Trans-suppression of Defense *DEFB1* Gene in Intestinal Epithelial Cells following *Cryptosporidium parvum* Infection is Associated with Host Delivery of Parasite Cdg7_FLc_1000 RNA

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Abstract

To counteract host immunity, *Cryptosporidium parvum* has evolved multiple strategies to suppress host antimicrobial defense. One such strategy is to reduce the production of the antimicrobial peptide beta-defensin 1 (*DEFB1*) by host epithelial cells but the underlying mechanisms remain unclear. Recent studies demonstrate that a panel of parasite RNA transcripts of low protein-coding potential are delivered into infected host cells and may modulate host gene transcription. Using *in vitro* models of intestinal cryptosporidiosis, in this study we analyzed the expression profile of host beta-defensin genes in host cells following infection. We found that *C. parvum* infection caused a significant downregulation of the *DEFB1* gene. Interestingly, downregulation of *DEFB1* gene was associated with host delivery of Cdg7_FLc_1000 RNA transcript, a *C. parvum* RNA that has previously demonstrated to be delivered into the nuclei of infected host cells. Knockdown of Cdg7_FLc_1000 in host cells could attenuate the trans-suppression of host *DEFB1* gene and decreased the parasite burden. Therefore, our data suggest that trans-suppression of *DEFB1* Gene in intestinal epithelial cells following *C. parvum* infection involves host delivery of parasite Cdg7_FLc_1000 RNA, a process that may be relevant to the epithelial defense evasion by *C. parvum* at the early stage of infection.

Keywords

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Disclosures

The authors disclose no conflict of interest.

Introduction

Cryptosporidium parvum, a zoonotic protozoan parasite, has been recognized as the leading cause of waterborne disease outbreaks worldwide (Checkley et al. 2015). This phylum *Apicomplexan* parasite is resistant to standard disinfection applied to drinking water and is a potential bioterror pathogen (O'Donoghue 1995). *C. parvum* infects human gastrointestinal epithelium and causes an acute, self-limited diarrheal disease in immunocompetent individuals but potentially life-threatening syndromes in immunocompromised patients, young children and elders (Kotloff et al. 2013; Checkley et al. 2015). Humans are infected by ingesting *Cryptosporidium* oocysts. Once ingested, oocysts excyst in the gastrointestinal tract, releasing infective sporozoites. The sporozoite attaches to the apical membrane of host epithelial cells and forms an intracellular but extracytoplasmic parasitophorous vacuole in which the organism resides. The internalized parasite then develops for its life cycle resulting in autoinfection and release of mature oocysts (Chen et al. 2002). Despite the magnitude and severity of cryptosporidial infection, there is currently no fully effective therapy (Striepen 2013).

Immunity against cryptosporidiosis involves parts of the innate and adaptive host immune responses. Mucosal epithelial cells recognize and respond to *C. parvum* infection through Toll-like receptors and initial host defense mechanisms, including release of inflammatory cytokines/chemokines and infiltration of immune effector cells at infection sites (Chen et al. 2002). Upon *C. parvum* infection, epithelial cells quickly initiate a series of innate immune reactions including production and secretion of various cytokines and chemokines, prostaglandin E2 (which stimulates mucin production), and antimicrobial peptides (defensins and cathelicidins) and nitric oxide, which can kill *C. parvum* or inhibit parasite growth (Laurent et al. 1998; Chen et al. 2005; Rogers et al. 2006). These chemokines/cytokines can mobilize and activate immune effector cells (e.g., lymphocytes, macrophages and neutrophils) to the infection sites (Chen et al. 2002; Rogers et al. 2006). These responses provide the front line of anti-*C. parvum* defense. However, complete elimination of infection requires the adaptive immune responses, particularly those mediated by CD4+ T cells (Schmidt et al. 2001; Chen et al. 2002). Interferon-γ, mainly released by activated CD4+ T cells (Schmidt et al. 2001), is also critical in the control of cryptosporidiosis (Chen et al. 2002). Eradication of *C. parvum* infection requires cell-mediated adaptive immunity in which CD4+ T cells play a key role (Chen et al. 2002). Such cell-mediated immune responses become effective about three weeks after initial infection (Schmidt et al. 2001). Therefore, it appears that *C. parvum* can survive host innate immune attack during the early stage of infection (O'Donoghue 1995; Chen et al. 2002; Checkley et al. 2015). How *C. parvum* may evade host innate immune defense at the early stage of infection is still unclear. One immune evasion strategy developed by the parasite is to reduce the production of the antimicrobial peptide beta-defensin 1 (*DEFB1*) by host epithelial cells via unknown mechanisms (Zaalouk et al. 2004).

We previously demonstrated that several *C. parvum* RNA transcripts of low protein-coding potential are selectively delivered into the nuclei of host cells through heat shock protein 70-mediated nuclear importing mechanisms during host-parasite interactions and may modulate gene transcription in infected epithelial cells (Puiu et al. 2004; Yamagishi et al. 2011; Wang

et al. 2017a). Specifically, delivery of parasite Cdg7_FLc_0990 RNA (GenBank ID: FX115678.1) (Puiu et al. 2004; Yamagishi et al. 2011) into infected intestinal epithelial cells causes trans-suppression of the LDL receptor related protein 5 (*LRP5*), solute carrier family 7 member 8 (*SLC7A8*), and interleukin 33 (*IL33)* genes through histone modification-mediated epigenetic mechanisms (Wang et al. 2017a, b). Delivery of the parasite Cdg7_FLc_1000 transcript (GenBank ID: FX115830.1) (Puiu et al. 2004; Yamagishi et al. 2011) causes trans-suppression of host sphingomyelin phosphodiesterase 3 (*SMPD3*) gene (Ming et al. 2017). Using a non-malignant human intestinal epithelial cell line for *C. parvum* infection *in vitro*, here, we report that downregulation of the *DEFB1* gene in intestinal epithelial cells following *C. parvum* infection involves the host delivery of Cdg7_FLc_1000 RNA transcript, a process relevant to the epithelial defense evasion by *C. parvum* at the early stage of infection.

