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ORIGINAL ARTICLE

The Involvement of NF-κB/Klotho Signaling in Colorectal Cancer Cell Survival and Invasion

Biao Xie[1] · Shaolin Nie[2] · Gui Hu[3] · Li Xiong[4] · Fan Hu[1] · Mei Li[1] · Tianshu Peng[5] · Jing Nie[6] · Yongheng He[1]

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Abstract

Lipopolysaccharide significantly increased invasion, cell proliferation, and phospho-NF-κB p65 and phospho-IGF-1R protein, but decreased klotho protein expression, cell apoptosis, and the percentage of sub G0/G1 cells in SW480 and HT29 colorectal cancer cells. In contrast, NF-κB inhibitor exhibited a counteract effect of lipopolysaccharide. Transfection of Toll-like receptor 4 shRNA significantly decreased phospho-NF-κB p65 and phospho-IGF-1R protein levels, invasion, but significantly increased klotho protein expression, cell apoptosis, and the percentage of sub G0/G1 in SW480 and HT29 cells. In conclusion, inflammation inhibits *klotho* gene expression in colorectal cancer cells through activation of Toll-like receptor 4 /NF-κB signal pathway.

Keywords LPS · NF-κB · Colorectal cancer · Klotho

Introduction

The statistics of WHO reported that colorectal cancer (CRC) is a third leading cause of death worldwide, accounting for 774,000 deaths in 2015 [1]. The statistics published in 2017 by the American Cancer Society revealed that the overall incidence and mortality of CRC are dramatically reduced during the last decade in USA, but CRC is still the third most commonly diagnosed cancer in Americans [2]. The 5-year relative survival rate for patients with stage I, IIA, IIB, IIIA, IIIB, IIIC, and IV colon cancer is about 92%, 87%, 63%, 89%, 69%, 53%, and 11%, respectively. The 5-year relative survival rate for patients with stage I, IIA, IIB, IIIA, IIIB, IIIC, and IV rectal cancers is about 87%, 80%, 84%, 71%, 58%, and 12%, respectively [3]. Despite the progress in screening, diagnosis, and therapy, the prognosis of CRC patients with advance disease is still poor. The partial reason for the poor prognosis of CRC might be that the molecular mechanisms which drive CRC development are not fully elucidated.

The relationship between inflammation and tumorigenesis of CRC is well-established and inflammatory bowel disease is widely thought an important risk factor [4]. However, the molecular mechanisms by which inflammation drives the development of CRC are not fully elucidated. NF-κB is a key inflammatory factor involving in the development of colitis-associated cancer [5]. NF-κB can stimulate the expression of various cytokines and chemokines during inflammatory response which are involved in the epithelial-to-mesenchymal transition (EMT) during colorectal carcinogenesis [6]. Lipopolysaccharide (LPS) is an activation factor of immune system, which can activate NF-κB and subsequently promote tumor development [7]. Toll-like receptor (TLR) 4 has been demonstrated to be one of the major receptors which mediate the activation of NF-κB induced by LPS and other microbial products [8].

Biao Xie and Shaolin Nie contributed equally to this work.

✉ Yongheng He
heyongheng1964@163.com

[1] Department One of Anorectal Surgery, The Second Affiliated Hospital of Hunan University of Chinese Medicine, 139 Renmin Road, Changsha, Hunan 410005, People's Republic of China

[2] Department of Colorectal Surgery, Hunan Province Tumor Hospital, Changsha 410013, Hunan, China

[3] Department of Gastrointestinal Surgery, The Third Xiangya Hospital, Central South University, Changsha 410013, Hunan, China

[4] Department of Hepatobiliary Surgery, the Second Xiangya Hospital, Central South University, Changsha 410008, Hunan, China

[5] Hunan Slack King Laboratory Animal Co., Ltd., Changsha 4100125, Hunan, China

[6] Department Four of Anorectal Surgery, The Second Affiliated Hospital of Hunan University of Chinese Medicine, Changsha, Hunan 410005, People's Republic of China

Klotho was initially identified as an anti-aging gene and is abundantly expressed in various human tissues, but its expression is aberrant in a number of cancers [9, 10]. Our previous studies demonstrated that klotho functions as a tumor suppressor to inhibit the proliferation of gastric cancer [11]. Klotho may participate in the progression of human cancers through inhibiting insulin/IGF1 and Wnt signaling [12]. A recent study demonstrated that high glucose decreased klotho expression, but activated NF-κB p65 signaling pathways in cultured mesangial cells and promoted the downstream inflammatory reaction [13]. However, the relationship between NF-κB signaling and klotho expression in colorectal cancer cells remains unknown. This study investigated the effects of LPS on *Klotho* gene expression in colorectal cancer cells and their relationship with NF-κB signaling as well as the biological significance of inflammation-klotho in the malignant phenotype of colorectal cancer.

Materials and Methods

Cell Culture

The SW480, SW620, HCT116, Colo205, HT29, and Caco-2 human colorectal cancer cell lines were obtained from the Shanghai Institute. Cells were cultured at 37 °C, 5% CO_2 in DMEM medium (GIBCO) containing 10% FBS (fetal bovine serum).

Cell Proliferation Analysis

A Cell Counting Kit-8 (CCK-8) assay (Beyotime, Shanghai, China) was used to measure the cell proliferation. SW480 and HT29 cells were seeded in 96-well plates (1×10^4 cells/well) and cultured for overnight. The cells were then incubated with 1 μg/ml of LPS with or without 1 μg/ml of CAPE for 48 h. CCK-8 solution (10 μl) was added to each well, followed by continuous culture for 2 h. The absorbance was measured at 450 nm by a multiplate reader. Cell proliferation was expressed as a percentage of the untreated cells.

Apoptosis and Cell Cycle Analysis

Apoptosis was detected by an annexin V-FITC / propidium iodide staining. Briefly, the treated cells were harvested by centrifugation at 2000 rpm × 5 min after trypsanization. After washing with phospholate buffer saline (PBS) for once, 5×10^5 cells were re-suspended in 600 μL of binding buffer and incubated with 5 μL of annexin V-FITC and then 5 μL of propidium iodide for 10 min at RT (room temperature) in the dark room. Apoptotic cells were counted using flow cytometry.

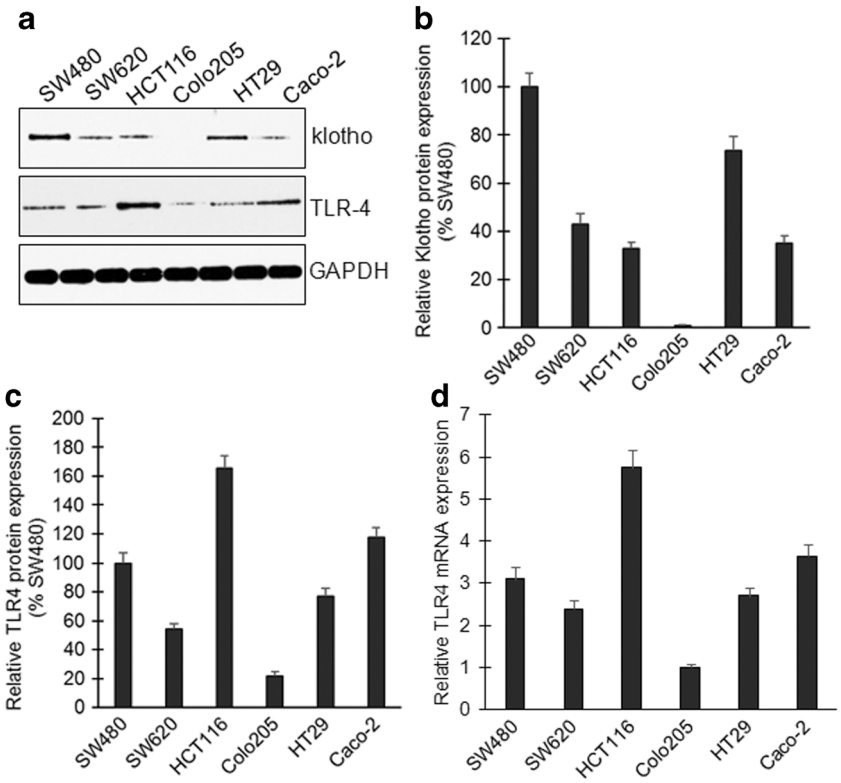

Fig. 1 The expression of klotho and TLR-4 protein in colorectal cancer cell lines. **a** Representative Western blots of klotho, TLR-4, and GAPDH protein in different cell lines. **b** Semi-quantitative analysis of klotho protein expression in (**a**). **c** Semi-quantitative analysis of TLR-4 protein expression in (**a**). **d** Real-time PCR of TLR-4 mRNA expression

Cell cycle was also analyzed by flow cytometry. Briefly, cells were treated, trypsinized, and resuspended as described above. After fixed in 75% ethanol (pre-cooled) overnight at 4 °C, cells were washed with 1 × PBS, and re-suspended in 800 μl of 1 × PBS containing 1% BSA. Cells were then incubated with 100 μl of PI dye and 100 μg of RNase A (10 mg / ml) in the dark at 37 °C for 30 min. Cell cycles were sorted using flow cytometry.

