Plasmodium Infection Suppresses Colon Cancer Growth by Inhibiting Proliferation and Promoting Apoptosis Associated with Disrupting Mitochondrial Biogenesis and Mitophagy in Mice

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Abstract

Background: Colon cancer is a common gastrointestinal tumor with a poor prognosis, which makes it urgent to explore new therapeutic strategies. The anti-tumor effect of *Plasmodium* infection has been reported in some murine models, but it is not clear whether it has an anti-colon cancer effect. In this study, we investigated the anti-colon cancer effect of *Plasmodium* infection and its related mechanisms using a mouse model of colon cancer.

Methods: An experimental model was established by intraperitoneal injection of *Plasmodium yoelii*-infected erythrocytes into mice with colon cancer. The size of tumors was observed dynamically in mice, and the expression of Ki67 detected by immunohistochemistry was to analyze tumor cells proliferation. Apoptosis was assessed by Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) staining, and the expression of apoptosis concerned proteins, including Bax, Bcl-2, Caspase-9, Cleaved Caspase-3, were detected by western blot and immunohistochemistry, respectively. Transmission electron microscopy (TEM) was used to observe the ultrastructural change of colon cancer cells. And the expression of mitochondrial biogenesis correlative central protein, PGC-1α, and mitophagy relevant crucial proteins, PINK1/Parkin, were detected by western blot.

Results: We found that *Plasmodium* infection reduced the weights and sizes of tumors and decreased the expression of Ki67 in colon cancer-bearing mice. Furthermore, *Plasmodium* infection promoted mitochondria-mediated apoptosis in colon cancer cells, as evidenced by the increased proportion of TUNEL-positive cells, the up-regulated expression of Bax, Caspase-9, and Cleaved Caspase-3 proteins, and the down-regulated expression of Bcl-2 protein. In colon cancer cells, we found destroyed nucleus, swollen mitochondria, missing cristae, and the decreased number of autolysosomes. In addition, *Plasmodium* infection disturbed mitochondrial biogenesis and mitophagy through the reduced expression of PGC-1α, PINK1, and Parkin proteins in colon cancer tissues.

Conclusions: *Plasmodium* infection can play an anti-colon cancer role in mice by inhibiting proliferation and promoting mitochondria-mediated apoptosis in colon cancer cells, which may relate to mitochondrial biogenesis and mitophagy.

Background

Colon cancer is one of the most frequent digestive system tumors, ranking fifth in new cases and fatalities among all malignancies worldwide [1]. At present, although surgical resection, radiotherapy, and conventional chemotherapy are used routinely in the treatment of colon cancer [2], the therapeutic effect is still not satisfactory, and it is urgent to explore new therapeutic strategies.

As early as the beginning of the last century, *Plasmodium*, one of the most important parasites, has been tried to treat diseases such as advanced syphilis [3]. Since then, the relationship between *Plasmodium* and cancer has also received some attention. According to the report, there was an inverse correlation between the incidence of malaria and the mortality rate of some cancer from 1955 to 2008 globally[4].

The antitumor effect of *Plasmodium* infection has been reported in some animal studies, including lung cancer [5], hepatocellular carcinoma [6], leukaemia [7], and melanoma [8]. However, it has not been reported whether *Plasmodium* infection could inhibit colon cancer.

In the reported studies, the antitumor mechanism of *Plasmodium* infection focused on host antitumor immune response [5], inhibiting tumor microenvironment signals [9], and anti-tumor angiogenesis [10]. However, what happens to tumor cells doesn't get enough attention. It is well known that the infinite proliferation ability and loss of apoptosis ability of tumor cells are the key to tumor growth [11]. Many therapies exert anticancer effects by promoting tumor cell apoptosis, especially mitochondria-mediated apoptosis [12, 13, 14]. Some parasites, such as *Toxoplasma gondii* [15], exerted an anti-tumor function by promoting apoptosis. However, it is not clear whether *Plasmodium* infection also plays an anti-tumor role by inhibiting proliferation and promoting apoptosis.

