

Original Article

B7-2 gene silencing by lentivirus-mediated delivery of shRNA reduces progression of experimental lupus nephritis

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Abstract: Over-activation of B7/CD28 co-stimulatory signal is believed to play an important role in promoting hyper-functional immune response which contributes to autoimmune diseases. In the present study, we generated the recombinant lentivirus transcribing short hairpin RNA (shRNA) specific for mouse B7-2 gene silencing and B7/CD28 signal inhibition to explore and evaluate a potential candidate approach to suppress B7/CD28 co-signal and ameliorate auto-immune diseases. A pristane-induced mouse model of lupus nephritis (LN) was generated to assess the effects of an immune intervention on the progression of systemic lupus erythematosus (SLE) and end-stage renal injury. Lentivirus-mediated B7-2 gene silencing attenuated the over-activity of splenic immune cells, including macrophages, dendritic cells, granulocytes, and B-cells in the experimental lupus nephritis model ten days after pristane was given. Production of auto-antibodies, e.g., antinuclear antibody (ANA), anti-double-strand-DNA antibody (anti-dsDNA), and development of proteinuria after treatment with B7-2 silencing lentivirus were lessened from the third or fourth month while auto-antibodies and proteinuria appeared to be decreased at the eighth month. In addition, secretion of interleukin-4 and interferon- γ cytokines, immune complexes accumulation/deposition in kidneys, and the renal inflammatory damage were relieved compared to those in mice of model group without B7-2 silencing lentivirus intervention. In conclusion, suppression of B7/CD28 signal by lentivirus-mediated B7-2 gene silencing can reduce the development of experimental lupus nephritis, suggesting it is a potentially useful strategy for decelerating the progression of lupus-like diseases.

Keywords: Lentivirus, RNA interference, B7-2, systemic lupus erythematosus, pristane

Introduction

Systemic lupus erythematosus (SLE) is a kind of chronic auto-immune disease with different degrees of inflammatory damage on joints, skin, kidneys, liver, brain, etc [1]. Despite etiology and pathogenesis of SLE remain to be elucidated, it is considered that over-activated antigen presenting cells (APCs) triggered by defect and cleared of apoptotic cells could provide abnormal co-stimulatory signals to induce T-cells hyper-function, which promotes B-cells to produce high levels of circulating antibodies to a wide spectrum of auto-antigens [2, 3]. Formation and/or deposition of auto-antigen-antibody immune complexes (ICs) in target organs ultimately result in the infiltration of in-

flammatory cells, tissue injury, and end-stage diseases [4, 5].

Co-stimulatory signal transmitted by binding B7 to CD28 or CTLA4 is well studied for its crucial role on regulatory T cell activation or tolerance [6]. In the pathogenesis of SLE, over-activated B7/CD28 co-stimulatory signal plays a critical role on inducing T- and B-cells hyper-function [7, 8], which makes it possible to alleviate SLE illness by inhibiting B7/CD28 signal [9]. As an important member of B7 family and an identified ligand of CD28 and CTLA4, B7-2 is proved to be over-expressed on APCs in SLE patients and generate hyper-activated co-stimulatory signal to promote abnormal immune response and the occurrence of SLE [7, 10].

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Table 1. Sequences of oligonucleotides encoding shRNA targeting mouse B7-2

Position*	Sequences					
139	5'-GATCC	GCTTGGCAATCCTTATCTTTG	TTCAAGAGA	CAAAGATAAGGATTGCCAAGC	TTTTTT	G-3'
	3'-G	CGAACCGTTAGGAATAGAAAC	AAGTTCTCT	GTTTCTATTCCTAACGGTTCG	AAAAAA	CTTAA-5'
425	5'-GATCC	GGACATGGGCTCGTATGATTG	TTCAAGAGA	CAATCATACGAGCCCATGTCC	TTTTTT	G-3'
	3'-G	CCTGTACCCGAGCATACTAAC	AAGTTCTCT	GTTAGTATGCTCGGGTACAGG	AAAAAA	CTTAA-5'
848	5'-GATCC	GGAGATTACAGCTTCAGTTAC	TTCAAGAGA	GTAAGTGAAGCTGTAATCTCC	TTTTTT	G-3'
	3'-G	CCTCTAATGTCGAAGTCAATG	AAGTTCTCT	CATTGACTTCGACATTAGAGG	AAAAAA	CTTAA-5'

*the start position of candidate target sequence in coding region of mouse B7-2 transcription.

Though previous studies reported that using anti-B7-2 antibody or CTLA4-Ig fusion protein to block B7-2/CD28 signal could effectively relieve the severity of SLE [11, 12], great efforts are still being made to explore more novel and economic potential strategies beyond protein based bio-drugs for SLE treatment.

RNA interference (RNAi), a sequence-specific post-transcriptional gene silencing process mediated by 21~23 bp double-stranded RNA (dsRNA), can trigger the degradation of homologous mRNA as well as down-regulation of corresponding protein [13] and kindly favor us by facilitating B7-2 expression and B7-2/CD28 signal in gene level. In the present study, we generated recombinant lentivirus that can mediate B7-2 gene silencing followed with the assessment of the silencing efficacy on B7-2 expression and antagonism on B7-2/CD28 signal. In addition, we administrated the recombinant lentivirus into a C57BL/6 mouse lupus nephritis model induced by pristane to specifically attenuate B7-2/CD28 co-stimulatory signal and evaluate its immune intervention effects on the development of LN. Our results suggest that B7-2 gene silencing by lentivirus may be a potentially useful strategy for ameliorating lupus-like diseases.

