ADMINISTRATION OF DENDRITIC CELLS MODIFIED BY RNA INTERFERENCE PROLONGS CARDIAC ALLOGRAFT SURVIVAL

JIANBIN XIANG, M.D.,[1] XIAODONG GU, M.D.,[1] YIMING ZHOU, M.D.,[1] XUN GONG, M.D.,[1] SHIGUANG QIAN, M.D.,[2] and ZONGYOU CHEN, M.D.[1]*

Systemic administration of immature donor-dendritic cells (DC) that are deficient in co-stimulatory molecules delays the onset of allograft rejection. However, it is not easy to control culture condition and guarantee that the administered DC are in the immature stages, which obviously affects their therapeutic effect. In this study, we attempted to inhibit expression of CD86 on DC using an RNA interference technology. The function of $CD86^{low}$ DC was determined by the influence on their capacity to stimulate T cell proliferation and by the effect of DC systemic administration on survival of cardiac allografts. $CD86^{low}$ DC stimulated low T cell proliferative responses in vitro and administration of $CD86^{low}$ DC prolonged survival of heart allografts in vivo. These results suggest that RNA interference is a useful approach to modify DC function, which has potentials for clinical application. © 2007 Wiley-Liss, Inc. Microsurgery 27:320–323, 2007.

Initiation of a T cell-mediated immune response requires antigen-presenting cells (APC) that present foreign peptides in association with MHC molecules to the T cell receptor (TCR) on T cells. Co-stimulatory signals are also required for T cell activation.[1] The engagement of antigen/MHC with TCR in the absence of co-stimulatory signals triggers T-cell apoptosis or induces T regulatory cells.[2] CD86 on activated APC binds to CD28 on T cells. Selective CD86 blockade obstructs the CD28-mediated co-stimulatory signal, resulting in inhibition of in vitro and in vivo immune responses. CD86 blockade by anti-CD86 mAb abrogated either acute or chronic graft-vs-host disease by preventing the activation of donor $CD4^+$ T cells.[3] Administering anti-CD86 mAb alone at right time also could induce long-term graft survival.[4]

Dendritic cells (DC) are the most potent APC. They rapidly up-regulate their surface expression of MHC and co-stimulatory molecules under stimulation, and transform from "tolerogenic" to "immunogenic."[5] Therefore, we applied RNA interference (RNAi), which is a double-strand RNA (dsRNA)-mediated gene silencing process against specific gene expression observed in many eukaryotic cells. Small interfering RNA (siRNA) provides a powerful way to regulate specific gene expression in mammalian cells.[6] We have ascertained siRNA sequences that can specifically and effectively knock down CD86 gene expression in previous studies.[7]

In this study, we use the siRNA strategy for blocking CD86 expression in DC and to examine the impact on the function of $CD86^{low}$ DC in mixed lymphocyte reaction and heterotopic heart transplantation.

MATERIALS AND METHODS

Animals

C3H ($H2^k$) and C57BL/6 (B6, $H2^b$) mice, 7–9 weeks old, were obtained from Shanghai Laboratory Animal Center of Chinese Academy of Sciences (Shanghai, China) and maintained in a specific pathogen-free facility at Fudan University (Shanghai, China).

Propagation of Bone Marrow-derived DC

DC were generated from bone marrow progenitor cells as previously described,[8] with some modifications. Briefly, bone marrow cells harvested from femurs and tibias of C3H mice were cultured in 24-well plates (1×10^6/well) in 1 ml RPMI 1640 (Gibco, Gaithersburg, MD) supplemented with 10% v/v FCS (referred to subsequently as complete medium), and 10 ng/ml recombinant GM-CSF (R&D Systems, Minneapolis, MN). All cultures were incubated at 37 °C in 5% humidified CO_2. Non-adherent granulocytes were removed after 48 h of culture and a fresh complete medium supplemented with GM-CSF was added. Medium was exchanged by half every 48 h. After 6 days of culture, 1 μg/ml LPS (Sigma, St. Louis, MO) was added to the culture for 18 h to allow for maturation.

siRNA Design and Synthesis

The siRNA sequences used for targeting CD86 (Genbank access number: NM_019388) were designed and selected according to the method of Elbashir et al.[9] The CD86 dsRNA consisted of the sense strand 5′CAGAGAAACUUGAUAGUGUdTdT3′ and the antisense strand 5′ACACUAUCAAGUUUCUCUGdTdT3′.

