Silencing CD40 in Dendritic Cells by siRNA

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Abstract

We have previously demonstrated that short interfering RNA (siRNA) is an effective method of gene-specific immune modulation in dendritic cells (DC) (*J Immunol 171:691*). In this study we investigated the feasibility of generating tolerogenic DC through silencing of CD40, one of the most important costimulatory molecules. Screening of effective siRNA specific to murine CD40 sequence was performed using the novel strategy of siRNA expression cassettes (SEC). Amongst the 4 designed SECs targeting the CD40 sequence (CD40-SEC), two SEC completely and partially suppressed CD40 expression on mature DCs. Functionally, CD40-SEC inhibited allostimulatory activity of DC in mixed lymphocyte culture (MLR). These data suggest that CD40-SEC is a useful tool for generating tolerogenic DC.

Keywords: Dendritic cells, Gene silencing, RNA interference, siRNA, CD40

Abbreviations: DC, dendritic cells; RNAi, RNA interference; siRNA, small interfering RNA, SEC, siRNA-expression cassette.

Introduction

Dendritic cells (DC) are most potent antigen presenting cells. Control of immunity by DC is based on the ability of this cell, once mature, to provide 3 signals to T cells during activation: Signal 1, the antigenic

signal that is communicated via the high expression of MHC molecules found on DC [1]; Signal 2, the "costimulatory" signal, comprising membrane-bound molecules such as CD40, CD80/86 and OX-40L [2], which are essential for T cell expansion and escape from anergy; and Signal 3, soluble cytokines that induce T cell differentiation into a Th1 phenotype (IL-12) or Th2 phenotype (IL-10) [3]. In the absence of costimulatory molecules provided by DC, the T cell can undergo apoptosis, anergy, Th2 differentiation, or differentiate into a T regulatory cell (Treg) [4].

CD40 is an integral membrane protein belonging to the tumor necrosis factor receptor superfamily that is inducible upon DC maturation [5]. On the other hand, CD40 acts bidirectionally, by also activating T cells subsequent to ligation of its ligand, CD40L (CD154) [6]. CD40L crosslinking on T cells has been demonstrated to increase IFN-γ production and proliferation [7]. In addition CD40 crosslinking on DC stimulates maturation and IL-12 production [8]. To date, blockade of the CD40-CD40L interaction is being very aggressively pursued as a tolerance-inducing strategy [9]. Among the presently known costimulatory molecules, such as CD80, CD86 and OX-40L, CD40 appears to be a key "switch" during the DC-T cell interaction. Indeed, blockade of this switch not only inhibits T cell response [10] but actively generates Treg cells [11]. Unfortunately, blockade of this interaction by administration of anti-CD40L antibody is not clinically feasible due to thrombotic complications [12].

RNA interference (RNAi) is a cellular defence mechanisms against viral double stranded RNA in which the host cell selectively inactivates endogenous mRNA transcripts that are homologous to exogenous double-stranded RNA (dsRNA). The initial suggestion that dsRNA may possess such a gene silencing effect was reported in 1996 in work with Petunias [13]. Experiments in *C. elegans* demonstrated that inhibition of gene expression by dsRNA is so potent that approximately 1-3 molecules of duplexed RNA per cell are effective at knocking down gene expression [14]. Mechanistically, RNAi is associated with activation of a ribonuclease III enzyme that cleaves the duplex into smaller, 21-23 base-pairs termed small interfering RNA (siRNA), which are capable of blocking gene expression in mammalian cells without triggering the nonspecific panic response [15]. siRNA has been used extensively in blocking various genes and is presently being evaluated as a therapeutic for cancer and viral disease [16, 17]. We are the first to demonstrate that short interfering RNA (siRNA) can be an effective method of gene-specifically inducing immune modulation in DC [18], suggesting another potential application of siRNA for transplant tolerance. In this paper we report the first successful silencing of the CD40 gene on DC.

