

CD40 Knocked Down Tolerogenic Dendritic Cells Decrease Diabetic Injury

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ABSTRACT

Background: Type-1 diabetes (T1D) is an autoimmune disease in which T lymphocytes destroy insulin-producing β -cells. Control of self-reactive T lymphocytes and recovery of diabetic injury is the end point of T1D. **Objective:** To investigate generation of tolerogenic dendritic cells (tolDCs) as an innovative method of diabetes therapy. **Methods:** Lentivirus vector production was achieved by GIPZ mouse CD40 shRNA, psPAX2 and pMD2G plasmids DNA. Purified bone marrow derived DCs were treated with CD40 shRNA, and expression of CD40 and mRNA level were evaluated by flow cytometry and Real-Time PCR, respectively. CD40 knock-down DCs were injected into STZ-induced diabetic mice. Blood glucose; glucose tolerance test and weight were analyzed in different groups. **Results:** Mice treated with CD40 shRNA transfected DCs showed considerable differences in blood glucose, glucose tolerance, and weight compared to other groups. Also cytokine assays indicated an increase in IL-13 production in the CD40 shRNA group. **Conclusion:** In streptozotocin-induced diabetic mice model, administration of tolerogenic dendritic cells could improve diabetic parameters.

Mahmoudzadeh A, et al. Iran J Immunol. 2017; 14(4):270-280.

Keywords: CD40, Lentiviral Vector, Tolerogenic Dendritic Cells, Type 1 Diabetes

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INTRODUCTION

Juvenile or insulin-dependent diabetes, nowadays known as Type 1 diabetes (T1D), results from autoimmune destruction of insulin-producing β -cells by T cells. Immune system attacks β -cells in the islets of the pancreas resulting in their destruction and decreasing the capability of the individual to maintain normal blood glucose level. Therefore, development of hyperglycemia and more seriously, diabetic ketoacidosis, and ultimately death may occur (1). This type of diabetes has been rising steadily in developed countries, and the prevalence of T1D is estimated at 171 million of the adult population (2). By 2020, the prevalence of T1D in children under the age of 5 will double, and in children younger than 15 years may reach 160,000 cases (3,4,5). There is no preventive methods for the development of diabetic complication and only using of insulin can bring blood glucose to normal range (6). Research on the novel treatment strategies is conducted worldwide, including:

- Intra-islet injections of precursor cells that can be differentiated into neo-islets/pancreatic β -cells upon appropriate stimulation (7,8).
- Regeneration of β -cells therapies aiming at full replacement of destroyed pancreatic islets by tissue-specific directed differentiation and genetic reprogramming (9,10).
- Immunotherapy techniques such as peptides activity, T cell receptor function and blocking adhesion molecules interactions. Generation of immune tolerance has been developed by regulatory T Lymphocytes and co-stimulatory molecules and cytokines by self-reactive T lymphocytes control (11-15).

Dendritic cells (DCs) are one of the most widely used cells for immunotherapies, probably due to their relative ease of production and lack of toxicity associated with administration and their clinical effectiveness (16). It has been shown that in NOD (Non-Obese Diabetic) mice, *in vitro* treatment of antisense oligonucleotides (AS-ODN) against co-stimulatory molecules maintenance DCs in immature state that can prevent or delay diabetes (17). In this study, we used a novel method to produce tolerogenic dendritic cells by lentiviral vector to deliver CD40 antisense molecule into dendritic cells. Administration of tolerogenic dendritic cells produced by this method could recover diabetic parameters; therefore, it could be a novel treatment method for diabetic patients.

MATERIALS AND METHODS

Animals. Male DBA/2J and BALB/c (H-2d) 6-8-week mice were purchased from Pasteur Institute of Iran, Tehran, Iran. Access to water and food as well as hygienic conditions was conducted according by Tarbiat Modares University animal ethic protocols.

