Therapeutic effect of hydroxychloroquine on colorectal carcinogenesis in experimental murine colitis

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A B S T R A C T

Chronic inflammation in the intestine is a strong risk factor for colitis-associated colorectal cancer (CAC). Hydroxychloroquine (HCQ) is widely used as an anti-inflammatory drug in the treatment of immune-mediated inflammatory disorders and various tumors. However, little is known regarding the effects of HCQ on colitis-associated tumorigenesis. In this study, mice treated with HCQ showed a significant reduction in early-stage colitis following azoxymethane (AOM)/dextran sodium sulfate (DSS) administration, as well as a remarkable inhibition of colonic tumorigenesis and tumor growth at late stages of CAC. Mechanistically, the therapeutic effects of HCQ were attributed to inhibition of inflammatory responses and production of mutagenic reactive oxygen species (ROS) in immune cells and subsequent promotion of apoptosis and cell cycle arrest in tumor cells. Furthermore, we found that HCQ inhibited the production of inflammatory cytokines and ROS in response to toll-like receptor 4 (TLR4) activation in macrophages. Our data presented herein may help guide the clinical use of HCQ as a prevention and treatment strategy for CAC.

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1. Introduction

Inflammation is a strong risk factor for many cancers. Colitis-associated colorectal cancer (CAC), which is associated with inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn’s disease (CD), accounts for 15–20% of colorectal cancer (CRC) cases [1]. In IBD, commensal microorganisms translocate from the bowel lumen to the submucosa, resulting in excessive activation of innate immune cells, such as macrophages and dendritic cells, in the colonic lamina propria [2]. Consistently, TLRs, particularly TLR4, the sensor of gram-negative bacteria, are over-expressed in human and murine colitis and colitis-associated neoplasia. TLR4-deficient mice have a lower risk of colon carcinoma [3]. Furthermore, in response to TLR signaling, reactive oxygen species (ROS) and reactive nitrogen species (RNS) are persistently released by macrophages and neutrophils in colonic lamina propria. These molecules elicit epigenetic changes and DNA damage in colonic epithelial cells, including inactivation of tumor suppressor genes and activation of oncogenes, which play important roles in the regulation of mutagenic environments in the colon [4,5]. Therefore, colitis is essential for CAC initiation. Inflammation is also involved in TLR4-dependent, colitis-promoted tumor growth via up-regulation of nuclear factor (NF)-κB and several tumorigenic pro-inflammatory cytokines (e.g., IL1β, IL6 and TNFα) in lamina propria myeloid cells. In turn, these cytokines can regulate the expression of genes associated with tumor cell survival and proliferation [6–8]. One study shows that knockout of NF-κB in myeloid cells results in reductions in both tumor size and tumor number in azoxymethane (AOM)/dextran sodium sulfate (DSS)-treated mice [9]. Therefore, inflammation could be an attractive target for preventing and treating CAC.

Chloroquine (CQ) and its hydroxyl analog, hydroxychloroquine (HCQ), have been used as anti-malarial agents for half a century [10]. They have also been used in the management of several inflammatory diseases, such as sepsis, bacterial infection and immune-mediated inflammatory disorders [11–13]. CQ/HCQ possesses several mechanisms of action. For example, their accumulations in lysosomes and autophagic vacuoles inhibit the growth of intracellular bacteria and target them for degradation in intracellular...
organelles [12,14]; down-regulate pro-inflammatory cytokines (e.g., IL1, IL6 and TNFα) [15]; control TLR4 and NF-κB activation [16], and modulate antigen presentation [17]. Indeed, CQ/HQC protect against many inflammatory diseases.

Accumulating evidences from both animal studies and clinical trials indicate that CQ/HQC could be used as effective agents in anti-cancer therapies [18–20]. The functions of CQ/HQC include inhibition of tumor cell proliferation and induction of cell death via apoptosis and necrosis [21]. Besides direct effects on tumor cells, CQ/HQC could also enhance the anti-cancer effects of ionizing radiation and chemo- or targeted-therapies through autophagy-dependent or independent mechanisms [22,23].

Interestingly, it has been reported that CQ has therapeutic potential in mouse models of IB and in patients with UC [24,25]. However, little work has been performed to establish the effects of CQ/HQC on CAC. Compared with CQ, HCQ has a lower risk of adverse gastrointestinal and ocular reactions [26]. Therefore, in the current study, we sought to explore the effect of HCQ on experimental murine CAC. We found that HCQ treatment dramatically inhibited the production of inflammatory cytokines and ROS in response to TLR4 activation in macrophages. Thus, HCQ not only reduces inflammation and ROS generation in lamina propria immune cells, as well as subsequent promotion of apoptosis and cell-cycle arrest in tumor cells. In vitro experiments revealed that HCQ inhibited the production of inflammatory cytokines and ROS in response to TLR4 activation in macrophages. Thus, HCQ not only reduces inflammation and mutagenic ROS-induced tumorigenesis but also inhibits CAC growth. These observations demonstrate that HCQ represents a promising agent for prevention and therapeutic intervention of CAC.