Western Blot

The treated cells were collected in Eppendorf tubes and homogenized with 200 μl of RIPA lyses buffer for 30 min on ice, followed by sonication for 10 s on ice. The lysate was collected after removing the debris (centrifuge at 14,000 rpm × 10 min at 4 °C). The total protein concentration was determined by BCA protein assay kit (Bio-Rad, Hercules, CA, USA). To perform Western blot, 20 μg of total protein was separated on 4–20% SDS-PAGE (sodium dodecyl sulphate-polyacrylamide *gel* electrophoresis) gels and then electrophoretically transferred on the PVDF (polyvinylidene difluoride) membranes. After blocking with 5% non-fat milk, the membranes were incubated with primary antibody for overnight at 4 °C, followed by HRP-conjugated secondary antibody for two hours after washing with PBST buffer for 10 min × 4 times. The immune reaction was visualized by incubating the membranes in ECL substrates. The antibodies for human klotho, phospho-IGF-

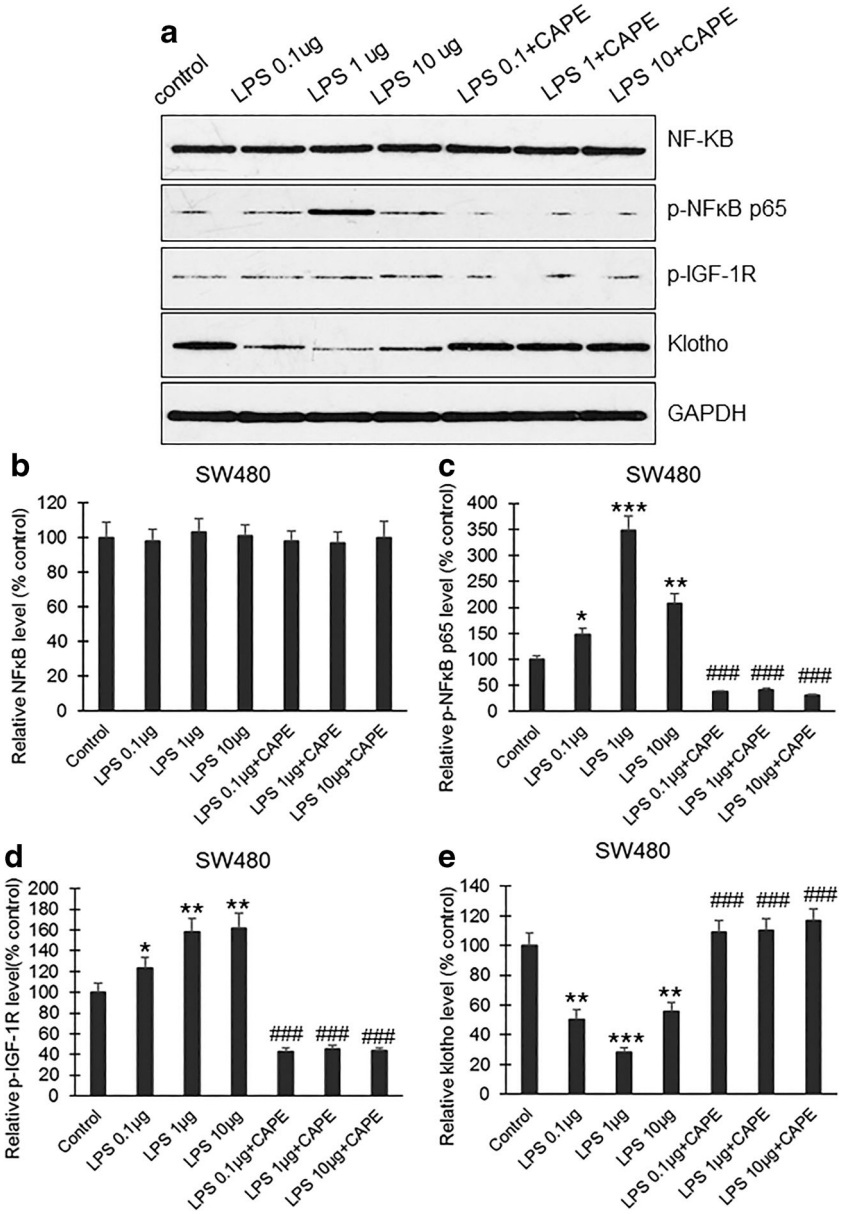

Fig. 2 The effects of LPS and CAPE on klotho-associated signaling in SW480 cells. **a** Representative Western blots of NF-κB, phospho-NF-κB p65 (p-NF-κB p65), phospho-IGF-1R (p-IGF-1R), klotho, and GAPDH protein expression underlying 0.1 to 10 μg/ml of NF-κB activator LPS with or without 0.1 to 10 μg/ml of NF-κB inhibitor CAPE. **b** Semi-quantitative analysis of NF-κB protein expression in (**a**). **c** Semi-quantitative analysis of p-NF-κB p65 protein levels in (**a**). **d** Semi-quantitative analysis of p-IGF-1R protein levels in (**a**). **e** Semi-quantitative analysis of klotho protein expression in (**a**). $^{*}p < 0.05$, $^{**}P < 0.01$, $^{***}p < 0.001$ vs. control group. $^{\#\#\#}p < 0.001$ vs. the responding LPS alone group. $N = 4$

IR, phospho-NF-κB p65 (Ser276), NF-κB, and GAPDH and HRP-conjugated anti-mouse and anti-rabbit antibody were purchased from Santa Cruz biotechnology (Santa Cruz, CA, USA).

Real-Time PCR

Total RNA was isolated from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed using RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas Biotechnology, Waltham, MA, USA). Real-time quantitative PCR was performed using SYBR Green PCR Master Mix (ABI company, Leuven, Belgium). TLR-4 gene was amplified using a forward primer: 5′- AGACCTGTCCCTGA ACCCTAT-3′ and a reverse primer: 5’-CGATGGAC TTCTAAACCAGCCA-3′. GAPDH gene was amplified using a forward primer: 5’-GGAGCGAGATCCCTCCAAAA T-3’and a reverse primer: 5’-GGCTGTTGTCATACTTCTCA TGG-3′ as internal control. Data were analyzed using the comparative $^{\Delta\Delta}$CT method.

