

## CD40 and Tolerance Induction

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### ABSTRACT

CD40 is recognized as a member of tumor necrosis factor receptor super family. It is expressed by the immune and non-immune cells. Its interaction with CD40 ligand (CD154) brings about a regulatory effect on the cellular and humoral immunity. The pathway of CD40-CD154 is influential in various diseases. Investigations on such diseases have revealed dimensional mechanisms whereby this route intensifies host protection. Moreover, through these mechanisms, pathogens subvert the signaling of the CD40, conditions in which the CD40-CD154 pathway promotes disease and also through the relevant modulation for immunotherapy.

This review focuses on the role of CD40-CD40L (CD154) interactions in dendritic cells (DCs) regulation, tolerogenic dendritic cells, role of CD40 in autoimmune disease, allograft rejection and induction of tolerance by down regulation of CD40. According to these roles, it is assumed that CD40 is a functional molecule in the pathologies of conditions like autoimmune diseases and allograft rejection caused by activated T and B cells.

**Keywords:** CD40; Tolerance

### INTRODUCTION

#### CD40 Definition and Characterization

CD40 is recognized as a member of tumor necrosis factor receptor super family. It is expressed by antigen presenting cells (APCs). Furthermore it is expressed by various non-hematopoietic cells; activated CD4<sup>+</sup> T cells primarily reveal its ligand CD154.<sup>1-2</sup> In the mid 1980s, CD40 was first discovered as a surface receptor of B cells which is able to cause polyclonal activation through engagement with its ligand. Moreover, CD40 ligand (CD154) was discovered as a T cell surface molecule able to induce contact dependent

differentiation of B cells.<sup>3</sup> For many years, CD40 and CD40L received an exclusive immunological connotation but soon it was realized that these molecules are expressed on a wider range of cells and have a variety of activities other than what originally described for T and B cell interactions.<sup>4-5</sup> Due to the expanded biological function of CD40/CD40L, intense studies have been conducted on the biological, clinical and therapeutic implications of these molecules.

CD40 is known as type I transmembrane protein with 45-50 kDa molecular weight which is ubiquitously expressed by both immune cells including B cells, monocytes, macrophages, and dendritic cells (DCs), and non-immune cells including epithelial, endothelial, and mesenchymal (fibroblasts, myofibroblasts, synoviocytes, stellate cells, etc), and platelets.<sup>5-6</sup>

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CD40L type II transmembrane protein is 39 kDa member of TNF gene super family which is expressed on the surface of activated CD4+ T cells and activated platelets. Moreover it is variably expressed by monocytic cells, natural killer cells, B cells, CD8+ T cells, mast cells and basophiles.<sup>6</sup>

CD40/CD154 interaction regulates many features of cellular and humoral immunity such as T cell-mediated activation of DCs, T cell priming, proliferation of B cells, immunoglobulin synthesis, isotype switching, and germinal center formation.<sup>7-9</sup> (Figure 1)

The relevance of CD40 in humans is clarified by the lack of functional CD154 that can undergo a congenital immunodeficiency called X-linked hyper IgM (X-HIM) syndrome.<sup>10</sup>

### Biological Effects of CD40/CD40L Interaction

Cognate interactions of CD40 and CD40L induce intracellular signals and expression of surface and secreted molecules influencing both humoral and cellular immunity, and inflammation. A switch in recombination and synthesis of immunoglobulin by B cells is one of the first reported and most investigated biological effects of CD40 ligation.<sup>6</sup>

It was found that patients suffering from X-linked hyper-IgM syndrome with severely compromised humoral immune response have CD40L genetic mutation.<sup>11</sup> Since these patients are unable to switch the IgM isotype, they have deficiency in production of antibody including the circulating IgG and IgA.

Moreover, binding of T cell CD40L to its ligand is critical in the maturation process of B cells to the memory cells and their activity and proliferating induction. These reactions could be regenerated *in vivo* in mice with a genetic defect in CD40/CD40L pathway.<sup>12,13</sup>

Soon, the different aspects of CD40/CD40L interactions in humoral immunity were demonstrated and the data indicated that these interactions were also vitally important to cell mediated immunity.<sup>13-14</sup>

Among the different cellular functions of CD40L, we can mention the co-stimulatory activity of APC regulation, inducing B cells to up-regulate B7.1 and B7.2, and stimulating DCs to increase the cell surface expression of other co-stimulatory molecules like CD54 and CD86 (B7.2).<sup>15,6</sup>

