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Tolerance Induction by CD40 Blocking through Specific Antibody in Dendritic Cells

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ABSTRACT

Blocking antibodies are valuable tools for inhibiting the specific receptor- ligand interactions. The interaction of co-stimulatory molecules on the antigen presenting cells with their ligands on T cells is an essential step for T cell activation. In the present study, the effect of blocking antibody against CD40 on its T cell stimulatory potential is investigated.

The DCs (dendritic cells) were collected from the mice spleens and then cultured in vitro. We used purified rat anti-mice CD40 (Clone HM40-3) (BD USA) as a blocking antibody and the appropriate titer of the blocking antibody was determined by flow cytometry. The DCs were then treated by antibody and used in MLR assay.

The results of these experiments showed that CD40 blockade were associated with the increase in the of IL-4 secretion, shifting the DCs to stimulate Th2 cytokine production by the allogenic T cells, while the secretion of IL-12 by DCs decreased. Similarly, the DCs with reduced CD40 expression poorly responded to alloantigen stimulation in the MLR.

Collectively, these results emphasize the importance of CD40 pathway in tolerogenic DCs generation and also support the idea that downregulation of CD40 is effective in inhibiting the allostimulatory function.

Key words: CD40; Dendritic Cell; Tolerance Induction

INTRODCUTION

The compounds which bind T cell surface molecules and interfere with T cell activation and its effector functions are thought to be effective for inhibition of organ rejection following transplantation as well as autoimmunity therapies. In murine models, the disruption of CD40 and CD28 pathways, either by biological agents or by genetic means, can undergo tolerance, leading to prolonged allograft survival and the prevention of autoimmune disease.[1-3]

One of the member of tumor necrosis factor receptor superfamily (TNFRSF) is a 48 kDa transmembrane glycoprotein surface receptor named CD40.[4] After CD40 discovery, the critical role of this protein in the regulation of DC function was not known for many years[4]. Finally several groups reported that CD40 signaling make some changes in DCs like MHC class II, CD80 and CD86 upregulation and convert them to the more effective antigen presenting cells (APCs).[5,6] In spite of many similarity between DCs and

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B cells in CD40 ligation, it became clear that their response to CD40 stimulation is completely different.[7]

The co-stimulatory function of CD40 is critical for the function of antigen presenting cell[8] as well as activated CD4 and memory CD8 T cells.[9]

The importance of a functional CD40–CD40L pathway is for effective T-cell activation, recruitment of leukocytes into the allograft, and for promoting the development of Th1 responses.[10,11]

In order to study the function of co-stimulatory molecules, it was determined that blocking antibody inhibited the interaction between the receptor and cognate ligand. It is the best technique for studying the co-stimulatory function.

Due to the central role of CD40-CD40Linteraction in DCs maturation and its effect on immune regulation, tolerance induction and generation of tolerogenic DCs, in the present study we investigated the effects of blocking antibody on CD40 and thus its influence on DCs function.

MATERIALS AND METHODS

Animals

8-10 week old male Balb/c and C57Bl/6 mice were obtained from the Pasteur Institute (Tehran, Iran). All the experiments were approved and conducted according the protocols of the Ethical Committee of Tarbiat Modares University (Tehran, Iran).

Purification of Splenic DCs

We isolated splenic DCs as described previously.[12]

Determination of Blocking Antibody Titer

We used the following antibody as a blocking antibody: purified rat anti-mice CD40 (Clone HM40-3) (BD USA). For determination of the appropriate titer for blocking antibody, we used flow cytometry and chose with the highest MFI (Mean fluorescent Intensity). For this purpose, different concentrations of the antibody were added to DCs and the concentration of antibody molecules on DCs was measured using flow cytometry. The appropriate titer was determined based on MFI plateau.

Flow Cytometry Analysis

In order to analyze the cell surface phenotype, we operated flow cytometry as describe previously.[12] The FITC-conjugated AnnexinV and PI staining were used for the determination of apoptosis /necrosis as described by other researchers.[13]

MLR

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

ELISA Assay

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

ELISPOT

The IL-4 and IFN-γ-producing cells were quantified by standard ELISPOT assay as described previously.[13] Briefly, the T cells were first cultured for 24 h with the control and blocking antibody treated DCs. Then the T cells were purified by nylon wool and ELISPOT assay was performed as recommended by the manufacturer (R&D, USA). The optimum time for incubation of 10^5 cells in ELISPOT was 36 h.

