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Cryptosporidium parvum upregulates miR-942-5p expression in HCT-8 cells *via* TLR2/TLR4-NF- κ B signaling

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Abstract

Background: Micro (mi)RNAs are small noncoding RNA molecules that function in RNA silencing and post-transcriptional regulation of gene expression. This study investigated host miRNA activity in the innate immune response to *Cryptosporidium parvum* infection.

Methods: *In vitro* infection model adopts HCT-8 human ileocecal adenocarcinoma cells infected with *C. parvum*. The expression of miR-942-5p was estimated using quantitative real-time polymerase chain reaction (qPCR). The TLRs-NF- κ B signaling was confirmed by qPCR, western blotting, TLR4- and TLR2-specific short-interfering (si)RNA, and NF- κ B inhibition.

Results: HCT-8 cells express all known toll-like receptors (TLRs). *Cryptosporidium parvum* infection of cultured HCT-8 cells upregulated TLR2 and TLR4, and downstream TLR effectors, including NF- κ B and suppressed I κ B α (nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, alpha). The expression of miR-942-5p was significantly upregulated at 4, 8, 12 and 24 h post-infection, and especially at 8 hpi. The results of TLR4- and TLR2-specific siRNA and NF- κ B inhibition showed that upregulation of miR-942-5p was promoted by p65 subunit-dependent TLR2/TLR4-NF- κ B pathway signaling.

Conclusions: miR-942-5p of HCT-8 cells was significantly upregulated after *C. parvum* infection, especially at 8 hpi, in response to a p65-dependent TLR2/TLR4-NF- κ B signaling. TLR4 appeared to play a dominant role.

Keywords: *Cryptosporidium parvum*, HCT-8, TLRs, NF- κ B, miR-942-5p

Background

Cryptosporidium is an emerging zoonotic pathogen that causes diarrhea in both immunocompetent and immunosuppressed hosts, and is second only to rotavirus as a cause of moderate-to-severe diarrhea in children under two years of age [1]. In immunocompromised patients, it can cause severe, life-threatening prolonged disease. In 2016, the disease burden of cryptosporidiosis in

children younger than five years was more than 12 million disability-adjusted life-years (DALYs) [2]. Thirty-eight *Cryptosporidium* valid species and approximate 60 *Cryptosporidium* genotypes have been identified or described in animals, humans, and environmental samples [3] but *C. parvum* and *C. hominis* are responsible for more than 90% of infections in humans. Despite recent efforts, effective prophylaxis and treatment are not available.

Micro (mi)RNAs are small noncoding RNA molecules found in plants, animals, viruses, and some parasite species. They function in RNA silencing and post-transcriptional regulation of gene expression [4]. Because *C. parvum* lacks

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key components needed for small RNA-mediated post-transcriptional gene silencing, it is an ideal model for investigating miRNA-mediated defenses against infection in epithelial cells [5]. At least seven host miRNAs, let-7i, miR-98, miR-513, miR-424, miR-503, miR-221 and miR-27b, are thought to be active in the innate immune defense against *Cryptosporidium* infection [6–13]. In biliary epithelial cells for example, *C. parvum* reduces the expression of let-7 family miRNAs, which target the synaptosome associated protein 23 (*SNAP23*) gene, and loss of *SNAP23* expression interferes with the release of exosomes carrying antimicrobial-peptides [7].

As with other intracellular pathogens, *Cryptosporidium* infection influences apoptosis. Microarray analysis of 51 apoptosis-associated genes indicated biphasic regulation by *Cryptosporidium*, with an anti-apoptotic state at 6 and 12 h post-infection (hpi) and a moderately pro-apoptotic state at 24, 48 and 72 hpi [14]. Inhibition of apoptosis in infected cells increases parasite survival and continuing apoptosis in uninfected bystander cells act to decrease the host immune response and may contribute to evasion of host defenses [15]. Previous studies have reported that *Cryptosporidium* inhibited of host-cell apoptosis by activating NF- κ B [16, 17]. Little is known about the regulation of host-cell apoptosis by miRNAs following *Cryptosporidium* infection. A previous study found that downregulation of miR-513 was followed by the upregulation of B7-H1 expression and decreased apoptosis [11].