In DCs, CD40 ligation induces the production of different cytokines, like IL-8, TNF- $\alpha$ , and MIP-1 $\alpha$ . Also, CD40 stimulation leads to IL-12 production. IL-

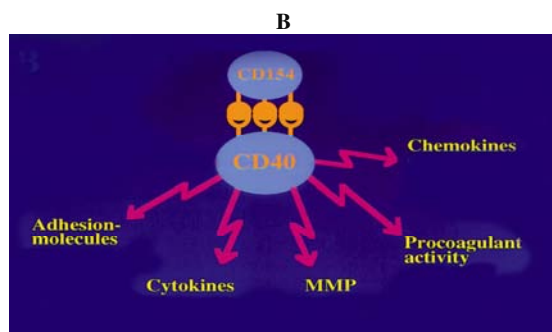
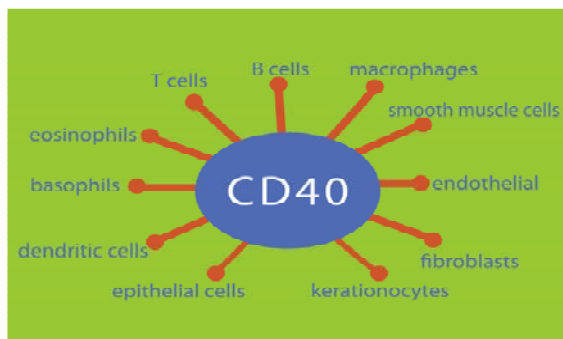
12 is a crucial cytokine in the polarization of Th1 immune responses.<sup>6</sup>

Moreover, induction of apoptosis in CD4+ T cells<sup>16</sup> and the generation of CD8+ T cell memory<sup>17</sup> is another function of CD40/CD40L interactions. The interaction of CD40 and CD40L seems to be bidirectionally, for instance CD40 expressing by APCs contributes to T cell activation. In animals with CD40L deficiency, in response to antigen exposure, CD4+ T cells proliferate poorly and produce little IL-4 and interferon (IFN)- $\gamma$ ; they also fail to generate antigen specific T cell responses.<sup>13</sup>

Considering above mentioned points, CD40 and CD40L not only are expressed on classical immune cells, but also they can be expressed on an entire host of non-immune cells including Epithelial Endothelial cells, Fibroblasts, Myofibroblasts, Synoviocytes and Platelets. CD40/CD40L system is an actual tool for communication of the immune cells and numerous types of non-immune cells with each other.

Hence, the usual outcome would be amplification of the immune and inflammatory responses. For example, CD40 ligation on the endothelial cells or on fibroblasts induce the sudden production of various chemokines including IL-8, MCP-1, MIP-1 $\alpha$  and  $\beta$ , RANTES (regulated on activation normal T expressed and secreted), Fractalkine, and cytokines like IL-1, IL-6, IL-12, and TNF- $\alpha$ , up-regulation of cell adhesion molecules, including ICAM-1, VCAM-1, and E-selectin, secretion of matrix metalloproteinases (MMPs), such as MMP-1, 2, 3 and 9, and tissue factor expression.<sup>18-20</sup>

In fibroblasts, CD40 engagement up-regulates cyclooxygenase-2 expressions and prostaglandin E2 production.<sup>6</sup> Besides, sCD40L makes the platelets active and causes the up-regulation of P-selectin expression as well as  $\beta$ -thromboglobulin and 5-hydroxytryptamine release;<sup>21</sup> however, the membrane bound form of CD40L leads to the release of biologically active RANTES stored in the platelet granules. Through inflammation and tissue destruction, the combination of the mediators' effects will be detrimental to the host.<sup>22</sup>



**Figure 1.** Expression and function of CD40 are shown (A) different cell types expressing CD40 and (B) potential consequences of the encounter of the CD154 with its receptor CD40. MMP: Matrix Metalloproteinases

### Polymorphisms and CD40

The correlation between single nucleotide polymorphisms (SNPs) and the regulation of CD40 expression in infection and autoimmunity-associated diseases, e.g. rheumatoid arthritis, coronary artery calcification, systemic lupus erythematosus are now being studied.<sup>23-24</sup>

Nearby the start codon at the 5'-untranslated region (5'-UTR), there is the only SNP known to influence the levels of CD40 protein<sup>25</sup> and this SNP (-1C>T; rs1883832) concurs with the Kozak consensus sequence.<sup>26</sup> By retarding the stabilization of mRNA-ribosome complex, it is known that the allele carrying T nucleotide is responsible for reduced levels of CD40.<sup>27</sup> A number of studies report that TT genotype is associated with higher risk of follicular lymphoma while CC genotype being responsible for high CD40 surface expression seen in Graves Disease (GD) in a familial sub-group of Caucasians and unrelated Koreans but not in all populations studied.<sup>26-28</sup>

