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Lentivector-mediated RNAi Efficiently Downregulates Expression of Murine TNF-α Gene *in vitro* and *in vivo*[*]

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Summary: In order to explore the role of TNF-α in Niemann-Pick type C (NPC) disease, lentiviral-delivered RNA interference (RNAi) was used to silence the expression of murine TNF-α gene *in vitro* and in npc mice. Interference efficiency of the lentivirus expressing TNF-α-siRNA, previously constructed with the concentration of 2×10^8 ifu/mL, was determined by RT-PCR and ELISA in BV-2 cells and astrocytes. At the same time, the constructed Lenti-TNF-α-siRNA was intracerebroventricularly infused into 4-week old npc mice for a 4-week period, and the mice were divided into 3 groups: Lenti-TNF-α-siRNA (n=6), control lentivirus (n=6), and NPC mice without any intervention (n=4). By using immunohistochemistry and real-time PCR, the down-regulation of the target genes was detected. The Lenti-TNF-α-siRNA downregulated the expression of murine TNF-α gene efficiently *in vitro* and the interference efficiency was 66.7%. Lentivirus could be expressed stably for long-term in the npc mice brain. Immunohistochemistry and real-time PCR revealed that, as compared with non-intervention group and Lenti-control group, Lenti-TNF-α-siRNA efficiently down-regulated the expression of murine TNF-α gene with the interference efficiency being 66.9%. TNF-α-siRNA downregulated the expression of TNF-α gene *in vitro* and *in vivo*, which provided a potential tool for studying and treating neurodegenerative diseases and TNF-α-related diseases.
Key words: lentivirus; TNF-α; neurodegenerative disease; RNA interference; npc mice

Niemann-Pick disease type C (NPC) is an autosoaml recessive inherited neurovisceral storage disease caused by mutations in NPC1 or NPC2 gene[1, 2]. The main characteristics of neuropathology are extensive progressive neuronal degeneration and death, lipid storage and neurofibrillary tangles (NFTs). Clinically, the patients show progressive loss of neuronal function and disturbance of intelligence, and die in the second or third decade. Balb/c nih npc-1 mice (npc mice) have natural mutation in the NPC1 gene and recapitulate the neurological and neuropathological phenotype of the human disease (except NFTs). Therefore, npc-1 mice are good models *in vivo* of NPC and even Alzheimer disease (AD)[3, 4].

Our previous studies showed that the glia cells in npc mice were abnormally proliferated, activated, and released TNF-α[5]. TNF-α plays an important role in the neurodegenerative process of neurodegenerative diseases including AD, Parkinson disease (PD), NPC, etc. It has been shown that the proliferated and activated glia cells release some bioactive substances such as TNF-α, IL-6 and glumatic acid, which in turn aggravate the damage of neurons[5, 6]. A recent small and open-label pilot study suggested that inhibition of the inflammatory cytokine TNF-α utilizing the perispinal administration of etanercept may lead to sustained cognitive improvement for 6 months in patients with mild, moderate, and severe AD[7]. In our study, the lentivirus carrying TNF-α-siRNA was used to infect the cultured BV-2 cells and astrocytes, and the interference efficiency was tested. In addition, the lentivirus was injected into the lateral ventricule of the 4-week-old npc mice. The expression of the lentivirus in brains of mice and the effects were observed.

1 MATERIALS AND METHODS

1.1 Npc-1 Mice

A breeding pair of heterozygous npc1 mice of the BALA/c^{nih} strain were obtained from the Jakson Laboratory (Bar Harbor, ME, USA) and bred to generate the homozygous mice for this study. Genotyping was done by PCR at 3-weeks old mice. The npc-/- mice were randomly divided into 3 groups: non-intervention (n=4), control lentivirus (n=6), and Lenti-TNF-α-siRNA (n=6).

