

Recombinant Sj16 from *Schistosoma japonicum* contains a functional N-terminal nuclear localization signal necessary for nuclear translocation in dendritic cells and interleukin-10 production

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Received: 16 August 2016 / Accepted: 2 September 2016 / Published online: 17 September 2016
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Abstract Sj16 is a *Schistosoma japonicum*-derived protein (16 kDa in molecular weight) that has been identified as an immune modulation molecule, but the mechanisms of modulation of immune responses are not known. In this report, we aimed to investigate the host immune regulation mechanism by recombinant Sj16 (rSj16) and thus illuminate the molecular mechanism of immune evasion by *S. japonicum*. The effect of rSj16 and rSj16 mutants on the biology of dendritic cells (DCs) was assessed by examining DC maturation, cytokine production, and expression of surface markers by flow cytometry and enzyme-linked immunosorbent assay. We found that rSj16 significantly stimulated interleukin (IL)-10 production and inhibited LPS-induced bone marrow-derived dendrite cell (BMDC) maturation in a dose-dependent manner. By using antibody neutralization experiments and IL-10-deficient (knockout) mice, we confirmed that the inhibitory effect of rSj16 on LPS-induced BMDCs is due to its induction of IL-10 production. To understand how

rSj16 induces the production of IL-10, we analyzed the protein sequence and revealed two potential nuclear localization signals (NLS) in Sj16. The N-terminal NLS (NLS1) is both necessary and sufficient for translocation of rSj16 to the nucleus of BMDCs and is important for subsequent induction of IL-10 production and the inhibition of BMDC maturation by rSj16. The results of our study concluded that the ability of rSj16 to inhibit DC functions is IL-10 dependent which is operated by IL-10R signal pathway. This study also confirmed that NLS is an important domain associated with increased production of IL-10. Our findings will extend the current understanding on host-schistosome relationship and provide insight about bottleneck of parasitic control.

Keywords *Schistosoma japonicum* · rSj16 · IL-10 · Nuclear localization signal

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Introduction

Schistosomiasis, which is caused by infection with parasitic flatworms, remains a public health burden in many developing countries in the tropics and subtropics (Uttinger et al. 2005). Effective control and treatment rely on chemotherapy by using praziquantel, which remains the only drug in the mainstay of current medical treatment (Harder 2002). However, praziquantel does not prevent reinfection, and resistant strains may develop in the future. Development of a vaccine is ultimately required, and, as yet, no effective vaccines are available (Mackinnon et al. 2008). The most important reason for the lack of a vaccine is that the mechanisms of immune evasion and immune modulation by schistosomes are not clear. Unlike in some other infectious diseases, natural infection by schistosomes hardly induces

any protective immunity. Based on their capacity to withstand to the immune system's attack, schistosomes can develop and ensure long-term survival within a host, allowing continued transmission. It is thought that coevolution of schistosoma together with the immune systems of their hosts has equipped the schistosome with many ingenious strategies to modulate the host immune environment, such as releasing active (cytokine-like) molecules (Maizels et al. 2004). The most accepted mechanism of immune modulation involves the establishment of a balanced immunological state in the host, often termed a “modified T helper type 2 (Th2) response” that facilitates the long reproductive phase of the parasite (Loes et al. 2012). Interleukin (IL)-10, known as cytokine synthesis inhibitory factor (CSIF), which is an important regulator of Th2 response, downregulates Th1 and Th17 response-driven inflammatory reactions and prevents tissue damage and immune pathology during parasite infection (Gazzinelli et al. 1996; Hawrylowicz 2005; O'Garra et al. 2008). Some experiments in patients have shown that there was a positive association between both schistosome egg-induced and adult-induced IL-10 and grade I fibrosis at a level that has also been described for severe fibrosis in *Schistosoma mansoni* infection (de Jesus et al. 2004). IL-10 has been implicated in the regulation of Th1 and Th2 responses and is considered to be a host-protective cytokine during schistosomiasis (Hoffmann et al. 2002). Thus, recognizing how schistosomes, especially some schistosome-derived proteins, induce high IL-10 production and modulate the immunity of the host will be useful for revealing the mechanisms of immune escape of *Schistosoma japonicum*.

Previously, our group has identified a 16-kD secretory protein, Sj16, from *S. japonicum*. Sj16 is produced and secreted by all stages of the parasite and was confirmed as an important protein in alleviating inflammation damage when cercariae penetrated into the skin and was intimately involved in the immune escape of the schistosoma (Hu et al. 2008b). By using recombinant Sj16 (rSj16) expressed in *Escherichia coli*, we demonstrated that this protein has a potent immunomodulatory effect and significantly alleviated rat adjuvant-induced arthritis (AA) (Sun et al. 2010). Recently, we have observed that treatment of immature dendritic cells (DCs) with rSj16 can significantly induce IL-10 production and Th2-type responses. The cells show an increase in expression of costimulatory molecules or cytokines, while lipopolysaccharide (LPS)-induced activation, including expressions of major histocompatibility complex (MHC)-II and costimulatory molecules, as well as IL-12 production, is suppressed. However, since there are no known homologs of Sj16 in metazoans outside the genus *Schistosoma*, the relationship between Sj16 function and IL-10 production is unclear. In the present study, by using antibodies and an IL-10^{-/-} mouse model, we confirmed that the inhibitory effects of rSj16 on DCs are IL-10 dependent; further, we identified the key structural domain in the protein and possible molecular mechanisms.

Materials and methods

Ethics statement

The animal experiments in this study were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Ministry of National Institutes of Health (GB 14922.2-2011). Procedures involving vertebrate animals were reviewed and approved by Sun Yat-sen University's Animal Care and Use Committee, and permission for sampling from vertebrates was obtained from the funding project committee and Sun Yat-sen University's Animal Care and Use Committee (no. 2014031).

Animals and cell lines

IL-10^{-/-} (knockout (KO) mice in the C57BL/6 background and Do11.10 OVA323–339-specific T cell receptor (TCR)-transgenic mice with C57BL/6 background were obtained from the Chinese Academy of Sciences (Shanghai, China). C57BL/6 mice weighing 20–25 g were obtained from the Center of Experimental Animals, Sun Yat-sen University (Guangzhou, China) and used as a wild-type (WT) control. All animals were kept (four per cage) under a 12/12-h light/dark cycle in germ-free surroundings with free access to a standard pellet diet and tap water. They were acclimatized for 3–4 days before experiments. Human cervical carcinoma cell line (HeLa cell line) was obtained from American Type Culture Collection (ATCC, USA).

Antibodies and reagents

LPS (*E. coli* 055:B5), CCK8, mouse β -actin protein, and complete Freund's adjuvant were purchased from Sigma-Aldrich (CA, USA). PE-conjugated anti-CD86 Ab, FITC-conjugated anti-CD80 Ab, PE-conjugated anti-CD40 Ab, antigen-presenting cell (APC)-conjugated anti-CD11c Ab, and PE-conjugated anti-MHC class II were purchased from eBioscience (UK). Mouse enzyme-linked immunoabsorbent assay (ELISA) kits for detection of IL-12p40, IL-12p70, IL-4, IL-6, IL-10, IL-17, and interferon (IFN)- γ were purchased from Biosource (San Francisco, CA, USA).

Expression of recombinant Sj16

Purified rSj16 protein was produced as previously described (Hu et al. 2008a). Briefly, pGEX-4T-1-Sj16 was transformed into *E. coli* BL21 (DE3). rSj16 was expressed as a glutathione S transferase-Sj16 (GST-Sj16) fusion protein upon isopropyl- β -D-thiogalactopyranoside induction. Recombinant protein was purified by using a GSTrapTM Fast-Flow resin column (Amersham Pharmacia, USA), followed by thrombin (Sigma) cleavage. The cleaved protein was excised from

sodium dodecyl sulfate polyacrylamide gel electrophoresis and confirmed by mass spectrometry. Protein concentration was determined by Bradford assay (Bradford 1976). The protein was treated with AffinityPak™ Detoxi-Gel™ Endotoxin Removing Gel (Thermo, USA) to remove endotoxin. The endotoxin level of rSj16 solution in 1 mg/mL was 0.01 EU/kg.

