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ORIGINAL ARTICLE

Tolerance Induction by CD40 Silenced Dendritic Cells through Antisense

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Abstract

Background: One of the valuable tools for inhibiting the specific gene expression is antisense technique. To determine T cell responses, co-stimulatory molecule expression on the antigen presenting cells is important. In the present study, the effects of high affinity antisense against CD40 mRNA on the function and phenotype of DCs (dendritic cells) were investigated.

Methods: The DCs were separated from the mice spleens and then cultured in vitro. By means of lipofectamine 2000, the antisense was delivered into the cells and the efficacy of transfection was estimated by flow cytometry. Also, the mRNA expression and protein synthesis were assessed by real time PCR and flow cytometry, respectively. The DCs were transfected with 6 µM antisense and 2 µl lipofectamine 2000.

Results: The percentage of CD40 expression in DCs was 38%. The results showed that CD40 expression is reduced in DCs to 22% and 24%. By annexine V and propidium iodine staining, we could evaluate the viability of the transfected cells. The inhibition of CD40 gene expression was associated with the increase in IL-4 secretion. This shifted the DCs to stimulate Th2 cytokine production from the allogenic T cells. In addition, in the MLR, the DCs without CD40 expression showed poor allostimulatory effects. This finding is valuable in the study of the co-stimulatory molecules of DCs.

Conclusion: These data demonstrate that direct interference of the cell surface expression of CD40 at transcriptional level by antisense confers tolerogenecity potential of DCs. This approach is a useful tool through which DCs become tolerogenic and can be studied as a potential therapeutic option for the autoimmune diseases and allograft rejection.

Keywords: Dendritic cell; Antisense; CD40; Tolerance induction

Introduction

Dendritic cells (DCs) are the potent professional antigen presenting cells (APCs) that can stimulate T cells and regulate immune responses.[1-3] Immune response induction by DCs makes them a useful tool in cancer research, while the prevention/suppression of the immune responses is an important approach in autoimmune or transplantation studies.[4-7]

In the maturation of DCs, CD40 triggering of DCs is an important step because they become able to activate CD4 and CD8 T cells. CD40 is a receptor belonging to the tumor necrosis factor receptor family and is constitutively expressed on the cell surface of APCs such as B cells, monocytes/macrophages, and DCs. Apart from classic antigen presenting cells (APCs), CD40 is expressed in the endothelial cells, fibroblasts, and smooth muscle cells. CD40 ligand is CD154, which is mainly expressed on the activated

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$CD4^+$ T helper cells.[8,9] CD40/CD154 interactions regulate the immune responses.[10] CD40 activation in DCs and macrophages will result in high co-stimulatory molecules expression on their surface and further release of pro-inflammatory cytokines. These cause more antigen presentation on the DC surface.[8,9]

In order to study the target genes and their function, several methods have been evolved such as knocking down and knocking out the gene expression, among which antisense oligonucleotide is a new and widely used technique.

Antisense method is a valuable tool for sequence-specific gene expression inhibition. It is also useful for functional genomics, target validation, and treatment of diseases.[11] Usually, antisenses contain 15-20 nucleotides and their sequences are target mRNA complement. Two different mechanisms can be assumed for antisense function. In the first mechanism, it is probable that RNase H enzyme is activated by antisense oligonucleotide and that it can cleave the RNA moiety of DNA RNA heteroduplex. So the target mRNA is degraded. In the second mechanism, steric blockade of the ribosomes is responsible for translation inhibition.[11,12]

Due to the central role of CD40-CD40L in DC maturation and its effect on immune regulation, in the present study, we studied the effects of CD40 antisense ODN and antibody blocking against CD40 on mouse DC function. By means of lipofectamine 2000, antisense ODN (oligo deoxy nucleotides) delivery into DCs was investigated. Antisense ODN treated DCs showed a reduction in the target mRNA and protein expression. Furthermore, IFN-γ expression in the T cells decreased. As a result, IL-4 production increased and IL-12 decreased. Also, they could not strongly stimulate T-cell proliferative responses. Accordingly, antisense technique is considered as one of the best methods for generation of tolerogenic DCs.

Materials and Methods

Eight to 10 weeks old male Balb/c and C57Bl/6 mice were provided from the Pasteur Institute of Iran, (Tehran, Iran). All of the cells were cultivated in RPMI medium (Gibco, USA), supplemented with 10% FCS, 3 mM L-glutamine, 5 µM 2ME, 100 IU/ml penicillin, and 100 µg/ml streptomycin, all prepared from Sigma (USA).

