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Oleanolic acid, a plant triterpenoid, significantly improves survival and function of islet allograft

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Abstract

Background—Oleanolic acid (OA) is a ubiquitous triterpenoid, with potent anti-oxidant and anti-inflammatory properties. Here we tested if these combined properties of OA can prevent non-immunologic primary nonfunctioning and immunologic phenomena ascribed to graft rejection hence prolong islet allograft survival.

Methods—Islet transplants were performed under kidney capsule of STZ induced diabetic C57BL/6 mice with BALB/c islets. Recipients were treated with 0.5mg/day of OA i.p. serum samples were collected once in two days and used for luminex, ELISA and donor specific antibody screening. Transplanted mice were sacrificed at different time intervals to obtain splenocytes and kidney samples for ELISPOT, MLR and immunohistochemical studies respectively.

Results—Post-transplant the decrement of blood glucose was significantly faster in mice receiving OA <2±1 days compared to untreated (4±2). OA prolonged survival of transplanted islets up to 23±3 days, and reversed diabetes even with 250 islets. Treatment group showed increased serum IL-10 (2fold) and decreased IP-10 and IL-4(3) in luminex. Significantly reduced frequency of IFN γ (4.5fold) IL-4(3.5), IL-2(2.3) and IL-17(4) producing T cell populations were found in ELISPOT. OA treated grafts had significant reduced and delayed infiltration of CD4+ and CD8+ T cells. OA also delayed donor specific antibody generation up to 19 days following transplantation. Combined treatment with CSA, OA further prolonged the islet allograft survival to 34 ± 3 days.

Conclusions—In conclusion OA is an attractive, dietary non-toxic plant triterpenoid, which suppresses the production of pro-inflammatory cytokines and delays graft specific immune responses to prolong islet allograft survival.

Keywords

Oleanolic acid; Islet allograft; grafts prolongation; anti-inflammatory; immunosuppressant

Introduction

Human pancreatic islet transplantation is a promising alternative therapy for selected patients with metabolic complications ie, brittle type 1 diabetes mellitus (T1DM) and this procedure

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can prevent long term complications associated with diabetes (1-3). However, continued function of islets following transplantation in diabetic patients has been limited and reports suggest that five year insulin free survival is less than 20% (4,5). Early events following transplantation leading to islet damage and poor engraftment is attributed not only to islet specific immunological responses but also to innate immune activation and cytokine mediated damage (6). Even under optimal conditions, 60% of transplanted islets are lost within 3 days after transplantation (6,7). Initial β -cell death resulting in poor islet engraftment is identical independent of the type of transplantation (7,8). Among a number of factors that lead to islet allograft failure, primary graft dysfunction of islet remains a significant issue (9,10). Further, revascularization of transplanted islets require up to 2 weeks. Prior as well as after revascularization the oxygenated recipient blood often leads to injury and inflammation of the islets (11). It has been demonstrated that β -cells are highly sensitive to membrane damaging agents from both recipient serum and ROS generated within the islets during isolation and transplantation (12,13). Further, instant blood-mediated inflammatory reactions negatively influence islet engraftment and survival (14,15). These inflammatory reactions together elicit strong adaptive immune response characterized primarily by macrophage-mediated islet destruction (16-18). In addition, β -cells are extremely sensitive to proinflammatory cytokines such as IL-1, IFN- γ and TNF- α , which also inhibits glucose-stimulated insulin secretion and promote islet degeneration (16-18). As is the case for other organ transplantation, islet grafts are also subjected to rejection.

There has been a renewed interest in recent years in the use of plant products for the treatment of diabetes and its complications (19). One of the best known bioactive triterpenoid is oleanolic acid (OA) present in herbs and is an integral part of human diet (20,21). The traditional uses of plants containing OA in folk medicine has been thought to be due to its anti-inflammatory (22), anti-oxidant (22,23), radical scavenging, hepatoprotective and anti-cancer properties (24-29). The mechanisms behind anti-inflammatory and anti-oxidant activities are attributed to the inhibition of enzymes such as phospholipases(30,31), cyclooxygenases (32), lipoxygenases (33,34), iNOS, C3 convertase, cytochrome P450 and leukocyte elastases (20-22,35). Hence we hypothesized that administration of OA will prevent early inflammation, delay or prevent adaptive immune responses leading to early as well as long term function of the transplanted islets.