Generation of murine dendritic cells

Bone marrow-derived dendrite cells (BMDCs) were prepared by using a revised method (Lutz et al. 1999). Briefly, bone marrow cells (DC precursors) were removed from the femur and tibia and resuspended in RPMI-1640, and the red blood cells were lysed with ammonium chloride buffer (0.15 M NH₄Cl, 10 mM KHCO₃, and 0.1 mM Na₂EDTA). The bone marrow cells were then washed three times with RPMI-10 before being cultured in 6-well plates (Corning) at 3 × 10⁶/mL in 3 mL of RPMI-1640 (Invitrogen, USA) containing 10 % (v/v) heat-inactivated fetal calf serum, supplemented with penicillin (100 U/mL), streptomycin (100 µg/mL), glutamine (4 mM), 50 µM 2-mercaptoethanol (all purchased from Sigma), 10 ng/mL murine granulocyte-macrophage colony-stimulating factor (GM-CSF), and 10 ng/mL murine IL-4 (R&D, USA). Then, the cells were incubated for 7 days at 37 °C in a 5 % CO₂-humidified atmosphere. On day 3 and day 5, another 3 mL of complete medium containing 10 ng/mL GM-CSF and 5 ng/mL IL-4 was added. On day 7, DC clusters were harvested and subcultured overnight to remove adherent cells. The nonadherent cells (immature DC) were collected and cultured alone or with different antigens for another 24 h: LPS (1.0 µg/mL, Sigma-Aldrich), LPS 1.0 µg/mL + rSj16 1.0 µg/mL, LPS 1.0 µg/mL + rSj16 5.0 µg/mL, LPS 1.0 µg/mL + rSj16 10.0 µg/mL, or LPS 1.0 µg/mL + actin 5 µg/mL (Sigma).

Characterization of the effect of rSj16 on mature DCs

To test the endocytosis of DCs in each group, 4 × 10⁵ DCs/well were incubated at 37 °C for 1 h with 0.7 mg/mL FITC-dextran (42,000 Da, Sigma), total 1.4 mg. After incubation, cells were washed twice with cold washing buffer (PBS containing 0.5 % bovine serum albumin). The percentage of endocytosed DCs was analyzed by flow cytometry (Jee Youn Kim et al. 2009). Then, the expression of cell surface markers on DCs was quantified by flow cytometry by using specific antibodies for CD11c, MHC-II, CD40, CD86, and CD80. Acquisition was performed by using a FACSCalibur flow cytometer (BD Biosciences). Supernatants from cultured DCs were tested for the production of IL-6, IL-23, IL-12p40, IL-12p70, and IL-10 by ELISA (Biosource).

Assays for Ag-specific CD4⁺ T cell response

In order to determine the T cell-polarizing function of differentially activated BMDC, an antigen-restricted assay was used incorporating CD4⁺ T cells from Do11.10 transgenic mice which express a TCR specific for OVA peptide. CD4⁺ T cells were positively selected with anti-CD4-coated microbeads by MACs from the spleens of naïve Do11.10 OVA323–339-specific TCR-transgenic mice (Miltenyi Biotec, USA). Purity was typically >99 %. BMDCs (1 × 10⁴) were added in graded doses to 1 × 10⁵ CD4⁺ T cells in U-bottomed 96-well plates (Janelidze et al. 2005). Cocultivation was performed in a final volume of 200 µL for 72 h. IL-4, IFN-γ, IL-12p70, IL-12p40, IL-17, and IL-10 in the culture supernatants were measured by ELISA.

Characterization of effects of rSj16 on mature DCs from IL-10 KO mice or WT mice with IL-10 production neutralized by IL-10 antibody or IL-10R antibody

At day 8, nonadherent, immature DCs from IL-10 KO mice were collected and cultured alone or with different antigens for another 24 h: LPS (1.0 µg/mL) or LPS 1.0 µg/mL + rSj16 10.0 µg/mL. Meanwhile, nonadherent, immature DCs from WT mice were collected and cultured alone or with stimulation for another 24 h: LPS (1.0 µg/mL), LPS 1.0 µg/mL + rSj16 10.0 µg/mL, LPS 1.0 µg/mL + rSj16 10.0 µg/mL, LPS 1.0 µg/mL + rSj16 10.0 µg/mL and IL-10 antibody 0.5 µg/mL, or LPS 1.0 µg/mL + rSj16 10.0 µg/mL and IL-10R antibody 3 µg/mL. The expression of cell surface markers on DCs was quantified by flow cytometry by using specific antibodies (as above). Supernatants from cultured DCs were tested for the production of IL-12p70 and IL-10 by ELISA.

Labeling of recombinant Sj16 with *N*-hydroxysuccinimide-fluorescein

Purified recombinant Sj16 was fluorescently labeled with *N*-hydroxysuccinimide (NHS)-fluorescein (Pierce, Thermo Scientific) according to the manufacturer's instructions. After protein labeling, unreacted NHS-fluorescein was removed by using Zeba™ Desalt Spin Columns (Pierce). Live cell imaging was performed with a Zeiss confocal laser scanning microscope and analyzed with ZEN 2009 Light Edition 5.5 software. In detail, BMDCs were added to a channel in 3.5-mm slide glass-bottomed dishes and incubated for 1, 2, 4, 6, or 12 h at 37 °C under 5 % CO₂ with NHS-fluorescein-labeled Sj16 (1 mg/mL). Nuclei of the cells were then stained with 4',6-diamidino-2-phenylindole (DAPI). For DAPI staining, BMDCs were washed with phosphate-buffered saline (PBS) and fixed with 3.7 % paraformaldehyde in PBS for 10 min at room temperature. Fixed cells were

washed with PBS and stained with 1 µg/mL DAPI solution for 15 min at room temperature. Then, the cells were rinsed twice with PBS. After staining with DAPI, the cells were analyzed with a confocal laser scanning microscope Zeiss LSM 710 (Carl Zeiss, Germany).

Nuclear localization signal sequence analysis and site-directed mutagenesis

The Sjl6 amino acid sequence was used for identification of nuclear localization signal (NLS) sequence motifs in web-based bioinformatic tools, including CELLO, ESLPred, Motif Scan, PSORTII, SignalP 3.0, and TargetP1.1. All programs were used with default settings. Site-directed mutagenesis was carried out on eGFP-sjl6 to convert two basic amino acids in the N-terminal (K59 and R60), middle (R63 and K64), or C-terminal (R78 and K79) parts of the putative NLS1 identified in residues 59–79 to the neutral amino acid alanine. Site-directed mutagenesis was also used to mutate two basic amino acids to alanine in the N-terminal (K95 and R96) and C-terminal (K106 and K107) parts of the putative NLS2 identified in residues 95–107. Mutagenesis was carried out by using the QuikChange II site-directed mutagenesis kit (Stratagene, USA) according to the manufacturer's instructions, by using *Pfu* high-fidelity polymerase and a plasmid encoding eGFP-sjl6 as template. Table 1 shows constructs and mutagenesis primers.

Putative NLS function verification

To determine whether putative NLSs facilitated localization of Sjl6 to host cell nuclei, HeLa cells were transiently transfected with eGFP-Sjl6 or a series of eGFP-Sjl6 constructs in which the putative NLSs were mutated as described in Table 1. After transfection, HeLa cells were fixed in 4 % paraformaldehyde, then washed three times with PBS for 5 min each. After washing, cells were permeabilized with 0.1 % Triton X-100 in PBS for 1 to 4 min. Cell nuclei were stained with DAPI; then, cells were rinsed with PBS and visualized with a Zeiss Axiovert inverted epi-fluorescence microscope. Images were captured with AxioCam and analyzed by using Axiovision software. Color images were exported from Axiovision and assembled by using Photoshop software. Pictures were adjusted to gain optimal contrast to visualize features of interest.