2. Experimental procedures

2.1. Mice and CAC model

Male C57BL/6 mice (6–8 weeks of age, 20–25 g, Shanghai Institute of Material Medicine, Chinese Academy of Science, China) were maintained in a pathogen-free facility and were handled after approval from the Animal Care and Use Committee of Zhejiang University. Mice received a single intraperitoneal injection (i.p.) of 10 mg/kg AOM (Sigma–Aldrich, USA); 7 days later, mice were given a single dose of 2.5% DSS (MP Biomedical, Santa Ana, USA) in autoclaved drinking water to induce colitis, and four rounds of 1.5–2.5% DSS in autoclaved drinking water for inducing colon tumorigenesis. Mice were too sensitive to the 1st dose of 2.5% DSS following AOM injection, which resulted in fatal bloody stools. Thus, DSS was given from low to high concentrations. In total, 1.5% DSS was given for the 1st and 2nd rounds, and 2% and 2.5% DSS were given for the 3rd and 4th rounds, respectively. Mice were euthanized by cervical dislocation on 19 and 120 days after AOM injection to study the early and late stage of CAC, respectively.

2.2. Mouse tissue processing

After mice were euthanized, the entire colon was removed and emptied of fecal contents, and the distance between the ileocecal junction and the proximal rectum was measured. After opening the colon longitudinally, gross tumors were quantified. Distal colon was fixed in 10% neutral-buffered formalin (Sigma–Aldrich, USA) and used for hematoxylin–eosin (H&E), immunohistochemical (IHC) and terminal deoxynucleotidyl transferase dUTP nick end labeling (Tunel) staining. The mid-colon was used for western blotting and RNA extraction. Lamina propria mononuclear cells (LPMCs) were isolated from proximal colon.

2.3. Clinical assessment of colitis

The severity of colitis was expressed as the disease activity index (DAI) and histologic activity index (HAI) as previously described [26]. Briefly, DAI was calculated by scoring body weight loss, stool consistency and blood in stool. HAI was defined as the sum of the degree of epithelial damage and infiltration. The macroscopic scoring was performed in a single-blind manner.

2.4. Isolation of tumor cells, intestinal epithelial cells, LPMCs and colonic lamina propria from mouse colonic samples

The colon was washed extensively in ice-cold phosphate buffered saline (PBS) and cut into one-mm pieces that were incubated in Hank’s Balanced Salt Solution (Sigma–Aldrich, USA) supplemented with 1.5 mM Dithiothreitol (DTT) (Sigma–Aldrich, USA), 30 mM Ethylene Diamine Tetraacetate Acid (EDTA) (Sigma–Aldrich, USA) with shaking at 37 °C for 30 min to remove mucosa. The mucosa was then washed in PBS and incubated for 30 min in 0.3 U/ml Dispase II (Sigma–Aldrich, USA) at 37 °C. The supernatant (released tumor cells and intestinal epithelial cells) was collected and washed twice in PBS. Colonic lamina propria was obtained from the remaining tissue. LPMCs were isolated from the tissue remaining after DTT and EDTA treatment. The tissues were flushed with PBS followed by incubation in 0.3 U/ml Collagenase D (Roche, Germany) with shaking at 37 °C for 60 min. The digested colons were passed through a 40 μM cell strainer (BD Falcon, USA), washed with PBS and re-suspended in DMEM/F12 medium (Sigma–Aldrich, USA), and then added to a 40–70% Percoll gradient solution (Sigma–Aldrich, USA) laid over a 70% Percoll solution. LPMCs were harvested from the 40 to 70% interface, more than 90% LPMCs are F4/80 positive macrophages, which were identified by flow cytometry (data not shown). F4/80 antibody was shown in Table 1.

2.5. Isolation of murine peritoneal macrophages (PMs)

PMs were obtained and cultured as previously described [27]. Briefly, cold PBS was injected into the peritoneal cavity and then collected after massaging the abdomen. Cells in PBS were centrifuged, re-suspended and plated in a 12-well plate at a density of 1 × 10⁶ cells/well. The medium was changed 2 h and 24 h after plating, and the PMs were used for further studies.

