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# A CELLULAR MIRNA, *LET-7I*, REGULATES TOLL-LIKE RECEPTOR 4 EXPRESSION AND CONTRIBUTES TO CHOLANGIOCYTE IMMUNE RESPONSES AGAINST *CRYPTOSPORIDIUM PARVUM* INFECTION*

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**Running Head**: *let-7* regulates cholangiocyte immunity

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**Toll-like receptors (TLRs) are important pathogen recognition molecules and are key to epithelial immune responses to microbial infection. However, the molecular mechanisms that regulate TLR expression in epithelia are obscure. MicroRNAs play important roles in a wide range of biological events through post-transcriptional suppression of target mRNAs. Here we report that human biliary epithelial cells (cholangiocytes) express *let-7* family members, microRNAs with complementarity to TLR4 mRNA. We found that *let-7* regulates TLR4 expression via post-transcriptional suppression in cultured human cholangiocytes. Infection of cultured human cholangiocytes with *Cryptosporidium parvum*, a parasite that causes intestinal and biliary disease, results in a decreased expression of primary *let-7i* and mature *let-7* in a MyD88/NF-κB dependent manner. The decreased *let-7* expression is associated with *C. parvum*-induced up-regulation of TLR4 in infected cells. Moreover, experimentally-induced suppression or forced expression of *let-7i* causes reciprocal alterations in *C. parvum*-induced TLR4 protein expression, and consequently, infection dynamics of *C. parvum in vitro*. These results indicate that *let-7i* regulates TLR4 expression in cholangiocytes and contributes to epithelial immune responses against *C. parvum* infection. Further, the data raise the possibility that microRNA-mediated post-transcriptional pathways may be critical to host-cell regulatory responses to microbial infection in general.**

Toll-like receptors (TLRs) are an evolutionarily conserved family of cell surface pattern recognition molecules that play a key role in host immunity through detection of pathogens (1,2). Most TLRs, upon recognition of discrete pathogen-associated molecular patterns, activate a set of adaptor proteins (e.g., myeloid differentiation protein 88 [MyD88]) leading to the nuclear translocation of transcription factors, such as NF-κB and AP-1, and thus transcriptionally regulate host-cell responses to pathogens, including parasites (3-5). TLRs may also recognize endogenous ligands induced during the inflammatory response (1-4). Evidence is accumulating that the signaling pathways associated with TLRs not only mediate host innate immunity but are also important to adaptive immune responses to microbial infection (6). Epithelial cells express TLRs and activation of TLRs triggers an array of epithelial defense responses, including production and release of cytokines/chemokines and anti-microbial peptides (1-8). Expression of TLRs by epithelia is tightly

regulated, reflecting the specific micro-environment and function of each epithelial cell type. This cell specificity is critically important to assure that an epithelium will recognize invading pathogens but not elicit an inappropriate immune response to endogenous ligands or commensal microorganisms (4).

Human bile is thought to be sterile under physiological conditions (9). Nevertheless, the biliary tract is connected and open to the intestinal tract and therefore, is potentially exposed to microorganisms from the gut. For example, duodenal microorganisms are believed to be a major source of bacterial infection in several biliary diseases (9,10). Indeed, *Cryptosporidium parvum*, a coccidian parasite of the phylum *Apicomplexa*, preferentially infects the small intestine yet can infect biliary epithelial cells (i.e., cholangiocytes) causing biliary tract disease. We and others previously reported that human cholangiocytes express all ten known TLRs and produce a variety of inflammatory cytokines/chemokines and antimicrobial peptides in response to microbial infection, suggesting a key but poorly understood role for cholangiocytes in epithelial defense (9-14). We also previously demonstrated that TLR2 and TLR4 signals mediate cholangiocyte responses including production of human beta-defensin 2 (HBD2) against *C. parvum* via TLR-associated activation of NF-κB (11). Dominant negative TLR/MyD88 expressing cholangiocytes have diminished defenses against *C. parvum* infection *in vitro* (11). Thus, TLRs and the mechanisms involved in their regulation are key elements for cholangiocyte defense against *C. parvum* infection.

MicroRNAs (miRNAs) are a newly identified class of endogenous small regulatory RNAs (15-17). In the cytoplasm, they associate with messenger RNAs (mRNAs) based on complementarity between the miRNAs and the target mRNAs. This binding causes either mRNA degradation or translational suppression resulting in gene suppression at a post-transcriptional level (15-17). MicroRNAs exhibit tissue-specific or developmental-stage-specific expression, indicating that their cellular expression is tightly regulated (15-17). Nevertheless, the molecular mechanisms underlying cellular regulation of miRNA expression are unclear. Recent studies have indicated that transcription factors, such as c-Myc and C/EBPα, appear to be involved in the expression of miRNAs, suggesting a role for transcription factors in regulation of miRNA expression (18,19). It has become clear that miRNAs play essential roles in several biological processes, including development, differentiation and apoptotic cell death (15-27). Also, an antiviral role for miRNAs has been described in plants (15). Direct evidence of the importance of miRNAs in vertebrates to control viral invasion has also recently emerged from studies using human retroviruses (20); a host-cell miRNA, miR-32, has been identified that can effectively suppress primate foamy virus type 1 (PFV-1) replication (20). Conversely, a PFV-1 derived protein, Tas, alters host-cell miRNA expression (20). Thus, host-pathogen interactions can influence host-cell miRNA-mediated post-transcriptional suppression, a process potentially involved in the regulation of epithelial defenses in response to microbial infection.

In work described here, we found that human cholangiocytes express members of the *let-7* family. We demonstrate that at least one of these, *let-7i*, directly regulates TLR4 expression. We also show that *C. parvum* infection of cholangiocytes decreases *let-7i* expression via a MyD88/NF-κB dependent mechanism. Moreover, a decrease of *let-7i* expression is associated with *C. parvum*-induced upregulation of TLR4 in infected cells, and consequently, the infection dynamics of *C. parvum in vitro*. Thus, a novel *let-7*-mediated regulatory pathway for TLR4 expression has been identified in cholangiocytes, a process that is involved in epithelial responses against microbial infection.

## EXPERIMENTAL PROCEDURES

***C. parvum and cholangiocyte cell lines*** - *C. parvum* oocysts of the Iowa strain were purchased from a commercial source (Bunch Grass Farm, Deary, ID). Before infecting cells, oocysts were excysted to release infective sporozoites as previously described (11,28). Freshly excysted sporozoites were tested for LPS activity using the Limulus Amebocyte Lysate (LAL) test kit (Bio-Whittaker, Walkersville, MD) as reported by others (29) and only LAL-negative sporozoites were used in the study. H69 cells are SV40

transformed normal human cholangiocytes originally derived from normal liver harvested for transplant. These cholangiocytes continue to express biliary epithelial cell markers, including cytokeratin 19, gamma glutamyl transpeptidase and ion transporters consistent with biliary function and have been extensively characterized (11,28).

***In vitro infection model*** - An *in vitro* model of human biliary cryptosporidiosis using H69 cells was employed as previously described (11,28). Before infecting cells, oocysts were excysted to release infective sporozoites (11,28). Infection was done in a culture medium consisting of DMEM-F12, 100 U/ml penicillin and 100 μg/ml streptomycin and freshly excysted sporozoites (1 X $10^6$ sporozoites/ per slide well or culture plate). Inactivated organisms (treated at 65˚C for 30 min) were used for sham infection controls. For some experiments, H69 cells were exposed to LPS (100 ng/ml, Invivogen) for 12 hours. For the inhibitory experiments, SN50, one specific inhibitor of NF-κB, was added in the medium at the same time as *C. parvum*. A concentration of 50 μg/ml, which showed no cytotoxic effects on H69 cells or on *C. parvum* sporozoites, was selected for the study. All the experiments were performed in triplicate.