For the isolation of splenic DCs, the gradient media (Nycodenz, Axis Shield, Norway) was used as explained before.[13]

The primers were designed by Beacon designer software (Primer Biosoft V. 7) for CD40 gene (NM_011611) and GADPH (NM_008084) as an internal control. Thermodynamic parameters and the secondary structure were determined by mfold software. The primers' positions in relation to exon–exon domains were evaluated by Spidey software (www.ncbi.nlm.gov/Spidey, USA) and their specificity was analyzed by BLASTn (www.ncbi.nlm.gov/BLASTn, USA).

RNeasy plus mini kit (Qiagen, USA) was utilized. The DCs and BCL1 cell line RNA were extracted according to the manufacturer's instruction. The purity of RNA was determined by measuring the optical density 260/280 and its integrity was measured by 1% agarose gel electrophoresis. The genomic DNA traces were removed by DNase digestion, using RNase-free DNase set (Roche, Germany) and then RNA reverse transcription was done by superscript III (Invitrogen, USA) and oligodT.

Based on the manufacturer's instruction, mRNA expression was quantitatively analyzed by real time PCR technique, using a My-IQ cycler system (Biorad, Milan, Italy) and IQ sybergreen supermix kit (Biorad, Milan, Italy). For DNA amplification, SYBR Green I Dye, forward and reverse primers, and the template cDNA were added to each tube. Melting curve was analyzed for PCR determination of specificity. For the target genes, the results were determined as fluorescent signal intensity and normalized to the internal standard gene GADPH.

The PCR conditions were:

95°C	3 Min	1X	
-95°C	30 Sec		
-60°C	30Sec	↓	40
-72°C	30 Sec		
-81°C	10 Sec		
-95°C	1 Min		
-65°C	1 Min		
-65°C	10 Sec	0.5	60

The antisense sequence used for silencing of murine CD40 was designed by Oligo analyzer software (www.idtdna.com). The BLASTn (NCBI, USA) were carried out to ensure that the sequences would not target another gene script. Antisenses were synthesized by MWG Company (Biotech, Germany) and used according to the manufacturer's instruction (final concentration 6 µM). The non-silencing antisense

was an irrelevant antisense which did not react with any of target genes. After an overnight incubation of the DCs in OPTIMEM media containing 200 IU GM-CSF (BD, USA), the cells were transfected by lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instruction.

To optimize these instructions, the mature DCs were washed and cultured in OPTIMEM media and then transfected with 2, 4, 6 and 8 μM antisense 1, 2 μl lipofectamine 2000 for 24, 48 and 72 h.

Antisense sequence 1:
ACTCTCTTTACCATCCTCCT
Antisense sequence 2:
CACAGATGACATTAG
Sense sequence 1:
TGAGAGAAATGGTAGGAGGA
Sense sequence 2[14]:
GTGTCTACTGTAATC
Non silencing antisense sequence:
CAAGCTCCGATTGCAGAAAG

The DCs were purified cultivated in 24-well plates. Then, they were stimulated by LPS (10 μg/ml; Sigma-Aldrich).

In order to analyze the cell surface phenotype, the following antibodies were used: PE-conjugated hamster anti-mice CD11c (Clone HL3) and FITC-conjugated rat anti-mice-CD40 (clone 3/23) together with their respective isotype controls (All from BD Pharminogen, USA).

The FITC-conjugated Annexin-V and PI staining method was used for determination of apoptosis/necrosis as previously described by other researchers.[15] The cells were collected on the 2nd day after transfection and were analyzed by flow cytometry.

Mixed lymphocyte reaction (MLR) was set up by the culturing of T cells isolated from popliteal lymph node of C57BL/6 mice (10^5 cell per well) with graded numbers of the allogenic antisense treated DCs for 54 h in 200 μl RPMI, supplemented with 10% FCS. The T cell proliferation was measured through [^{3}H]-Thymidine incorporation.

Antisense-treated DCs (10^5, Balb/C origin) were cultured with the allogenic T cells (10^6 C57bl/6 origins) for 48 h. The supernatants were harvested and assessed for T cell cytokines (IFN-γ**,** IL-4) by ELISA. Antisense-treated DCs (5×10^5 cells per well) were cultured with anti-CD40 stimulatory antibody (BD, USA) for 48 h. Then, secretion of IL-12 P70 in the culture medium was measured, using Quantikine ELISA set according to the manufacturer's instruction (R&D systems, USA).