2.6. Cell culture

The mouse macrophage cell line, Raw264.7 cell, was obtained from the Cell Bank of the Chinese Academy of Science (Shanghai, China) and used between the 10th passage and 30th passage. Raw264.7 cells and mouse PMs were cultured in DMEM/F12 medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco, CA, USA) in a humidified 5% CO₂ incubator at 37 °C. The medium was changed every two days.

2.7. IHC staining

After being deparaffinized and hydrated, tissue slides were subjected to antigen retrieval using 0.01 M sodium citrate buffer (pH 6.0), followed by blocking of endogenous peroxidases with 3% hydrogen peroxide. The sections were incubated with the following primary antibodies overnight at 4 °C: Ki67 (Cell Signaling Technology, Cat. No. 12202, Rabbit IgG, dilution 1:400) and c-PARP (Abcam, ab32064, Rabbit IgG, dilution 1:100). Biotinylated goat anti-rabbit secondary antibody (ZSGB-BIO, Beijing, China) was used followed by incubation with streptavidin–horseradish-peroxidase
Table 1

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2.8. Tunel staining

Tunel assay was performed according to instrument of the manufacturer (Roche, Germany). Briefly, paraffin sections were rehydrated and dewaxed. Proteinase K was added and incubated for 20 min at 37 °C. The slides were washed in PBS for three times, followed by Tunel reaction mixture for 60 min at 37 °C. After washing slides in PBS, 4', 6-diamidino-2-phenylindole (DAPI) was added and incubated for 10 min at 37 °C. Slides were analyzed by fluorescence microscope. To quantify the percent of Tunel positive cells, at least five random selected fields were counted. Single blind method was used in the experiment.

2.9. Western blotting

Western blotting was performed as previously described [28]. Briefly, total cell lysates were prepared and resolved by SDS–PAGE and transferred to PVDF membranes (Millipore, Billerica, MA, USA). Membranes were blocked with 5% dry milk dissolved in TBST and probed with primary and secondary antibodies. Antibodies used in this study were shown in Table 1. Signals were visualized with enhanced chemiluminescence (Biological Industries, Kibbutz Beth HaEmek, Israel).

2.10. RNA extraction, reverse transcription and real-time PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, California, U.S.) following the manufacturer's instructions. RNA was quantified using a NanoDrop 2000c spectrophotometer (Thermo Scientific, USA). cDNA from RAW264.7 cells and PMs was synthesized using HiFi-Script 1st Strand cDNA Synthesis kit (Cwbiotech, China), and cDNA from murine colon tissue was synthesized using an RNeasy Mini Kit (Qiagen, Germany). Quantitative real-time PCR analyses were performed with SYBR Green Master Mix (Cwbiotech, China). Expression of mRNA was normalized to GAPDH. Primer sequences are listed in Table 2.

2.11. Measurement of intracellular ROS

Macrophages (1 × 10^6 cells/well on 12-well plates) were pre-treated with HCQ (JK Chemical, Beijing, China) for 2 h and then stimulated with LPS (Sigma–Aldrich, USA) for 12 h. Cells were rinsed with PBS followed by addition of 10 μM chloromethyl-2', 7'-dichlorofluorescein diacetate (DCFH-DA, Sigma–Aldrich, USA) for another half an hour at 37 °C. Afterward, cells were washed twice with PBS to remove any extra dye, and the change in DCFH-DA fluorescence was detected with a fluorescence reader (BD FACSCaliburTM system, USA) with excitation and emission wavelengths of 488 and 525 nm, respectively.

2.12. Ethics statement

The institutional animal ethics committee of Zhejiang University approved the animal study with approval NO. zju-2013-06-02-011. The methods were carried out according to the approved guidelines.

2.13. Statistical analysis

The data were analyzed by two-tailed Student's t-test, and one-way and two-way ANOVA followed by Bonferroni’s post hoc test, and log-rank test. The results were expressed as mean value ± SD. Statistical significance was set at *P* < 0.05.