Effects of rSjl6 and rSjl6 mutants on dendritic cells

rSjl6, rSjl6/NLS1 mutant 1 (M1), rSjl6/NLS1 M2, and rSjl6/NLS1 M3 were expressed in *E. coli* and purified as described (Hu et al. 2008a). Purified rSjl6 mutants were fluorescently labeled with NHS-fluorescein, and live cell imaging was performed as described above. To further compare the

effects of rSjl6 and the three rSjl6 mutants on LPS-induced immature DC, the expression of cell surface markers on DCs was quantified by flow cytometry by using specific antibodies for CD11c, MHC-II, CD40, CD86, and CD80. Supernatants from cultured DCs were tested for the production of IL-12p70 and IL-10 by ELISA.

Statistical analysis

Data are presented as means ± SD and were evaluated by using two-way ANOVA. Analysis of variance by Tamhane tests for repeated measurement was used where applicable. A value of $P < 0.05$ was considered statistically significant. Calculations were performed by using the SPSS 13.0 statistical package.

Results

rSjl6 inhibited the LPS-induced functional maturation of DCs

To examine whether the ability of DCs to capture antigens was affected by rSjl6, FITC-conjugated dextran particles, which were mainly taken up via the mannose receptor, were used. Unstimulated DCs (immature DC, control group) are physiologically immature and efficiently internalize antigens; however, this ability is significantly decreased during the maturation process (when immature DCs are treated with stimulators such as LPS (Granucci et al. 1999)), as the mature DCs acquire potent APC functions. After incubation of immature DCs with rSjl6 in the presence of LPS, the rSjl6-treated DCs exhibited a higher degree of endocytotic capacity for dextran-FITC compared with untreated controls and showed inhibition of LPS-induced functional mature DCs (Fig. 1).

rSjl6 inhibits LPS-induced phenotypic maturation of and influences cytokine production by DCs

Antigen presentation is an important function of mature DCs. Functional maturation of BMDCs and optimal antigen presentation require the expression of MHC-II and costimulatory molecules on DCs (B. P 2005; Pulendran and Banachereau 2001). Thus, we tested the expression of MHC-II, CD40, CD80, and CD86 on the cell surface by using flow cytometric analysis. LPS treatment was accompanied by a significant upregulation of CD80, CD86, CD40, and MHC-II surface markers of mature DCs. However, rSjl6 significantly suppressed the expression of surface markers in a dose-dependent manner

Table 1 Summary of Sjl6/ Δ NLS mutants

	Amino acid sequence (sj16 residues 59–110)
Sjl6/wild type	k r s f r k g r h h i y k v m d k y r <u>k</u> e d l g m k m d v a k i l g r r i e k r m e y i a k l k l d k
Sjl6/NLS1 M1	a a s f r k g r h h i y k v m d k y r <u>k</u> e d l g m k m d v a k i l g r r i e k r m e y i a k l k l d k
Sjl6/NLS1 M2	k r s f a a g r h h i y k v m d k y r <u>k</u> e d l g m k m d v a k i l g r r i e k r m e y i a k l k l d k
Sjl6/NLS1 M3	k r s f r k g r h h i y k v m d k y a a e d l g m k m d v a k i l g r r i e k r m e y i a k l k l d k
Sjl6/NLS2 M4	k r s f r k g r h h i y k v m d k y r <u>k</u> e d l g m k m d v a k i l g a a i e k r m e y i a k l k l d k
Sjl6/NLS2 M5	k r s f r k g r h h i y k v m d k y r <u>k</u> e d l g m k m d v a k i l g r r i e k r m e y i a a l k l d k

(Fig. 2). These results indicate that treatment with rSjl6 suppresses the maturation of immature BMDCs by LPS.

Stimulation of DCs also induces the production of a wide range of cytokines. For example, LPS can induce IL-12 production in DCs (Jiang et al. 2002). As Fig. 3 shows, the expression levels of IL-12p40, IL-6, and IL-23 were markedly increased upon exposure of DCs to LPS. However, in the presence of rSjl6, the expressions of IL-12p70, IL-6, and IL-23 were suppressed in a dose-dependent manner. In contrast, the expression of IL-10 was increased in the presence of rSjl6.

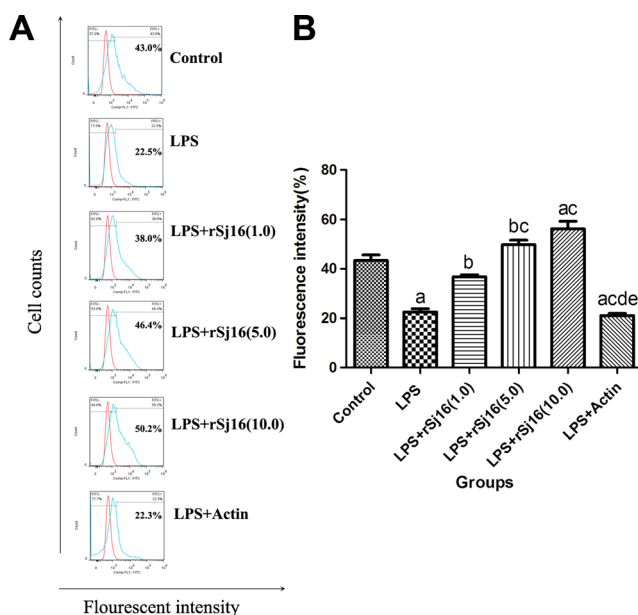


Fig. 1 Effects of rSjl6 on endocytosis by DCs. Immature DCs (imDCs) were generated from mouse BM cells by treating them with 10 ng/mL GM-CSF and 10 ng/mL IL-4 for 8 days and further treatment with LPS 1.0 μ g/mL [LPS], LPS 1.0 μ g/mL + rSjl6 1.0 μ g/mL [LPS + rSjl6(1.0)], LPS 1.0 μ g/mL + rSjl6 5.0 μ g/mL [LPS + rSjl6(5.0)], LPS 1.0 μ g/mL + rSjl6 10.0 μ g/mL [LPS + rSjl6(10.0)], LPS 1.0 μ g/mL + actin 10.0 μ g/mL [LPS + actin], or no treatment [control] for 24 h. Nonadherent and loosely adherent cells were harvested and treated with 0.7 mg/mL dextran-FITC for 1 h at 37 °C. After washing, DCs were analyzed by flow cytometry. Results in **a** are representative of more than three separate experiments. Results in **b** are representative of the mean of the three separate experiments. Differences were analyzed for significance by one-way ANOVA and Tukey's test. **a** $P < 0.05$ vs. control group, **b** $P < 0.05$ vs. LPS group, **c** $P < 0.05$ vs. LPS + rSjl6(1.0) group, **d** $P < 0.05$ vs. LPS + rSjl6(5.0) group, and **e** $P < 0.05$ vs. LPS + rSjl6(10.0) group