***Immunofluorescent microscopy*** - After exposure to *C. parvum* as described above, cells were fixed with 2% paraformaldehyde and permeabilized with 0.2% (v/v) Triton-X100 in PBS. For double-immunofluorescent labeling of TLR4 with *C. parvum*, fixed cells were incubated with a monoclonal antibody TLR4 (Imgenex) mixed with a polyclonal antibody against *C. parvum* (a gift from Dr. Guan Zhu, Texas A&M University, College Station, TX) followed by anti-mouse and anti-rabbit secondary antibodies (Molecular Probes) as we previously reported (11,28). In some experiments, 4', 6-diamidino-2-phenylindole (DAPI, 5 μM) was used to stain cell nuclei. Labeled cells were assessed by confocal laser scanning microscopy.

***Western blot*** - A previously reported semi-quantitative Western blot approach was used to assess TLR4 expression in cells (11). Briefly, total cell lysates were obtained from the cells after exposure to *C. parvum* and blotted for TLR4 and actin. Antibodies to TLR4 (Imgenex) and actin (Sigma-Aldrich) were used. TLR4 levels were expressed as their ratio to actin (11).

***Microarray analysis of endogenous miRNA expression*** - H69 cells were grown to confluence and total RNAs were isolated using the Trizol kit (Invitrogen). MicroRNA expression profile in H69 cells was performed with a recently developed semi-quantitative microarray approach with the GenoExplorer™ microRNA Biochips by Genosensor (Tempe, Arizona). 5s rRNA was detected as the control and data were analyzed with the software provided by the company (Genosensor).

***let-7i precursor and antisense oligonucleotide*** - To manipulate cellular function of *let-7i* in H69 cells, we utilized an antisense approach to inhibit *let-7i* function and precursor transfection to increase *let-7i* expression. For experiments, H69 cells were grown to sub-confluence and then incubated in culture medium containing *let-7i* antisense 2-methoxy oligonucleotide (Ambion, 20 ng/ml) for 12 h. For *let-7i* precursor transfection, H69 cells were grown to 60-70% confluence and were transfected with the *let-7i* precursor (Ambion, 20 ng/ml) using the NeoFx transfection agent (Ambion). Those cells were usually used for experiments 12 h after transfection.

***Northern blot*** - Total RNA from cultured cells was isolated using the conventional method of acid phenol:chloroform extraction using TRI Reagent (Sigma-Aldrich) and subsequent alcohol precipitation. The total RNA was then used as the starting material for the enrichment of miRNAs. MicroRNAs were enriched using the mirVana miRNA Isolation kit (Ambion, Austin, TX).

For Northern blot detection, 1 μg of miRNAs was separated on a 15% polyacrylamide gel in 1X TBE. Following electrophoresis, miRNAs were transferred to nylon membrane using a semi-dry transfer and then UV cross-linked to the membrane using 120 mJ for 30 seconds. The probes for the detection of miRNAs and the 5s rRNA were synthesized using an *in vitro* transcription approach with [α-$^{32}$P]UTP. The templates for the *in vitro* transcription of *let-*

*7i* were: *let-7i* (5'-TGAGGTAGTAGTTTGTGCTGTCCTGTCTC-3'), and 5s rRNA (5'-GTTAGTACTTGGATGGGAGACCGCCCTGTCTC-3'). The membranes were incubated with 2.5 X $10^6$ cpm per blot overnight at 42°C. Subsequently, stringent washes were performed and the membranes were exposed to autoradiography film for 24-48 h.

**In situ *hybridization*** - Cells were grown on 4-well chamber slides and fixed with 4% formaldehyde/5% acetic acid for 15 min after a short washing with PBS. After treatment with pepsin (0.1% in 10 mM HCl) for 1 min, cells were dehydrated through 70%-, 90% and 100% ethanol. Treated cells were then hybridized with the fluorescent probes (10 μM) in probe dilution (EXIQON, Vedbaek, Denmark) for 30 min at 37°C followed by confocal microscopy (LSM 510, Carl Zeiss). FITC-tagged antisense probe specific to *let-7i* was obtained from Ambion. Fluorescent intensity in the cytoplasm of cells was measured and calculated with an analysis system of the LSM 510 provided by Carl Zeiss.

***Luciferase reporter constructs and luciferase Assay*** - Complementary 59 mer DNA oligonucleotides containing the putative let-7 target site within the 3'UTR region of human TLR4 mRNA were synthesized with flanking SpeI and HindIII restriction enzyme digestion sites (sense, 5'-GATACTAGTATCGGGCCCAAGAAAGTCATTTCAACTCTTACCTCATCAAGTAAGCTTACA -3'; antisense 5'-TGTAAGCTTACTTGATGAGGTAAGAGTTGAAATGACTTTCTTGGGCCCGATACTAGTATC). The sense and antisense strands of the oligonucleotides were annealed by adding 2 μg of each oligonucleotide to 46 μl of annealing solution (100 mM potassium acetate, 30 mM HEPES-KOH, pH 7.4 and 2 mM magnesium acetate) and incubated at 90°C for 5 min and then at 37°C for 1 h. The annealed oligonucleotides were digested with SpeI and HindIII and ligated into the multiple cloning site of the pMIR-REPORT Luciferase vector (Ambion, Inc.). In this vector, the post-transcriptional regulation of luciferase was potentially regulated by miRNA interactions with the TLR4 3'UTR. Another pMIR-REPORT Luciferase construct containing TLR4 mRNA 3'UTR with a mutant (ACCTCAT to ACCGAAT) at the putative seed region for let-7 binding was also generated as a control. We then transfected cultured cholangiocytes with each reporter construct, as well as let-7i antisense oligonucleotide or precursor, followed by assessment of luciferase activity 24 h after transfection. Luciferase activity was then measured and normalized to the expression of the control TK Renilla construct as previously reported (11).

***Quantitative RT-PCR*** - A quantitative RT-PCR approach (LightCycler) to measure *C. parvum* infection was established by modification of a previous report (11). Briefly, total RNA was harvested from the cells after exposure to *C. parvum* and reversed transcribed to cDNA and amplified using Amplitaq Gold PCR master mix (Roche). Primers specific for *C. parvum* 18s ribosomal RNA (forward: 5'-TAGAGATTGGAGGTTGTTCCT-3' and reverse: 5'-CTCCACCAACTAAGAACGGCC-3') were used to amplify the cDNA specific to the parasite. Primers specific for human plus *C. parvum* 18s (11) were used to determine total 18s cDNA. Data were expressed as copies of *C. parvum* 18s vs total 18s. Similarly, primers specific for human TLR4 (forward: 5'-TACTCACACCAGAGTTGCTTTCA-3' and reverse: 5'-AGTTGACACTGAGAGAGGTCCAG-3') and *let-7i* primary transcript (Pri-*let-7i*) (forward: 5'-CCTAGAAGGAATTGAGGGGAGT-3' and reverse: 5'-TGGCATTTAACTGCTGAAAGAA-3') were used to amplify the cDNA specific to TLR4 and Pri-*let-7i*. Data of quantitative RT-PCR analysis were expressed as copies of targets vs. 18s.

