Original Article

Downregulating immunogenicity of Schwann cells *via* inhibiting a potential target of class II transactivator (*CIITA*) gene

Yi Yang[*], Wenda Dai[*], Zhengrong Chen, Zuoqin Yan, Zhenjun Yao, Chi Zhang[**]

Orthopedic department, Zhongshan Hospital, Fudan University, Shanghai, China.

Summary   **Immunological rejection induced by allogeneic Schwann cells remains a problem for construction of artificial nerves. Class II transactivator (*CIITA*) gene is a chief regulator of major histocompatibility complex class II (MHC II) molecules which contributes to the immunogenicity of Schwann cells. This study aimed to downregulate MHC II expression by suppressing CIITA expression, therefore reducing the immunogenicity of Schwann cells. Recombinant siRNA expression vectors targeting the *CIITA* gene were produced and subsequently transfected into rat RSC96 Schwann cells. Interferon (IFN)-γ was used to augment immunological rejection of RSC96 cells. The mRNA levels of CIITA and MHC II were assessed by fluorescence quantitative PCR. The protein levels of MHC II were determined using flow cytometry assays. Finally, the immunogenicity of RSC96 cells was analyzed using mixed lymphocytes reactions. Results indicated the expression of MHC II molecules was at a low level in cultured RSC96 cells, while significantly elevated after treatment with IFN-γ. Concurrent treatment with the constructed CIITA siRNAs efficiently downregulated the mRNA levels of CIITA and MHC II in RSC96 cells at 48 h post-transfection. MHC II protein levels were also significantly reduced after CIITA siRNAs transfection. Correspondingly, the immunogenicity of RSC96 cells was significantly downregulated post-transfection. These studies suggest suppressing *CIITA* gene was efficient in reducing MHC II expression and thus decreasing the immunogenicity of rat Schwann cells.**

Keywords: Schwann cells, class II transactivator (*CIITA*) gene, major histocompatibility complex class II (MHC II), RNAi

1. Introduction

Reconstruction of peripheral nerve defects remains a great challenge for surgeons. Nerve autografts are considered the golden standard for clinical treatments in repairing large lesion gaps in the peripheral nervous system; the disadvantages include limited availability of donor nerves and donor site morbidity (*1-3*). Therefore, intensive research has been focused on artificial nerves. However, obtaining an adequate number of autologous Schwann cells for constructing artificial nerves requires much time, which contributes to delaying repair of the peripheral nerve injuries and has a negative impact on

nerve regeneration. As for allogeneic Schwann cells, immunological rejection remains a problem when contributing to construction of artificial nerves (*4-6*).

Schwann cells contribute to immunogenicity of artificial nerve *via* expressing major histocompatibility complex (MHC) I and MHC II antigens (*7,8*). Immunosuppressive agents have been applied to prolong the survival of Schwann cells; however, it has been found that there was extensive loss of regenerated axons in the allograft when immunosuppression was withdrawn (*9*).

Mosahebi *et al.* (*4*) demonstrated that the increase of expression of MHC II at 3 weeks in the conduits containing allogeneic Schwann cells corresponded to an increase of infiltration of T-lymphocytes as well as macrophages. Furthermore, there was a corresponding reduction in X-gal staining at 3 weeks pointing to a rejection process of allogeneic Schwann cells. Therefore, downregulation of the *MHC II* gene is important for survival of Schwann cells.

Class II transactivator (CIITA) is referred to as a

* Both contributed equally to this work.
**Address correspondence to:*
Dr. Chi Zhang, Orthopedic Department, Zhongshan Hospital, Fudan University, Shanghai 200032, China.
E-mail: zhang.chi@zs-hospital.sh.cn

chief regulator of MHC II transcription (*10*). It is also important for both constitutive expression of MHC II in B-cells or dendritic cells as well as cytokine-induced expression of MHC-II in a variety of other cell types including fibroblasts and vascular endothelial cells. Recent research (*11*) showed that disruption of function of CIITA played a beneficial role in preventing normal allogeneic T-cell responses and thus can prolong survival of *CIITA*-deficient hearts as compared to wild-type grafts. Therefore, we believe that *CIITA* is a potential target gene to downregulate immunogenicity of Schwann cells.

The aim of the present study is to investigate the feasibility of downregulating immunogenicity of Schwann cells *via* inhibiting a potential target of the *CIITA* gene, which might contribute to suppress immunological rejection of allogeneic Schwann cells.