Materials and methods

Materials

Lipofectamine™2000, RPMI-1640 medium and fetal bovine serum (FBS) were purchased from Life Technologies (Grand Island, NY, USA). Recombinant murine GM-CSF and IL-4 were purchased from Peprotech (Rocky Hill, NJ, USA). Phycoerythrin (PE) conjugated anti-mouse CD11b, CD11c, Gr1, CD21, IgG; Fluorescein isothiocyanate (FITC) conjugated anti-mouse B7-1, B7-2, IgG; Mouse IFN- γ and IL-4 ELISA kits

were purchased from eBioscience (San Diego, CA, USA).

DAPI staining solution was purchased from Boster (Wuhan, Hubei, CHN). Vectashield mounting medium (Vector Laboratories, CA, USA). ANA and anti-dsDNA antibody detection kits were purchased from H&J NovoMed (Beijing, China). Pristane, lipopolysaccharide (LPS), polybrene were purchased from Sigma-Aldrich (St. Louis, MO, USA) 293T cell was purchased ATCC (Manassas, VA, USA). Lentiviral vector system was purchased from Genepharma (Shanghai, China). Female C57BL/6 mice, aged 6~8 weeks, were obtained from Laboratory Animal Center of Soochow University (Suzhou, Jiangsu, CHN). All experiments involving mice were conducted with approval of the Institutional Animal Care and Use Committee of Soochow University.

shRNA design and lentiviral expression vector construction

On the basis of published criteria [14, 15], three candidate 21-mers target sequences (start position at 139, 425, and 848, respectively) selected from coding sequence of mouse B7-2 transcription were used to design shRNA templates. Oligonucleotides encoding shRNA consists of sense and reverse complement strand of candidate target sequence linked by a spacer (TTCAAGAGA), six thymidines served as a transcription terminator of polymerase III and terminal EcoRI and BamHI restriction sites (**Table 1**). A random scrambled heterology of target sequences was used to design a negative control shRNA for the identification of RNAi specificity. The oligonucleotides were annealed and then ligated into lentiviral expression plasmid between EcoRI and BamHI restriction sites to generate recombinant lentiviral expression vectors. Sequencing of recombinant expression plasmids was performed with an automat-

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ed DNA sequencer (3730XL, ABI, USA) to confirm the validity of the inserted sequences.

Production of lentivirus

Lentivirus was produced as previously described [16, 17] by co-transfecting 293T cells with a 4-plasmid system including recombinant lentiviral expression plasmid and three packaging plasmids with the help of Lipofectamine™2000. We collected the resulting culture supernatant containing lentiviral particles at 48 h and 72 h post-transfection and then centrifuged the pooled supernatant via ultra-centrifugation for 2 h at 50,000 g (Optima L-90K, Beckman, USA).

Infectious titer of the preparations was determined using inverted fluorescence microscope (Ti-s, Nikon, JPN) by detecting the percentage of GFP positive 293T cells after transfected with serial dilutions of concentrated lentivirus.

Transduction of lentivirus in cultured dendritic cells and inhibition of B7/CD28 signal in vitro

Bone marrow cells isolated from C57BL/6 mice were induced to differentiate into DCs for 8 days in RPMI-1640 medium supplemented with 10% FBS, GM-CSF (20 ng/ml), IL-4(10 ng/ml) and LPS (1 µg/ml, used for last 2 days) as previously described [18]. The resulting cell populations were greater than 80% positive of CD11c/MHCII/B7-1/B7-2, having obvious dendrites of typical mature DCs and ability to trigger T-cells proliferation. On the 6th day of culture, DCs were transfected at density of 1×10^6 cells/ml by lentivirus-negative at different multiplicity of infection (MOI; 0, 5, 10, 20, 40, 60) for 12 h in the presence of polybrene (5 ng/ml), then cultured in fresh medium containing GM-CSF, IL-4 for 60 h and LPS for remaining 48 h. Cells were then collected for flow cytometry analysis (FACSCalibur, BD, USA) of lentiviral transduction efficiency. Three candidates of lentivirus-shRNA and lentivirus-negative control were used to transfect DCs collected on day 6 for flow cytometry analysis of the expression of B7-2 to choose the B7-2 RNAi lentivirus based on the silencing efficacy. A mixed lymphocyte reaction system consists of 2×10^4 transfected DCs and 2×10^5 mouse splenic T cells per well in 96-well plate was cultured for 3 days to analyze antagonism on B7/CD28 signal and T-cells pro-

liferation of the chosen B7-2 RNAi lentivirus by MTT cell viability assay.

Establishment of pristane-induced mouse lupus nephritis model and immune intervention by lentivirus

C57BL/6 mice were intraperitoneally injected with 0.5 ml pristane one time to develop the lupus nephritis model. Mice were divided into 4 groups including the model control, cyclophosphamide (CTX) treatment, lentivirus-negative/lentivirus-shRNA treatment, and a negative control with a single intraperitoneal injection of 0.5 ml normal saline. Mice in CTX group were treated with i.p. with 60 mg/kg CTX per time on days 1, 8, 22, 29, 43, 50, 64, 71, 85, 92; Mice of the lentivirus treatment group were intravenously injected in the tail with 0.5×10^8 TU lentivirus-shRNA or lentivirus-negative per time on day 1 and day 60.