Generation of Dendritic Cells. DBA bone marrow progenitor cells were selected generate DCs, as previously described by Moazzeni *et al.* (18). Briefly, 5×10^6 bone marrow cells were cultured in 4 ml of RPMI-1640 medium supplemented with fetal calf serum (10%), Penstrep (100 U/ml and 100 μ g, respectively) and 2 mM L-glutamine (all from Gibco, U.S.A.). All cultures were produced in six-well plates. Recombinant IL-4 (mouse Interleukin 4) and GM-SCF (mouse granulocyte macrophage colony-stimulating factor) were added to plates, 10 ng/ml and 20 ng/ml respectively) (both from Peprotec, U.K.). The plates incubated in 37°C and 5% CO₂. After 96 h (Day 3), non-

adherent cells were harvested, and fresh RPMI-1640 was added. On day 5, half of medium aspirated and fresh medium and cytokines was added. Non-adherent cells were harvested after 7 days. DCs were stimulated with 5 µg/ml LPS (Sigma, U.S.A.) for 8-10 h. Purity of the DCs was determined by flow cytometry using PE labeled anti CD11c (Abcam, U.K.).

Plasmids DNA Purification. The bacteria of GIPZ mouse CD40 shRNA (with GFP), non-silencing CD40 shRNA, psPAX2 and pMD2G plasmids in glycerol were purchased from ThermoFisher Scientific, and 5 µl of these bacteria was cultured in 5 ml (25 g/l) of LB broth. After 12-16 h, plasmid DNA purification was performed by plasmid DNA purification Macherey-Nagel (MN, Germany) kit. Briefly, 5-15 ml of saturated LB culture was centrifuged and the precipitate was added to cell lysis buffer. The clarified lysate was placed in NucleoSpin plasmid column, and after centrifugation, the eluted DNA was collected by adding elution buffer. Then, the concentration of plasmid DNA was measured by nanodrop.

Virus Packaging. Lentiviral vector was generated in HEK-293T cells by transfecting calcium phosphate. Briefly, in 10 cm² plates, 5×10⁶ HEK-293T cells were plated in 10 ml of the medium, and were transfected on the following day with 21 µg CD40 shRNA and CD40 non-silencing plasmid vectors, 10.5 µg pMD2G plasmid and 21 µg psPAX2 plasmid. 14-16 h post transfection, medium aspirated and 10 ml fresh pre-heated virus medium was added. In 2 and 3 days post transfection cell supernatant was collected and filtered through a 0.4-mm pore size. Collected supernatant centrifuged at 25000 rpm/90 min at 4°C by ultracentrifuge (Beckman, U.S.A.) for concentration. Concentrated viral vector titer was checked using HEK-293 T cells.

Titration of Vector. For titration serial dilutions was used in HEK-293T cells. Simply, 6 ×10⁴ HEK 293T cells/wells were plated in 250 µl medium on 24-well plates, 4 wells were inoculated by virus, and one well was kept as a negative control. After 24 h, fresh medium (1 ml) was added to each well. After 72 h, GFP expressing cells of each well were detected by an invert fluorescence microscope and quantified by flow cytometry to determine transducing units (TU) per milliliter.

Transduction of Lentiviral Vector to DCs. In 10 cm² plate 5×10⁴ purified BM-derived DCs with concentrated lentiviral vectors (2×10⁶ TU/ml) were transduced. MOI 10-40 for 6 h in the presence of polybrene (1 µg/λ) (Sigma, U.S.A.) transduction was conducted. Afterwards, supernatants of the cells were replaced with 1 ml of fresh medium. GFP expression by invert fluorescence microscope was visualized after 72 h.

Flow Cytometry. Immature DCs, mature DCs and mature transduced DCs with CD40 shRNA and CD40 non-silencing shRNA lentivectors (2×10⁵ in 50 µl PBS) were incubated with fluorescein isothiocyanate (FITC)-conjugated rat anti mouse for CD40, and appropriate isotype controls (From BD, U.S.A.) for 30 min on ice. After staining, the cells were washed in wash buffer (PBS containing 2% FBS), and were fixed in 2% paraformaldehyde. The tubes were subjected to flow cytometric analysis.

Real Time PCR. Total RNA isolation of animal cells was done as mentioned by the manufacture protocol. Purity and integrity of RNA were determined by measuring the optical density in 260/280 nm and electrophoresis in 1% agarose gel. RNA transcribed to cDNA By superscript III reverse transcriptase and oligo dT (Invitrogen, USA). CD40 and beta-actin mRNA expression for quantitative analysis was performed by Real-Time PCR using My-IQ cycler system and IQ syber green supermix kit (Biorad, Milan, Italy). Forward and reverse primers and the template cDNA amplifications were carried out in the presence of SYBR Green I Dye. Beta-actin gene was internal standard and target

genes were measured as fluorescent signal intensity normalized with it. The primers were designed for CD40 gene and beta-actin as an internal control and blasted for sequence checking using Oligo 6.1 software. The primers were synthesized by Macrogen Company (South Korea).