Materials and Methods

Construction of CD40-SEC and DC transfection

SECs were generated using the Silencer Express Kit (Ambion Inc, Austin TX). Sense and anti-sense hairpin siRNA template oligonucleotides for the precursor SEC were designed according to user's instruction. The oligonucleiotides contain 19-mer hairpin sequences specific to the mRNA target, a loop separating the two complementary domains, two 3'-end overhang nucleotide. 3 PCR reactions were performed to generate SEC using a promoter element (mouse U6) as template, a promoter PCR primer, and gene specific sense and anti-sense oligonucleotides. The first reaction forms half of the hairpin siRNA (sense oligonucleotides) using following primers:
CD40-SEC1, 5'-
ACACTACACAAATGTTCCACTGGGCTGAGAACCGGTGTTTCGTCCTTTCCACAAG-3'; CD40-SEC2, 5'-
CCTCTACACAAAAGGTACAGACAGTGTCTGACCGGTGTTTCGTCCTTTCCACAAG-3'; CD40-SEC3, 5'-
AAACTACACAAATTTCTGTAGGACCTCCAAGCCGGTGTTTCGTCCTTTCCACAAG-3'; CD40-SEC4, 5'-
GTGCTACACAAACACTGAGATGCGACTCTCTCGGTGTTTCGTCCTTTCCACAAG-3'. The primers used for second PCR were: CD40-SEC1, 5'-
CGGCGAAGCTTTTTCCAAAAAATTCTCAGCCCAGTGGAACACTACACAAATGTT-3'; CD40-SEC2, 5'-
CGGCGAAGCTTTTTCCAAAAAATCAGACACTGTCTGTACCTCTACACAAAAGGT-3'; CD40-SEC3, 5'-
CGGCGAAGCTTTTTCCAAAAAACTTGGAGGTCCTACAGAAACTACACAAATTTG-3'; CD40-SEC4, 5'-
CGGCGAAGCTTTTTCCAAAAAAAGAGAGTCGCATCTCAGTGCTACACAAACACT-3'.

DC culture and gene silencing

DC were generated from bone marrow progenitor cells as previously described [18]. Briefly, bone marrow cells were flushed from the femurs and tibias of B6 mice (Jackson Lab, Bar Harbor ME), washed and cultured in 12 well plates ($2*10^6$ cell per plate) in 2 ml of complete medium (PRMI-1640 supplemented with 2mM L-glutamine, 100U/ml of penicillin, 100 ug of streptomycine, 50 uM 2-mercatoethanol, and 10% fetal calf serum, recombinant GM-CSF(10ng/ml, Peprotech,Rocky Hill,NJ) and recombinant mouse IL-4 (10ng/ml, Peprotech). All cultures were incubated at 37C° in 5% humidified CO_2.

Gene silencing was performed by transfecting CD40-SEC. After 6-7 days culture, DC were seeded at $1*10^6$cells in 24 well plates and transfected using liposome method in 24-well plates. In brief, a transfection mixture

containing 300ng CD40-SEC and 5 µl GenePorter in 0.5 ml serum-reduced PRMI-1640 was added to each well. Cells were treated in the transfection mixture for 5 hr followed by addition of 20% serum medium. Subsequently cells were incubated overnight and stimulated with LPS (10ng/ml) and TNF-α (10ng/ml).

Flow cytometry

The efficacy of gene silencing was assessed at the protein level. Forty-eight hrs after CD40-SEC transfection, DC were harvested and stained with FITC-conjugated anti-CD40 mAb(BD PharMingen, San Diego, CA) as described elsewhere [18]. Expression of CD40 was measured on a FACS Calibur flowcytometer (Perkin Elmer) and data were analyzed using CellQuest and WinDMI software.

Mixed leukocyte reaction

One way mixed leukocyte reaction (MLR) cultures were performed in triplicate in 96-well, round bottom microculture plates. T cells were purified from BALB/c splenocytes using nylon wool columns and used as responders ($1*10^5$/well). siRNA treated DC($5\text{-}50*10^3$/well from C57BL/6 mice) were used as stimulators. Seventy two-hour MLR was performed and the cells were pulsed with 1 µCi [^{3}H]thymidine for the final 18 hours. The cultures were subsequently harvested onto glass fibre filters (Wallac, Turku, Finland). Radioactivity was counted using a Wallac Microbeta liquid scintillation counter and the data were analyzed with Prism software.

Results and Discussion

Preparation of CD40-SEC

An important consideration in the design of siRNA is choosing the appropriate sequence of the duplex that would most effectively silence the mRNA transcript whose inhibition is desired. It is known that efficacy of silencing varies with segments of the transcript that are targeted [19]. At present no clear-cut rules exist for choosing the best segment to silence, however several guidelines exist: it is suggested that the target region should be at a least 70 to 100 nucleotides away from the translational initiation site of the transcript and that the AU:GC content should be as close to 50% as possible [20]. In addition, the siRNA should target coding sequences since the process of RNAi occurs only in the cytoplasm [21]. Using these guidelines we have generated four siRNAs along the CD40 gene. The target sites chosen were as follows: CD40-SEC(1):5'-AATTCTCAGCCCAGTGGAACA-3'; CD40-SEC(2): 5'-AATCAGACACTGTCTGTACCT-3'; CD40-SEC(3):5'-

AACTTGGAGGTCCTACAGAAA-3'; CD40-SEC(4): 5'-AAAGAGAGTCGCATCTCAGTG-3'.