Cell Invasion Assay

The treated cells were harvested by centrifugation at 2000 rpm × 5 min after digestion with 0.25% EDTA-free trypsin. After washing with PBS once, cells

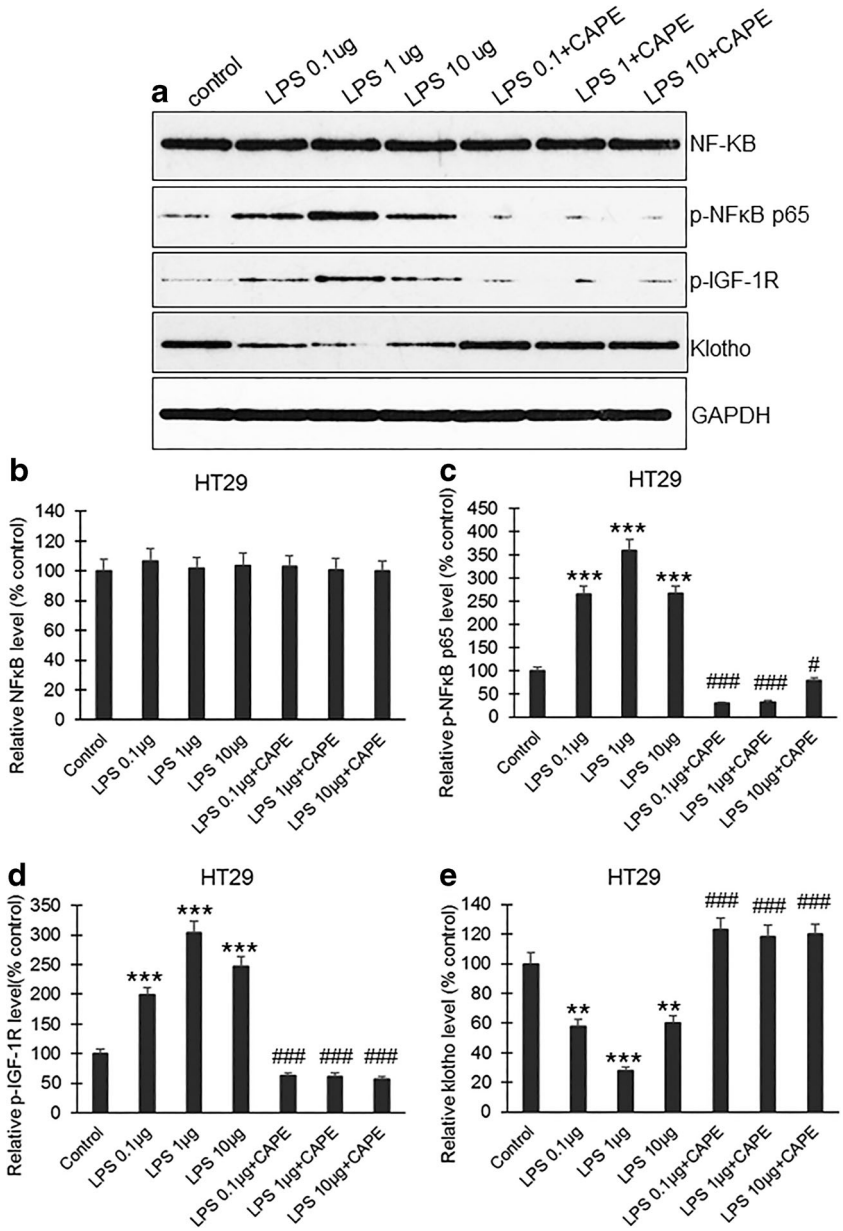

Fig. 3 The effects of LPS and CAPE on klotho-associated signaling in HT29 cells. HT29 cells were treated with 0.1 to 10 μg/ml of NF-κB activator LPS with or without 0.1 to 10 μg/ml of NF-κB inhibitor CAPE. **a** Representative Western blots. **b**–**e** Semi-quantitative analysis of NF-κB (**b**), phospho-NF-κB p65 (p-NF-κB p65) (**c**), phospho-IGF-1R (p-IGF-1R) (**d**), and klotho (**e**) protein levels in (**a**). $^{**}P < 0.01$, $^{***}p < 0.001$ vs. control group. $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ vs. the responding LPS alone group. $N = 4$

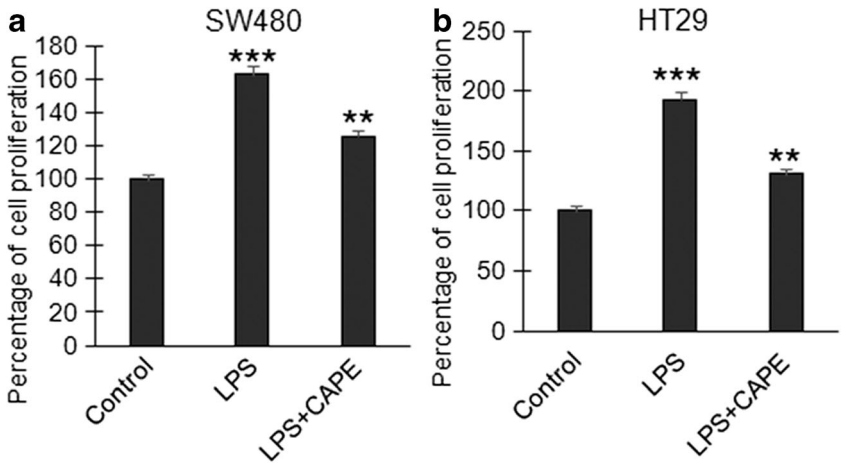

Fig. 4 The effects of NF-κB activation on cell proliferation in SW480 and HT29 cells. Cells were treated as described above. **a** The percentage of proliferation in SW480 cells. **b** The percentage of proliferation in HT29 cells. $^{**}P < 0.01$, $^{***}p < 0.001$ vs. control group. $N = 4$

weresuspended in DMEM medium containing 1% BSA at 5×10^4/ml. One ml of DMEM medium (10% FBS) was added to 6-well plates and two ml cells were added into the Transwell chamber. After cells were cultured for 24 h, the membrane was removed from the lower chamber. Matrigel was wiped with a cotton swab from

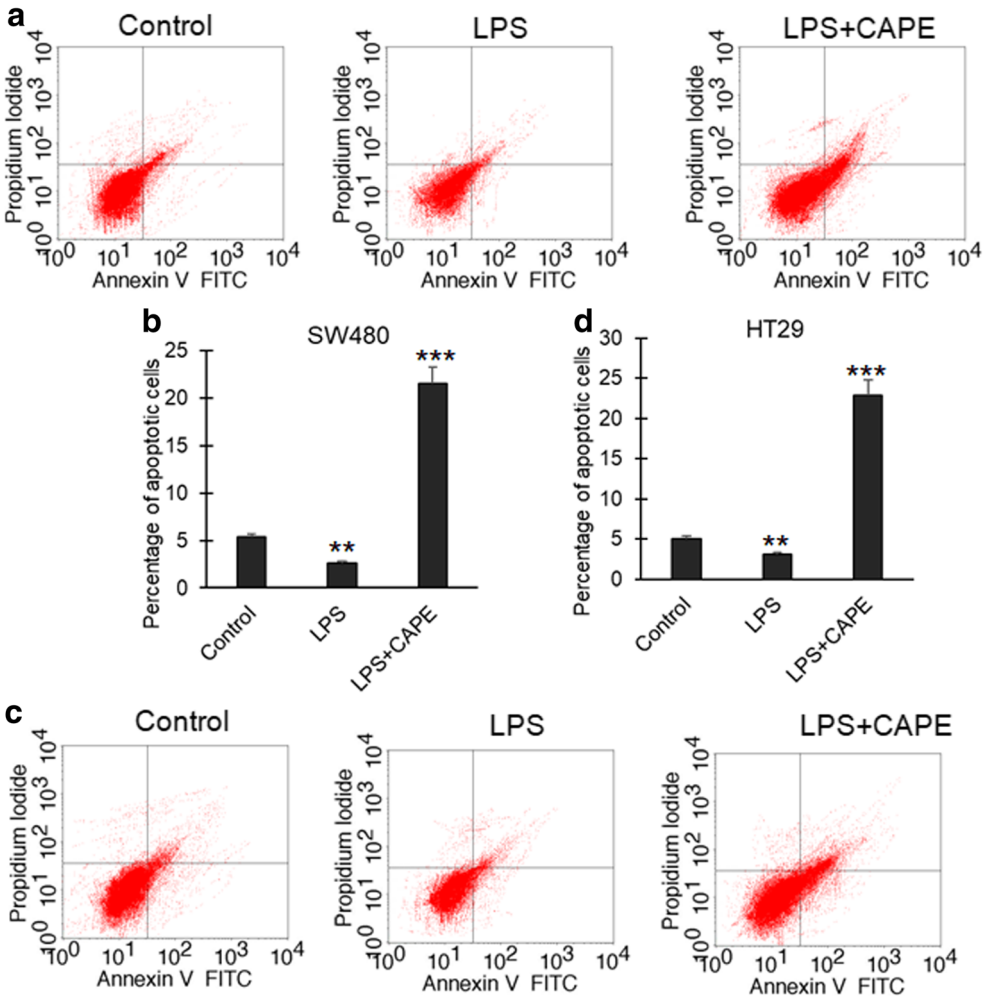

Fig. 5 The effects of NF-κB activation on cell apoptosis in SW480 and HT29 cells. Cells were treated as described above. **a** Representative flow cytometry of apoptotic SW480 cells. The right up and down quadrant indicates the apoptotic cells. **b** The percentage of apoptotic SW480 cells. **c** Representative flow cytometry of apoptotic HT29 cells. **d** The percentage of apoptotic HT29 cells. $^{**}P < 0.01$, $^{***}p < 0.001$ vs. control group. $N = 4$

the membranes and cells were fixed with 95% alcohol for 10 min. After hematoxylin staining for 10 min, cells were counted under inverted microscope.