Analysis of mouse splenocytes activation

Spleen cells were dissociated from mice of aforementioned five groups on the 10th day. Single cell suspensions were collected and stained with fluorescein labeled antibodies for 30 min at 4°C as follows: (1) PE-CD11b & FITC-B7-2; (2) PE-CD11c & FITC-B7-2; (3) PE-CD21 & FITC-B7-2; and (4) PE-Gr1. Stained cells were washed with PBS and then subjected to flow cytometry analysis.

Measurement of ANA, anti-dsDNA antibody production and IFN-γ, IL-4 secretion

Blood was sampled from retro-orbital plexus of anesthetized mice to prepare serum. Serum ANA and anti-dsDNA antibody production was assessed by indirect immunofluorescence assay according to the manufacturer's instruction. Briefly, mouse serum diluted in PBS was incubated with HepG2 cells or Crithidia mellificae which were immobilized on slides for 30 min. After washing, FITC labeled anti-mouse IgG antibody was added to incubate with slides for another 30 min. Then slides were washed, dried, and mounted with Vectashield. Images were taken with a confocal microscope (C1-si, Nikon, JPN) and scored as follows. ANA, score (0) dilution ratio 1:100, negative; (1) 1:100~300, positive; (2) 1:300~1000, positive; (3) >1:1000, positive. Anti-dsDNA antibody, (0) 1:10, negative; (2) 1:10~30, positive; (3)

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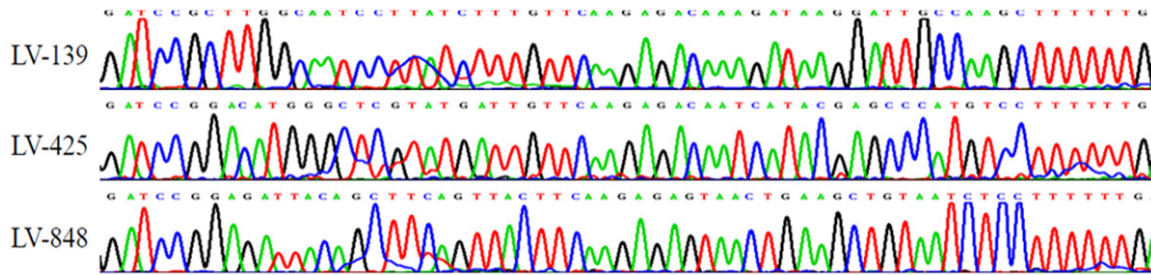


Figure 1. DNA sequencing of inserted shRNA encoding sequences in recombinant lentiviral expression vectors for mouse B7-2 gene RNAi. Recombinant clones of each target specific lentiviral expression vector were chosen and subjected to DNA sequencing. Recombinant lentiviral expression vectors targeting three sequences were named LV-139, LV-425 and LV-848 respectively.

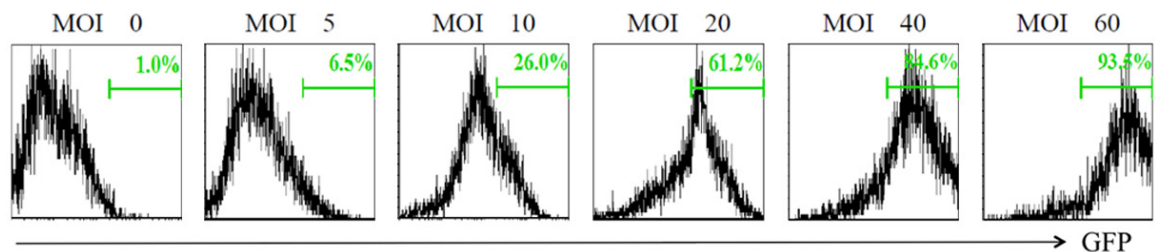


Figure 2. Infection efficiency of recombinant lentivirus on mouse BM-DCs. BM-DCs were transfected with lentivirus-negative at different MOI for 12 h and then cultured in fresh complete RPMI-1640 medium for 60 h to express the transgene of GFP. Flow cytometry analysis was then performed to measure the positive rate of GFP expressing cells, which means infection efficiency on DCs.

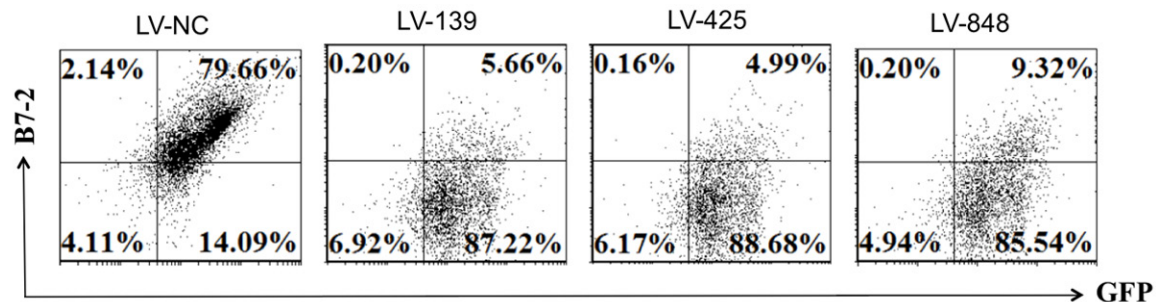


Figure 3. Silencing efficacy of recombinant lentivirus on the expression of B7-2 in mouse BM-DCs. BM-DCs were transfected with recombinant lentivirus at MOI of 60 to analyze the interference efficiency on B7-2 expression by flow cytometry. Horizontal and vertical axis of each figure represent the expression of GFP and B7-2 separately.