CD40-Forward 5'-ACTGTGAACCCAATCAAGG-3'
 CD40-Reverse 5'-TCTCCATAACTCCAAAGCC-3'
 Beta-Actin- Forward 5'- CTTCTTGGGTATGGAATCCTG-3'
 Beta-Actin –Reverse 5'-GTGTTGGCATAGAGGTCTTTAC-3'

Cytokine Assay. 10^5 DBA mice mature DCs, non-silencing CD40 shRNA and CD40 shRNA were cultured separately with the allogeneic T lymphocytes (10^6 cells from Balb/C) for 48 h. T cell cytokine (IL-13) production was assessed in supernatants using flowCytomic sandwich immunoassay (eBioscience, U.S.A.). Briefly, antibody coated beads were added and incubated with the samples. Biotin-conjugated, optimally paired antibodies specific for bound analytes were added, and then Streptavidin-PE was subjoined to detect biotin-conjugated antibody. After flow cytometry, the concentration of cytokine was analyzed by FlowCytomix™ Pro software.

Induction of Diabetic Mouse Model with Multiple Low Dose Streptozotocin. The final dose of 50 mg/kg of Streptozotocin (STZ) in sodium nitrate solution was injected in five consecutive days to induce diabetes. Since this solution is unstable, it should be administered within 15 to 20 min after preparation. The mice must be kept fasting at least for 4-6 h, and then streptozotocinin sodium nitrate solution was injected to them via intra peritoneal route. After 4-5 weeks, the tail vein blood glucose was measured using glucometer. Glucose levels higher than 250 mg/dl were considered an indicator of diabetic mice.

Injection of Tolerogenic Dendritic Cells. After induction of tolerogenic dendritic cells with CD40 shRNA, 10^6 tolerogenic dendritic cells were injected via intravenous and intra peritoneal route in two consecutive days. In addition, non-tolerogenic dendritic cells with CD40 non-silencing shRNA were injected via intravenous route in two consecutive days (Table 1).

Table 1. Laboratory tests including Blood Glucose, Glucose Tolerance Test and Weight and grouping of mice are shown.

Number of Groups	Name of Groups	Laboratory Tests
1	Control	Blood glucose, Glucose Tolerance Test, weight
2	Diabetic with STZ	Blood glucose, Glucose Tolerance Test, weight
3	Non-Silencing CD40 shRNA(ip)	Blood glucose, Glucose Tolerance Test, weight
4	CD40- shRNA(ip)	Blood glucose, Glucose Tolerance Test, weight
5	CD40- shRNA(iv)	Blood glucose, Glucose Tolerance Test, weight

Measurement of Blood Glucose. One week after intravenous and intra-peritoneal injection of tolerogenic DCs and non-tolerogenic DCs with CD40 non-silencing shRNA, blood glucose in all groups (Table 1) (five mice in each group) was measured up to five consecutive weeks using a glucometer.

Glucose Tolerance Test. Glucose was injected intra peritoneally dosing 2 g/kg to determine the glucose tolerance of all groups (Table 1). After 14-12 h fasting, blood glucose of each group was measured by glucometer from the tail vein blood in 0, 15, 30, 60, 90 and 120 min.

Weight Measurement. Before and during the study, different mice groups (Table 1) were weighed once a week using a digital scale.

Statistical Analysis. All data were reported as mean \pm SD and analyzed using ANOVA (one-way) and Tukey's post test. p value (less than or equal to 0.05) was considered significant. All statistical analyses were conducted using SPSS V.16 software.

RESULTS

CD40 shRNA Inhibited CD40 Expression in DCs.

To investigate the effects of shRNA cytotoxicity and alternation of CD40 gene expression, the cells viability and surface expression of CD40 were analyzed 48 h after transduction using 0.04% trypan blue staining and flow cytometric analysis, respectively. DCs viability was over 90%, indicating no cytotoxic effect of shRNA.