Cell Transfection

Cells were seeded in 6-well plates and transfected with TLR-4-shRNA expression vector (2 μg/ml) using Lipofectamine ™ 2000 reagent by following the user manual when cells were at 70% confluency. The TLR-4-shRNA expression vectors were constructed by cloning of a mini gene of TLR-4 into the pGPU6/GFP/Neo vector at Bam HI and Bbs I sites. Four minigenes (TLR-4-shRNA) were tested and the most effective minigene was constructed into pGPU6/GFP/Neo vector, called pGFP-shTLR-4. The TLR-4 minigene included TLR-4-shRNA1 (5′- CACCGGTTTAGAAGTCCATCGTTTGT TCAAGAGACAAACGATGGACTTCTAAACCTTTTTTG -3′), TLR-4-shRNA2 (5′- CACCGCCTGTGCAATTTGAC CATTGTTCAAGAGACAATGGTCAAATTGCACAGGCT TTTTTG -3′), TLR-4-shRNA3 (5′- CACCGGACCTCTCT CAGTGTCAACTTTCAAGAGAAGTTGACACT GAGAGAGGTCCTTTTTTG -3′), and TLR-4-shRNA4 (5′- CACCGCTTCTTGCTGGCTGCATAAATTCAAGA GATTTATGCAGCCAGCAAGAAGCTTTTTTG -3′).

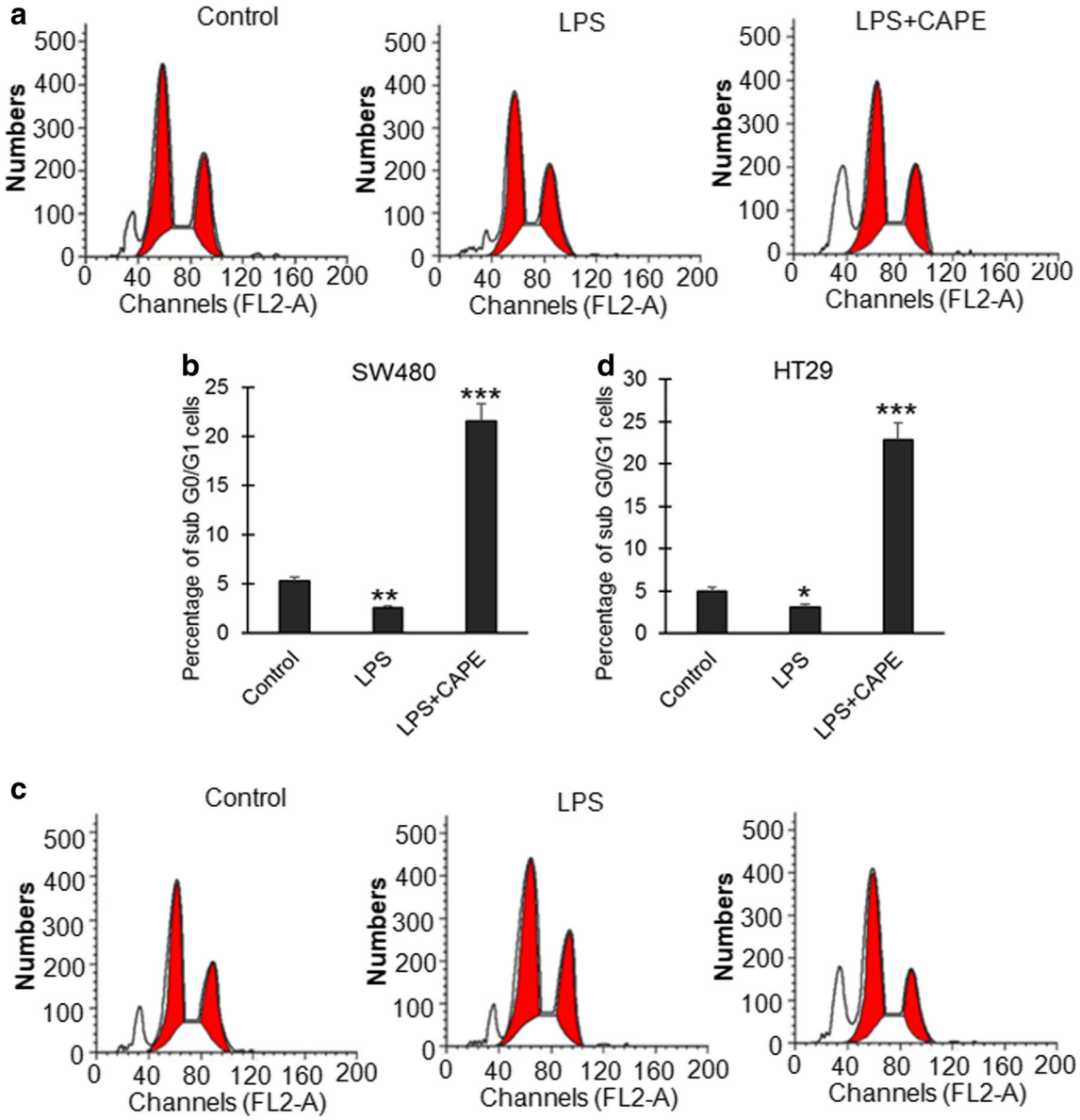

Fig. 6 The effects of NF-κB activation on the percentage of sub G0/G1 cells of SW480 and HT29 cells. **a** Representative flow cytometry assay of cell cycle in SW480 cells. **b** The percentage of sub G0/G1 cells in SW480 cells. **c** Representative flow cytometry assay of cell cycles in HT29 cells. **d** The percentage of sub G0/G1 cells in treated HT29 cells. $^{**}P < 0.01$, $^{***}p < 0.001$ vs. control group. $N = 4$

NF-κB activator (LPS, 1 μg / ml) and NF-κB inhibitor (Caffeic Acid Phenethyl Ester, called CAPE, 200 μM) were added 24 h after transfection and cells were continuously incubated for 24–48 h.

Statistical Analysis

Data were analyzed using SPSS v17.0 statistical software. The χ^2 test was used to analyze the differences of count data, while the independent sample t-test was used to analyze the measurement data. A $P < 0.05$ was considered statistically significant.

Results

The Effects of NF-κB Activation on Klotho Gene Expression and its Downstream Signaling in SW480 and HT29 Cells

The expression of klotho and TLR-4 protein were investigated in 6 colorectal cancer cell lines using Western blot assay. The mRNA expression of TLT-4 was measured by real-time PCR. SW480 cells exhibited a highest klotho protein expression and third *TLR-4* mRNA and protein expression. HT29 cells exhibited a secondary highest klotho protein expression and fourth highest *TLR-4* mRNA and protein expression (Fig. 1). Therefore, SW480 and HT29 were selected for the further experiments in this study.

In SW480 cells, 0.1 to 10 μg/ml of NF-κB activator LPS with or without 0.1 to 10 μg/ml of NF-κB inhibitor CAPE exhibited no effects on NF-κB expression (Fig. 2a, b). Different doses of LPS significantly increased phospho-NF-κB p65 (Fig. 2a, c) and phospho-IGF-1R protein levels (Fig. 2a, d), but significantly decreased klotho protein expression (Fig. 2a, e). NF-κB inhibitor CAPE exhibited a counteract effect of LPS on NF-κB p65 and IGF-1R phosphorylation, and klotho protein expression (Fig. 2). In HT29 cells, LPS and CAPE exhibited similar effects on NF-κB, phospho-NF-κB p65, phospho-IGF-1R, and klotho protein levels to that in SW480 cells (Fig. 3).

The Effects of NF-κB Activation in Cell Invasion, Cell Cycle, and Apoptosis in SW480 and HT29 Cells

The SW480 and HT29 cells were treated with 1 μg/ml of LPS with or without 1 μg/ml of CAPE for 48 h.