Statistical Analysis

The results are expressed as mean ± standard deviation (S.D). Statistical analysis was performed using the Mann-Whitney U-test. The p-values less than 0.05 were considered as significant.

RESULTS

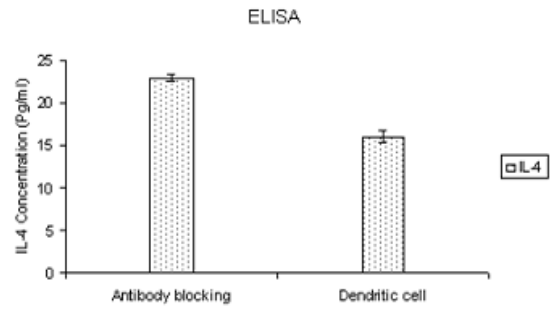
Blocking Antibody Treated DCs Promoted Th2 Differentiation

Based on the fact that CD40 can polarizes T cells to Th1 phenotype,[14] in the present study, the allostimulation ability of the blocking antibody treated DCs to alter cytokine production from the responding T cells was evaluated. From the MLR culture

supernatants, IFN-γ and IL-4 were assayed by ELISA and also from the cultured cells, the mentioned cytokines were assessed by ELISPOT. Finally, the ELISA and ELISPOT results were compared with each other.

The results showed that low levels of IFN-γ and high levels of IL-4 were secreted from the T cells incubated with the blocking antibody treatment (Figures 1 and 2).

The difference between ELISA results was significant for IL-4 (blocking antibody, $p<0.001$) and IFN-γ (blocking antibody, $p<0.01$). The differences between ELISPOT results for IL-4 and IFN-γ were not significant. In contrast, the cytokine profile of the T cells incubated with the untreated DCs showed a high level of IFN-γ and a low level of IL-4.



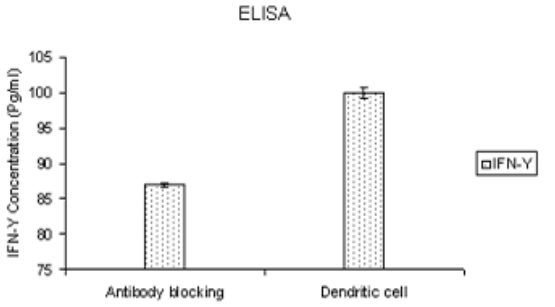
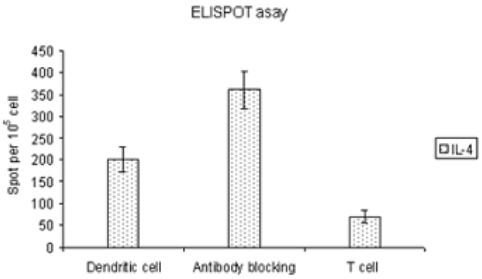


Figure 1. The blocking antibody treated DCs promote Th2 polarization. The blocking antibody specifically blocks CD40, up-regulates IL-4 and downregulates IFN-γ. The unmanipulated DCs, the DCs treated with blocking antibody, were separately cultured with allogenic T cells. The supernatants were harvested from the cultures and analyzed for IL-4 and IFN-γ production. The data are shown as mean ±SD of four independent experiments.



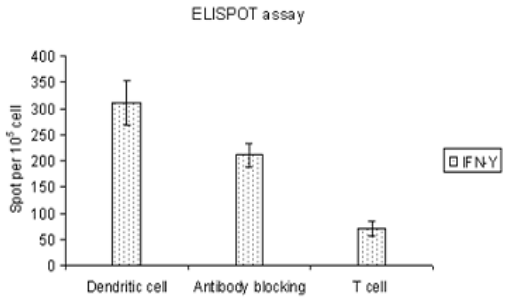


Figure 2. The frequency of IFN-γ and IL-4 producing T cells by ELISPOT assay. The T cells were harvested after 1 day of co-culture with the allogenic DCs, blocking antibody treated DCs plated into ELISPOT plate. The frequency of cytokine producing cells was measured after 36 h. The data are shown as mean ±SD of four independent experiments.

Therefore, the researchers conclude that the blocking antibody treated DCs have the ability to polarize naïve T cells into Th2 cells.