### CD40 and DC Subsets

In mice and human, there are a number of DC subsets. Analysis of CD40 expression and its relevant function for each subset is limited by the growing expanse of DC subsets.<sup>29</sup> In response to TLR9 (CpG) or TLR4 (LPS) agonists, human peripheral blood plasmacytoid DCs (CD4+, CD123+, BDCA1+, BDCA4+) and myeloid DCs (mDCs: CD4+CD11+CD33+) up-regulate CD40.<sup>30</sup> In vitro, CpG stimulated pDCs up-regulate CD40 to a greater extent in comparison with LPS stimulated mDCs, while the pDC subset does not induce CD40 up-regulation.<sup>30,31</sup> In response to the TLR7 ligand Imiquimod, both pDCs and mDCs up-regulate CD40 to a similar levels.<sup>31</sup> That is why in the peripheral blood DCs, the level of CD40 expression is not subset specific. In fact the signaling pathway and the receptor utilized for DC activation cause CD40 expression.<sup>32</sup>

Basal expression of CD40 and other co-stimulatory markers such as CD80 and CD86 in the mDCs of human lung are higher than basic expression in pDCs.<sup>32</sup> Besides, in the human thymus, mature DC subsets differentiated by CD11b expression have a different CD40 expressing pattern.<sup>33</sup> It is essential to investigate and compared the CD40 expression in subsets of DC localized to other tissues, particularly in the mucosal tissues, spleen and lymph nodes.

To define DC subsets in mice, broad classifications of human DC populations were utilized<sup>32</sup> and it was concluded that in the basal level of CD40 expression, there is no difference between mouse splenic CD8<sup>+</sup>CD4<sup>+</sup> and CD8<sup>-</sup>CD4<sup>-</sup> mDCs.<sup>34</sup> CD11c<sup>hi</sup>B220<sup>-</sup> DC subsets of the liver express higher basal level of CD40 than CD11c<sup>int</sup>B220<sup>-</sup> subsets. On the B220<sup>+</sup>CD11c<sup>int</sup> population, there is little or no CD40 expression.<sup>35</sup> By means of murine cytomegalovirus (MCMV), activation of DC subsets induces high up-regulation of CD40 in the CD11c<sup>hi</sup>B220<sup>-</sup> population, while in CD11c<sup>int</sup>B220<sup>+</sup> DCs no up-regulation is seen.<sup>35</sup> Also, on hepatic DC subsets CD8 expression is associated by the higher basal expression of CD40 compared to CD8<sup>-</sup> DC populations.<sup>35</sup> Various investigators compared CD40 expression on pDC versus mDC in response to HCV, Streptococcus pyogenes and influenza A and the results indicated that the differences in the up-regulation of CD40 expression appear to be more dependent on the type of stimulation rather than the DC subset examined.<sup>32</sup>

### DC Function and CD40 Expression Levels

'Regulatory DCs' are a type of DC subsets which are distinct from 'immature' or 'tolerogenic' DCs that present signal 1 (e.g. antigen peptide-MHC complex) but not a co-stimulator signal 2 and induce anergy in the cognate lymphocytes.<sup>36</sup> 'Regulatory DCs' in mice and human induce little or no proliferation in naïve CD4<sup>+</sup> T cells and they produce IL-10 and TGF- $\beta$  not Th1 or Th2 cytokines.

These regulatory DCs lead to Tregs (regulatory T cells) generation<sup>37-38</sup> and are found in human and mice. They are composed of a heterogeneous and wide range of DCs that are induced under multiple stimuli and types of pathogens.<sup>39</sup>

A novel research described one special population of regulatory DCs in mice which is identified specifically as CD11cint and CD45RBhi. This DC population is found in the spleen and lymph nodes. It can promote differentiation of Treg cells through secreting IL-10. One of the CD45RB<sup>+</sup> regulatory DCs properties is that even after infection or TLR stimulation, they do not up-regulate CD40. In human regulatory DCs, further subsets have been recognized with similar effectors functions as the CD11cintCD45RBhi murine DCs (e.g Tregs induction, low capacity of CD4<sup>+</sup> effectors T cells inducing IL-10 secretion).<sup>32</sup> However, these markers have not been used for the identification of these human DC subsets, and there is no study to have compared the functional similarities of these subsets in mice and human.<sup>32</sup>