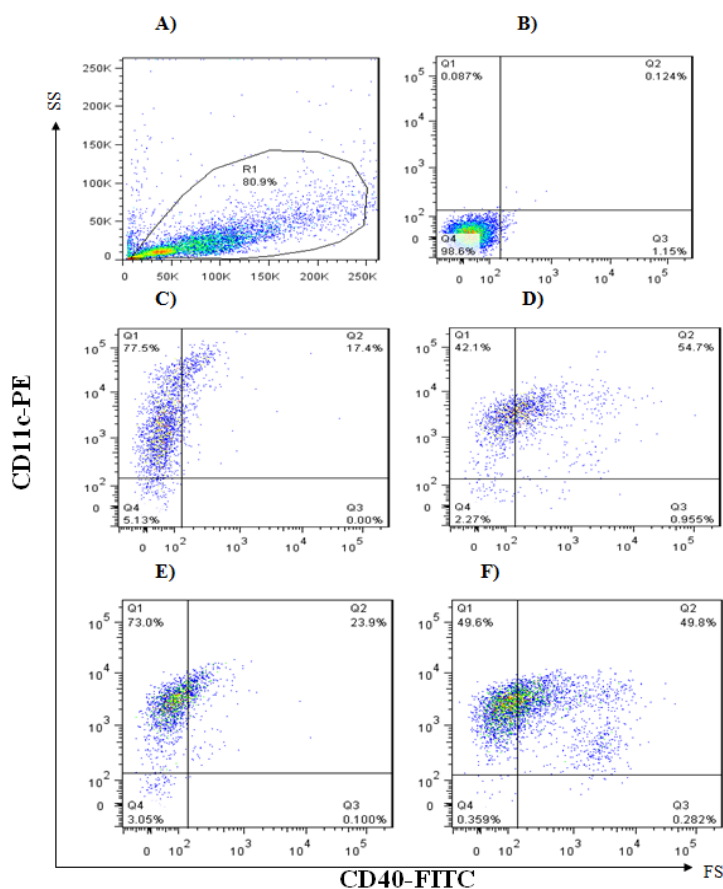


Figure 1. CD40 expression affected by CD40 shRNA. The gating strategy for flow cytometry(A), negative control(B), Immature DCs(C), mature DCs (D), CD40 shRNA (E), and non-silencing CD40 shRNA (F). As shown, the statistical analysis by ANOVA and Tukey HSD tests indicate that in CD40 shRNA group, compared to mature DC and non-silencing CD40 shRNA, the expression of CD40 was decreased significantly ($p < 0.05$). This figure also shows the results of one experiment with flow cytometry.

All experiments were performed in duplicate and the results were shown as the mean \pm SD for each group. Considerable differences in CD40 shRNA group (23.35 ± 1.76), in comparison with immature (11.9 ± 2.4), mature DCs (53.2 ± 1.27), and non-silencing CD40 shRNA (48.5 ± 1.8) were seen ($p < 0.05$) (Figure 1).

CD40 shRNA Down Regulated CD40 mRNA Expression in DCs.

To investigate CD40 mRNA down regulation in DCs, Real-Time PCR was conducted. To detect down regulation of mRNA CT marker and normalization, $2^{-\Delta\Delta CT}$ method was used. The results in duplicate experiments with ΔCT indicated considerable differences of CD40 mRNA down regulation in the CD40 shRNA group (33.17 ± 0.53) compared to DCs before transfection (27.11 ± 0.03) and non-silencing CD40 shRNA (28.86 ± 0.19) groups ($p < 0.05$). However, there was no considerable difference in beta-actin as an internal control of these groups.

Cytokines Assay.

In polarize T cells toward Th1 phenotype, CD40 is a key co-stimulatory molecule. In our study, allostimulation of DCs transduced by CD40 shRNA could alter cytokines profile from Th1 to Th2 was not assessed. Supernatants of mature DCs, dendritic cells treated with non-silencing CD40 shRNA and CD40 shRNA (10^5 cells from DBA mice) co-cultured with the allogeneic T cells (10^6 cells from Balb/C) for 48 h and IL-13 cytokine assay were conducted. Our results from triplicate experiments indicate considerable differences in the CD40 shRNA group (3427.5 ± 161.92) compared to mature DCs (993 ± 26.87) and non-silencing CD40 shRNA (1262 ± 21.21) groups ($p < 0.05$) (Figure 2).

