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RESEARCH

The *Plasmodium* Circumsporozoite Protein, a Novel NF-κB Inhibitor, Suppresses the Growth of SW480

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Abstract The blocking of NF-κB activation is a promising strategy for the treatment of colorectal cancer. The circumsporozoite protein (CSP), a key component of the sporozoite stage of the malaria parasite, was recently reported to block NF-κB activation in hepatocytes. Thus, we investigated the effect of the CSP on the growth of the human colon cancer cell line, SW480. We demonstrated that transfection with a recombinant plasmid expressing CSP inhibited the proliferation of SW480 in a dose-dependent manner and induced the apoptosis of SW480. A NF-κB gene reporter assay showed that both the CSP and its nuclear localization signal (NLS) motif could equally suppress the activation of NF-κB following the stimulation with human recombinant TNF-α in the SW480. Furthermore, western blot analysis indicated that NLS did not affect the phosphorylation and degradation of IκB, but could sharply inhibit the nuclear translocation of NF-κB in TNF-α stimulated SW480. Hence, our data suggest that the CSP might be explored as a new NF-κB inhibitor for the treatment of colorectal cancer.

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Introduction

Colorectal cancer is the most common fatal malignancy in the world [1]. Although great progress has been made in the development of colorectal cancer therapies, not all patients are successfully treated[2, 3]. An example of a beneficial treatment is the use of 5-fluoruracil (FU) formulated with oxaliplatin and irinotecan for colorectal cancer. Additionally, monocolonal antibodes against vascular endothelial growth factor (VEGF) and the epidermal growth factor receptor (EGFR) have been used to treat metastatic colorectal carcinoma [2, 3]. However, due to the resistance of some patients to these therapeutic regimans, it is necessary to develop new approaches to replace or complement the current therapies.

Nuclear factor κB (NF-κB) is important in the regulation of the innate immune response, inflammation, cell survival, and lymphoid organ development [4]. It is assembled by dimerization of two of the five subunits, p65 (RelA), c-Rel, Rel-B, p50/NF-κB1, and p52/NF-κB2. Without stimulation, most NF-κB dimers are bound to a specific inhibitory protein, termed IκB, in the cytoplasm. Following stimulation by proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α) or lipopolysaccharide (LPS), the phosphorylated IκB kinase (IKK) degrades the inhibitory protein IκB, and releases NF-κB. NF-κB (p50/p65 or p52/Rel-B) then translocates to the nucleus and results in activation of target genes, including cytokines, chemokines, and anti-apoptotic genes [5].

In addition to its regulatory role in physiological processes, growing evidence supports a critical role of NF-κB activation in tumor initiation, progression and metastasis [6]. The relationship of NF-κB activation with tumorigenesis is well documented in both colitis-associated and hepatitis-associated cancer models, and the specific switching off of NF-κB in both hepatocytes and intestinal epithelial cells leads to a dramatic decrease in tumor incidence [7, 8]. During tumor progression, infiltrating immune cells consisting mainly of tumor-associated macrophages (TAMs), activate the NF-κB of cancer cells through the secretion of proinflammatory cytokines (TNF-α, IL-1, IL-6 or IL-23) [9, 10]. NF-κB was also constitutively activated in a variety of primary tumors and cancer cell lines, such as hepatocellular carcinoma [11], breast cancer [12], prostate cancer cells [13], pancreatic adenocarcinoma cells [14], and colorectal carcinoma [15]. The constitutive activation of NF-κB in cancer cells was demonstrated to result in anti-apoptosis, cell growth, angiogenesis and metastasis of tumor cells [12–18]. In contrast, blocking NF-κB activation prevents inflammation-mediated tumor growth and metastasis [19–21] and sensitizes tumor cells to chemotherapy and radiation [22–25]. As NF-κB is critical for tumor growth, the inhibition of NF-κB activation was regarded as a promising strategy for antitumor therapy [26]. The effects of NF-κB inhibitors, including curcumin, DHMEQ and KINK-1, on cancer cell growth have been investigated in different types of tumors [27–30].