Allostimulatory Activity of Blocking Antibody Treated DCs Decreased

Stimulation of alloreactive T cells by DCs in the MLR, at least in part, can characterize the DCs function.[15] For this purpose, the DCs treated with blocking antibody together with T cells from C57Bl/6 (DC/T ratio, 1/10) were cultured into the 96 well U shape plates for 54 h. Then, the cells were pulsed with [^{3}H]-thymidine for 18 h and harvested. Finally, the filters were counted in β counter. The blocking antibody treated DCs showed a 23% reduction allogenic T cell stimulatory index. The difference between blocking antibody treated and untreated groups was significant ($p< 0.001$).

Blocking Antibody Treated DCs Decreased IL-12 Production of DCs

CD40-CD154 interaction triggers the secretion of a number of cytokines such as IL-12. That is why the measurement of IL-12 levels can help to assume the functional loss of CD40 receptor.[16] According to this fact, a protocol was designed to evaluate the effect of blocking antibody against CD40 on IL-12 secretion. In this research, the control groups were the untreated DCs and IFN-γ stimulated DCs.

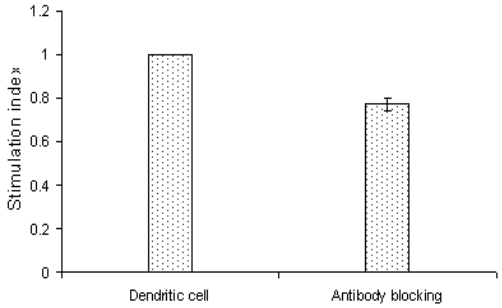


Figure 3. Blocking antibody inhibited the DCs allostimulatory ability. The unmanipulated DCs and the DCs treated with blocking antibody were separately cultured with allogenic T cells for 54 h. The proliferation was determined using [^{3}H]-thymidine incorporation. The data are shown as mean ±SD of four independent experiments.

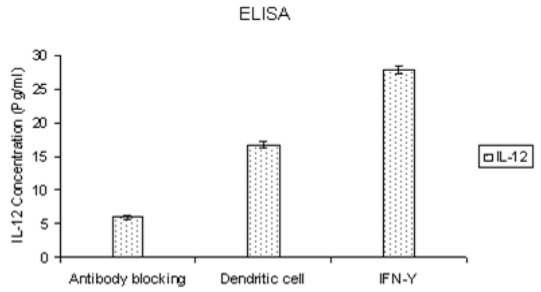


Figure 4. Blocking antibody specifically blocked CD40 and down-regulated IL-12. The cells were first treated with blocking antibody. The stimulatory antibody against CD40 (10μg/ml) was added to the unmanipulated (DCs), the DCs treated with blocking antibody and treated with IFN-γ (as a positive control). After 36 h, the supernatants were collected and analyzed for IL12p70 production. The data are shown as mean ±SD of four independent experiments.

It was shown that the blocking antibody treated DCs, in comparison with the control group, produced IL-12. As shown in Fig. 4, blocking antibody against CD40 reduced IL-12 p70 heterodimer production (as determined by ELISA) by 70% compared to the untreated DCs. The difference between untreated groups was significant ($p < 0.001$).

DISCUSSION

For the inhibition of specific T cell responses and to achieve the tolerance induction, it is possible to execute the manipulation of DCs. Indeed, these cells were selected because they were among the most potent APCs to present antigens with high efficiency and with the capacity to activate large numbers of T cells.

One effective method to inhibit the co-stimulatory function and the induction of T-cell hypo-responsiveness is using the fusion proteins and antibodies designated for cell-surface molecules, like anti-CD40L and CTLA4-Ig. The short half life of this compound causes multiple antibody treatments, since they inhibit T cell co-stimulation by DCs,[17] the inhibition of alloantigen specific T-cell proliferation by DCs makes them a proper applicant for a better allograft response.[18]

The tolerogenicity of allogeneic nonspecific antigen presenting cells like B cells is induced by blocking the CD40L interaction through anti-CD40L monoclonal antibodies.[19] This can explain why blocking of CD40-CD40L interaction is a good way to inhibit the allograft rejection. On the other hand, the first clinical trial in the primary renal allograft recipients with hu5C8, a humanized anti-CD154, was launched in 1990 with great anticipation that this regimen might result in operational tolerance.