1:30~100, positive; (4) >1:100, positive. The concentrations of IFN- γ and IL-4 cytokines in serum were measured using IFN- γ and IL-4 sandwich ELISA kits according to the manufacturer's instruction.

Detection of renal immune complexes deposition

Direct immunofluorescence studies were carried out to detect deposits of IC. In brief, kidney

tissues obtained from mice humanely killed at the 8th month were frozen in OTC medium and cut into 5 μ m-thick slices, followed by being dried for 30 min and fixed with acetone at 4°C for 10 min. Slices were washed in PBS and then incubated with PE conjugated goat anti-mouse IgG (1:100 dilution) and DAPI staining solution according to the manufacturer's instruction. After mounting with Vectashield, ICs were observed under a confocal microscope.

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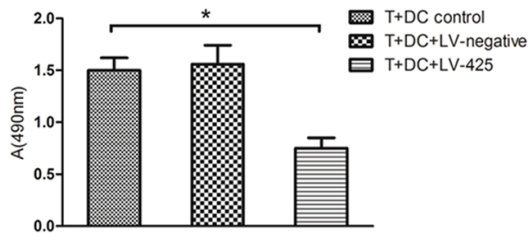


Figure 4. Antagonism of lentivirus-shRNA (425) on T cells proliferation induced by B7/CD28 signal. 2×10^4 DCs transfected by LV-425 and 2×10^5 splenic T cells was co-cultured for 72 h followed by MTT cell viability. *: $P < 0.05$, compared to T cells + DC control group.

Assay of proteinuria development

24 h urine specimens were collected to analyze the positive rate of proteinuria and concentration of urinary protein using Albustix reagent strips (Bayer, GER) according to the manufacturer's instruction by comparing results to color chart and scored as follows: (0) 0 mg/L; (1) 150~300 mg/L; (2) 300~1,000 mg/L; (3) 1,000~3,000 mg/L; (4) 3,000~20,000 mg/L; (5) >20,000 mg/L.

Evaluation of lupus nephritis

Kidney tissues obtained from mice at the time of sacrifice were bisected. One half of each kidney was fixed in 10% neutral-buffered formalin for 24 h, followed by being embedded in paraffin wax and cut into 5 μ m-thick sections. Sections were then dewaxed through a series of graded alcohol rehydration steps. Slices were stained with hematoxylin-eosin and observed under a light microscope. The other half of each kidney was cut into 1 mm³ specimens followed by being fixed in 2.5% glutaraldehyde for 4 h and in 1% osmium tetroxide for 1 h. After specimens being dehydrated using series of graded acetone, embedded in epoxy resin, and cut into 70 nm-thick sections. Then sections were sputter-coated with gold and examined using a transmission electron microscope (TEM) (S-450, HITACHI, JPN).

Statistical analysis

Results are expressed as mean \pm standard deviation and analyzed using SPSS19.0 software. The statistical significance of difference in values and frequency between groups were evaluated by one way ANOVA. Categorical data was subjected to Redit analysis. Statistical cal-

culations were performed with GraphPad Prism 5.0 software. $P < 0.05$ was considered as statistically significant difference.

Results

Lentivirus-mediated B7-2 silencing inhibits B7/CD28 signal in vitro

The results of DNA sequencing showed that recombinant lentivirus were constructed with correctly inserted shRNA encoding sequences as expected (**Figure 1**). According to our typical procedures for lentivirus production and titration, the titer of the preparation was approximately $1 \sim 3 \times 10^8$ transduction units/ml. BM-DCs were cultured and infected by lentivirus-negative (LV-NC) at different MOI (0, 5, 10, 20, 40, and 60). The data demonstrated an increasing efficiency of infection and an effective transduction with above 90% GFP positive cells being detected when MOI reached to 60 (**Figure 2**). Based on these results, lentivirus-shRNA directing three targets of B7-2 gene were subsequently used to transfect mouse BM-DCs for the purpose of determining the silencing efficacy on B7-2 expression. Evidence shown in **Figure 3** revealed that transduction of lentivirus-shRNA in DCs suppressed expression of B7-2 significantly at MOI of 60, with the inference efficiency of 92.8% (LV-139), 93.7% (LV-425), and 88.2% (LV-848), respectively. Considering the better silencing effect of lentivirus-425, it was chosen for further assessment of antagonism on B7/CD28 signal and T cells proliferation by MTT cell viability assay. We found that T cells proliferation was notably inhibited ($P < 0.05$) owing to the transduction of lentivirus-425 in DCs (**Figure 4**).