3. Results

3.1. HCQ suppresses AOM/DSS-induced murine colitis

To determine whether HCQ could suppress acute colitis, mice were challenged with 3.5% DSS for 5 days for the induction of lethal acute colitis. HCQ was given during the whole experimental period. Disease progression was assessed until day 20. Results showed that HCQ significantly reduced the mortality of mice with DSS-induced acute colitis (Fig. 1A). Next, to explore whether HCQ could suppress colitis in the initiation of CAC as well, we generated an AOM/DSS mouse model for evaluating tumor-associated colitis by injecting mice with the carcinogen AOM, followed by one cycle of oral DSS administration (Fig. 1B). AOM/DSS-treated mice showed the typical symptoms of colitis, including dramatic weight loss, diarrhea and rectal bleeding. Administration of HCQ (50 mg/kg, i.p. for 19 days) significantly ameliorated these symptoms, as body weight and DAI value decreased more slowly relative to AOM/DSS-treated mice on day 17 and day 19, respectively (Fig. 1C and D). Treatment with HCQ decreased the AOM/DSS-induced DAI from 10.8 ± 1.2 to 9.3 ± 1.3 (Fig. 1D). In addition, HCQ also alleviated colitis-induced colonic shortening (Fig. 1E and F). H&E staining and histological analysis also revealed that AOM/DSS induced severe colitis in challenged mice by eliciting epithelial damage and infiltration of macrophages and neutrophils into the colonic lamina propria.
However, these pathological changes were remarkably reversed by HCQ treatment (Fig. 1G). Quantitative analysis showed that HCQ treatment reduced HAI scores of AOM/DSS-challenged mice from 6.7 ± 0.8 to 5.4 ± 1.1 (Fig. 1H). Collectively, these results indicate that HCQ ameliorates colitis in the murine CAC model.

3.2. HCQ prevents colitis-associated tumorigenesis and suppresses growth of CAC in mice

We next sought to assess the role of HCQ in the tumorigenesis of CAC. To establish AOM/DSS recurring inflammation-driven colorectal cancer, AOM was given before four cycles of DSS administration (Fig. 2A). The mice lost weight after each DSS exposure and subsequently recovered weight by drinking water. As shown in Fig. 2B, HCQ treatment (50 mg/kg, i.p. for 120 days) could prevent severe body weight loss and promote a more rapid recovery compared to the control group. When euthanized on day 120 after AOM injection, 100% of mice had developed tumors. Smaller and fewer tumors were observed in the mucosa of HCQ/AOM/DSS-treated mice, particularly in the distal colon and rectum (Fig. 2C). Histopathological analysis revealed that most of these tumors were adenomas with low-grade intraepithelial neoplasia (IEN) in the HCQ/AOM/DSS-treated group but adenocarcinomas in the AOM/DSS-treated group (Fig. 2D and Table 3). HCQ treatment significantly decreased the tumor number in AOM/DSS-challenged mice (Fig. 2E), demonstrating that HCQ could suppress tumorigenesis of CAC.

Moreover, the average tumor load, defined as the total diameters of all tumors in a given mouse, was remarkably reduced by

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Fig. 1. HCQ alleviates colitis at an early stage of CAC. (A) HCQ improved the survival rate in DSS-induced fatal colitis. Mice were challenged with 3.5% DSS with or without HCQ treatment for 5 days, and disease progression was assessed daily. n = 16 in each group. Kaplan–Meier survival curves were compared by the log-rank test. (B) Schematic overview of AOM and DSS protocol for induction of colitis. Mice were injected with AOM followed by 1 cycle of 2.5% DSS for 7 days. HCQ was given by i.p. injection daily. Mice were euthanized on day 19 after AOM injection. (C) Weight loss was monitored throughout the process of the AOM/DSS model. (D) Disease activity index was evaluated on day 19 after AOM injection. (E) Colon lengths were measured. (F) Representative H&E staining from mouse colon. Bar = 100 μm. (H) The histological activity index was assessed. n = 5 for Ctrl and HCQ groups, n = 11 for AOM + DSS and AOM + DSS + HCQ groups. Differences were calculated using a one-way analysis of variance (ANOVA) followed by Bonferroni’s post hoc test. Values are presented as mean ± SD. **P < 0.01, ***P < 0.001.
HCQ (Fig. 2F). Tumor size was also decreased in HCQ-treated mice (Fig. 2G). Consistently, tumors with diameters above 4 mm were observed in 14% of HCQ/AOM/DSS-treated mice, much lower than in 25% in AOM/DSS-challenged mice (Fig. 2H), indicating a suppressive role of HCQ in tumor growth. Taken together, the above results demonstrate that HCQ not only prevents colitis-associated tumorigenesis but also suppresses tumor growth in mice.