It is still under investigation to find out whether CD40 signaling is linked to the function of regulatory DCs subsets or not. According to the fact that CD40 has a low expression on regulatory DCs, it would be attractive to know whether TNFR family members other than CD40 such as RANK (a recently identified member of the TNF receptor superfamily termed receptor activator of NF- $\kappa$ B) control regulatory DCs. Moreover, it can be suggested that reduced expression of co-stimulatory molecules on these DCs may lead to the decline of its potential to induce the effectors of CD4<sup>+</sup> T cells and distort Tregs differentiation. The studies show that in the periphery, steady-state DCs without anti-CD40 activation promote CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs differentiation from naïve CD4<sup>+</sup>CD25<sup>-</sup> T cells.<sup>40</sup> Splenic CD8 $\alpha$ <sup>+</sup> DC subsets with a fusion protein of an anti-DEC205 antibody were investigated and accordingly this antibody was conjugated to the hemagglutinin antigen from the

influenza virus (anti-DEC-HA) which by itself does not alter maturation status of steady-state DCs.<sup>41</sup>

Recently, a study has been done on murine DCs, displaying a 'semi-mature' phenotype which play a role in regulating T cell responses in collagen-induced rheumatoid arthritis (CIA).<sup>42</sup> Murine BMDCs were stimulated by naked plasmid DNA versus LPS and it was concluded that treatment with DNA plasmid induced cytokines and co-stimulatory molecules expression (including CD40). Since the expressions have been shown as intermediate between LPS and unstimulated BMDCs, the mentioned DCs were designated as "Semi-mature".

From the functional viewpoint, it seems that there is a kind of similarity between 'semi-mature' DCs and LPS-stimulated mature DCs in the potency to induce Treg, and accordingly in protection against CIA. It has also been demonstrated that the influence of semi-mature DCs to induce Treg showed common qualities with that of LPS stimulated DCs with elevated levels of CD40. It is also noteworthy that comparison shows that levels of IL-10 and TGF transcription in the peripheral lymph nodes were greater in animals transferred with semi-mature DCs versus LPS- Stimulated DCs at early time sets. Wherever both 'semi-mature' and LPS-stimulated DCs stimulate Treg differentiation, it is proposed that an environment is encountered with high levels of immunomodulatory cytokines IL-10 and TGF- $\beta$  may induce alternate T cell programming leading to the protection against CIA. DC treatment with cytokine and pharmacological agents will influence the expression level of CD40 and other co-stimulatory molecules contributing to DC maturation status. Totally, in mouse models pharmacological treatments like Vitamin D regulate surface CD40 expression and maturation of DCs which induce Tregs and increase the secretion of IL-10 resulting in increased tolerance to transplants and decreased diabetes.<sup>32</sup> (Figure 2)

### CD40 and Pathogenic Condition

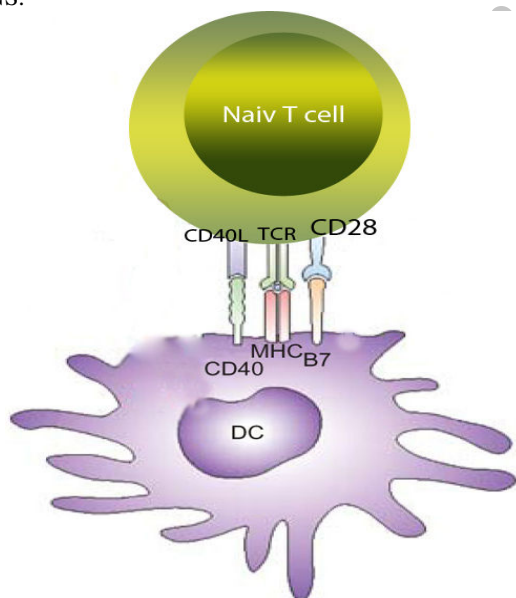
#### CD40 and Neuroinflammatory Diseases

In an inflammatory demyelinating disease of the brain and spinal cord namely Multiple Sclerosis (MS), CD4<sup>+</sup> and CD8<sup>+</sup> T cells, B cells, macrophages, and activated microglia infiltrate the CNS and destroy the myelin leading to motor and sensory dysfunction.<sup>43</sup> Examining the human post-mortem brain lesions and studying the mouse models with experimental autoimmune encephalomyelitis (EAE), in which immunization of mice with myelin

components causes CNS infiltration by the immune cells, helped the researchers to elucidate the different aspect of MS pathogenesis.<sup>29, 43</sup>

