

Protection of rat pancreatic islet function and viability by coculture with rat bone marrow-derived mesenchymal stem cells

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The maintenance of viable and functional islets is critical in successful pancreatic islet transplantation from cadaveric sources. During the isolation procedure, islets are exposed to a number of insults including ischemia, oxidative stress and cytokine injury that cause a reduction in the recovered viable islet mass. A novel approach was designed in which streptozotocin (STZ)-damaged rat pancreatic islets (rPIs) were indirectly cocultured with rat bone marrow-derived mesenchymal stem cells (rBM-MSCs) to maintain survival of the cultured rPIs. The results indicated that islets cocultured with rBM-MSCs secreted an increased level of insulin after 14 days, whereas non-cocultured islets gradually deteriorated and cell death occurred. The cocultivation of rBM-MSCs with islets and STZ-damaged islets showed the expression of IL6 and transforming growth factor- β 1 in the culture medium, besides the expression of the antiapoptotic genes (*Mapkapk2*, *Tnfp1* and *Bcl3*), implying the cytoprotective, anti-inflammatory and antiapoptotic effects of rBM-SCs through paracrine actions.

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Pancreatic islet transplantation has the potential to replace insulin-secreting β -cell mass and restore insulin-regulated glucose homeostasis in patients with type 1 diabetes mellitus. Despite the recent developments in the isolation of pancreatic islets, only a part of the total islet can be successfully recovered by the present protocols.¹ Even more, a functionality loss was detected in more than a half of these isolated islets.² To be successful in the β -cell islet transplantation, many problems associated with maintenance of viable, functional islets isolated from cadaveric sources should be solved. The isolation of islets is performed through mechanically enhanced enzymatic digestion of the pancreas, which dissociates the islets from the surrounding acinar and ductal tissues. During this process, islets are exposed to a number of insults including ischemia, oxidative stress and cytokine injury that may result in cellular damage and functional impairment, which ultimately leads to a reduction in the viable islet mass recovered.³ Therefore, different strategies were investigated for improving the results of experimental and clinical islet isolation.^{3–16} Among these attempts, the antiapoptotic

and protective effects of some compounds, functioning as cytoprotectives, such as nicotinamide,³ lisofylline,⁵ exendine-4,⁶ interleukin (IL)4,¹⁰ prolactin¹² and protein kinase C epsilon,¹³ were examined on islet cells *in vivo* and/or *in vitro*. In other studies, many approaches of cellular application were used.^{4,7,9,11,14–16}

The first study in promoting the survival and function of islet β -cells in tissue culture was realized by Rabinovitch *et al.*⁴ They cocultured (direct) islet cells from newborn rat pancreases with fibroblasts from a mouse embryonic cell line (3T3-L2). They concluded that fibroblastic cells produce a macromolecular factor(s) capable of enhancing the survival of functional islet β -cells in tissue culture.⁴ Similar approaches were tested using pancreatic ductal cells (DECs),¹⁶ MNK-1 cells,⁷ bone marrow (BM) mononuclear cells⁹ and rat pancreatic extracts (RPEs)¹⁵ on the viability and function of pancreatic islets. In all these studies, the islets were directly cocultured with the cell components. As a result, cells, extracts and even stem cells could have a promising effect on the protection of islets from damage during culture.

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Abbreviations: Tubb3, β -tubulin; Actb, actin- β ; Acta2, α -smooth muscle actin; Bcl3, B-cell leukemia; rBM-MSCs, bone marrow-derived mesenchymal stem cells; Bmp4, bone morphogenetic protein 4; DEC, pancreatic ductal cells; Des, desmin; DTZ, dithizone; Eno2, enolase- γ ; Fos, FBJ osteosarcoma oncogene; FDA, fluorescein diacetate; FN, fibronectin; Gapdh, glyceraldehyde 3-phosphate dehydrogenase; Gfap, glial fibrillary acidic protein; IHUMSCs, human umbilical cord mesenchymal stem cells; IFNG, interferon γ ; ICCs, islet-like cell clusters; IL6, interleukin 6; IL6ra, interleukin 6 receptor- α ; IL10, interleukin 10; LPS, lipopolysaccharide; MAPKAPK2, mitogen-activated protein kinase-activated protein kinase 2; Myog, myogenin; Nes, nestin; PBS, phosphate-buffered saline; PI, propidium iodide; RPE, rat pancreatic extract; rPIs, rat pancreatic islets; Sparc, secreted acidic cysteine-rich glycoprotein/osteonectin; Spp1, secreted phosphoprotein/osteopontin; STZ, streptozotocin; Tnfp1, TNFAIP3 (tumor necrosis factor, α -induced protein 3)-interacting protein 1; TGF- β 1, transforming growth factor- β 1; Vim, vimentin

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Here, we designed a novel approach to maintain the viability and functionality of the cultured rat pancreatic islets (rPIs) by indirect coculturing of streptozotocin (STZ) injured and untreated rPIs with rat bone marrow-derived mesenchymal stem cells (rBM-MSCs). The rBM-MSCs in the coculture islets maintained their viability and functionality as shown by the increased level of insulin secretion in comparison with monocultures. A gradual destruction was observed in monocultured islets, especially in those injured with STZ. Ultimately, cell death occurred after 14 days of incubation.

Results

Light microscopy of cultured islets. Examinations of cultured islets under an inverted microscope showed that they displayed a regular cellular structure (Figure 1a). There were no disintegrated islets.

Staining of islets with dithizone. After cultivation, some islets were immediately separated and treated with dithizone (DTZ). All the islets stained with DTZ became crimson red. No other cell types were observed with the islets, indicating that only islets were present in the preparation (Figure 1b).

Cell culture of rBM-MSC. MSCs attached to the culture flasks sparsely and a majority of the cells displayed a fibroblast-like, spindle-shaped morphology during the early days of incubation. These cells began to proliferate after 3–4 days, and gradually grew to form small colonies that were termed colony-forming units (Figure 1c). By days 6–8, the number of cellular colonies with different sizes had increased. As growth continued, colonies gradually

expanded in size and the adjacent ones interconnected with each other. These primary cells reached monolayer confluence after plating for 12–15 days in their first passages. In later passages, most of these MSCs exhibited large, flattened or fibroblast-like morphology (Figure 1d). No changes in morphology or growth pattern were observed throughout 25 passages.