3.3. HCQ suppresses inflammatory responses and ROS production in lamina propria cells

As described above, HCQ exhibited a protective effect on colitis and tumorigenesis in a murine CAC model. It is well established that colonic inflammation is mainly mediated by lamina propria immune cells, and the expression of tumorigenic and pro-inflammatory genes is modulated by signal transduction pathways involving NF-κB, MAPK and STAT3 proteins. To understand whether these pathways and molecules were regulated by HCQ, myeloid cells present in the colonic lamina propria were isolated at different stages of CAC and analyzed by western blotting. Indeed, significant activation of the NF-κB, MAPK and STAT3 pathways were observed in AOM/DSS-challenged colonic lamina propria at day 19 and day 120 of the AOM/DSS experimental protocol. However, the phosphorylation of STAT3, inhibitor of NF-κB (IkBα), NF-κBp65, mitogen-activated protein kinase 1/2 (MEK1/2) and extracellular signal-regulated kinase 1/2 (ERK1/2) were suppressed observably following treatment with HCQ (Fig. 3A and B). To further explore the effect of HCQ on production of inflammatory...
cytokines, mRNA in colonic lamina propria at day 19 of CAC was extracted and analyzed by quantitative RT-PCR. As shown in Fig. 3 C, HCQ remarkably reduced AOM/DSS-induced up-regulation of inflammatory cytokines, including IL1β, IL6, TNFα, COX2, IFNβ, interferon-inducible protein 10 (IP10), regulated on activation normal T cell expressed and secreted (RANTES) and monocyte chemoattractant protein 1 (MCP1).

ROS are involved in signal transduction and genomic instability in the initial stages of CAC and are hallmark of CAC pathogenesis. To investigate whether HCQ plays a role in regulation of ROS production, colonic lamina propria cells were isolated and labeled with DCFH-DA. Intracellular ROS were then measured with a fluorescence reader. As shown in Fig. 3D, the intensity of ROS in the AOM/DSS-treated group was at least 1.7 times higher than that of the control group. However, the level of intracellular ROS dropped to 1.1 times that of the control group when combined with HCQ treatment, demonstrating that HCQ suppressed AOM/DSS-induced ROS generation in colonic lamina propria cells.

3.4. HCQ inhibits the production of inflammatory cytokines and ROS in response to TLR4 activation in macrophages

A previous study reported that TLR4 knockout mice were protected from CAC due to down-regulation of inflammatory cytokines, mRNA in colonic lamina propria at day 19 of CAC was extracted and analyzed by quantitative RT-PCR. As shown in Fig. 3 A and B, Analysis of the indicated proteins in colonic lamina propria on day 19 (A) and day 120 (B) of the AOM + DSS-induced CAC model. (C) Relative expression of inflammatory cytokines from murine colon on day 19 after CAC induction. (D) Relative fluorescence intensity of ROS isolated from intestinal lamina propria cells at day 19 after CAC induction. The experiment was repeated three times. The results are expressed as mean ± SD. Differences were calculated using a one-way ANOVA followed by Bonferroni’s post hoc test. *P < 0.05, **P < 0.01, ***P < 0.001.

Fig. 3. HCQ suppresses inflammatory responses and ROS production in lamina propria cells. (A and B) Analysis of the indicated proteins in colonic lamina propria on day 19 (A) and day 120 (B) of the AOM + DSS-induced CAC model. (C) Relative expression of inflammatory cytokines from murine colon on day 19 after CAC induction. n = 5 for Ctrl and HCQ groups, n = 11 for AOM + DSS and AOM + DSS + HCQ groups. (D) Relative fluorescence intensity of ROS isolated from intestinal lamina propria cells at day 19 after CAC induction. n = 3 per group. The experiment was repeated three times. The results are expressed as mean ± SD. Differences were calculated using a one-way ANOVA followed by Bonferroni’s post hoc test. *P < 0.05, **P < 0.01, ***P < 0.001.
cytokines in the mucosa and subsequent inhibition of EGFR phosphorylation in colonic epithelial cells [3]. TLR4, as the canonical receptor recognizing Gram-negative bacteria, is primarily expressed in innate immune cells such as macrophages [29,30]. Consistent with previous reports [30], microarray data from the Gene Expression Omnibus (GEO) showed that TLR4 was highly expressed in IBD patients (Fig. 4), indicating an association between TLR4 expression and IBD. To explore the potential role of TLR4 in HCQ-mediated prevention of CAC, the mouse macrophage cell line, Raw264.7, and primary mouse PMs were pre-incubated with various concentrations of HCQ for 2 h, followed by stimulation with the TLR4 activating ligand, lipopolysaccharide (LPS) for an additional 2 h. Critical TLR4-related molecules were analyzed by western blotting. As shown in Fig. 5A and B, TLR4 and its downstream signaling proteins, including myeloid differentiation factor 88 (MyD88)-dependent pathway members TANK-binding kinase 1 (TBK1)-dependent pathway members (p-TBK1-p-IRF3), were up-regulated in LPS-stimulated RAW264.7 cells and PMs. However, these LPS-activated signals were completely inhibited by HCQ. Notably, HCQ dampened the TLR4 signal pathway in a dose-dependent manner, as HCQ showed the strongest inhibition at the highest tested concentration of 20 μM (Fig. 5C and D).