Lentivirus-mediated B7-2 silencing suppresses expression of B7-2 and activation of splenocytes

Ten days after pristane was injected, the expression of CD11b, CD11c, Gr1 and CD21 on splenic cells from mice of model control group were up-regulated compared to negative control ($P < 0.05$) (**Figure 5A**) indicates pristane induced activation of macrophages, dendritic cells, granulocytes, and B cells. Treatment with LV-425 significantly suppressed the expression of B7-2 on these cells ($P < 0.05$) (**Figure 5B**) and diminished the percentage of activated macrophages, dendritic cells, granulocytes, and B

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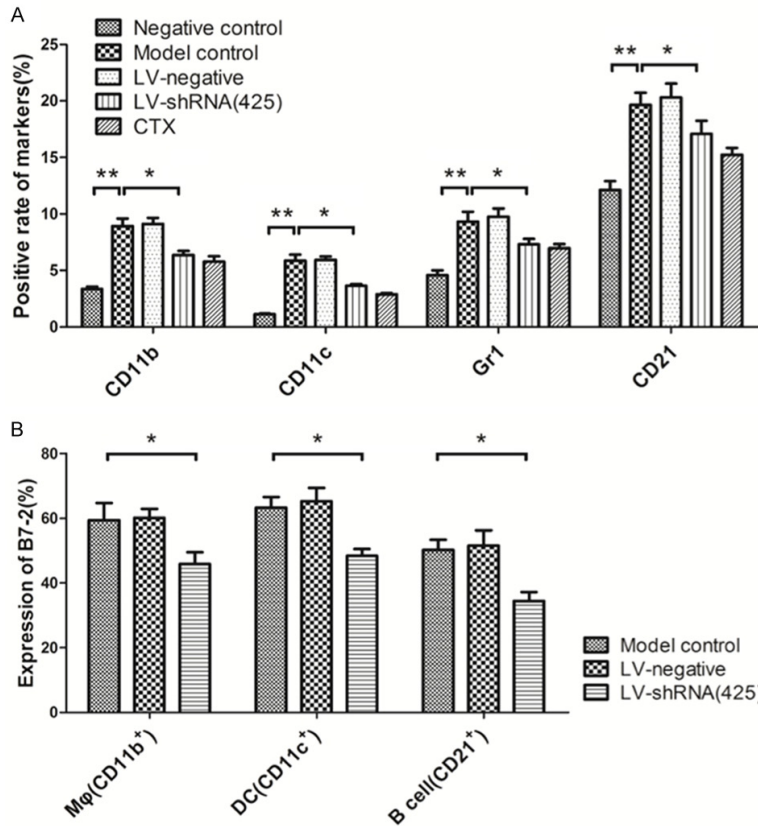


Figure 5. Splenocytes activation and B7-2 expression on splenic macrophages, dendritic cells, and B cells. Spleen cells were sampled on the 10th day from mice and then performed to flow cytometry analysis of the positive rates of CD11b, CD11c, Gr1, CD21 and the expression of B7-2 on Mφs (CD11b⁺), DCs (CD11c⁺), B cells (CD21⁺). *: $P < 0.05$, compared to Model control; **: $P < 0.05$, compared to negative control.

cells significantly ($P < 0.05$) (Figure 5A). These results suggest lentivirus-shRNA (425) can silence B7-2 expression and suppress the immune response of splenic cells induced by pristane.

Lentivirus-mediated B7-2 silencing reduces the level of auto-antibodies

ANA and anti-dsDNA antibody in mouse serum were detected in 30% and 10%, respectively, 3 month after administration with Pristane. Another two months later, the positive rates of ANA and anti-dsDNA antibody were increased to 80% and 70%, respectively. At the 8th month, almost all mice in model control group showed ANA and anti-dsDNA antibody positive in serum (Figure 6A and 6B). Immunofluorescence patterns of ANA examination demonstrated positive homogeneous, speckled, cytoplasmic, and nucleolar staining (Figure 6C), indica-

ting a wide spectrum of auto-antibodies were produced in the pristane treated mice. Mice treated with lentivirus-shRNA (425) demonstrated a significant decline in ANA and anti-dsDNA antibody production compared to model control group ($P < 0.05$) (Figure 6A and 6B).

Lentivirus-mediated B7-2 silencing reduces the secretion of IFN-γ and IL-4

To determine the effects of B7-2/CD28 signal inhibition induced by lentivirus mediated B7-2 silencing on the change of cytokines secretion, the concentrations of IFN-γ and IL-4 in sera were assessed by ELISA assay. As shown in Figure 7, treatment with lentivirus-shRNA (425) significantly suppressed the levels of both IFN-γ and IL-4 in lupus mice induced by pristane ($P < 0.05$).

Lentivirus-mediated B7-2 silencing reduces proteinuria production

Four months after pristane injection, 30% of mice developed proteinuria with a range of urinary protein concentration from 300~3,000 mg/L. The positive rates of proteinuria and urinary protein concentration gradually elevated which were suppressed by lentivirus-shRNA (425) administration. At the 8th month, the degree of proteinuria in lentivirus-shRNA (425) treatment group was significantly attenuated compared to that in model control group ($P < 0.05$, Figure 8).

Lentivirus-mediated B7-2 silencing alleviates renal ICs deposition and the severity of nephritis

As shown in Figure 9A, immunofluorescence assay on frozen sections of renal tissues in model control group revealed ICs continuously or linearly distributed in glomerular capillary loops and mesangium. In addition, ultrastruc-