Flow cytometry: identification of rBM-MSCs. Both previous and present data indicated that rBM-MSCs expressed CD29 and CD90, but not CD45 (data not shown).¹⁷ rBM-MSCs maintained their phenotype throughout passages. Therefore, there was no evidence of hematopoietic precursors in the cultures. In contrast, the expression of other surface antigens agreed with the previous reports regarding murine MSCs, and indicated that the cells used in this study had the characteristics of MSCs reported in other studies.^{17–19}

Immunocytochemistry: identification of rBM-MSCs. Immunohistochemical studies were performed to characterize the progeny of the rBM-MSCs by using a large panel of antibodies specific to known antigens associated with different phenotypes. The immunocytochemical staining results are shown in Supplementary Figures 1 and 2, and Supplementary Table 1.

The antigenic phenotype of MSCs is not unique and no single marker has been found to be specific for MSCs. However, in the past, certain antigens such as surface and cytoplasmic proteins have been used in attempts to characterize MSCs. Expression of some of these antigens was also examined for the rBM-MSCs in this study. Under standard

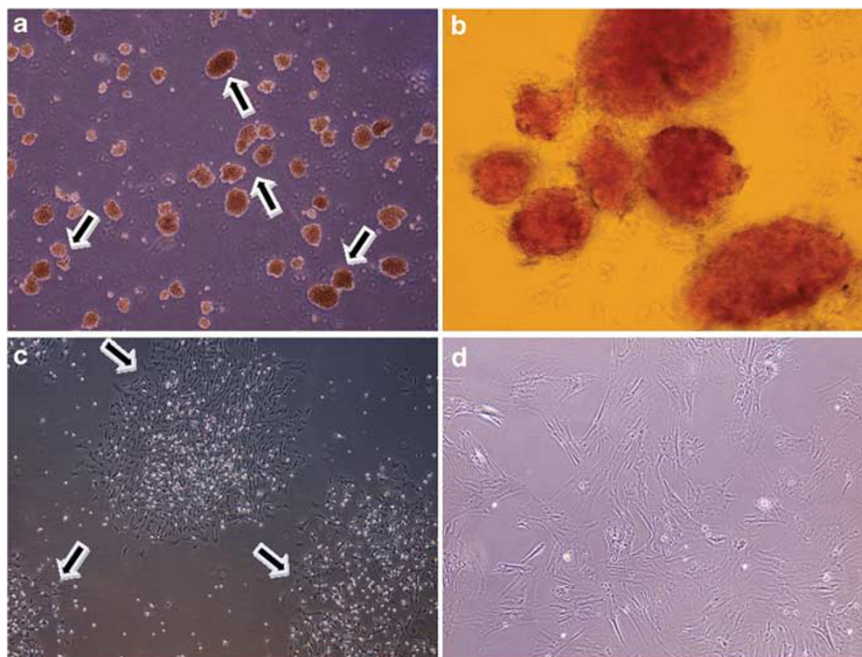


Figure 1 Morphology of PIs and rBM-MSCs. (a) Free-floating rat islets (arrows). (b) Rat islets stained with DTZ for specificity. (c) During the onset of growth (P_0 -13th day), isolated cells from the bone marrow formed single-cell-derived colonies (arrows). In later passages, most of these MSCs exhibited large, flattened or fibroblast-like morphology (P_1 -12th day) (d) (Original magnification: a, c $\times 40$; b, d $\times 200$)

culture conditions, α -smooth muscle actin (*Acta2*), actin- β (*Actb*), osteopontin (secreted phosphoprotein 1, *Spp1*), glial fibrillary acidic protein (GFAP), FBJ osteosarcoma oncogene (*Fos*), CD45, osteocalcin, osteonectin (secreted acidic cysteine-rich glycoprotein, *Sparc*), myosine IIa (Supplementary Figure 1), β -tubulin (*Tubb3*), nestin (*Nes*), fibronectin (FN) and BMP2 (Supplementary Figure 2), vimentin (*Vim*), desmin (*Des*), bone morphogenetic protein 4 (*BMP4*), myogenin (*Myog*), type I and II collagen, and CD105 (data not shown) were expressed. rBM-MSCs of this study did not express some surface markers including CD31 (endothelial) (Supplementary Figure 2C), CD34 (data not shown), CD45 (hematopoietic markers) (Supplementary Figure 1F) and CD71 (Supplementary Figure 2D).

Gene expression profiles of rBM-MSCs. Supplementary Table 2 summarizes the results of the RT-PCR analysis of rBM-MSCs. The expressions of some of the key transcripts for embryonic stem cell genes (*Pou5f1*, *Zfp42*) indicated the self-renewal capacity of rBM-MSCs. The cells were also found to be positive for *Actb*, glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*), *Vim*, *Acta2*, *Des*, *Myog*, *Nes*, *Gfap*, *Eno2*, *Tubb3*, *Sparc*, *Bmp4* and *Spp1*, showing that their expression is the indication of the differentiation to other tissues (Figure 2).

The expression of myogenic (*Des*, *Myog*, *MyosinIIa*, *Acta2*), neurogenic (enolase- γ (*Eno2*), *MAP2a,b*, *Fos*, *Nes*, *Gfap*, *Tubb3*) and osteogenic (*Sparc*, *Osteocalcin*, *Spp1*, *BMP2*, *BMP4*, *type I Collagen*) markers by BM-MSCs without induction is the explanation for the possible reason of the fact that these cells can readily differentiate into functional multiple lineages both *in vitro* and *in vivo*.

It was also shown that rBM-MSCs expressed myogenic, neurogenic and osteogenic genes under basal conditions designating the diverse immunophenotypic characteristics of MSCs that might explain the reason why these cells could easily differentiate into cells of bone, cartilage, muscle and neural tissues. Consistently, the expression of precursor markers of mesoderm and ectoderm by MSCs indicates that they could differentiate into cells of three germ layers under permissive *in vitro* conditions.

MSCs were investigated for the presence of molecules involved in immunomodulation or suppression. Mitogen-activated protein kinase-activated protein kinase 2 (MAPKAPK2) is related to kinases that mediate a wide range of biological functions in response to mitogens and stress stimuli. *Tnip1* (TNFAIP3 (tumor necrosis factor, α -induced protein 3)-interacting protein 1), which is previously known as A20, is another antiapoptotic gene (stress gene) like *Mapkapk2* expressed by both cell lines (Supplementary Table 2, Figure 2). In addition, rBM-MSCs expressed *Bcl3* (B-cell leukemia) (antiapoptotic gene) and interleukin 6 receptor- α (*IL6ra*) but failed to express *IL10* and interferon- γ (*Ifng*) (Supplementary Table 2, Figure 2). *IL6ra*, also known as CD126, is a type I cytokine receptor. Interleukin 6 (*IL6*) is a potent pleiotropic cytokine that regulates cell growth and differentiation and has an important role in immune response.