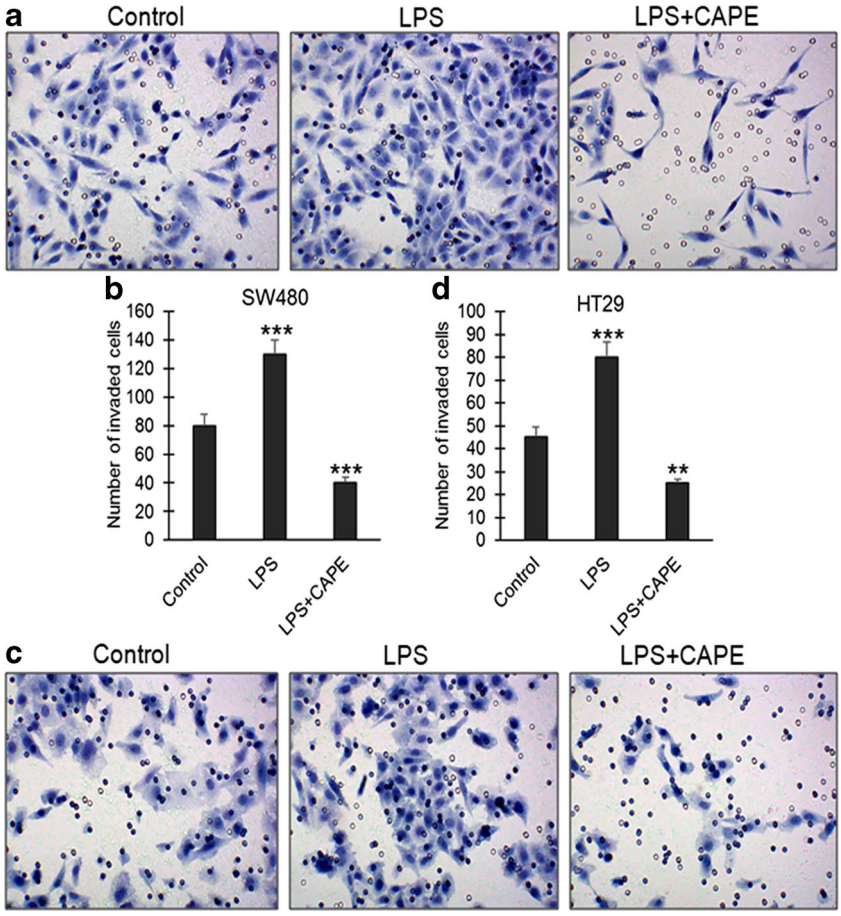

Fig. 7 The effects of NF-κB activation on cell invasion in SW480 and HT29 cells. **a** Representative photographs of cell invasion in SW480 cells. **b** The number of cell invasion in SW480 cells. **c** Representative photographs of cell invasion in HT29 cells. **d** The number of cell invasion in HT29 cells. $^{**}P < 0.01$, $^{***}p < 0.001$ vs. control group. $N = 4$

LPS significantly increased the cell proliferation (Fig. 4), but decreased the percentage of cell apoptosis in SW480 and HT29cells (Fig. 5) ($p < 0.01$) compared to control cells. In contrast, LPS plus CAPE significantly decreased cell proliferation (Fig. 4), but increased the percentage of cell apoptosis (Fig. 5) in SW480 and HT29 cells ($p < 0.001$) compared with LPS alone treated cells. Flow cytometry showed that LPS significantly decreased the percentage of sub G0/G1 in SW480 (Fig. 6a, b, $p < 0.01$) and HT29 cells (Fig. 6c, d) ($p < 0.05$) compared to control cells. LPS plus CAPE significantly increased the percentage of sub G0/G1 cells in both cell lines compared to cells treated with LPS alone (Fig. 6) ($p < 0.001$). Also, LPS significantly increased invasion in SW480 (Fig. 7a, b) and HT29 (Fig. 7c, d) cells ($p < 0.001$) compared to control cells. In contrast, LPS plus CAPE significantly inhibited cell invasion in both cell lines compared to cells treated with LPS alone ($p < 0.01$, $p < 0.001$).

The Effects of TLR-4 Knockdown on Klotho Gene Expression and its Downstream Signaling in SW480 and HT29 Cells

A TLR-4 shRNA expression vector was constructed (pGFP-shTLR-4) to test the effects of TLR-4 on klotho-associated signaling. Transfection of pGFP-shTLR-4 vectors for 48 h established over 85% transfection efficacy in SW480 (Fig. 8a) and HT29 (Fig. 9a) cells. Western blot showed that transfection of pGFP-shTLR-4 with or without LPS stimulation for 24 h significantly decreased phospho-NF-κB p65 (Fig. 8b, d) and phospho-IGF-1R protein levels (Fig. 8b, e), but significantly increased klotho protein expression (Fig. 8b, f) in SW480 cells ($p < 0.001$). Similarly, transfection of pGFP-shTLR-4 with or without LPS stimulation for 24 h significantly decreased phospho-NF-κB p65 (Fig. 9b, d) and phospho-IGF-1R protein levels (Fig. 9b, e), but increased klotho expression (Fig. 9b, f) in HT29 cells ($p < 0.01$, $p < 0.001$). Transfection of pGFP-shTLR-4 with or without LPS

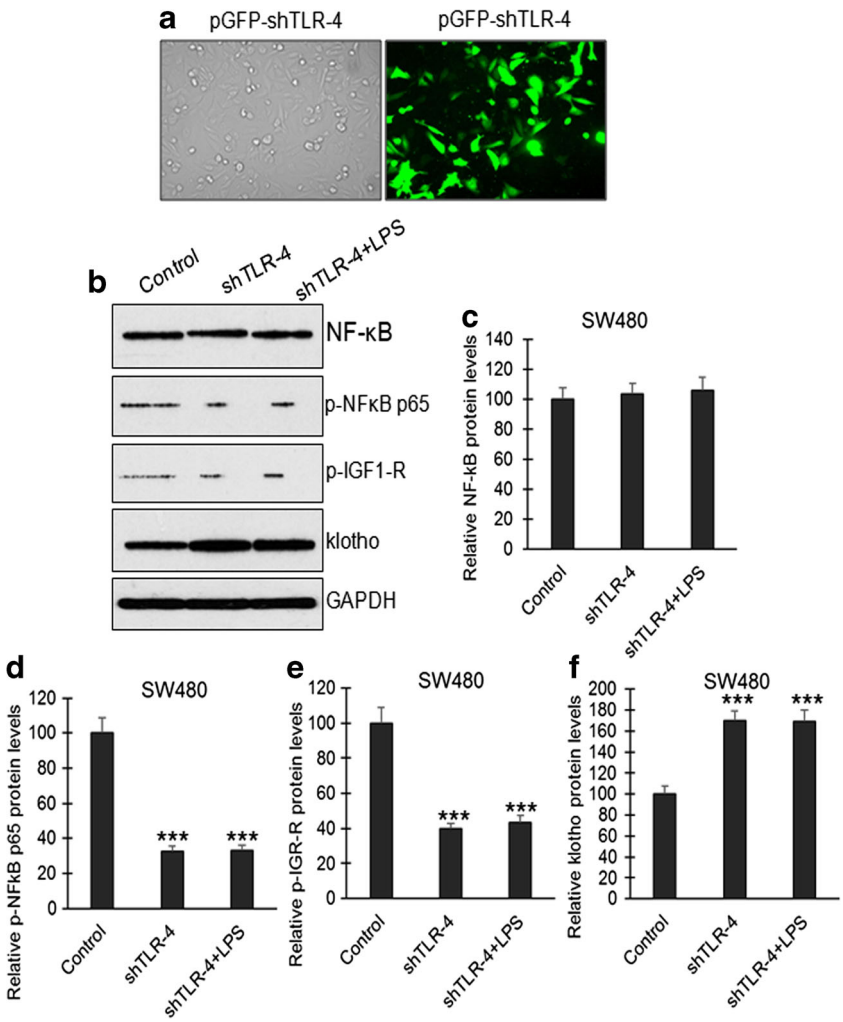

Fig. 8 The Effects of TLR-4 knockdown on the expression of klotho-associated signal molecules in SW480 cells. **a** The efficacy of pGFP-shTLR-4 transfection in SW480 cells. **b** Representative Western blots of NF-κB, phospho-NF-κB p65 (p-NF-κB p65), phospho-IGF-1R (p-IGF-1R), klotho, and GAPDH protein expression in SW480 cells underlying pGFP-shTLR-4 transfection (shTLR-4) with or without LPS stimulation. C-F) Semi-quantitative analysis of NF-κB (**c**), p-NF-κB p65 (**d**), p-IGF-1R (**e**), and klotho (**e**) protein levels in (**a**). $^{**}P < 0.01$, $^{***}p < 0.001$ vs. control group. $N = 4$