rSjl6 attenuates the interaction of naïve T-lymph cells with BMDCs

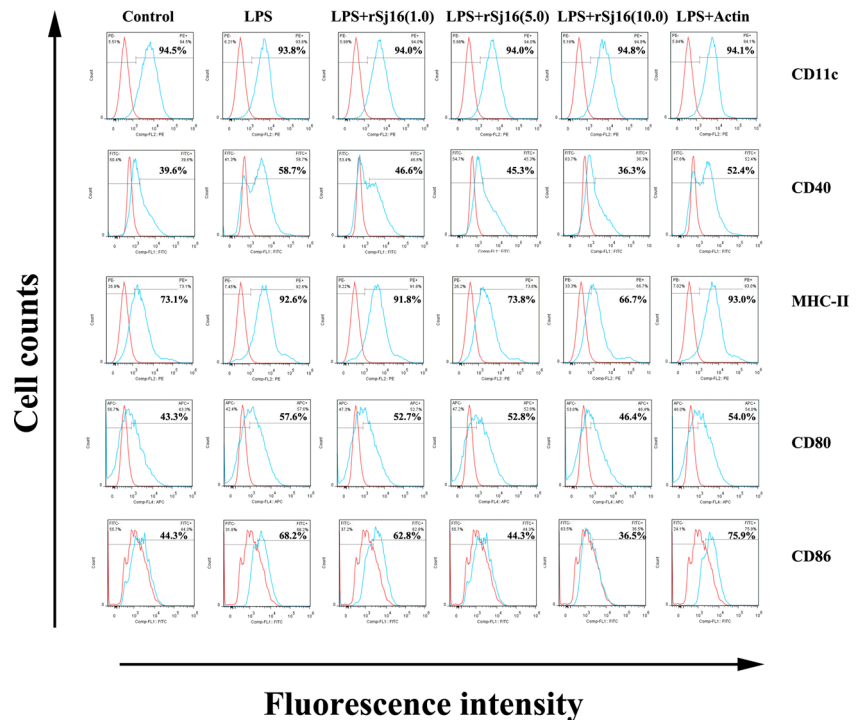
Activation of CD4⁺ naïve T cells is an important functional characteristic of mature DCs. To determine whether or not the suppression of LPS-induced BMDC maturation by rSjl6 actually plays a role in the function of BMDCs, we next examined the ability of rSjl6-stimulated BMDCs to stimulate naïve CD4⁺ T cell proliferation and cytokine production. Compared with unstimulated DCs (control group), LPS-pretreated DCs (mature DCs) could strongly activate T cells to immature proliferate and produce high levels of IL-12p70, IL-12p40, IL-17, IFN- γ , and IL-10 (Fig. 4). In contrast, rSjl6 could inhibit the ability of LPS-stimulated DCs to activate T cells (Fig. 4a) and the expression of IL-12p40, IL-17, and IFN- γ in T cells in a dose-dependent manner (Fig. 4b (a–d and f)). Similar to the result in Fig. 3, rSjl6 enhanced the expression of IL-10 in T cells (Fig. 4b (e)).

On loss of IL-10, the inhibition of LPS-induced DC maturation by rSjl6 disappears

Since IL-10 production is an important characteristic of the inhibitory effect of rSjl6 on LPS-induced DC maturation, we were interested to know whether the inhibition of LPS-induced DC maturation by rSjl6 is IL-10 dependent. After blocking IL-10 production with an IL-10 neutralizing antibody, rSjl6 could not significantly suppress the expression of MHC-II, CD86, and CD80 in LPS-induced BMDC (Fig. 5). Similar results were also observed after blocking IL-10R with an IL-10R neutralizing antibody. Moreover, by using IL-10^{-/-} mice, we found, consistent with the antibody neutralization results, that the inhibitory effect of rSjl6 on LPS-induced phenotypic maturation completely disappeared.

Influence on IL-12 production is another important characteristic of the inhibitory effect of rSjl6 on LPS-induced DC maturation. In cells deficient in IL-10 or with IL-10 production blocked by neutralizing antibody, the IL-10 production significantly decreased or disappeared. However, IL-12p70 production was increased after LPS stimulation (Fig. 5c, d), consistent with the results of others that IL-10 can suppress IL-12 production. IL-12p70 production by LPS-induced

Fig. 2 rSj16 inhibited LPS-induced phenotypic maturation of DCs. Immature DCs (imDCs) were generated from mouse BM cells by treating them with 10 ng/mL GM-CSF and 10 ng/mL IL-4 for 8 days and further treatment with LPS 1.0 $\mu\text{g}/\text{mL}$ [LPS], LPS 1.0 $\mu\text{g}/\text{mL}$ + rSj16 1.0 $\mu\text{g}/\text{mL}$ [LPS + rSj16(1.0)], LPS 1.0 $\mu\text{g}/\text{mL}$ + rSj16 5.0 $\mu\text{g}/\text{mL}$ [LPS + rSj16(5.0)], LPS 1.0 $\mu\text{g}/\text{mL}$ + rSj16 10.0 $\mu\text{g}/\text{mL}$ [LPS + rSj16(10.0)], LPS 1.0 $\mu\text{g}/\text{mL}$ + actin 10.0 $\mu\text{g}/\text{mL}$ [LPS + actin], or no treatment [control] for 24 h. Nonadherent and loosely adherent cells were harvested and stained with antibodies against CD11c, MHC-II, CD80, CD86, and CD40. Results are representative of more than three separate experiments



BMDCs could not be suppressed by the presence of rSj16. All these results indicate that rSj16-mediated suppression of LPS-induced DC activation is IL-10 dependent and occurs through the IL-10R signal pathway.

An N-terminal NLS is required for translocation of rSj16 to the host nucleus

As Fig. 6b shows, eGFP fused to wild-type Sj16 showed strong and exclusively nuclear staining in HeLa cells, which suggested that rSj16 may have one or more nuclear localization sequences. Thus, we used bioinformatic tools to predict the putative nuclear localization sequence(s). One putative nuclear localization sequence (NLS1) was identified at the N-terminus and another (NLS2) at the C-terminus of Sj16 by using Motif Scan software (Fig. 6a). To determine which cluster is responsible for the nuclear localization, site-directed mutagenesis was performed in basic residues within each basic amino acid cluster of the putative NLSs. We generated respective mutants in which one of the two or three clusters had mutations to alanine. Sj16/NLS1 M1, Sj16/NLS1 M2, and Sj16/NLS1 M3, in which NLS1 was mutated, were detected only in the cytosol. A total of 83.6 % of cells had exclusively nuclear localization of the wild-type Sj16 protein, whereas only 6.5 % of Sj16/NLS1 M1, 2.4 % of Sj16/NLS1 M2, and 5.9 % of Sj16/NLS1 M3 were exclusively nuclear. However, Sj16/NLS1 M4 and Sj16/ Δ NLS M5, mutated in NLS2, showed similar staining to wild-type Sj16 (Fig. 6b, c). Therefore, substitution of basic amino acids within NLS1 disrupted the nuclear localization of Sj16 in

HeLa cells and effected a diffused cytoplasmic distribution, indicating that all the basic amino acid clusters in NLS1 are essential for the nuclear localization of Sj16.

Exogenous Sj16 protein translocates to the nuclei of dendritic cells

Since Sj16 is a secreted protein with a functional NLS, we investigated whether Sj16 is internalized by primary cells; the capacity of mouse BMDCs to take up Sj16 was assessed. BMDC cells incubated for different times with 10 $\mu\text{g}/\text{mL}$ purified recombinant Sj16 labeled with NHS-fluorescein displayed a strong, time-dependent increase in fluorescence in the nucleus as determined by confocal laser scanning microscopy (Fig. 7a). However, compared with rSj16, the rSj16 mutants could not directly enter BMDC nuclei on the same timescale (Fig. 7b).

The N-terminal NLS is important for rSj16-mediated inhibition of mouse DCs

To determine whether the inhibition of maturation and function of LPS-induced mouse DCs and the triggering of IL-10 release by rSj16 require nuclear localization of the parasite protein after internalization, we compared the inhibitory effect of wild-type protein and the three rSj16/NLS1 mutants. As Fig. 7c, d shows, wild-type rSj16 significantly inhibited the expression of MHC-II, CD80, CD86, and CD40 and induced IL-10 production. In contrast, the rSj16/ Δ NLS mutants showed little or no ability to inhibit expression of MHC-II,