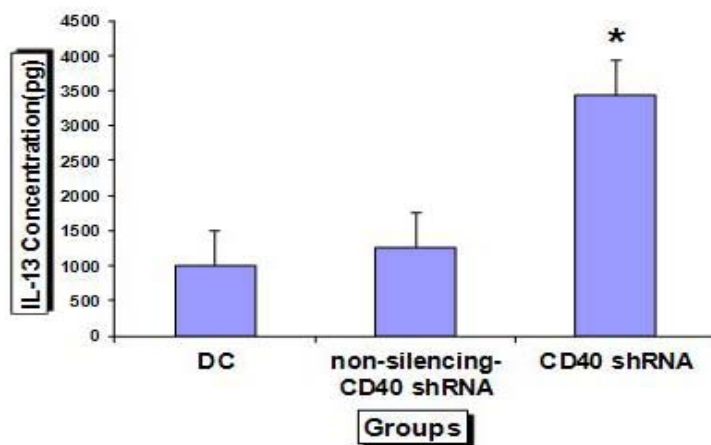


Figure 2. Th2 polarization by DCs transduced with CD40 shRNA. Up-regulation of Th2 cytokine (IL-13) and down regulation of CD40 specifically carried out by CD40 shRNA. Analysis of IL-13 production in harvested supernatants. The data are shown as means \pm SD for three separated experiments. * Means the considerable differences between groups.

Measurement of Blood Glucose. Using a glucometer, the blood glucose level of mice was measured for five weeks after injection of 10^6 DCs to all groups (Table 1). Our results from triplicate experiments (Mean \pm SD) indicate considerable differences between CD40 shRNA iv and ip injected groups in the fourth (297.6 ± 13.6 mg/dl and 301.6 ± 14.2 mg/dl, respectively) and fifth (258 ± 4.8 mg/dl and 265.2 ± 4.4 mg/dl, respectively) weeks after injection to be significantly lower than that of non-silencing

CD40 shRNA in fourth (371.8 ± 7.9 mg/dl) and fifth (420.8 ± 17.2 mg/dl) weeks and STZ-diabetic mice in fourth (360.4 ± 11 mg/dl) and fifth (425.4 ± 17.8 mg/dl) weeks ($p < 0.05$) (Figure 3).

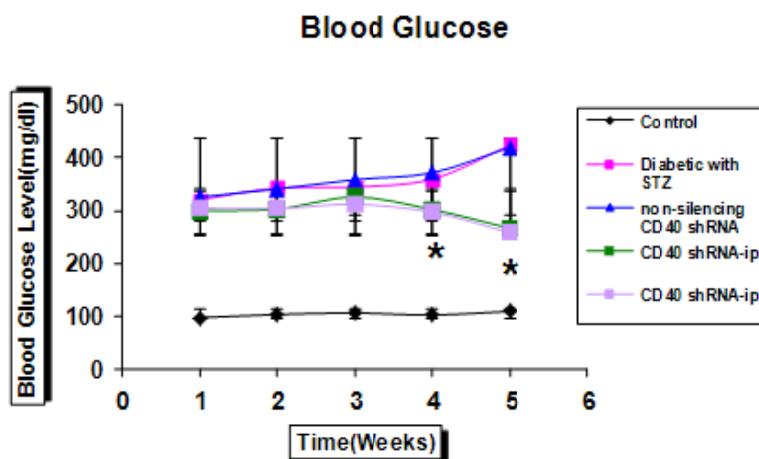


Figure 3. Blood glucose level in different groups at 1-5 weeks. Statistical analysis by ANOVA and Tukey HSD tests indicated that in CD40 shRNA groups (iv and ip) there were considerable differences ($p < 0.05$) with other groups in 4th and 5th weeks. The data are in means \pm SD.* Means the considerable differences between groups.

Glucose Tolerance Test (GTT).

To evaluate glucose injection response in the experimental and control groups, glucose tolerance test was performed. For this purpose, 2 g/kg glucose was injected via intraperitoneal route, and beta-cell responses along with decreased blood glucose levels at intervals of 0, 15, 30, 60, 90 and 120 min were measured. As Table 1 shows the results of triplicate experiments (Mean \pm SD), indicating considerable differences in CD40 shRNA (iv and ip) injected groups at all times in comparison to non-silencing CD40 shRNA and STZ diabetic groups ($p < 0.05$) (Figure 4).