In this communication, we demonstrate that administration of OA significantly reduces early islet cell loss following transplantation leading to restoration of normoglycemia following transplantation of even suboptimal number of islets in chemically induced diabetic animals. We also demonstrate that the beneficial effect of OA is mediated by its potent anti-inflammatory properties resulting in marked reduction in the cellular infiltration and cytokine release. Further, this reduction in the early inflammatory cascade by OA also reduced and delayed the cellular infiltration into the graft, thereby significantly enhancing islet allograft survival and is synergistic with a commonly used immunosuppressive agent ie, calcineurin inhibitor in increasing the islet allograft function.

Materials and Methods

Animals and induction of diabetes

Six to 8 week old male C57BL/6 (H2^b) and Balb/c (H2^d) mice were purchased from Jackson Laboratories All animal studies were performed in accordance with the Animal Studies Committee, Washington University, St Louis, MO guidelines. Streptozotocin (STZ, 200mg/kg body weight) was administered to chemically induce diabetes in mice.

Mouse islet isolation and transplantation

Islets from murine pancreata were isolated by collagenase digestion and transplanted under kidney capsule as described earlier (36,37). In brief, donor pancreata were digested using collagenase-XI (Roche, Indianapolis, 2mg/mL). Islets washed and purified on Ficoll gradient were handpicked and cultured for 24hrs before transplantation. Blood glucose was measured using tail snip. Primary graft function was defined as a reduction of blood glucose to <200mg/dL post-transplant, and rejection as rise in blood glucose to >250mg/dL.

Serum insulin content

The concentrations of serum insulin were measured using an insulin quantitation ELISA kit (Mercodia Inc., Winston Salem NC). Serum samples are obtained from 60 µl of blood collected from transplanted mice every other day by retro-orbital puncture, using alternative eyes with the help of anticoagulant coated capillary tubes. In brief, standard insulin solution and mouse serum samples (25µL) were added into monoclonal anti-insulin coated ELISA plate with enzyme conjugate solution and incubated for 2hrs at room temperature. After washing 6 times, TMB substrate (200µL) was added and incubated for another 5min. The reaction was stopped by adding 50µL of stopping solution and read at 450nm on ELISA reader.

Isolation of peritoneal macrophages, LPS challenge and ROS determination

Thioglycolate (3%) treated or untreated mice macrophages obtained by peritoneal wash with 5mL of DMEM and plated at 1×10^5 cells/well. Adherent cells with fresh medium were rested overnight and replaced with medium containing 100ng/mL LPS. After incubation, cells were resuspended in 100µL PBS. 2.5µL of a 1mM solution of 2,7-dihydrodichlorofluorescein diacetate (H₂DCFDA, Invitrogen) in PBS (fresh from 10mM stock) was added and incubation for 30min at 37°C. The cells were washed with PBS and fluorescence was measured at an excitation emission wavelength of 485 and 525nm respectively.

Detection of cytokine levels in serum by Luminex

Serum levels of cytokines were analyzed using multiplex bead immunoassay (Biosource International Inc, CA) as per the manufacturer's protocol and as described in our previous publications (38). Briefly, primary antibody coated beads with standards and samples were incubated for 2hrs at room temperature. The wells were washed and incubated with biotinylated antibodies for 30min. The streptavidin-R-phycoerythrin (RPE) solution was added and read using the Luminex xMAP system.