1.2 Lentivirus

The previously constructed lentivirus stocks were generated. The sequence of TNF-α-siRNA was GTAC-

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TTAGACTTTGCGGAG, and that of the control-siRNA was TTCTCCGAACGTGTCACGT. They were cloned into linearized pSIH1-H1-copGFP siRNA vector containing H1 promotor that is a dependent form of pol Ⅲ and infects both dividing and non-dividing cells. The recombinants were transfected by lipofectamine into 293T cells to generate lentivirus with the following concentrations: 1×10^8 ifu/mL for Lenti-control-siRNA; 2×10^8 ifu/mL for Lenti-TNF-α-siRNA. The Lenti-TNF-α- siRNA was diluted to 1×10^8 ifu/mL in accordance with the control.

1.3 Antibodies

TNF-α (goat IgG), glial fibrillary acidic protein (GFAP, rabbit IgG) and OX42 (CD11b, mouse IgG1) were purchased from Santa Cruz Biotechnologies Inc. (USA). Alexa Fluor 596 (red, 1:500) and Alexa Fluor 488 (green, 1:500) were the products of Amersham Biosciences and Molecular Probes (USA).

1.4 Lentiviral Infection and Functional Identification *in vitro*

One day before infection, 1×10^6 BV-2 cells and astrocytes were cultured in 6-well plates and the cells were 30% confluent at the time of infection. Twenty μL Lenti-TNF-α-siRNA and control lentivirus were added respectively to each well containing cells and medium. The GFP expression was detected under an inverted fluorescent microscope 72 h after infection. Part of the BV-2 cells was harvested and the level of TNF-α mRNA was detected by RT-PCR, using a pair of primers: for TNF-α, forward, ACCAGAGCGGCAAGAAGAACCA T; reverse, CATCAGACAT CGGAGGCAGGAAG. At 48 h after infection, part of the BV-2 cells was experienced oxygen deprivation for 24 h, and TNF-α production in the culture supernatant was assessed by ELISA.

1.5 Intracerebroventricular Injection

Intracerebroventricular injection was done on 4-week-old mice by a microsyringe. The mice were anesthetized with ketamine, the bregma was exposed, and the puncture point located 1.5 mm behind the bregma, 1 mm right to the middle line and 3 mm deep. Four microliters of lentivirus were injected into the lateral cerebral ventricle and were delivered at a rate of 0.5 μL/min. Four weeks later, the animals were anesthetized and decapitated, and the whole brains were removed carefully. The right halves of the brains were embedded in OCT glue, and quickly frozen in isopentane for immunohistochemistry. The frozen tissues were cut into 10-μm thick sections per sagittal section by using a cryostat at –18°C. The left halves of the brains were frozen at –80°C for RT-PCR and immunoblotting analysis.

1.6 Immunohistochemistry

Immunohistochemical analysis of mouse brain sections was done as described before[8]. The brain sections were fixed in 4% PFD, incubated with 0.25% Triton-X100, and blocked. The sections were incubated with primary antibodies at 4°C overnight. Flourescent secondary antibodies were then added to the slides for 60 min at room temperature. The sections were counterstained with DAPI and photographed under the flourescence microscope.

1.7 Statistical Analysis

Statistical analyses were carried out with Student's *t* -test using Microsoft Excel. Significant differences were accepted when *P* values were less than 0.05.

2 RESULTS

2.1 Stable TNF-α Gene Silencing *in vitro*

To examine the efficiency of TNF-α-siRNA, cultured BV-2 cells and astrocytes were divided into 3 groups. The first group was npc mice without any intervention, and the control group and the experimental groups were injected with either control lentivirus or Lenti-TNF-α-siRNA expressing nonsense sequence or TNF-α hairpin RNA sequences, respectively. Extensive expression of GFP fusion protein at 72 h after infection indicated that the virus infected the cultured cells successfully (fig. 1A and B). At 72 h after infection, TNF-α mRNA expression was analyzed by RT-PCR (fig. 1C). Compared with the control lentivirus, Lenti-TNF-α-siRNA significantly inhibited the mRNA level of TNF-α. TNF-α mRNA in BV-2 cells was decreased by 33.35% when compared to the control siRNA. Because the transfection efficiency was 50%, so the interfering efficiency was 33.35%/50%=66.7% (fig. 2D). At 48 h after infection with lentivirus, cells were experienced oxygen deprivation for 24 h and TNF-α in the culture supernatant was assessed by ELISA (fig. 1E). TNF-α level of the cells infected with TNF-α-siRNA was decreased significantly, compared with that in the control lentivirus and the blank cells ($P<0.05$).