2. Materials and methods

2.1 C. parvum, *cell lines, and infection models*

C. parvum oocysts of the Iowa strain were purchased from a commercial source (Bunch Grass Farm, Deary, ID). INT cells (FHs 74 Int, CCL-241™) and HCT-8 (CCL-244™) were purchased from ATCC (Manassas, VA). The murine intestinal epithelial cell line (IEC4.1) was a kind gift from Dr. Pingchang Yang (McMaster University, Hamilton, Canada) and cultured in Dulbecco's modified Eagle's Medium (DMEM-Mediatech Inc., Manassas, VA) supplemented with 5% fetal bovine serum (Hyclone), 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamicin and 1 ng/ml epidermal growth factor. Models of intestinal cryptosporidiosis using cultured cell lines were employed as previously described (Zhou et al. 2012; Wang et al. 2017a). Briefly, before infecting cells, *C. parvum* oocysts were treated with 1% sodium hypochlorite on ice for 20 min followed by extensive washing with DMEM-F12 medium and confirmed negative for the Limulus Amebocyte Lysate test (Bio-Whittaker). Real-time PCR and immunofluorescence microscopy were used to assay *C. parvum* infection as previously reported (Zhou et al. 2012; Wang et al. 2017a).

2.2 siRNAs and plasmids

Custom-designed siRNA oligos against Cdg7_FLc_1000 and a scrambled siRNA were synthesized by Integrated DNA Technologies (Coralville, Iowa) and transfected into cells with Lipofectamine RNAimax (Invitrogen) (Ming et al. 2017). siRNA sequence for Cdg7_FLc_1000: sense, 5'-GAGAUAACUAACGCCACCUU-3' and antisense, 5'-AGGUGGCGUUAGUUAUCUCUU-3'; non-specific control: sense, 5'-UUCUCCGAACGUGUCACGUUU-3' and antisense, 5'-ACGUGACACGUUCGGAGAAUU-3'. The plasmids for Full-Cdg7_FLc_1000 and Full-Cdg7_FLc_0990 were generated by RT-PCR amplification of the corresponding cDNA (Ming et al. 2017; Wang et al. 2017a, b), using RNA from *C. parvum* sporozoites (Iowa strain) and cloned into the pcDNA3.1(+) vector according to the manufacturer's protocol (Invitrogen). Full-Cdg7_FLc_1000 or Full-Cdg7_FLc_0990 were transfected into cells with lipofectamine 2000 (Invitrogen); pcDNA3.1(+) Empty vector transfected as control. The primer sequences for plasmid generation are listed as following: Cdg7_FLc_1000: forward (*Nhe I*), 5'-CGGCTAGCAGTTTTTACATTTTGTATCTCAGTT-3' and reverse (*KpnI*), 5'-

GGGGTACCTGAGCGAAATTAGAGTAGTCTGA-3'; Cdg7_FLc_0990: forward (*XbaI*), 5'-GCTCTAGAAGATTTATTAAGGTTTTATTTT-3' and reverse (*KpnI*), 5'-GGGGTACCAAAAATACAAGAAGGAGTTATG-3'.

2.3 Agilent microarray analysis

The Agilent SurePrint G3 Human Gene Expression Microarray and the LC Sciences service to process the samples were applied to genome-wide analysis. Briefly, INT cells were grown to 80% confluence and then exposed to *C. parvum* infection or transfected with Full-Cdg7_FLc_1000 or Full-Cdg7_FLc_0990. Total RNA of harvested cells was isolated with the RNeasy Mini kit (Qiagen). A mixture of equal amounts of total RNAs from each group was used as the reference pool. A total of 2 μg RNA from each sample was then labeled with the Agilent Gene Expression Hybridization Kit (Agilent). After hybridization, the slides were scanned with the Agilent Microarray Scanner (Agilent). The Feature Extraction software (version10.7.1.1, Agilent Technologies) was used to analyze array images to get raw data and Genesrping software was employed to finish the basic analysis with the raw data. To begin with, the raw data was normalized with the quantile algorithm. The probes that at least 1 out of all samples have flags as "detected" were treated as positive signals and chosen for further data analysis. Quantified positive signals were then extracted and analyzed by the LC Sciences, in accordance with MIAME guidelines.

2.4 Whole cell extracts, nuclear extracts, and quantitative real-time PCR

Whole cell extracts and nuclear extracts were obtained using the standard approach as previously described (Wang et al. 2017a). For quantitative analysis of mRNA and *C. parvum* RNA expression, comparative real-time PCR was performed as previous reported (Wang et al. 2017a; Ming et al. 2017) using the SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA). Briefly, RNA was extracted using TRI-reagent, treated with DNA-free Kit (Ambion) to remove any remaining DNA. Quantified 500 ng RNA was reverse-transcribed using T100 thermal cyclers (Bio-Rad). Real-time PCR was then performed using 25 ng of template cDNA for each RNA gene of interest. Each sample was run in triplicate. The relative abundance of each RNA was calculated using the   Ct method and normalized to *GAPDH* (total mRNA). The sequences for all the primers are as follows: *DEFB1*, forward, 5'-GCCTCAGGTGGTAACTTTCTC-3' and reverse, 5'-CGTCATTTCTTCTGGTCACTC-3'; *GAPDH*, forward, 5'-TGCACCACCAACTGCTTAGC-3' and reverse, 5'-GGCATGGACTGTGGTCATGAG-3'; C-X-C motif chemokine ligand 2 (*CXCL2*), forward, 5'-AAAGGGGTTCGCCGTTCT-3' and reverse, 5'-TGGCAGCGCAGTTCAGTG-3'; NFKB inhibitor zeta (*NFKBIZ*), forward, 5'-ACTCGGAACTTGGAGAACG-3' and reverse, 5'-CAGGACTGCCTAACTGACAG-3'; *CXCL8*, forward, 5'-TGCAGCTCTGTGTGAAGGTG-3' and reverse, 5'-ACAACCCTCTGCACCCAGTT-3'; *LRP5*, forward, 5'-GGCCAGTGTGTCCTCATCAAA-3' and reverse, 5'-AGACACCACCCATGACGAAGA-3'; *SLC7A8*, 5'-TTGCCCTGTCCACATTTGG-3' and reverse, TGGAGATGCATGTGAAGAGCA-3'; *U2*, forward, 5'-CTGATACGTCCTCTATCCGAGG-3' and reverse, TGCAATACCAGGTCGATGCGT-3'; *Defb1*, forward, 5'-CAGGTGTTGGCATTCTCA-3' and reverse, 5'-CCATCGCTCGTCCTTTAT-3'; *Gapdh*, forward, 5'-AACAGGGTGGTGGACCTCAT-3' and reverse, 5'-AGTTGGGATAGGGCCTCTCTT-3'; *Cdg7_FLc_1000*, forward, 5'-CTTCAAAAGGGACAGACAAACGGC-3' and reverse, 5'-