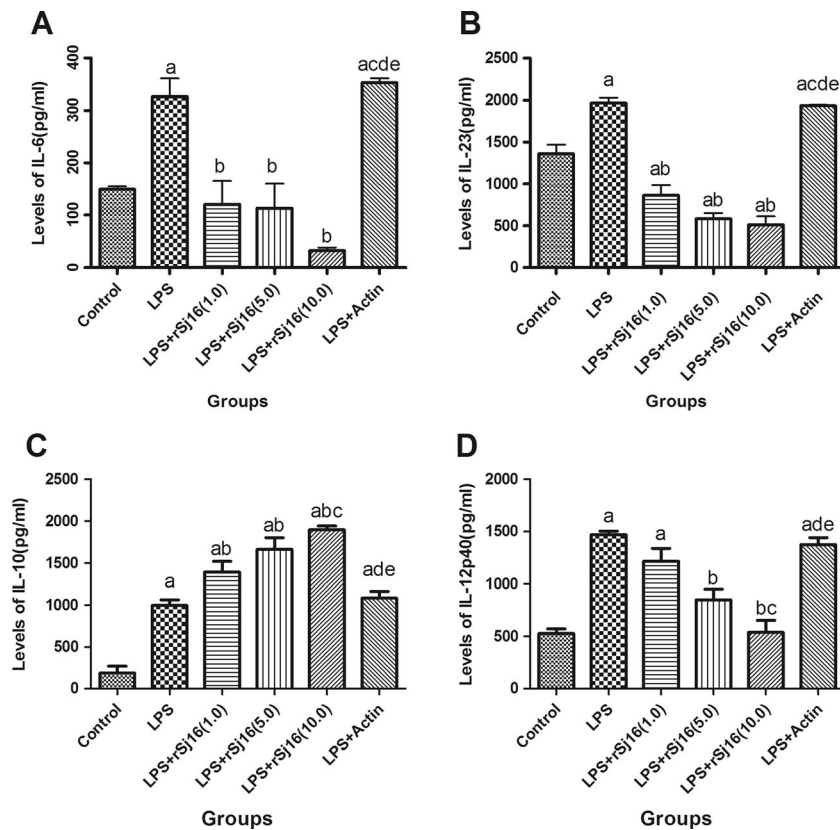


Fig. 3 Influence of rSj16 on production of cytokines by LPS-induced BMDCs. Immature DCs (imDCs) were generated from mouse BM cells by treating them with 10 ng/mL GM-CSF and 10 ng/mL IL-4 for 8 days and further treatment with LPS 1.0 $\mu\text{g}/\text{mL}$ [LPS], LPS 1.0 $\mu\text{g}/\text{mL}$ + rSj16 1.0 $\mu\text{g}/\text{mL}$ [LPS + rSj16(1.0)], LPS 1.0 $\mu\text{g}/\text{mL}$ + rSj16 5.0 $\mu\text{g}/\text{mL}$ [LPS + rSj16(5.0)], LPS 1.0 $\mu\text{g}/\text{mL}$ + rSj16 10.0 $\mu\text{g}/\text{mL}$ [LPS + rSj16(10.0)], LPS 1.0 $\mu\text{g}/\text{mL}$ + actin 10.0 $\mu\text{g}/\text{mL}$ [LPS + actin], or no treatment [control] for 24 h. Supernatants from cultured DCs were collected and

tested for the production of IL-6 (a), IL-23 (b), IL-10 (c), and IL-12 (d) by using ELISA. Results show one of three independent experiments, and data are expressed as the mean value \pm SD for triplicate ELISA. Differences were analyzed for significance by one-way ANOVA and Tukey's test. **a** $P < 0.05$ vs. control group, **b** $P < 0.05$ vs. LPS group, **c** $P < 0.05$ vs. LPS + rSj16(1.0) group, **d** $P < 0.05$ vs. LPS + rSj16(5.0) group, and **e** $P < 0.05$ vs. LPS + rSj16(10.0) group

CD80, CD86, and CD40 to induce IL-10 production in the same conditions. These results suggest that the N-terminal NLS (NLS1) is not only required for translocation of rSj16 to the host nucleus but is also correlated with the subsequent rSj16-mediated inhibition of mouse BMDCs.

Discussion

The effects of infection with helminths such as *S. japonicum* on host immune responses have been clearly described as Th2 responses and anti-inflammatory/regulatory responses (van Riet and Yazdanbakhsh 2007). CD4^+ T cells cannot directly recognize antigens and induce Th2 response, but rather require processing and presentation bound to MHC-II molecules on the surface of antigen-presenting cells (ML. K 2003). Thus, DC, as a unique APC, plays a central role in providing information on the nature of the invading/residing pathogen by integrating signals received and conveying them to T cells by expressing a variety of factors that affect T cell

differentiation (Kim et al. 2009; Terrazas and Gómez-García 2010). Previously, some researchers have reported that schistosomes fail to conventionally activate DC, resulting in contrasting maturation phenotypes, and efficiently control the specific immune response either in vitro or during active infection (MacDonald et al. 2001; Carvalho et al. 2009). This shows that some proteins produced by schistosomes may have a modulatory function on DCs.

In the present study, we describe the inhibitory effect of recombinant Sj16, a secreted protein from *S. japonicum*, on maturation of bone marrow-derived DCs. rSj16 significantly inhibited the phenotypic and functional maturation of DCs. DCs without any stimuli express in an immature stage that is characterized by high efficiency in taking up and processing antigens. Meanwhile, when DCs encounter some stimulus like LPS, they quickly change to a mature state that is characterized by the loss of antigen uptake capacity and migration to regional lymph nodes where they exert their function as potent APCs (Heufler et al. 1996; Macatonia et al. 1995; Zhou LJ 1996). Our results demonstrated that rSj16 inhibited the LPS-induced

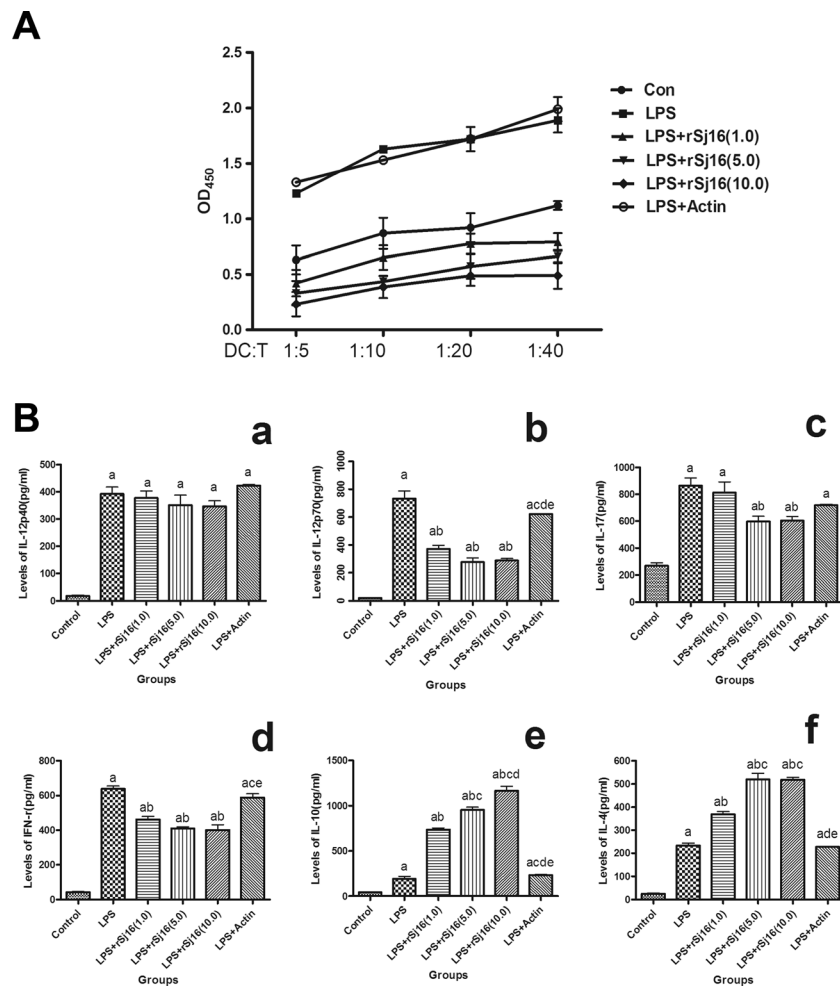


Fig. 4 rSj16-pulsed BMDCs inhibit proliferation of CD4⁺ T cells and induce an antigen-specific Th2 response in naive mice. Immature DCs (imDCs) were generated from mouse BM cells by treating them with 10 ng/mL GM-CSF and 10 ng/mL IL-4 for 8 days and further treatment with LPS 1.0 $\mu\text{g}/\text{mL}$ [LPS], LPS 1.0 $\mu\text{g}/\text{mL}$ + rSj16 1.0 $\mu\text{g}/\text{mL}$ [LPS + rSj16(1.0)], LPS 1.0 $\mu\text{g}/\text{mL}$ + rSj16 5.0 $\mu\text{g}/\text{mL}$ [LPS + rSj16(5.0)], LPS 1.0 $\mu\text{g}/\text{mL}$ + rSj16 10.0 $\mu\text{g}/\text{mL}$ [LPS + rSj16(10.0)], LPS 1.0 $\mu\text{g}/\text{mL}$ + actin 10.0 $\mu\text{g}/\text{mL}$ [LPS + actin], or no treatment [control] for 24 h. DCs (1×10^4) and CD4⁺ T cells (1×10^5) purified from Do11.10 mice were cocultured at different ratios as indicated. After 3 days of culture, CCK8