## RESULTS

### *Human cholangiocytes express let-7 family members, miRNAs that mediate TLR4 expression*

H69 cells, SV40-transformed human cholangiocytes derived from normal liver harvested for transplantation (10), were used to test the miRNA expression profile in human cholangiocytes. Employing a recently developed

semi-quantitative microarray approach that detects 385 human miRNAs provided and performed by Genosensor (Tempe, Arizona), we detected a distinct expression profile of miRNAs in our cells (Table 1). Using *in silico* computational target prediction analysis, we identified that of those miRNAs expressed in H69 cells, at least three of the *let-7* family, *let-7b*, *let-7i* and *let-7g*, have complementarity to TLR4 mRNA within the 3'UTR region (Fig. 1*A*). *let-7b* and *7g* are contained within known ESTs, *let-7i* is also potentially within an intron of an EST (DA092355). However, we were unable to amplify this EST from H69 cells (forward: 5'-GGTCACGTGGTGAGGAGTAGC-3' and reverse: 5'-CATTCTTGTCATATTGAAAATACGC-3'), while the EST was amplified from human cerebellum (data not shown). Additionally, *let-7i* has promoter elements immediately upstream of the precursor transcript (30), suggesting that *let-7i* expression is potentially regulated independent of EST DA092355. We therefore selected *let-7i* to test a potential role of miRNA-mediated post-transcriptional suppression in regulated TLR4 expression. To further confirm the expression of *let-7* in H69 cells, we used an antisense probe complementary to *let-7i* (Ambion) for Northern blot analysis. Using this technique, we cannot eliminate the possibility that other *let-7* family members are detected. However, the pattern of expression of the let-7 family can be discerned. *let-7* miRNAs were detected in our cells by Northern blot analysis, whereas a scrambled control probe showed no signal (Fig. 1*B*). To further assess the expression and intracellular distribution of *let-7* miRNAs in H69 cells, we used a fluorescein isothiocyanate (FITC)-tagged antisense oligonucleotide complementary to *let-7i* (Ambion) for *in situ* hybridization analysis followed by confocal microscopy. The antisense probe complementary to *let-7i* was visualized predominantly in the cytoplasm with smaller amounts in the nuclei of cells (Fig. 1*C*). Furthermore, cells transfected with a *let-7i* precursor (Ambion) showed a significantly increased *let-7* signal as assessed by both Northern blot analysis (Fig. 1*B*) and *in situ* hybridization (Fig. 1*D* and *F*). Cells transfected with a *let-7i* antisense 2-methoxy oligonucleotide (Ambion) showed a significant decrease of *let-7* signal by *in situ* hybridization (Fig. 1*E* and *F*) and a mild decrease by Northern blot (Fig. 1*B*).

Having established the approaches to manipulate intracellular *let-7* levels in cholangiocytes, we next tested whether alteration of *let-7* cellular levels affects TLR4 protein content in H69 cells. We transfected cells with the *let-7i* precursor or the *let-7i* antisense 2-methoxy oligonucleotide for 12 h and then measured TLR4 protein expression in cells by quantitative Western blotting. We found a dose-dependent increase of TLR4 protein content in cultured cholangiocytes after treatment with the *let-7i* antisense 2-methoxy oligonucleotide (Fig. 2*A*). In contrast, overexpression of *let-7i* with the precursor decreased TLR4 protein content in a dose-dependent manner (Fig. 2*A*). Since transfection of *let-7i* precursor and antisense 2-methoxy oligonucleotide is limited to a portion of the cell population, we tested whether alteration of TLR4 protein content occurs only in directly transfected cells. To accomplish this, the *let-7i* precursor or antisense 2-methoxy oligonucleotide was tagged with FITC using the *mir*Vana miRNA Probe Construction kit (Ambion) to label transfected cells. Expression of TLR4 in cultured cells was visualized by immunofluorescent microscopy. As shown in Fig. 2*B* and *C*, an increase of TLR4 protein (Fig. 2*C*, in red) was detected only in cells directly transfected with *let-7i* antisense 2-methoxy oligonucleotide (Fig. 2*B*, in green) compared with non-transfected cells. Quantitative analysis showed a significant increase of TLR4 specific fluorescence in cells transfected with *let-7i* antisense 2-methoxy oligonucleotide and a significant decrease of TLR4 specific fluorescence in cells treated with *let-7i* precursor compared with non-transfected control cells, respectively (Fig. 2*D*). Taken together, these data reveal that human cholangiocytes express multiple endogenous miRNAs, including members of the let-7 family, that have complementarity to TLR4 mRNA, and that modulation of at least one of these, *let-7i*, can mediate TLR4 protein expression in cholangiocytes *in vitro*.

***let-7i mediates TLR4 expression via translational suppression***

MicroRNAs mediate post-transcriptional suppression via either mRNA cleavage or

translational suppression. To test whether *let-7i* can induce cleavage of TLR4 mRNA, we measured the mRNA level of TLR4 in cultured cholangiocytes transfected with either *let-7i* antisense 2-methoxy oligonucleotide or *let-7i* precursor by quantitative RT-PCR. No significant difference of TLR4 mRNA was found in cells transfected with either *let-7i* antisense 2-methoxy oligonucleotide or *let-7i* precursor (Fig. 3*A*). To directly address whether *let-7i* binds to the 3'UTR region of TLR4 mRNA resulting in a translational suppression, we generated a pMIR-REPORT Luciferase construct containing TLR4 mRNA 3'UTR region with the putative *let-7* binding site (Fig. 3*B*). In addition, another pMIR-REPORT Luciferase construct containing TLR4 mRNA 3'UTR region with a mutation at the putative *let-7* binding site (ACCTCAT to ACCGAAT) was generated as a control (Fig. 3*B*). We then transfected cultured cholangiocytes with each reporter construct, as well as *let-7i* antisense 2-methoxy oligonucleotide or precursor, followed by assessment of luciferase activity 24 h after transfection. As shown in Fig. 3B, a significant decrease of luciferase activity was detected in cells transfected with the TLR4 3'UTR construct under the basal conditions (i.e., no antisense or precursor treatment). No change of luciferase was observed in cells transfected with the mutant TLR4 3'UTR construct. Importantly, *let-7i* precursor significantly decreased luciferase reporter translation and in contrast, *let-7i* antisense 2-methoxy oligonucleotide markedly increased luciferase reporter translation. A mutation in the binding sequence eliminated *let-7i* precursor induced decrease of reporter translation. Taken together, the above data suggest that the seed region for *let-7* binding within the TLR4 3'UTR is critical for TLR4 translational regulation in H69 cells. Furthermore, manipulation of cellular levels of let-7i results in alterations of TLR4 protein expression by suppressing translation via interactions with the 3'UTR region of TLR4 mRNA rather than mRNA cleavage.

***LPS stimulation and C. parvum infection decrease let-7i expression in cholangiocytes via a MyD88/NF-κB signaling-dependent mechanism***

Having demonstrated that *let-7i* mediates TLR4 translation in cholangiocytes, we then tested whether this regulation is of physiological or pathophysiological significance. Expression of TLR4 protein in epithelial cells is finely regulated and alterations of TLR4 expression have been reported in intestinal and airway epithelial cells following microbial infection (1, 31). Therefore, we measured *let-7i* expression in cholangiocytes upon LPS stimulation and following infection by *C. parvum*, a parasite that infects both intestinal and biliary epithelium. We previously demonstrated that *C. parvum* infection activates TLR signals in infected cholangiocytes in culture, including activation of the adaptor protein, MyD88, and nuclear translocation of NF-κB in directly infected cells (28). Here, a probe complementary to *let-7i* detected significantly less signal by northern blot analysis following LPS stimulation (Fig. 4*A*) or following *C. parvum* infection (Fig. 4*E*). However, given the sequence similarities between mature *let-7* transcripts, we could not definitively differentiate between family members. Therefore, to further demonstrate *let-7i* modulation following *C. parvum* infection and LPS stimulation, we performed quantitative RT-PCR for the primary transcript of *let-7i* using primers based on the 3' sequence of the primary *let-7i* transcript obtained from 3'-RACE (data not shown). A decrease of Pri-*let-7i* expression was confirmed in cells upon LPS stimulation (Fig. 4*B*) or following *C. parvum* infection (Fig. 4*F*). No change for miR-24, a randomly selected miRNA expressed in cholangiocytes (Table 1) as a control, was detected after infection (data not shown).