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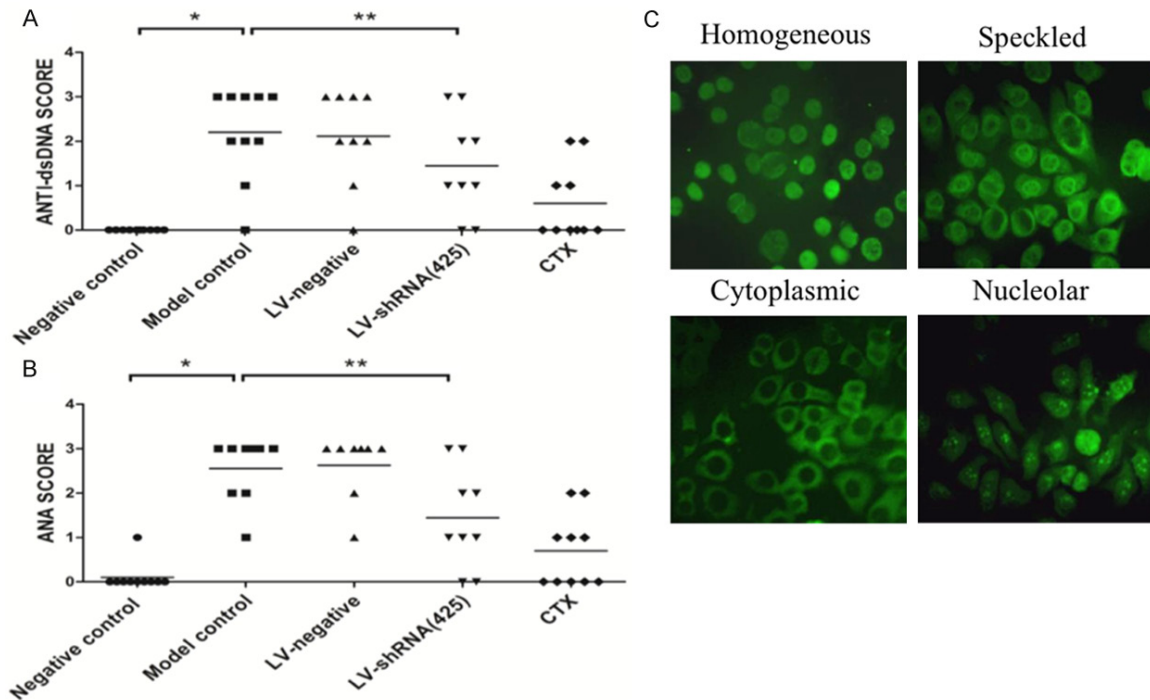


Figure 6. Production of ANA and anti-dsDNA antibody in mouse serum. Mouse serum collected at the 8th month was diluted in PBS and incubated with HepG2 cells or *Crithidia mellifica*, followed by detection with FITC labeled goat anti mouse IgG antibody using immunofluorescence assay and scored as described in part 2.7. *: $P < 0.05$, compared to Negative control; **: $P < 0.05$, compared to Model control. Immunofluorescence patterns of ANA are shown in Figure 7C with a magnification of 400x.

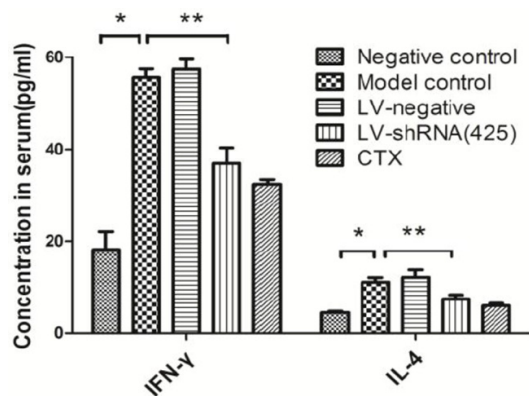


Figure 7. Concentration of IFN- γ and IL-4 in mouse serum. Quantitation of IFN- γ and IL-4 in serum sampled at the 8th month was performed by sandwich Elisa. *: $P < 0.05$, compared to Negative control; **: $P < 0.05$, compared to Model control.

tural analysis (**Figure 9B**) demonstrated large amorphous electron dense deposits formed in the basement membrane, mesangial, and sub-endothelial region. The inflammatory injury of kidney caused by these ICs were confirmed by the results of renal pathological examination. As shown in **Figure 9B**, distended appearance

of glomerular basement membrane (GBM), thickened capillary walls, mesangial interposition and expansion, and podocytes fusion were found in pristane treated mice. Results of H&E staining as shown in **Figure 9C** showed infiltration of inflammatory cells and hyper-cellularity in mesangial/glomerular regions, volume enlargement of renal glomerulus and glomerular capsules; diffuse glomerular necrosis could be found in model control group as well. After treatment with lentivirus-shRNA (425), ICs deposition was reduced as only slight linear and granular fluorescent signal could be detected (**Figure 9A**). The ultrastructural of renal glomerulus appears to be normal compared with negative control as well (**Figure 9B**). Additionally, lentivirus-shRNA (425) treatment attenuated the infiltration of inflammatory cells and diffuse glomerular necrosis (**Figure 9C**). These results demonstrate that B7-2 gene silencing by lentivirus significantly alleviates the development of lupus nephritis.

Discussion

T-cells response is regulated by a two signal pathways: one is the specific signal provided by

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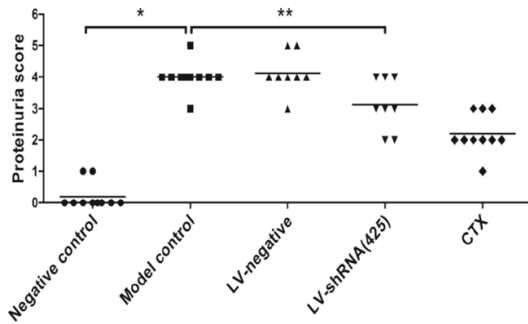


Figure 8. Assay of proteinuria development. 24 h urine specimens at 8th month were collected to analyze the positive rate of proteinuria development and concentration of urinary protein using Albustix reagent strips. *: $P < 0.05$, compared to Negative control; **: $P < 0.05$, compared to Model control.

engagement of T-cell receptor (TCR) with antigenic peptide-MHC molecules presented on APCs, the other is co-stimulatory signal [19, 20]. B7, including B7-1 and B7-2, is an important co-stimulatory molecule expressed on the surface of APCs [21]. Ligation of B7 with its ligands, e.g., CD28 expressed on T-cells, provides an essential co-stimulatory signal to assist the specific signal for normal T-cells immune response. However, hyper-activation of B7/CD28 signaling pathway under a pathological state can induce the hyper-function and abnormal immune response of T- and B-cells, which may cause the occurrence of autoimmune diseases [3, 22].