The circumsporozoite protein (CSP) is the main surface protein of sporozoite of the malaria parasite. Recently, it was shown that the transfer of the CSP from the parasitophorous vacuole to the cytoplasm is essential for the development of the malaria parasite in the hepatocyte, due to its blockage of NF-κB activation in hepatocytes [31]. Thus, we investigated the effect of the CSP on the growth of the human colon cancer cell line, SW480, and found that the CSP, via its NLS motif, suppressed the proliferation and survival of SW480 through competition with the nuclear translocation of NF-κB.

Materials and Methods

Recombinant Plasmid Construction

The full-length CSP coding sequence was reverse transcribed and amplified using PCR from the *Plasmodium yoelii* 265BY sporozoite with the primers P_{CSP3}(5′-CCCaagcttGGG AAGAAGTGTACCATTTTAG-3′, the *Hind*III restriction site is underlined) and $P_{CSP1284}$ (5′-CGggatccCGTTAATTAAAGAATACTAATAC-3′, the *BamH* I restriction site is underlined). The amplified fragment, which was 1281 bp in length, was cloned into the pFLAG-CMV8 plasmid, resulting in the recombinant plasmid pFLAG-CMV8-CSP. A second plasmid, pFLAG-CMV8-CSP NLS, was constructed through ligation of the annealing nuclear location signal (NLS) oligonucleotide to the *Hind*III and *BamH* I restriction sites of pFLAG-CMV8. To construct the recombinant plasmid pFLAG-CMV8-CSPΔNLS, the CSP lacking the NLS was obtained using the method of overlapping PCR and then cloned into the *Hind*III and *BamH* I restriction sites of pFLAG-CMV8. The correct orientation of all the recombinant plasmids was confirmed using sequencing.

Cell Line and Culture

The human colon cancer cell line SW480 was obtained from the ATCC and maintained in Dulbecco's Modified Eagle's Medium supplemented with 10 % fetal bovine serum, 100 U/mL of penicillin, and 100 μg/mL of streptomycin.

Preparation of Rabbit Anti-CSP Serum

The B cell epitope QGPGAQGPGAQGPGAP of the CSP was synthesized and purified via HPLC and then conjugated with keyhole limpet hemocyanin (KLH). The resulting conjugated peptide was emulsified with Freund's adjuvant and immunized in a SPF grade rabbit at 0, 2 and 4 weeks. After the final immunization, rabbit serum was collected, and the titer of antibody against the epitope was detected using ELISA.

Indirect Immunofluorescence Assay (IFA)

After transfection with pFLAG-CMV8-CSP or a control plasmid using Lipofectamine 2000™ for 24 h, SW480 cells, which were grown on a coverslip, were incubated with rabbit anti-CSP serum (1:10) for 1 h. After washing twice with PBS, the cells were labeled with goat anti-rabbit FITC-IgG (H+L) for 20 min and observed under fluorescence microscopy.

Alamar Blue Assay

SW480 were transfected with or without pFLAG-CMV8-CSP (0.2, 0.4, 0.8, 1.2 or 1.6 μg), and 24 h later, 120 μL of SunBio™Am-Blue was added to each well and incubated for 2 h at 37°C. The fluorescence was read at wavelength of both 570 and 600 nm. Cell proliferation was expressed as OD570-OD600 .

Dual Luciferase Assay

SW480 were transfected using Lipofectamine™ 2000 in 24-well plates. Each well received 200 ng pBIIx-luc report vector containing two κB sites upstream of the c-fos

promoter (gift from Dr. Sankar Ghosh, Yale University) and 2 ng TK-RL (Promega), together with 600 ng pFLAG-CMV8-CSP, pFLAG-CMV8-CSP NLS or pFLAG-CMV8-CSPΔNLS. After 24 h, the cells were stimulated in the presence or absence of 100 ng/ml human recombinant TNF-α (hTNF-α, Peprotech) and/or LPS for 6 h. Next, cells were lysed, and both firefly and Renilla luciferase activities were determined using the Dual Luciferase Assay kit (Promega). The data were expressed as the ratio of firefly luciferase to Renilla luciferase.