To test whether MyD88 or NF-κB are involved in the *C. parvum*-induced decrease of *let-7* expression in cholangiocytes, we measured expression of *let-7* in cells either stably transfected with a functionally deficient dominant negative (DN) mutant of MyD88 or treated with a specific NF-κB inhibitor, SN50, after exposure to *C. parvum*. Transfection of cells with MyD88-DN and inhibition of NF-κB with the inhibitor, SN50, partially inhibited the LPS or *C. parvum*-induced decrease of *let-7* expression (Fig. 4*A* and *E*). Decreased *let-7* expression in cholangiocytes upon LPS stimulation was further confirmed by *in situ* hybridization using a FITC-tagged probe

complementary to *let-7i* (Fig. 4*C*, in green). Transfection of cells with MyD88-DN and inhibition of NF-κB with SN50 inhibited the LPS-induced decrease of *let-7* expression (Fig. 4*C* and *D*).

Activation of MyD88/NF-κB signals in cholangiocytes following *C. parvum* infection is limited to directly infected cells (28). If *C. parvum* infection decreases *let-7* expression via activation of the MyD88/NF-kB signals, decrease of *let-7* expression should be limited to directly infected cells. Indeed, a decrease of *let-7* signal as visualized by *in situ* hybridization was detected only in cells directly infected by *C. parvum*, not in bystander non-infected cells (Fig. 4*G* and *H*). Transfection of cells with MyD88-DN and inhibition of NF-κB with SN50 inhibited the *C. parvum*-induced decrease of *let-7* expression (Fig. 4*G* and *H*). Equally important, these results exclude LPS contamination in our *C. parvum* sporozoite preparation since decreased expression of *let-7* induced by *C. parvum* is limited only to directly infected cells (Fig. 4*G*) while in the control experiment LPS decreases *let-7* expression in all cells (Fig. 4*C*). Taken together, the above data suggest that LPS and *C. parvum* infection cause decreased *let-7* expression in infected cholangiocytes via a MyD88/NF-κB dependent mechanism.

***Decrease of let-7i is involved in expression of TLR4 in cholangiocytes following C. parvum infection or LPS stimulation***

To test whether *let-7i*-mediated TLR4 expression occurs during microbial infection of cholangiocytes, we first measured TLR4 mRNA and protein expression in cells after exposure to *C. parvum* for 12 h. Expression of TLR4 mRNA was not different in sham-infected cells compared to *C. parvum* infected cells as assessed by both RT-PCR (Fig. 5*A*) and quantitative RT-PCR (Fig. 5*B*). In contrast, protein expression of TLR4 was significantly increased in cells exposed to *C. parvum* (Fig. 5*C*), and this effect was abrogated in cells stably transfected with MyD88-DN after exposure to *C. parvum* (Fig. 5*C*). To further clarify the relationship between *C. parvum* infection and TLR4 expression, we measured TLR4 expression in *C. parvum*-infected cells by immunofluorescent microscopy. Consistent with activation of MyD88/NF-κB signals in directly infected cells, increased expression of TLR4 (arrowheads in Fig. 5*D*) was found only in cells directly infected by the parasite (arrows in Fig. 5*D*) not in bystander non-infected cells.

To test the potential role of *let-7i* in *C. parvum*-induced upregulation of TLR4 protein in infected cells, we assessed the effects of experimentally manipulated *let-7i* cellular levels on *C. parvum*-induced TLR4 upregulation in cholangiocytes. Cells were first transfected with *let-7i* precursor or antisense 2-methoxy oligonucleotide, respectively, and then exposed to *C. parvum*. We found a significant increase of TLR4 protein content in infected cells compared with control cells (Fig. 5*E*). Cells treated with *let-7i* antisense 2-methoxy oligonucleotide showed a further increase of TLR4 protein content following *C. parvum* infection (Fig. 5*E*). In contrast, transfection of cells with the *let-7i* precursor diminished the *C. parvum*-induced increase of TLR4 protein (Fig. 5*E*). Similarly, a significant increase of TLR4 protein content was found in LPS-treated cells. Cells pre-treated with *let-7i* antisense 2-methoxy oligonucleotide showed a further increase of TLR4 protein content and transfection of cells with the *let-7i* precursor diminished the LPS-induced increase of TLR4 protein (data not shown).

***let-7i and associated TLR4 protein expression are involved in cholangiocyte immune responses against C. parvum infection***

To test directly whether *let-7i* and *let-7i*-associated TLR4 regulation are involved in cholangiocyte defense responses against *C. parvum* infection, we then assessed the number of parasites detected over time in cultured cholangiocytes transfected with *let-7i* precursor or antisense 2-methoxy oligonucleotide. Cells were first transfected with *let-7i* precursor or antisense 2-methoxy oligonucleotide and then exposed to *C. parvum*. After incubation with the same number of *C. parvum* sporozoites for 2 h to allow sufficient host-cell attachment and cellular invasion (28), cells were washed with culture medium to remove non-attached and non-internalized parasites. Cells were then harvested for RNA isolation to measure initial parasite host-cell attachment and invasion in cholangiocytes using a quantitative RT-PCR approach we

previously reported (11). Some cells were also further cultured for an additional 48 h to assess parasite propagation/survival in cells. We found that the parasite burden detected after initial exposure to *C. parvum* for 2 h were similar in all the cells, including those transfected with *let-7i* precursor or antisense oligonucleotide, suggesting that *let-7i* does not affect parasite initial host-cell attachment and cellular invasion (Fig. 6*A*). Consistent with our previous studies (11), a significant increase in parasite burden was found in MyD88-DN stably transfected cells 48 h after initial infection. Interestingly, we also detected a significantly higher parasite burden in *let-7i* precursor treated cells than in the control cells. In contrast, a significantly lower parasite burden was detected in *let-7i* antisense 2-methoxy oligonucleotide treated cells (Fig. 6*B*). An increased parasite burden was further confirmed by immunofluorescent microscopy in MyD88-DN stably transfected cells or cells transfected with the *let-7i* precursor (Fig. 6*C*). A decreased parasite burden was confirmed in *let-7i* antisense 2-methoxy oligonucleotide treated cells (Fig. 6*C*). These results suggest that the machinery for miRNA-mediated post-transcriptional gene regulation exists in cholangiocytes and is potentially involved in *C. parvum*-induced epithelial innate immune responses.

## DISCUSSION

The key findings in this report are: **i**) *let-7* family members are expressed in cholangiocytes and mediate TLR4 expression via translational regulation; **ii**) *C. parvum* infection and LPS stimulation decrease *let-7* expression in cholangiocytes via a MyD88/NF-κB dependent mechanism; **iii**) the *C. parvum*-induced decrease of *let-7* expression is associated with upregulation of TLR4 in cholangiocytes; and **iv**) experimentally-induced suppression or induction of *let-7i* causes reciprocal alterations in cholangiocyte immune response to *C. parvum* infection *in vitro*. These data suggest that *let-7* regulates *C. parvum*-induced upregulation of TLR4 in cholangiocytes and contributes to epithelial defense responses against *C. parvum*.