2.2 Lentivirus Injection into the Npc Mice

To further study the silencing effect *in vivo*, lentivirus was injected into the lateral cerebral ventricles of npc mice. The mice were killed 4 weeks after injection. Infected cells in the brain displayed extensive GFP throughout the whole brain, including the cerebellum, pons and cotex (fig. 2A, B, and C). The infected cells showed lasted, stable GFP expression 1, 2, 3, 4 weeks after injection (fig. 2D, E, F, and G, shown only in pons). We detected the kinds of the infected cells by immunohistochemistry. The Neun-immunoreactive neurons were infected by the lentivirus in the brain (fig. 2H, I, and J). Similarly, OX42-immunoreactive microglias were infected with the lentivirus in the brain (fig. 2K, L, M, and N). The data indicated that the lentivirus infected dividing and non-dividing cells.

2.3 The Silencing Effect in Npc Mice

Flourescence microscopy and double immunofluorescence detection were used to assess the expression of TNF-α in the mouse brain. Double-labeling of OX42 and TNF-α indicated that the expression of TNF-α in the mice injected with TNF-α-siRNA (fig. 3A) was significantly decreased as compared with that of the mice injected with control lentivirus (fig. 3B). Similar results were obtained in double-labeling of GFAP and TNF-α (fig. 3C, and D). TNF-α mRNA of infected cells in the brain was analyzed by real-time RT-PCR (fig. 3E). TNF-α mRNA level in the cells infected with lenti-TNF-α-siRNA was decreased significantly compared to those cells infected with the control lentivirus (fig. 3F). The interference efficiency of lenti-TNF-α-siRNA was 66.9% ($\Delta\Delta ct=0.58$, $2^{-0.58}=0.66896$) and 69.26% ($\Delta\Delta ct=0.53$, $2^{-0.53}=0.69255$) as compared with the control lentivirus and the mice without intervention.