Activation of TLR4 and its downstream pathways elicit robust inflammatory responses and ROS production in macrophages [29,31]. Our results clearly showed that MyD88-dependent cytokines, including IL1β, IL6, TNFα and COX2, and TBK1-dependent cytokines, including IFNβ, IP10, RANTES and MCP1, were significantly up-regulated in LPS-stimulated RAW264.7 cells and PMs. However, HCQ suppressed the expression of these cytokines in a dose-dependent manner (Fig. 6A-D). Similarly, HCQ reversed the up-regulation of ROS in LPS-stimulated RAW264.7 cells and PMs (Fig. 6E and F). Previous studies have reported that activation of the TLR4 downstream transcription factor, NF-κB, resulted in the release of intracellular ROS by macrophages [32,33], given that HCQ inhibited TLR4 and its downstream signaling NF-κB (Fig. 5A and B). Therefore, it is reasonable to speculate that HCQ-mediated ROS suppression is dependent on NF-κB signaling. As expected, pretreatment with the NF-κB inhibitor, BAY11-7082, dramatically reduced ROS in LPS-stimulated RAW264.7 cells (Fig. 6G). Thus, inhibition of TLR4 and NF-κB is at least in part, responsible for HCQ-mediated down-regulation of ROS in LPS-activated macrophages.

3.5. HCQ inhibits growth of CAC by induction of cell cycle arrest and apoptosis in tumor cells

As shown in Fig. 2F–H, HCQ treatment resulted in much smaller tumors in AOM/DSS-induced CAC, suggesting a potential function of HCQ in regulation of tumor cell proliferation or apoptosis. To assess the effect of HCQ on cell proliferation, IHC staining of Ki-67 was performed in colon tissue. The results clearly showed that Ki-67 was mainly expressed at the base of colonic crypts in Ctrl and HCQ-injected mice. Following AOM/DSS treatment, Ki-67 labeling extended to most of the tumor area. Strikingly, HCQ treatment decreased the positive staining rates of Ki-67, indicating that HCQ suppresses tumor cell proliferation in the AOM/DSS-induced CAC model (Fig. 7A and B). Furthermore, western blotting of tumor cell lysates demonstrated that AOM/DSS-treatment resulted in up-regulation of cyclin D1 and cyclin D3, which were inhibited by HCQ, suggesting an inhibitory effect of HCQ on the cell cycle of late G1 to G1/S phase transition (Fig. 7C).

Next, to determine whether HCQ contributes to apoptosis, we examined the expression of pro-apoptotic proteins in each group tumors. Compared to the AOM/DSS group, significant up-regulations of Bad, Bax, cleaved-caspase 3 (c-caspase 3) and cleaved-PARP (c-PARP) were observed in the HCQ-combined-treated group, indicating a much higher apoptotic rate in these tumors (Fig. 7C). These results were further confirmed by IHC staining of c-PARP (Fig. 7D and E), as well as Tunel assay (Fig. 7F and G). We also determined the expression of the anti-apoptotic proteins Bcl-2 and Bcl-xl in isolated tumors. Consistently, significant reductions of Bcl-2 and Bcl-xl were found in HCQ-treated CAC tumors (Fig. 7C).

Taken together, the above results demonstrate that HCQ could suppress growth of AOM/DSS-induced CAC through induction of cell cycle arrest and apoptosis in tumor cells.

4. Discussion

TNFs antagonists are ubiquitously used as induction and post-remission therapies for patients with IBD and an effective preventative therapy for colitis-associated tumorigenesis, which elicit an anti-inflammatory effect on IBD and CAC [34,35]. COX and its analog, HCQ, the two most frequently used 4-aminoquinolone antimarial drugs, due to their ability to directly inhibit inflammatory cytokines [13], have been used to treat autoimmune diseases for decades, such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA). With regard to the therapeutic effects of CQ/HCQ in IBD, one previous study has demonstrated that short-duration CQ treatment is safe for mild to moderately active UC [25]. Similarly, in our study, HCQ significantly reduced the mortality of mice with DSS-induced acute colitis (Fig. 1A). Furthermore, HCQ ameliorated colitis in the early stage of AOM/DSS-induced murine CAC (Fig. 1B–H) and subsequently suppressed tumorigenesis and tumor growth (Fig. 2). Interestingly, the effects of HCQ on tumorigenesis were more striking compared to the effect on acute colitis following the first cycle of DSS administration. There are two reasonable possibilities for this difference. One is that acute colitis was induced only by one cycle of DSS administration, while CAC formed after four cycles of DSS administration, HCQ ameliorated CAC dramatically in that the anti-inflammatory effect of HCQ accumulated over time. In addition, HCQ suppressed tumor proliferation and promoted tumor apoptosis, which also attributed to the inhibition of CAC at the late stage.