CTTGATCTCGCCCATAGCCACTT-3'; *18S*, forward, 5'-TAGAGATTGGAGGTTGTTCCT-3' and
reverse, 5'-CTCCACCAACTAAGAACGGCC-3'.

2.5 Statistical Analysis

All values are given as mean ± standard error of the mean (SEM). Means of groups were
from at least three independent experiments and compared with Student's *t* test (two-tailed
unpaired) or the ANOVA test when appropriate. The array data were analyzed and compared
statistically by LC Sciences using the Agilent Feature Extraction Software, in accordance
with MIAME guidelines. *p* values < 0.05 were considered statistically significant.

3. Results

3.1 Expression levels of host DEFB genes in cultured INT cells following C. parvum infection revealed by genome-wide array analysis

To explore the expression profile of *DEFB* genes in intestinal epithelial cells following *C. parvum* infection, we analyzed our previous genome-wide array data on non-malignant
human intestinal epithelial cells (INT cells) following *C. parvum* infection for 8 and 48 h
(the GEO database with the accession number: GSE87047) (Wang et al. 2017a). Out of a
total of 35 human *DEFB* genes available in the array chip, signals for 12 *DEFB* genes were
passed the threshold set by the array chip internal control in either the un-infected control or
the infected cells and thus, they treated as the detectable *DEFB* genes in INT cells (Fig. 1A,
B). Expression levels for other *DEFB* genes were then treated as undetectable in the un-
infected control or *C. parvum* infected INT cells. Out of these detectable *DEFB* genes,
DEFB1 was downregulated in cells following infection at 8 h and 48 h. Expression level for
DEFB132 was downregulated in INT cells following infection for 8 h but no significant
difference was found between the infected cells following infection for 48 h and the control
(Fig. 1A, B). Expression level for *DEFB124* was upregulated in INT cells following
infection for 48 h but no significant difference was found comparing the infected cells
following infection for 8 h with the control (Fig. 1A, B). Consistent with results from
previous studies (Wang et al. 2017a; Ming et al. 2017; Deng et al. 2004; Yang et al. 2009),
upregulation of several epithelial cell defense genes, such as interleukin-8, C-X-C motif
chemokine ligand 5, and nitric oxide synthase 2, was detected in cells following *C. parvum*
infection (data not shown).

3.2 Expression levels of host DEFB genes in INT cells expressing C. parvum Cdg7_FLc_1000 RNA revealed by genome-wide array analysis

We previously demonstrated that nuclear delivery of *C. parvum* RNA transcripts of low
protein-coding potential, such as Cdg7_FLc_0990 and Cdg7_FLc_1000, is involved in the
trans-suppression of specific host genes in *C. parvum*-infected intestinal epithelial cells
(Wang et al. 2017a, b; Ming et al. 2017). We then explored the expression levels of *DEFB*
genes in INT cells expressing Cdg7_FLc_0990 or Cdg7_FLc_1000. We analyzed the
previous genome-wide data using the same array chip as described above on INT cells
following transfection of the Full-Cdg7_FLc_0990 or Full-Cdg7_FLc_1000 for 48 h (the
GEO database with the accession number: GSE87047) (Wang et al. 2017a, b; Ming et al.
2017). No significant difference in the expression levels of *DEFB* genes was detected in

cells transfected with the Full-Cdg7_FLc_0990, compared with cells transfected with the empty vector as the control (Fig. 2A, B). In contrast, downregulation of *DEFB1* gene was observed in cells transfected with the Full-Cdg7_FLc_1000 (Fig. 2A, B). The expression level of *DEFB104B* showed a tendency to increase (with a P value close to 0.05) in INT cells after Full-Cdg7_FLc_1000 transfection (Fig. 2A, B). Notably, although the expression levels of the *DEFB104B* gene showed a tendency to increase in cells following infection, there was no statistic difference between cells of the non-infected control and cells following *C. parvum* infection for 8 and 48 h (Fig. 1A, B).

3.3   Trans-suppression of host DEFB1 gene following C. parvum infection involves host delivery of Cdg7_FLc_1000 RNA

Given the downregulation of *DEFB1* gene in cells expressing Cdg7-FLc_1000, we then questioned whether trans-suppression of host *DEFB1* gene following infection involves host delivery of Cdg7-FLc_1000 RNA. We first measured the expression levels of *DEFB1* gene at the RNA level in INT and HCT-8 cells following *C. parvum* infection for various periods of time using real-time PCR. As shown in Fig. 3A, expression levels of DEFB1 RNA in INT and HCT-8 cells following infection for 24, 36, and 48 h were significantly lower than that in the un-infected control. Accordingly, suppression of *DEFB1* gene was also detected in INT and HCT-8 cells following transfection of the Full-Cdg7-FLc_1000 for 24, 36, and 48 h (Fig. 3B). Moreover, suppression of *DEFB1* gene was further confirmed in murine intestinal epithelial (IEC4.1) cells following *C. parvum* infection or after transfection of the Full-Cdg7_FLc_1000 (Fig. 3C). For control, induction of the *CXCL2*, *NFKBIZ* and *CXCL8* genes in HCT-8 cells following infection was detected (Fig. 3D). Consistent with results of previous reports (Wang et al. 2017a, b), suppression of *SLC7A8* and *LRP5* genes was detected in cells transfected with the Full-Cdg7-FLc_0990; in contrast, no changes were detected in the expression level of *DEFB1* in the transfected cells (Fig. 3D), further supporting the specificity of trans-suppression of *DEFB1* by Cdg7-FLc-1000.