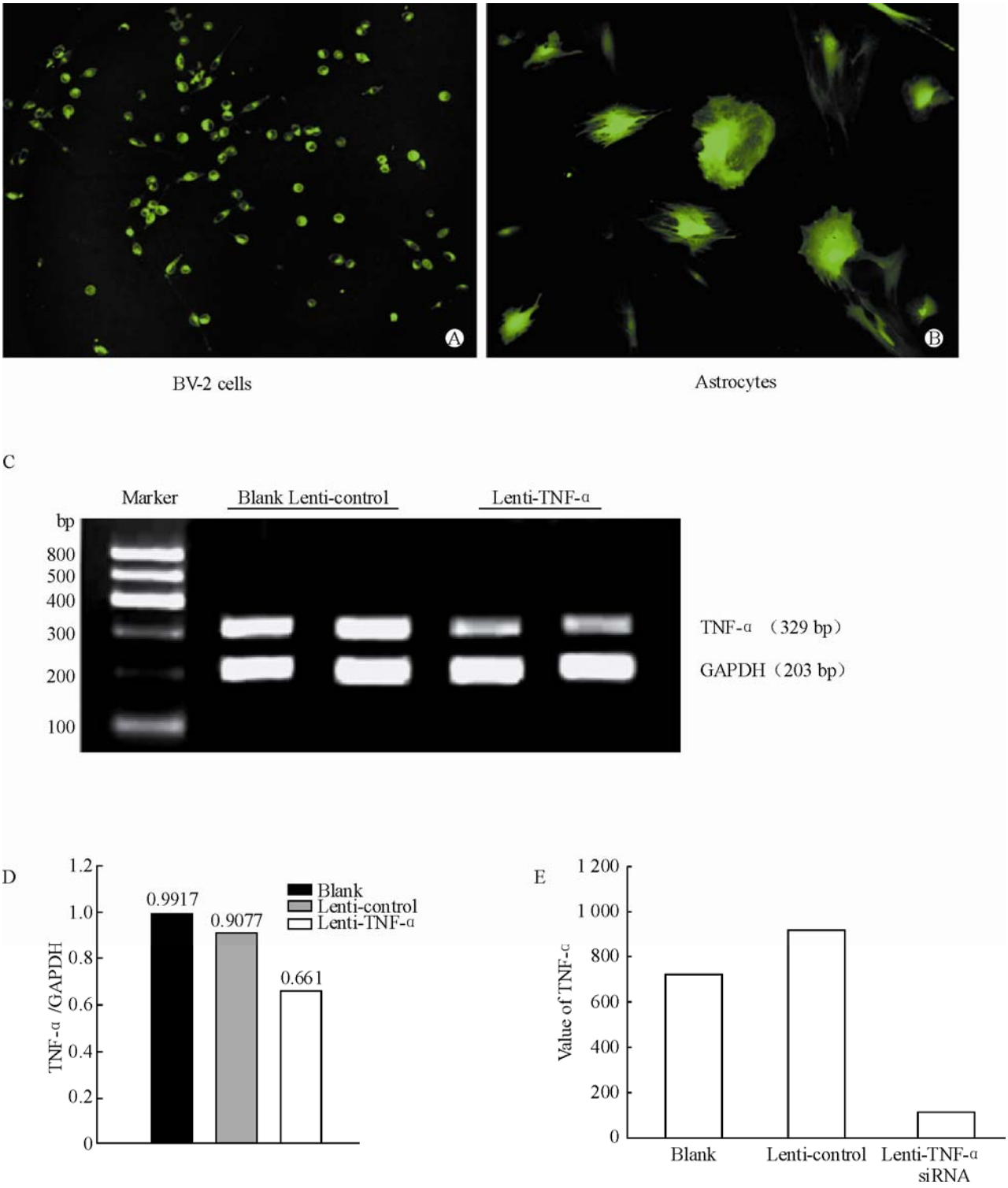

Fig. 1 *In vitro* inhibition of TNF-α gene expression by TNF-α-siRNA

A: Cultured BV-2 cells are infected with either control lentivirus or Lenti-TNF-α-siRNA. Seventy-two h later, cells are viewed under an inverted fluorescent microscope. Data are from one cell-well and are representative of 5 others. B: Similar results of cultured astrocytes. Photomicrographs of A and B are taken at 40 magnification. C: Seventy-two h after infection, total RNA of the cells is isolated using the guanidine isothiocyanate method. TNF-α mRNA is assayed by RT-PCR. The data show the RP-PCR from the 3 groups. The first lane is DNA size marker, the second is blank cells, the third lane is cells infected with the inactive siRNA, and the last two from cells infected with Lenti-TNF-α-siRNA. D: Band intensity is analyzed, ratio of TNF-α/GAPDH is adopted, and the interference efficiency of Lenti-TNF-α-siRNA is 66.7%. E: At 48 h after infection with lentivirus, cells experienced oxygen deprivation for 24 h and TNF-α production in the culture supernatant is assessed by ELISA. TNF-α level of the cells infected with TNF-α-siRNA is decreased significantly compared to that of the control lentivirus and the blank cells, $P<0.05$