Immediately after antisense treatment, the dose-dependent CD40 protein decrease on the BCL1 cell line was determined by flow cytometry.[14] The DCs were isolated from Balb/c mice and the purity of isolation was evaluated by direct flow cytometry (the data are not shown). There is no uptake of antisense by the cells in the absence of lipofectamine 2000 (the data are not shown). As previously shown, the optimum concentration of lipofectamine 2000 was determined.[16]

The optimum concentration of lipofectamine 2000 was 2 μl, antisense 6 μM and the efficacy of transfection was 77% for the DCs. It was important for us to know if antisense or the transfection procedure itself could change DC viability or function. Thus, we stained the splenic DCs with Annexin-V and propidium iodide (PI) and analyzed them, using flow cytometry. As previously shown, the toxicity was determined in transfected cells versus the untreated cells.[16]

For cell transfection, the effect of transfection reagents on the cellular functions or its probable side effects on CD40 gene is an important concern. To know whether lipofectamine 2000 has any effect on CD40 gene expression, at presence or absence of lipofectamine 2000, its expression was determined by real time PCR.

Transfecting the DCs with CD40 antisense, we could determine the specificity of the inhibition of antisense mRNA. Through real time PCR and using primers flanking, the antisense target sequence, CD40 transcripts were detected. In fact, the efficacy of CD40 gene knockdown was evaluated by RT-PCR approaches.

For DC transfection, 6 μM antisense and 2 μl lipofectamine 2000 were incubated with the cultivated DCs for 40 h. The expression of genes encoding GADPH, G6PD and ADA were determined in order to find the best internal control. The data of the present study were normalized, using GADPH. The Pffafl equation was used for the relative changes in the expression of CD40 gene ($2^{-\Delta\Delta CT}$).

After 48 h, the cell surface CD40 was analyzed by flow cytometry (two color flow cytometry) to assess the effect of antisense transfection on the protein expression.

CD40 mRNA expression was measured by RT-PCR and presented as ΔCt values.

A lower ΔCt means that there is a higher expression of the gene.

DC phenotype was assessed by the expression analysis of CD40 and CD11c after 48 h by two color

flow cytometry. Considering the fact that an important co-stimulatory molecule named CD40 can polarize T cells to Th1 phenotype,[17] in the present study, we evaluated the allostimulation ability of the antisense transfected DCs in altering cytokine production from responding T cells. From the MLR culture supernatants, IFN-γ and IL-4 were assayed by ELISA.

The unmanipulated DCs (10^4) (control), the transfected DCs with the nonsilencing antisense CD40, the transfected DCs with antisense1CD40, and the transfected DCs with antisense2 CD40 were subsequently cultured with allogenic T cells (10^5) for 48 h.

The alloreactive T cells, stimulation by DCs in the MLR, at least in part, can characterize DC function.[3] To understand the effects of different factors such as antisense transfection, and the ability of splenic DCs on stimulation of allogenic T cells proliferation, we transfected the DCs from Balb/c mice with antisense. For determination of optimum ratio DC/T, we evaluated (1:40, 1:20, 1:10, 1:5 and 1:2.5) and found that 1:10 was the best ratio.

The BALB/C-derived DC (10^5 /well) were untransfected, transfected with nonsilencing antisense, transfected with antisense-CD40, transfected with sense CD40, and treated only with lipofectamine 2000 for 48 h. The allogenic T cells (10^6/well) were incubated with antisense, sense, and nonsilencing treated DC at the indicated numbers for 54 h.

CD40-CD154 interaction triggers the secretion of numerous cytokines such as IL-12. That is why the measurement of IL-12 levels can help us to assume the functional loss of CD40 receptor.[14] So we have to know whether antisense-CD40 can block the production of IL-12 protein or not. In this research, the control groups were the DCs transfected with nonsilencing antisense, untreated DC and IFN-γ stimulated DCs.

DCs ($5*10^5$) were unmanipulated (control), transfected with antisense -CD40, transfected with nonsilencing antisense, and treated with IFN-γ as a positive control. The stimulatory antibody against CD40 (10 μg/ml) was added 48 h after the supernatants were collected.