We then measured the effects of Cdg7_FLc_1000 knockdown on *DEFB1* expression in cells following infection. Because conventional genetic tools are very difficult, if not impossible, to modify *C. parvum* genes (Striepen 2013; Vinayak et al. 2015), we designed an siRNA to Cdg7_FLc_1000 and transfected host cells for 12 h, followed by the exposure to *C. parvum* infection. A non-specific scrambled siRNA was used for control. The increase of Cdg7_FLc_1000 RNA levels in the nuclear extracts (Fig. 4A) and whole cell extracts (Fig. 4B) of HCT-8 cells induced by *C. parvum* infection was partially suppressed by pre-treatment of the siRNA to Cdg7_FLc_1000. Accordingly, suppression of DEFB1 RNA expression induced by *C. parvum* infection was significantly attenuated through pretreatment of the siRNA to Cdg7_FLc_1000 in HCT-8 cells (Fig. 4C). Taken together, these data suggest that suppression of *DEFB1* gene in host cells induced by *C. parvum* infection may be mediated through host delivery of Cdg7_FLc_1000.

3.4   Inhibition of Cdg7_FLc_1000 delivery decreases C. parvum infection burden in cultured human intestinal epithelial cells

To test whether Cdg7_FLc_1000-mediated trans-suppression is involved in intestinal epithelial defense responses against *C. parvum* infection, we assessed the effects of

Cdg7_FLc_1000 knockdown on the parasite infection burden in cultured cells. HCT-8 cells were transfected with the specific siRNA to Cdg7_FLc_1000, and then exposed to a constant number of *C. parvum* oocysts for 4 h to allow sufficient host-cell attachment and cellular invasion (Zhou et al. 2012; Chen and LaRusso 2000; Zhou et al. 2009). After extensive washing with culture medium to remove non-attached and non-internalized parasites, cells were either collected to detect the parasite attachment/invasion or cultured for an additional 28 h for the measurement of infection burden at intracellular development stage. Parasite burden was assessed in the samples using a real-time PCR approach as we previously reported (Ming et al. 2017; Zhou et al. 2012). The parasite burden in cells after exposure to *C. parvum* for 4 h, reflecting parasite attachment/invasion, was similar between cells transfected with the control siRNA and cells transfected with the siRNA to Cdg7_FLc_1000 (Fig. 5A), confirming that the siRNA treatments do not affect initial parasite host cell attachment and cellular invasion. Cells transfected with the siRNA to Cdg7_FLc_1000 displayed a decreased parasite burden at 32 h after infection as compared to cells treated with the non-specific scrambled siRNA (Fig. 5B). Similar results were obtained with INT cells (data not shown). Decrease of parasite burden at 32 h after initial infection in HCT-8 cells treated with the siRNA to Cdg7_FLc_1000 was further assessed and confirmed by immunofluorescent microscopy (Fig. 5C).

4. Discussion

Increasing evidence suggests that RNA molecules may play important regulatory roles in diverse biological processes, including regulation of gene transcription (Prasanth and Spector 2007; Ulitsky and Bartel 2013). Recent genomic research has revealed the expression of novel non-protein coding RNA genes in the protozoan group of parasites, such as *P. falciparum* and *C. parvum* (Abrahamsen et al. 2004; Puiu et al. 2004; Yamagishi et al. 2011; Liao et al. 2014; Vembar et al. 2014). The interactions between *Cryptosporidium* and intestinal epithelial cells involves exchanges of distinct effector molecules from both sides of the host cell and the parasite at the host-parasite interface (Sibley 2004). Several *C. parvum* proteins have been demonstrated to be delivered into host epithelial cells at the host-parasite interface and are involved in parasite intracellular development (Sibley 2004; O'Connor et al. 2007). Our recent observation of selective delivery of *C. parvum* RNA transcripts of low protein-coding potential into infected host epithelial cells (Wang et al. 2017a) and consequent trans-suppression of host genes through distinct mechanisms (Ming et al. 2017; Wang et al. 2017b) support the notion that cryptosporidial infection causes transcriptional gene suppression with pathological significance in infected cells through nuclear transfer of specific parasite RNAs. In this study, our data suggest that trans-suppression of host *DEFB1* gene in cultured human intestinal epithelial cells following *C. parvum* infection involves nuclear delivery of parasite Cdg7_FLc_1000 RNA, a process that may be relevant to the epithelial defense evasion by *C. parvum* at the early stage of infection.

One of the interesting findings of this study is the downregulation of *DEFB1* genes in cells following *C. parvum* infection *in vitro*. Out of the 12 *DEFB* genes detectable with the microarray approach in INT cells, *DEFB1* was persistently downregulated in cells following infection. Our finding is consistent with data from a previous report on the reduced production of the antimicrobial peptide of DEFB1 by murine and human intestinal epithelial

cells following *C. parvum* infection *in vitro* and *in vivo* (Zaalouk et al. 2004). Downregulation of *DEFB132* was detected in infected cells but only in cells following infection for 8 h. Downregulation of *DEFB1* gene was further confirmed using real-time PCR analysis in cultured INT and HCT-8 cells following infection. In contrast, only *DEFB124* was found to be upregulated in INT cells following infection for 48 h. Expression level of *DEFB104B* (also known as *HBD-4*), one of the most common inducible *DEFB* genes (Semple and Dorin 2012; Jarczak et al. 2013), showed a tendency to increase but without statistical significance. Of note, upregulation of *DEFB104B* induced by *C. parvum* has previously been demonstrated in human biliary epithelial cells and malignant human intestinal epithelial cell lines following infection *in vitro* (Zaalouk et al. 2004; Chen et al. 2005; Hu et al. 2013). Many effector molecules released from immune cells, such as IFN-γ from NK cells and many inflammatory cytokines from macrophages and lymphocytes, can regulate the expression of defense genes in the intestinal epithelial cells during microbial infection *in vivo* (Lantier et al. 2013; McDonald et al. 2013). Coupled with the fact that multiple *DEFB* genes are expressed in the intestine, future studies should characterize the *DEFB* gene expression profiles in epithelial cells using intestinal tissues from patients with intestinal cryptosporidiosis and elucidate the role of each *DEFB* gene in intestinal defense against *cryptosporidium* infection *in vivo* using conditional knock-out mice.