Differentiation potential of rBM-MSCs

Adipogenic differentiation. rBM-MSCs at passage 3 (P_3) were used in the adipogenic differentiation experiments, in which 80–90% sub-confluence was found to be optimal for cell differentiation that lasted for 2–3 weeks. At the beginning of the third week, lipid droplets enlarged and invaded the entire cytoplasm of the adipocyte-differentiated rBM-MSCs (Supplementary Figure 3).

Osteogenic differentiation. Histo- and/or immunohistochemical techniques for the detection of bone-specific markers were applied to cells grown in osteogenic differentiation medium and in control medium to assess the osteogenic differentiation of rBM-MSCs. Proliferation and differentiation of both cultures were compared using phase contrast microscopy. In both control and osteogenic cultures, cells proliferated and reached almost complete confluency after 8–10 days of incubation. Later, the cellular aggregates were observed in osteogenic differentiation culture plates and gradually increased until the completion of the experiment. These aggregates were characterized by the presence of amorphous material deposits (Supplementary Figure 4A). The staining of these nodular aggregates in osteogenic cultures by Alizarin red S showed that the amorphous deposits observed at the microscope were actually calcium deposits. Alizarin red positive nodular aggregates were already present at day 15 (Supplementary Figure 4B) and control cultures showed only minimal background staining (Supplementary Figure 4D). Cells that were incubated for 15 days were also strongly stained positive for BMP4 (Supplementary Figure 4C), osteopontin (Supplementary Figure 4C), osteocalcin (data not shown) and extracellular located matrix proteins BMP2 (data not shown).

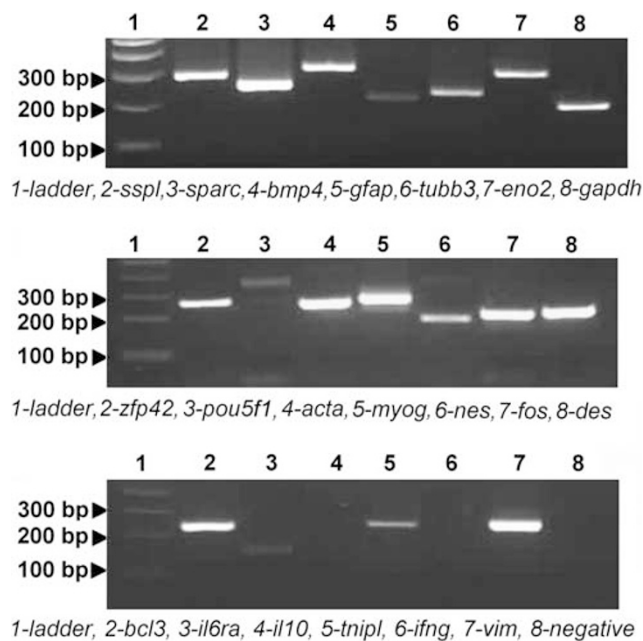


Figure 2 Agarose gel electrophoresis of RT-PCR products. Representative panel of RT-PCR analysis of rBM-MSCs. Internal control: glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*). Negative control: PCR mix without template

Antiapoptosis ability of rBM-MSCs on STZ-induced pancreatic islets. After coculture experiments, quantitative analysis of apoptosis was conducted by FACS (AnnexinV/PI) and microscopic evaluations on the islets stained with fluorescein diacetate (FDA)/propidium iodide (PI).

For determination of cell viability in normal and STZ-induced pancreatic islets cocultured with rBM-MSCs, FDA/PI staining was performed. In Figure 3, the images of rPIs cultured under different conditions are given. Dead

cells stained red and viable cells stained green. The percentage viability of islet cells was calculated as described in the Materials and Methods section. As shown in Figure 4c, STZ induced a significant decrease in the viability rates of islets compared with untreated islets ($10\% \pm 2.8$, $60\% \pm 2.81$, respectively; $P < 0.001$). In contrast, coculture with rBM-MSCs induced a significant increase in viability rates in STZ-induced islets ($72.5\% \pm 4.01$; $P < 0.001$).

