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Induction of $CD4^+CD25^+$ T Cells and Control of Cardiac Allograft Rejection by CD40/CD40L Costimulatory Pathway Blockade in Mice

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ABSTRACT

Background. Rejection is a major problem in organ transplantation. We speculated that dendritic cells (DCs) with low expression of CD40 may inhibit T-cell responses providing protective effects on organ transplantions.

Methods. RNA interference (RNAi) is an effective approach to degrade RNA levels of a target gene. We used RNAi to reduce CD40 levels in mouse bone marrow-derived DCs. Allogeneic T-lymphocyte proliferation stimulated by DCs was assessed in mixed lymphocyte reactions. A mouse model of heterotopic abdominal heart transplantation was performed to evaluate survival times and histologic grade of acute rejection at 7 days after transplantation. $CD4^+CD25^+$ regulatory T cells (Tregs) in peripheral blood were quantified by flow cytometry.

Results. Mouse DCs with reduced CD40 expression lowered the proliferation of recipient T cells. More DCs with silenced CD40 delayed cardiac allograft rejection.

Conclusions. Reduction of CD40 in DCs induced peripheral Tregs and delayed cardiac allograft rejection.

IMMUNOSUPPRESSIVE agents are used to prevent rejection of transplanted organs or tissues. Although these drugs prolong transplant survival, they are associated with remarkable morbidity. Furthermore, they do not offer long-term graft survival. Therefore, induction of antigen-specific tolerance has been suggested to be an alternative approach.[1]

Rejection of a transplanted organ is the body's natural response. The recipient's immune system kills donor organs through activation of effector T cells. In contrast, regulatory T cells (Tregs) may also be involved in transplant tolerance.[2] For activation of naïve T cells, 3 signals are required; the first, by TCR/antigen which alone is insufficient to trigger productive T-cell responses; the second, by costimulatory molecules; and the third, by cytokines via their receptors, such as interleukin (IL)-2/IL-2R, which promote long-term proliferation of activated T cells.[3] The second signal drives clonal expansion, survival, and differentiation of activated T cells into distinct functional subsets.[4] Without the activation of costimulatory molecules, T cells become anergic. CD40/CD40L, a newly discovered costimulatory pathway,[5] includes CD40 expressed on antigen-presenting cells (APCs) and CD40 ligand expressed on activated T cells. Dendritic cells (DCs), one of the most potent APCs, present alloantigens to T cells. We speculated that RNA interfering (RNAi)-mediated CD40 silencing could inhibit T-cell responses providing protection for a transplanted organ.

RNAi is an approach that specifically inhibits the expression of target genes by inducing RNA degradation. RNAi can be mediated through 2 types of molecules: Chemically synthesized double-stranded small interfering RNA or vector-based, short hairpin RNA (shRNA). shRNA used with lentiviral vectors is a promising option, because it may provide both persistent and stable gene silencing and can be produced in large quantities.

In this study, we constructed CD40 shRNA lentiviral vectors to modify CD40 levels in mouse bone marrow-

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derived DCs (BMDCs). Mouse DCs with decreased expression of CD40 block the CD40/CD40L costimulatory pathway exhibiting protective effects on grafts in vivo.

MATERIALS AND METHODS

Animals

Six- to 8-week-old C57BL/6 and BALB/c mice purchased from the Experimental Animal Center in the Academy of Military Medical Sciences (Peking, China) were kept at the animal facility. The procedures were performed according to our Animal Care and Use Committee.

DC Culture

C57BL/6 murine BMDCs were generated from bone marrow (BM) precursors as previously described.[6] Mouse BM cells extracted from the femora and tibiae were placed in a culture flask with RPMI-1640 (GIBCO, Grand Iste, NY) supplemented with Glutamax (HyClone, Logan, Ut), 10% fetal calf serum (FCS; HyClone), 1% 2-mercaptoethanol (2-ME; Sigma, St Louis, Mo), 200 U/mL penicillin and 100 U/mL streptomycin. The next day, nonadherent cells were resuspended in fresh culture medium with added cytokines- granulocyte-macrophage colony-stimulating factor (GM-CSF; 20 ng/mL) and IL-4 (1 ng/mL; Peprotech, Rocky Hill, NJ). On day 4, fresh cytokines and new culture medium were added; the cells were re-incubated. From days 6 to 9, the cells were cultured with tumor necrosis factor (TNF, 1 ng/mL; Peprotech). On day 10, harvested nonadherent cells were routinely tested for CD80, CD40, and CD11c expression using flow cytometry.