In the brain of MS patients, most CD154+ cells are CD4<sup>+</sup> T cells, and most of the CD40<sup>+</sup> cells are either CD11b+ macrophages/microglia or B cells.<sup>2</sup> In their peripheral blood, T cells induce more IL-12 production by either normal or MS-derived APCs in a CD40-dependent manner.<sup>44</sup>

Compared with healthy controls, MS patients have a higher frequency of CD154+ T cells which decrease following treatment with interferon- $\beta$ .<sup>45</sup> In EAE, during acute disease and relapse CD40 is expressed in the spinal cord, while CD154 is expressed highly only during relapse. Expressions of these molecules are in association with Th1 cytokine production within the CNS.<sup>46</sup> As a result of the defect in APC activation, CD154<sup>-/-</sup> mice do not develop EAE.<sup>2</sup> At the time of EAE induction, an anti-CD154 mAb antagonist treatment inhibits induction of the disease<sup>2</sup>, by skewing the immune responses towards non-pathogenic Th2 responses<sup>47</sup> or stopping the Th1 cells retention or expansion within the CNS.<sup>48</sup> If it is administered at the peak of acute disease, mice have fewer relapses of shorter duration, associated with decreased Th1 cell differentiation and fewer inflammatory cells within the CNS.<sup>49</sup>



**Figure 2. Schematic presentation of CD40 and CD40L/CD154 expression on DCs and lymphocytes. Model of CD40-CD40L/CD154 interactions between T cells and DCs**

It has been commented that if anti-CD154 treatment is given >7 days post-immunization, it would be ineffective.<sup>2</sup> Therefore, during the priming stages of EAE CD40 signals appear to be more crucial than during the established disease. Microglia express CD40 and following IFN- $\gamma$  treatment, it is up-regulated.<sup>50</sup> In microglia, CD40 signaling leads to the production of TNF- $\alpha$  and IL-12, microglial activation, and neuronal cell death.<sup>50, 51</sup> Experiments using bone-marrow chimeras showed that for EAE progression, CD40 expression on microglia is essential.<sup>51</sup> Mice lacking the expression of CD40 have less severe EAE disease only in the radio resistant CNS compartment, with fewer CNS-infiltrating encephalitogenic T cells and less demyelination.<sup>51</sup> Thus, CD40 signals are crucial for both initial priming of T cell responses in the periphery and optimal T cell expansion and/or retention in the target tissue. MS has a strong genetic component but the CD40 gene does not lie within any of the identified genomic risk regions.<sup>43, 52</sup>

A small case-control study in a heterogeneous population of MS vs. Huntington's disease patients showed that there is no association between Kozak sequence SNP (-1C/T) and MS susceptibility or disease course<sup>53</sup> although this SNP has been associated with Graves' disease.<sup>54</sup>

#### **CD40 and Rheumatoid Arthritis (RA)**

About 1% of the world's population is affected by RA, a chronic inflammatory disease resulting in joint destruction if untreated.<sup>55</sup> Innate and adaptive immune cells infiltrate the joint space and lead to local production of pro-inflammatory Th1 and Th17-type cytokines, chemokines, and matrix metalloproteinases by infiltrating the monocytes and synovial cells.<sup>56</sup> Proliferation of the synovial cells results in synovium thickening and underlying cartilage and bone degradation.<sup>55</sup> CD40 expressing on the smooth muscle fibroblasts from normal and RA patients<sup>57</sup> and RA synovial cells<sup>2</sup> can be up-regulated by pro-inflammatory cytokines including IFN- $\gamma$  and TNF- $\alpha$  and in these cells CD40 signals lead to fibroblast proliferation, up-regulation of an adhesion molecule<sup>57</sup>, and secretion of pro-inflammatory cytokines and chemokines such as IL-6, GM-CSF, and MIP-1 $\alpha$ .<sup>57</sup> If fibroblast-like synovial cells are cultivated with activated T cells from RA patients, they secrete the elevated levels of IL-15, TNF- $\alpha$ , and IL-17, as well as IL-8 and MCP-1 in a CD40-dependent manner.<sup>57-59</sup>

When CD40-activated monocytes are cultured with fibroblast-like synovial cells, the same results are observed<sup>2</sup> and these results show that CD40 signaling on both monocytes and synovial fibroblasts arrange a complex network of pro-inflammatory cytokine and chemokine secretion which leads to joint destruction.<sup>55-56</sup> It was seen that in fibroblast-like synovial cells, CD40 signaling causes RANKL expression which induces osteoclast-mediated bone resorption.<sup>60</sup>