2. Material and Methods

2.1. *Cell lines and cell culture*

The rat RSC96 Schwann cells were purchased from the Cell Bank of Chinese Academy of Science (Shanghai, China). Schwann cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Life Technologies, Carlsbad, CA, USA), with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 90% 4.5 g/L glucose and 10% fetal bovine serum, at 37°C in a humidified incubator with 5% CO_2.

2.2. *Construction of siRNA expression vectors*

According to the recommendations of Ambion (Life Technologies) and Genscript (GenScript, Piscataway,

NJ, USA) on the RNAi target sequence, ten pairs of DNA oligonucleotides were designed and synthesized for hairpin RNA expression by Sangon (Shanghai, China) (Table 1). Oligonucleotides were dissolved in sterile, nuclease-free H_2O to a concentration of 3 mg/mL, and kept at −20°C. Oligonucletides were subsequently assembled using an annealing reaction by mixing 1 μL of each oligonucleotide (sense + antisense) with 48 μL annealing buffer. The mixture was incubated at 90°C for 4 min, at 70°C for additional 10 min, and then was slowly cooled to 10°C.

To linearize 1 μL of the pSUPER vector with *Bgl* II and *Hin*d III restriction enzymes, oligonucleotides were inserted into the linearized pSUPER vectors at a molar ratio of 3:1 with the aid of T4 DNA ligase. Colonies were picked randomly. Then the recombinant plasmids were transformed into Top10F competent cells (Novegen, Darmstadt, Germany). The plasmids were extracted and purified, and digested with *Eco* RI and *Hin*d III to confirm the presence of insert.

2.3. *Vectors transfected into rat Schwann cells*

The RSC96 cells were plated at a density of 2×10^5/L per well. When the cell confluence reached 80%, a given amount of each siRNA was mixed with LipofectAMINE 2000 (Life Technologies) for 20 min at room temperature according to the manufacturer's instructions. The mixtures were applied to the cells. After incubation for 9 h at 37°C in a humidified incubator with 5% CO_2, 10^6 U/L IFN-γ was added. Seven groups were set as follows: (*i*) siRNA 1 with interferon (IFN)-γ (10^6 U/L) added, (*ii*) siRNA 2 with INF-γ (10^6 U/L) added, (*iii*) siRNA 3 with INF-γ (10^6 U/L) added, (*iv*) siRNA 4 with INF-γ (10^6 U/L) added, (*v*) nonspecific vector control

Table 1. Interference target gene sequence and oligo-DNA sequence of recombinant vectors

Target gene sequences	Name	Oligo DNA sequences
CTCAACTCAgAgACAgACA 1.Position in gene seqTence: 524 GC content: 42.9%	M1 sense	5'-gATCCCCCTCAACTCAgAgACAgACATTCAAgAgATgTCTgTC TCTgAgTTgAgTTTTTA-3'
	M1 antisense	5'-AgCTTAAAAACTCAACTCAgAgACAgACATCTCTTgAATgTC TgTCTCTgAgTTgAgggg-3'
TCAggAgAgAAgCCTCAgA 2.Position in gene seqTence: 761 GC content: 47.6%	M2 sense	5'-gATCCCCTCAggAgAgAAgCCTCAgATTCAAgAgA TCTgAggCT TCTCTCCTgATTTTTA-3'
	M2 antisense	5'-AgCTTAAAAATCAggAgAgAAgCCTCAgATCTCTTgAATCTgAg gCTTCTCTCCTgA ggg-3'
GTggTCCTCTggTAgCCAT 3.Position in gene seqTence: 1291 GC content: 52.4%	M3 sense	5'-gATCCCCgTggTCCTCTggTAgCCATTTCAAgAgAATggCTACC AgAggACCACTTTTTA-3'
	M3 antisense	5'-AgCTTAAAAAgTggTCCTCTggTAgCCATTCTCTTgAAATggCT ACCAgAggACCACggg-3'
CAgTCCTCCTggAgCCTTA 4.Position in gene seqTence: 2234 GC content: 52.4%	M4 sense	5'-gATCCCCCAgTCCTCCTggAgCCTTATTCAAgAgATAAggCTCC AggAggACTgTTTTTA-3'
	M4 antisense	5'-AgCTTAAAAACAgTCCTCCTggAgCCTTATCTCTTgAATAAggC TCCAggAggACTgggg-3'
CACAgAgTCCATgTCACAA Irrelevant sequence control	NTC sense	5'-gATCCCCCACAgAgTCCATgTCACAATTCAAgAgATTgTgACA TggACTCTgTgTTTTTA-3'
	NTC antisense	5'-AgCTTAAAAACACAgAgTCCATgTCACAATCTCTTgAATTgTg ACATggACTCTgTgggg-3'

with INF-γ (10^6 U/L) added, (*vi*) negative control with INF-γ (10^6 U/L) added, (*vii*) negative control without IFN-γ added. Cells were then cultured for an additional 48 h at 37°C before further analysis. The best siRNA sequence was chosen for *CIITA*.