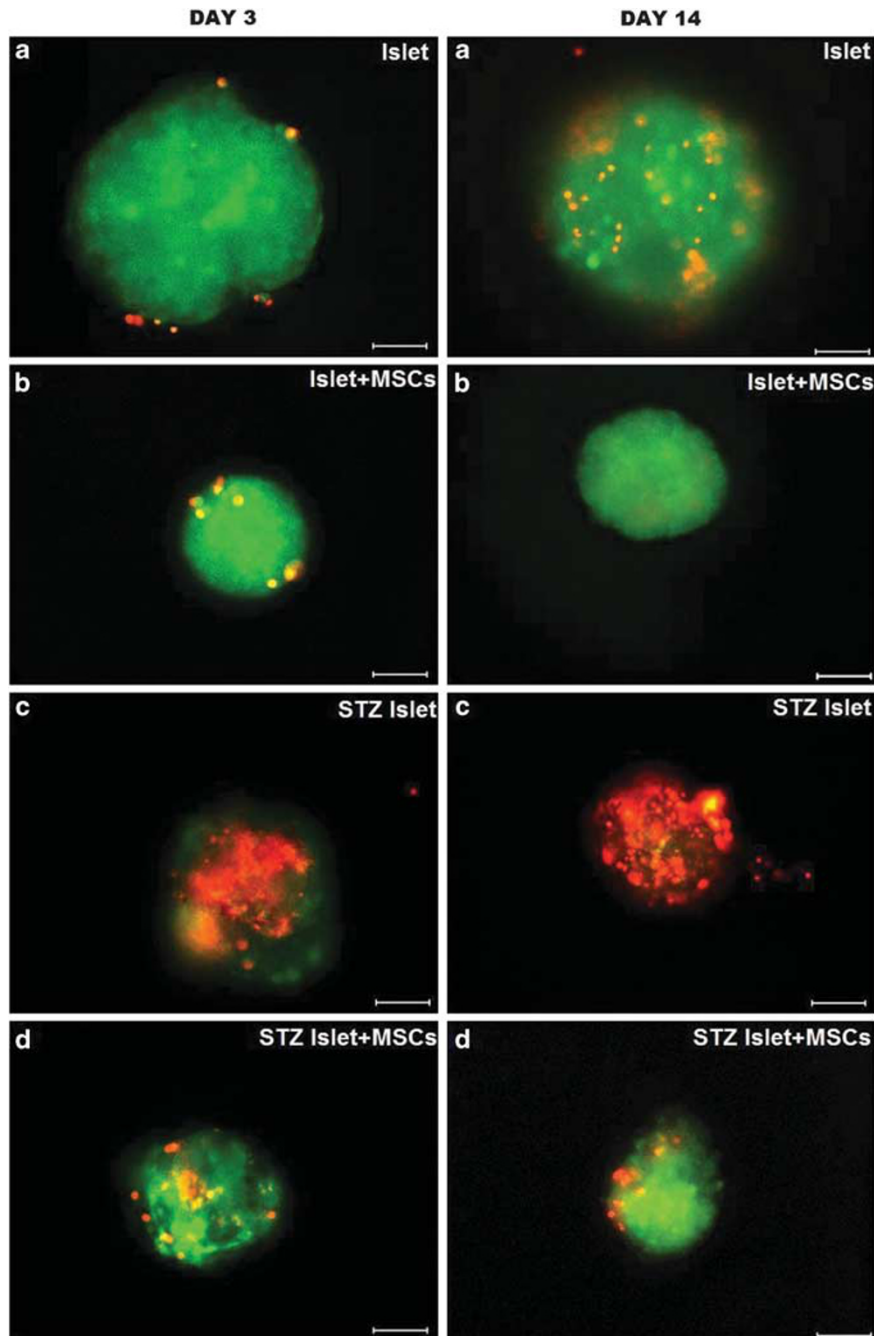


Figure 3 Cell viability. For determination of cell viability in normal (a, b) and STZ-induced pancreatic islets cocultured with rBM-MSCs (c, d), PI/FDA staining was performed. The small bright spots in the fluorescent micrographs correspond to the intense red staining of PI-positive (dead) cells. The diffusely stained regions correspond to the FDA-stained (live) cells (Scale bars = 50 μm)

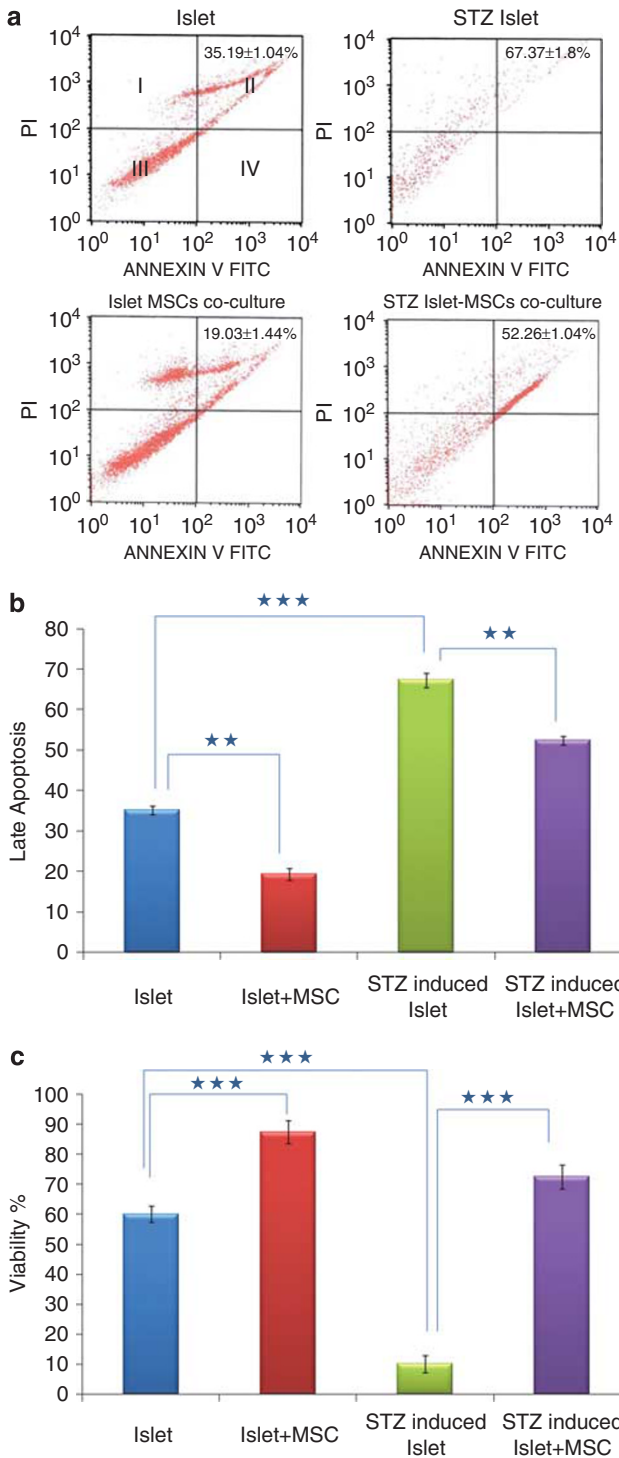


Figure 4 Detection of apoptosis. (a) Apoptosis was quantified by FACS analysis after staining with Annexin V and PI at the end of the experiment (day 14) (islets were incubated with trypsin to obtain dispersed cells). Viable cells were Annexin V⁻/PI⁻ (III), early apoptotic cells were Annexin V⁺/PI⁻ (IV), late apoptotic cells were Annexin V⁺/PI⁺ (II) and necrotic cells were Annexin V⁻/PI⁺ (I). Representative examples are shown in b. (c) We also performed PI/FDA staining for determination of cell viability in normal and STZ-induced pancreatic islets cocultured with rBM-MSCs and calculated as described in Methods. STZ induced a significant decrease in the viability rates in islets compared with untreated islets (10% ± 2.8, 60% ± 2.81, respectively). Coculture with rBM-MSCs induced a significant increase in viability rates in STZ-induced islets (72.5% ± 4.01; *P* < 0.001). (mean ± S.D., *n* = 3 each, ***P* < 0.01, ****P* < 0.001)