One of the important observations in our study is that *let-7i* regulates TLR4 expression via translational suppression in human cholangiocytes. Expression of *let-7* family members in cultured normal human cholangiocytes was evident by microarray, and Northern blot analyses, while specific detection of *let-7i* was evidenced by PCR. By *in situ* hybridization, *let-7* is seen in the nuclei and to a greater extent in the cytoplasm of cultured cholangiocytes, similar to the intracellular distribution of other miRNAs (32). Experimentally-induced suppression or induction of *let-7i* caused reciprocal alterations in TLR4 protein, respectively. Importantly, the change in TLR4 protein levels was limited to cells directly transfected with either the antisense or precursor. While no significant difference of TLR4 mRNA levels in cells transfected with either *let-7i* antisense or precursor was found, *let-7i* precursor significantly decreased luciferase reporter translation using a luciferase reporter plasmid containing the 3'UTR region of TLR4 mRNA with the putative *let-7* binding site. Additionally, *let-7i* antisense markedly increased luciferase reporter translation and a mutation in the binding sequence blocked *let-7i* precursor- induced decrease of luciferase reporter translation. Taken together, our data indicate that *let-7i* is expressed in cholangiocytes and regulates TLR4 expression via translational suppression. It is likely that other members of the *let-7* family, in particular, *let-7b* and *let-7g*, also regulate TLR4 expression in cholangiocytes, however the specific expression profiles, based on primary, precursor, and mature transcript expression, need further investigation.

Expression of TLRs by epithelia is tightly regulated to assure that an epithelium will recognize invading pathogens but not elicit an inappropriate immune response to endogenous ligands or commensal microorganisms (4). Upregulation of TLRs, such as TLR4, has recently been reported in intestinal and airway epithelial cells in response to microbial infection with *Helicobacter pylori* and *Salmonella enterica* (31,33). Here, we found that *C. parvum* infection decreases *let-7i* expression and more importantly, this process is involved in *C. parvum*-induced up-regulation of TLR4 in infected cholangiocytes. When cultured human cholangiocytes were exposed to *C. parvum*, we found a significant increase of TLR4 protein content in infected cells compared with control cells. A decrease of *let-7*

expression in infected cells was confirmed by multiple approaches including Northern blot and *in situ* hybridization analyses, as well as specific analysis of Pri-*let-7i* expression with PCR. Importantly, manipulation of *let-7i* levels significantly altered *C. parvum*-induced TLR4 expression. Cells treated with *let-7i* antisense oligonucleotide showed a further increase of TLR4 protein expression following *C. parvum* infection. In contrast, increasing *let-7i* cellular levels with the precursor reduced the *C. parvum*-induced increase of TLR4 protein. Furthermore, *let-7i* associated TLR4 expression appears to be involved in LPS-stimulated TLR4 expression in cholangiocytes. Expression of TLRs upon LPS stimulation differs in cell types (34,35). We found that at an early time point, LPS stimulation increases TLR4 protein level in cultured cholangiocytes. Manipulation of *let-7i* levels influences LPS-stimulated TLR4 expression. Thus, *let-7i* mediated post-transcriptional regulation may be involved in host-cell responses to microbial infection in general, including but not limited to *C. parvum*-biliary infection.

MicroRNAs have distinct expression patterns in different cell types, suggesting that cellular expression of miRNAs is tightly regulated (15-17). The role of transcription factors in miRNA expression is still unclear. c-Myc appears to be involved in the expression of the miR-17 cluster (18). It has been reported that C/EBPα regulates miR-223 during human granulocytic differentiation (19). More recently, the NF-κB signaling pathway has been implicated in the induction of miR-146 expression in human monocytes upon LPS stimulation (36). Here, we found that NF-κB may play a critical role in *C. parvum*-induced *let-7i* suppression in cholangiocytes. We previously demonstrated nuclear translocation of NF-κB components via TLR-mediated pathogen recognition in *C. parvum*-infected cells (11). Knockout of MyD88 or inhibition of NF-κB by specific inhibition partially blocked *C. parvum*-induced *let-7i* decrease, suggesting that TLR/NF-κB signals are involved in *C. parvum*-induced decreased expression of *let-7i*. This is further supported by the observation that a decrease of *let-7i* is limited to directly infected cells, consistent with the nuclear translocation of NF-κB in directly infected cells (28). Whereas in most cases, NF-κB binding to a DNA element drives transcription of associated genes, recent studies indicated that various genes are also down-regulated by NF-κB binding (37,38). Thus, TLR/NF-κB signals may be involved in *C. parvum*-induced *let-7i* suppression in cholangiocytes.

TLRs are key to epithelial innate immunity through detection of invading pathogens and subsequent activation of associated intracellular signaling pathways leading to the release of cytokines/chemokines and anti-microbial peptides (1,2). We previously demonstrated that TLR2 and TLR4 are involved in cholangiocyte immune response to *C. parvum* infection via activation of NF-κB and subsequent secretion of antimicrobial peptides, such as HBD2 (11). To determine whether *let-7i*-mediated TLR4 expression is involved in cholangiocyte immune responses against *C. parvum* infection, we tested *C. parvum* infection dynamics in cultured cholangiocytes transfected with either *let-7i* antisense oligonucleotide or precursor. No change of *C. parvum* initial host-cell attachment and cellular invasion was detected in both *let-7i* antisense and precursor transfected cells, suggesting that initial parasite attachment/invasion may not be associated with basal *let-7i* levels. In contrast, a significantly higher parasite burden was detected in cells transfected with *let-7i* precursor than in the control cells at 48-96 h after initial exposure to an equal number of parasites. A decreased parasite burden was detected in cells transfected with the *let-7i* antisense oligonucleotide. These data suggest that *let-7i* and associated TLR4 expression are involved in cholangiocyte immune responses against *C. parvum* infection.

In conclusion, our data indicate that human cholangiocytes express *let-7i*, a cellular miRNA that directly regulates TLR4 expression and contributes to epithelial immune responses against *C. parvum* infection. It will be of interest to extend these studies to other members of *let-7* family, as well as to determine the mechanisms by which TLR/NF-κB signals regulate *let-7i* miRNA expression and the role of miRNAs in epithelial innate immunity *in vivo*.

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## FOOTNOTES

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The abbreviations used are: miRNAs, microRNAs; mRNAs, messenger RNAs; TLRs, Toll-like receptors; HBD, human β-defensin; MyD88, myeloid differentiation protein 88; DAPI, 4', 6-diamidino-2-phenylindole; FITC, fluorescein isothiocyanate.

## FIGURE LEGENDS

TABLE 1. **Expression of miRNAs in H69 cells as assessed by microarray analysis**. H69 cells were grown to confluence and total RNAs were isolated for miRNA microarray analysis with the GenoExplorer™ microRNA Biochips by Genosensor. Data were expressed as the fluorescent intensity for each miRNA, representing the mean values from three independent analyses. Expression of 5s rRNA was used as the control.