Results

The optimum concentration of lipofectamine 2000 was 2 μl, antisense 6 μM and the efficacy of transfection was 77% for the DCs. No toxicity was observed in transfected cells versus the untreated cells. In the presence of lipofectamine 2000, we did not observe any changes in CD40 mRNA expression in comparison with the control group (the data are not shown).

Comparing untreated cells and sense strand treated cells, transfection of the splenic DCs showed a strong cellular CD40 mRNA decrease, indicating a high knockdown efficiency of antisense delivery by lipofectamine 2000 (Figure 1).

According to the results, the reduction of CD40 expression in DCs by two antisenses was 22% (from 38.2% to 16.5%) and 24% (from 38.2% to14.8%) (Figure 2).

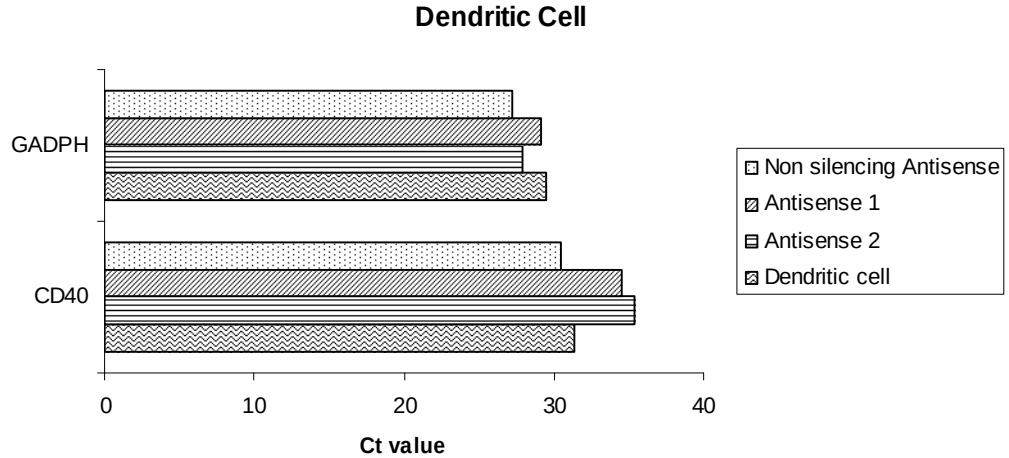

Figure 1: CD40 mRNA expression in the DCs

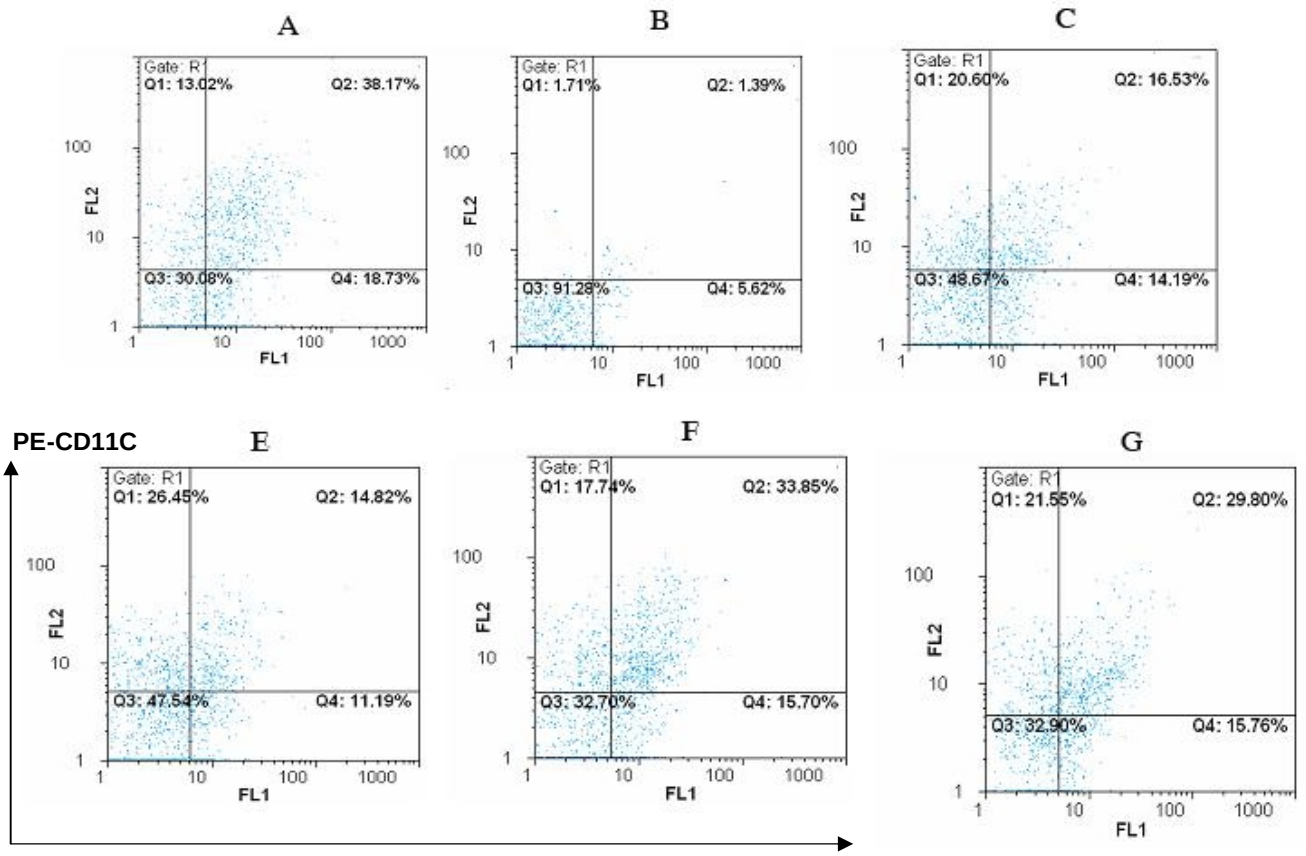

Figure 2: The effect of CD40 antisense transfection on DCs

DC phenotype which was assessed by the expression analysis of CD40 and CD11c after 48 h by two color flow cytometry was shown in Figure 2 (A: The Untreated DCs, B: Isotype control, C:CD40 antisense1 treated, D: CD40 antisense2 treated, E: Nonsilencing antisense, and F: CD40 sense treated).