Several pieces of evidence implicate that nuclear delivery of the parasite Cdg7_FLc_1000 RNA transcript into infected cells modulates transcription of the *DEFB1* gene. First, downregulation of the *DEFB1* gene was observed in cells following infection. Intriguingly, genome-wide analysis of the gene expression profile revealed significant suppression of *DEFB1* gene in cultured human intestinal epithelial cells overexpressing the Cdg7_FLc_1000 RNA. Second, using a specific siRNA to knockdown Cdg7_FLc_1000 in host cells during *C. parvum* infection attenuated the downregulation of the *DEFB1* gene. Moreover, the association between Cdg7_Flc_1000 delivery and trans-suppression of *DEFB1* appears to be specific, as transfection of another nuclear delivery parasite Cdg7_FLc_0990 RNA (Wang et al. 2017a, b), failed to downregulate the expression level of *DEFB1*. How nuclear delivery of Cdg7_FLc_1000 RNA may suppress *DEFB1* transcription in infected cells remains unclear.

Despite significant induction of antimicrobial peptides, release of inflammatory chemokines/cytokines and local inflammatory reactions at infection sites, *C. parvum* can survive host immune attack at the early stage of infection. How *C. parvum* evades host innate immune defense at the early stage of infection is still unclear. Intracellular pathogens, including protozoan parasites, have evolved strategies to evade host immune attack. The suicide of host cells following invasion by intracellular pathogens is an ancient defense mechanism (Carmen and Sinai 2007). Many intracellular pathogens have developed strategies to inhibit the induction of host cell death (Carmen et al. 2006; Petersen et al. 2006). Indeed, *C. parvum* infection induces apoptotic resistance in infected epithelial cells (Chen et al. 2001; Liu et al. 2009). Our data from the current study suggest a new mechanism by which *C. parvum* may attenuate epithelial cell antimicrobial defense, i.e., trans-suppression of host defense *DEFB1* gene through delivery of the parasite Cdg7_FLc_1000 RNA transcript into infected cells. Nuclear transfer of Cdg7_FLc_1000 RNA into infected host cells has also been demonstrated to attenuate intestinal epithelial cell migration via trans-suppression of host

cell *SMPD3* gene (Ming et al. 2017). Therefore, host delivery of parasite RNAs, such as Cdg7_FLc_1000, may provide several benefits to *C. parvum* for its intracellular development within intestinal epithelial cells after internalization. Targeting host delivery of specific parasite RNA transcripts may be of therapeutic value and merits further investigation.
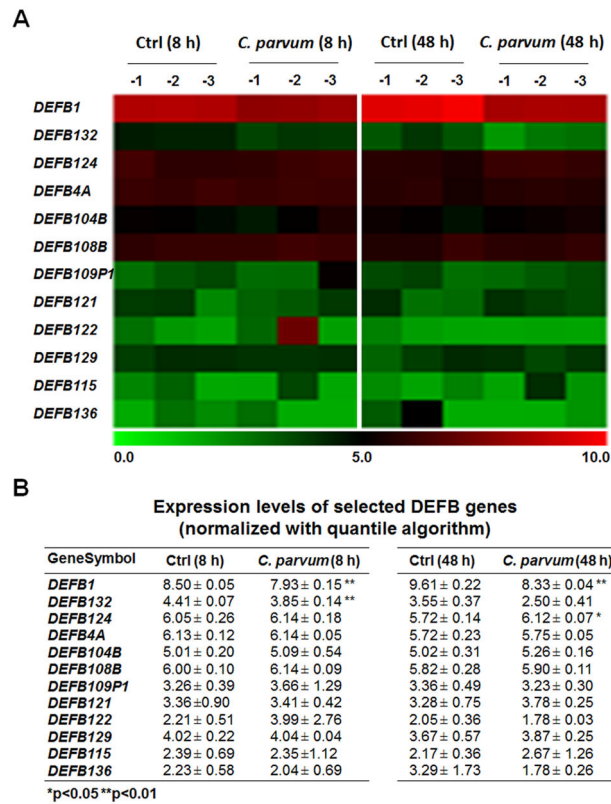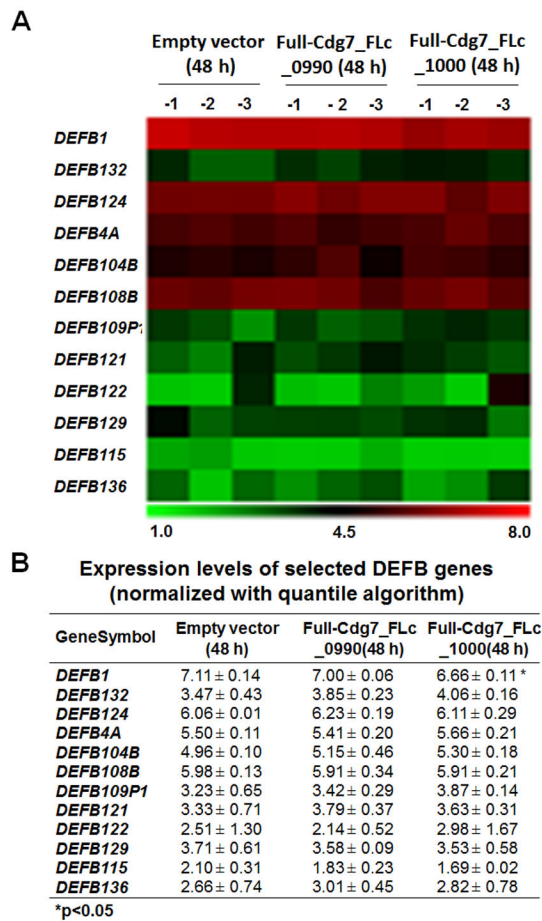
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A