Our previous study of the miRNAs expressed in HCT-8 cells infected with *C. parvum* found that miR-942-5p, miR-181d, miR-3976, miR-18b-3p, miR-34b-5p and miR-3591-3p may regulate apoptosis in the early phase of infection [18]. This study investigated the upregulation of miR-942-5p in cultured HCT-8 human ileocecal adenocarcinoma cells following activation of the TLR2/TLR4-NF- κ B signaling pathway by *C. parvum*.

Methods

Cryptosporidium oocysts and HCT-8 cells

Cryptosporidium parvum subtype IIdA19G1 oocysts were maintained in infected neonatal calves and stored in 2.5% K₂Cr₂O₇ solution at 4 °C after purification. As previously described, oocysts were excysted in 0.25% trypsin and 0.75% sodium taurocholate for 1 h with mixing every 5 min, followed by incubation at room temperature for 30 min [19, 20]. HCT-8 human ileocecal adenocarcinoma cells (American Type Culture Collection, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 4 mmol/l L-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin at 37 °C in a humidified 5% CO₂ incubator [18]. Cell monolayers in 24-well cell culture dishes were

inoculated with 2.5×10^6 purified sporozoites per well in DMEM. The sporozoite:host-cell ratio was 10:1.

Real-time quantitative PCR (qPCR)

HCT-8 cells were washed three times with phosphate buffered saline (PBS) before adding 1 ml TRIzol reagent (Invitrogen, Waltham, MA, USA) to each well. Total RNA was isolated following the kit manufacturer's instructions subsequent to treatment with Recombinant DNase I (Takara, Kyoto, Japan). RNA was reverse transcribed to cDNA with SuperScript IV Reverse Transcriptase (Invitrogen) by oligo (dT) and random primers. The cDNA was amplified using the TB Green *Premix Ex Taq* II (Takara, Kyoto, Japan) and the gene-specific primers shown in Table 1. GAPDH or β -actin genes were internal references for toll-like receptors (TLRs), the U6 gene was the internal reference for miR-942-5p. miR-942-5p was reverse transcribed to cDNA using the stem-loop primer (5'-GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACC ACA TGG C-3') and the primer (5'-CGC TTC ACG AAT TTG CGT GTC AT-3') for U6. PCR included one 30 s cycle at 95 °C, 40 cycles of 5 s at 95 °C, 10 s at 55 °C, and 15 s at 72 °C, and a final 15 s cycle at 95 °C, 1 min at 60 °C, and infinite at 25 °C. The Cq values were analyzed using the comparative Cq ($\Delta\Delta$ Cq) method and the amount of target was obtained by normalizing to internal reference and comparing with the control group.

Western blotting

HCT-8 cells were grown to 80% confluence in 6-well culture plates and exposed to *C. parvum* sporozoites. The cells were lysed with a total protein extraction kit (Solarbio Life Sciences, Beijing, China), and the protein concentrations were determined with a Pierce Bicinchoninic Acid (BCA) Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. The proteins in 30 μ g samples of lysate were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto nitrocellulose membranes. Membranes were incubated with TLR4, NF- κ B, I κ B α , and β -actin primary monoclonal antibodies (Abcam, Cambridge, UK), and then with 0.2 μ g/ml horseradish peroxidase (HRP)-conjugated secondary antibodies. The blots were read by an electrochemiluminescence (ECL) substrate (Thermo Fisher Scientific).