Immunohistochemistry

Tissues embedded in Freez Tissue matrix (OCT) and cut at 5 µm thickness. The sections fixed in cold alcohol for 2min (-20 °C), air dried and treated with 3% H₂O₂ in EtOH and blocked with Biotin/Avidin system components (Avidin/Biotin Blocking Kit, Vector laboratories). The sections were incubated overnight with rat anti-mouse mAbs (5.0µg/mL, BD Pharmingen,) or isotype control Ab (Chemicon International, Billerica, MA). Washed sections were treated with biotin-conjugated goat anti-rat IgG (BD Pharmingen) followed by streptavidin-HRP (BD Pharmingen). The presence of positive cells was detected with the DAB substrate Kit (BD Pharmingen).

Analysis of T cell frequency by ELISPOT

Splenocytes isolated from transplanted mice at the time of allograft rejection were stimulated with irradiated donor splenocytes and number of T cells secreting IFN-γ, IL-10 and IL-17 was enumerated by ELISPOT (39,40). Briefly, MultiScreen plates were coated overnight with 5.0 µg/mL capture mouse cytokine-specific mAb (BD Biosciences). Subsequently, 3×10^5 recipient

splenocytes were cultured in triplicate in the presence of irradiated donor splenocytes (1:1 ratio). After 48-72hrs, 2.0µg/mL biotinylated mAb (BD Biosciences) was added to the wells and incubated overnight. The plates were washed and 100µL HRP-labeled streptavidin was added. After 2hrs, the assay was developed by 3-amino-9-ethylcarbazole substrate reagent (BD Biosciences) and the spots were analyzed.

Detection of donor specific Abs in the recipient by FACS

Serums from sensitized mice were assayed for alloantibodies by their ability to bind BALB/c splenocytes using flow cytometry. Donor splenocytes (0.5 million) were incubated in 50µL FACS buffer containing 1:20 recipient serum for 30min at 4°C. Anti-mouse PE labeled secondary Ab was added and further incubated for 30min. The cells were washed and read on FACS caliber machine with instrumental setting for mouse splenocytes.

Immunosuppression with OA with low-dose cyclosporine

C57BL/6 mice were transplanted with 500 BALB/c islets. The animals were treated with either OA alone (25mg/Kg, n=4) or CSA alone (25mg/Kg, n=4) or a combination of OA and CSA (both 25mg/Kg body weight, n=4) everyday. Blood glucose levels were monitored once in 2 days as described earlier.

Chemicals and reagents

Streptozotocin (STZ), thioglycolate, DMSO and OA were purchased from Sigma Chemical Company (St. Louis, MO, USA). RPMI-1640 medium was from GIBCO Invitrogen, Life Technologies, Inc. (Grand Island, USA). Blood glucose was monitored using the One Touch II portable glucometer (Lifescan Inc, Milpitas, CA, USA) via tail snipping. The OA stock solution is made in DMSO, by constant stirring in hot water bath (50°C) followed by quick spin and sonication (Barnson sonifier-250, Danbury, CT). 3mg aliquots were made and stored at -20°C until diluted with sterile PBS/ culture medium.

Statistical analysis

Data are expressed as means $\pm$ standard deviation (SD). Statistical differences between means were analyzed using a paired or unpaired Student's *t*-test, or subjected to analysis of variance (ANOVA) and post-hoc test. A value of $p < 0.05$ was considered significant.

Results

Administration of OA to allograft recipients reverses diabetes earlier and prolongs graft survival

In order to determine the effect of OA following islet transplantation, diabetic BL/6 mice transplanted with 500 BALB/c islets were treated i.p with OA (0.5mg/day in 100 µl PBS-DMSO) or vehicle (100 µl PBS-DMSO) from day -1 onwards. As shown in figure 1, OA significantly reduced time taken to reverse diabetes following transplantation compared to control (< 2 vs 5 ± 2 days). More importantly, OA prolonged allograft survival up to 23 ± 3 days whereas vehicle treated control mice rejected the allograft on 6 ± 2 days after a short period of normoglycemia, reflecting acute allograft rejection. In order to confirm that the normoglycemia was achieved by the transplanted islets, a group of mice were nephrectomized the kidney in which islets were placed on day 18 following transplantation. Nephrectomy resulted in elevation of the blood glucose level promptly demonstrating that normoglycemia was induced through the transplanted islets (Fig.1a).