	Ctrl (8 h)			C. parvum (8 h)			Ctrl (48 h)			C. parvum (48 h)		
	-1	-2	-3	-1	-2	-3	-1	-2	-3	-1	-2	-3

DEFB1
DEFB132
DEFB124
DEFB4A
DEFB104B
DEFB108B
DEFB109P1
DEFB121
DEFB122
DEFB129
DEFB115
DEFB136

0.0   5.0   10.0

B

Expression levels of selected DEFB genes
(normalized with quantile algorithm)

| GeneSymbol	Ctrl (8 h)	C. parvum (8 h)	Ctrl (48 h)	C. parvum (48 h)
DEFB1	8.50 ± 0.05	7.93 ± 0.15 **	9.61 ± 0.22	8.33 ± 0.04 **
DEFB132	4.41 ± 0.07	3.85 ± 0.14 **	3.55 ± 0.37	2.50 ± 0.41
DEFB124	6.05 ± 0.26	6.14 ± 0.18	5.72 ± 0.14	6.12 ± 0.07 *
DEFB4A	6.13 ± 0.12	6.14 ± 0.05	5.72 ± 0.23	5.75 ± 0.05
DEFB104B	5.01 ± 0.20	5.09 ± 0.54	5.02 ± 0.31	5.26 ± 0.16
DEFB108B	6.00 ± 0.10	6.14 ± 0.09	5.82 ± 0.28	5.90 ± 0.11
DEFB109P1	3.26 ± 0.39	3.66 ± 1.29	3.36 ± 0.49	3.23 ± 0.30
DEFB121	3.36 ± 0.90	3.41 ± 0.42	3.28 ± 0.75	3.78 ± 0.25
DEFB122	2.21 ± 0.51	3.99 ± 2.76	2.05 ± 0.36	1.78 ± 0.03
DEFB129	4.02 ± 0.22	4.04 ± 0.04	3.67 ± 0.57	3.87 ± 0.25
DEFB115	2.39 ± 0.69	2.35 ± 1.12	2.17 ± 0.36	2.67 ± 1.26
DEFB136	2.23 ± 0.58	2.04 ± 0.69	3.29 ± 1.73	1.78 ± 0.26

*$p < 0.05$ **$p < 0.01$

Fig. 1. Genome-wide array analysis of DEFB genes in INT cells following *C. parvum* infection.
Alterations in *DEFB* gene expression profile from genome-wide transcriptome analysis in INT cells following *C. parvum* infection. INT cells were exposed to *C. parvum* infection for 8h and 48 h, followed by the microarray analysis; non-infected cells were used as the control. The mean value of the expression level for selected *DEFB* genes, which passed the filtering criteria variation across the samples and normalized with quantile algorithm using the Genesrping software, was presented as the heatmap (**A**) and listed in the table (**B**). *$P < 0.05$ and **$P < 0.01$ versus non-infected cells.

A

Empty vector (48 h) — -1 -2 -3
Full-Cdg7_FLc_0990 (48 h) — -1 -2 -3
Full-Cdg7_FLc_1000 (48 h) — -1 -2 -3

DEFB1
DEFB132
DEFB124
DEFB4A
DEFB104B
DEFB108B
DEFB109P1
DEFB121
DEFB122
DEFB129
DEFB115
DEFB136

1.0   4.5   8.0

B

Expression levels of selected DEFB genes
(normalized with quantile algorithm)

| GeneSymbol	Empty vector (48 h)	Full-Cdg7_FLc _0990(48 h)	Full-Cdg7_FLc _1000(48 h)
DEFB1	7.11 ± 0.14	7.00 ± 0.06	6.66 ± 0.11 *
DEFB132	3.47 ± 0.43	3.85 ± 0.23	4.06 ± 0.16
DEFB124	6.06 ± 0.01	6.23 ± 0.19	6.11 ± 0.29
DEFB4A	5.50 ± 0.11	5.41 ± 0.20	5.66 ± 0.21
DEFB104B	4.96 ± 0.10	5.15 ± 0.46	5.30 ± 0.18
DEFB108B	5.98 ± 0.13	5.91 ± 0.34	5.91 ± 0.21
DEFB109P1	3.23 ± 0.65	3.42 ± 0.29	3.87 ± 0.14
DEFB121	3.33 ± 0.71	3.79 ± 0.37	3.63 ± 0.31
DEFB122	2.51 ± 1.30	2.14 ± 0.52	2.98 ± 1.67
DEFB129	3.71 ± 0.61	3.58 ± 0.09	3.53 ± 0.58
DEFB115	2.10 ± 0.31	1.83 ± 0.23	1.69 ± 0.02
DEFB136	2.66 ± 0.74	3.01 ± 0.45	2.82 ± 0.78