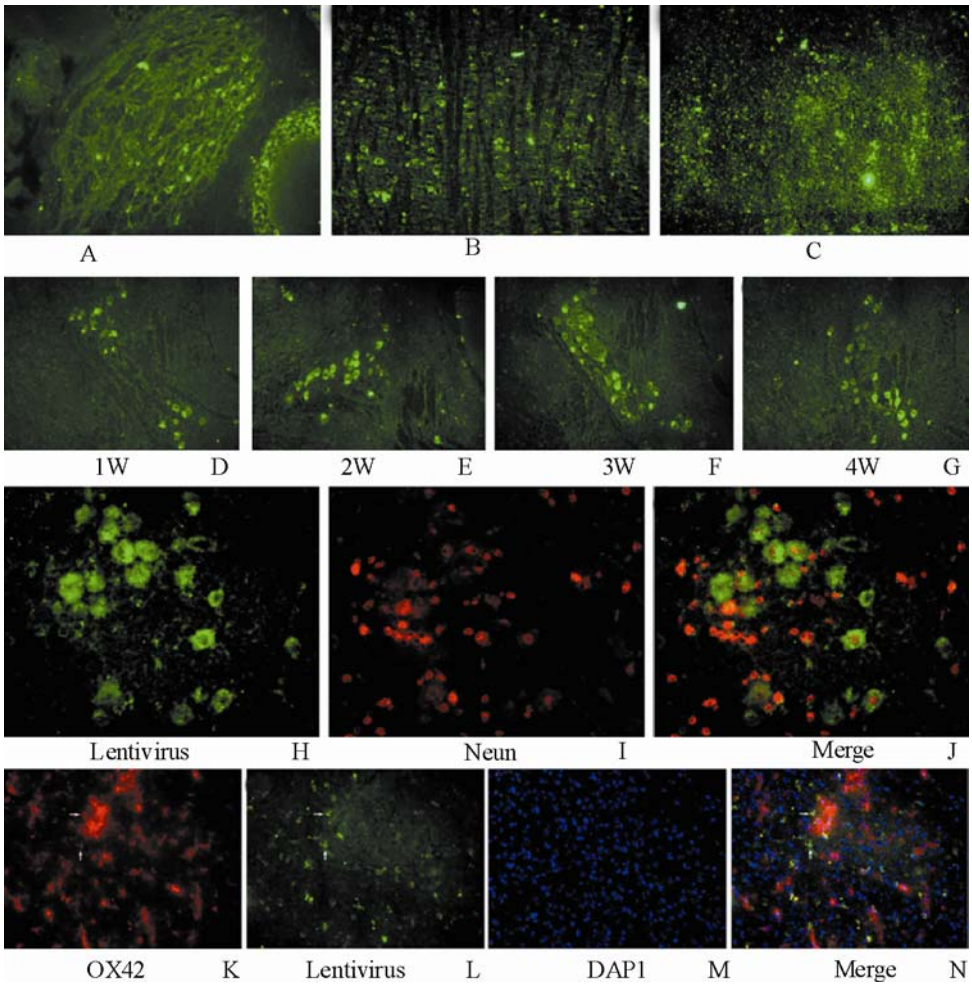

Fig. 2 The extensive and stable expression of lentivirus in brain of npc mice

Mice are divided into 3 groups. The first group without any intervention, and the second and the third groups were injected with either the control lentivirus or Lenti- TNF-α-siRNA, respectively. A, B, and C: the cerebellum, pons and cotex of mice injected with lentivirus, respectively. The data are from one mouse and are representative of others. D, E, F, and G displayed the pons of mice at 1-4 weeks postinjection. H: The GFP expression of lentivirus. I: Immunofluorescence antibody staining for Neun. J: Merge of H and I, displaying Neun-immunopositive neurons are infected with lentivirus. K: The GFP expression of lentivirus. L: Immunofluorescence antibody OX42 staining for microglia. M: DAPI staining of the nuclear. N: Merge of K, L, and M, displaying co-localization of OX42 and GFP expression. All of the photomicrographs are taken at 40 magnification

3 DISCUSSION

TNF-α is a pro-inflammatory/apoptosis cytokine. The previous studies showed that TNF-α played an important role in neurodegenerative diseases such as AD, PD, NPC and ALS. In the pathogenesis of AD, TNF-α is produced by activated microglia[8], mainly in response to Aβ(1-40) and Aβ(1-42) peptides, oxidative stress, glutamate, and LPS[9-11]. It induces neuronal death and damages inflammatory tissue through its receptor TNF-R1[12]. This pathway may be a target for intervention to save the degenerative neurons. Therefore, it may be an effective approach to treat neurodegenerative diseases through silencing TNF-α gene expression.

RNAi has been widely used for controlling gene expression and exploring gene function in the whole genome. RNAi has been successfully used to knockdown definite gene in mammalian cells[13]. Retrovirus and adenovirus are difficult to stably express transfected genes in non-dividing cells such as hematopoietic stem cells, hepatocytes and nerve cells[14]. Lentiviral vector is based on human immunodeficiency virus-1 (HIV-1), which is able to infect dividing and non-dividing cells, integrate into genomic DNA and provide stable, long-term expression of the target gene[15]. Lentiviral vector is the most effective means for gene therapy in nervous system disease.