Short-interfering (si)RNA

SiRNAs targeting TLR-2 and TLR4 mRNAs were designed by the Sangon Biotech (Shanghai, China). HCT-8 cells were grown to 60–70% confluency in 12-well cell culture plates and transfected with siRNAs using Lipofectamine 3000 (Thermo Fisher Scientific). The

Table 1 Primer sequences used in qPCR

Target mRNA	Primers	
	Forward	Reverse
TLR1	GGTGTGGCTGTGACTGTGA	TGGAGTTCCTTAAGGGTATG
TLR2	GATGCCTACTGGGTGGAGA	AGACGGAAATGGGAGAAGT
TLR3	CCAAGCCTTCAACGACTG	TTGCGTGTTCCAGAGCC
TLR4	CCGCTTCTGGTCTTATCAT	TCTGCTGCAACTCATTTTCAT
TLR5	CAACCTTACAGCGAACC	AAACATCCCAACAGAGC
TLR6	CAGTTAATACTTTAGGGTGCT	CGTTTCTATGTGGTTGAGGG
TLR7	CCTTTCCAGAGCATAACAGC	GGACAGAACTCCCACAGAGC
TLR8	CAGAGCATCAACCAAGCAA	GCTCCGTAGCCTCAAATAC
TLR9	GTGCAGCCGGAGATGTTT	CGTGAATGAGTGCTCGTGTTAG
TLR10	GCCCACCAATCTCTTCCA	GCCCACATTTACGCCTATCCTT
GAPDH	AGAAGGCTGGGGCTCATTG	AGGGGGCATCCACAGCTTTC
U6	GCTTCGGCAGCACATATACAAAAT	CGCTTCACGAATTTGCGTGTTCAT
β -actin	AGCGAGCATCCCCAAAGTT	GGGCACGAAGGCTCATCATT

extent of inhibition was determined by qPCR assays of TLR2 and TLR4 expression at 48 h post-transfection. The siRNAs that caused the greatest inhibition of TLR2, TLR4 expression were TLR2, GGA AGA UAA UGA ACA CCA ATT (sense) and UUG GUG UUC AUU AUC UUC CTT (antisense); TLR4, CCA GGU GCA UUU AAA GAA ATT (sense) and UUG GUG UUC AUU AUC UUC CTT (antisense). The siRNA oligonucleotides had no significant overlap with homologous gene sequences. Nonspecific siRNAs containing the same nucleotides in an irregular sequence were used as controls. The siRNAs were labeled with Cy3 using a silencer siRNA labeling kit (Thermo Fisher Scientific) for identification of transfected cells by confocal microscopy. HCT-8 cells were infected with *C. parvum* sporozoites 6 h after siRNA transfection. Total RNA was extracted at 0, 4, 8, 12, 24 and 48 hpi.

Inhibitors

Pyrrolidine dithiocarbamate (PDTC) and SC-514 (MedChemExpress, Monmouth Junction, NJ, USA) were used to inhibit NF- κ B activation [21, 22]. HCT-8 cells were pretreated with inhibitor for 2 h prior to *C. parvum* infection. PDTC and SC-514 were used at concentrations of 3.286 μ g/ml and 22.43 μ g/ml, which were not cytotoxic in either HCT-8 cells or *C. parvum* sporozoites.

Data analysis

Data are represented as the mean \pm standard deviation (SD) from three independent experiments. Each independent experiment was conducted by three replicates of qPCR and the mean value was used for data

analysis. One-way ANOVA or t-test was carried out using the software of GraphPad Prism version 8.02 (<https://www.graphpad.com/>).