The NF-κB, MAPK and STAT3 pathways have profound effects on CAC. Florian R. Greten et al. reported that inhibition of the NF-κB pathway via knock-out of IKKβ in myeloid cells leads to a remarkable decrease in tumor incidence and tumor size in murine
Fig. 5. HCQ down regulates the expressions of TLR4-associated proteins in macrophages. (A and B) Raw264.7 cells and PMs were pretreated with various concentrations of HCQ for 2 h followed by exposure to 100 ng/ml LPS for 2 h. The expression of TLR4 and its downstream signaling mediators were detected by immunoblot. (C and D) Immunoblot bands were quantified by ImageJ densitometric analysis and normalized to GAPDH. One representative experiment of three independent experiments is shown. PMs, peritoneal macrophages.
CAC model [9]. p38 MAPK-deficient mice show decreased tumorigenesis in the AOM/DSS model due to inhibition of myeloid cell recruitment and suppression of inflammatory responses [36]. In addition, STAT3 mediates crosstalk between inflammation and tumorigenesis, as IL-6 released by myeloid cells promotes STAT3 transcription in epithelial cells during CAC tumorigenesis [6]. Our study found that HCQ significantly inhibited NF-$\kappa$B, MAPK and STAT3 pathways in lamina propria cells, implying HCQ-mediated suppression of tumorigenic inflammation in mouse colon (Fig. 3A and B). The accumulation of inflammatory cytokines in colon is another important contributor to CAC tumorigenesis. Many reports have demonstrated that inflammatory cytokines, including IL-6, TNF$\alpha$, IL1$\beta$, COX2, IP10, RANTES and MCP1, are over-expressed in murine CAC and that blockade of these inflammatory cytokines is an effective CAC therapy [6,7,37–40]. Our study clearly showed that the expressions of the aforementioned pro-tumorigenic cytokines in colonic lamina propria were dramatically inhibited by HCQ (Fig. 3C). Thus far, our data demonstrate that HCQ inhibits CAC tumorigenesis through suppression of inflammation in the lamina propria.

As the products of cellular metabolism in inflammation, ROS can damage DNA by decreasing the efficiency of DNA repair and
Fig. 7. HCQ inhibits growth of CAC by induction of cell cycle arrest and apoptosis in tumor cells. (A) Immunohistochemical staining of Ki-67 using paraffin-embedded sections. Bar = 100 μm. (B) Quantification of Ki-67 staining by Image Pro Plus 5.0. (C) Expression of indicated proteins isolated from mouse colonic epithelia of Ctrl or HCQ treated groups and tumors of AOM + DSS or AOM + DSS + HCQ challenged groups. (D) Immunohistochemical staining of c-PARP using paraffin-embedded sections. Bar = 100 μm. (E) Quantification of c-PARP staining by Image Pro Plus 5.0. (F) The apoptosis was confirmed using Tunel assay. Bar = 200 μm. (G) Quantification of apoptotic cells by counting Tunel positive cells. The results are expressed as mean ± SD. **P < 0.01, ***P < 0.001, as determined with a one-way ANOVA followed by Bonferroni’s post hoc test.
inducing lipid peroxidation. The genetic instability induced by elevated ROS promotes tumorigenesis in inflammatory-associated diseases [41]. ROS, generated in part by activated leukocytes, acts as carcinogens in patients suffering from IBD [42,43]. Moreover, antioxidants, such as N-acetylcysteine, can provide protection against CAC in mouse models [44]. Our study has confirmed these findings, in that ROS in lamina propria immune cells were upregulated during the initial stage of AOM/DSS-induced CAC. However, following HCQ treatment, ROS productions were inhibited significantly (Fig. 3D), which is in accordance with in vitro data (Fig. 6E and F). Mechanically, the inhibition of TLR4 and NF-kB is, at least partially, responsible for HCQ-mediated down-regulation of ROS in LPS-activated macrophages.