It has been reported that intraperitoneal injection of anti-TNF-α siRNA inhibited TNF-α gene expression[16]. So far, lentivector-mediated TNF-α-siRNA has not been reported to be used. We have previously constructed Lentivirus of TNF-α-siRNA at the concentration of 2×10^8 ifu/L. In this study, we used Lenti-TNF-α-siRNA to infect BV-2 cells and astrocytes. We found infection of Lenti-TNF-α-siRNA and the down-regulated expression

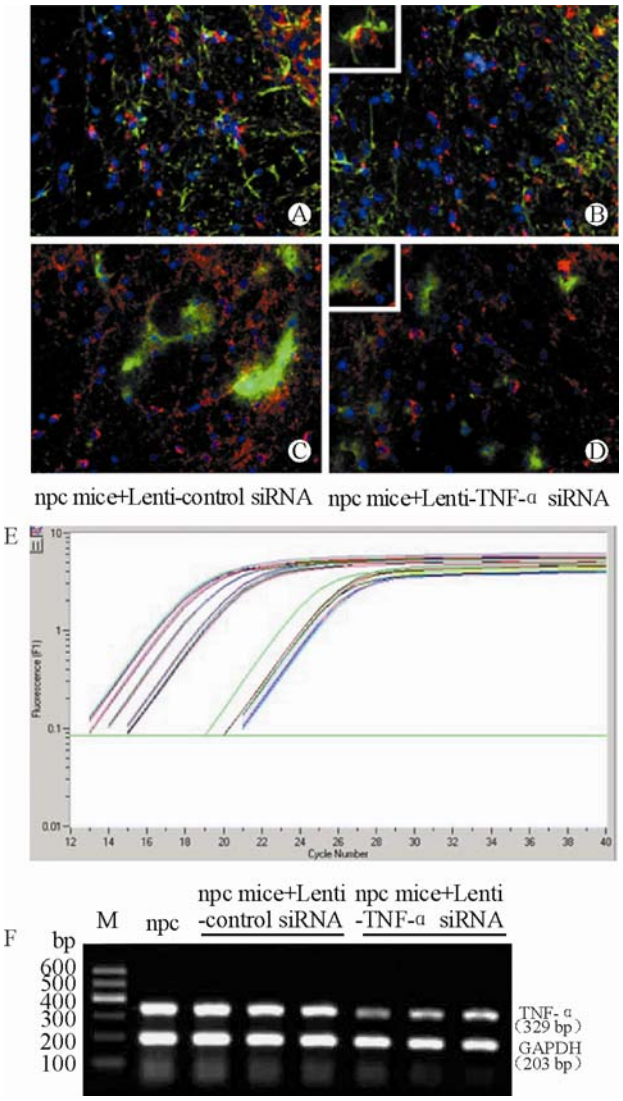

Fig. 3 Lenti-TNF-α-siRNA silencing TNF-α gene efficiently
A and B: Double-labeling of TNF-α and GFAP of mice injected with either control lentivirus or Lenti-TNF-α -siRNA. C and D: Double-labeling of OX42 and GFAP of mice injected with either control lentivirus or Lenti-TNF-α-siRNA. All of the photomicrographs were taken at 40 magnification. E: Real-time RT-PCR amplification of β-actin TNF-α and GAPDH mRNA from control lentivirus and Lenti-TNF-α-siRNA injected mice brains. Total RNA (100 ng) from each mouse in 3 groups was amplified using the MasterAmp™ Real-Time RT-PCR Kit. TNF-α mRNA was assayed by ΔΔct, as the results, the interference efficiency of Lenti-TNF-α-siRNA was 66.9% compared to control lentivirus. F: Electrophoresis of the samples on 1.5% (w/v) agarose gels and visualized by ethidium bromide staining

of murine TNF-α gene efficiently *in vitro*, and the interference efficiency was 66.7%, determined by RT-PCR and ELISA. Lenti-TNF-α-siRNA was also intracerebroventricularly infused into 4-week-old npc mice and the expression of lentivirus in the brains of the mice was observed. The co-localization of Neun or OX42 and lentivirus indicated that lentivirus could infect dividing and non-dividing cells, as the previous study reported[14]; Lenti-TNF-α-siRNA was expressed in mouse brains 1－4 weeks after injection. Double labeling of GFAP and TNF-α as well as OX42 and TNF-α indicated that Lenti-TNF-α-siRNA efficiently down-regulated the expression of murine TNF-α gene, compared with mice with control lentivirus injection and those without intervention. The interference efficiency was 66.9%. At the same time, the expression of GFAP and OX42 was also down-regulated. Further studies to explore the mechanisms by which lentivirus inhibited the activation of glias are required.