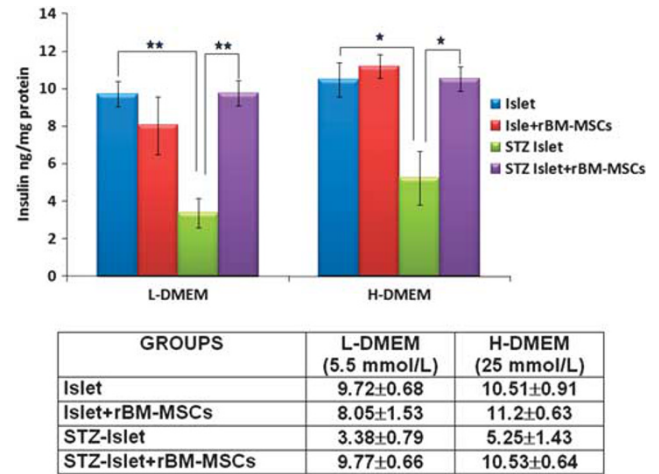


Figure 5 Insulin release in response to glucose stimulation of islets in different groups. On day 14, normal islets, islets cocultured with rBM-MSCs, STZ-induced islets and STZ-induced islets cocultured with rBM-MSCs secreted insulin into the medium and the insulin was increased under glucose challenge. However, the insulin secretion level of STZ-induced islets was decreased more significantly than in other groups, similar to under H-DMEM conditions. Interestingly, injured islets cocultured with rBM-MSCs released insulin into culture medium under glucose stimulation, similar to normal islets. Data represent the mean ± S.E. of values of three independent measurements. Data were analyzed for significant change by using two-way ANOVA and paired *t*-test (**P* < 0.05; ***P* < 0.01)

Cell apoptosis was measured by Annexin V-FITC, which binds to phosphatidylserine residues that are redistributed from the inner to the outer leaflet of the cell membrane as an early event in apoptosis. After loss of membrane integrity, PI can enter the cell and intercalate into DNA.²⁰ Figure 4a and b shows the percentages of Annexin V-PI-stained cells of islets. The average percentages of Annexin V⁺/PI⁺ (late apoptotic cells) were highest in STZ-induced islets (67.34% ± 1.8) and the percentage in islets cocultured with rBM-MSCs was the lowest (19.3% ± 1.4). Moreover, there was a statistical significance between STZ-induced islets and STZ-induced islets cocultured with rBM-MSCs (52.26% ± 1.04) (*P* < 0.01) as well as the rate of Annexin V⁻/PI⁻ (viable cells), Annexin V⁺/PI⁻ (early apoptotic cells) and Annexin V⁻/PI⁺ (necrotic cells) (data not shown). Therefore, this suggested that islets cocultured with rBM-MSCs had a superior tolerance to STZ-induced cytotoxicity.

Insulin content. The content of total cellular insulin in experimental groups was evaluated and it was found that STZ-induced islets showed a significant decrease in cellular insulin content in comparison to the untreated islets when induced with L-DMEM and H-DMEM (3.38 ± 0.79 ng/mg protein and 9.72 ± 0.68 ng/mg protein in L-DMEM, and 5.25 ± 1.43 ng/mg protein and 10.51 ± 0.91 ng/mg protein in H-DMEM, with respect to STZ-induced islets and untreated islets; *P* < 0.01) (Figure 5). The proportion of insulin release to insulin content in STZ-induced islets cocultured with rBM-MSCs was estimated to be approximately 9.77 ± 0.66 ng/mg protein in L-DMEM and 10.53 ± 0.64 ng/mg protein in H-DMEM (Figure 5).

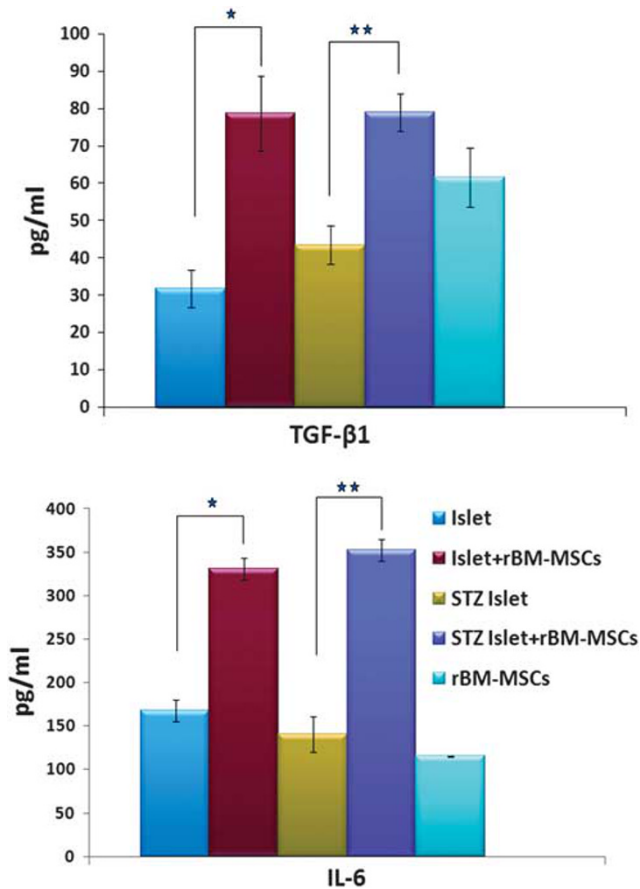


Figure 6 Cytokine secretion evaluated by ELISA. The expressions of TGF- β 1 and IL-6 were quantified in various monocultures including islets, STZ-induced islets and rBM-MSCs and cocultures including islets + rBM-MSCs and STZ-induced islets + rBM-MSCs. Data represent the mean \pm S.E. of values of three independent measurements. Data were analyzed for significant change by using two-way ANOVA and paired *t*-test (* $P < 0.05$; ** $P < 0.01$)

Cytokine secretion. Transforming growth factor- β 1 (TGF- β 1), IL4 and IL6 were found to be secreted by rBM-MSCs independent of the cocultured islets. The analysis of three cytokines (TGF- β 1, IL4 and IL6) in all three cell culture supernatants showed that all of the monocultures have detectable concentrations of these cytokines. Only the concentrations of IL4 were not altered in cocultures (data not shown). The coculturing of rBM-MSCs with islets and STZ islets led to an increase in secretion of IL6 and TGF- β 1 in comparison to monocultures of rBM-MSCs, islets and STZ islets (Figure 6).

