of the mice in groups of Control, Gel, Gel+MSC, increased rapidly, while Gel+Islet and Gel+MSC+Islet transplanted mice progressed slowly, which indicates a suboptimal glycemic control as a result of an increase in islet's survival. Glucose levels were better maintained in the Gel+MSC+Islet group than the others, over a 32 day period.

Graft losses were coincided with the day in which blood glucose levels exceed 400 mg/dl. Mice were later euthanized to confirm diabetes recurrence, and the graft survival period was calculated as number of days, before diabetes recurrence. Co-embedded islets with MSCs, improved the graft outcome, when compared with encapsulated islets alone. The average blood glucose level for the Gel+ MSC + Islet group was significantly lower on day 1 to 32, after transplantation (Gel+MSC+Islet vs. Gel+Islet; $p<0.05$) (Figure 3).

3.4. Effect of AT-MSCs on cytokine production in the supernatant of MLNs and spleen cell culture

Release of cytokines into culture supernatants from mononuclear cells derived from MLNs and spleen were measured using ELISA. Levels of anti-inflammatory cytokines in the supernatant of mononuclear cells derived from MLNs, including TGF-β1 and IL-4 in Gel+MSC and Gel+MSC+Islet groups, were significantly higher than other corresponding groups (Gel+MSC vs. Control or Gel; $p< 0.01$and Gel+MSC+Islet vs. Gel+Islet; $p< 0.001$). In other counterparts, results obtained from proinflammatory cytokines measurement, such as IFN-γ and IL-17. Obtained results were indicated a significant decrease in IL-17 levels in both Gel+MSC and Gel+MSC+Islet groups in comparison with control, Gel and Gel+Islet groups, respectively ($p< 0.001$). IFN-γ levels significantly decreased only in the Gel+MSC+Islet group in comparison with the Gel+Islet group; $p< 0.001$ (Figure 4).

Similar results were obtained in analyses of similar cytokines in the supernatant of mononuclear cells derived from spleen. In fact, results of the measurement of cytokine levels in the

supernatant of mononuclear cells derived from mice spleen indicated that there were a significant increase in TGF-β1 and IL-4 production in groups transplanted with allografts containing AT-MSCsGel+MSC and Gel+MSC+Islet groups . On the other side, IFN-γ and IL-17 production significantly decreased, in same groups (Figure 5).

3.5. Flow cytometric investigation of Treg cells in mononuclear cells derived from MLNs and Spleen

Representative data of flowcytometric analyses of $CD4^+CD25^+$ $FOXP3^+$ cells (Tregs) derived from MLNs and spleens of mice transplanted with or without AT-MSCs, are presented in figure 6 and figure 7, respectively. Dot plots showing representative proportions of Tregs in MLN cells and splenic tissues, 32 days after transplantation, are divided into five groups. Statistical analysis in the graphs of figure 8 shows the percent of T-regulatory cells (%Treg) in MLNs (A) and spleen (B). graphs show that in MLNs analysis, the percentage of Tregs significantly increased, from 0.705% to 6.99% in Gel versus the Gel+MSC group and from 1.04% to 4% in Gel+Islet versus Gel+MSC+Islet group ($P<0.05$) (Figure 8A). But, there were no significant variation in %Treg among the same groups, in the splenic tissues (Figure 8B).

3.6. Real time –PCR

Co-transplantation of AT-MSCs significantly increased the transcript levels of IDO and iNOS in the Gel+ MSC+ Islet group, in comparison with the Gel and Gel+Islet and Gel+MSC groups. Similar results were also obtained for transcript levels of PDX1 which significantly increased in Gel+MSC+Islet group as compared to Gel and Gel+Islet and Gel+MSC groups (Figure 10).

4. DISCUSSION

AT-MSCs has a good potential for regenerative medicine because of their multipotent activity, self-renewing capacity, differentiation into various types of tissues and possessing a very important property, which enables easy accessibility to these cells in adult patients (5, 8, 9). Because of proven immunomodulatory effects of MSCs, Therapeutic administration of MSCs in different autoimmune disorders has long been of interest to researchers (25). One of the most popular amongst these autoimmunities is T1DM, which also were the target of cell therapies with MSCs. Researches on rodent models of T1DM showed that, the systemic administration of

MSCs has the potential to revert hyperglycemia and increase insulin production through the process of pancreatic islets' recovery, and also has the ability of exerting beneficial immunomodulatory effects (26-32). Moreover, studies on local MSCs transplantation also showed their immunomodulatory effects on infiltrating and resident immune cells (33, 34). In this regard, it has been reported that the underlying effecter mechanism of MSCs effectiveness might be attributed to both cell-cell contacts (35) and released soluble factors (36, 37).

In our previous study, proliferation potency of mononuclear cells derived from T1DM mice spleen decreased in the presence of AT-MSCs. In fact, it revealed that AT-MSCs have *in vitro* immunomodulatory effects on T1DM mice (21). Our work provides a new insight into the function of locally administered AT-MSCs in allograft transplantation. Using co-transplantation of AT-MSCs with pancreatic islets, we have shown that AT-MSCs are capable of improving graft function due to immunomodulatory and protective effects of these cells. In this study, composites formed in a 3D Life hydrogel transplanted into the abdominal cavity of T1DM mice model. *In vitro* experiments showed that these composites enable adequate glucose sensing and a significant insulin release, which have been indicating of any significant differences between free islets and those imbedded into hydrogel. In addition, AT-MSC's presence showed direct linkage with the significant increase of insulin secretion in different concentrations of glucose (control medium, 150 and 300 mg/dl), in Gel+MSC+Islet group, in comparison with Gel+Islet group (Figure 1). These data were consistent with that of Niels O.S. Câmara *et al.* showing that AT-MSCs are able to prevent hyperglycemia and β cell destruction via immunomodulatory functions and regulatory T cells (Treg) expansion, in NOD mice (29). Likewise, several studies showed that co-transplantation of islets with MSCs enhanced graft revascularization (38, 39). This gives rise to probable role of Treg cells as an underlying mechanism.

Many studies were carried out on the transplantation, to establish a relationship between the levels of Tregs and graft function status (40-42). Ge et al. have shown that MSCs induce *in vivo* $CD4^{+}CD25^{+}FoxP3^{+}$ Treg production in renal allograft recipients with Indoleamine 2, 3-Dioxygenase (IDO) expression and thereby, improve immunological tolerance (43). Results of flowcytometric analysis of MLNs derived cells, as shown in Figure8 (A), were indicative of a significant increase in Treg population with the addition of MSCs to graft composite, in Gel versus the Gel+MSC group and in Gel+Islet versus the Gel+MSC+Islet group. It is noteworthy that, there were no significant increase in Treg cells population in flowcytometric analysis of the spleen cells. This might be as a result of the local transplantation of the graft in the abdominal

cavity that has more effect on the local immune tissues or infiltrating immune cells (33). Proving the presence of Treg cells, it seemed to be important to evaluate their functionality by investigation of related soluble factors such as IDO, iNOS and cytokines. So, these results have been confirmed using Real Time-PCR analyses evaluating transcript levels of IDO and iNOS of hydrogel containing graft on one side and levels of TGF-β1 and IL-4 in the supernatant of MLNs derived mononuclear cells on the other side. Obtained data showed significant increase of this inhibitory elements in grafts containing AT-MSCs as compared to those lacking these cells. On the other hand, IL-17 and IFN-γ as representatives of inflammatory cytokines decreased in groups with allografts containing AT-MSCs in consistent with the increase of Treg cells populations. To explain the mechanism of this decrease we can refer to Obermajer et.al's recent study in which they proposed a novel mechanism in which MSCs directly converted Th17 into Treg cells, and thereby, induced immunosuppression (44).