An increasing number of studies have shown that there is a close link between mitochondria and cancer development [16, 17]. Mitochondria is fundamental for cell growth and proliferation in cancer, and metabolic imbalances or increased resistance to mitochondrial apoptosis are prominent features for cancer cell [18]. Indeed, mitochondria maintain cellular homeostasis by regulating mitochondrial biogenesis and mitophagy [19, 20]. Mitochondrial biogenesis can generate new functional mitochondria to increase the number of mitochondria through transcriptional level regulation [21], and inhibition of mitochondrial biogenesis can inhibit tumor cell proliferation [22]. Mitophagy is a form of autophagy by which the damaged or superfluous mitochondria are phagocytosed and degraded [23, 24, 25], and inhibition of mitophagy can aggravate mitochondrial damage and promote mitochondria-mediated apoptosis [26, 27]. Because mitochondria play a key role in cell proliferation and death, plenty of studies have proved that targeting mitochondria could restrain tumor growth or promote apoptosis [28, 29]. However, whether mitochondrial biogenesis and mitophagy are involved in the anti-tumor effect of *Plasmodium* infection requires further exploration.

In this study, we used a murine colon cancer model to investigate the anti-colon cancer effect of *Plasmodium yoelii* infection and its potential mechanism. Our results suggested that *Plasmodium* infection could inhibit tumor growth in mice by suppressing proliferation and promoting apoptosis, which might relate to the inhibition of mitochondrial biogenesis and mitophagy in colon cancer cells. Those results will contribute to developing novel strategies for colon cancer treatment.

Methods

Source of animals, cells, and parasites

BALB/c mouse, which featured females, 6-8 weeks old and 18-20 g, were purchased from Chang Zhou Cavens Laboratory Animal Ltd and maintained under a controlled temperature of 20-25 ℃, relative humidity of 40-50%. All experiments were reviewed and approved by the Experimental Animal Management and Ethics Committee of Bengbu Medical College, Bengbu, China (approval no: 2021-322).

The nonlethal *Plasmodium yoelii* 17XNL (*P. yoelii* 17XNL) strain was donated by Professor Yaming Cao of China Medical University and preserved by our laboratory.

The CT26.WT cell, mouse colon cancer cell lines, were purchased from Guangzhou Cellcook Biotechnology Company and cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, USA) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (100 units/ml penicillin and 100 μg/ml streptomycin) under 37 ℃ and 5%CO_2 in an incubator.

Establishment of the murine colon cancer model and infection with the *P. yoelii*

A colon cancer model was established as described Masayoshi [30]. As the subcutaneous model of colon cancer-bearing mice, ten BALB/c mice were established by subcutaneous inoculation of 5×10^6 CT26.WT cells below the axilla of the right forelimb in the mouse. From the date of tumor formation (six days after tumor cell injection), ten mice were divided into two groups randomly (five animals per group). CT26.WT +P.y as the experimental group, each mouse was intraperitoneally injected with 1×10^6 *P. yoelii* infected erythrocytes. The control group (CT26.WT) of the mouse was intraperitoneally injected with 1×10^6 *P. yoelii* uninfected erythrocytes. The tumor growth was observed every two days from the date of tumor emergence.

The dynamical observation of tumor growth

When the tumor was measurable in tumor-bearing mice (on the sixth day after CT26.WT cells inoculation), the long diameter *a* (mm) and short diameter *b* (mm) of the tumor was measured with vernier caliper every two days, and the tumor volume was calculated according to the formula, $V=(ab^2)/2$. On the 18th day after inoculating *Plasmodium*-infected erythrocytes (on the 24th day after modeling), the tumor-bearing mice were sacrificed to harvest tumors, weigh, and photograph for further analysis.

Immunohistochemistry staining

The tumor specimens were fixed with 4% paraformaldehyde solution for paraffin embedding and sectioning. After xylene dewaxing, gradient ethanol hydration, and antigen high-pressure repair, they were stained with primary antibody (Abcam, USA; Cell Signal Technology, USA;) (Ki67 antibody, 1:400; Bax antibody, 1:400; Bcl-2 antibody, 1:500; Caspase-9 antibody, 1:300; Caspase-3 antibody, 1:300; Cleaved Caspase-3 antibody, 1:2000), and incubated at 37 ℃ for one hour. The second antibody was cultivated at 37 ℃ for 30 minutes. After DAB coloration and observation under a high magnification microscope (×400), the positive results were expressed in the brown position. Image-Pro Plus 6.0 software was used for statistical analysis, and average optical density value was used to reflect protein expression level.