was added into the cells and OD450 was measured by using a microplate spectrophotometer. **a** rSj16-pulsed BMDCs inhibit proliferation of CD4⁺ T cells. **b** rSj16-pulsed BMDCs induce an antigen-specific Th2 response in naive mice. Culture medium was collected 24 h after mixing DCs, and T cells and IL-12p70 (**a**), IL-12p40 (**b**), IL-17 (**c**), IFN- γ (**d**), IL-10 (**e**), and IL-4 (**f**) levels were measured by using ELISA kits. Significance was determined by ANOVA. **a** $P < 0.05$ vs. control group, **b** $P < 0.05$ vs. LPS group, **c** $P < 0.05$ vs. LPS + rSj16(1.0) group, **d** $P < 0.05$ vs. LPS + rSj16(5.0) group, and **e** $P < 0.05$ vs. LPS + rSj16(10.0) group

augmented surface expression of CD40, CD80, CD86, and MHC-II in DCs, while the endocytotic capacity of rSj16-treated DCs was profoundly increased. Furthermore, rSj16 changed cytokine production by LPS-stimulated DCs, decreasing the production of IL-12, IL-6, and IL-23 and significantly increasing the production of IL-10. It is accepted that DC maturation is related to detectable IL-12 secretion (Marovich et al. 2000). A high level of IL-12 in the microenvironment enhances the secretion of other proinflammatory cytokines and chemokines and induces the recruitment and/or activation of other effectors, which ultimately, as dominant cytokines, lead to a Th1 response and other potent inflammatory reactions by inducing secretion of IFN- γ (Gately et al. 1998; Kang and Kim

2005; Nakahara et al. 2006). IL-6 and IL-23 are important inducers of Th17 response; IL-6 programs Th17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways (Zhou et al. 2007). In contrast, IL-10 has been reported to be a global suppressor of inflammatory responses and an important immunoregulator of Th cell response (Moore et al. 2001). Thus, rSj16 may modulate DC functions by influencing DC maturation that will ultimately influence T cell activation and impair Th1 and Th17 responses. Our results also confirm the function of rSj16-stimulated DC on naive T cells; we found that rSj16-stimulated DC inhibited consequent CD4⁺ T proliferation and promotion of T cells to Th2 response by increasing the production of IL-10 and IL-4.

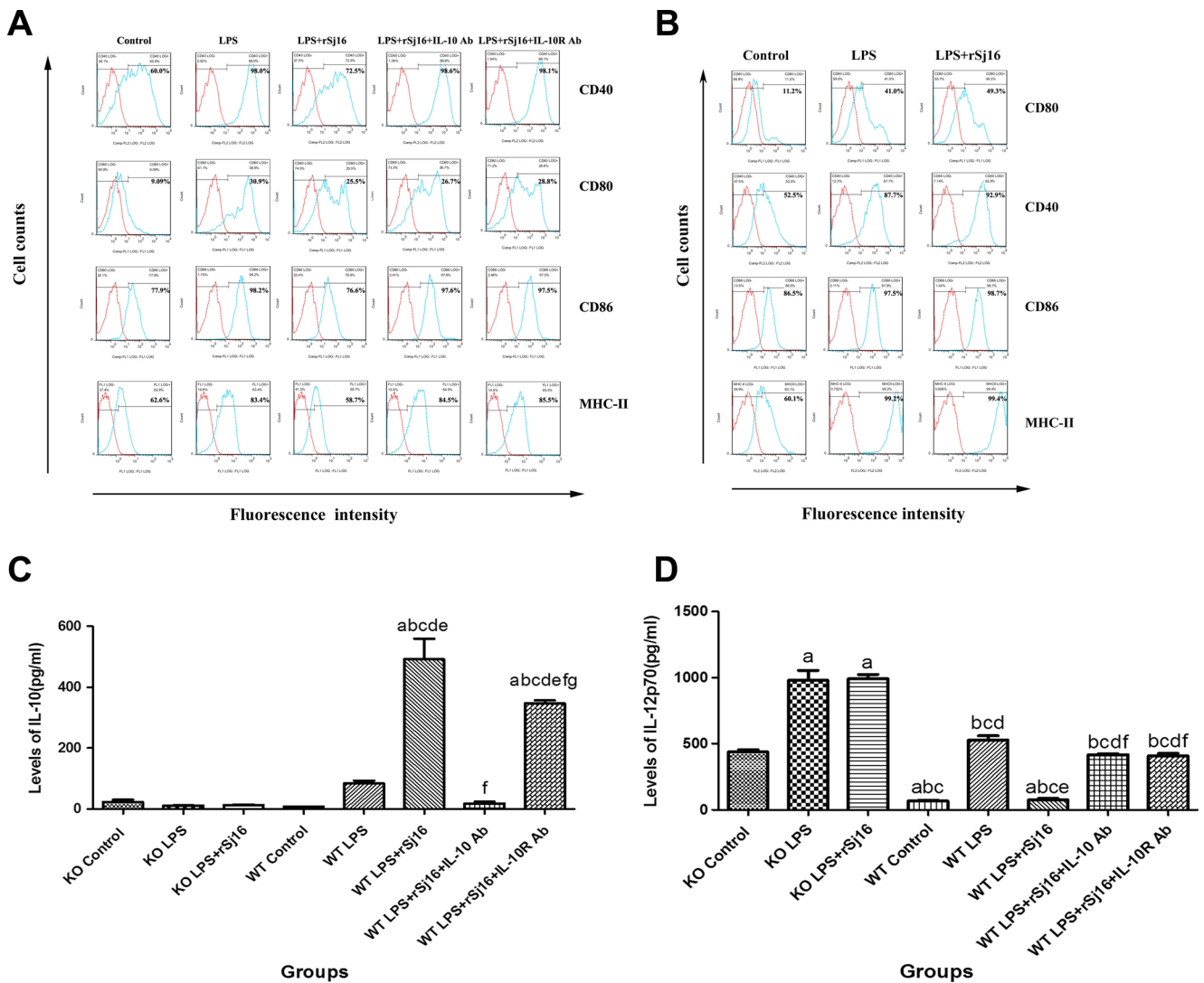


Fig. 5 In the absence of IL-10, rSj16 could not inhibit LPS-induced DC maturation. **a** Immature DCs (imDCs) were generated from wild-type mouse BM cells by treating them with 10 ng/mL GM-CSF and 10 ng/mL IL-4 for 8 days and further treatment with LPS 1.0 $\mu\text{g}/\text{mL}$, LPS 1.0 $\mu\text{g}/\text{mL}$ + rSj16 10.0 $\mu\text{g}/\text{mL}$, LPS 1.0 $\mu\text{g}/\text{mL}$ + rSj16 10.0 $\mu\text{g}/\text{mL}$ + anti-IL-10 antibody 0.5 $\mu\text{g}/\text{mL}$, LPS 1.0 $\mu\text{g}/\text{mL}$ + rSj16 10.0 $\mu\text{g}/\text{mL}$ + anti IL-10R antibody 0.5 $\mu\text{g}/\text{mL}$, or no treatment [control] for 24 h. **b** Immature DCs (imDCs) were generated from IL-10^{-/-} mouse BM cells by treating them with 10 ng/mL GM-CSF and 10 ng/mL IL-4 for 8 days and further treatment with LPS 1.0 $\mu\text{g}/\text{mL}$, LPS 1.0 $\mu\text{g}/\text{mL}$ + rSj16 10.0 $\mu\text{g}/\text{mL}$, or no treatment [control] for 24 h. Nonadherent and loosely