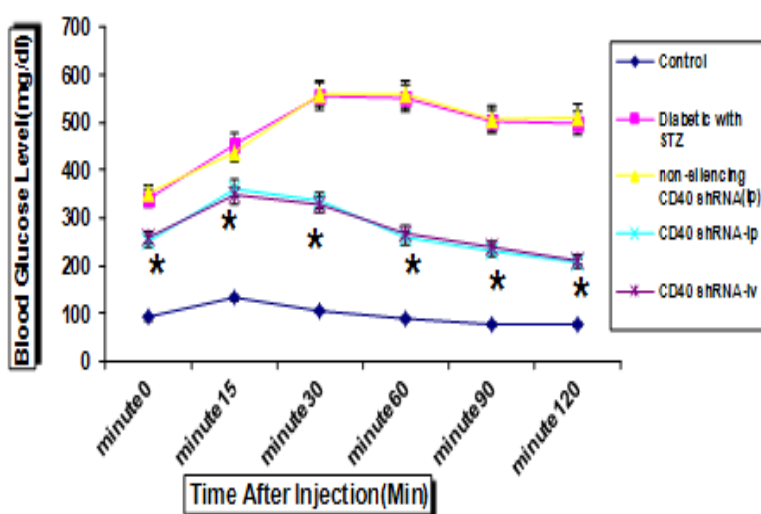


Figure 4. Glucose Tolerance Test in different groups performed within 0-120 min. Statistical analysis by ANOVA and Tukey HSD tests indicate that in CD40 shRNA groups (iv and ip) there were considerable differences ($p < 0.05$), compared to other groups at all test times. The data are shown as means \pm SD.* Means the considerable differences between groups.

Weight Changes.

All groups (Table 1), including DCs containing CD40 shRNA, non-silencing CD40 shRNA, control and diabetic with STZ groups were weighed weekly during the first week after intravenous and intraperitoneal injection. Results of triplicate experiments (Mean \pm SD) in Figure 5 indicate considerable differences between CD40 shRNA and non-silencing CD40 shRNA, control and STZ-diabetic mice groups within 4 and 5 weeks ($p < 0.05$) (Figure 5).

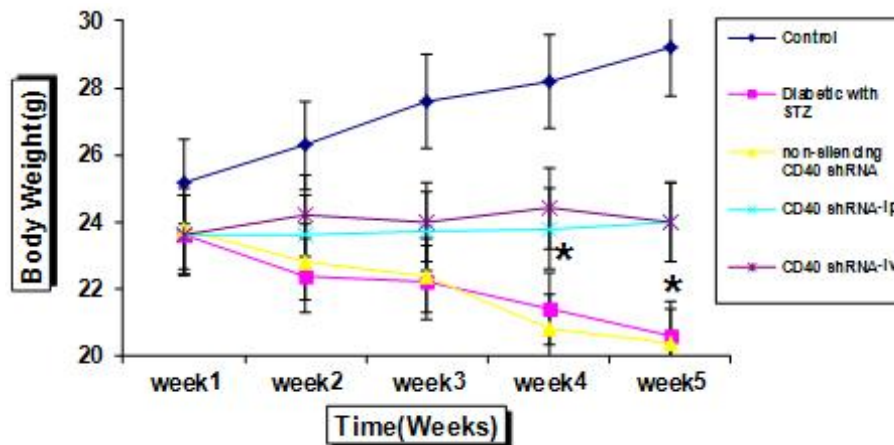


Figure 5. Body weights in different groups in weeks 1-5. Statistical analysis by ANOVA and Tukey HSD tests indicate that in CD40 shRNA groups (iv and ip) there were considerable differences ($p < 0.05$), compared to other groups in 4 and 5 weeks. The data are shown as means \pm SD. * Means the considerable differences between groups.