TUNEL staining

The tumor tissue specimens were fixed with 4% paraformaldehyde and sectionalized with paraffin embedding. Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) assay

performed using the One-Step TUNEL Apoptosis Assay Kit, DAPI Staining Solution, and Antifade Mounting Medium (Beyotime Biotechnology), according to the manufacturer's protocol. Under a fluorescence microscope (×400), the normal nucleus is blue and the apoptotic nucleus is green. All images were acquired using the Nikon Eclipse 50i microscope system and Image-pro Plus 6.0 software with standard image processing. The apoptosis rate was calculated by the number of TUNEL positive nuclei/number of DAPI stained nuclei.

Western blot

RIPA buffer (Beyotime Biotechnology, China) was used to extract total protein from the tumor tissue, and the BCA method was used for quantitative detection of protein. The proteins were isolated in 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE gel) and transferred onto the PVDF membrane. After being sealed with 5% defatted milk at room temperature, the PVDF membrane was incubated with the specific primary antibodies against PGC-1α, PINK1, Parkin (ABclonal) (rabbit polyclonal antibody, 1:1000), Bax, Bcl-2, Caspase-9, Caspase-3, Cleaved Caspase-3 (Cell Signal Technology, USA) (rabbit anti-mouse monoclonal antibody, 1:1000), at 4 ℃ overnight. After being incubated with the second antibody (Cell Signal Technology, USA) (HRP-conjugated goat anti-rabbit IgG, 1:2000) was added and incubated at room temperature for an hour, the protein bands were visualized by enhanced chemiluminescence reagent (Merck Millipore, USA) and detected using BioImageing Systems (BIO-RAD ChemiDoc^{TM}MP Imaging System, USA). The results were quantified by Image-J software. The relative protein expression was expressed by the gray value of target protein /GAPDH gray value.

Morphological observation by transmission electron microscopy (TEM)

The tumor tissue samples were harvested and fixed at 4 ℃ for 2-4 hours with glutaraldehyde. Then they were fixed at room temperature (20 ℃) for 2 h with 1% osmium and 0.1M phosphoric acid buffer PB, rinsed with 0.1M phosphoric acid buffer PB. After dehydration in graded ethanol and acetone, the samples were embedded Epon 812. The ultrathin sections were stained with uranium acid and lead uranium and then observed under TEM (Hitachi, Japan).

Statistical analysis

For parameter data in each experiment, the unpaired two-tailed Student's t-test was used to analyze the differences between groups. Statistical analyses were performed with GraphPad Prism software (version8). All experiments were repeated more than three times, and the data were expressed as the mean ± standard error of the mean (SEM). A *P*-value less than 0.05 was considered statistically significant.

Results

Inhibition of colon cancer growth by *Plasmodium* infection in mice

The mouse model of colon cancer was established by subcutaneous inoculation. On the sixth day after tumor cells inoculation, erythrocytes infected with *P. yoelii* were injected intraperitoneally into tumor-being mice (Fig. 1b). At first, there was no significant difference in tumor size between the *Plasmodium* infection group (5.905±1.357 mm^3) and the control group (3.487±0.758mm^3) on day six (Fig. 1a). From the 15th day of tumor cells inoculation, tumor size in the *P. yoelii*-infected group was not only significantly reduced compared to the un-infection group, but tumor growth was remarkably slow in the CT26.WT+P.y group. On the 18th day after being inoculated with *Plasmodium*-infected erythrocytes, the tumors were strikingly smaller in size and weight than the control group (Fig. 1b, c). The results indicated that *Plasmodium* infection could inhibit the growth of colon cancer in mice.

Plasmodium Infection suppressed the proliferation of colon cancer cells in mice

To determine the effect of *Plasmodium* infection on the proliferation of colon cancer cells in mice, we examined the expression of Ki67, a cell proliferation marker, using immunohistochemistry staining. We found that the expression of Ki67 was decreased obviously in the *P. yoelii* infection group than the control group (Fig. 2a). There were statistically significant differences in the percentage of Ki67 positive cells between the two groups (Fig. 2b). It was suggested that *Plasmodium* infection could suppress the proliferation of colon cancer cells *in vivo.*

Apoptosis of colon cancer cells induced by *Plasmodium* infection in mice

Apoptosis of tumor cells was detected by TUNEL assay, in which enhanced green fluorescence represented the TUNEL positive cells. A small number of apoptotic cells were found in the non-infected *Plasmodium* group (CT26.WT), while a large number of TUNEL positive cells were detected in the infected *Plasmodium* group (CT26.WT+P.y) (Fig. 3a). The CT26.WT+P.y group displayed a variation in the proportion of cell apoptosis compared with the CT26.WT group (Fig. 3b). It indicated that *Plasmodium* infection could accelerate the apoptosis of colon cancer cells.