In the meantime, there were only one exception about IFN-γ levels which only significantly increased in the Gel+Islet group in comparison with the others, but in the case of Gel+MSC group, we observed an insignificant decrease. As showed in Figure 4 and 5, this cytokine significantly increased both in MLNs and spleen analysis, when AT-MSCs were not imbedded. Nevertheless, MSCs need an increasing IFN-γ production in the early stages of activation, prepared by diabetes induction using STZ and allograft transplantation. After activation, they suppressed IFN-γ production following the anti-inflammatory process. Results, reported by Chinnadurai, indicated the role of IFN-γ in the licensing of MSCs to exert immunomodulatory effects, such as proliferation and cytokine inhibition on activated T cells (45). Furthermore, Jang IK et.al suggested a correlation between IDO and this IFN-γ licensed MSC inhibitory effect on T cells (46).

. In addition, there were a significant increase at the transcript levels of PDX1 in the presence of AT-MSCs- (Figure 9C)., PDX1 is known as a factor which is important for development of islet cells during emberyonic development and after that keeping their function as mature islet cells (47, 48). So our results suggest a conservatory role of AT-MSCs for islets which may be through PDX1 pathway.

Results of all abovementioned cellular and molecular analyses in different groups receiving allografts are in consistence with cell infiltration in histopathology reports and blood glucose levels in recipient mice models of T1DM as it has been reported in other studies (49). We can conclude that AT-MSCs could be recommended as a promising candidates in engineered-cell

therapy for islet transplantation based on the significant ability of co-transplanted AT-MSCs to suppress the intensive allogeneic inflammatory process coupled with their ease of access.

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Conflict of interest

The authors declare no conflict of interest.

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References

1. Shapiro AM, Lakey JR, Ryan EA, Korbutt GS, Toth E, Warnock GL, et al. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. The New England journal of medicine. 2000 Jul 27;343(4):230-8. PubMed PMID: 10911004.
2. Ding Y, Xu D, Feng G, Bushell A, Muschel RJ, Wood KJ. Mesenchymal stem cells prevent the rejection of fully allogenic islet grafts by the immunosuppressive activity of matrix metalloproteinase-2 and -9. Diabetes. 2009 Aug;58(8):1797-806. PubMed PMID: 19509016. Pubmed Central PMCID: 2712800.
3. Reading JL, Sabbah S, Busch S, Tree TI. Mesenchymal stromal cells as a means of controlling pathological T-cell responses in allogeneic islet transplantation. Current opinion in organ transplantation. 2013 Feb;18(1):59-64. PubMed PMID: 23222174.
4. Jalili RB, Rayat GR, Rajotte RV, Ghahary A. Suppression of islet allogeneic immune response by indoleamine 2,3 dioxygenase-expressing fibroblasts. Journal of cellular physiology. 2007 Oct;213(1):137-43. PubMed PMID: 17477384.
5. Porada CD, Zanjani ED, Almeida-Porad G. Adult mesenchymal stem cells: a pluripotent population with multiple applications. Current stem cell research & therapy. 2006 Sep;1(3):365-9. PubMed PMID: 18220880.
6. Abbott A. Cell culture: biology's new dimension. Nature. 2003 Aug 21;424(6951):870-2. PubMed PMID: 12931155.
7. Peeters P, Vanholder R. Transplantation as a therapeutic option for diabetic nephropathy. Acta clinica Belgica. 2004 May-Jun;59(3):125-33. PubMed PMID: 15462508.
8. Abdi R, Fiorina P, Adra CN, Atkinson M, Sayegh MH. Immunomodulation by mesenchymal stem cells: a potential therapeutic strategy for type 1 diabetes. Diabetes. 2008 Jul;57(7):1759-67. PubMed PMID: 18586907. Pubmed Central PMCID: 2453631.

9. Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, et al. Pluripotency of mesenchymal stem cells derived from adult marrow. Nature. 2002 Jul 04;418(6893):41-9. PubMed PMID: 12077603.
10. Girlanda R. Complications of Post-Transplant Immunosuppression. In: Andrades JA, editor. Regenerative Medicine and Tissue Engineering: Intech Open; 2013.
11. Jacobs SA, Roobrouck VD, Verfaillie CM, Van Gool SW. Immunological characteristics of human mesenchymal stem cells and multipotent adult progenitor cells. Immunology and cell biology. 2013 Jan;91(1):32-9. PubMed PMID: 23295415. Pubmed Central PMCID: 3540326.
12. Hoogduijn MJ, Betjes MG, Baan CC. Mesenchymal stromal cells for organ transplantation: different sources and unique characteristics? Current opinion in organ transplantation. 2014 Feb;19(1):41-6. PubMed PMID: 24275893.
13. Sekiya I, Larson BL, Smith JR, Pochampally R, Cui JG, Prockop DJ. Expansion of human adult stem cells from bone marrow stroma: conditions that maximize the yields of early progenitors and evaluate their quality. Stem cells. 2002;20(6):530-41. PubMed PMID: 12456961.
14. Kawada H, Fujita J, Kinjo K, Matsuzaki Y, Tsuma M, Miyatake H, et al. Nonhematopoietic mesenchymal stem cells can be mobilized and differentiate into cardiomyocytes after myocardial infarction. Blood. 2004 Dec 01;104(12):3581-7. PubMed PMID: 15297308.
15. Boltze J, Arnold A, Walczak P, Jolkkonen J, Cui L, Wagner DC. The Dark Side of the Force - Constraints and Complications of Cell Therapies for Stroke. Frontiers in neurology. 2015;6:155. PubMed PMID: 26257702. Pubmed Central PMCID: 4507146.
16. Liu H, Lin J, Roy K. Effect of 3D scaffold and dynamic culture condition on the global gene expression profile of mouse embryonic stem cells. Biomaterials. 2006 Dec;27(36):5978-89. PubMed PMID: 16824594.
17. Schmeichel KL, Bissell MJ. Modeling tissue-specific signaling and organ function in three dimensions. Journal of cell science. 2003 Jun 15;116(Pt 12):2377-88. PubMed PMID: 12766184. Pubmed Central PMCID: 2933213.
18. Xu J, Miao G, Zhao Y, Wei J. Subcutaneous transplantation may not be an appropriate approach for the islets embedded in the collagen gel scaffolds. Transplantation proceedings. 2011 Nov;43(9):3205-8. PubMed PMID: 22099758.
19. Brosius F. Low-Dose Streptozotocin Induction Protocol (mouse). Diacomp Protocols, Diabetic Complications Consortium. The University of Michigan Medical Center2015.
20. Mohammadi Ayenehdeh J, Niknam B, Hashemi SM, Rahavi H, Rezaei N, Soleimani M, et al. Introducing a New Experimental Islet Transplantation Model using Biomimetic Hydrogel and a Simple High Yield Islet Isolation Technique. Iranian biomedical journal. 2016 Oct 18. PubMed PMID: 27752182.
21. Rahavi H, Hashemi SM, Soleimani M, Mohammadi J, Tajik N. Adipose tissue-derived mesenchymal stem cells exert in vitro immunomodulatory and beta cell protective functions in streptozotocin-induced diabetic mice model. Journal of diabetes research. 2015;2015:878535. PubMed PMID: 25893202. Pubmed Central PMCID: 4393922.
22. Taguchi Y, Tasaki Y, Terakado K, Kobayashi K, Machida T, Kobayashi T. Impaired insulin secretion from the pancreatic islets of hypothyroidal growth-retarded mice. The Journal of endocrinology. 2010 Aug;206(2):195-204. PubMed PMID: 20488945.
23. Marchetti P, Scharp DW, McLear M, Gingerich R, Finke E, Olack B, et al. Pulsatile insulin secretion from isolated human pancreatic islets. Diabetes. 1994 Jun;43(6):827-30. PubMed PMID: 8194670.
24. Hanson MS, Park EE, Sears ML, Greenwood KK, Danobeitia JS, Hullett DA, et al. A simplified approach to human islet quality assessment. Transplantation. 2010 May 27;89(10):1178-88. PubMed PMID: 20182409. Pubmed Central PMCID: 2948961.
25. Klinker MW, Wei CH. Mesenchymal stem cells in the treatment of inflammatory and autoimmune diseases in experimental animal models. World journal of stem cells. 2015 Apr 26;7(3):556-67. PubMed PMID: 25914763. Pubmed Central PMCID: 4404391.