Construction of CD40 shRNA Lentiviral Vectors

According to GenBank mouse CD40 gene sequences, we designed 4 pairs of small hairpin RNA specific for CD40 (named Psc-1, -2, -3, and -4). Psc-2 (TGGTAAAGAGAGTCGCATC) the most effective one in pretests, was cloned into the pGC-LV vector. The 293T cells were co-transfected with pGC-LV, pHelper1.0 and pHelper2.0 to produce CD40 shRNA lentivirus.

Gene Transfection

One day before transfection, DCs (10^5 cells per well) were placed in a 24-well plate for incubation at 37°C with 5% CO_2. DCs were infected by recombinant lentiviral pGC-LV CD40 RNAi at a multiplicity of infection (MOI) of 10. After 8 hours incubation, the supernate was replaced with an equal volume of RPMI 1640 supplemented with 20% FCS, 20 ng/mL GM-CSF, and 1 ng/mL IL-4. The cells were collected at 48–72 hours after transfection to determine gene expression.

Quantitative Real-Time Polymerase Chain Reaction

We examined the CD40 expression of DCs by quantitative real-time polymerase chain reaction (qRT-PCR) before and after transfection to determine the effect of RNAi. Briefly, total RNA was extracted and collected from cells using the RNeasy Micro Kit (Qiagen, Hilden, Germany). RNA expression levels were quantified with a LightCycler instrument (Roche, Nutley, NJ) using the LightCycler FastStart DNA Master SYBR Green I (Roche). According to the manufacturer's instructions, each gene was amplified with specific primers as summarized in Table 1. For the PCR reactions, the following settings were used: Preincubation (2 min at 95°C) followed by 3-step cycling amplification of 44 cycles; denaturation (15 sec at 95°C), annealing (30 sec at 60°C), and extension (30 sec at 68°C). Results were then quantified using the relative quantification approach using software provided by the manufacturer (Roche).

Table 1. PCR Primers

	Product Length (bp)	Primer
CD40	121	Upstream 5′-TGTGACTCAGGCGAATTCTC-3′ Downstream 5′-CAGTGTCTGATTCTGCGGTG-3′
β-Actin	287	Upstream 5′-ATCCGTAAAGACCTCTATGC-3′ Downstream 5′-ACGCAGCTCAGTAACAGTC-3′

Flow Cytometry

Phenotypic analysis of DCs was performed by flow cytometry using an ACSCalibur scan (Becton Dickenson, Franklin Lakes, NJ). The cells were stained with anti-mouse CD11c-fluoroscein isothiocynate (FITC) anti-mouse CD40-PE or anti-mouse CD80-PEcy5 monoclonal antibodies (mAbs; Biolegend). Tregs were analyzed on by flow cytometry after triple staining with mAbs against with Foxp3-FITC, CD4-PE, and CD25-CY5(Biolegend). Antibodies of the same isotypes were used as controls.

One-Way Mixed Lymphocyte Culture

CD40RNAi transfected DCs from C57BL/6 mice were resuspended at 1×10^5 cells/mL in RPMI1640 for 30 min incubation with mitomycin C (25 ng/L). Cells washed twice with RPMI1640 served as stimulators. BALB/c splenic cells (1×10^7 cells/mL) served as responders; DCs and T cells were mixed at a 1:1 ratio. The mixed lymphocyte cultures were incubated in 96-well plates (200 μL each well). After 3 days of culture at 37°C in a 5% CO_2 humidified atmosphere, thiazolyl blue tetrazolium bromide solution (final concentration of 0.5 g/L) was added for 4 hours followed by extraction with 100 μL dimethylsulfoxide for 10 minutes. Optical density (OD) values were measured at 570 nm. At the same time, we established the negative control group: BALB/c mouse DC cells and BALB/c mouse spleen T cells. The stimulation index (SI) was calculated by the following formula: SI = OD of experimental group/OD of negative control group.

Heart Transplantation

Recipient BALB/c (H-2^d) mice were primed with 10^6 donor CD40RNAi DCs from C57BL/6 mice (H-2^b) by tail vein injection on days 3 and 7 before transplantation. Abdominal heterotopic vascularized heart transplantation was performed using previously described techniques.[7] The donor heart harvested after heparinization was preserved in saline at 4°C. In the recipient, a midline laparotomy was performed; the donor's ascending aorta was sutured end-to-side to the recipient's abdominal aorta and the donor's pulmonary artery, to the recipient's inferior vena cava. The complete loss of a palpable heart beat indicated rejection.

Histologic Analysis of Cardiac Grafts

On posttransplant day 7, recipient mice were humanely killed to examine the grafted hearts. For histologic analysis, samples were fixed in 10% formalin, embedded in paraffin, sectioned, and stained