In order to generate siRNA in a quick and rapid manner, we used SEC. Basically, SEC is a PCR product that contains a Pol III promoter and a DNA sequence that, once transcribed, forms hairpin siRNA [22]. Three PCR reactions were performed to generate SEC with the first reaction to form half of the hairpin siRNA (sense). The products of first PCR were used as templates for the second PCR. The second reaction generates the precursor SEC (second half of the siRNA hairpin); the last reaction adds the terminator and restriction sites for cloning to the SEC (Figure 1B). The products of SEC showed clear-cut bands that were equivalent to the designed molecular size (Figure 2).

Since the SEC can be designed with restriction sites, it is possible to clone effective SEC sequences into expression plasmids in order to raise large quantities of SEC. While the SEC technique does not allow permanent transfection of cells with siRNA, the expediency and low cost of this procedure lends itself to mass screening of siRNA libraries as well as identification of siRNA target sites. Therefore SEC is a rapid method to

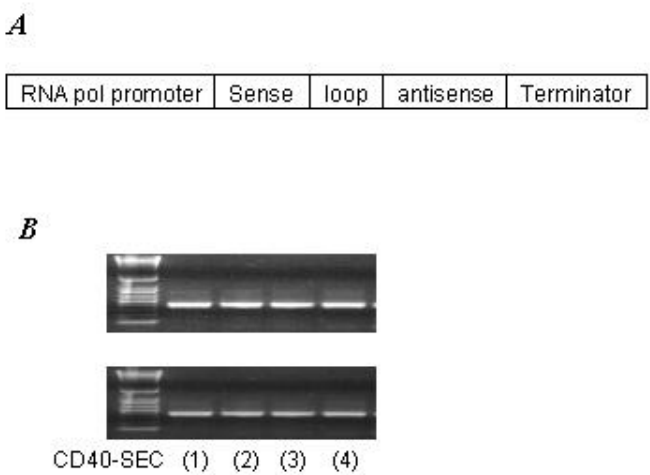

Figure 1. Figure 3. siRNA expression cassettes (SEC) construct
A: *Schematic depiction of SEC. SEC are generated as PCR products consisting of a hairpin siRNA template flanked by promoter and terminator sequences. Once the SEC is transfected into cells, the hairpin siRNA is expressed from the PCR product and leads to gene silencing.* ***B:*** *Generation of 4 SEC targeting CD40. 3 PCR reactions were performed to generate SEC. The first reaction formed half of the hairpin siRNA (sense), the second reaction generated the precursor SEC (second half of the siRNA hairpin) (upper panel), the last reaction added the terminator and restriction sites for cloning to the SEC (lower panel).*

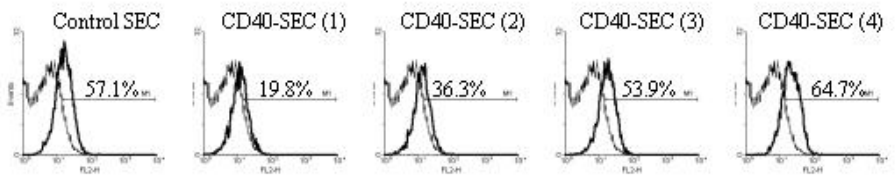

Figure 2. Screen target sites in CD40 with SEC. *CD40-SEC(four different target sites) were added into DC culture at 150ng/ml at day 6 followed by transfection of 300ng SEC at day 7. DC were activated with LPS with a period of 20 hours. Expression of CD40 was assessed by flow cytometry after staining with FITC-labeled anti-mouse CD40 mAb.*

screen effective siRNA sequences. The most effective siRNA (e.g., CD40-SEC(1)) could be further cloned into an expression vector for in vivo and in vitro experiments (data not shown).

Suppression of CD40 on DC by CD40-SEC

To assess the efficacy of gene silencing by SEC, purified SECs specific to CD40 were transfected into 7-day cultured DC following activation by LPS and TNF-α. As seen in Figure 2, mature DC demonstrated high expression of CD40 that was not affected by treatment of non-specific control SEC. The silencing effects of the 4 CD40-SECs used possessed significant variation. At the protein level, CD40-SEC(1) inhibited >70% expression of CD40, while CD40-SEC(2) only induced marginal inhibition. CD40-SEC(3) and CD40-SEC(4) had no silencing effects (Figure 2). The silencing effects of CD40-SECs were also detected at the mRNA level by RT-PCR and RNA protection assay, respectively (data not shown). These data are the first indication that manipulation of CD40 expression can be induced in DC through siRNA. The observation that the reduction in CD40 levels are not "all-or-none" after siRNA-mediated silencing is important. This suggests that through the careful selection of siRNA sequences can be used to achieve a "graded" biological response, something not commonly done with antibodies or other blocking reagents.