26. Ezquer F, Ezquer M, Contador D, Ricca M, Simon V, Conget P. The antidiabetic effect of mesenchymal stem cells is unrelated to their transdifferentiation potential but to their capability to restore Th1/Th2 balance and to modify the pancreatic microenvironment. Stem cells. 2012 Aug;30(8):1664-74. PubMed PMID: 22644660.
27. Boumaza I, Srinivasan S, Witt WT, Feghali-Bostwick C, Dai Y, Garcia-Ocana A, et al. Autologous bone marrow-derived rat mesenchymal stem cells promote PDX-1 and insulin expression in the islets, alter T cell cytokine pattern and preserve regulatory T cells in the periphery and induce sustained normoglycemia. Journal of autoimmunity. 2009 Feb;32(1):33-42. PubMed PMID: 19062254.
28. Jurewicz M, Yang S, Augello A, Godwin JG, Moore RF, Azzi J, et al. Congenic mesenchymal stem cell therapy reverses hyperglycemia in experimental type 1 diabetes. Diabetes. 2010 Dec;59(12):3139-47. PubMed PMID: 20841611. Pubmed Central PMCID: 2992776.
29. Bassi EJ, Moraes-Vieira PM, Moreira-Sa CS, Almeida DC, Vieira LM, Cunha CS, et al. Immune regulatory properties of allogeneic adipose-derived mesenchymal stem cells in the treatment of experimental autoimmune diabetes. Diabetes. 2012 Oct;61(10):2534-45. PubMed PMID: 22688334. Pubmed Central PMCID: 3447906.
30. Fiorina P, Jurewicz M, Augello A, Vergani A, Dada S, La Rosa S, et al. Immunomodulatory function of bone marrow-derived mesenchymal stem cells in experimental autoimmune type 1 diabetes. Journal of immunology. 2009 Jul 15;183(2):993-1004. PubMed PMID: 19561093. Pubmed Central PMCID: 3895445.
31. Madec AM, Mallone R, Afonso G, Abou Mrad E, Mesnier A, Eljaafari A, et al. Mesenchymal stem cells protect NOD mice from diabetes by inducing regulatory T cells. Diabetologia. 2009 Jul;52(7):1391-9. PubMed PMID: 19421731.
32. Ezquer FE, Ezquer ME, Parrau DB, Carpio D, Yanez AJ, Conget PA. Systemic administration of multipotent mesenchymal stromal cells reverts hyperglycemia and prevents nephropathy in type 1 diabetic mice. Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation. 2008 Jun;14(6):631-40. PubMed PMID: 18489988.
33. Ottoboni L, De Feo D, Merlini A, Martino G. Commonalities in immune modulation between mesenchymal stem cells (MSCs) and neural stem/precursor cells (NPCs). Immunology letters. 2015 Dec;168(2):228-39. PubMed PMID: 25986012.
34. Mastro-Martinez I, Perez-Suarez E, Melen G, Gonzalez-Murillo A, Casco F, Lozano-Carbonero N, et al. Effects of local administration of allogenic adipose tissue-derived mesenchymal stem cells on functional recovery in experimental traumatic brain injury. Brain injury. 2015;29(12):1497-510. PubMed PMID: 26287760.
35. Sheng H, Wang Y, Jin Y, Zhang Q, Zhang Y, Wang L, et al. A critical role of IFNgamma in priming MSC-mediated suppression of T cell proliferation through up-regulation of B7-H1. Cell research. 2008 Aug;18(8):846-57. PubMed PMID: 18607390.
36. Lu X, Liu T, Gu L, Huang C, Zhu H, Meng W, et al. Immunomodulatory effects of mesenchymal stem cells involved in favoring type 2 T cell subsets. Transplant immunology. 2009 Dec;22(1-2):55-61. PubMed PMID: 19695330.
37. Sato K, Ozaki K, Oh I, Meguro A, Hatanaka K, Nagai T, et al. Nitric oxide plays a critical role in suppression of T-cell proliferation by mesenchymal stem cells. Blood. 2007 Jan 01;109(1):228-34. PubMed PMID: 16985180.
38. Kono T, Ahn G, Moss DR, Gann L, Zarain-Herzberg A, Nishiki Y, et al. PPAR-gamma activation restores pancreatic islet SERCA2 levels and prevents beta-cell dysfunction under conditions of hyperglycemic and cytokine stress. Molecular endocrinology. 2012 Feb;26(2):257-71. PubMed PMID: 22240811. Pubmed Central PMCID: 3275161.
39. Hong SJ, Traktuev DO, March KL. Therapeutic potential of adipose-derived stem cells in vascular growth and tissue repair. Current opinion in organ transplantation. 2010 Feb;15(1):86-91. PubMed PMID: 19949335.

40. Nikoueinejad H, Amirzargar A, Sarrafnejad A, Einollahi B, Nafar M, Ahmadpour P, et al. Dynamic changes of regulatory T cell and dendritic cell subsets in stable kidney transplant patients: a prospective analysis. Iranian journal of kidney diseases. 2014 Mar;8(2):130-8. PubMed PMID: 24685736.
41. Bouvy AP, Klepper M, Kho MM, Boer K, Betjes MG, Weimar W, et al. The impact of induction therapy on the homeostasis and function of regulatory T cells in kidney transplant patients. Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association. 2014 Aug;29(8):1587-97. PubMed PMID: 24771499.
42. Nguyen MT, Fryml E, Sahakian SK, Liu S, Michel RP, Lipman ML, et al. Pretransplantation recipient regulatory T cell suppressive function predicts delayed and slow graft function after kidney transplantation. Transplantation. 2014 Oct 15;98(7):745-53. PubMed PMID: 24879386.
43. Ge W, Jiang J, Arp J, Liu W, Garcia B, Wang H. Regulatory T-cell generation and kidney allograft tolerance induced by mesenchymal stem cells associated with indoleamine 2,3-dioxygenase expression. Transplantation. 2010 Dec 27;90(12):1312-20. PubMed PMID: 21042238.
44. Obermajer N, Popp FC, Soeder Y, Haarer J, Geissler EK, Schlitt HJ, et al. Conversion of Th17 into IL-17A(neg) regulatory T cells: a novel mechanism in prolonged allograft survival promoted by mesenchymal stem cell-supported minimized immunosuppressive therapy. Journal of immunology. 2014 Nov 15;193(10):4988-99. PubMed PMID: 25305313.
45. Chinnadurai R, Copland IB, Patel SR, Galipeau J. IDO-independent suppression of T cell effector function by IFN-gamma-licensed human mesenchymal stromal cells. Journal of immunology. 2014 Feb 15;192(4):1491-501. PubMed PMID: 24403533.
46. Jang IK, Yoon HH, Yang MS, Lee JE, Lee DH, Lee MW, et al. B7-H1 inhibits T cell proliferation through MHC class II in human mesenchymal stem cells. Transplantation proceedings. 2014 Jun;46(5):1638-41. PubMed PMID: 24935340.
47. Kimmel RA, Dobler S, Schmitner N, Walsen T, Freudenblum J, Meyer D. Diabetic pdx1-mutant zebrafish show conserved responses to nutrient overload and anti-glycemic treatment. Scientific reports. 2015 Sep 18;5:14241. PubMed PMID: 26384018. Pubmed Central PMCID: 4585597.
48. Marty-Santos L, Cleaver O. Pdx1 regulates pancreas tubulogenesis and E-cadherin expression. Development. 2016 Jan 01;143(1):101-12. PubMed PMID: 26657766. Pubmed Central PMCID: 4725206.
49. Eggenhofer E, Steinmann JF, Renner P, Slowik P, Piso P, Geissler EK, et al. Mesenchymal stem cells together with mycophenolate mofetil inhibit antigen presenting cell and T cell infiltration into allogeneic heart grafts. Transplant immunology. 2011 Apr 15;24(3):157-63. PubMed PMID: 21194567.