Discussion

Therapeutic effects of MSCs are believed to occur not only by direct differentiation into injured tissue but also by production of paracrine and autocrine factors. MSCs at the injured tissue environments can promote the secretion of a variety of cytokines and growth factors that have both paracrine and autocrine activities.^{21,22} On this line, several studies have been performed. For instance, in probing the mechanism of treating effects of MSCs transplanted into the infarcted heart,

several researchers noticed that MSCs undergoing hypoxia environments stimulated the infarcted heart local microenvironment to secrete more amounts of cardioprotective vital growth factors to inhibit cardiomyocyte apoptosis compared with MSCs *in vitro* cultured under normoxia.²³ Gatto *et al.*¹⁶ reported that DECAs are putative stem cells for islets, which might secrete specific factors supporting islet growth and function. Therefore, the effects of direct coculturing with DECAs on the viability of fresh and cryopreserved human pancreatic islets were studied. The results indicated that the coculture process represents a valuable tool to improve the survival and functional activity of islets.¹⁶ Miki *et al.*⁷ showed that coculture of the mouse, rat and pig islets with MNNK-1 cells that served as a feeder cell in the coculture maintained their viability and insulin secretion with glucose responsiveness.⁷ Luo *et al.*⁹ examined the reparative effects of allogeneic BM mononuclear cells that were placed in culture with isolated human islets. Coculture with BM showed the ability of damage repair by human islets resulting in functional insulin release from islets in the long term. They suggested that BM increases survival of islets and function with the eventual formation of pancreatic endocrine tissue capable of sustaining β -cell function.⁹ Chao *et al.*¹¹ designed an approach to maintain the cultured rat pancreatic islet-like cell cluster (ICC) survival and function, when cocultured with human umbilical cord mesenchymal stem cells (HUMSCs). Their findings suggest that coculture with HUMSCs may have a significant potential to protect ICCs from damage.¹¹ Similarly, Xu *et al.*¹⁵ studied the impact of RPE on cytokine secretion by MSCs and examined the influence of administration of conditioned media of MSCs treated with RPE on blood glucose levels in diabetic rats. Their report indicated that RPE enhanced the secretion of cytokines by MSCs. MSCs in the pancreatic microenvironment might exert indirect salutary effects through paracrine mediators on injured pancreatic cells in an STZ-induced diabetic animal model. The secreted factors may exert their therapeutic benefits by preventing apoptosis of pancreatic β -cells.¹⁵ More recently, Solari *et al.*¹⁴ examined the immunoregulatory effect of autologous MSCs on sub-optimal numbers of islets co-transplanted into an omental pouch to enhance insulin secretion and sustained normoglycemia in a model of STZ-induced diabetic rats. They have shown that allogeneic islets with autologous MSCs induced insulin secretion and promoted long-term islet allograft survival.¹⁴

In this study, we used a novel approach to maintain viability and functionality of the cultured rPIs. In this approach, cultures were separated by a membrane preventing any direct interaction between islets and rBM-MSCs. In routine cultures, STZ induction reduced the viability and functional activity of rPIs compared with non-inductive islets (fresh tissue). However, it was found that rBM-MSCs in coculture had the ability to maintain islet viability and functionality, proven by prolonged insulin secretion by islets for 14 days. Unlike protected islets, a gradual destruction was observed in unprotected islets and a significant loss of function was observed in islets injured by STZ ultimately leading to cell death after 14 days of incubation.

It has also been shown that *in vitro* expanded and purified rat MSCs spontaneously secrete TGF- β 1, hepatocyte growth factor and IL6, but not IFNG, IL4, IL5 or IL10.^{24,25} It was

considered rather that MSCs promote tissue repair by secreting soluble factors that modulate inflammation and angiogenesis.²⁶ In this study, the cocultivation of rBM-MSCs with islets and STZ islets led to an increase in IL6 and TGF- β 1 into the culture supernatant in comparison with monocultures of rBM-MSCs, islets and STZ islets. Secretion of these cytokines by MSCs could have a role in their anti-inflammatory or antiapoptotic function.

Oh *et al.*²⁷ showed that human MSCs secreted small amounts of IL6 while secreting large amounts of TGF- β 1 into the culture medium.²⁷ Moreover, increased IL6 secretion was shown when hMSCs were cocultured with chemically damaged human corneal epithelial cells (hCECs). This is in agreement with the data reporting upregulation of IL6 in stimulated MSCs.^{28,29} In a recent study, it has been suggested that suppressive effects of MSCs can be through IL6 by inhibiting lymphocyte apoptosis.³⁰ Their findings pointed out that MSCs inhibit apoptosis of lymphocytes, and that soluble factors, mainly IL6 secreted by MSCs after direct interaction with lymphocytes, have an important role in their antiapoptotic function. In another report, IL6 mediated antiapoptotic effects and drug resistance mechanisms through both STAT3 and bcl-xL pathways in prostate cancer cells were revealed.³¹ In this study, IL6 and its specific receptor (IL6RA) were expressed by rBM-MSCs, and these data might show the antiapoptotic effect of MSCs. In addition, it has been shown that MSCs suppress various immune functions through a release of immunosuppressive soluble factor, TGF- β 1.³²

SPP1 is a highly hydrophilic and negatively charged sialo-protein of approximately 298 amino acids that contains a Gly-Arg-Gly-Asp-Ser motif. SSP1 exists both intra- and extracellularly. It has been shown that *Ssp1* is expressed in the pancreatic islets of the rat and that its pancreatic levels are acutely upregulated in response to STZ-induced diabetes. It was also shown that SSP1 protects the islets from STZ-induced no production.³³ SSP1 also prevents IL-1 β -induced rat islet dysfunction.⁸ In this study, it was shown that rBM-MSCs expressed SSP1 at the levels of gene and protein without stimulation. Therefore, it was considered that SSP1 could be secreted by rBM-MSCs into coculture medium and could protect islets from destruction. Moreover, it has been observed that FN treatment significantly inhibited lipopolysaccharide (LPS)-induced apoptotic activities in FN-treated MSCs-derived hepatocyte-like cells and improved the survival rates of LPS-induced SCID mice.³⁴ This study showed that FN was expressed by rBM-MSCs at both the gene and protein levels.