Higher circulating insulin in OA treated islet transplant recipients

In order to determine the mechanism by which OA reduces the blood glucose levels we measured the levels of insulin in serial serum samples from the mice that received syngeneic or allogeneic graft. The levels of insulin in the OA treated mice in both allo and syngeneic models were significantly higher (day 8 to day 10, $p < 0.01$) when compared to mice which were transplanted but not given OA. This data suggest that OA administration allows engraftment of larger number of islets most likely due to lack of cytokine damage of the transplanted islets resulting in increased serum levels of insulin (Fig.1b).

OA administration restores normoglycemia with suboptimal number of islet allograft

Based on the previous observation that insulin concentration is significantly higher in OA administered mice in both allo or syngeneic grafts and sugar levels being normal within 2-3 days of transplant, we tested whether suboptimal number of islets could restore normoglycemia with OA. OA administered group of mice achieved normoglycemia with 250 islets and remained normoglycemic till 12 days following transplantation (Fig.1c) whereas control mice though reduced the blood glucose level failed to restore normoglycemia. However, optimal number of islets (500 islets) transplanted from the same preparation restored normoglycemia.

Inhibitory effect of OA on ROS generation in islets and macrophages

The early damage to islets during isolation and immediately following transplantation are mostly attributed to ROS generated within the islets and macrophages of the host, followed by specific cytokines that contribute towards mobilization of host cells and islet cell damage. In order to study the effect of OA on ROS generation, 200 islets were stimulated with 28mM glucose/1 μ M rotenone respectively with or without 100 μ M OA. In murine islets, OA prevented the formation of glucose and rotenone induced ROS generation by 2.5 and 2.3 folds respectively ($p < 0.01$) (Fig.2a). The macrophages isolated from normal mice when stimulated with LPS in presence of OA showed significantly less ROS (4.5 folds, $p < 0.05$) generation (Fig.2b). Similarly macrophages isolated from OA treated transplanted mice showed significantly less ROS generation ($p < 0.01$) compared to macrophages isolated from untreated control in the presence of LPS (Fig.2c).

OA administration significantly decreases the production of pro-inflammatory cytokines

Ischemia-reperfusion injury has been shown to result in increased production of pro-inflammatory cytokines and result in islet cell death (6,41). In order to test whether OA will reduce the levels of inflammatory cytokines, serum collected on 10th day following transplantation from control and treatment groups were analyzed for serum levels of IP-10, IL-4 and IL-10 using Luminex. OA treatment results in a 3 fold reduction of IP-10 and IL-4 ($p < 0.01$) with a concomitant 2 fold increase in IL-10 ($p < 0.01$) (Fig.3).

OA administration increases the production of serum VEGF

Early engraftment of islets and/or significant reduction of early islet cell loss due to lack of inflammatory cytokines may account for the early reversal of diabetes in OA treated mice following transplantation. To test the mechanism we analyzed one of the important cytokine needed for islet engraftment ie, VEGF. Our preliminary data demonstrate that VEGF concentration is significantly higher in OA treated mice serum ($p < 0.01$) (Fig.3) raising the possibility that both mechanisms may be operative for the noted beneficial effect of OA following islet transplantation.

Low frequency of donor reactive cells in the recipient spleen following treatment with OA

In order to study the effect of OA on the initiation of alloimmune responses following islet transplantation, we determined the frequency of donor specific IFN- γ , IL-2, IL-4 and IL-17 secreting T cells by ELISPOT and MLR. We observed a significant reduction ($p < 0.01$) in the frequency of IFN- γ , IL-4, IL-17 and IL-2 producing T cells by 4.3, 3.4, 4.1 and 2.1 fold respectively in OA treated mice compared to control (Fig.4a). There was also a significant reduction (2.5 fold, < 0.01) in the lymphocyte proliferation following allogeneic stimulation in OA group (Fig.4b).