In the bone marrow and synovium of RA patients, it is thought that a nurse-cell like population supporting B cell survival will up-regulate CD40 in response to IFN- $\gamma$  treatment, but the functional consequences of CD40 signaling in these cells is unknown.<sup>2</sup> In response to CD40 ligation, the adherent fraction of the synovial tissue cells, including macrophages and DCs, secrete TNF- $\alpha$ .<sup>61</sup> In ex vivo cultures DC-derived TNF- $\alpha$  directly leads to collagen destruction.<sup>62</sup>

Some of the RA patients who are most prone to severe disease have detectable anti-citrulline antibody containing peptides (anti-CCP Abs).<sup>63</sup> For inducing anti-CCP Ab secretion by B cells from either healthy controls or RA patients, CD40 signals are needed but ex vivo they are only secreted by B cells from anti-CCP seropositive patients and thus it is concluded that these cells have already received CD40 signals within the synovial compartment.<sup>63</sup> In comparison with healthy controls, on the circulating and synovial T cells of the RA patients CD154 expression is up-regulated faster and with a higher degree, inducing more Ig production by B cells, required for IL-12 production by the synovial DC and macrophages.<sup>61</sup> On T cells, CD154 over expression is attributed to higher disease activity and fewer remissions.<sup>64</sup>

#### **CD40 and Allograft Rejection**

It has been demonstrated that CD40 blockade by means of anti-CD154 will inhibit acute rejection and assist long-term allograft acceptance in several murine transplant models.<sup>65-67</sup> Administration of anti-CD154 regimen followed by 5 monthly doses allowed rhesus monkeys to extend the renal allografts survival, including survival for more than one year after therapy was stopped.<sup>68,69</sup> In an islet-cell transplant model, similar data were obtained.<sup>70,71</sup> But it should be noted that the above therapy induced prolonged immunosuppression rather than true tolerance. Besides, in both cases, animals withdrawn from therapy ultimately rejected their grafts. In many murine studies,

a similar conclusion was accomplished where a short-course of anti-CD154 alone was suboptimal in promoting the permanent engraftment in both islet and cardiac transplant models<sup>65, 67</sup> or contributed to the development of chronic rejection in the cardiac allografts.<sup>72</sup> CD154-/- mice do not reject cardiac allografts but are subjected to developing chronic allograft vasculopathy.<sup>73</sup> In this model, CD40-blockade-resistant CD8 cells might be the cause of development of chronic vasculopathy.<sup>73</sup>

#### **Different Mechanisms of CD40 Down Regulation**

In vitro tolerogenic dendritic cell generation may be practical with DCs manipulation by means of the following three methods:<sup>74</sup>

- 1- Physiological mediators
- 2- Pharmacological mediators
- 3- Genetic engineering

The third method is another approach to generate tolerogenic DCs through genetic engineering. Several genes, including co-stimulatory molecules and cytokines, can be transferred through viral and nonviral delivery systems, and the expression of an immunosuppressive molecule may induce DC tolerogenicity. Thus, genetic manipulation can be experienced by three systems including viral vectors, anti-sense and siRNA.<sup>75-77</sup> For example, the interaction of CD80 and CD86 on DCs with CD28 on T cells will be blocked by the suppressive recombinant molecule CTLA4-Ig. An adenoviral vector harboring the CTLA4-Ig gene transducing murine DCs showed reduced cell surface staining for CD86 but not for MHC class II and these cells induced alloantigen-specific T-cell hyporesponsiveness.<sup>78</sup>

As described above, there are numerous methods that can be applied to knock down a gene product. The choice of technique is based on the specific aim; experiment duration, stability and localization of the protein, cell accessibility, or species of the organism all have an effect. To achieve this purpose, antisense oligonucleotides are worthwhile. They can be applied for therapeutic purposes, functional genomics and target validation.<sup>79</sup> In 1978, Zamecnik and Stephenson could comprehend the oligonucleotide ability to inhibit viral replication in cell culture.<sup>79</sup> After that, antisense technology was developed as an efficient therapeutic device.<sup>80</sup>

A novel mechanism to target for gene targeting is RNA interference (RNAi). Compared to antisense