FIGURE 1: **Complementarity of *let-7* family miRNAs expressed in cholangiocytes to the 3'UTR region of TLR4 mRNA and manipulation of *let-7i* expression in cultured human cholangiocytes**. (**A**) Complementarity of *let-7* family miRNAs detected in H69 cells to the 3'UTR region of TLR4 mRNA. Using *in silico* computational target prediction analysis, we identified that the expressed *let-7* family members, *let-7b*, *let-7i* and *let-7g*, have complementarity to the 3'UTR region of TLR4 mRNA. (**B** to **F**) Manipulation of *let-7i* expression in H69 cells with specific *let-7i* precursor or antisense oligonucleotide as assessed by Northern blot analysis (**B**) or by *in situ* hybridization (**C** to **F**). *let-7* miRNAs were detected in H69 cells by Northern blot analysis using a probe complementary to *let-7i* (**B**). The control probe showed no signal. Whereas cells treated with a *let-7i* precursor (Ambion) showed an increased signal indicating an increase of *let-7*, cells treated with a *let-7i* antisense 2-methoxy oligonucleotide (Ambion) showed a mild decrease of *let-7* signal (**B**). 5s rRNA was probed to confirm equal loading of mRNA. A FITC-tagged anti-sense oligonucleotide complementary to *let-7i* was used to visualize *let-7* miRNAs. The anti-sense probe was visualized predominantly in the cytoplasm with a limited detection in the nucleus (**C**). Furthermore, cells treated with the precursor showed an increased fluorescence (**D**), whereas cells treated with the antisense

oligonucleotide showed a significant decrease of fluorescent signal (**E**). (**F**) Quantitative analysis of fluorescent intensity of the FITC-tagged *let-7i* anti-sense oligonucleotide. A total of about 200 cells were randomly selected for each group and each data bar represents mean ± SD from three independent experiments. *, $P < 0.05$, ANOVA vs. basal non-transfected control cells (Basal). Bars = 5 μm.

FIGURE 2: ***let-7i* mediates TLR4 expression in cultured cholangiocytes**. (**A**) Effects of *let-7i* on TLR4 protein expression by Western analysis. H69 cells were transfected with a *let-7i* precursor or *let-7i* antisense 2-methoxy oligonucleotide for 12 h followed by Western blot for TLR4. A dose-dependent increase of TLR4 protein content was detected after treatment with *let-7i* antisense oligonucleotide. In contrast, overexpression of *let-7i* with the *let-7i* precursor decreased TLR4 protein content in a dose-dependent manner. (**B** and **C**) TLR4 expression in cells transfected with a *let-7i* antisense oligonucleotide as assessed by immunofluorescence. H69 cells were transfected with a FITC-conjugated antisense oligonucleotide complementary to *let-7i* for 12 h followed by immunofluorescent staining for TLR4. An increased expression of TLR4 protein (in red, **C**) was detected in directly transfected cells (arrows, in green, **B**) compared with non-transfected cells. (**D**) Effects of *let-7i* on TLR4 protein expression by quantitative fluorescent analysis. H69 cells were transfected with either *let-7i* precursor or *let-7i* antisense oligonucleotide for 12 h followed by quantitative analysis of immunofluorescent signals for TLR4. A total of about 200 cells were randomly selected for each group and each data bar represents mean ± SD from three independent experiments. *, $P < 0.05$, ANOVA vs. with non-transfected control cells (Basal). Bars = 5 μm.

FIGURE 3: ***let-7i* mediates TLR4 protein expression via post-transcriptional suppression.** (**A**) Effects of *let-7i* on TLR4 mRNA content. H69 cells were transfected with either *let-7i* precursor or *let-7i* antisense oligonucleotide for 12 h followed by quantitative RT-PCR for TLR4 mRNA. Data were normalized to the 18s rRNA level and expressed as copies of TLR4 mRNA/$10^6$ copies 18s rRNA. (**B**) Targeting of *let-7i* to the 3'UTR region of TLR4 mRNA. A reporter construct with the potential binding site for *let-7* in the 3'UTR region of TLR4 was generated. H69 cells were transiently co-transfected for 24 h with the reporter construct and either *let-7i* antisense oligonucleotide or *let-7i* precursor. Luciferase activities were measured and normalized to the control TK *Renilla* luciferase level. Bars represent the mean ± SD from three independent experiments. *, $P < 0.05$, ANOVA vs. cells transfected only with the reporter construct (3'UTR Ctrl); #, $P < 0.05$, ANOVA vs. cells transfected with the reporter construct plus *let-7i* precursor (3'UTR + *let-7i* precursor).

FIGURE 4: **LPS stimulation and *C. parvum* infection decrease *let-7i* expression in cholangiocytes in a NF-kB dependent manner**. (**A** to **H**) Expression of *let-7i* in cholangiocytes after treatment with LPS (**A** to **D**) or infection by *C. parvum* (**E** to **H**). H69 cells, as well as cells stably transfected with a MyD88 functionally-deficient dominant negative mutant construct (MyD88-DN) or an empty control vector, were exposed to LPS (100 ng/ml) for 4 h or *C. parvum* for 12 h followed by Northern blot (**A** and **E**), quantitative RT-PCR (**B** and **F**) or *in situ* hybridization (**C** and **G**) analysis for *let-7i*. For Northern blot analysis, 5s rRNA was blotted to confirm that equal amount of total RNA was used. *let-7* signals, detected with the *let-7i* antisense probe, from three independent experiments were measured using a densitometric analysis and expressed as the ratio to 5s rRNA (**A** and **E**). Quantitative RT-PCR analysis was performed with specific primers to *let-7i* primary transcript and expressed as copies/18s rRNA (**B** and **F**). For *in situ* hybridization analysis, an FITC-tagged anti-sense probe complementary to *let-7i* (Ambion) was used to detect *let-7* family miRNAs. Cells were also stained with DAPI to label the nuclei in blue. Representative confocal images are shown in **C** and **G**. *C. parvum* was stained red using a specific antibody (arrowheads in **G**). **D** and **H** are quantitative analyses of *let- 7* expression detected with the fluorescently tagged anti-sense

oligonucleotide complementary to *let-7i* in the cytoplasm of cultured cells by *in situ* hybridization after treatment with LPS (**D**) or infection by *C. parvum* (**H**), respectively. A total of about 200 cells were randomly selected for each group and each data bar represents mean ± SD from three independent experiments *, $P < 0.05$, ANOVA vs. no-LPS treated control cells (Ctrl, in **A**, **B** and **D**) or sham infected cells (Sham Inf. Ctrl, in **E**, **F** and **H**). Bars = 5 μm.

FIGURE 5: ***let-7i* is involved in *C. parvum*-induced upregulation of TLR4 in cholangiocytes.** (**A** and **B**) TLR4 mRNA expression in cells after exposure to *C. parvum* for 12 h by RT-PCR (**A**) and quantitative RT-PCR (**B**). No significant difference in TLR4 mRNA levels was detected between the sham-infected cells and cells exposed to *C. parvum.* (**C**) Upregulation of TLR4 protein in cells following *C. parvum* infection. Whereas a low expression of TLR4 protein was detected in control sham-infected cells, a significant increase of TLR4 protein was found in cells exposed to the parasite. A decreased expression of TLR4 was found in cells transfected with MyD88-DN after exposure to *C. parvum*. (**D**) Upregulation of TLR4 protein in directly infected cells by immunofluorescent microscopy. TLR4 was stained in green, *C. parvum* in red and the nuclei in blue. Increased expression of TLR4 protein (arrowheads) was found only in cells directly infected by the parasite (arrows), not in bystander non-infected cells. (**E**) Effects of manipulation of *let-7i* levels on *C. parvum*-induced TLR4 upregulation in cholangiocytes as assessed by Western blot and quantitative densitometric analysis. Cells treated with *let-7i* antisense 2-methoxy oligonucleotide showed a further increase of TLR4 protein content following *C. parvum* infection compared with infection control cells. In contrast, transfection of cells with the *let-7i* precursor diminished the *C. parvum*-induced increase of TLR4 protein. *, $P < 0.05$ compared with sham infected cells (Sham inf. Ctrl). **, $P < 0.05$, ANOVA vs. with cells transfected with the empty vector (**C**) or cells without oligonucleotide or precursor treatment (**E**). Bars = 5 μm.