adherent cells were harvested and stained with antibodies against CD11c, MHC-II, CD80, CD86, or CD40. Histograms and mean fluorescence intensities (MFIs) are shown. Results are representative of more than three separate experiments. Meanwhile, supernatants from cultured DCs were collected and tested for the production of **c** IL-10 and **d** IL-12 by using ELISA. Differences were analyzed for significance by Student's *t* test. **a** $P < 0.05$ vs. knockout (KO) control group, **b** $P < 0.05$ vs. KO LPS group, **c** $P < 0.05$ vs. KO LPS + rSj16 group, **d** $P < 0.05$ vs. wild-type (WT) control group, **e** $P < 0.05$ vs. WT LPS group, **f** $P < 0.05$ vs. WT LPS + rSj16 group, and **g** $P < 0.05$ vs. WT LPS + rSj16 + IL-10 antibody group

Previous reports have shown that production of IL-10 increased significantly when the body encountered some pathogens, such as schistosoma, or suffered from some autoimmune diseases. IL-10 was initially described as an important anti-inflammatory molecule in limiting an overexuberant immune response and preventing autoimmunity; recently, it has been concluded that IL-10 production seems to be associated with many immune cells, affirming its crucial role as a feedback regulator of diverse pathogen infection immune responses (Margarida Saraiva 2010). IL-10 plays an

important role as a negative regulatory factor involved in inhibiting antigen-presenting cell functions, prolonging the status of immune tolerance, influencing the clearance of parasites, and causing immune escape when the host encounters parasite infection (David Sacks 2002). Combined with our previous study showing that the effect of rSj16 on immune cells is the production of a large amount of IL-10, we hypothesized that the mechanism of production of IL-10 may be key to the immunomodulatory effects of rSj16 (Hu et al. 2012; Sun et al. 2010; Sun et al. 2012a; Sun et al. 2012b).

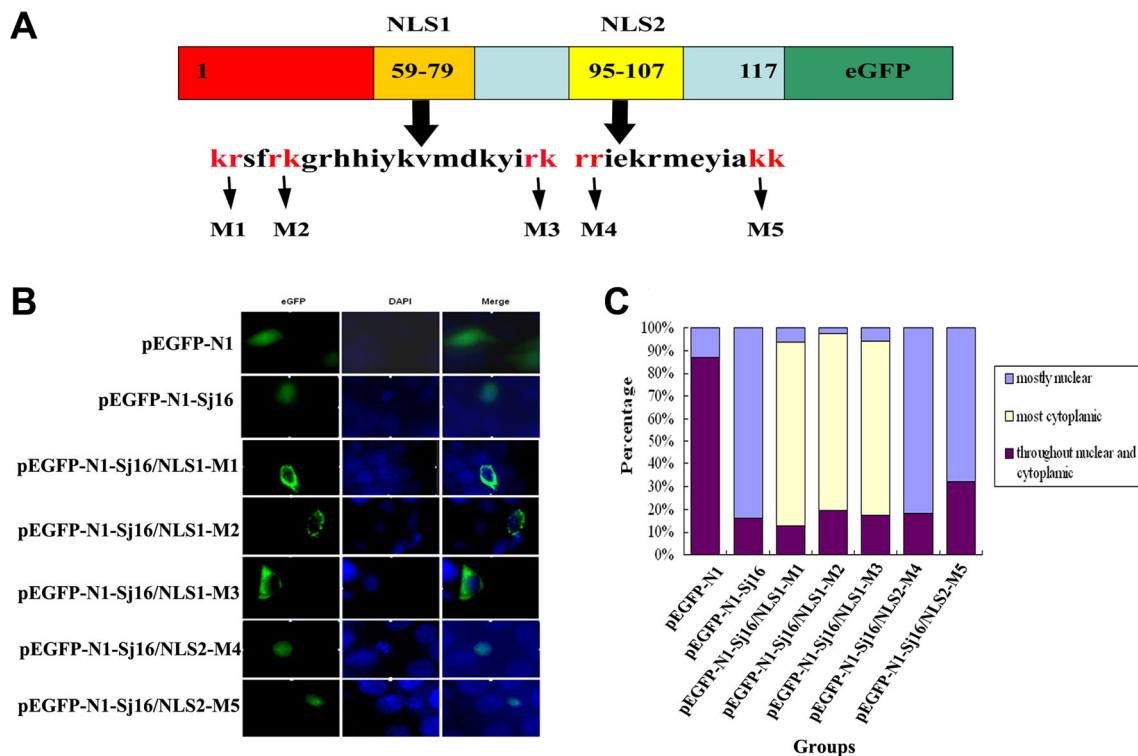


Fig. 6 Sj16 contains two putative nuclear localization signals (NLSs). **a** Schematic representation of wild-type Sj16 that includes a signal peptide (SP), two putative NLSs, and the C-terminus fused with eGFP. **b** Fluorescence microscopy analysis of cellular localization of eGFP-Sj16 fusion proteins. HeLa cells were transiently transfected with DNAs encoding the indicated eGFP-Sj16 fusion proteins and five eGFP-Sj16/NLS mutants (three separate experiments). The *left row* shows the eGFP

signal (*green*), the *middle row* shows the DAPI signal (*blue*), and the *right row* shows merged eGFP and DAPI images. **c** Quantitative analysis of the intracellular localization of eGFP empty vector, pEGFP-N1-Sj16 fusion, and five NLS mutants. Percentages are representative of at least 100 cell counts each from a minimum of three separate experiments. Mostly nuclear (*blue bars*), mostly cytoplasmic (*purple bars*), throughout the nucleus and cytoplasm (*yellow bars*)

To confirm our hypothesis, we tested the impact of IL-10 in the inhibitory effect of rSj16 on LPS-induced BMDC maturation by using an anti-IL-10 antibody or anti-IL-10R antibody. When IL-10 production was blocked during rSj16 treatment, there was no decrease in the expression of MHC-II, CD86, CD80, or CD40 or in the production of IL-12, in LPS-induced BMDCs. Meanwhile, results with IL-10^{-/-} mice are consistent with the antibody neutralization results. Thus, we confirmed that the inhibitory effect of rSj16 on LPS-induced BMDC maturation disappeared with the loss of IL-10. All results indicated that rSj16-mediated suppression of LPS-induced DC activation is IL-10 dependent and occurs through the IL-10R signal pathway. As far as we know, this is the first observation that Sj16, a secretory protein from *S. japonicum* with an anti-inflammatory effect, regulates the immune response directly through upregulation of IL-10 expression in antigen-presenting cells. To date, few reports have shown that IL-10 plays an important role in the immune escape of pathogens and that the absence of IL-10 leads to better clearance of some pathogens with no immunopathology (Brooks DG et al. 2006). Thus, identification of the close relationship between IL-10 and a schistosoma-derived protein, and recognition of how this protein increases the production of IL-10, will be useful in exploring mechanisms of immune escape.

By using host cell surface receptors to gain entry into host cells is a common strategy among pathogens, especially viruses (Soeiro et al. 1999). Relatively large pathogens such as helminths cannot enter host cells directly, so it is possible that they use similar strategies involving hijacking of host cell receptors for internalization and subsequent nuclear translocation of switch factors that may influence the host cells indirectly (Kaur et al. 2011).