Annexin V-FITC Apoptosis Assay

After SW480 were transfected with or without 0.8 μg pFLAG-CMV8-CSP for 24 h, the cells were stimulated with 100 ng/mL hTNF-α for 6 h. Next, the cells were collected and incubated with Annexin V-FITC (Beyotime, China) and propidium iodide (PI) for 10 min on ice, and analyzed using flow cytometry.

Western Blot

After transfection with or without pFLAG-CMV8-CSP NLS for 24 h, SW480 cells were stimulated with 100 ng/mL hTNF-α for 30 min. The cells (2×10^6) were washed with cold phosphate-buffered saline (PBS) and suspended in 0.4 mL hypotonic lysis buffer containing protease inhibitors for 30 min The cells were lysed with 12.5 μL 10 % Nonidet P-40. The homogenate was centrifuged, and the supernatant containing the cytoplasmic extracts was stored at −80°C. The nuclear pellet was resuspended in 25 μL ice-cold nuclear extraction buffer. After 30 min of intermittent mixing, the extract was centrifuged, and the supernatants containing nuclear extracts were collected. Protein concentration in cytoplasmic extracts and nuclear extracts were then determined with the BCA protein assay kit (Beyotime, China). Equal amounts of cytoplasmic and nuclear extracts were separated using SDS-PAGE, transferred to a PVDF membrane, and probed with the NF-κB p105/p50 antibody (Cell Signaling Technology) followed by ECL reagent (Pierce).

Statistical Analysis

All data were analyzed using a Student's *t*-test, and a $p<0.05$ was considered statistically significant.

Results

The CSP Suppresses the Proliferation of SW480

For the CSP to promote the development of sporozoite in hepatocytes, it has to be transferred from the parasitophorous vacuole into the cytoplasm [31]. To mimic this process, the full length of the CSP coding sequence was amplified from the *Plasmodium yoelii* 265BY sporozoite, and cloned into the pFLAG-CMV8 plasmid. The resulting recombinant plasmid, pFLAG-CMV8-CSP, was then transfected into the human colon cancer cell line SW480, and its intracellular distribution was determined using indirect immunofluorescence assay using anti-CSP serum. CSP was expressed primarily in the cytoplasm, although also present in the membrane, of SW480 cells after transfection of recombinant plasmid pFLAG-CMV8-CSP (Fig. 1a).

Although the presence of the CSP in the cytoplasm is essential for the development of the malaria sporozoite [31], its effect on cancer cell proliferation had not been investigated. Alamar blue is a sensitive oxidation reduction indicator that fluoresces and undergoes a color change upon reduction in living cells; additionally, the alamar blue assay was reported to be slightly more sensitive than the MTT assay in determining the effect of anti-proliferation compounds on various human cancer cell lines [32]. Thus, the alamar blue assay was used to investigate the effect of the CSP on SW480 proliferation. As shown in Fig. 1b, transfection with 0.4 μg of the pFLAG-CMV8-CSP significantly suppressed the proliferation of SW480, compared with the proliferation following transfection with the control pFLAG-CMV8. When the concentration of the transfected pFLAG-CMV8-CSP was increased from 0.4 to 1.2 μg, cell viability and proliferation decreased in a dose-dependent manner. Following transfection with 1.2 μg of pFLAG-CMV8-CSP, cell proliferation was less than 30 % (0.105 vs. 0.375 OD value). Thus, our data demonstrated the inhibitory role of the CSP on the proliferation of SW480.