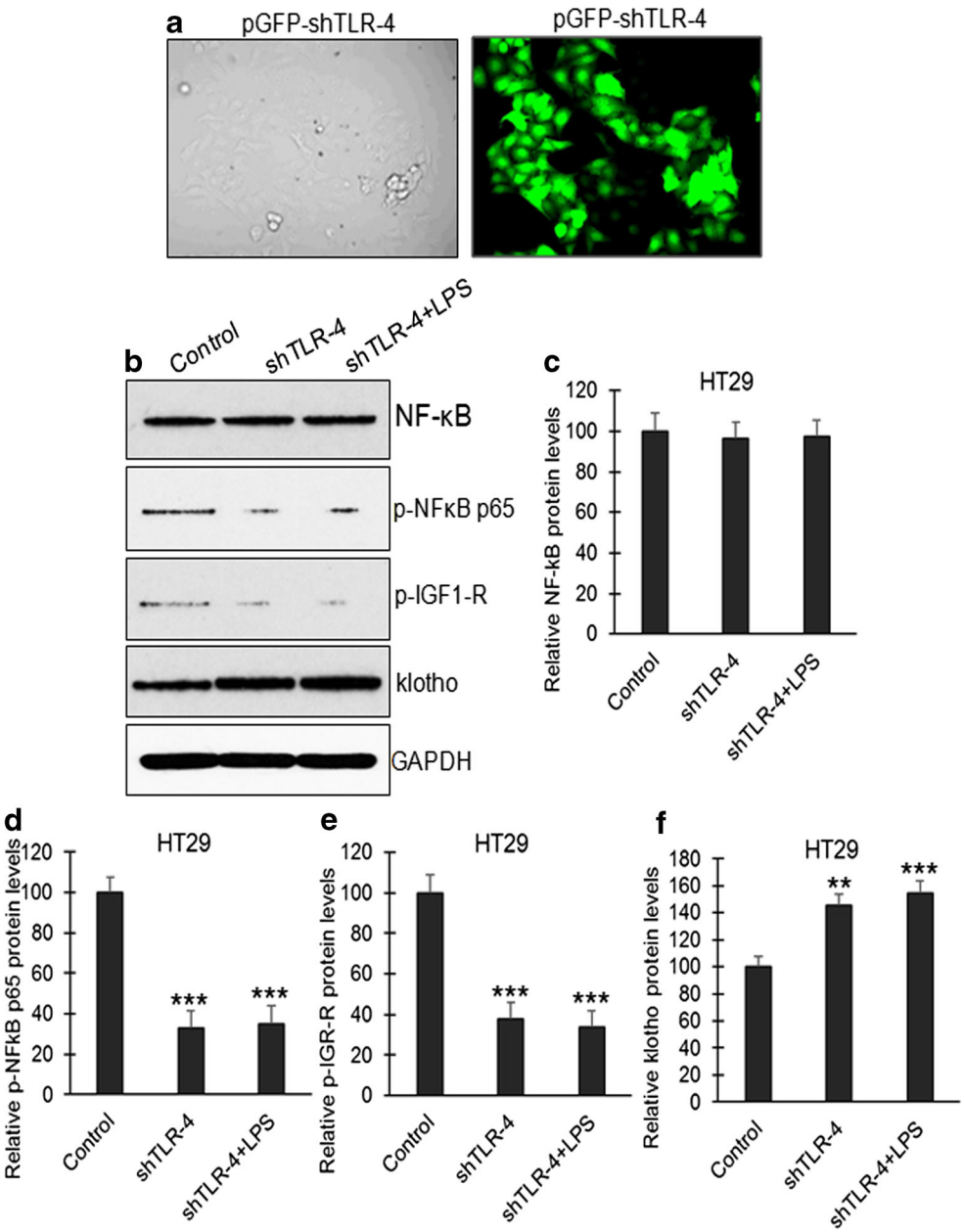

Fig. 9 The Effects of TLR-4 knockdown on the expression of klotho-associated signal molecules in HT29 cells. **a** pGFP-shTLR-4 transfection in HT29 cells. **b** Representative Western blots of NF-κB, phospho-NF-κB p65 (p-NF-κB p65), phospho-IGF-1R (p-IGF-1R), klotho, and GAPDH protein expression in HT29 cells underlying pGFP-shTLR-4 transfection (shTLR-4) with or without LPS stimulation. **c**–**f** Semi-quantitative analysis of NF-κB (**c**), p-NF-κB p65 (**d**), p-IGF-1R (**e**), and klotho (**e**) protein levels in (**a**). $^{**}P < 0.01$, $^{***}p < 0.001$ vs. control group. $N = 4$

stimulation showed no effects on NF-κB expression in both cell lines (Figs. 8b, c and 9b, c).

The Effects of TLR-4 Knockdown on Cell Invasion, Cell Cycle, and Apoptosis in SW480 and HT29 Cells

Transfection of pGFP-shTLR-4 with or without LPS stimulation for 48 h significantly increased the percentage of cell apoptosis in SW480 (Fig. 10a, b) and HT29 (Fig. 10c, d) cells ($p < 0.001$). Flow cytometry showed that transfection of pGFP-shTLR-4 with or without LPS stimulation for 48 h significantly increased the percentage of sub G0/G1 in SW480 (Fig. 11a. 11B, $p < 0.001$) and HT29 cells (Fig. 11c, d) ($p < 0.001$). Also, transfection of pGFP-shTLR-4 with or without LPS stimulation for 48 h significantly decreased invasion in SW480 (Fig. 12a, b) and HT29 (Fig. 12c. 12D) cells ($p < 0.05$).

Discussion

The roles of inflammation in tumorigenesis of colorectal cancers have been well-documented. However, the molecular