The mitochondrial apoptosis activation by *Plasmodium* infection

To further explore the mechanism of *Plasmodium* infection-induced apoptosis, we used western blot to detect the expression of apoptotic proteins in the mitochondrial pathway. We observed that the expression levels of pro-apoptotic proteins containing Bax, Caspase-9, and Cleaved Caspase-3 were up-regulated, while the expression of anti-apoptotic protein Bcl-2 was down-regulated after *Plasmodium* infection compared with the control group (Fig. 4a, b).

In addition, the effects of *Plasmodium* infection on the expression of mitochondrion-mediated apoptosis proteins were observed through immunohistochemistry staining (Fig. 4c, d). Consistent with the results of western blot, we found that the expression of Bax, Caspase-9, and Cleaved Caspase-3 in the *P. yoelii*-treated model was increased noticeably. Nevertheless, there were less brown areas of Bcl-2 expression in the CT26.WT+P.y group than the CT26.WT group. These results suggested that *Plasmodium* infection

involved in regulating the expression of apoptotic proteins in the mitochondrial pathway leading to mitochondrion-mediated apoptosis.

The mitochondria and nucleus of colon cancer cells changed by *Plasmodium* infection in tumor-being mice

As shown in Fig. 5, we used TEM to further observe changes in the ultrastructure of colon cancer cells among the two groups. After *Plasmodium* treatment, colon cancer cells displayed severe edema, nuclear atypia, chromatin clumpy agglutination, and nuclear disintegration. Mitochondria were severely swollen and enlarged, the matrix in the membrane dissolved, and the cristae disappeared and vacuolated in the CT26.WT+P.y group compared to the control group. Moreover, under TEM, we could see only one autolysosome in the *P. yoelii*-treated group in this field, whereas there were three autolysosomes in the control group. The results revealed that *Plasmodium* infection-induced tumor cell damage was associated with mitochondrial and nuclear damage in colon cancer cells.

Plasmodium infection disrupted the mitochondrial biogenesis in the colon cancer model

To assess the effect of parasite infection on mitochondrial biogenesis, we used western blot to analyze a key regulator of mitochondrial biogenesis, PGC-1α. And the result showed that the expression of PGC-1α protein in the parasite-treated group was reduced evidently in contrast to the control group (Fig. 6). It was demonstrated that *Plasmodium* infection could disrupt mitochondrial biogenesis in colon cancer.

Plasmodium infection attenuated mitophagy in colon cancer-bearing mice

PINK1/Parkin-mediated pathway is the most important pathway of mitophagy, which is crucial for maintaining mitochondrial function and integrity [31]. To evaluate the effect of *Plasmodium* infection on mitophagy in colon cancer, we, therefore, examined the expression of mitophagy-related proteins. Western blot results showed that the levels of PINK1 and Parkin were declined after *Plasmodium* infection (Fig. 7). The results expressed that *Plasmodium* infection could attenuated mitophagy leading to mitochondrial dysfunction in colon cancer.

Discussion

As one of the most ordinary malignant tumors, colon cancer does pose an extreme threat to human health [32]. Due to the high incidence and mortality of colon cancer, researchers who have devoted themselves to reducing mortality and improving the quality of life for patients have been seeking new therapeutic methods to inhibit the growth, recurrence, and metastasis of colon cancer [33]. Previously, researchers and our team all have found that *Plasmodium* infection had antitumor effects on some cancer in some mouse models, but it is not clear whether it has similar influences on colon cancer yet. Therefore, in this study, we investigated the anti-colon cancer effect of *Plasmodium* infection in mice.

In this study, we have found that *Plasmodium* infection decelerated the growth of tumors and reduced the size and weight of the tumors in the murine colon cancer model. These results indicated that *Plasmodium* infection could play an anti-colon cancer role in mice.