In summary, we have demonstrated that TNF-α-siRNA down-regulated TNF-α expression *in vitro* and *in vivo*. TNF-α down-regulation mediated by lentivirus may provide a novel means for treating NPC and other neurodegenerative disorders.

REFERENCES

1 Vanier MT, Suzuki K. Recent advances in elucidating Niemann-Pick C disease. Brain Pathol, 1998,8:163-174
2 Elleder M, Jirasek A, Smid F, *et al*. Niemann-Pick disease type C. Study on the nature of the cerebral storage process. Acta Neuropathol, 1985,66:325-336
3 German DC, Quintero EM, Liang CL, *et al*. Selective neurodegeneration, without neurofibrillary tangles, in a mouse model of Niemann-Pick C disease. J Comp Neurol, 2001,433:415-425
4 Bu B, Klunemann H, Suzuki K, *et al*. Niemann-Pick disease type C yields possible clue for why cerebellar neurons do not form neurofibrillary tangles. Neurobiol Dis, 2002,11:285-297
5 Wu YP, Mizukami H, Matsuda J, *et al*. Apoptosis accompanied by up-regulation of TNF-alpha death pathway genes in the brain of Niemann-Pick type C disease. Mol Genet Metab, 2005,84(1):9-17
6 Bezzi P, Domercq M, Brambilla L, *et al*. CXCR4-activated astrocyte glutamate release via TNF-alpha: amplification by microglia triggers neurotoxicity. Nat Neurosci, 2001,4(7):702-710
7 Tobinick E. Perispinal etanercept for treatment of Alzheimer's disease. Curr Alzheimer Res, 2007,4(5):550-552
8 Butovsky O, Koronyo-Hamaoui M, Kunis G, *et al*. Glatiramer acetate fights against Alzheimer's disease by inducing dendritic-like microglia expressing insulin-like growth factor 1. Proc Natl Acad Sci USA, 2006, 103(31):11 784-11 789
9 Nelson TJ, Alkon DL. Oxidation of cholesterol by amyloid precursor protein and beta-amyloid peptide. J Biol Chem, 2005,280(8):7377-7387
10 Takeuchi H, Jin S, Wang J, *et al*. Tumor necrosis factor-alpha induces neurotoxicity via glutamate release from hemichannels of activated microglia in an autocrine manner. J Biol Chem, 2006,281(30):21 362-21 368
11 Culbert AA, Skaper SD, Howlett DR, *et al*. MAPK-activated protein kinase 2 deficiency in microglia inhibits pro-inflammatory mediator release and resultant neurotoxicity. Relevance to neuroinflammation in a transgenic mouse model of Alzheimer disease. J Biol Chem, 2006,281(33):23 658-23 667
12 Alvarez A, Cacabelos R, Sanpedro C, *et al*. Serum TNF-α levels are increased and correlate negatively with free

IGF-I in Alzheimer disease. Neurobiol Aging, 2007,28(4): 533-536
13 Smith-Arica JR, Bartlett JS. Gene therapy: recombinant adeno-associated virus vectors. Curr Cardiol Rep, 2001, 3(1):43-49
14 Bovia F, Salmon P, Matthes T, *et al*. Efficient transduction of primary human B lymphocytes and nondividing myeloma B cells with HIV-1-derived lentiviral vectors. Blood, 2003,101(5):1727-1733
15 Rubinson DA, Dillon CP, Kwiatkowski AV, *et al*. A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. Nat Genet, 2003,33:401-406
16 Sørensen DR, Leirdal M, Sioud M. Gene silencing by systemic delivery of synthetic siRNAs in adult mice. J Mol Biol, 2003,327(4):761-766

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