DISCUSSION

Use of cell therapy such as DCs in T1D has been reviewed in several studies and good reasons for this therapy have been mentioned. First, the most readily available sources for generation of DCs such as bone marrow progenitors by using granulocyte-macrophage colony-stimulating factor (GM-CSF) culture conditions to acquire a pre-DCs phenotype. However, GM-CSF-generated pre-DCs are undifferentiated and phenotypically and functionally unstable upon *in vivo* injection. IL-4 is absolutely required to fully commit them into bona fide DCs by preventing GM-CSF induced macrophage outgrowth, contaminating DCs cultures. Bone Marrow Dendritic Cells (BMDCs) transduced by lentiviral vector encoding IL-4 are able to prevent the disease in old (12-week-old) NOD recipients (19,20). As indicated in our study, this technique was used to generate DCs. The purity and viability (77.6% and over 90%, respectively) were determined by anti CD11c antibody and 0.04% trypan blue. Second, typical migratory of tolerogenic DCs in body and as same as this type of migration by therapeutic DCs from inoculation sites to target tissues (21,22). Bioluminescence imaging demonstrated that peripheral lymph node was preferentially targeted by bone marrow derived DCs following intravenous administration. In other words, no

differences exist in immunological responses between ip and iv injection methods. As observed, there were no differences between ip and iv CD40 shRNA DCs in blood glucose, glucose tolerance test and weight (23,24). Third, DCs are amenable to manipulate *in vitro* by actively silencing, over expressing the genes or supplementing the culture conditions. Earlier, antibodies and low molecular weight pharmacological drugs were used for gene silencing. However, in the past two decades, by enhancing the knowledge of gene functions, other branches of science have tried to silence these genes and investigate the effects. Antisense oligonucleotide and recently RNA interference (RNAi) have been the alternative methods (25,26). One of the most important studies using lentiviral vectors in DCs of rats was conducted by Mei *et al.* (2009). In their study, gene expression of CD40 was targeted, and the results suggested that the CD40 expression was decreased (27). In 2003, Belousova *et al.* used adenovirus vectors for CD40 gene silencing in dendritic cells and human embryonic kidney cell line HEK-293. Results indicated the high capacity of adenovirus vectors to infect DCs and CD40 containing tumor cells (28). Perone showed that Galectin-1 gene transfer to DCs by adenovirus could delay the onset of type I diabetes in NOD mice (29). Another study demonstrated that using oligonucleotides against CD40, CD80, and CD86 followed by NF- κ B targeting could prevent diabetes in NOD mice (30). Our results are in complete agreement with other studies in which CD40 expression of DCs were down regulated. Therefore, we can conclude that the antisense can effectively reduce the level of mRNA by interfering with its function. Fourth, up regulation of co-stimulatory molecules such as MHC-II and cytokines production affected DCs maturation. Acquiring a variety of immune effector phenotypes by naïve CD4⁺ T helper (Th) cells strongly depends on cytokines release such as Th1 response depending on release of IL-12. Th2 cells are stimulated through OX40 ligation by DCs, produce mainly IL-4, IL-5 and IL-13, and promote B cells activation, which can be involved in auto immunity (32,33). A number of studies indicate that down regulated co-stimulatory capacity of DCs promote a tolerogenic therapeutic outcome to ensure that tolerogenic dendritic cells phenotype is stable. Thus, low co-stimulation in this way persistently keeps pro-inflammatory cytokine production in low-to absent situation. Tolerogenic dendritic cells express low levels of MHCII, CD40 and the co-stimulatory molecules CD80 and CD86 as well as low levels of inflammatory cytokines (e.g. IL-12, IL-1 β , TNF), unlike higher levels of regulatory/suppressive cytokines (e.g. IL-4, IL-10, IL-13 and TGF- β) (34). More recently described induction of anergy by tolerogenic dendritic cells depends on direct cell contact, cytokines release and up-regulation of the number and function of immune cell subset, especially regulatory T cell population (Foxp3⁺ CD25⁺ CD4⁺) (35,36). Our results were consistent with previous studies, because suppressive cytokines such as IL-13 were significantly different in CD40 shRNA group, compared to other groups ($p < 0.05$). Therefore, the results suggest that differentiation of T cells to Th2 depend on down regulate of CD40 co-stimulatory.

ACKNOWLEDGEMENTS

This work was supported by a grant (94-212) from Transplant Research Center affiliated to Shiraz University of Medical Sciences. The authors also thank Tarbiat Modares University.

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