[1]Department of General Surgery, Huashan Hospital, Fudan University, Shanghai, China
[2]Department of Immunology and General Surgery, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, OH
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*Correspondence to: Zongyou Chen, M.D., Department of General Surgery, Huashan Hospital, Fudan University, 12 Wulumuqi Road, Shanghai 200040, China. E-mail: zongyouc@hotmail.com
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The sequence of the siRNA has been shown to be the most effective in the preliminary experiments.[7] The scrambled control dsRNA consisted of the sense strand 5′UUCUCCGAACGUGUCACGUdTdT3′ and the antisense strand 5′ACGUGACACGUUCGGAGAAdTdT3′. All siRNA were synthesized and annealed by Shanghai GeneChem Limited Company (Shanghai, China).

siRNA Transfection

Transfection was conducted when DC were 30–50% confluent according to the manufacturer's protocol. Twelve microliter of 20 μM siRNA was mixed in 50 μl of Opti-MEN (Gibco), while 3 μl of LipofectAMINE 2000 (Invitrogen, Garlsbad, CA) was incubated in 50 μl of Opti-MEN at room temperature for 5 min. Then the diluted siRNA and LipofectAMINE 2000 were incubated for a further 20 min at room temperature for complex formation. Five hundred microliter medium was sucked from each well and the complexes were added. The final siRNA concentration was 400 nmol/L.

Flow Cytometry

Phenotypic analysis of siRNA-treated DC was performed on a FACScan (BD Biosciences, San Jose, CA) and analyzed using CellQuest software (BD Biosciences). The FITC-conjugated anti-CD80 and anti-CD86 mouse monoclonal antibodies (R&D Systems) were used. Appropriate species-matched irrelevant isotype antibody was used as control.

Mixed Lymphocyte Reacton (MLR)

One-way MLR was performed using γ-irradiated (20 Gy) DC derived from C3H bone marrow as stimulators and nylon wool-purified B6 splenic T cells (2×10^5) as responders. Cultures were established in triplicate in 96-well, round-bottom microculture plates (200 μl/well) and maintained in complete medium for 3 days at 37 °C in 5% humidified CO_2. [^{3}H]-TdR (0.5 μCi/well)was added for the final 18 h of culture. Cells were harvested onto glass fiber disks using an automated system, and incorporation of [^{3}H]-TdR into DNA was assessed by Wallac 1450 liquid scintillation counter (PerkinElmer, Boston, MA). Results were expressed as mean cpm ± SD.

Heterotopic Heart Transplantation

Cervical vascularized heart transplantation was performed from C3H donors to size-matched B6 recipients using a cuff technique. The operative time was about 60 min, and the cold ischemic time for donor heart was less than 30 min. Graft survival was assessed by daily palpation. The operation was successful when grafts continued to beat for more than 3 days. Rejection was defined as total cessation of cardiac contraction. To assess the effect of donor-derived DC on allograft survival, the recipients received 2×10^6 DC (C3H) i.v. 7 days before heart transplantation. No immunosuppressive therapy was used during the entire experiments.

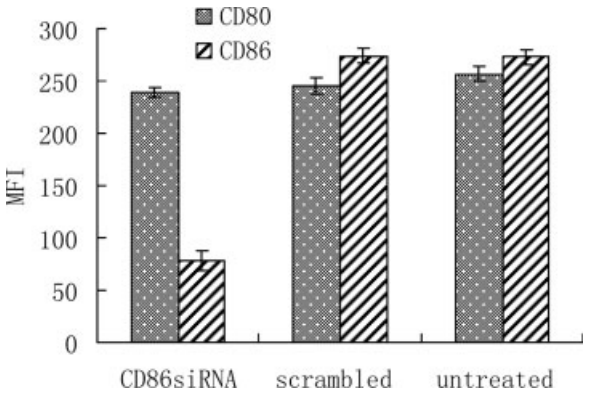

Figure 1. Expressin of CD80 and CD86 after transfection with relevant siRNA.

Statistical Analysis

Statistical analyses were performed with Stata 8.0 software (Stata, College Station, TX). The data were given as mean ± SD. Statistical comparisons between groups were preformed using a one-way ANOVA followed by a Scheff's test, as appropriate. Graft survival between groups was compared using the log-rank test for comparison of survival curves. Differences among groups were considered significant when $P < 0.05$.

RESULTS

Inhibition of CD86 Expression on DC by siRNA

As shown in Figure 1, transfection with anti-CD86 siRNA markedly inhibited expression of CD86 on DC ($P < 0.05$ compared with scrambled siRNA or no-treated DC), but did not affect CD80 expression ($P > 0.05$ compared with scrambled siRNA or no-treated DC), indicating the effectiveness and specificity of the anti-CD86 siRNA used in this study.