OA delays the infiltration of donor specific CD4+ and CD8+ cells into the graft

In order to identify the kinetics and specificity of cells infiltrating the islet allograft, we performed immunohistochemical analysis of the transplanted islets at varying time intervals in mice with and without OA treatment using mAbs specific for CD4+ and CD8+ cells. The control graft showed a significant increase in the number of graft infiltrating CD4+ and CD8+ cells on day 12 after transplant. In contrast, there were very little infiltrations of CD4+ and CD8+ cells in the OA treated grafts until day 19. However, at the time of rejection of OA treated grafts (23 days), the CD4+ and CD8+ cells were seen in the graft (Fig.5).

OA administration delays formation of donor specific Abs in the recipient

Studies from our lab, and others, have shown that development of donor specific Abs correlates with the rejection of transplanted islets (42-44). Serum collected at different time intervals were tested against the donor splenocytes by FACS. The serum of the control mice without OA treatment showed development of antibodies resulting in binding to the donor lymphocytes on day 12 following transplant. This corresponded to the increase in blood glucose levels indicating rejection of islets. In contrast, serum from the OA treated mice showed no reactivity on day 12 when the islets were still functioning. However presence of antibodies was seen on day 19, and on day 23 all the mice developed hyperglycemia and donor lymphocyte specific antibody binding were found in equal amounts to that of control (day 12) animals (Fig.6).

Synergistic effect of OA with low-dose CSA in prolonging islet allograft survival

Results presented above demonstrate that OA abrogates early proinflammatory cytokine release resulting in reduction of islet cell loss and possibly better engraftment due to stimulation of angiogenic growth factors such as VEGF. Although significantly delayed in rejection kinetics, OA administration alone did not prevent rejection of the islet allograft. Therefore, we tested whether administration of OA will act in a synergistic manner with the commonly used immunosuppressive agent, cyclosporine A. Mice transplanted with allogeneic islets when treated with CSA or OA alone rejected on 25 ± 4 or 23 ± 3 days respectively. When CSA treatment was combined with OA allograft survival was significantly prolonged for 34 ± 3 days ($p < 0.01$) (data not shown). This data strongly suggest that OA can be combined with other immunosuppressive agents to improve islet graft survival.

Discussion

Isolated islets, when transplanted into a type 1 diabetic patient, are challenged by a series of insults leading to cell death. Some of the major impediments of engraftment and survival are revascularization of the islets, instant blood mediated inflammatory reaction and an attack by inflammatory agents produced by infiltrating cells. It is generally believed that inflammatory cytokines secreted by macrophages are highly toxic to β cells resulting in islet cell apoptosis (45,46). To prevent innate and immune-mediated islet destruction, immunosuppressive agents are administered following transplantation and it has been shown that agents such as corticosteroids and rapamycin are toxic to islets (47-49). Therefore it is important to find other

agents which are non-toxic which will reduce early islet cell loss and facilitate early islet engraftment following transplantation. In this study we demonstrate that OA, a plant product, can be a unique non toxic, natural compound that results in protection of islets due to its anti-inflammatory, anti-oxidant potentials in addition to islet stimulatory activity (50-52).

In the present study, we demonstrated that monotherapy with OA resulted in early achievement of normoglycemia and prolongation of islet allograft survival in mice (Fig.1a). Blood glucose reached normal levels in control mice only 4 days after transplantation where as OA treatment resulted in significant reduction in the blood glucose level on day 1 post transplantation. This could be attributed either to an increased survival of islets due to lack of cytokines generated by the inflammatory cells, due to early engraftment of transplanted islets. This effect could also be attributed to stimulation of these islets upon treatment with OA. Our results demonstrating that even a sub-optimal number of islets can result in normoglycemia with OA administration (Fig.1c) support the view that OA prevents early islet cell loss due to reduction in the pro-inflammatory cytokines. However, it is also possible that OA increases an important angiogenic growth factor ie VEGF resulting in early engraftment of islets (Fig. 3). Therefore, it is reasonable to conclude that both of the above mentioned mechanisms may be operative for the beneficial effect of OA following islet transplantation.