The results showed that the lower levels of IFN-γ and the higher levels of IL-4 were secreted from the T cells incubated with antisense CD40-treated DC (Figure 3). The difference was significant for IL-4 ($P<0.001$) and IFN-γ ($P=0.003$). In contrast, the cytokine profile of T cells incubated with the untransfected DCs and the nonsilencing antisense transfected DCs showed a high level of IFN-γ and a low level of IL-4. The antisense-CD40 treated DCs had the ability to polarize naive T cells to Th2 cells.

The DC antisense1, antisense 2 treated caused allogenic T cell stimulatory index a 20% and 22% reduction, respectively. The differences between antisense treated and untreated group was significant ($p<0.001$**).** Nonsilencing antisense transfected cells showed no reduction in the stimulatory index (Figure 4).

For determination of optimum ratio DC/T which we evaluated (1:40, 1:20, 1:10, 1:5 and 1:2.5) found that 1:10 was the best ratio.

The DC which were untransfected, transfected with nonsilencing antisense, transfected with antisense-CD40, transfected with sense CD40, and treated only with lipofectamine 2000 showed that the data were representative of two independent experiments.

It is shown that the transfected DCs in comparison with the control group treated cells produce less IL-12 (70% reduction). The differences between antisense treated and untreated group was significant ($p<0.001$) (Figure 5).