Infinite proliferation is one of the important characteristics of tumor cells [34]. Inhibition of cell proliferation could effectively inhibit tumor growth [35]. Ki67 protein, a nuclear antigen related to cell proliferation, is expressed in G1, G2, S, and M period, but no expression in the G0 period [36]. As one of the most reliable indicators for detecting cell proliferation activity of tumor cells, the function of Ki67 protein is closely associated with the process of cell mitosis [37, 38]. In this study, the expression of Ki67 in colon cancer tissues was distinctly decreased after parasite treatment, suggesting that *Plasmodium* infection could suppress the proliferation of colon cancer cells and further inhibit tumor growth in tumor-bearing mice.

Apoptosis, the most common form of programmed cell death, is one of the keys to maintaining healthy cell homeostasis [39]. Apoptosis resistance is another crucial characteristic of tumor cells [40]. As an important method for prevention and treatment, induction of apoptosis is widely used in antitumor drug research [41]. There are three pathways of apoptosis in mammalian cells, containing the Bcl-2 regulatory mitochondrial pathway, the death receptor pathway, and the endoplasmic reticulum pathway [42, 43, 44]. Mitochondrial apoptosis is the primary form of apoptosis [45]. When mitochondria are damaged, Bax is transported into mitochondria to initiate apoptosis, triggering Cytochrome C releasing in the mitochondria, which further activates the Caspase cascade resulted in apoptosis [46, 47, 48].

In the tumor-being mice infected with *P. yoelii*, we found the increased proportion of TUNEL-positive cells, the up-regulated expression of pro-apoptotic factors Bax, Caspase-9, and Cleaved Caspase-3, and the down-regulated expression of anti-apoptotic factor Bcl-2 in colon cancer tissues, which indicated *Plasmodium* infection promoted mitochondria-mediated apoptosis. Since inadequate apoptosis is one of the typical characteristics of cancer, promoting apoptosis will lead to the death of cancer cell. Thus, we have reason to believe that *Plasmodium* infection promoting mitochondria-mediated apoptosis could inhibit tumor growth through promoting mitochondria-mediated apoptosis and play an anti-colon cancer role in tumor-bearing mice like some anti-tumor drugs [49, 50].

Mitochondrion, a subcellular organelle, which plays a major role in cell energy control and metabolism, is essential for the survival and growth of cells [51, 52]. As regulators involved in the pathways of mitochondrial biogenesis, mitophagy, carcinogenesis, and tumor cell death, including mitochondria-mediated apoptosis, mitochondria may be considered as potential therapeutic targets for cancer [53]. In this study, we found that the mitochondria and nucleus of colon cancer cells were damaged severely in the *Plasmodium*-treated mice, mainly with the mitochondrial cristae disappearance and vacuolation. The results implicated that the antitumor effect of *Plasmodium* infection may relate to mitochondria. The maintenance of mitochondrial homeostasis depends on the interaction between mitochondrial biogenesis and mitophagy [54]. PGC-1α protein dominated mitochondrial biogenesis and protected tumor cells from apoptosis [55, 56]. Those studies both *in vivo and in vitro* certified that down-regulated

expression of PGC-1α induced apoptosis through the mitochondrial pathway [57]. Recent study has revealed a potential relationship between mitochondrial biogenesis and apoptosis [58]. In the early stage of apoptosis, mitochondria produced energy to sustain homeostasis, leading to enhanced mitochondrial biogenesis [59]. As the massive production of reactive oxygen species (ROS), mitochondrial energy metabolism was unbalanced, mitochondrial biogenesis was weakened, mitochondrial membrane potential was decreased, Cytochrome C was released, and the Caspase pathway was activated, leading to apoptosis [60, 61]. In this study, we found that the expression of PGC-1α protein in colon cancer cells was reduced after *P. yoelii* infection. The results suggested that *Plasmodium* infection could inhibit mitochondrial biogenesis in colon cancer cells. Inhibition of mitochondrial biogenesis could promote apoptosis and inhibit proliferation, which may be one of the mechanisms underlying the antitumor effects of *Plasmodium* infection.