SLE is a kind of autoimmune diseases characterized by a variety of auto-antibodies accompanied by immune complex formation/deposition and inflammatory lesions in multiple tissues and organs [1, 23-25]. Up to now, several researchers have demonstrated that loss of immune tolerance caused by the abnormality of B7/CD28 co-stimulatory signal and the over-activity of T- and B-cells plays a significant role in the pathological process of SLE [6, 26-28]. To restore normal immune tolerance and relieve the severity in SLE, different kinds of therapeutic approach at protein level using specific anti-B7 antibody or fusion protein of Ig with CTLA4 to bind to B7 and inhibit B7/CD28 signal are well studied [29-31].

RNA interference [32], which is a highly efficient regulatory process that causes gene silencing in most eukaryotic cells, provides a new attractive approach for B7 gene-specific inhibi-

tion and B7/CD28 signal suppression. In the present study, we constructed and produced recombinant lentivirus, which is capable of transcribing short hairpin RNA (shRNA) targeting B7-2 gene, to silence its expression followed by BM-DCs being transfected by lentivirus. The results showed an effective transduction of lentivirus on DCs with the percentages of GFP positive cell more than 86% and a high silence efficacy of lentivirus-shRNA on B7-2 expression (>90%), indicating lentivirus-shRNA (425) directing B7-2 is a useful candidate for suppressing B7/CD28 signal pathway. A mixed lymphocyte culture system containing splenic T-cells and BM-DCs transduced with LV-425 was set up to assess the antagonism on B7/CD28 signal, and we observed a markedly inhibition of T-cells proliferation induced by B7/CD28 signal.

To evaluate the intervention effects of lentivirus-shRNA (425) on B7/CD28 signal and the progress of SLE, a C57BL/6 mouse model of lupus nephritis was induced by a single intraperitoneal injection of pristane. The pristane, an isoprenoid alkane primarily derived from mineral oil, is believed as membrane-active compound capable of interacting with phospholipid bilayers of cell membranes and inducing cell apoptosis [33]. A large amount of cell debris, including nucleosomes and its components as a result of excessive cell apoptosis, induce chronic inflammation and immune activation [33-35]. In this study, we observed the up-regulation of CD11b, CD11c, Gr1 and CD21 on splenic cells of mice 10 days after pristane injection, which indicates the activation of macrophages, dendritic cells, granulocytes, and B cells. While in mice treatment with lentivirus-shRNA (425), the activation of macrophages, dendritic cells, granulocytes, and B cells and B7-2 expression were down-regulated markedly compared to model control, we conclude that lentivirus based shRNA directing B7-2 specifically silenced its expression and directly or indirectly decreased the activation of APC/T/B cells mediated by B7-2/CD28 signaling pathway.

In the setting of hyper-activation of immune cells, auto-antigens initiate the generation of cytokines and auto-antibodies contributes to the development of lupus [36, 37]. To address the changes of cytokines and auto-antibodies in pristane-induced mouse lupus nephritis and

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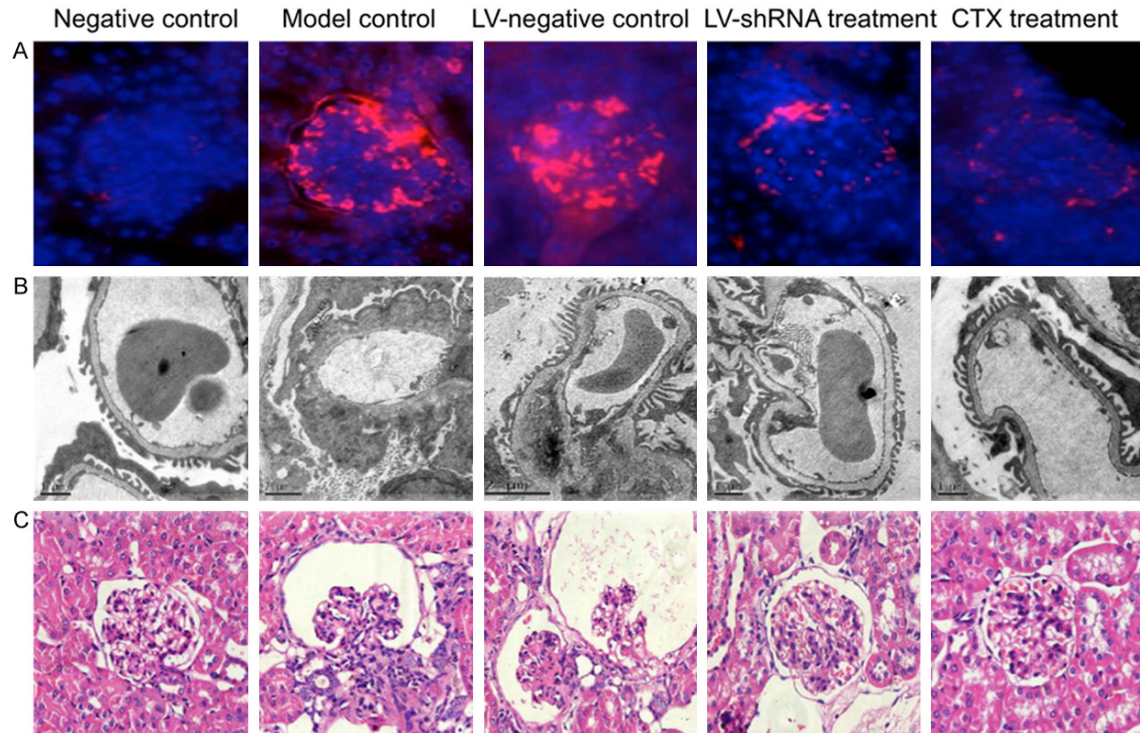