CD86^{low} DC Stimulate Low Proliferative Responses in Allogeneic T Cells

To examine the effect of anti-CD86 siRNA on DC allostimulatory activity, irradiated DC derived from C3H bone marrow that had been transfected with anti-CD86 siRNA were cultured with B6 splenic T cells at various ratios in a 3-day MLR. As shown in Figure 2, in the "No treatment" group, DC stimulated profound proliferative responses in allogeneic T cells, whereas DC transfected with anti-CD86 siRNA induced significantly less allogeneic T cell proliferation ($P < 0.05$). DC transfected with scrambled control siRNA retained high allostimulatory

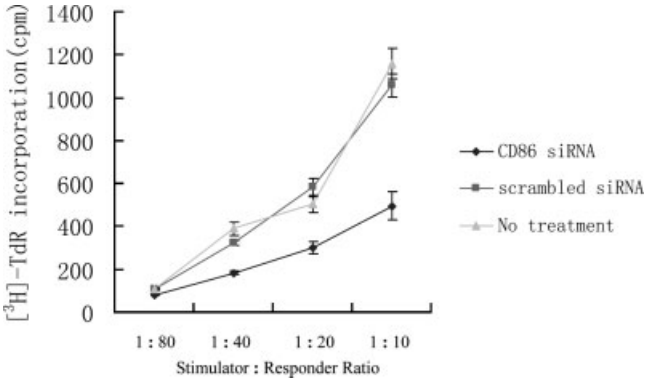

Figure 2. Role of CD86^{low} DCs in initiation of allogeneic T cells immune response.

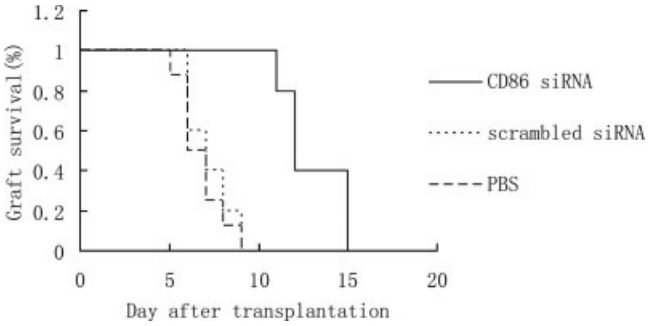

Figure 3. Role of CD86^{low} DCs on cardiac allograft survival.

activity ($P > 0.05$ compared with "No treatment" group), indicating the specificity of anti-CD86 siRNA.

Effect of CD86^{low} DC on Survival of Cardiac Allografts

To determine the influence of anti-CD86 siRNA on DC stimulatory activity in vivo, 2×10^6 DC from C3H that were transfected with anti-CD86 siRNA were intravenously injected into B6 recipients 7 days before receiving a C3H heart allograft in the absence of immunosuppressive therapy. The recipients treated with DC transfected with scrambled control siRNA or treated with PBS as controls. Survival of cardiac allografts was significantly prolonged in the anti-CD86 siRNA treated group ($n = 5$, $P < 0.05$ compared with scrambled siRNA group, $n = 5$, or PBS group, $n = 8$), suggesting that DC treated with anti-CD86 siRNA demonstrated immunosuppressive activity (Fig. 3).

DISCUSSION

An attractive potential approach in transplantation is to modify donor-derived DC to induce an immunological hyporesponsive state in the recipient. Immature DC can inhibit alloantigen-specific T cell responses and prolong graft survival,[10] and DC genetically engineered to express CTLA4-Ig,[11] FasL,[12] IL-10, or TGF-β[13] also have similar effects. However, the tolerogenic properties of these DC are inconsistent because of the late maturation by inflammatory cytokines in vivo. Antibodies specific for cell-surface molecules prevent DC costimulation of T cells, but they have a limited half-life and require multiple antibody treatments.[14] In this study, we introduced RNA interference which is superior to genetic engineering or antibody blocking approaches due to the following reasons[15]: (1) blocking efficacy is more potent, only a few copies of dsRNA are able to conduct multiple rounds of gene-specific mRNA cleavage; (2) targeting gene expression is more specific, even a single nucleotide mismatch between the target mRNA and siRNA is sufficient to prevent silencing; (3) this technique is relatively simple; (4) simultaneously targeting multiple genes is possible for increasing efficacy; (5) inhibitory effects can be passed for multiple generations, the silencing effect sustained long enough for measure phenotypic changes in the cells, although a dilution effect is evident in mammalian cells.[16]

In this study, the molecules expression was analyzed by flow cytometry after transfection with anti-CD86 siRNA, showing that siRNA suppressed the expression of CD86 on DC and did not affect the expression of CD80. Our data clearly demonstrated that CD86^{low} DC significantly inhibited allostimulatory activity in MLR in vitro and prolonged the survival of cardiac allografts in vivo. These results support that CD86 is an important co-stimulaory molecules expressed on DC. CD86 is the initial costimulatory ligand based on its more abundant and earlier expression pattern.[17] DCs with suppressed CD86 expression can be skewed from "immunogenic" to "tolerogenic."

In summary, our research demonstrated that CD86^{low} DC inhibited allogeneic T cell proliferation and prolonged the survival of heart allografts through an RNA interference technique. This RNA interference strategy has great clinical therapeutic potential for the therapy of allograft rejection.

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