Table1. Primer sets used in this study

Gene	Forward	Reverse
β_2m	5' -GCCTTCACCCCAGAGAAAGG-3'	5`-GCGGTTGGGATTTACATGTTG-3`
IDO	5' -ACGAGTGTGTGAATGGTCTG-3'	5`-ATCAGTGGGCTTCTTCTTCG-3`
iNOS	5'- TGGATTTGGAGCAGAAGTGC-3'	5`-GTCATCTTGTATTGTTGGGCTG-3`
Pdx1	5' - ATTCTTGAGGGCACGAGAG-3'	5`-TTGGTCCCAGGAAAGAGTC-3`

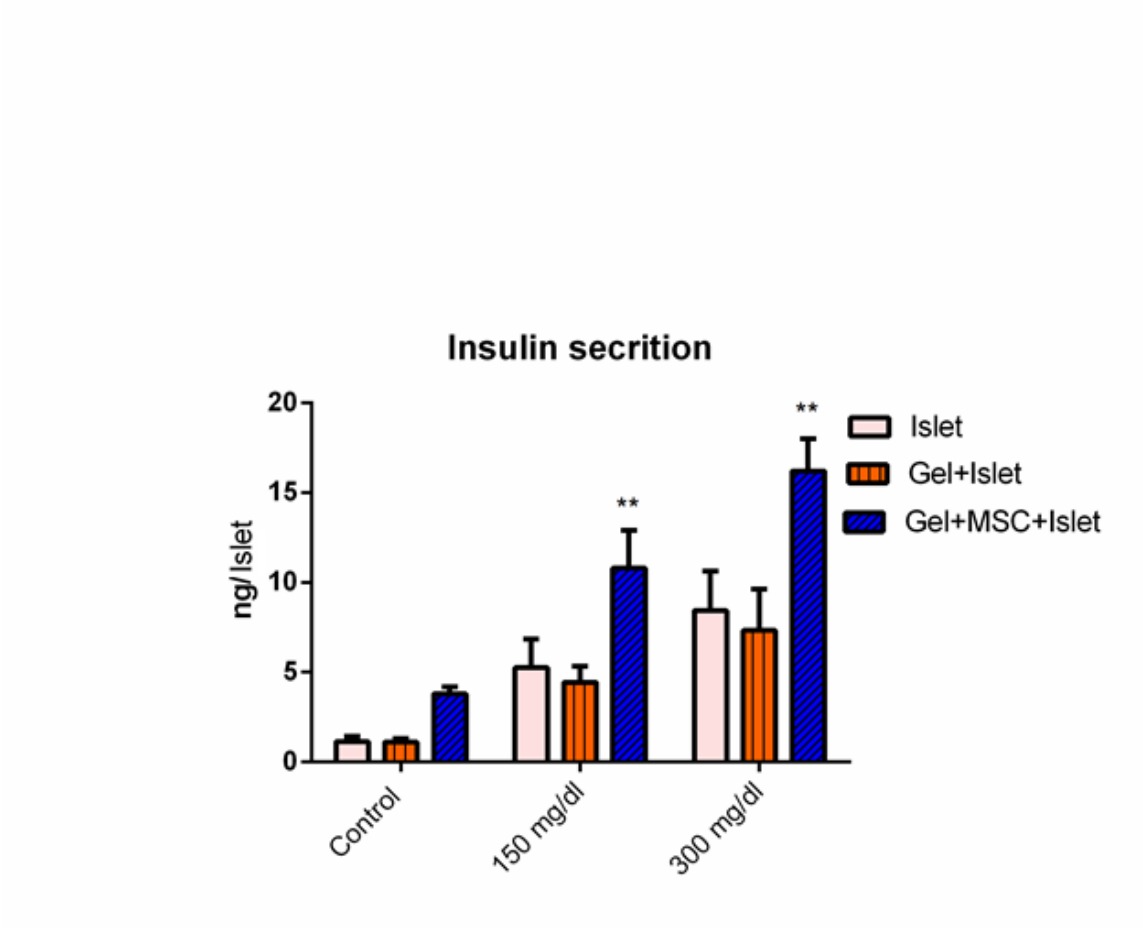


Figure 1. Comparison of the effects of Gel and/or AT-MSCs presence on glucose mediated insulin secretion from islets. Insulin secretion in supernatants of three groups of islets, including free islets without Gel (Islet), 10 islets imbedded into 100 µl hydrogel (Gel+Islet) and hydrogel composite of 10 islets and 2×10^5 MSCs/100 µl hydrogel (Gel+MSC+Islet) assessed after 60 minutes. Insulin secretion in different glucose concentration solutions, including control (no extra glucose), 150 mg/dl and 300 mg/dl are shown here. There were no significant alterations between free islets and Gel+Islet groups; whereas, there were significant changes in insulin secretion between these groups as compared to Gel+MSC+Islet group in both 150 mg/dl and 300 mg/dl (Gel+MSC+Islet vs. Islet and Gel+Islet; $P<0.01$). Data are presented as mean values of 6 independent experiments ±SD. Statistically significant differences were tested with one-way ANOVA and the *Tukey's Multiple Comparison Test*; *P<0.05, **P<0.01, ***P<0.001. ANOVA, analysis of variance; SD, standard deviation.