Genes with known antiapoptotic functions, and those expressed under stress include *Mapkapk2*, *Trnp1* and *Bcl3*. The expressions of these genes by stem cells were examined in this study. MAPKAPK2 is a direct substrate of p38 MAP kinase in response to cellular stress such as mechanical stress, heat shock, osmotic stress, UV irradiation, bacterial LPS, several inflammatory cytokines and growth factors.³⁵ TNIP1 was originally described as an antiapoptotic TNF- α -induced gene in endothelial cells.³⁶ BCL3, in complex with p52, can promote transcription of the genes encoding the cell cycle regulator cyclin D1 and the antiapoptotic BCL2 protein.³⁷ The presented data showed the expression of all three genes in rBM-MSCs. Expression of these genes by

rBM-MSCs might imply the role of rBM-SCs and highlight the antiapoptotic influence mechanisms that could have a protective role on pancreatic islet cells.

Overall, the underlying mechanisms modulating pancreatic islet viability might be attributed to the paracrine mediators IL6, TGF- β 1, SSP1 and FN secreted by MSCs. Coculturing with rBM-MSCs might have a significant potential to protect STZ-induced injured pancreatic islets through paracrine actions such as cytoprotective, anti-inflammatory and antiapoptotic effects. In this study, an indirect coculture approach was used for the first time to protect and improve islet viability and its functionality, although several other studies, in which islets were directly cocultured with cellular extracts or with various cell types (e.g., fibroblasts, pancreatic ductal cells, MNNK-1 cells BM-mono-nuclear cells) to reach the same target, bring about the requirement for separation afterwards.^{4,7,9,11,14–16} The uniqueness of our approach is the elimination of mixing the different cell populations or contaminating cell extracts by placing a separator (insert) between the islets and the cells/cell extracts. Therefore, the approach used in this study would be very important in allogeneic islet transplantations and clinical practices.

Materials and Methods

Animals. Wistar male rats (8–10 weeks old) were obtained from the Experimental Animal Center of Kocaeli University (Kocaeli, Turkey). Animal housing and experiments were approved by the local animal care committee according to the institutional guidelines and national animal welfare. They were housed under standard conditions for a week before use.

Isolation of islets. Islets were isolated as described³⁸ and cultured in RPMI 1640 (Invitrogen/GIBCO, Grand Island, NY, USA) with glucose 2 g/l supplemented with 10% fetal bovine serum (FBS; Invitrogen/GIBCO), 100 IU/ml penicillin-100 μ g/ml streptomycin (Invitrogen/GIBCO) and glutamine (2 mmol/l; Invitrogen/GIBCO) at 37°C in a humidified air atmosphere containing 5% CO₂.

Islets were then tested for their specificity by DTZ staining. DTZ staining was carried out by adding 10 μ l DTZ stock solution to islets suspended in 1 ml Krebs-Ringer bicarbonate buffer (pH 7.4) with HEPES (10 mM) (KRBH) and incubated at 37°C for 10–15 min. The stained islets appeared as bright red under the inverted microscope (CKX41, Olympus, Tokyo, Japan). Non-islet tissue remained unstained.

Isolation and culturing of rBM-MSCs. To establish the MSC culture, the animals were anesthetized with Ketalar (Pfizer, Kirklareli, Turkey) and killed by cervical dislocation method. Under sterile conditions, both femur and tibiae from each rat were excised. Muscle and the entire connective tissue were detached. The ends of the bones were removed and a 21-gauge needle, inserted into the shaft of the BM, was extruded by flushing with RPMI 1640 supplemented with 10% FBS and 100 IU/ml penicillin-100 μ g/ml streptomycin that was also used as the growth medium. Marrow plug suspension was dispersed by pipetting, successively filtered through 70 μ m mesh nylon filter (BD Biosciences, Bedford, MA, USA), and centrifuged at 200 g for 10 min. Supernatant containing thrombocytes and erythrocytes was discarded, and the cell pellet was resuspended in the medium. The cells from one rat were seeded onto two 25 cm² plastic tissue culture flasks (BD Biosciences) and incubated at 37°C in a humidified atmosphere containing 5% CO₂ for 3 days. The mesenchymal stem cells were isolated on the basis of their ability to adhere to the culture plates. On the third day, red blood cells and other non-adherent cells were removed and fresh medium was added to allow further growth. The adherent cells grown to 70% confluency were defined as passage zero (P₀) cells. The P₀ MSCs were washed with Ca²⁺-Mg²⁺ free phosphate-buffered saline (PBS) (Invitrogen/GIBCO) and detached by incubating with 0.25% trypsin-EDTA solution (Invitrogen/GIBCO) for 5–10 min at 37°C. Complete medium was added to inactivate the trypsin. The cells were centrifuged at 200 g for 10 min, resuspended in 1 ml complete medium and counted manually in duplicate using the Thoma chamber. Then the cells were plated as P₁ in 75 cm² flasks (BD Biosciences) at densities of 1 \times 10⁶ cell/flask. Complete medium was replaced once every 3 days

over a 10–14 day period. For each passage, the cells were plated similarly and grown to a confluency of 70%.

Phenotype identification of rBM-MSCs

Flow cytometry. To confirm that rBM-MSCs maintain their phenotypic characteristics after growth in culture, undifferentiated MSCs were subjected to flow cytometry analysis. Flow cytometry was performed on a FACS Calibur (BD Immunocytometry Systems, San Jose, CA, USA) and the data were analyzed with the Cell Quest software (BD Immunocytometry Systems). Immunophenotyping of rBM-MSCs was performed with antibodies against rat antigens CD29 (Integrin- β_1 chain; Ha2/5; FITC), CD45 and CD90 (Thy-1/Thy-1.1-FITC), as well as their isotype controls (IgG2a κ ; FITC) (BD Biosciences, San Diego, CA, USA).

Immunohistochemistry. The immunohistochemistry studies on the P₀, P₁, P₃, P₄, P₇, P₉ and P₁₀ cells were performed as previously described by us and used primary antibodies were listed in Supplementary Table 1.¹⁷ The mounted cells were examined under a light/fluorescence microscope (Leica DMI 4000B, Wetzlar, Germany).