However, anti-CD154 therapy can cause thrombotic events which undergo discontinuation of the trial as well as the cessation of clinical development of anti-CD154 in both transplantation and autoimmune diseases. That is why the interest has shifted to targeting CD40, which should not be associated with thrombotic events and preclinical studies in autoimmune diseases and organ transplantation are being conducted.[19,20]

In the present study, for immune modulation, we applied the specific antibody against CD40 in the DCs and in order to evaluate the function of CD40 in

generation of tolerogenic DCs, we applied different methods like ELISA and ELISPOT.

The results indicated that ELISPOT in comparison with ELISA showed more Th1/Th2 shift (For antisense in ELISPOT, the ratio was 2.65 while in ELISA, it was 1.6). The difference between these two ratios can be described by the fact that ELISPOT assay is a more sensitive method and can detect every single cell in the culture, while ELISA is a technique to measure the rate of cytokines production in the supernatant.

We previously used siRNA and antisense to knockdown CD40 in order to induce tolerogenic DCs.[12,21] We reasoned that choosing the best system in CD40 down-regulation was the most important step in tolerogenic DC generation. Previous research showed that siRNA was more effective in downregulation of CD40.[12]

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.[22] Thus, we can conclude that IL-12 reduction affects allogeneic T cell proliferation. Moreover, IL-12 level decrease will cause more IL-10 production. Also, IL-10 can inhibit T cell proliferation.[23] Again we can assume that the ability of IL-10 itself may reduce the IL-12 level from APCs.[24]

Th2 promoting DCs produce low levels of IL-12 and reduce allostimulatory function. In the activated T cells one of the factors affecting cytokine production is IL-12 and IL-10 balance.[25] Haase C et al.[26] reported that the CD40 co-stimulatory molecule is critical for naïve allogeneic T cells activation in vivo but not in vitro. Cong et al. showed that CD40L blocking inhibits IL-12p40 production by 80–90%.[27] Their results indicate that CD40-CD40L interactions are directly involved in the induction of IL-12 production, and this is in agreement with our data.

Taylor et al.[28] reported that in allo-MLR cultures, tolerance induction via blockade of the CD40-CD40L co-stimulatory pathway leads to the acquisition of potent suppressor function that inhibits naïve and primed alloresponses both in vitro and in vivo.

Zhang L et al.[29] reported that different levels of CD40 surface expression on the LPS-matured DCs had different impacts on naïve T cell activation in vitro. He reported that levels of CD40 downregulation correlated with the suppression of naïve OVA specific T cell proliferation.

Li L et al.[30] reported that in vitro blocking CD40 co-stimulatory pathway lead to the generation of anergic cell, decreases T cell proliferation and ameliorate collagen induced arthritis. Zhi-juan L et al.[31] reported that in vitro blocking CD28/B7 co-stimulatory pathway suppresses MLR and allograft rejection response.

We confirmed this finding using siRNA, antisense and blocking antibody against CD40 in an allogeneic MLR in vitro. The results showed that siRNA had more effects in reduction of allostimulatory function, decreased IL-12, IFN-γ production and increase IL-4.[12] Probably, quantitative expression of CD40 would affect DC function in vitro.[26] siRNA showed more potential in downregulation of CD40 and duration, followed by antisense.[21]

We provided functional data to further demonstrate that quantitative expression of CD40 on DC has different functional outcomes on allogenic T cell responses in vitro.

The mechanism by which anti-CD40 antibody or CD40 antisense treated DCs generate tolerogenic DCs requires further investigation. Based on the current information, however, it is reasonable to suggest that blocking antibody or antisense act by inhibiting the upregulation of CD80 and CD86 induces apoptosis in the effecter cells or by inhibition of IDO production. Additionally, the inhibition of CD40 ligation could also inhibit the production of the important biological effectors molecules such as nitric oxide and IL-12 by DCs.

In conclusion, DCs are specialized regulators of the innate and adaptive immunity. Advances in using different techniques to down-regulate costimulatory molecules provide a unique tool to induce tolerance. This approach will allow us to examine the importance of quantitative expression of costimulatory molecule(s), such as CD40, in determining different functional properties of DC to tolerance induction, activation or polarization antigen-specific Th1/Th2 immune responses.

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