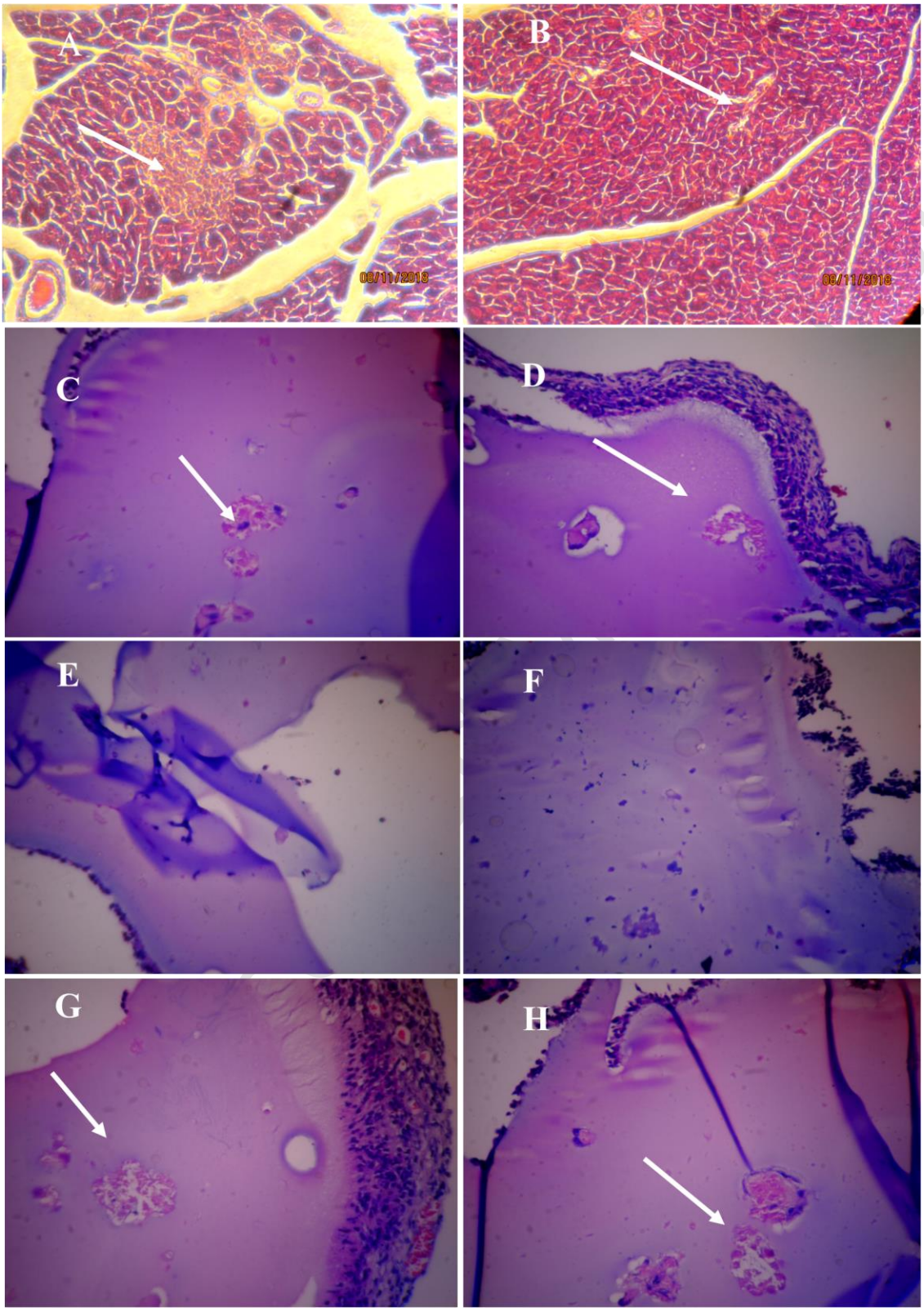


Figure 2. Leukocyte infiltration decreased in the presence of AT-MSCs in graft's Histopathological analyses with Hematoxylin-Eosin staining. Langerhans islets (arrows) are shown in: (A) normal mouse pancreas in between normal pancreatic acini and normal pancreatic ducts. (B) Diabetic pancreas showing ruptured and destructed islet of Langerhans with damage in β-cells. For islets' assessment in the body (C, D), some grafts were isolated into two time points after transplantation. Figures are representative of result of intact islets placed into them [10 days (C) and 20 days (D) post-Tx]. Finally, all grafts in transplanted groups (Gel, Gel+MSC, Gel+Islet and Gel+MSC+Islet) were isolated after 32 days (E-H respectively). Staining of grafts showed that immune cells could not infiltrate into the grafts (E-H) and leukocyte infiltration decreased when AT-MSC were transplanted (F and H), in comparison with corresponding groups (E and G), respectively. Scale bars represent 100μm.

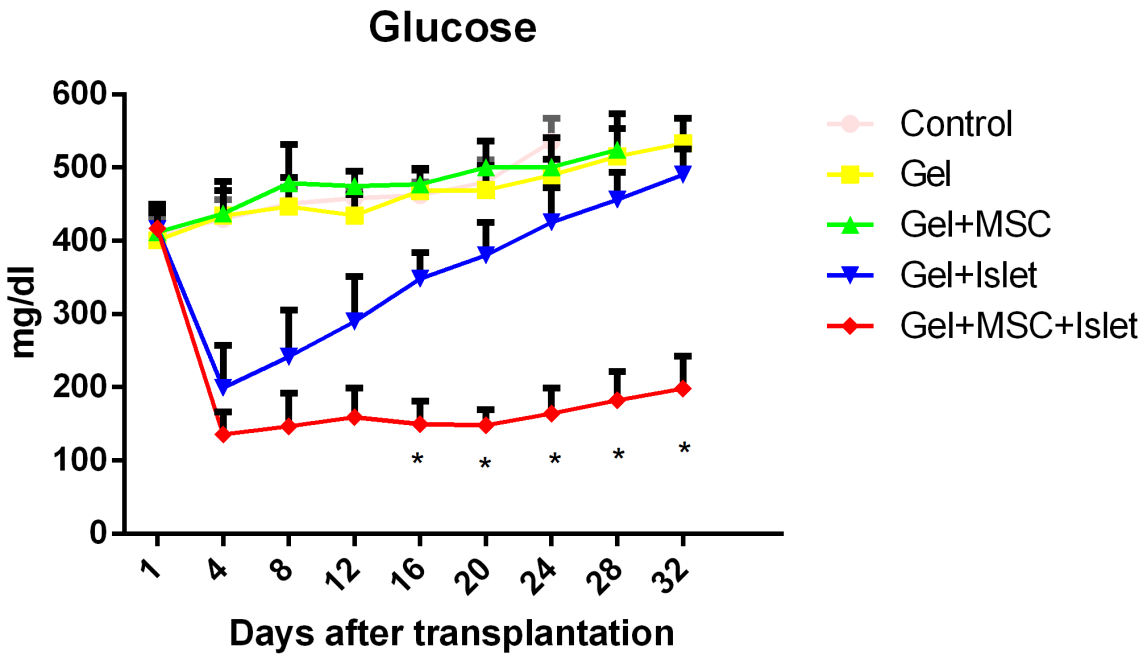


Figure 3. Comparison of blood glucose levels in T1DM mice transplanted with Islet and/or AT-MSCs. Blood glucose have been measured for diabetic mice transplanted with two pieces of 100 µL hydrogel, containing 100 islets embedded alone or co-embedded with 1×10^5 MSCs into every piece of hydrogel. Blood glucose levels of Gel+MSC+Islet group tended to show an improved glycemic state more than the Gel+Islet group. The Gel+MSC+Islet group's glucose levels significantly maintained under 300 mg/dl, since 16^{th} days after transplantation for a longer period of time in comparison with other groups (Gel+MSC+Islet vs. Gel+Islet; $P<0.05$). Data are presented as mean values of n=6 animals/group±SD. Statistical differences were tested via *Multiple t Test*; *P<0.05, **P<0.01, ***P<0.001. SD, standard deviation.

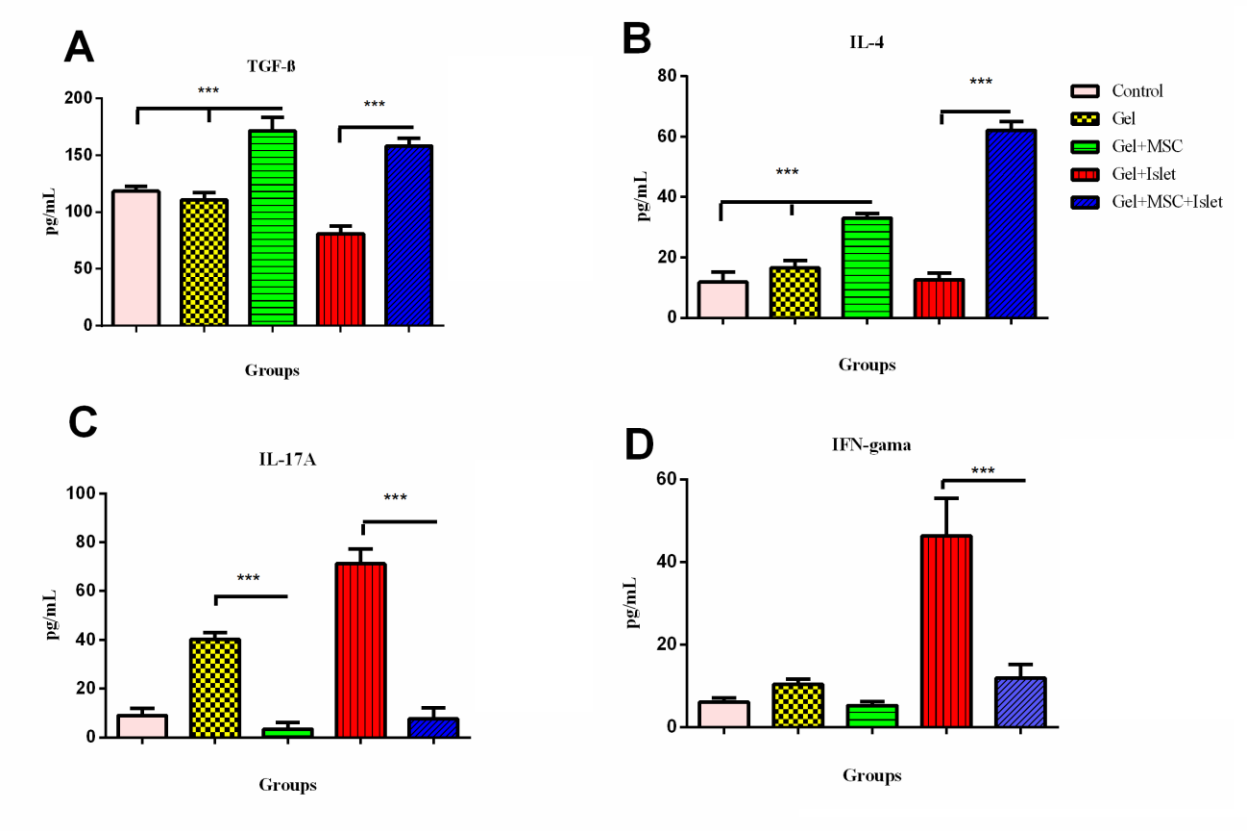


Figure 4. **Cytokine secretion analyses of the supernatant of mononuclear cells derived from Mesenteric Lymph Nodes (MLNs).** Inhibitory effects of AT-MSCs at passage 3 co-transplanted