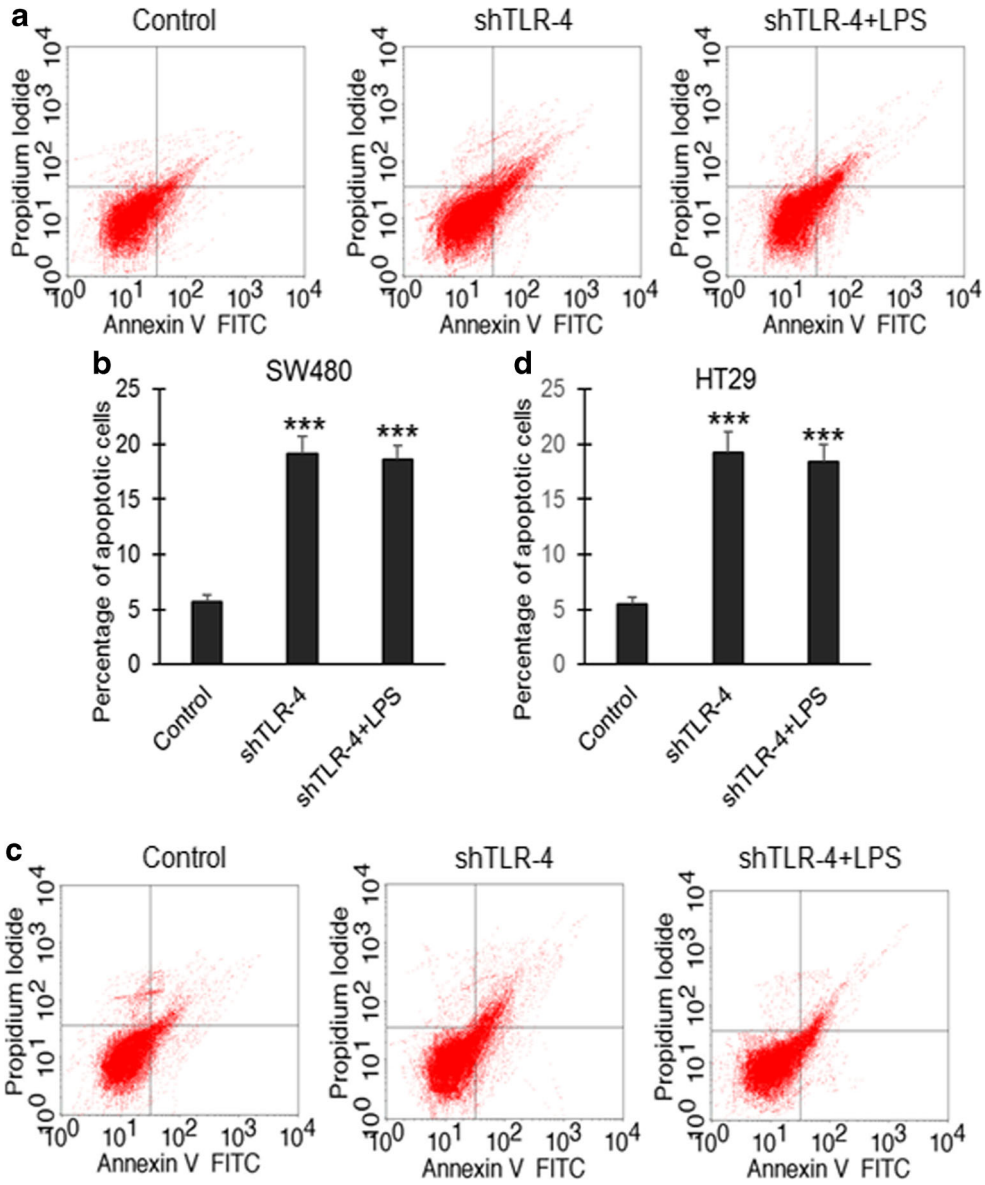

Fig. 10 The effects of TLR-4 knockdown on cell apoptosis in SW480 and HT29 cells. Cells were transfected with pGFP-shTLR-4 with or without LPS stimulation. **a** Representative flow cytometry of apoptotic SW480 cells. The right up and down quadrant indicates the apoptotic cells. **b** The percentage of apoptotic SW480 cells. **c** Representative flow cytometry of apoptotic HT29 cells. **d** The percentage of apoptotic HT29 cells. $^{***}p < 0.001$ vs. control group. $N = 4$

mechanisms involving in the inflammation-induced tumor cell growth, survival, and invasion remain to be fully elucidated. The findings in this study suggest the involvement of TLR-4/ NF-κB/klotho/IGF-1R signal pathway in LPS-induced CRC cell survival and invasion. This study provides a potential therapeutic approach to treat CRC through targeting this inflammation-activated pathway.

The roles of TLR- 4 in LPS and other microbial products induced activation of NF-κB have been well-characterized [8]. In addition, TLR-4 can induce the expression of many genes and activation of many proteins during inflammatory responses, including NF-κB, activator protein 1, Signal Transducers and Activators of Transcription family of transcription factors (STAT1) and Interferon regulatory factors (IRF's), which are the key players in regulating the inflammatory response [14]. This study revealed that LPS increased NF-κB phosphorylation in CRC cells, as well as the survival, and invasion of CRC cells in two cell lines. In contrast, knockdown of TLR-4 expression significantly decreased NF-κB phosphorylation, survival, and invasion. Also, administration of the NF-κB inhibitor CAPE can reverse the effects of LPS in the survival and invasion of CRC cells. However, neither LPS, nor TLR-4 knockdown affected NF-κB protein expression, suggesting that

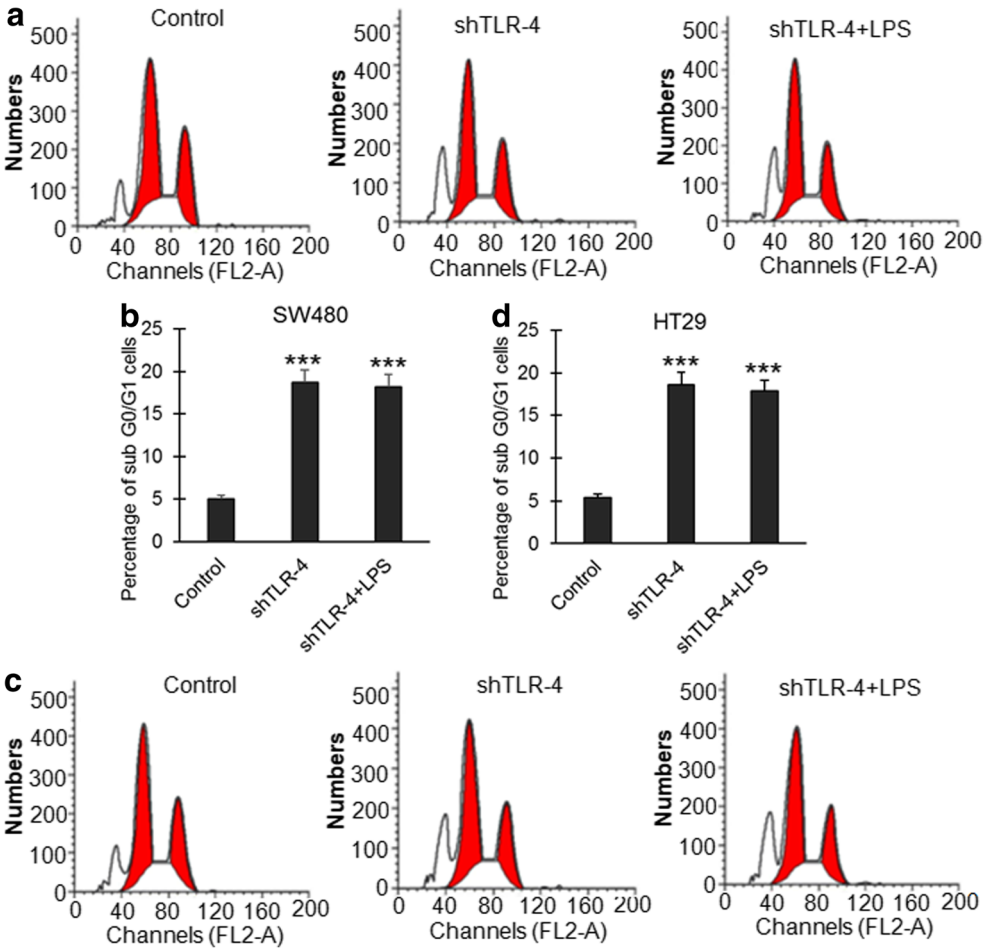

Fig. 11 The effects of TLR-4 knockdown on the percentage of sub G0/G1 cells of SW480 and HT29 cells. **a** Representative flow cytometry assay of cell cycle in SW480 cells. **b** The percentage of sub G0/G1 cells in SW480 cells. **c** Representative flow cytometry assay of cell cycles in HT29 cells. **d** The percentage of sub G0/G1 cells in treated HT29 cells. $^{*}P < 0.001$ vs. control group. $N = 4$

inflammatory factors activated TLR-4/NF-κB signaling. NF-κB is an important nuclear transcription factor involving in tumor inflammation [15]. Thus, our study confirmed the roles of inflammation in the survival and invasion of CRC through a LPS initiated TLR-4 / NF-κB signaling.

Klotho has been characterized as a tumor suppressor and its inhibitory effects in the proliferation of gastric tumor cells have been reported [11]. The lack of klotho expression has been observed in several tumors [12, 16]. However, the expression of klotho in human CRC patients and its effects in CRC cell survival and invasion have not been reported. Klotho can participate in the progression of human cancers through inhibiting insulin/IGF1 signaling [12]. However, how the inflammation regulates klotho gene expression is still unclear. This study found that when NF-κB signaling was inhibited, klotho protein expression was significantly increased in CRC cells. In contrast, when NF-κB signaling was activated, klotho protein expression was significantly decreased. Also, the increase or decrease in klotho protein expression was accompanied by the decrease or increase in IGF-1R phosphorylation. Our previously studies found that klotho protein inhibited the proliferation, survival, and invasion of gastric cancer and liver cancer cells, mainly through the inhibition of IGF-1R phosphorylation and its downstream signaling pathways [17, 18]. This study found that p-IGF-1R levels, and cell invasion were significantly increased, whereas cell apoptosis was significantly decreased after activation of TLR-4/ NF-κB signaling. Our findings suggest that inflammatory factors like LPS activate TLR-4/ NF-κB signaling and subsequently inhibits klotho expression and activate IGF-1 receptor. Thus, *klotho* gene may be an intermediate factor of inflammation in colorectal cancer.