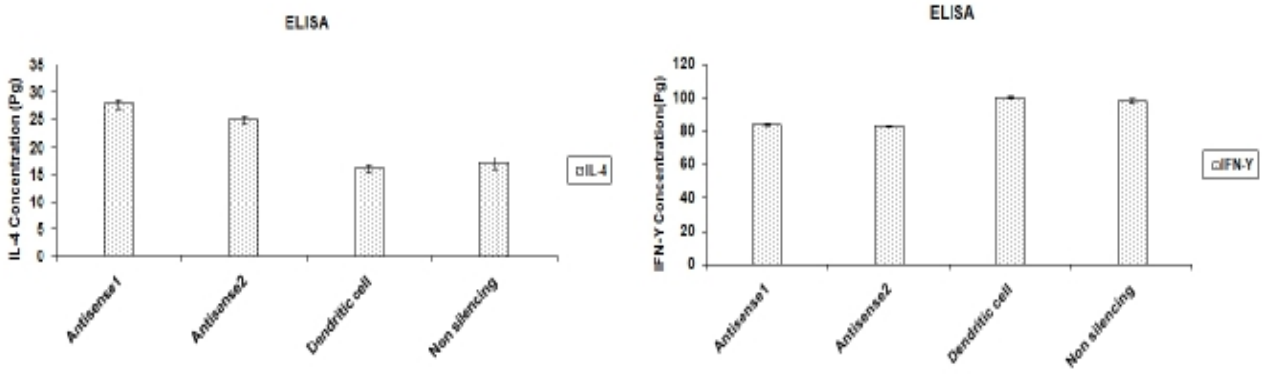

Figure 3: The antisense -CD40-transfected DCs treated DCs promote Th2 polarization

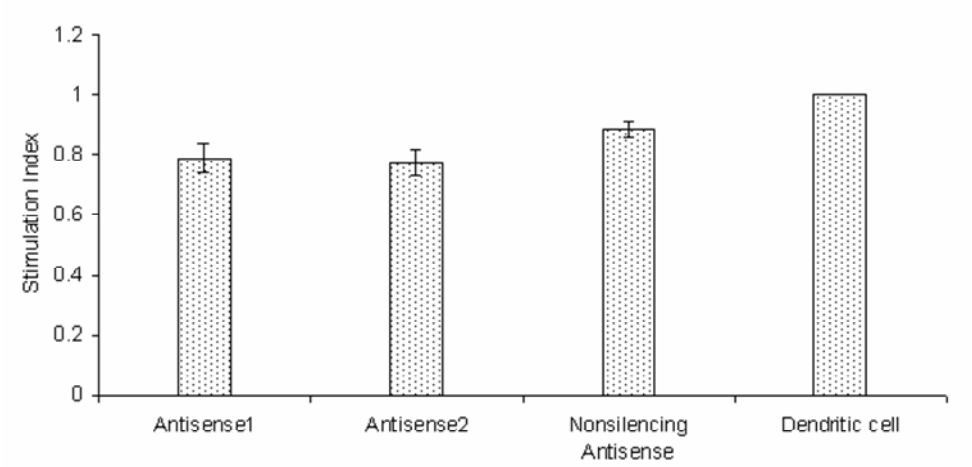

Figure 4: Antisense-CD40 transfection inhibited the DCs allostimulatory ability.

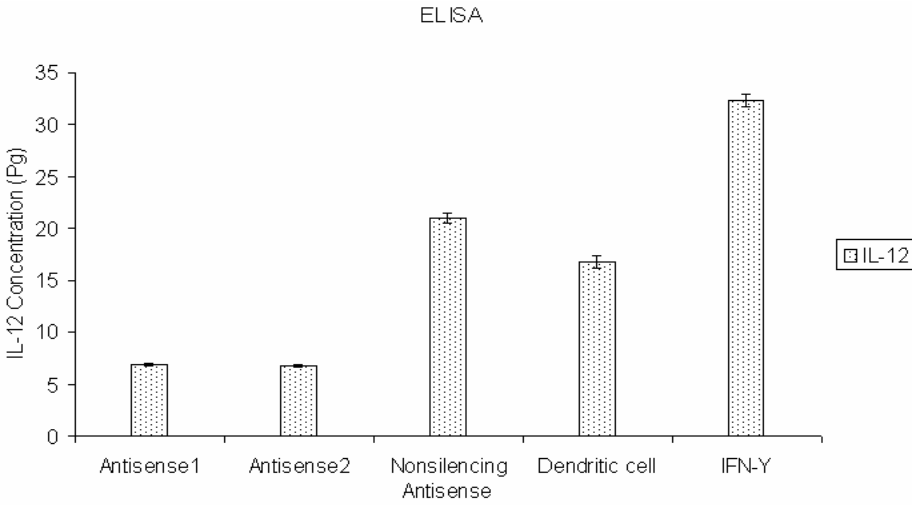

Figure 5: Antisense-CD40 specifically blocks CD40 and down-regulates IL-12.