with islets on cytokine production were assessed in mononuclear cells isolated from the mesenteric lymph nodes of mice. Levels of TGF-β1, IL-17, IFN-γ and IL-4 in supernatant of cultured cells were measured using ELISA. Transplantation of AT-MSCs alone or co-transplantation of them with allograft caused a significant increase in the levels of TGF-β1 (Gel+MSC vs. Gel; P<0.01 or Control; P<0.01 and Gel+MSC+Islet vs. Gel+Islet; P<0.001) and IL-4(Gel+MSC vs. Gel; P<0.001 or Control; P<0.001 and Gel+MSC+Islet vs. Gel+Islet; P<0.001) (A and B respectively). As their counterpart, proinflammatory cytokine levels significantly decreased in IL-17 (Gel+MSC vs. Gel and Gel+MSC+Islet vs. Gel+Islet; P<0.001) and IFN-γ (Gel+MSC+Islet vs. Gel+Islet; P<0.001) when AT-MSCs were transplanted (C and D respectively). Data are presented as mean values of n=6 animals/group±SD. Statistically significant differences were tested with one-way ANOVA and the *Tukey's Multiple Comparison Test*; ***P<0.001, **P<0.01. ANOVA, analysis of variance; SD, standard deviation.

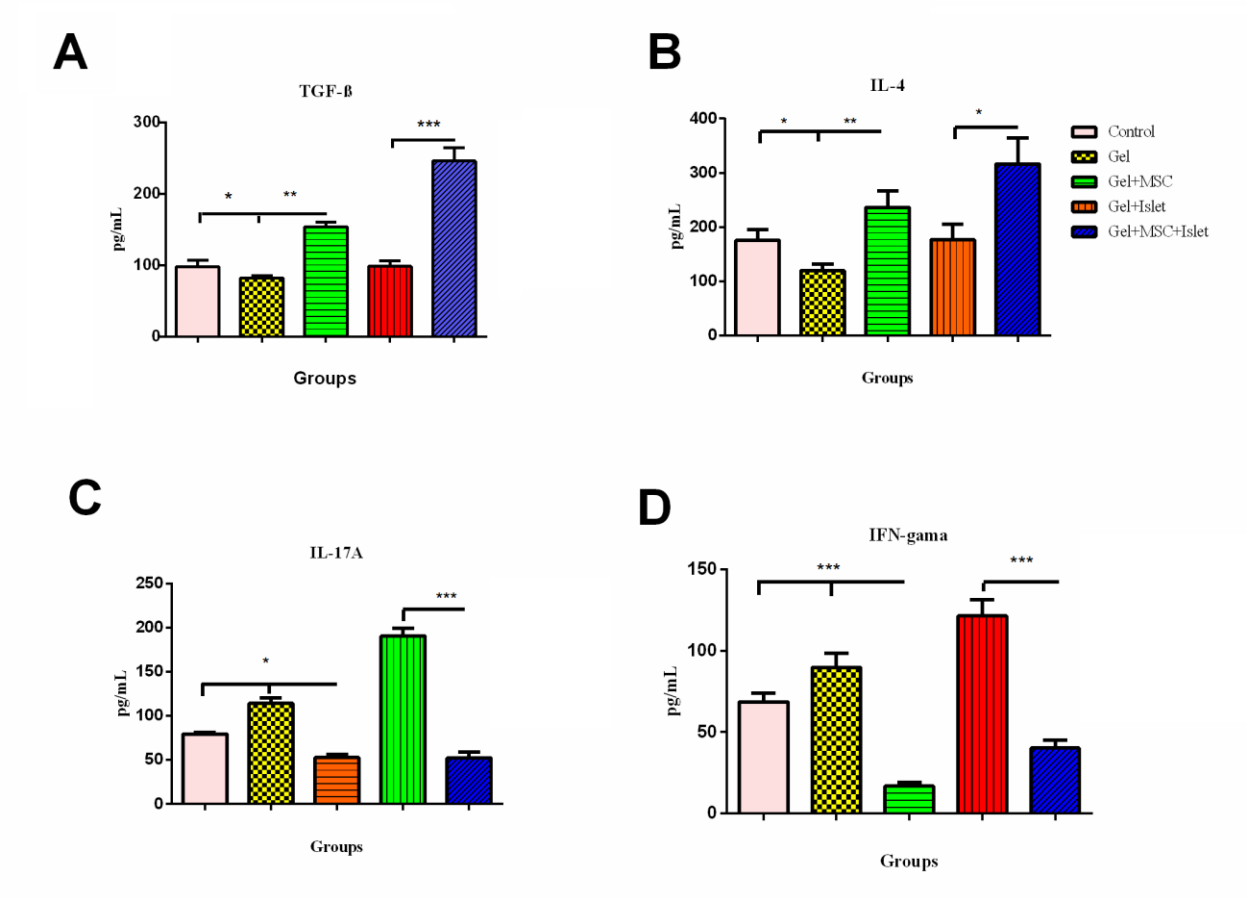


Figure 5. **Cytokine secretion analyses of the supernatant of mononuclear cells derived from spleen.** Inhibitory effect of AT-MSCs at passage 3 co-transplanted with islets on cytokine production were assessed in mononuclear cells isolated from spleen of mice. Levels of TGF-β, IL-4 IL-17 and IFN-γ in the cell culture supernatants of spleen were measured using ELISA. Transplantation of AT-MSCs alone or co-transplantion of them with allograft showed a significant increase in the levels of TGF-β1 (Gel+MSC vs. Gel; $P<0.01$ or Control; $P<0.05$ and Gel+MSC+Islet vs. Gel+Islet; $P<0.001$) and IL-4 (Gel+MSC vs. Gel; $P<0.01$ or Control; $P<0.05$ and Gel+MSC+Islet vs. Gel+Islet; $P<0.05$) (A and B respectively). As their counterparts, proinflammatory cytokine levels significantly decreased in IL-17 (Gel+MSC vs. Gel or Control; $P<0.05$ and Gel+MSC+Islet vs. Gel+Islet; $P<0.001$) and IFN-γ (Gel+MSC vs. Gel or Control; and Gel+MSC+Islet vs. Gel+Islet; $P<0.001$) when AT-MSCs were transplanted (C and D respectively). Data are presented as mean values of n=6 animals/group±SD. Statistically significant differences were tested with one-way ANOVA and the *Tukey's Multiple Comparison Test*; ***$P<0.001$,**$P<0.01$,*$P<0.05$. ANOVA, analysis of variance; SD, standard deviation.