The CSP Induces the Apoptosis of SW480

To investigate whether the CSP could also induce the apoptosis of SW480, an Annexin V-FITC apoptosis assay was performed after cells were transfected with 0.8 μg of pFLAG-CMV8-CSP and then stimulated with hTNF-α. As shown in Fig. 2, the apoptosis rate of SW480 transfected with the control plasmid pFLAG-CMV8 was 3.5 %, comparable with that tansfected with pFLAG-CMV8 or pFLAG-CMV8-CSP, or stimualted with hTNF-α alone. However, the apoptosis rate of SW480 transfected with the pFLAG-CMV8-CSP was significantly higher than that transfected with pFLAG-CMV8, after stimulation with hTNF-α (17.4 % vs. 3.5 %, $p<0.01$). Hence, our data indicated that the CSP induced the apoptosis of SW480.

The CSP Suppresses the TNF-α-Induced Activation of NF-κB in SW480 Through its NLS Domain

As NF-κB is important for tumor cell survival and proliferation [7], the effect of the CSP on NF-κB activation of

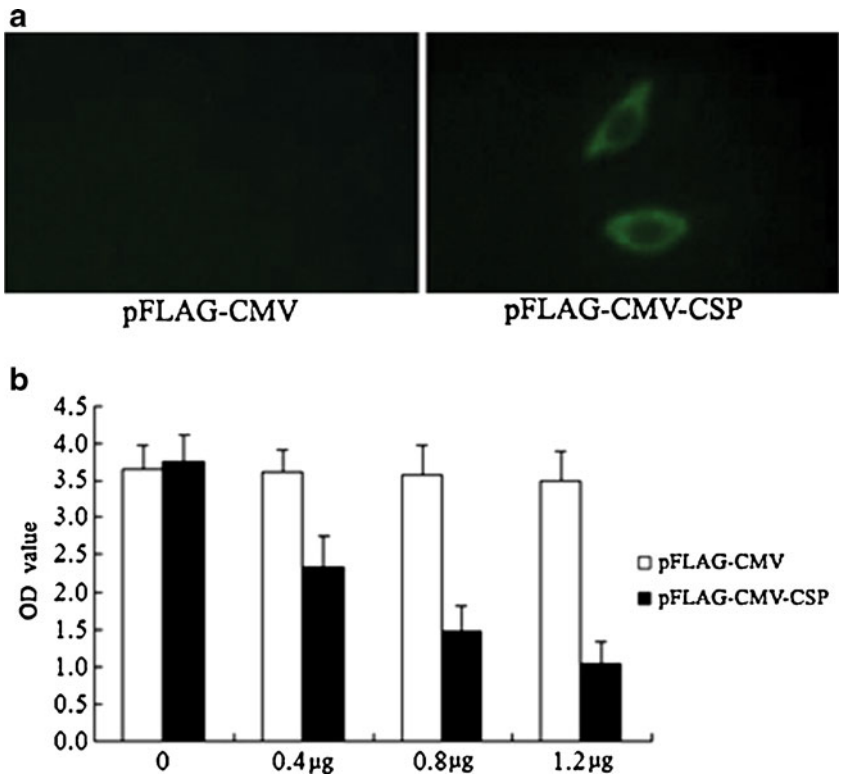

Fig. 1 The effects of pFLAG-CMV8-CSP on the proliferation of SW480. **a** After transfection with pFLAG-CMV8 or pFLAG-CMV8-CSP for 24 h, SW480 cells grown on a coverslip were stained with rabbit anti-CSP serum and goat anti-rabbit FITC-IgG(H+L). **b** After SW480 transfection with or without 0.4, 0.8 or 1.2 μg of or pFLAG-CMV8 or pFLAG-CMV8-CSP, 120 μL Sun-Bio™Am-Blue was added to each well and incubated for 2 h at 37°C. At this point, the fluorescence was read at wavelengths of both 570 and 600 nm. OD570-OD600 was expressed as cell proliferation. Experiments were performed in triplicate, and data are expressed as the Mean±SD. (*$p < 0.05$)