*p<0.05

Fig. 2. Genome-wide array analysis of DEFB genes in INT cells expressing specific parasite RNA transcripts.
Alterations in *DEFB* gene expression profile from genome-wide transcriptome analysis in INT cells expressing specific RNA transcripts. INT cells were transfected with the Full-Cdg7_FLc_1000 or Full-Cdg7_FLc_0990 for 48h, followed by the microarray analysis; cells transfected with the empty vector were used as the control. The mean value of the expression level for these *DEFB* genes was presented as the heatmap (**A**) and listed in the table (**B**). *P<0.05 *t* test versus cells transfected with the empty vector.
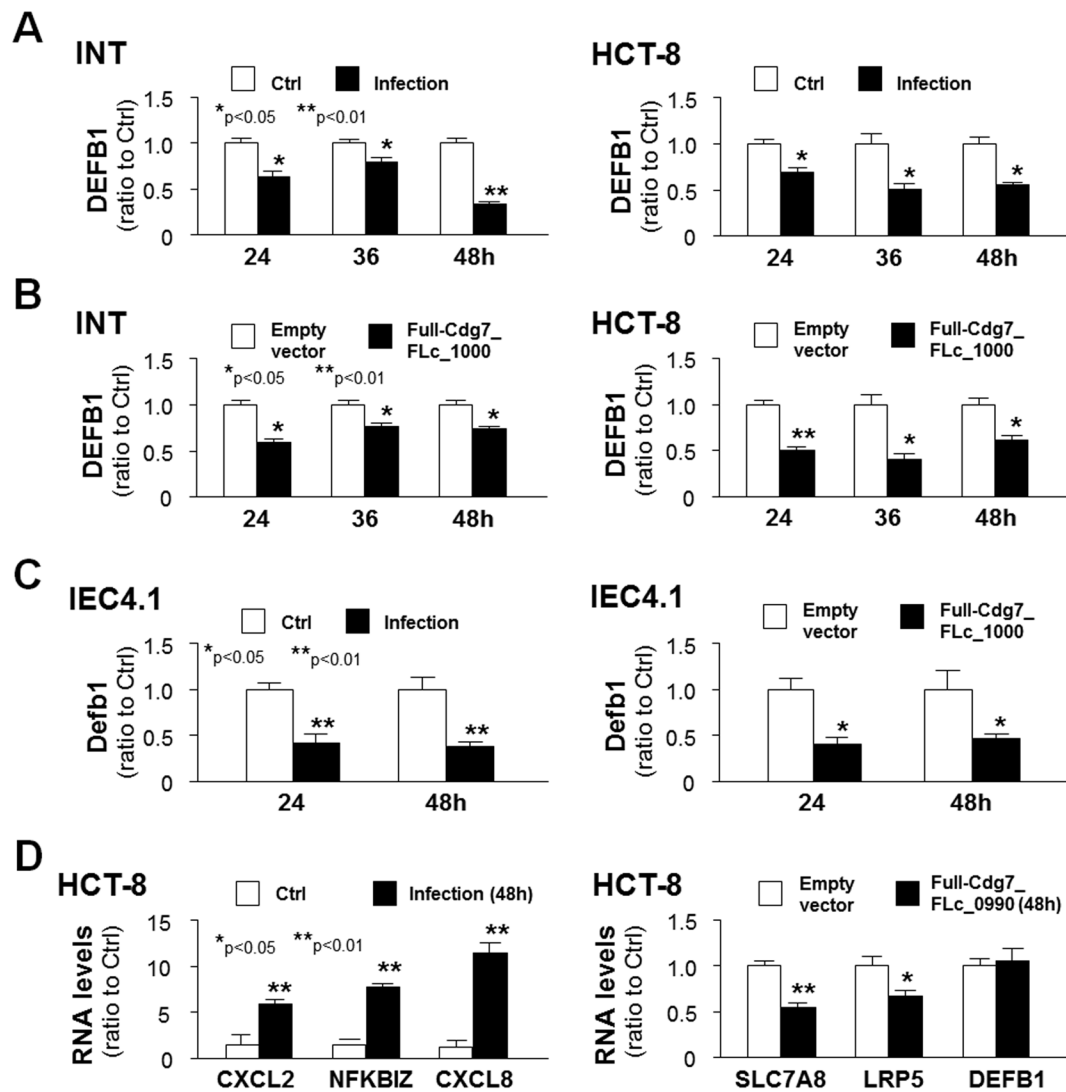
Fig. 3. Trans-suppression of *DEFB1* gene in cultured human and murine intestinal epithelial cells following *C. parvum* infection measured with real-time PCR.

(**A**) Downregulation of the *DEFB1* gene in INT and HCT-8 cells following *C. parvum* infection. INT and HCT-8 cells were exposed to *C. parvum* infection for 24, 36, and 48 h, followed by real-time PCR analysis of DEFB1 RNA levels. (**B**) Downregulation of the *DEFB1* gene in INT and HCT-8 cells following *C. parvum* infection or transfection with the Full-Cdg7_FLc_1000. INT and HCT-8 cells were exposed to *C. parvum* infection or transfection with the Full-Cdg7_FLc_1000 for 24, 36, and 48 h, followed by real-time PCR analysis of DEFB1 RNA levels. (**C**) Murine intestinal epithelial (IEC4.1) cells were exposed to *C. parvum* infection or transfection with the Full-Cdg7_FLc_1000, followed by real-time PCR analysis of Defb1 RNA levels. (**D**) Expression levels of selected genes induced by *C. parvum* infection or suppressed by Cdg7_FLc_0990 transfection in HCT-8 cells. HCT-8 cells were exposed to *C. parvum* infection for 48 h or transfection with the Full-Cdg7_FLc_0990 for 48 h, followed by real-time PCR analysis of selected genes. **P<0.05 and **P<0.01 *t* test versus non-infected cells or cells transfected with the empty vector.
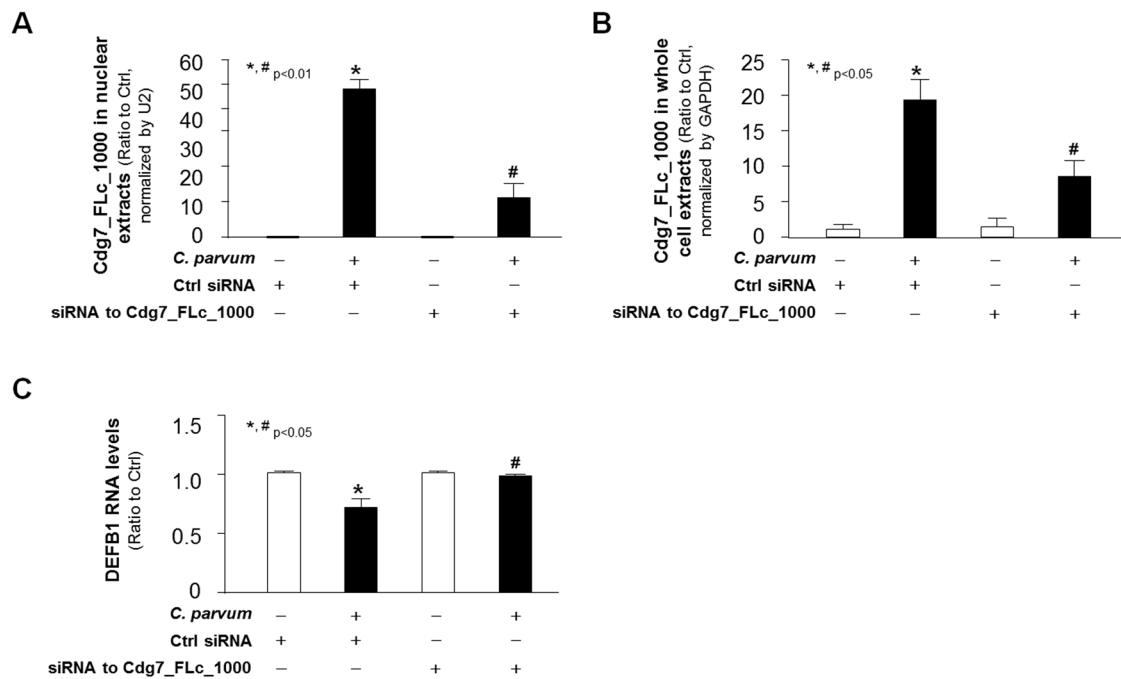
Fig. 4. Inhibition of Cdg7_FLc_1000 in host cells by the siRNA treatment attenuated the downregulation of *DEFB1* following *C. parvum* infection.