Results presented in Fig.1 also demonstrate that OA significantly prolonged islet allograft survival (23 days), three times the control graft survival. Our contention was that OA significantly inhibited pro-inflammatory cytokines produced by the macrophages, antigen presenting and other islet infiltrating cells. To test this we analyzed the ROS generated in the islets and also from host macrophages. Results presented in Fig 4b and 4c demonstrate that OA significantly inhibited LPS induced ROS generation in macrophages isolated from mice peritoneum. In addition there was significantly less ROS generation upon LPS stimulation from macrophages isolated from OA treated islet transplanted mice. Further, OA also reduced ROS generation upon stimulation of isolated islets *in vitro* when stimulated with glucose or rotenone, a mitochondrial complex-1 inhibitor (Fig.2a). Therefore, we believe that OA not only protects transplanted islets from ROS generated during isolation but also from ROS generated by the host antigen presenting cells.

Another significant finding is that OA markedly reduced graft infiltrating cells, including CD4 and CD8 T cells following transplantation (Fig.5). Even on day 19, there were fewer cellular infiltrations. Infiltration was noted only at 23 days, the time of rejection. Measurement of serum cytokines from these animals on day 10 post transplantation demonstrated a significant reduction of pro inflammatory cytokines IP-10 and IL-4 in OA animals in comparison to control (Fig.3). ELISPOT analysis of antigen reactive cells also demonstrated marked reduction in cells producing IL-4, IFN- γ and IL-2 (Fig.4a). Taken together, our data demonstrate that OA significantly reduces graft infiltrating cells leading to reduced levels of pro-inflammatory cytokine and delay in activating an antigen specific immune response.

Our results also demonstrate that OA is not an immunosuppressive agent since animals treated with OA eventually rejected the allograft at 24 days, whereas control animals rejected the allograft at 8 days (Fig. 1). Therefore, we analyzed antigen specific proliferative response on day 10 post transplantation and as shown in Fig 4b, OA administration reduced antigenic proliferation by 50%. As discussed above, at this time point, there was significant reduction in serum cytokines as well as Th-1 cytokine (IFN- γ and IL-2) production following antigen stimulation. We propose that this is due to lack of inflammatory cells infiltrating the graft during the early post transplant period following OA administration which leads to lack of support in propagating an adaptive immune response as evidenced by the low number of graft infiltrating cells at this time point. However, specific immune response prevails and the graft rejects in a delayed manner. It is of interest that not only the frequency of alloreactive cells

were less in OA treated mice (Fig.4a and b) but also there was a marked reduction in specific antibody which was detectable only at day 19 post transplantation (Fig.6). This finding is further supported by our results of low levels of both IL-4 and IL-17, known to be involved in B cell proliferation and germinal center formation (53,54). Therefore, it is possible that OA administration may have a significant effect in antibody production, not only allo antibodies but also auto antibodies which have been associated with type 1 diabetes (55). We are currently testing this in NOD mice, a model of type 1 diabetes. OA administration significantly reduces IL-17 may also have implications in other immuno-regulatory events. Most notably, IL-17 is involved in inducing proinflammatory responses such as IL-1 β , TGF- β , TNF- α , MCP-1, and prostaglandins (e.g. PGE₂) from cells especially macrophages (53,56). Therefore, our finding that IL-17 levels are reduced in OA treated animals may provide a mechanism for the noted lack of inflammatory cellular infiltrate into the graft.

Finally, we determined whether OA can function in a synergistic manner with other immunosuppressive agent. Our preliminary results suggest that OA when given in combination with a calcineurin inhibitor, a commonly used immunosuppressant, can significantly improve allograft survival (data not shown). OA or CSA alone resulted in an allograft survival of 23-25 days. However, combination of these agents resulted in a significant improve of allograft survival to 34 days ($p < 0.01$). Further studies are currently ongoing to test the beneficial effect of OA with other agents which are currently employed in clinical islet transplantation.