During intestinal inflammation, TLR4, as the primary receptor for Gram-negative bacteria, was strongly up-regulated in patients with CD and UC [45], which is consistent with our study (Fig. 4). In addition, the TLR4 variant Asp299Gly has been shown to be associated with IBD [46]. Furthermore, TLR4-deficient mice were protected from CAC tumorigenesis [3]. Due to its critical role in triggering IBD and CAC, TLR4 pathway appears to be a promising target for CAC. Herein, we found that HCQ significantly decreased the expression of TLR4, and inhibited its downstream signaling mediators, inflammatory cytokines and ROS in LPS-activated Raw264.7 cells and PMs (Figs. 5 and 6). However, TLR4 signaling activation is implicated in many other cells besides macrophages [45,47]. Masayuki Fukata et al. demonstrated that TLR4 in colonic epithelia is more important than that in myeloid cells during the development of CAC. Using bone marrow chimeras in the AOM/DSS model, they found that the incidence of dysplasia is remarkably reduced when TLR4 knockout, rather than wild type, mice are used as recipients. Histologically, TLR4 expressed in epithelial cells recruits COX2-expressing macrophages and neutrophils, resulting in tumorigenesis [47]. In our study, only macrophages were used in the mechanisms of HCQ in CAC. However, the effect of HCQ on TLR4 in intestinal epithelia cells during CAC pathogenesis remains elusive. Furthermore, the exact interaction between HCQ and TLR4 in macrophages also requires further investigation. HCQ indeed ameliorates CAC, at least partially, through inhibiting TLR4 and subsequent macrophage-mediated inflammatory responses and ROS production.

Besides TLR4 stimulated inflammation, HCQ could also suppress various kinds of inflammations. It has been reported that TLR7 and TLR9 ligand induced inflammations are inhibited by CQ/HCQ in macrophages [48,49]. CQ inhibits TLR3-induced biological response and TLR2-mediated antigen cross-presentation in immune cells [50,51]. Inflammation plays indispensable role in the pathogenesis of CAC, including TLRs [3,52]. Considering the indispensable role of TLR4 in pathogenesis of CAC [3,52], we explored the effect of HCQ on TLR4 activated key pathways in macrophages in the current study. In addition, we also detected the effect of HCQ on TNFα stimulated inflammation. Results preliminarily showed that HCQ significantly inhibited the mRNA level of IL1β and IL6 in TNFα-stimulated RAW264.7 cells (data not shown). Taken together, besides TLR4, we cannot exclude the involvements of other inflammatory mediators in HCQ treated CAC. Nevertheless, we found that HCQ inhibited tumorigenesis of CAC, at least partially, through suppression of TLR4 signaling in macrophages.

As mentioned above, HCQ alleviated tumorigenesis via suppressing inflammation and ROS in lamina propria immune cells. Nevertheless, a direct suppressive effect of HCQ on tumor cell phenotype cannot be excluded. It is worth noting that tumors in HCQ-treated mice were significantly smaller than tumors in control mice (Fig. 2E, G and H), suggesting an anti-proliferative or pro-death effect of HCQ on tumor cells. Indeed, it has been reported that CQ directly inhibits growth of the colon cancer cell line, CT26, by induction of apoptosis [53]. Other studies have also demonstrated that CQ can sensitize CRC cells to chemoradiation and anti-angiogenic therapy [54,55]. In the current study, we found that HCQ has inhibitory effects on the in vivo progression of CAC through induction of tumor cell cycle arrest and apoptosis (Fig. 7). However, the exact molecular mechanism of HCQ-mediated tumor cell cycle arrest and apoptosis remains elusive and requires further in vitro and in vivo investigations.

It is well known that HCQ inhibits autophagy by impeding lysosomal acidification, therefore blocking autophagosome fusion and degradation [56]. Autophagy is a protector in tumor initiation and a booster in tumor progression, meanwhile, autophagy involves in inflammatory diseases by restricting inflammation and necrosis [57–59]. Recently, some reports have shown that novel therapies ameliorate CAC by regulating autophagy in tumor and immune cells [60–62]. As a vital autophagy inhibitor, whether HCQ modulates CAC through the autophagy pathway is unknown in this study. However, we found that another autophagy inhibitor, 3-methyladenine (3-MA), had the opposite effect on inflammatory responses in LPS-stimulated macrophages, compared to the inhibitory effect of HCQ (data not shown). On the contrary, autophagy inducers, such as rapamycin, and serum starvation, could decrease the expression of inflammatory cytokines in LPS-stimulated macrophages in vitro, similar to HCQ (data not shown). Given the definite role of 3-MA, rapamycin and starvation in autophagy, we speculate that HCQ suppresses inflammation in an autophagy-independent manner. Accordingly, some reports have also demonstrated that CQ has multiple autophagy-independent functions [23,63,64].

In the current study, we identified a protective role of HCQ on CAC initiation and progression. This HCQ-mediated CAC suppression is based on the down-regulation of inflammation and ROS in colonic lamina propria immune cells, and on the induction of cell cycle arrest and apoptosis in tumor cells. Our data presented here may help guide clinical use of HCQ as a preventive or curative strategy for patients with IBD or CAC.

Conflict of interest

No potential conflicts of interest were disclosed.

Acknowledgments

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