2.4. *Fluorescence quantitative PCR*

Total RNA of the cells was isolated and collected with RNase-free DNase Set (Qiagen, Valencia, CA, USA). RT-PCR was performed using the RNA PCR kit (TaKaRa, Ver.3.0) and employing 0.4 mg total RNA as the template per time point. For CIITA mRNA amplification, the primers 5'-GCCTGAGATGACCC TGCTGTA-3' and 5'-CAGTTCAAGGTCCAGCATG GT-3' were used. For MHC II mRNA amplification, the primers 5'-GCATACGGCTCGTGATCAGA-3' and 5'-CCCACGTCGCTGTCGAA-3' were used. Cycling conditions were as follows: 90 sec at 95°C; followed by 40 cycles of 5 sec at 95°C, 30 sec at 58°C, and 1 min at 95°C, 1 min at 58°C; a touchdown (0.5°C/cycle) annealing for 10 sec, with the last cycle concluding with a reaction for 7 min at 72°C. The obtained PCR products were separated using 1.5% agarose gel electrophoresis, analyzed by AlphaImager 2000 (Alpha Innotech Corporation, San Leandro, CA, USA), and quantitated by a digitalized software (Kodak Digital Science™ ID Image Analysis Software; Eastman Kodak Co., Rochester, NY, USA).

2.5. *Flow cytometry*

RSC96 Schwann cells were collected and washed in phosphate buffered saline (PBS); cell concentration was adjusted to $5 \times 10^6 - 1 \times 10^7$/mL. Cells were stained with specific MHC II antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and then washed with PBS. After washing, the cells were stained with goat anti-rat fluorescent antibody, and fixed in 10 mL of ice-cold 75% ethanol. After 24 h of incubation at 20°C, cells were washed twice in PBS and resuspended in 3 mL of PBS for 5 min. Three-color flow cytometry was employed using an Enzymatic Amplification Staining Kit (Flow-Amp Systems, Tebu-bio, Le Perray en Yvelines, France) (*12*).

2.6. *Mixed lymphocytes reaction*

Normal peripheral blood mononuclear cells (PBMCs) were isolated from heparinized, vacutainer-collected peripheral blood using Ficoll-Hypaque density gradient centrifugation at 2,000 rpm for 10 min. Stimulative cells were subgrouped as (*i*) Schwann cells without IFN-γ treatment; (*ii*) IFN-γ-treated Schwann cells (negative control); (*iii*) IFN-γ-treated Schwann cells transfected with siRNA for 24 h; (*iv*) IFN-γ-treated Schwann cells transfected with siRNA for 48 h. Cell concentration

was 10^6 cells/100 μL. PBMCs and stimulative cells were mixed and cultured for 3 days. Then mononuclear cell proliferation was detected by MTT assay. Briefly, 20 μL of MTT (Sigma-Aldrich, St. Louis, MO, USA) at 5 mg/mL was added into each well and incubated for 4 h in a 37°C, 5% CO_2 and 90% humidity incubator. The medium was then removed and 150 μL DMSO (Fisher Scientific, Loughborough, UK) was added to each well to extract and solubilize the formazan crystal by incubating for 10 min. Finally, the plate was read at 570 nm using a microplate photometer (Multiskan Ascent, Thermo Fisher Scientific, Waltham, MA, USA).

2.7. *Statistical analysis*

Data are displayed as mean ± SD, SPSS 11.5 (SPSS Inc., Chicago, IL, USA) and one-way analysis of variance (ANOVA) tests were used for statistical analysis. $p < 0.05$ was considered statistically significant.

3. Results

3.1. *CIITA and MHC II mRNA expression after siRNA transfection*

The mRNA level of CIITA and MHC II were measured by fluorescence quantitative PCR. IFN-γ treatment of Schwann cells elevated the expression of *CIITA* and *MHC II* genes. Forty eight hours after transfection, the mRNA level of CIITA and MHC II were significantly decreased in all siRNA groups, compared to the control group (Table 2). However, the difference between non-specific vector controls and the negative control group was not significant ($p > 0.05$). Among the four siRNA groups, the siRNA group 2 was the most efficient, mRNA levels of CIITA and MHCII decreased by an average of 88.32 ± 0.93% and 86.54 ± 0.69%, respectively. Thus we chose siRNA 2 as interference vector in the subsequent experiments.