Reverse transcription-polymerase chain reaction. Total RNA was isolated from rat islets and P₃ cell cultures (rBM-MSCs) by the High Pure RNA Isolation Kit (Roche, Mannheim, Germany) and cDNA was synthesized by using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche). The PCR mix consisted of 1 \times PCR buffer, 0.2 mM dNTPs, 0.5 μ M of each primer (Supplementary Table 2), 1.25 mM MgCl₂ and 1.5 U of Taq DNA polymerase. The PCR products were analyzed by agarose gel (2%) electrophoresis. Sequences and annealing temperatures of each primer pair are listed on Supplementary Table 2. The studied genes were selected: (i) to show that MSCs have diverse immunophenotypic characteristics and could be characterized by studying the expression of *Spp1*, *Sparc*, *Bmp4*, *Gfap*, tubulin beta 3 (*Tubb3*), *Eno2*, smooth muscle α -actin (*Acta2*), actin- β (*Actb*), *Myog*, *Des*, *Nes*, *Fos* and *Vim* genes; (ii) to relate the antiapoptotic and anti-inflammatory effects of MSCs cocultured with STZ-injured islets following the expression of apoptosis-related genes, such as *IL6ra*, TNFAIP3 interacting protein 1 (*Tnip1*), *Bcl3*, *Irfng* and *IL10*. In the study, the housekeeping gene, *Gapdh*, was selected as the internal control.

In vitro differentiation of rBM-MSCs. The *in vitro* studies of adipogenic and osteogenic differentiations were performed according to the methods described previously by Karaoz et al.¹⁷

Apoptosis induction in pancreatic islets

In vitro STZ treatment to apoptosis induction. STZ was prepared 1 min before use by dissolving in 0.1 mol/l sodium citrate (pH 4.5) at 4°C a concentration of 200 mmol/l. A volume of 10 μ l of this solution was added to the islets to obtain 1 mmol/l STZ. Islets were then incubated overnight (14 h).³⁹ In the next experimental set STZ-induced islets + MSCs group, STZ induction was performed in the presence of MSCs. Later, STZ-exposed islets were transferred into the upper section of the wells separated by an insert that was previously prepared by the addition of MSCs.

rBM-MSCs/rPIs indirect coculture experiments. All cocultures were conducted in triplicate in 12-well plates (Becton Dickinson Labware Europe, Le Point De Claix, France) using 0.4- μ m pore size, high pore density and polyethylene terephthalate track-etched cell culture inserts (Becton Dickinson Labware Europe). rBM-MSCs (1×10^5) were seeded in the lower chamber of the coculture system, and incubated in RPMI 1640 medium including 10% FBS and 1% antibiotic. Once cultured MSCs reached confluence, pancreatic islets (normal and STZ-induced) (20 per well) were then placed on the upper surface of the membrane of the cell culture inserts. Cocultured cells and islets were maintained for 14 days in RPMI 1640 at 37°C and 5% CO₂ in a humidified atmosphere with the medium being changed every 2 days. Control MSCs without pancreatic islets were prepared in the same conditions as cocultured rBM-MSCs.

Apoptosis detection in pancreatic islets with/without coculture

Apoptosis detection by FDA-PI viability staining. Islet preparations were assessed for islet cell viability using cell membrane exclusion dyes. Viability of islets was assessed using fluorescein diacetate (FDA) (Sigma-Aldrich, St. Louis, MO, USA) and PI (Sigma-Aldrich) staining.³ Briefly, islets in the inserts were transferred in PBS to a Petri dish (35 mm). FDA and PI stock solutions were added to the sample at a final concentration of 0.67 and 75 μ M, respectively. Using a fluorescent microscope (Leica DMI 4000B), a procedure for the assessment of intact pancreatic

islet viability was used. After 30 s of adding the stain, it was placed in a Petri dish containing 2.0 ml of PBS. The sample was placed on the fluorescent microscope, visualized and photographed using the filter FITC for fluorescein ($\text{em} \sim 530 \text{ nm}$) and rhodamine ($\text{em} > 600 \text{ nm}$). Dead cells were stained red and viable cells were stained green. The objective of the test was to assess, as accurately as possible, the percentage of islet cells that were viable. The cells were assigned to categories as follows: **0**: few or no cells were green and the majority were red (average viability = non-viable); **1**: approximately 75% of the cells were red (average viability = 25%); **2**: approximately 50% of the cells were red (average viability = 50%); **3**: approximately 25% of the cells were red (average viability = 75%); **4**: few or no cells were red (average viability = 100%).

Calculations. Total viable = 0.25 (sum of category 1) + 0.5 (sum of category 2) + 0.75 (sum of category 3) + (sum of category 4)

Total number of islets = (sum of category 1)

Percent viable = (total viable \times 100)/total number of islets

Apoptosis detection by flow cytometry. Following coculture experiments, islets were incubated for 5 min with trypsin-EDTA. 0.25% trypsin, 1 mM EDTA.4Na in Hanks' balanced salt solution without Ca⁺² and Mg⁺² at 37°C, and islet cells were gently dispersed.²⁰ After washing with PBS, apoptotic cell percentage was detected by flow cytometry with Annexin-V-FITC Apoptosis Detection Kit (BD Pharmingen).

Measurement of insulin secretion by ELISA. At the end of the 14th day, inserts including islets were transferred into new 12-well plates and the culture media were switched to serum-free low glucose (L)-DMEM (5.5 mmol/l) containing 0.5% BSA for 2 h, washed twice with PBS, and then stimulated by high glucose (H)-DMEM (25 mmol/l) for 2 h. At the end of this second 2 h incubation, the culture media were collected and frozen at -70°C until they were assayed for insulin content. To estimate total cellular insulin levels, the total cell protein content was tested using BCA Protein Assay Kit (Sigma Aldrich). Insulin release was detected by rat insulin enzyme-linked immunosorbent assay (ELISA kit, Linco) according to the manufacturer's instructions.

Cytokine quantification. Secretions of rat interleukin (IL4, IL6, IL10) and transforming growth factor (TGF- β 1) were determined in supernatants by ELISA (ELISA kit, Invitrogen, Camarillo, CA, USA) according to the manufacturer's recommendations. The minimum detectable dose (MDD) was 2 pg/ml for IL4, 7 pg/ml for IL6, 5 pg/ml for IL10, and 15.6 pg/ml for TGF- β 1. All the experiments were performed in triplicate.

Statistical analysis. All experiments were repeated a minimum of three times. Data are reported as means \pm S.D. All statistical analyses were performed using SPSS 10.0 (SPSS Inc., Chicago, IL, USA). Data were analyzed using one-way ANOVA and paired *t*-test. Differences between the experimental and control groups were regarded as statistically significant when $P < 0.05$.

Conflict of Interest

The authors declare no conflict of interest.

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