Autophagy is a process in which intracellular components are degraded into autophagosomes and combined with the lysosomes to form autolysosomes, resulting in the degradation of their encapsulated contents [62, 63]. More and more studies have demonstrated that autophagy is a self-protective mechanism for cells [64, 65]. Mitophagy, a type of mitochondria-specific autophagy, is a self-protective process that dysfunctional mitochondria are degraded selectively [66]. When mitochondria are damaged, PINK1 protein hydrolysis is restrained and expressed steadily on the mitochondrial outer membrane. Then, PINK1 recruits Parkin to the outer membrane of mitochondria and ubiquitinates multiple mitochondrial outer membrane proteins to mediate mitophagy, eventually removing damaged mitochondria [67, 68]. In the study, we found that the number of autolysosomes was decreased and the expression of PINK1/Parkin proteins were declined in colon cancer after *Plasmodium* infection. The results meant that *Plasmodium* infection could inhibit mitophagy in colon cancer-bearing mice leading to disruption of mitochondria and mitochondrial dysfunction. Many studies have shown that suppression of autophagy, including mitophagy, could accelerate apoptosis resulting in tumor cell death, ultimately inhibiting tumor growth [69, 70]. Therefore, in colon cancer cells, *Plasmodium* infection could inhibit mitophagy, thus promoting apoptosis and ultimately inhibiting tumor growth, which might be one of the anti-colon cancer mechanisms of *Plasmodium* infection.

In this study, although we found that *Plasmodium* infection might inhibit proliferation and promote apoptosis by controlling mitochondrial biogenesis and mitophagy, it is not clear why *Plasmodium* infection could inhibit mitochondrial biogenesis and mitophagy. This suppression function may be connected with the subsequent effect of cytokines induced by immune effects after *Plasmodium* infection, the impact of components or metabolites of *Plasmodium* itself, or the result of changes in non-coding RNA expression caused by *Plasmodium* infection. The mechanism of *Plasmodium* infection controlling mitochondrial biogenesis and mitophagy remains to be further explored.

Conclusions

In this study, our findings firstly demonstrated that *Plasmodium* infection had significant anti-colon cancer effects in mice by inhibiting tumor cells proliferation and promoting mitochondrial apoptosis,

which might relate to the inhibition of mitochondrial biogenesis and mitophagy (Fig. 8). The results presented here might inspire a new approach for the treatment of colon cancer.

Abbreviations

P. yoelii
Plasmodium yoelii
RPMI 1640
Roswell Park Memorial Institute 1640 medium
FBS
fetal bovine serum
TUNEL
Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling
TEM
transmission electron microscopy
SDS-PAGE gels
Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM
Standard error of the mean.

Declarations

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Availability of data and materials

The datasets supporting the findings of this article are included within the article and its additional file.

Authors' contributions

QF, ZT, JL, and XY conceived and designed the study. XY, YC, LL, YX, HC, CL, DC, KW, JX, and RF performed the experiments. XY, ZT, HX, and QF analyzed the data. XY wrote the manuscript. QF critically revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments were reviewed and approved by the Experimental Animal Management and Ethics Committee of Bengbu Medical College, Bengbu, China (approval no. 2021-322).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

P. yoelii infection suppressed tumor growth in a murine colon cancer model. Balb/c mice were subcutaneously injected CT26.WT mice colon cancer cells under the right forearm. On the sixth day, the tumor-bearing mice were intraperitoneally injected with either *P. yoelii*-infected erythrocytes or uninfected erythrocytes. (**a**) Tumor volume was measured over time from the date of tumor formation. Day15 (t-test, $t_{(8)}$=10.01, P<0.0001); Day18 (t-test, $t_{(8)}$=6.515, P=0.0002); Day21 (t-test, $t_{(8)}$=6.290, P=0.0002); Day24 (t-test, $t_{(8)}$=6.290, P=0.0002). The mice were sacrificed on day 24, and tumors were harvested for weighing, photographing (**b**), and further analysis. (**c**) Weight of the Tumors mass (t-test, $t_{(8)}$=6.618, P=0.0002). CT26.WT denotes the control group and CT26.WT+P.y denotes the experimental group. The results are shown as mean ± SEM (n= 5). ***P<0.001, ****P<0.0001.

Figure 2

Plasmodium infection inhibited the proliferation of tumor cells in colon cancer tissues. Immunohistochemical staining (**a**) demonstrating that *P. yoelii* infection impacted colon cancer proliferation in mice (×400). The percentage of Ki67 expression in tumor tissues of the two groups (t-test, $t_{(8)}$=17.51, P<0.0001) (**b**). Brown areas represent positive expression. Scale bar=50 μm. The results are shown as mean ± SEM (n= 5). ****P<0.0001.