In conclusion results presented here demonstrate that OA is an attractive, non-toxic natural compound with ability to suppress the production of pro-inflammatory cytokines and ROS generation following islet transplantation. OA administration also increased angiogenic growth factor VEGF. These unique properties of OA facilitates not only early engraftment of transplanted islets but also delayed the activation of adaptive immune response resulting in significant prolongation of islet allograft survival. OA administration also delayed specific antibody production following transplantation most likely due to its ability to block both IL-4 and IL-17 synthesis. Preliminary studies also suggest that OA can be effectively used in combination with other immunosuppressive agents like CSA to prolong islet allograft survival.

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Abbreviations

CSA	Cyclosporine A
DAB	3, 3'- Diaminobenzidine
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunospot assay
H₂DCFDA	2,7-dihydrodichlorofluorescein diacetate
HRP	Horseradish peroxidase
IFN	Interferon
IL	Interleukin
iNOS	Inducible nitric oxide synthase
LPS	Lipopolysaccharide
OA	Oleanolic acid
ROS	Reactive oxygen species
STZ	

	Streptozotocin
TMB	3, 3', 5, 5'-Tetramethylbenzidine
TNF	Tumor necrosis factor
VEGF	Vascular endothelial growth factor

Fig.1. Effect of administration of OA on islet graft survival and serum insulin content
 Diabetic C57BL/6 mice were transplanted with BALB/c islets. OA (25 mg/Kg) was administered daily from day - 1. The blood glucose level was monitored using glucometer by tail snip method every day. Serum collected on alternative days from the day of transplant was analyzed by insulin ELISA (n=6). Control animals received vehicle only (n=6).

Fig.2. Effect of OA on ROS generation in islets and macrophages

200 freshly isolated islets were incubated with either 28mM glucose/1μM rotenone along with/ without 100μM OA for 30min. Adherent macrophages (1×10^5) from transplanted OA treated and control mice on 10th day, or from untransplanted naïve mice treated with thioglycolate (Fig.2b and c) were incubated with medium containing 100ng/mL of LPS, 2.5μL of 1mM H₂DCFDA was added and the fluorescence was measured. Data is representative of 3 tests performed in triplicates (* = $P < 0.05$, ** = $P < 0.01$).

Fig.3. Analysis of cytokines in transplanted mice serum by luminex

Serum collected on 10th day after transplant was used for comparative screening of various cytokine levels using luminex kit according to the protocol provided by the company. The data are representative of three separate experiments done in triplicates, and the SD bars are illustrated for the luminex assay (** = $p < 0.01$).

Fig.4. Determination of frequency of donor specific T cells by ELISPOT and MLR

C57BL/6 splenocytes on 10th day of transplant (3×10^5) from OA treated and control mice were stimulated with irradiated BALB/c splenocytes (3×10^5) and analyzed for production of cytokines. For MLR (Fig.4b) the cells were cultured in medium containing tritium for 48hrs and analyzed for proliferation. The data are representative of three separate experiments done in triplicates, and the SD bars are illustrated for these assays (**= $p < 0.01$).

Fig.5. Immunohistochemical staining for graft infiltrating T cells

Sections of grafts taken from islet transplanted mice at different time intervals were stained for infiltrating CD4 and CD8 cells. Image in A and B = control on 12th day, C and D = OA on 12th day, E and F = OA on 19th, G and H = OA on 23rd day.

Fig.6. Analysis of donor specific Abs in recipient serum by FACS

Donor splenocytes (0.5 million) were incubated in 50 μ l FACS buffer containing 1:20 times diluted recipient serum for 30min at 4C°. PE labeled secondary Ab was added and further incubated for 30min. The cells were washed thrice with FACS buffer and read on FACS caliber machine with instrumental setting for mouse splenocytes. A= Control 12th day, B= OA 12th day, C= OA 19th day, D= OA 23rd day.