In this study, by using standard approaches, we have demonstrated for the first time that a protein secreted by *S. japonicum* contains two putative NLS motifs: one (NLS1) at the N-terminus and the other (NLS2) at the C-terminus. Although NLS sequences are not conserved, they generally belong to one of two arginine/lysine-rich patterns. The classical NLS motif contains one monopartite or two bipartite clusters of basic amino acids (Chen et al. 2005; Dingwall and Laskey 1991; Pemberton and Paschal 2005). The typical monopartite NLS is a cluster of basic residues starting with proline and followed by five residues, of which at least three are either lysine or arginine. The bipartite pattern begins with two basic residues followed by a ten-residue spacer and finally a cluster in which at least three of five residues are lysine or arginine. Besides the classical NLS motifs, Hsu et al.

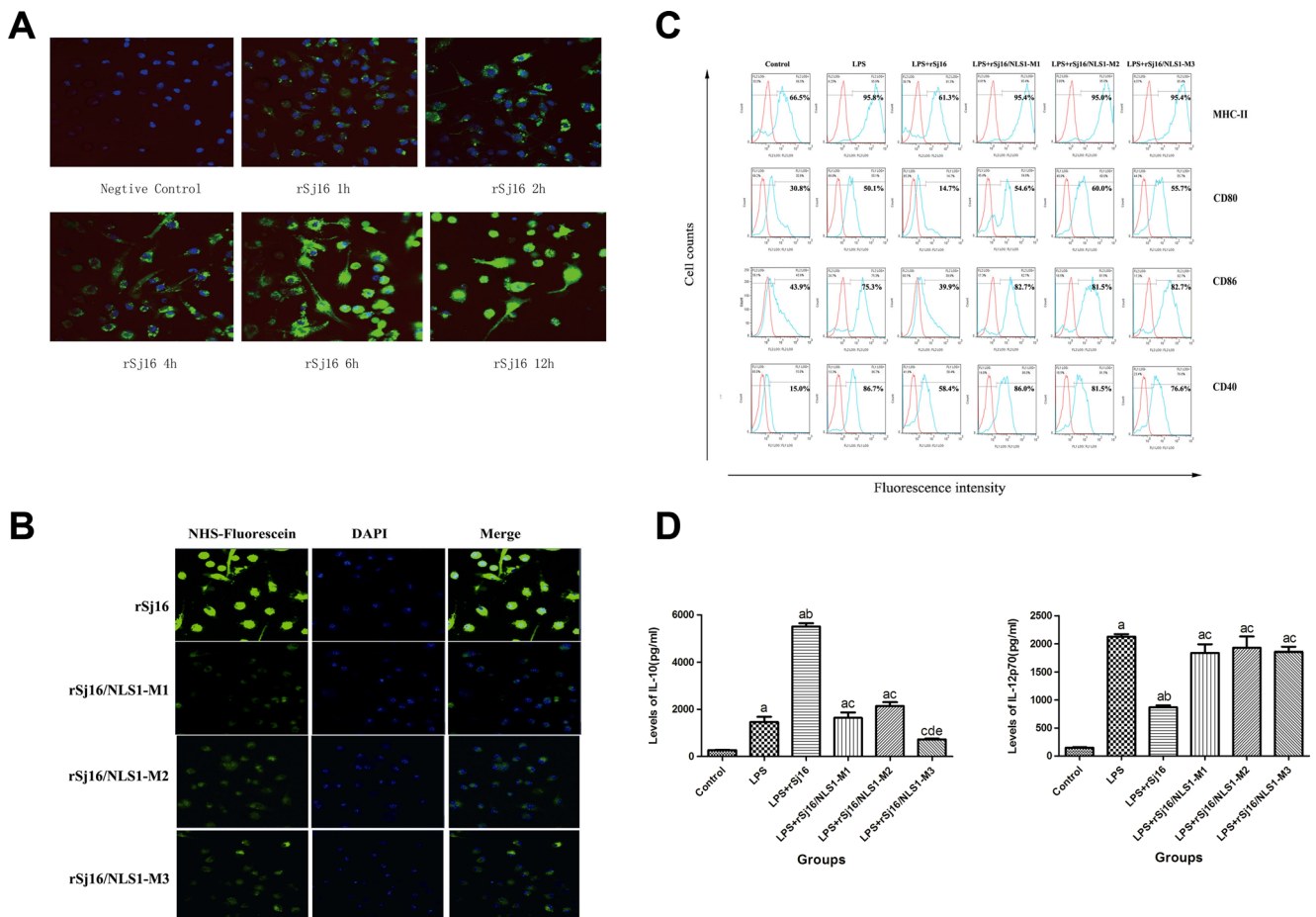


Fig. 7 The N-terminal NLS is important for translocation to the nuclei of BMDCs and rSj16-mediated inhibition of mouse DCs. **a** Subcellular localization of exogenously added recombinant rSj16 labeled with *N*-hydroxysuccinimide (NHS)-fluorescein in BMDCs at 1, 2, 4, 6, and 12 h. The cells were then stained with DAPI for 10 min and visualized by confocal microscopy. **b** Subcellular localization in BMDCs of exogenously added recombinant rSj16 and rSj16 mutants labeled with *N*-hydroxysuccinimide (NHS)-fluorescein at 6 h. The cells were then

stained with DAPI for 10 min and visualized by confocal microscopy. **c** Surface marker detection and **d** production of cytokines in immature BMDCs alone (control) or after treatment with LPS 1.0 $\mu\text{g}/\text{mL}$ (LPS), LPS 1.0 $\mu\text{g}/\text{mL}$ + rSj16 10.0 $\mu\text{g}/\text{mL}$ (LPS + rSj16), LPS 1.0 $\mu\text{g}/\text{mL}$ + rSj16/NLS1 M1 10.0 $\mu\text{g}/\text{mL}$ (LPS + rSj16/NLS1 M1), LPS 1.0 $\mu\text{g}/\text{mL}$ + rSj16/NLS1 M2 10.0 $\mu\text{g}/\text{mL}$ (LPS + rSj16/NLS1 M2), or LPS 1.0 $\mu\text{g}/\text{mL}$ + rSj16/NLS1 M3 10.0 $\mu\text{g}/\text{mL}$ (LPS + rSj16/NLS1 M3). Results are representative of more than three separate experiments

identified a novel tripartite NLS containing three clusters of basic amino acids (Hung 2006). In Sj16, NLS2, [(K/R)₂-X₁₀₋₁₂-(K/R)₂], is a classical bipartite cluster, and NLS1 is a novel kind of nonclassical tripartite NLS, [(K/R)₂-X₂-(K/R)₂-X₁₀₋₁₃-(K/R)₂], which has not been reported before. As far as we know, examples of secretory proteins that contain two NLS motifs are relatively rare (Ishwinder et al. 2011). This raised the question of whether the two NLS motifs have similar effects, or a connected effect, on the nuclear localization activity. Mutations in these two NLSs showed that NLS1 is necessary and sufficient for translocation of the protein to the nucleus in host cells. We also confirmed that the inhibition of maturation and function of LPS-induced mouse DCs and the triggering of IL-10 release by rSj16 require NLS1 of the parasite protein. Thus, these findings suggest that perhaps only the N-terminal NLS functions when the protein contains two or more NLSs.

Though we have shown that introduction of *E. coli*-expressed Sj16 protein to the culture medium of DCs results in its internalization and subsequent targeting to the nucleus, whether NLS1 is related to the function of rSj16 in LPS-induced BMDCs remains unknown. However, we assessed the function of rSj16 and rSj16 NLS mutants in LPS-induced BMDCs. After mutation of the key amino acids of NLS1, rSj16 could not enter the nucleus of BMDCs and did not inhibit MHC-II, CD80, CD86, and CD40 expression or promote IL-10 production by LPS-induced BMDCs.

Acknowledgments These experiments were supported by grants from the National High Technology Research and Development Program of China (no. 2015AA020934), National Natural Science Foundation of China (grant no. 81201309 and 30972574), grant from the Doctoral Program of Higher Education of China (grant no. 20120171120049),

and grant from the National Science Foundation of Guangdong Province (grant no. S2012040007256).

Compliance with ethical standards

Conflict of interests The authors declare that they have no competing interests.

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