3.2. *MHC II protein expression after siRNA transfection*

The effect of transfection on MHC II expression on Schwann cells was assessed by flow cytometry. Results are shown in Table 3. The expression of MHC II of Schwann cells was at a low level without IFN-γ treatment, however, the expression of MHC II increased significantly after exposure to IFN-γ. Forty eight hours after transfection, the protein expression of MHC II was decreased significantly compared to the control group ($p < 0.01$).

3.3. *Mixed lymphocyte reaction*

Schwann cells without IFN-γ treatment stimulated a low level of PBMCs proliferation, while IFN-γ-treated Schwann cells stimulated a higher level of PBMCs proliferation. After transfection with CIITA siRNA for

Table 2. Levels of expression of CIITA mRNA and MHCII mRNA in Schwann cells after transfection with different siRNA sequences

Group	CIITA copies (copies/10^6 GAPDH)	CIITA mRNA vs. negative control (%)	MHC II copies (copies/10^6 GAPDH)	MHC II mRNA vs. negative control (%)
1. Negative control with INF-γ (10^6 U/L) added	$5.63 \pm 0.31 \times 10^4$	100	$2.66 \pm 0.31 \times 10^4$	100
2. Nonspecific vector control with INF-γ (10^6 U/L) added	$5.47 \pm 0.26 \times 10^4$	98.3 ± 1.0	$2.47 \pm 0.66 \times 10^4$	93.2 ± 5.6
3. siRNA 1 with IFN-γ (10^6 U/L) added	$1.56 \pm 1.35 \times 10^4$ *	28.4 ± 3.4	$8.52 \pm 0.73 \times 10^3$ *	32.7 ± 1.1
4. siRNA 2 with INF-γ (10^6 U/L) added	$6.25 \pm 0.51 \times 10^3$ **	12.3 ± 0.6	$3.19 \pm 0.12 \times 10^3$ **	12.5 ± 1.8
5. siRNA 3 with INF-γ (10^6 U/L) added	$8.77 \pm 0.38 \times 10^3$ **	16.4 ± 0.8	$4.67 \pm 0.38 \times 10^3$ **	18.5 ± 2.8
6. siRNA 4 with INF-γ (10^6 U/L) added	$1.51 \pm 0.86 \times 10^4$ *	27.5 ± 2.4	$7.51 \pm 0.64 \times 10^3$ *	27.7 ± 2.8
7. Negative control without IFN-γ added	$7.46 \pm 0.11 \times 10^2$	1.4 ± 0.4	$8.35 \pm 0.23 \times 10^2$	3.6 ± 0.6

Mean ± SD, $n = 3$. * $p < 0.05$, ** $p < 0.01$.

Table 3. Flow cytometry analysis of MHC II molecules expression by Schwann cells transfected with CIITA siRNA

Group	MHC II molecules on Schwann cells	Inhibitory rate (%)
1. Schwann cells without IFN-γ treatment	135 ± 25	---
2. IFN-γ-treated Schwann cells (negative control)	847 ± 84	---
3. IFN-γ-treated Schwann Cells were transfected with siRNA for 24 h	646 ± 106	23.6 ± 11.2
4. IFN-γ-treated Schwann Cells were transfected with siRNA for 48 h	152 ± 32	81.6 ± 3.1*

Mean ± SD, $n = 3$. * $p < 0.05$.

Table 4. Inhibitory rate of PBMCs proliferation after siRNA in mixed lymphocytes reactions assay

Group	OD$_{570}$	Inhibitory rate (%)
1. Schwann cells without IFN-γ treatment	0.32 ± 0.07	---
2. IFN-γ-treated Schwann cells (negative control)	1.43 ± 0.65	---
3. IFN-γ-treated Schwann cells were transfected with siRNA for 24 h	0.76 ± 0.25	43.2 ± 22.9
4. IFN-γ-treated Schwann cells were transfected with siRNA for 48 h	0.47 ± 0.17	75.9 ± 20.8*

Mean ± SD, $n = 3$. * $p < 0.05$.

24 h, the proliferation of PBMCs was slightly inhibited compared to the control group (inhibitory rate $43.2 \pm 22.9\%$, $p > 0.05$). After RNAi for 48 h, the proliferation was decreased significantly (inhibitory rate $75.9 \pm 20.8\%$, $p < 0.05$). Results are presented in Table 4 and Figure 1.