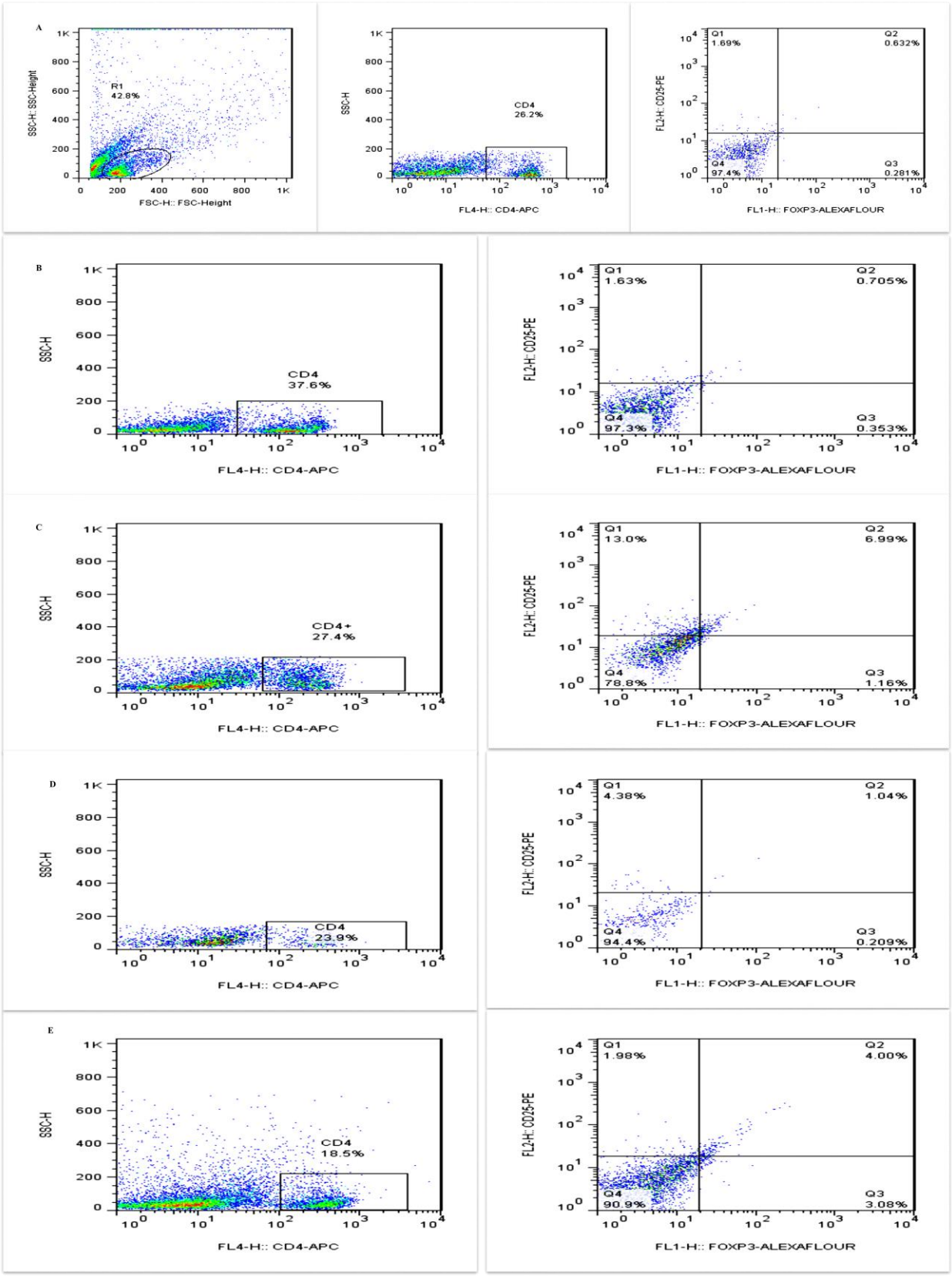


Figure 6. Assessment of Treg cells population in Flowcytometric analysis of mononuclear cells derived from MLNs. A: On the top left, a plot with side scatter on the Y axis and forward scatter on the X-axis were depicted by the gated cluster of mononuclear cells from the MLNs of control mice (diabetic mice without any treatment). Thereafter, a plot with side scatter on the Y axis and CD4-APC on the X-axis (CD4 gating) are shown. Gated CD4+ cells became the subject of further analyses for FOXP3 and CD25 at the top right plot, to indicate regulatory T cells (Treg) percentages of controls. B, C, D and E graphs are representatives of results for other transplantation groups (Gel, Gel+MSC, Gel+Islet and Gel+MSC+Islet, respectively). Treg cells population is significantly comparable between Gel+MSC, Gel, and control groups. Same result were obtained in the Gel+MSC+Islet group as compared to Gel+Islet group. MLNs; Mesenteric Lymph Nodes.

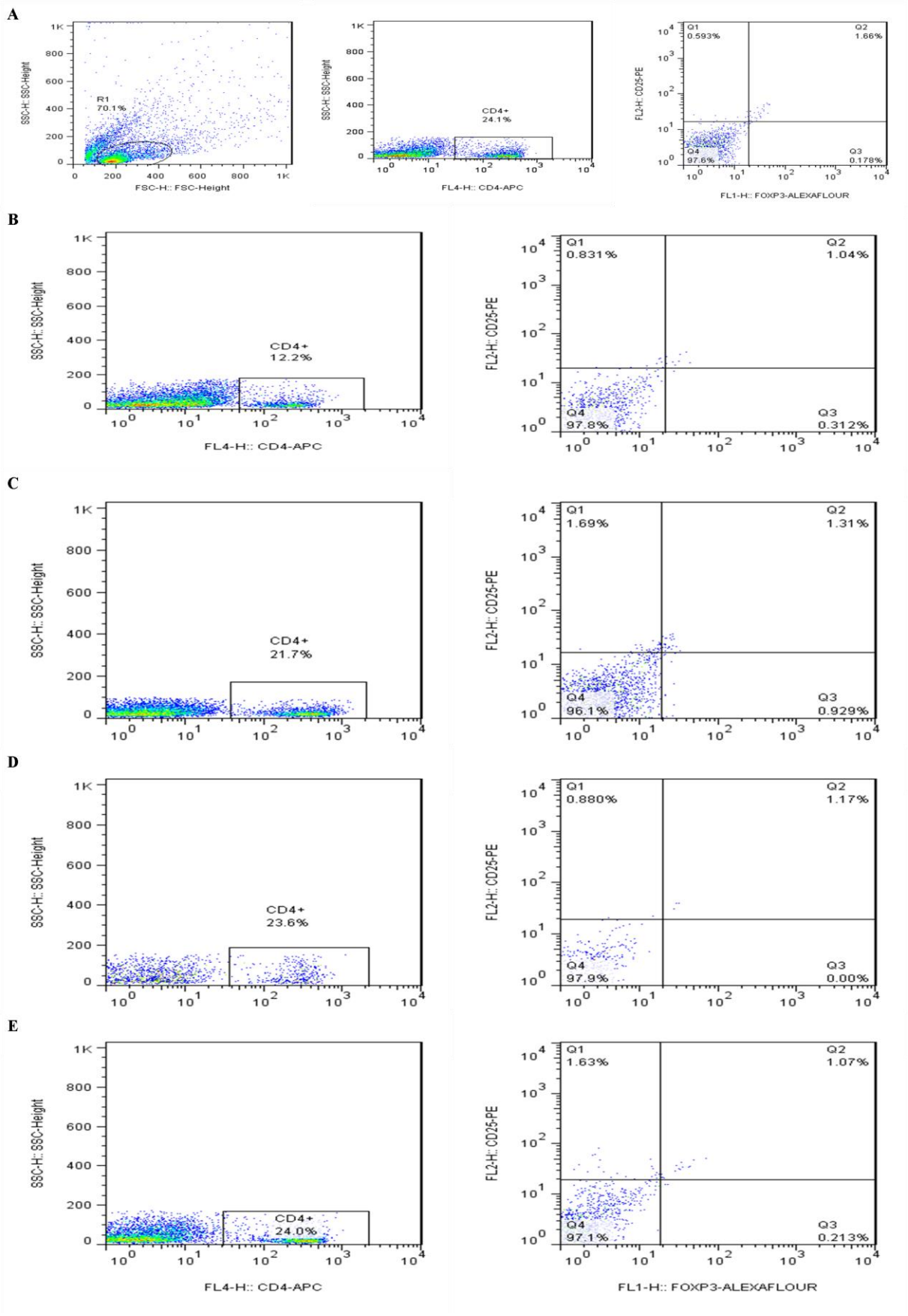


Figure 7. Assessment of Treg cells population inFlowcytometric analyses of mononuclear cells derived from mice spleen. A: On the top left, with side scatter on Y axis and forward scatter on X-axis depicted by gated cluster of the mononuclear cells from spleen tissue of the control mice (diabetic mice without any treatment), thereafter, a plot with side scatter on Y axis and CD4-APC on X-axis, are shown. The gated CD4+ cells were then, further analyzed for FOXP3 and CD25 on the top right plot to indicate regulatory T cells (Treg) percentage in controls. The graphs B, C, D and E represent results for other transplantation groups, respectively (Gel, Gel+MSC, Gel+Islet and Gel+MSC+Islet). There was no significant variation in %Treg among the groups in the spleen tissue.