SW480 was investigated. A NF-κB reporter gene assay showed that transfection with pFLAG-CMV8-CSP suppressed the NF-κB activation in SW480 induced with hTNF-α (Fig. 3a). The CSP, a critical protein for invasion of sporozoites into hepatocytes, includes important motifs including the regionI, regionII plus and region III [33] (Fig. 3b). Recently, the CSP was also reported to have a NLS motif with a sequence of VRVRKNVN [31](Fig. 3b). To determine the region responsible for the NF-κB inactivation by the CSP, the recombinant plasmids pFLAG-CMV8-CSP NLS (containing only the NLS motif) and pFLAG-CMV8-CSPΔNLS (CSP lacking the NLS motif) were constructed (Fig. 3b), and their inhibitory role on the activation of NF-κB in SW480 induced with hTNF-α was observed. As shown in Fig. 3c, transfection of pFLAG-CMV8-CSPΔNLS did not inhibit the NF-κB activation in SW480 stimulated with hTNF-α, compared with that tansfected with pFLAG-CMV8 or pFLAG-CMV8-CSP. However, both pFLAG-CMV8-CSP and pFLAG-CMV8-CSP NLS could reduce the NF-κB activity of SW480 more than 3.5 fold. Thus, our data supported the essential role of NLS for inactivation of NF-κB by the CSP.

NLS Outcompetes the Nuclear Translocation of NF-κB in SW480, Following Induction with TNF-α

Proteins with NLS are imported to the cell nucleus through the nuclear pore complex [34]. We postulated that the CSP may inhibit the nuclear translocation of NF-κB through its NLS motif. Both cytoplasmic and nuclear extracts were probed with NF-κB p105/p50 antibody following separation on SDS-PAGE. As shown in Fig. 4, although the cytoplasmic NF-κB p50 was at a comparable level in SW480 transfected with either pFLAG-CMV8 or pFLAG-CMV-CSP NLS, the level of NF-κB p50 in nuclear extract of SW480 transfected with pFLAG-CMV-CSP NLS was much lower than that transfected with pFLAG-CMV8. Hence, our data suggested NLS did not affect the phosphorylation of IκB, and the following release of NF-κB from the inhibitory complex but that it might inhibit the nuclear translocation of the activated NF-κB.

Discussion

The aim of the present study was to determine whether the CSP could inhibit the growth of the human cancer cell SW480. Our data demonstrated that the CSP was found distributed primarily in the cytoplasm of SW480 after transfection and that it suppressed the proliferation and induced the apoptosis of SW480. The inhibitory role of the CSP on the growth of SW480 might be dependent on outcompeting NF-κB nuclear translocation through its NLS motif.

The CSP plays an important role in the sporozoite invasion of hepatocytes and is regarded as an immunodominant protective antigen of irradiation-attenuated sporozoite [33, 35]. We found that the CSP significantly suppressed the growth of human colon cancer cell line SW480 in a dose-