4. Discussion

Schwann cells are critical for nerve regeneration, and are the major cells contributing to the immunological rejection of nerve allografts because of MHC expression (*13*). Schwann cells can act as nonprofessional antigen presenting cells (APC) under certain conditions, and can activate T cells *in vitro* in an antigen-specific and MHC-restricted manner (*14,15*). Allogeneic Schwann cells seem to induce the upregulation of inflammatory cytokines such as IFN-γ, which are known to participate in immunological rejection. The authors have observed low level expression of MHC II molecules on cultured rat Schwann cells, and the present study confirmed that rat Schwann cells can be induced by IFN-γ to express a high level of MHC II molecules.

The expression of classical and nonclassical *MHC II* genes is regulated primarily by CIITA (*10*), which is achieved by three independent CIITA promoters (pI, pIII, and pIV), promoter pIV can be activated by IFN-γ. CIITA is the chief regulator of *MHC II* gene transcription and MHC II-restricted antigen presentation. The present study confirmed that there was a clear correlation between *MHC II* genes and the *CIITA* gene, the expression of MHC II molecules and the *CIITA* gene on Schwann cells increased simultaneously followed by IFN-γ induction. The *CIITA* gene is an ideal target for inhibiting the expression of *MHC II* genes.

RNA interference can inhibit the expression of the *CIITA* gene. In the present study, the pSUPER plasmid was used as the vector. The plasmid contained the RNA polymerase III H1 promoter, and could transcribe the hairpin RNA with a 9-nt stem-loop structure which can produce the target sequence. After transfection with pSUPER recombinant vectors (*16*), the induced MHC II expression on cell surface was significantly inhibited, and the CIITA mRNA level was also decreased. The expression of CIITA and MHC II were significantly inhibited after transfection for 48 h, indicating that 48 h was the optimal time for RNA interference. We also found that the inhibitory degree of *MHC II* gene expression was lower than that of the *CIITA* gene; therefore, we speculated that the *MHC II* gene may be also regulated by an additional regulation approach
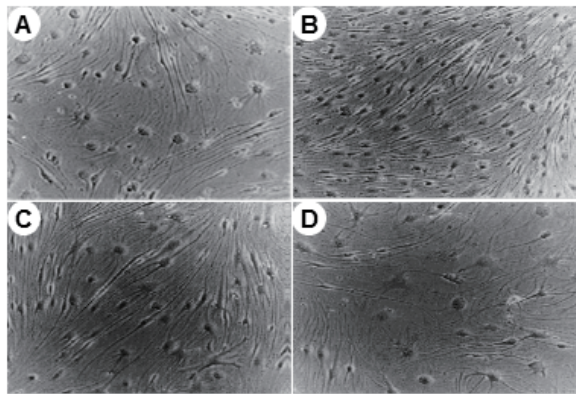
Figure 1. Mixed lymphocytes reaction. Untreated Schwann cell stimulated low level PBMCs proliferation **(A)**; IFN-γ-treated Schwann cell stimulated high level of PBMCs proliferation **(B)**; Proliferation of PBMCs was slightly decreased after siRNA for 24 h **(C)**; Proliferation of PBMCs was significantly decreased after siRNA for 48 h **(D)**.

except for *CIITA*.

Mixed lymphocyte reaction is an assay to detect response of alloreactive T cells to foreign MHC molecules and is used as a predictive test for T cell-mediated graft rejection (*17-20*). In the present study, a one-way mixed lymphocyte reaction was applied. PBMCs were used as reaction cells, and Schwann cells were used as stimulative cells. Alloantigen recognition of mixed lymphocyte reaction *in vitro* occurs *via* a direct recognition pathway, which plays a dominant role in acute rejection. The results of mixed lymphocyte assays demonstrated that the immunogenicity of rat Schwann cells increased after exposure to IFN-γ, and there was a decline in the immunogenicity after 48 h of transfection, which confirmed that the immunogenicity of Schwann cells depended on the expression of MHC II molecules. In direct recognition, alloreactive T cells are stimulated by donor APCs which express the allogeneic MHC, however, in the present study, PBMCs were stimulated successfully without the existence of any professional donor APCs. Therefore, we believethat Schwann cells may be able to take up antigens and act as non-professional APCs with IFN-γ treatment.

In conclusion, the pSUPER recombinant vectors targeting the *CIITA* gene can specifically suppress gene expression of CIITA and MHC II and thus significantly downregulate the immunogenicity of rat Schwann cells *in vitro*. Further experimentation will be needed to determine whether the decline in the immunogenicity of Schwann cells can be sustained.

Acknowledgments:

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