Figure 3

Apoptosis in colon cancer induction by *Plasmodium* infection. (**a**) Apoptosis was observed under a fluorescence microscope (×400). (**b**) Quantitative estimation of the proportion of apoptotic cells in each experimental group (t-test, $t_{(8)}$=22.31, P<0.0001). Green fluorescence indicates the nuclei of TUNEL-positive cells, blue represents DAPI stained nuclei, and Merge indicates the two fluorescence superpositions. The results are shown as mean ± SEM (n= 5). Scale bar=50 μm. **** P<0.0001.

Figure 4

The mitochondrial pathway regulation by *Plasmodium* infection in colon cancer-being mice. Western blot analysis (**a**) shown the expression of mitochondrial-mediated proteins (Bax, Bcl-2, Caspase-9, and Caspase-3). Quantification of Western blot signals from (**b**). Bax (t-test, $t_{(8)}$=9.438, P<0.0001); Bcl-2 (t-test, $t_{(8)}$=3.822, P=0.0051); Caspase-9 (t-test, $t_{(8)}$=5.462, P=0.0006); Caspase-3 (t-test, $t_{(8)}$=3.836, P=0.0050); Cleaved Caspase-3 (t-test, $t_{(8)}$=11.36, P<0.0001). Immunohistochemistry Staining (**c**) and quantification of apoptosis protein expression (**d**) in the mitochondrial pathway. Bax (t-test, $t_{(8)}$=7.222, P<0.0001); Bcl-2 (t-test, $t_{(8)}$=10.92, P<0.0001); Caspase-9 (t-test, $t_{(8)}$=7.465, P<0.0001); Caspase-3 (t-test,

$t_{(8)}$=10.23, *P*<0.0001); Cleaved Caspase-3 (t-test, $t_{(8)}$=7.458, *P*<0.0001). The results are presented as mean ± SEM (n= 5). Scale bar=50 μm. ** *P*<0.01, *** P<0.001, **** *P*<0.0001.

Figure 5

Changes of tumor cells ultrastructure after *Plasmodium* infection. The ultrastructural changes of tumor cells in the two groups were observed under TEM (**a**) (magnification×2000) (**b**) (magnification×6000). The chromatin of the nucleus was massive agglutination and the organelles were severely swollen in CT26.WT+P.y group. The mitochondria (M, red arrow) were swollen, the cristae disappeared and vacuolated, and the chromatin clumpy agglutination and nuclear disintegration (N: nucleus). The number of autolysosomes (ASS, blue arrow) in the CT26.WT+P.y group was more than that in the CT26.WT group. (**a**) Scale bar=5 μm. (**b**)Scale bar=1 μm.

Figure 6

Plasmodium infection inhibited mitochondrial biogenesis in colon cancer. Western blot analysis (**a**) showed the expression of PGC-1α protein was decreased by *Plasmodium*-treated colon cancer tissues. GAPDH was detected as a loading control. The density ratio of PGC-1α/ GAPDH is shown on the right (**b**). PGC-1α (t-test, $t_{(10)}$=10.16, *P*<0.0001). The results are shown as the density mean ± SEM (n= 5). **** *P*<0.0001.

Figure 7

Plasmodium infection inhibited mitophagy in the murine colon cancer model. The expression of proteins associated with PINK1/Parkin pathway were inhibited after *P. yoelii* infection via Western blot analysis (**a**). Quantification of proteins expression normalized to GAPDH (**b**). PINK1 (t-test, $t_{(10)}$=7.340, *P*<0.0001); Parkin (t-test, $t_{(10)}$=4.829, *P*=0.0007). *P. yoelii* infection abated the levels of PINK1/Parkin. The results are shown as the density mean ± SEM (n= 5). ** *P*<0.01, *** *P*<0.001.

Figure 8

The anti-colon cancer mechanisms of *Plasmodium* infection *in vivo*. In the murine colon cancer model, *Plasmodium* infection inhibited the proliferation of tumor cells and induced mitochondria-mediated apoptosis. Furthermore, *Plasmodium* infection disturbed mitochondrial biogenesis leading to proliferation inhibition and apoptosis promotion. Mitophagy was attenuated by *Plasmodium* infection

contributing to mitochondrial dysfunction. *Plasmodium* infection inhibited the growth of tumors to exert the tumor suppression function.

Supplementary Files

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