HCT-8 cells were treated with an siRNA to Cdg7_FLc_1000 for 12 h and then exposed to *C. parvum* infection for additional 24 h. Content of Cdg7_FLc_1000 in the nuclear extracts (**A**) and whole cell extracts (**B**) from cells following infection, as well as the expression level of DEFB1 RNA in the infected cells (**C**), was quantified by real-time PCR. Data represent three independent experiments. *P<0.05 ANOVA versus non-infected cells treated with the control siRNA; # P<0.05 ANOVA versus infected cells treated with the control siRNA.
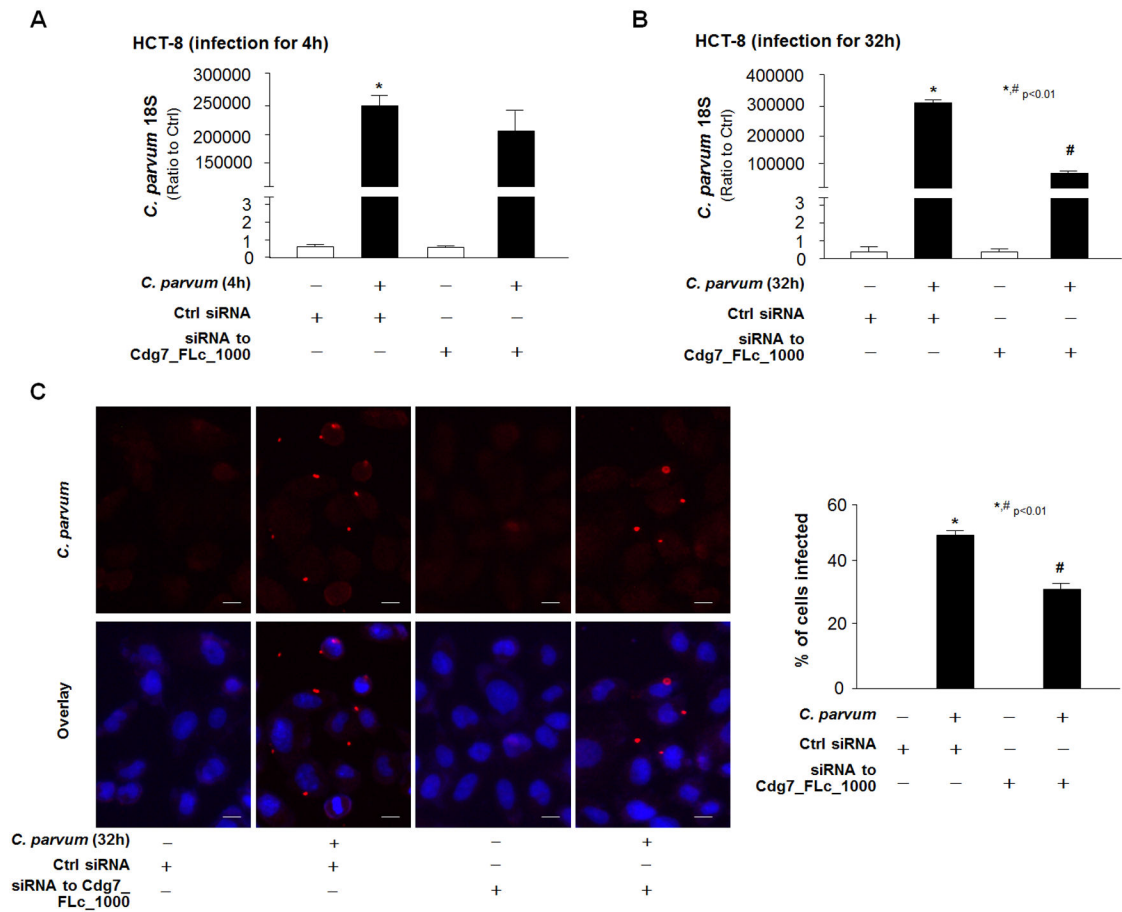
Fig. 5. Inhibition of Cdg7_FLc_1000 in host cells by the siRNA treatment decreased parasite infection burden in HCT-8 cells.

Cells were treated with an siRNA to Cdg7_FLc_1000 for 12 h and then exposed to *C. parvum* infection for 4 h or 32 h. Parasite infection burden was measured by using real-time PCR for the *C. parvum* 18s (**A** and **B**) or by immunofluorescent staining (**C**). Data represent three independent experiments. *P<0.01 ANOVA versus non-infected cells treated with the control siRNA; # P<0.01 ANOVA versus infected cells treated with the control siRNA. Bar = 10 μm.