Results

Cryptosporidium parvum activation of TLR2 and TLR4 in HCT-8

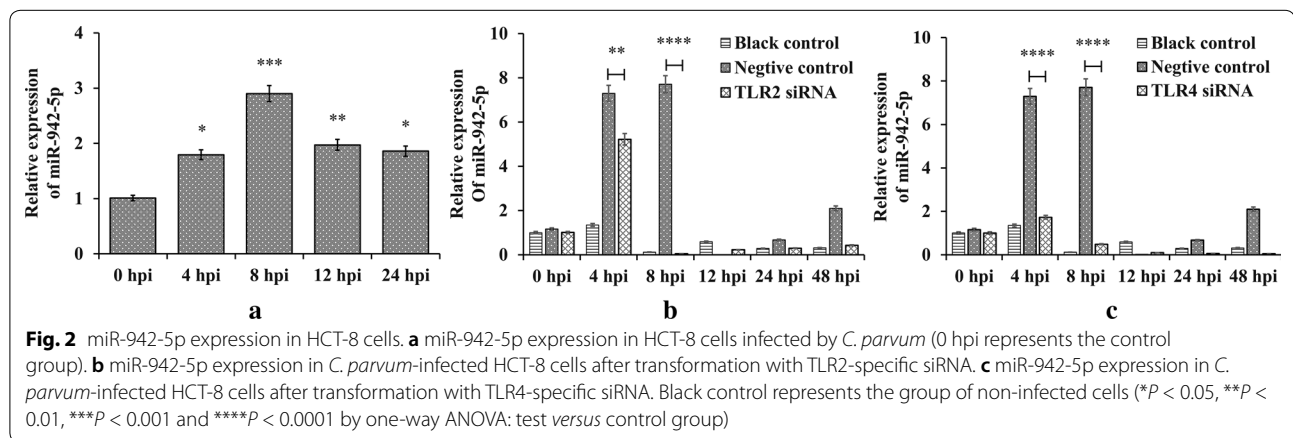
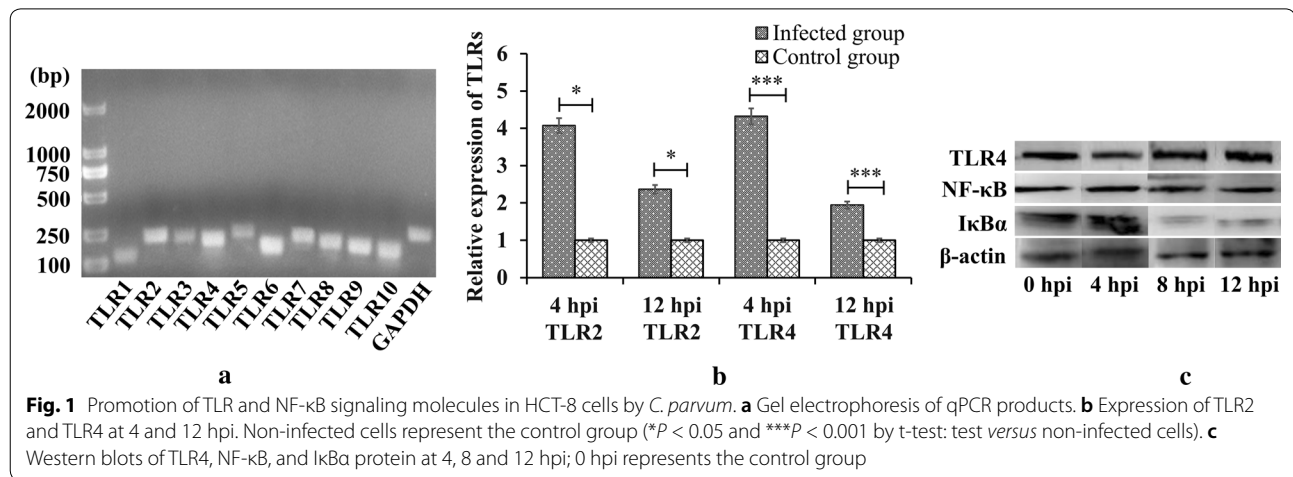
TLR1 to TLR10 expression was assayed by qPCR at 8 and 12 hpi. All were expressed in HCT-8 cells (Fig. 1a), but significant differences in infected and uninfected cells were observed only for TLR2 and TLR4. The difference was the greatest for TLR4 (TLR2 4 h: $t_{(4)} = 4.961$, $P = 0.0077$; TLR2 12 h: $t_{(4)} = 4.052$, $P = 0.0155$; TLR4 4 h: $t_{(4)} = 22.31$, $P = 10^{-9}$; and TLR4 12 h: $t_{(4)} = 12.18$, $P = 0.0003$ by t-test: test versus non-infected cells) (Fig. 1b). Activation of the TLR/NF- κ B signaling pathway was confirmed in western blots, which showed that expression of TLR4 and NF- κ B increased and that of I κ B α decreased at both 8 and 12 hpi (Fig. 1c).