Inhibition of T cell response by CD40-silenced DC

Due to the dependency of T cell activation of CD40 expression by DC, several investigators have suppressed the CD40-CD40L interaction to achieve a variety of immune-inhibitory effects. For example, murine studies demonstrated antibodies to CD40 result in: 1) Abrogation of T cell responses [9]; 2) Inhibition of cytotoxic T cell generation [23]; 3) Suppression of inflammatory cytokine production by T cells [24]; and 4) Generation of Treg [25]. However, most work on CD40-CD40L block-

ade has been performed by using anti-CD40L mAb to block CD40L on the T cell [9] Unfortunately this approach is not clinically feasible due to thrombotic complications arising from the non-specific activation of platelets by anti-CD40L antibodies [12]. Since conventional anti-CD40 mAbs are agonists that stimulate DC maturation, the blockade of CD40 on DC is yet to be developed. While a large body of studies have been performed blocking CD40L, little work has been carried out investigating the role of blocking CD40 on DC.

In order to assess the inhibitory function of CD40-silenced DC, an allogeneic MLR was performed. CD40-SEC(1) and CD40-SEC(2) induced inhibition of allogeneic T cell response in comparison to the non-specific control SEC (Figure 3). Interestingly, although the suppression of CD40 was not detected (Figure 2) by flow cytometry in DC that was treated with CD40-SEC(3) and CD40-SEC(4), DC treated with CD40-SEC(3) caused partial inhibition of T cell responses in MLR (Figure 3). The discrepancy between level of protein expression and allostimulatory activity may be the result of pre-stored pools of CD40, a phenomenon previously described in DC [26].

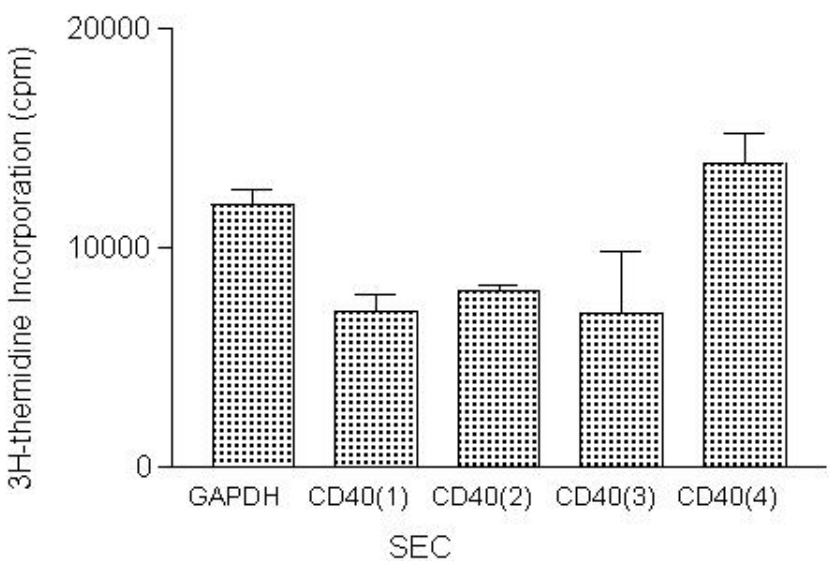

Figure 3. CD40-SEC transfected DCs inhibit T cell activation*. C57BL/6-derived DC (1*106) were transfected with control SEC or CD40-SEC (500ng/ml) for 24-48 hours. Allogenic (BALB/c) T cells(1*105/well) were incubated with SEC-treated DC at the ratio of 1:10 for 72 hours. Proliferation was determined using [3H] thymidine incorporation. Data are representation of three independent experiments*

Conclusion

Immune modulation through targeting the CD40-CD40L interaction has been a long-sought goal. Unfortunately the fact that antibodies to CD40 induce thrombosis has limited the applicability of this approach. In this paper we are the first to demonstrate that the technique of siRNA can be used on DC for manipulating levels of CD40 expression, as well as decreasing the functional ability of DC to stimulate allogeneic T cells. This methods described in this report of silencing CD40 on DC could be applied for the generation of tolerance-promoting DC for use in autoimmunity and transplantation.

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