Figure 9. Evaluation of ICs deposition and the severity of nephritis. A. ICs deposition assay demonstrated obvious fluorescent signal was found continuously and linearly distributed in glomerular capillary loops and mesangium in mice kidneys of model control and LV-negative control groups, and treatment with lentivirus-shRNA (425) or CTX substantially reduced the IC deposits as only slight linear and granular fluorescent signal could be detected. B. Ultra-structural analysis of kidneys by TEM showed clear changes of ultrastructure in model control group including GBM and capillary walls thickening, mesangial interposition and expansion, podocytes fusion, electron dense deposits formation and alleviation of pathological changes when intervened by lentivirus-shRNA (425) or CTX. C. H&E staining of renal tissues showed mice model induced by Pristane developed marked diffused glomerulonephritis with infiltration of inflammatory cells, enlargement of glomerulus and necrosis of glomerular capillary loops. Mice treated by lentivirus-shRNA (425) or CTX showed obvious reduction in severity of nephritis ($\times 400$).

the intervening effects on those changes, we examined the production of inflammatory cytokines and auto-antibodies representing T- and B-cell function, respectively. IFN- γ was approved of vital role for the expression auto-antibodies in pristane induced lupus nephritis model [38]. Other works demonstrated both Th1/Th2 cytokines participate in the pathogenesis in the experimental SLE [39]. Our results revealed that both of IFN- γ and IL-4 were up-regulated at the 8th month and diminished concentrations of IFN- γ and IL-4 in serum were found after treatment with lentivirus-shRNA (425), which confirmed the inhibition of up-regulated Th1 and Th2 cytokines secretion induced by pristane. Considering the results may just reflect a state of Th1/Th2 at specific time point, further studies should be considered to comprehensively evaluate the dynamic changing process of Th1/Th2 cytokines.

Auto-antigens caused by disturbances of apoptotic and necrotic cell clearance would induce the production of auto-antibodies in the context of hyper-function of immune cells and abnormal Th1/Th2 response. As nucleosomes (including its constituents) being reported as the main auto-antigens [40, 41], we chose ANA and anti-dsDNA antibody as markers [42] to examine the status of auto-antibodies expression in the present study. Our data showed that ANA and anti-dsDNA antibody emerged after the third month with the positive rates and increased titers. The staining type of ANA demonstrates the existence of homogeneous, speckled, cytoplasmic, and nucleolar auto-antibodies which is consistent with previous report [43]. Auto-antibody levels are diminished after treatment with lentivirus-shRNA (425). We conclude inhibition of B7-2/CD28 signal by lentivirus-mediated gene silencing suppresses

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T-cells activation and T-cell depended B-cells function.

Deposition of immune complex caused by binding of auto-antibodies to chromatin fragments released from apoptotic cells in kidneys and cross-reacting with intrinsic antigens of resident renal cells will lead to acquired renal DNase1 deficiency and positive feedback the ICs deposition [44-46]. In our study, obvious ICs was found deposited in glomerular and mesangial region of mice in model group, and lentivirus-shRNA (425) showed significant effects of decreasing the ICs deposition with less immunofluorescence signal and less electron dense deposits in kidney tissues. Formation of IC deposits in kidneys contributes to increase chemokine receptor and cytokine expression of resident renal cells and then precedes inflammatory cell infiltration (e.g., T cells, macrophages, and neutrophils), proteinuria development and end-stage glomerular injury. Our results demonstrate lentivirus-shRNA (425) reduced the progress and severity of proteinuria production in the pristane induced lupus nephritis. In addition, renal pathological and ultrastructural analysis further confirmed that lentivirus-shRNA (425) treatment relieved inflammatory cell infiltration and glomerular necrosis. In this study, we also found the intervening treatment with lentivirus-shRNA (425) was inferior to CTX since CTX could lead to nonspecific immune suppression. Also, other alternative co-stimulatory pathways, such as B7-1/CD28 and OX40/OX40L, could promote the activation of immune response after lentivirus-shRNA (435) treatment [47].

In conclusion, specific lentivirus-based shRNA targeting B7-2 is capable of inhibiting B7-2 mediated signaling pathway to decrease the activation of immune response and ameliorate pathological injury of kidney in a murine model of lupus nephritis. This study lays the groundwork for using lentivirus-based shRNA targeting specific molecules to suppress co-stimulatory signals as a useful therapeutic approach for SLE treatment.

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Disclosure of conflict of interest

None.

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