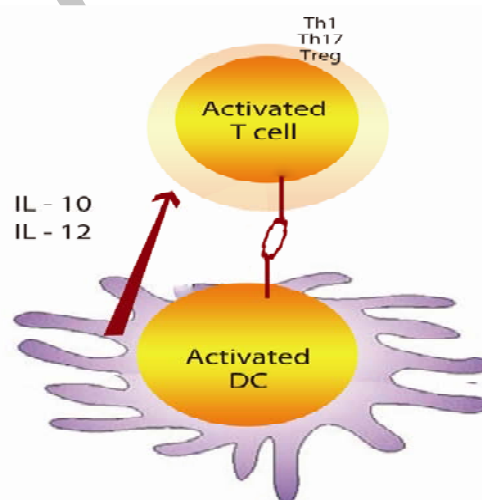
oligonucleotides, it is more efficient to suppress a specific sequences of a target gene and also easier to use in vitro. That is why RNAi is a suitable technique for high-throughput analyses and functional studies in vitro, including mammalian cells.<sup>81-84</sup> Now, siRNA is a widespread molecular therapeutic device with the potential to prevent gene expression in various diseases.<sup>83-85</sup> In this way, CD40 is one of the candidate molecules for generating TDCs and is inducible upon DC maturation.<sup>86</sup> During the DC-T cell interaction, CD40 is a key “switch” molecule among the co-stimulatory molecules.<sup>87</sup> Hence, to induce TDCs, CD40 gene silencing is achieved by antisense oligonucleotides (ASO)<sup>76</sup> or small interfering RNA (siRNA).<sup>77</sup> An ASO is a single-stranded deoxyribonucleotide (typically 20 bp in length) that is complementary to the target mRNA. ASO hybridization to the target mRNA can cause specific inhibition of gene expression by various mechanisms, depending on the chemical make-up of the ASO and location of hybridization, leading to decreased levels of the target transcript translation.<sup>88</sup> Knocking down the antisense oligonucleotide-induced protein is usually achieved by induction of RNase H endonuclease activity that cleaves the RNA– DNA heteroduplex resulting in the target mRNA degradation where as leaving the ASO intact.<sup>89</sup> On the other hand, RNA interference (RNAi) is a cellular defense mechanism against viral double stranded RNA where the host cell selectively inactivates endogenous mRNA transcripts that are homologous to exogenous double-stranded RNA (dsRNA). RNAi contributes to ribonuclease III enzyme activation which cleaves the duplex into smaller, 21-23 base-pairs termed siRNA, capable to block gene expression in mammalian cells without triggering the nonspecific panic response.<sup>82</sup> Thus, DNA and siRNA delivery to mammalian cells is an efficient method to treat different diseases progress by single gene defects.

Modulation of transcription usually affects gene expression, while new molecules acting on translation inhibit the expression of a single target gene. With regard to the fact that each mRNA molecule can generate multiple copies of a protein, it is better to target the mRNA rather than the protein to block the protein function. The main problem of mRNA strategies would be the effectiveness, e.g. efficient delivery, enhanced stability, minimization or elimination of sequence- and substance-dependent side

effects, and sensitive sites identification in target mRNAs.<sup>90</sup> Thus, exploring a new technique to induce immune tolerance is of great impact and can result in donor specific tolerance in the transplantation recipient. Although many studies demonstrate a limited capacity of DCs to be transfected with DNA, RNA transfection has been indicated to be a suitable alternation.<sup>91</sup>

In the previous study, we have shown that in the efficiency of DCs transfection, no significant difference was identified by Lipofectamine2000 and siRNA or antisense oligodeoxynucleotide.<sup>92</sup> Furthermore, it has been concluded that siRNA has been more effective in down regulation of CD40 in protein and mRNA level. The differences between this agent in decreasing IFN- $\gamma$  and increasing IL-4 in ELISA test were not significant, although siRNA had more effect on increasing IL-4 and on decreasing IFN- $\gamma$ .<sup>92</sup>

Of course, with ELISPOT in both cases (IFN- $\gamma$  and IL-4) displayed another result. siRNA showed a greater influence and the difference was significant ( $P < 0.001$ ).



**Figure 3. Model of CD40–CD40L/CD154 crosstalk interactions between T cells and DCs. Engagement of CD40 on DCs induces positive signaling that leads to the production of IL-12 that skews towards Th1 differentiation in CD4+ T cells. In addition to IL-12, CD154 signaling in T cells induces IFN- $\gamma$  production. CD40 signaling on DCs induces secretion of IL-12 which promotes Th1 differentiation, IL-10 which induces Tregs, or other cytokines that induce Th17 differentiation.**