Discussion

To inhibit the specific T cell responses and conquer the allograft rejection, it is possible to manipulate DCs by genetic engineering. This cell is selected because it is one of the most potent APCs to present the antigens with high efficiency and to activate the large number of T cells. The induction of T-cell hyporesponsiveness was carried out by the DCs which were not able to represent the co-stimulatory molecules.[18] Thus, the inhibition of alloantigen specific T-cell proliferation by the DCs makes them suitable applicants to have a better allograft response.[19,20]

For the activation of naive T cells and making a good antigen presenting cell, the DCs should be maturated. In the maturation step, different cell surface molecules like CD40 or MHC class II, CD80 and CD86 are highly expressed. Several studies have shown that if this process is prevented, tolerance will be induced and unwanted immune responses will be inhibited.[21,22]

By means of antisense oligonucleotides against CD40, CD80 and CD86 transcripts, the co-stimulatory molecules were down-regulated and the result was tolerogenic DC production.[23] That's why DCs are engineered for tolerance induction.[24] Immature DCs express a small amount of MHC and co-stimulatory molecules on the cell surface.[25,26]

For tolerance induction in the immunotherapy, it is useful to administer immature DCs. So, T-cells responses are deleted and non-responsiveness will happen. Non-responsive T-cells with suppressive phenotype are called regulatory T cells. They suppress autoaggressive immune responses efficiently.[27] In the recent studies, by antisense oligonucleotide, co-stimulatory molecules were suppressed.[19,20,22,23]

In the conventional therapeutic approaches, the compounds which are not usually selected by rational strategies are used and sometimes the results show low specificity of them. However, antisense therapy allows us design a highly specific oligonucleotide rationally to degrade the target mRNA.[28]

Several ways have been examined for targeting co-stimulatory molecules and antisense oligonucleotides are one of the recent tools utilized in the treatment of type 1 diabetes mellitus and allograft rejection. In the present study, for immune modulation we applied the antisense oligonucleotides against CD40 mRNA in the DCs. Antisense ODNs targeting CD40 could specifically decrease both CD40 gene expression and CD40 protein. Also, IL-4 production increased and a reduction in allostimulatory activity was seen. The production of IL-12 by APCs is very important for MLR proliferative response and if anti-IL-12 antibodies are added, the proliferation will be suppressed.[29] In this study, antisense ODNs treatment did not change the maturation state of the splenic DCs. So it can be concluded that IL-12 reduction affects allogeneic T cell proliferation.

Also, a decrease in IL-12 level will cause more IL-10 to be produced and this cytokine is an inhibitor for T cell proliferation.[30] Again, it is probable that the ability of IL-10 itself can reduce IL-12 level from the APCs.[31] Th2 promoting DCs have a reduced allostimulatory function and produce a low level of IL-12.[32] IL-12 and IL-10 balance is one the factors affecting cytokine production of the activated T cells.[33,7] IL-12 strongly promotes Th1 cell differentiation while IL-10 inhibits IL-12 production and interferes with the ability of DCs for the induction of Th1 responses.[34] Liang et al.[35] reported that antisense targeting CD80, CD86 could generate tolerogenic dendritic cell and decrease allostimulatory function. Cong et al.[36] reported that blocking of CD40L inhibited IL-12p40 production by 80–90%. These data indicate that CD40-CD40L interactions were directly involved in the induction of IL-12p40 production. This finding is in agreement with our results.

For example, in a heterotopic heart transplant model in the mice, CD80 and CD86 specific antisenses were delivered into the DCs, graft survival being prolonged. This indicates that allospecific T cells were reduced.[37] In the NOD mouse model, the DCs were transfected by antisense oligonucleotides against CD40, CD80 and CD86. The results clearly demonstrated an expansion in the regulatory T cell population and a significant delay in the onset of diabetes.[23] In the studies on the chronic IBD patients, antisense oligonucleotides targeting CD40 interfered in CD40/CD154 pathway. Thus, it can be considered as an attractive strategy for chronic IBD treatment.[38] Also, Qian S. et al. reported that NF-κB antisense oligonucleotides was able to promote DC tolerance by blocking the NF-κB DNA binding activity.[39] In vitro, CD40 gene knocked down dendritic cells induce T cell differentiation to Th2 cell preferentially. Since CD40 antisense treated DCs have a poor allostimulation activity and Th2-promoting characteristics, the resultant DCs have the phenotype of tolerogenic DC and are suitable tools for treatment Th1- mediated autoimmune diseases and the inhibition of allograft rejection.

In conclusion, our results support the view that CD40 pathway has a critical role in the regulation of DC-T cell interaction and generation of tolerogenic DCs, and that the treatment of DCs with antisense

against co-stimulatory molecules is an effective method for the inhibition of DC maturation and allostimulatory function.

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Conflict of interest: None declared.

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