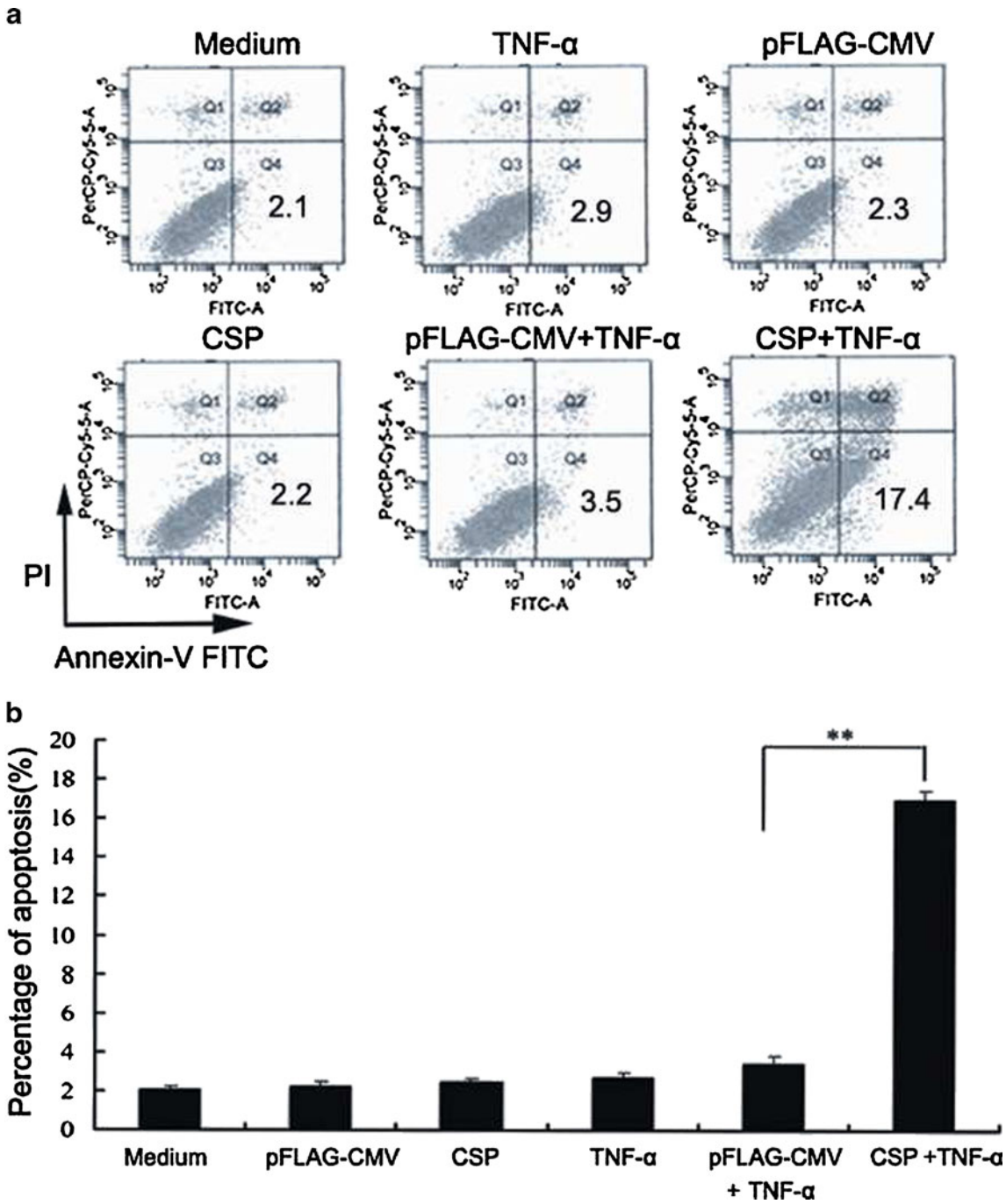

Fig. 2 The effects of the CSP on the apoptosis of SW480. **a** After transfection of SW480 with or without 0.8 μg pFLAG-CMV8 or pFLAG-CMV8-CSP for 24 h, the cells were cultured alone or stimulated with 100 ng/mL hTNF-α for 6 h. Next, the cells were harvested and labeled with Annexin V-FITC and PI and analyzed using flow cytometry. **b** The percentage of apoptotic SW480 following transfection with or without 0.8 μg pFLAG-CMV8 or pFLAG-CMV8-CSP followed hTNF-α stimulation were compared. The experiments were performed in triplicate, and the data are expressed as the M±SD. (**$p < 0.01$)

dependent manner (Fig. 1b) and induced its apoptosis (Fig. 2). It remains to be established whether antiapoptotic genes [36], such as Bcl-2, cFLIP, survivin and IAP-1, are also involved in this process, and whether the CSP could also suppress the growth of other human colon cancer cell lines. However, we have demonstrated a novel role of the CSP in inhibiting the growth of tumor cells.