FIGURE 6: ***let-7i* is involved in cholangiocyte immune responses against *C. parvum* infection *in vitro*.** (**A**) Effects of manipulation of *let-7i* levels on *C. parvum* attachment and invasion of cholangiocytes *in vitro* as assessed by quantitative RT-PCR. H69 cells stably transfected with a control empty vector or MyD88-DN construct, as well as *let-7i* antisense or precursor, were exposed to an equal number of *C. parvum* for 2 h followed by extensive washing and continued culture. A similar number of parasites was detected as assessed by quantitative RT-PCR in all the cells, including those transfected with *let-7i* precursor or antisense oligonucleotide, after initial exposure to *C. parvum* for 2 h. *, $P < 0.05$, ANOVA vs. cells transfected with an empty vector control. (**B**) Effects of manipulation of *let-7i* levels on *C. parvum* burden in cholangiocytes *in vitro* 48 h after initial exposure to the parasite by quantitative RT-PCR. A significant increase in parasite number was found in MyD88-DN stably transfected cells and cells transfected with the *let-7i* precursor 48 h after initial infection. In contrast, a significantly lower number of parasites were detected in *let-7i* antisense 2-methoxy oligonucleotide treated cells. (**C**) Effects of manipulation of *let-7i* levels on *C. parvum* burden in cholangiocytes *in vitro* 48 h after initial exposure to the parasite as assessed by immunofluorescent microscopy. *C. parvum* parasites were stained in red and nuclei in blue. Bars = 5 μm.

**Table 1. MicroRNAs detected in H69 cells by microarray analysis**

| miRNAs | Fluorescent intensity | miRNAs | Fluorescent intensity |
|---|---|---|---|
| miR-001 | 730 ± 120 | miR-199a | 152 ± 2.7 |
| miR-009 | 140 ± 9.1 | miR-214 | 864 ± 16 |
| miR-15a | 218 ± 8.7 | miR-216 | 163 ± 2.0 |
| miR-15b | 188 ± 7.0 | miR-296 | 169 ± 4.5 |
| miR-24 | 230 ± 2.1 | miR-299 | 171 ± 5.5 |
| miR-27b | 232 ± 1.7 | miR-302a | 186 ± 10 |
| miR-29a | 178 ± 3.6 | miR-302d | 164 ± 5.6 |
| miR-29b | 146 ± 3.6 | miR-324-3p | 150 ± 1.7 |
| miR-93 | 141 ± 3.6 | miR-337 | 155 ± 3.6 |
| miR-100 | 211 ± 2.3 | miR-338 | 251 ± 9.6 |
| miR-103 | 151 ± 1.5 | miR-339 | 182 ± 10 |
| miR-122a | 158 ± 3.2 | miR-340 | 172 ± 8.0 |
| miR-124a | 710 ± 230 | miR-342 | 164 ± 7.0 |
| miR-125a | 193 ± 4.6 | miR-368 | 162 ± 11 |
| miR-125b | 3490 ± 120 | miR-370 | 175 ± 1.2 |
| miR-128a | 330 ± 40 | miR-371 | 168 ± 7.4 |
| miR-130a | 165 ± 9.8 | miR-372 | 298 ± 19 |
| miR-130b | 202 ± 7.5 | miR-373 | 322 ± 18 |
| miR-133a | 1880 ± 120 | miR-373* | 205 ± 21 |
| miR-149 | 181 ± 11 | miR-374 | 720 ± 80 |
| miR-154 | 161 ± 4.0 | *let-7b* | 388 ± 8.5 |
| miR-181b | 174 ± 2.1 | *let-7i* | 161 ± 11 |
| miR-194 | 153 ± 2.5 | *let-7g* | 172 ± 4.0 |
| miR-197 | 174 ± 4.6 | rRNA-5s | 210 ± 30 |