In the MLR test, siRNA showed more effect and the differences between the stimulatory index of siRNA and antisense were significant ( $P < 0.045$ ). With regard to comparison of unmanipulated DCs, in CD40 siRNA and CD40, antisense treated DCs the production of IL-12p70 hetero dimer, were reduced by 75% and 60%, respectively. In our trial knock down with siRNA showed more influence and the differences between siRNA and the antisense effects were significant ( $P < 0.04$ ). This differences between siRNA and antisense also showed on the unmanipulated DCs ( $P < 0.001$ ).<sup>93,94</sup>

In 2003, Xu et al. reported that compared to antisense, a lower concentration of siRNA is necessary. Also, they concluded that antisense DNAs and siRNAs have different preferences for mRNA target sites.<sup>95</sup> Kurreck and his coworkers in 2003 compared the siRNA and antisense efficiency in knocking down VR1 and demonstrated the higher efficiency of siRNA in this process.<sup>96</sup> Besides, Hiroi et al. showed that in the suppression of target mRNA, siRNA is more effective at lower doses and more efficient than antisense.<sup>97</sup> Moreover, Miyagishi and his coworkers compared siRNA with antisense and ribozyme.<sup>98</sup> They reported that siRNA is an effective and highly efficient tool in mRNA suppression at lower doses as well.<sup>99</sup> However, other studies done by two different groups found the opposite results. They also demonstrated that siRNA-mediated gene silencing is highly specific and the gene expression pattern depends on the gene being targeted.<sup>99-100</sup> The superiority of siRNA to antisense seems to be a controversial question. For example, Bertrand et al. proved that in comparison with antisense oligonucleotide stabilized by two PS linkages at both ends, siRNA is more efficient in GFP suppression in cell culture and in vivo.<sup>101</sup> However, another study demonstrated that, the efficiency of antisense is related to the activity of RNase H-dependent 2-O-methoxyethyl/PS ONs targeted to the same sites<sup>102</sup>, while in these experiments, the potency, maximal effect, specificity, duration of action, and efficiency of both types of antisense agents were similar.

It should be noted that designs of some of the siRNAs used in the studies were not optimal. That is why further studies are needed to determine a more efficient method of RNAi or conventional antisense approaches. Although several molecular methods including antisense oligonucleotides and monoclonal antibodies have been developed for in vitro gene

suppression, RNAi appears to be more useful. As a matter of fact, from different points of view RNAi is an efficient and specific method not only because a few copies of siRNAs are enough to activate the RNA-induced silencing complex, but also because they can cleave the sequences with identity to one of dsRNA strands. The same results were achieved by our previous experiment demonstrated that for immune modulation of DCs, RNAi can successfully target the expression of Th1- polarizing CD40; hence, it is followed by antisense<sup>92</sup>.

The first RNAi clinical applications was aimed to treat the age-related macular degeneration (AMD), leading to blindness or limited vision in millions of adults annually.<sup>103</sup> At the present time, RNAi based therapies are also being advanced for viral infection, including human immunodeficiency virus (HIV), hepatitis B and C viruses (HBV and HCV), and respiratory syncytial virus (RSV). The directions of neurodegenerative diseases treatment and cancers are also well under way. Antivirus siRNA-based therapy for RSV is in a phase I clinical trial and AMD in phase II. Also, it is important to note that Vitravene (Fomivirsen), the first treatment for retinitis due to CMV is based on antisense is approved by FDA.<sup>104</sup>

It is hoped that these investigations can open a new horizon in modern medicine and also set the ground in safe systemic RNA therapies to enhance the therapeutical process of dimensional types of human diseases in the coming years.

### Concluding Remarks

For the first time, CD40 was detected as a co-stimulatory molecule expressed on APCs, having impressive effect on activation of T and B cells. It is noteworthy that this molecular pair regulates APC and effectors lymphocytes. (Figure 3) According to the growing numbers of different DC and T cell subsets, we also realize that CD40-CD154 interactions have an impressive effect on regulation of the above mentioned subsets.

As we continue to understand how CD40 causes the generation of the tolerogenic DCs and regulates T- cell response and the role of CD40 in generation of Treg, we hope to induce tolerance through the inhibition of IL-12 production and knock down of co-stimulatory molecules. Finally, we can also elucidate the molecular mechanism of co-stimulatory molecule precisely; therefore a new horizon in application of these



mechanisms and efficiency of these methods is gained so that the best method in generation of tolerogenic DC will be selected. Therefore the influence of CD40 and CD154 on cell interactions should be further clarified. Moreover, insights into the functions of CD40-CD154 interactions and knockdown mechanisms will hopefully advance our understanding of the immune cell crosstalk and the complexity of their interdependent regulatory interactions.

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