NF-κB activation is critical for the proliferation, survival and metastasis of colorectal cancer cells [15], thus we investigated whether the inhibition of the CSP on SW480 growth was associated with its blockage of NF-κB activation. We found that transfection of pFLAG-CMV-CSP or pFLAG-CMV-CSP NLS remarkably reduced the NF-κB activity, at comparable levels, of SW480 induced with

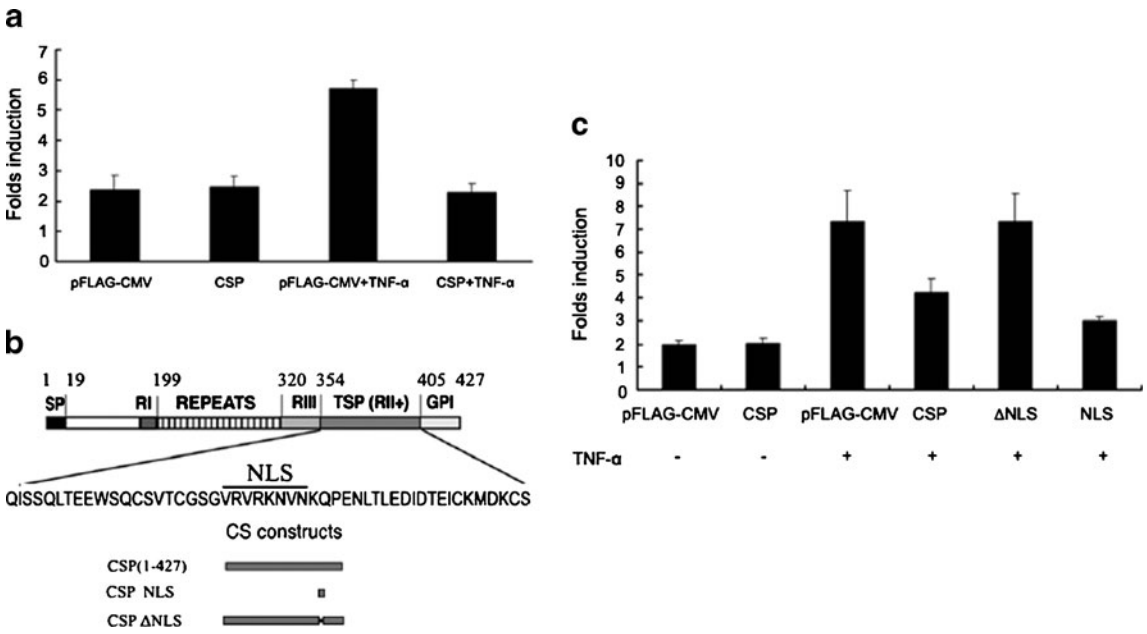

Fig. 3 The effects of the CSP on NF-κB activation in SW480. **a** After transfection of SW480 with or without pFLAG-CMV8-CSP, pBIIx-luc and TK-RL for 24 h, the cells were stimulated with or without 100 ng/mL hTNF-α for 6 h, and both firefly and Renilla luciferase activities were detected. The data are expressed as the ratio of firefly luciferase to Renilla luciferase. **b** Schematic representation of the *P. yoelii* CSP: SP, signal peptide; RI, RII+, RIII and REPEATS are conserved regions of CS; GPI, glycosylphosphatidylinositol attachment site, the number indicates the amino acid position. Bottom: the location of the NLS in the CSP and the representation of the constructed recombinant plasmids. **c** After transfection of SW480 with pBIIx-luc and TK-RL, together with or without pFLAG-CMV8, pFLAG-CMV8-CSP or pFLAG-CMV8-CSP NLS or pFLAG-CMV8-CSPΔNLS for 24 h, the cells were incubated with or without 100 ng/mL hTNF-α for 6 h, and both firefly and Renilla luciferase activities were detected. The data are expressed as the ratio of firefly luciferase to Renilla luciferase

hTNF-α (Fig. 3b,c). Furthermore, the NLS motif of the CSP, but not other sequences, inhibited the nuclear translocation of tumor cell NF-κB (Fig. 4). Thus, our data indicated that the inhibitory role of the CSP on the growth of SW480 might be attributed to outcompeting nuclear translocation of tumor cell NF-κB by its NLS motif. However, other mechanisms of the CSP on tumor growth could not be excluded, as the CSP was previously reported to modulate non-NF-κB target genes [31] and inhibit protein synthesis by binding to the ribosome [37].