Figure 1

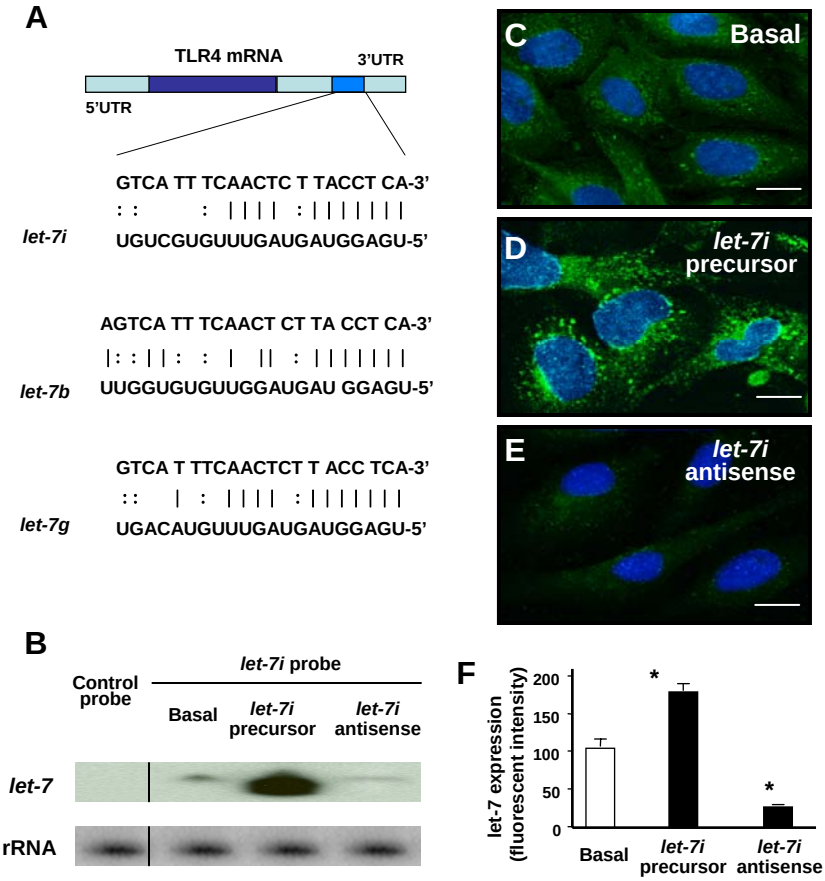

**Figure 2**

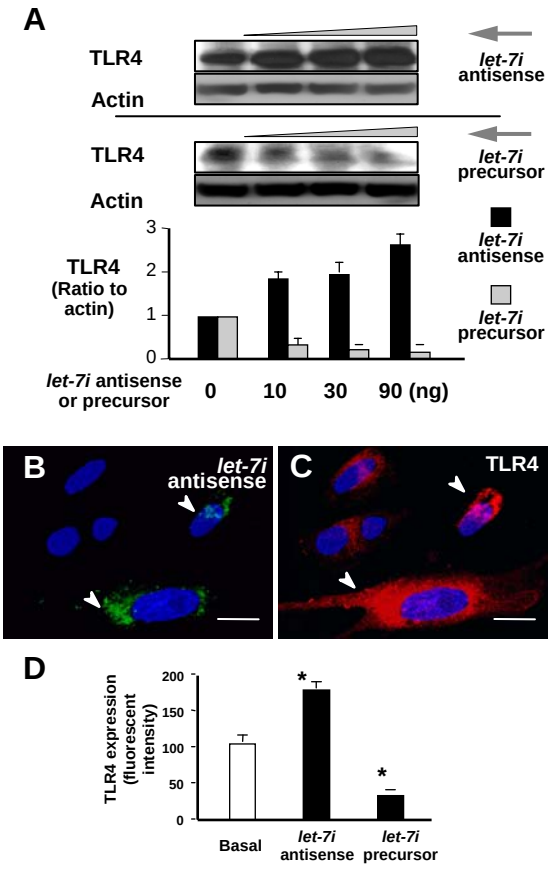

**Figure 3**

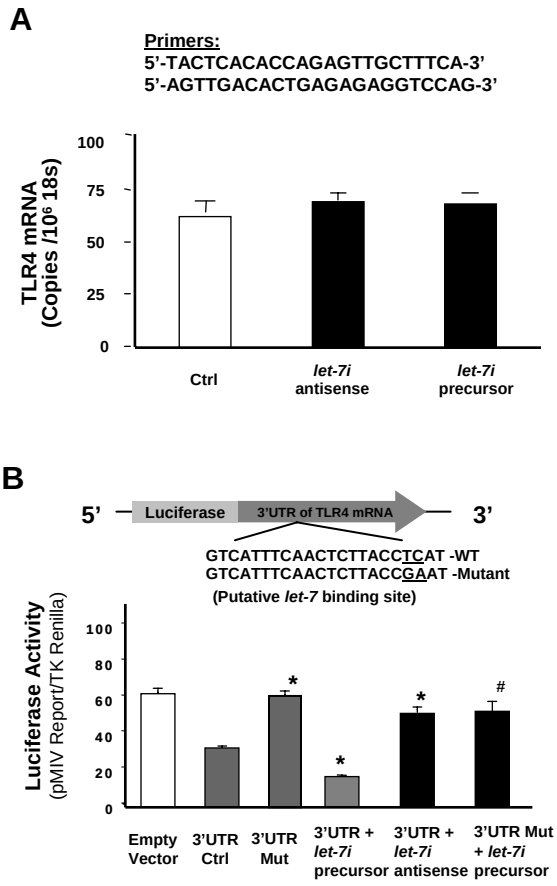

Figure 4

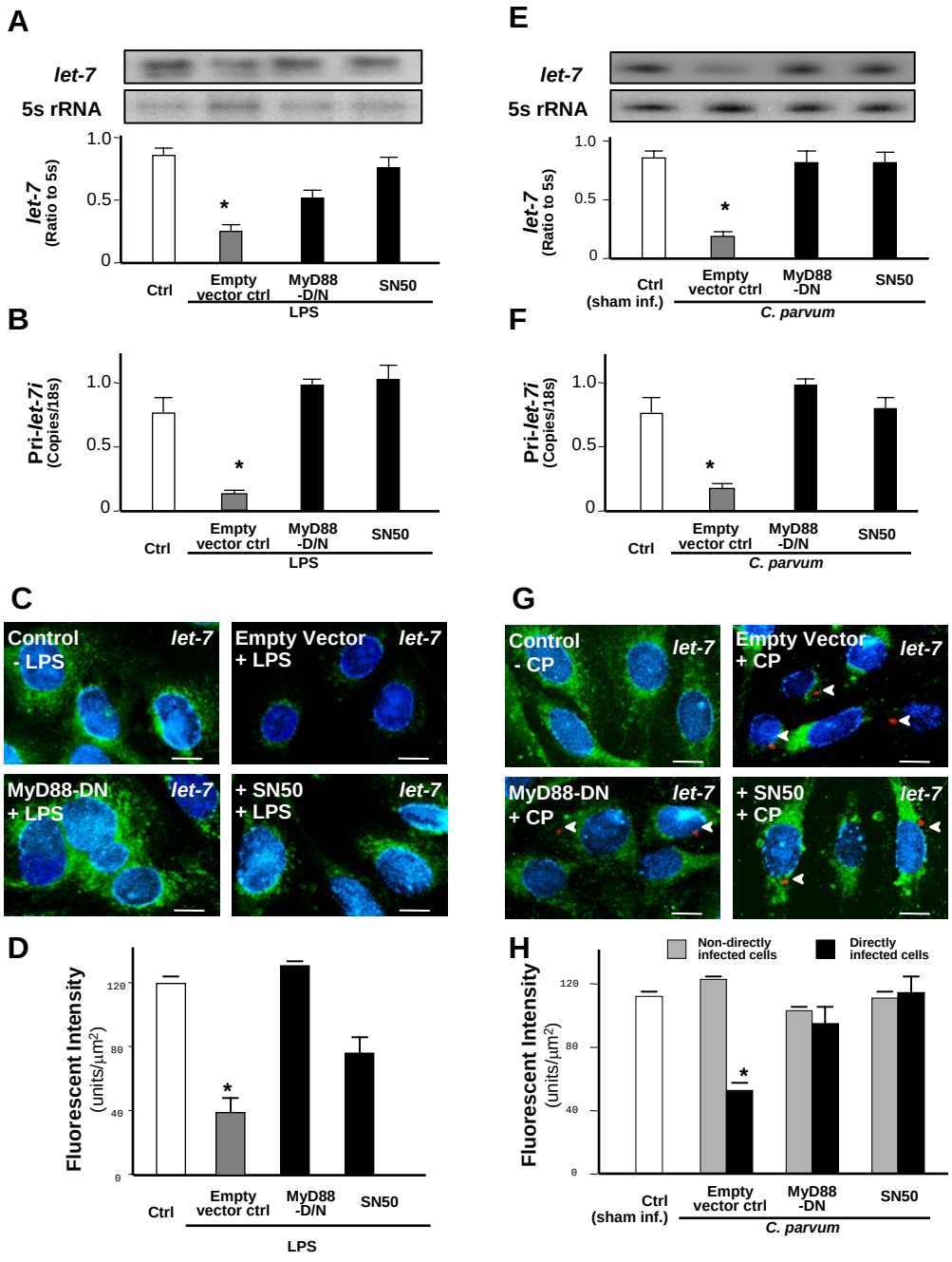

Figure 5

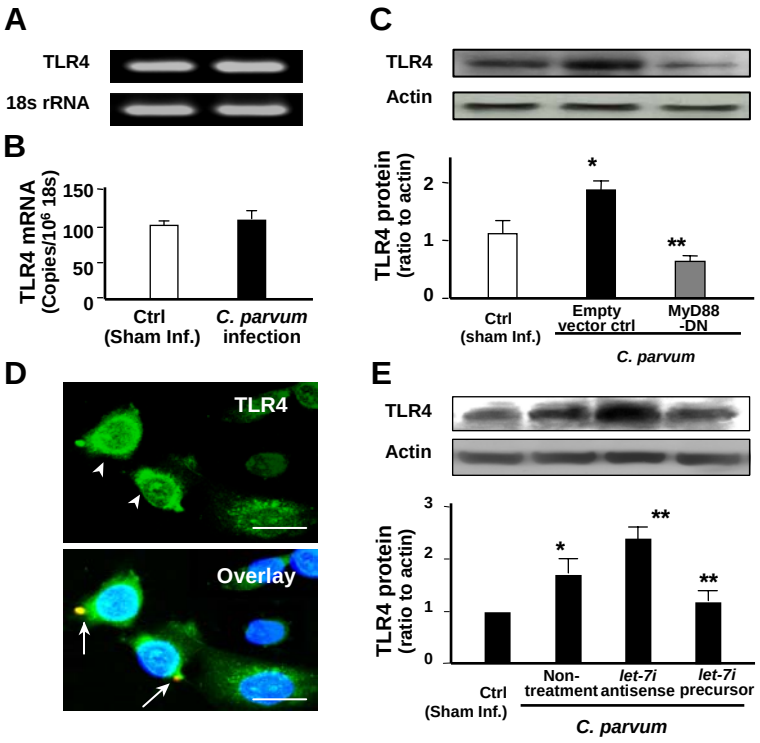

**Figure 6**