In conclusion, our study first proposed that inflammation inhibits *klotho* gene expression in colorectal cancer cells through activation of TLR-4/NF-κB signal pathway and highlighted a potential therapeutic approach to treat colorectal cancer through targeting *klotho* gene expression.

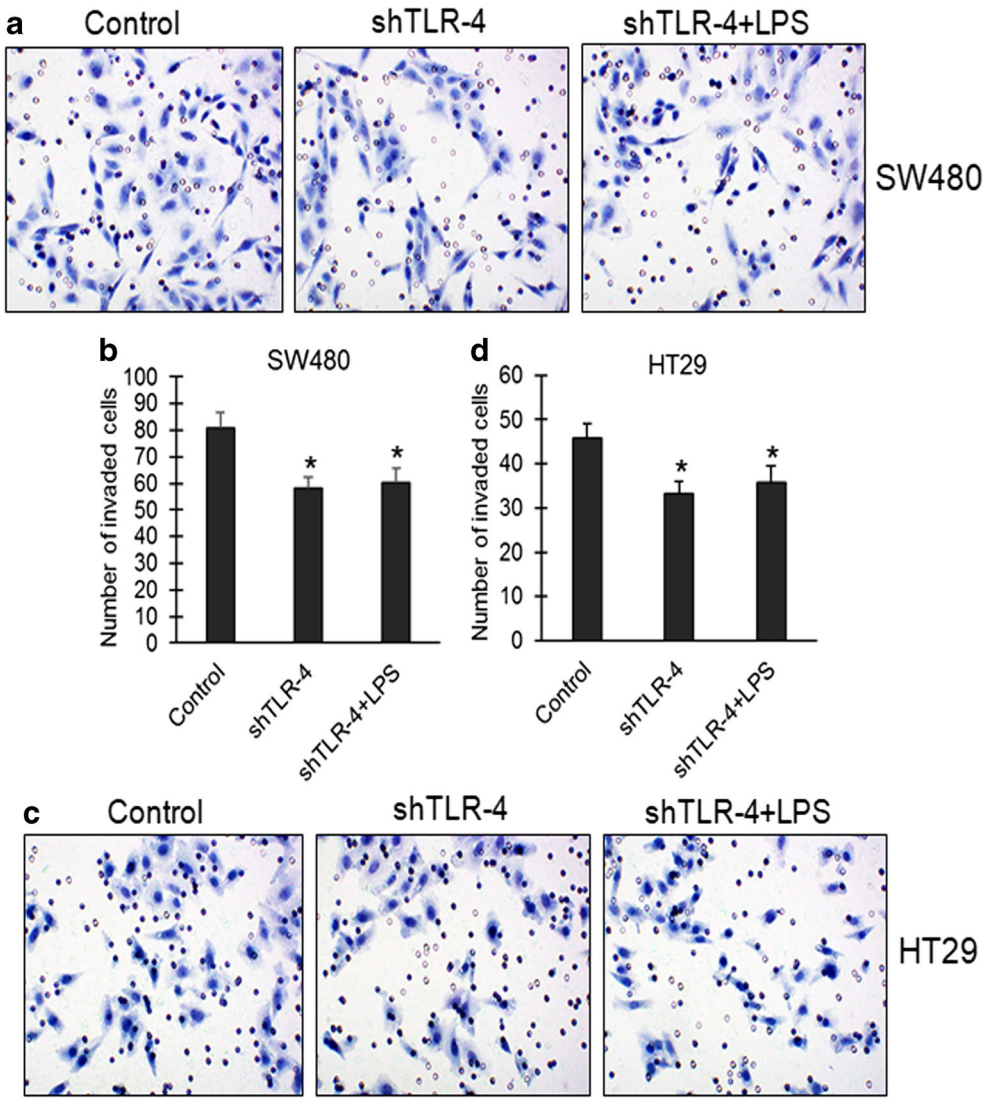

Fig. 12 The effects of TLR-4 knockdown on cell invasion in SW480 and HT29 cells. **a** Representative photographs of cell invasion in SW480 cells. **b** The number of cell invasion in SW480 cells. **c** Representative photographs of cell invasion in HT29 cells. **d** The number of cell invasion in HT29 cells. $^{*}P < 0.05$ vs. control group. $N = 4$

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Compliance with Ethical Standards

Conflict of Interest All authors declared no conflict of interest.

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References

1. Cancer-World Health Organization. http://www.who.int/mediacentre/factsheets/fs297/en/
2. Colorectal Cancer Statistics (2017) CA: Cancer J Clin. https://doi.org/10.3322/caac.21395
3. American Cancer Society (2014) Colorectal cancer facts & figures 2014-2016. Atlanta, Ga: American Cancer Society
4. Terzić J, Grivennikov S, Karin E, Karin M (2010) Inflammation and colon cancer. Gastroenterology 138:2101–2114.e5
5. Viennois E, Chen F, Merlin D (2013) NF-κB pathway in colitis-associated cancers. Transl Gastrointest Cancer 2:21–29
6. Zhu QC, Gao RY, Wu W, Qin HL (2013) Epithelial-mesenchymal transition and its role in the pathogenesis of colorectal cancer. Asian Pac J Cancer Prev 14:2689–2698
7. Sims GP, Rowe DC, Rietdijk ST, Herbst R, Coyle AJ (2010) HMGB1 and RAGE in inflammation and cancer. Annu Rev Immunol 28:367–388
8. Zhang G, Ghosh S (2000) Molecular mechanisms of NF-kappaB activation induced by bacterial lipopolysaccharide through toll-like receptors. J Endotoxin Res 6:453–457
9. Zhou X, Wang X (2015) Klotho: a novel biomarker for cancer. J Cancer Res Clin Oncol 141:961–969
10. Tang X, Wang Y, Fan Z, Ji G, Wang M, Lin J, Huang S, Meltzer SJ (2016) Klotho: a tumor suppressor and modulator of the Wnt/β-catenin pathway in human hepatocellular carcinoma. Lab Investig 96:197–205

11. Wang L, Wang X, Wang X, Jie P, Lu H, Zhang S, Lin X, Lam EK, Cui Y, Yu J, Jin H (2011) Klotho is silenced through promoter hypermethylation in gastric cancer. Am J Cancer Res 1:111–119
12. Xie B, Chen J, Liu B, Zhan J (2013) Klotho acts as a tumor suppressor in cancers. Pathol Oncol Res 19:611–617
13. Wu C, Lv C, Chen F, Ma X, Shao Y, Wang Q (2015) The function of miR-199a-5p/klotho regulating TLR4/NF-κB p65/NGAL pathways in rat mesangial cells cultured with high glucose and the mechanism. Mol Cell Endocrinol 417:84–93
14. Roy A, Srivastava M, Saqib U, Liu D, Faisal SM, Sugathan S, Bishnoi S, Baig MS (2016) Potential therapeutic targets for inflammation in toll-like receptor 4 (TLR4)-mediated signaling pathways. Int Immunopharmacol 40:79–89
15. Liu B, Sun L, Liu Q, Gong C, Yao Y, Lv X, Lin L, Yao H, Su F, Li D, Zeng M, Song E (2015) A cytoplasmic NF-κB interacting long noncoding RNA blocks IκB phosphorylation and suppresses breast cancer metastasis. Cancer Cell 27:370–381
16. Xie B, Zhou J, Yuan L, Ren F, Liu DC, Li Q, Shu G (2013) Epigenetic silencing of klotho expression correlates with poor prognosis of human hepatocellular carcinoma. Hum Pathol 44:795–801
17. Xie B, Zhou J, Shu G, Liu DC, Zhou J, Chen J, Yuan L (2013) Restoration of klotho gene expression induces apoptosis and autophagy in gastric cancer cells: tumor suppressive role of klotho in gastric cancer. Cancer Cell Int 13:18
18. Shu G, Xie B, Ren F, Liu DC, Zhou J, Li Q, Chen J, Yuan L, Zhou J (2013) Restoration of klotho expression induces apoptosis and autophagy in hepatocellular carcinoma cells. Cell Oncol (Dordr) 36: 121–129