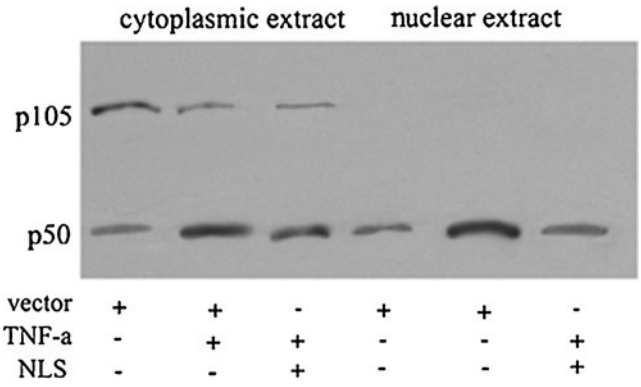

Fig. 4 The effects of the CSP NLS motif on the activation and nuclear translation of NF-κB. After transfection of SW480 with pFLAG-CMV8, pFLAG-CMV8 NLS for 24 h, the cells were stimulated with hTNF-α for 30 min. The cells were then harvested, and both the cytoplasmic and nuclear extracts were isolated, separated on a 4 % polyacrylamide gel and probed with NF-κB p105/p50 antibody

Inhibition of NF-κB activation is a promising antitumor strategy [6, 26]. Several NF-κB inhibitors have been reported to treat various tumors. Curcumin, which inhibits at a step prior to IκB phosphorylation, may suppress the growth of human multiple myeloma cells and head and neck squamous cell carcinoma [28, 38], as well as sensitize colorectal cancer and breast cancer cells to radiation and chemotherapy [22–25]. Another compound, DHMEQ, inhibited the growth of breast carcinoma, thyroid carcinoma, and multiple myeloma by suppression of nuclear translocation of NF-κB [39–41]. As both compounds do not specifically target the tumor cells, they also have a toxic effect on normal tissues, including the immune system, when administrated in vivo [26]. Recently, a small-molecule inhibitor of IKKβ, KINK-1, was reported to increase the susceptibility of melanoma cells to chemotherapy through specifically inhibiting the canonical pathway of NF-κB activation involved in tumor progression [29]. Although KINK-1 has no unwanted side effects on adaptive immunity mediated by the noncanonical pathway, its side effects on innate immunity cannot be completely avoided [42]. Here, we reported a novel protein-based NF-κB inhibitor, the CSP, a main surface protein of the malarial sporozoite, suppressed the growth of SW480. Evidence has suggested that gene therapy specificity on tumor cells can be achieved by placing the therapeutic gene

downstream of a tumor-specific promoter, such as hTERT [43, 44]. Thus, it would be possible to specifically suppress the NF-κB activation in tumor cell, but not the immune system through engineering the CSP into a vector containing a tumor-specific promoter. However, whether the design would work requires further research.

In summary, our data demonstrated that the *Plasmodium yoelii* BY265 CSP suppressed the growth of the human colon cancer cell SW480, potentially via its NLS domain outcompeting NF-κB nuclear translocation. Although further investigations regarding the influence of the CSP on the invasion ability of colon cancer cells as well as its antitumor efficiency in vivo are necessary, the potential ability of the CSP to act as a novel NF-κB inhibitor for the treatment of colorectal carcinoma should be explored.

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Conflict of interest The authors declare that they have no conflict of interest.

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