Upregulation of miR-942-5p by *Cryptosporidium parvum*

The qPCR results showed that miR-942-5p expression was significantly increased at 4, 8, 12 and 24 hpi, with the greatest difference compared with the control at 8 hpi ($F_{(4,10)} = 21.00$, 4 h: $P = 0.0121$, 8 h: $P = 10^{-9}$, 12 h: $P = 0.0032$, and 24 h: $P = 0.0073$ by one-way ANOVA: test versus control group) (Fig. 2a).

Both TLR2 and TLR4 contribute to upregulation of miR-942-5p

Our previous study found that miR-942-5p was strongly upregulated in HCT-8 cells following *C. parvum* infection, compared with uninfected cells [18]. In this study,



qPCR assays revealed that the transcription of the *miR-942* gene was significantly decreased after blocking either TLR2 or TLR4 ($F_{(2, 23)} = 58.32$, $P = 10^{-9}$ by one-way ANOVA: test versus control group for Fig. 2b; $F_{(2, 27)} = 89.08$, $P = 10^{-9}$ for Fig. 2c) (Fig. 2b, c). TLR4 appeared to have a stronger influence on miR-942-5p transcription than TLR2.

NF- κ B p65 is required for the transcription of miR-942-5p

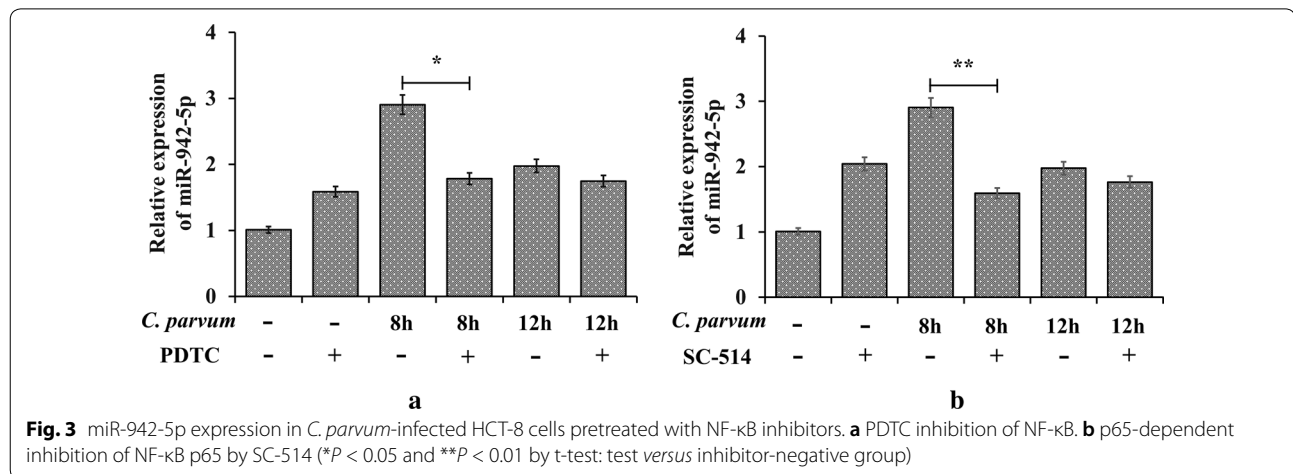
Inhibition of NF- κ B by PDTC resulted in downregulation of miR-942-5p expression at 8 and 12 hpi compared with controls ($t_{(4)} = 4.200$, $P = 0.0137$ by t-test: test versus inhibitor-negative group) (Fig. 3a). Inhibition of p65-associated transcriptional activation of the NF- κ B pathway by SC-514, a nuclear factor kappa-B kinase-2 (IKK-2) inhibitor that prevents NF- κ B-dependent gene expression, blocked the *C. parvum*-induced increase of miR-942-5p ($t_{(4)} = 5.436$, $P = 0.0056$ by t-test: test versus inhibitor-negative group) (Fig. 3b). Promoter binding of the NF- κ B p65 subunit was thus required for the

transcription of *miR-942* gene induced by *C. parvum* in HCT-8 cells.