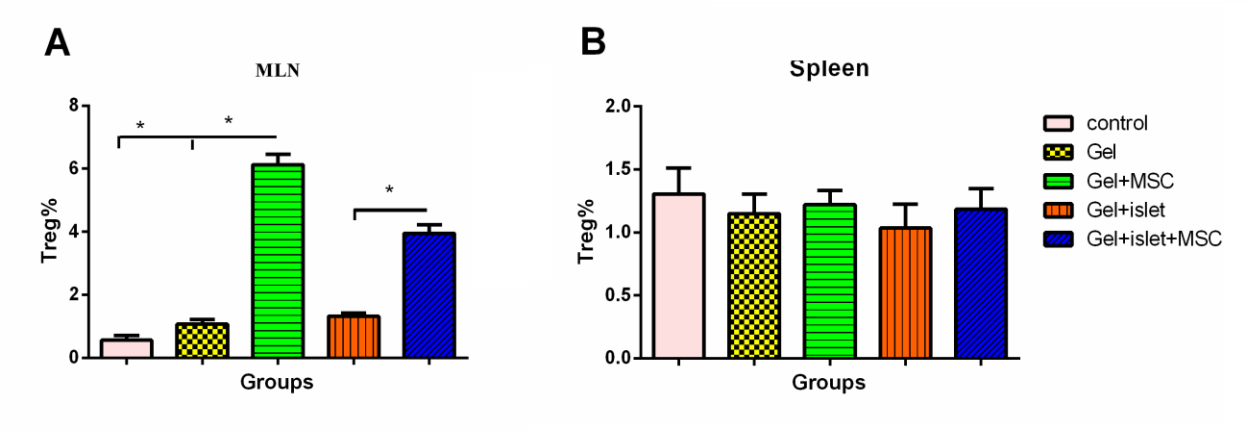
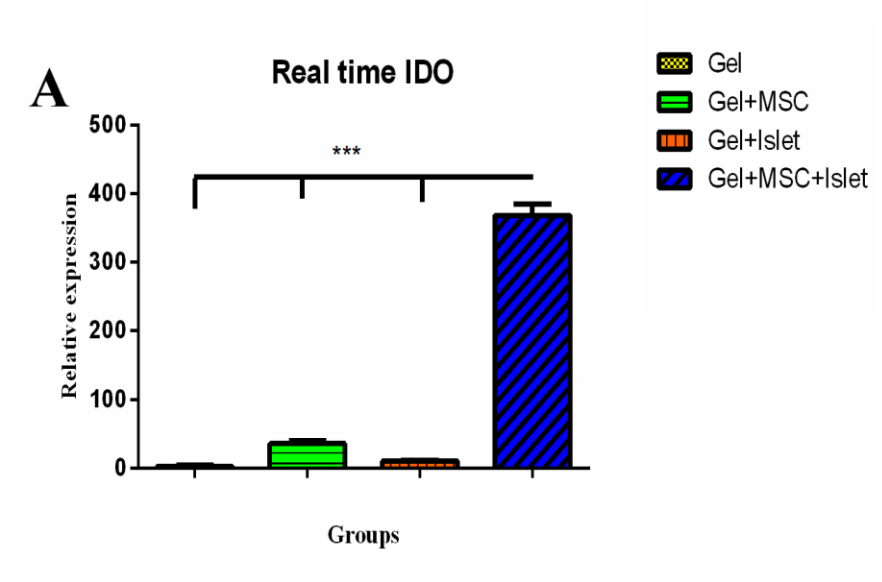
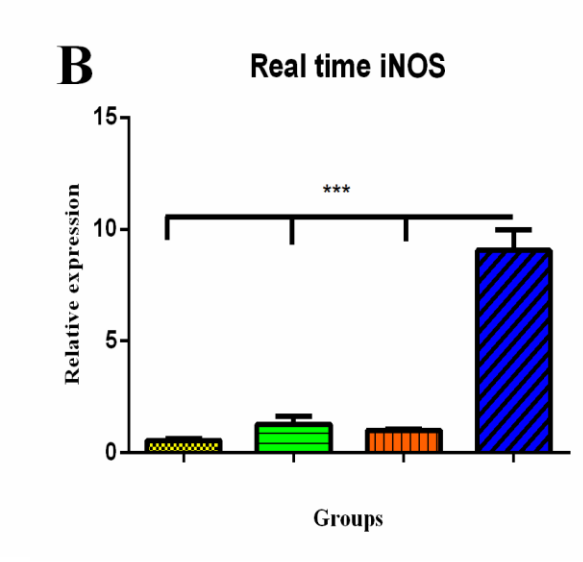


Figure 8. Comparison of regulatory T cells populations (%Treg) in mononuclear cells derived from MLNs and spleen of mice. A is %Treg cells from MLNs and B is the same from the spleen tissue. Graphs show that Treg cells population significantly increased in Gel+MSC (Gel+MSC vs. Gel or Control; $P<0.05$; 6.99% vs.0.705% or 0.622%) and Gel+MSC+Islet (Gel+MSC+Islet vs. Gel+Islet; $P<0.001$; 4% vs.1.04%) groups in MLNs (A). The same order were repeated in the mononuclear cells isolated from spleen, but there were no significant variation in the percentage of Treg cells among these groups in mononuclear cells isolated from mice spleen. Data are presented as mean values of n=6 animals/group±SD. Statistically

significant differences were tested with one-way ANOVA and the *Tukey's Multiple Comparison Test*; *P<0.05.ANOVA, analysis of variance; SD, Standard Deviation; MLNs, Mesenteric Lymph Nodes.





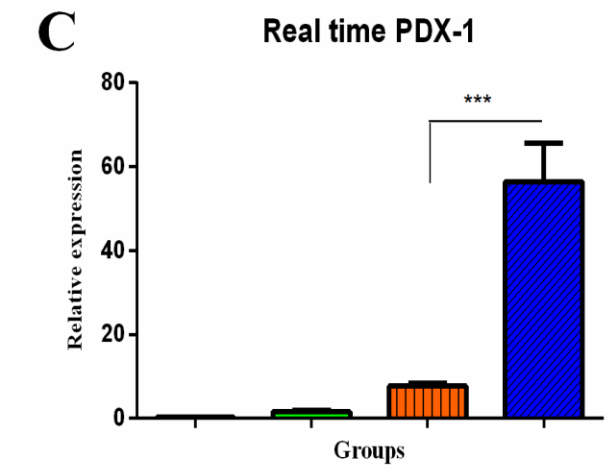


Figure 9. Transcript levels of IDO, iNOS and PDX1 in allografts suggestive of underlying inhibitory mechanisms in the presence of AT-MSCs. Co-transplantation of AT-MSCs significantly increased the transcript levels of IDO and iNOS in the Gel+MSC+Islet, as compared to the grafts without AT-MSCs and ever to grafts with AT-MSCs alone (Gel+MSC+Islet vs. Gel or Gel+Islet or ever Gel+MSC; $P<0.001$) (A and B respectively). Similar results were also observed at transcript levels of PDX1, which significantly increased ate the presence of AT-MSCs (Gel+MSC+Islet vs. Gel+Islet; $P<0.001$) (C). Data are presented as mean values of n=6 animals/group±SD. Statistically significant differences were tested with one-way ANOVA and the *Tukey's Multiple Comparison Test*; ***$P<0.001$. ANOVA, analysis of variance; SD, standard deviation.