Discussion

Cultured HCT-8 cells expressed all known TLRs (TLR1-TLR10) and *C. parvum* infection induced the upregulation of TLR2 and TLR4, but not other TLRs, as was previously found in H69 human choanocyte cells [23]. Upregulation of TLR4 was stronger than that of TLR2 (Fig. 1b), but activation of either receptor recruited downstream components, with increased NF- κ B expression and decreased expression of I κ B α , an NF- κ B inhibitor. Nuclear translocation of NF- κ B activated transcription. TLR2- and TLR4-induced activation of NF- κ B has previously been reported in H69 cells infected by *C. parvum* [23].

The upregulation of miR-942-5p after *C. parvum* infection was dependent on TLR2/TLR4-NF- κ B signaling. TLR4 may have had a stronger effect than TLR2, especially at 4 hpi, but both TLR2 and TLR4 contributed to



the upregulation of miR-942-5p expression (Fig. 2b, c). There are few data on the difference in the contributions of TLR2 and TLR4 during *C. parvum* infection, but TLR4-NF-κB signaling has been reported more frequently. TLR2 may be involved in *C. parvum*-induced stabilization of iNOS mRNA expression in biliary epithelial cells [13]. Post-transcriptional suppression of TLR4 expression by *let-7i* has been shown to contribute to immune responses to *C. parvum* infection in cultured human cholangiocytes, and mu-miR-92a-2-5p, which targets TLR2, relieves *Schistosoma japonicum*-induced liver fibrosis [6, 24].

A microarray analysis found that miR-942-5p was strongly upregulated during the early phase of *C. parvum* infection, and in this study qPCR confirmed that *C. parvum* infection was followed by significant upregulation of miR-942-5p at 4, 8, 12 and 24 hpi (Fig. 2a). Bioinformatics analysis indicated that miR-942-5p may be involved in the regulation of host-cell apoptosis. Previous studies have shown that miR-942 regulated cell apoptosis in response to microbial infection. For example, downregulation of miR-942 enhanced the apoptosis of HLCZ01 cells in response to hepatitis C virus infection [25]. Targeting of the *IFI27* gene by miR-942-5p has been shown to inhibit apoptosis role in HCT-8 cells during the early phase of *C. parvum* infection (our unpublished data).

Conclusions

HCT-8 cells expressed all known TLRs, and TLR2 and TLR4 were upregulated following *C. parvum* infection with activation of downstream signaling. miR-942-5p was significantly upregulated after *C. parvum* infection, especially at 8 hpi, in response to a p65-dependent TLR2/TLR4-NF-κB signaling. TLR4 appeared to play a dominant role.

Abbreviations

HCT-8 cells: HCT-8 human ileocecal adenocarcinoma cells; qPCR: real-time quantitative polymerase chain reaction; siRNA: short-interfering RNA; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells; IκBα: nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, alpha; PBS: phosphate buffered saline; TLRs: toll like receptors; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; PDTC: pyrrolidine dithiocarbamate.

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Authors' contributions

RW and LZ conceived and designed the experiments; GZ, YZ, ZN, CW, FX and JL performed the experiments; GZ, SZ, MQ, FJ and CN analyzed the data; RW, GZ and YZ wrote the paper. All authors read and approved the final manuscript.

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Availability of data and materials

Data are available from the authors upon reasonable request.

Ethics approval and consent to participate

The present study was approved by the Independent Ethics Committee of Henan Agricultural University (Approval No. IEC-HENAU-20180323-06).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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