Release of Luminal Exosomes Contributes to TLR4-Mediated Epithelial Antimicrobial Defense

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Abstract

Exosomes are membranous nanovesicles released by most cell types from multi-vesicular endosomes. They are speculated to transfer molecules to neighboring or distant cells and modulate many physiological and pathological procedures. Exosomes released from the gastrointestinal epithelium to the basolateral side have been implicated in antigen presentation. Here, we report that luminal release of exosomes from the biliary and intestinal epithelium is increased following infection by the protozoan parasite Cryptosporidium parvum. Release of exosomes involves activation of TLR4/IKK2 signaling through promoting the SNAP23-associated vesicular exocytotic process. Downregulation of let-7 family miRNAs by activation of TLR4 signaling increases SNAP23 expression, coordinating exosome release in response to C. parvum infection. Intriguingly, exosomes carry antimicrobial peptides of epithelial cell origin, including cathelicidin-37 and beta-defensin 2. Activation of TLR4 signaling enhances exosomal shuttle of epithelial antimicrobial peptides. Exposure of C. parvum sporozoites to released exosomes decreases their viability and infectivity both in vitro and ex vivo. Direct binding to the C. parvum sporozoite surface is required for the anti-C. parvum activity of released exosomes. Biliary epithelial cells also increase exosomal release and display exosome-associated anti-C. parvum activity following LPS stimulation. Our data indicate that TLR4 signaling regulates luminal exosome release and shuttling of antimicrobial peptides from the gastrointestinal epithelium, revealing a new arm of mucosal immunity relevant to antimicrobial defense.

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Introduction

Eukaryotic cells release membrane vesicles into their extracellular environment under physiological and pathological conditions [1]. These vesicles mediate the secretion of a wide variety of proteins, lipids, mRNAs, and microRNAs (miRNAs), interact with neighboring cells, and thereby traffic molecules from the cytoplasm and membranes of one cell to other cells or extracellular spaces [1,2]. There is increasing evidence that secreted vesicles play an important role in normal physiological processes, development, and viral infection and other human disease [3–6]. Exosomes represent a specific subtype of secreted membrane vesicles that are around 30–100 nm in size, formed inside the secreting cells in endosomal compartments called multi-vesicular bodies (MVBs) [2]. Exosomes are produced by a variety of cells (e.g., reticulocytes, epithelial cells, neurons, tumor cells) and have been found in bronchoalveolar lavage, urine, serum, bile, and breast milk [2,7,8]. The composition of exosomes is heterogenic, depending on the cellular origin of the exosome. Exosomes do not contain a random array of intracellular proteins, but a specific set of protein families arising from the plasma membrane, the endocytic pathway, and the cytosol, especially those of endosomal origin, such as CD63, ICAM-1, and MHC molecules [2,9–13].

Secretion of exosomes is regulated by various stimuli, including the activation of P2X receptor by ATP on monocytes and neutrophils, thrombin receptor on platelets, and Toll-like receptor (TLR) 4 by LPS on dendritic cells [2,14,15]. Formation of intraluminal vesicles of MVBs and targeting of transmembrane proteins to these vesicles involve a complex intracellular sorting network, including the endosomal sorting complex required for transport (ESCRT) machinery [2,15]. Fusion of MVBs with plasma membrane is an exocytotic process that requires the association of v-SNAREs (from the vesicles) and t-SNAREs (at the membrane) to form a ternary SNARE (SNAP receptor) complex. The SNARE complex brings the two membranes in apposition, a necessary step in overcoming the energy barrier required for membrane fusion [16]. Several Rab family proteins, including Rab11 and Rab27b, are key regulators of the exosome secretion pathway and are involved in MVB docking at the plasma membrane [17].

Epithelial cells along the mucosal surface provide the first line of defense against luminal pathogen infection in the
Exosomes are secreted membranous nanovesicles produced by a variety of cells. Exosomes shuttle various molecules to transfer them to neighboring or distant cells, and have been implicated as mediators in cell-cell communications to modulate physiological and pathological procedures. Here, we report that luminal release of exosomal vesicles is an important component of Toll-like receptor 4 (TLR4)-associated gastrointestinal epithelial defense against infection by Cryptosporidium parvum, an obligate intracellular protozoan that infects gastrointestinal epithelial cells. Activation of TLR4 signaling in host epithelial cells following C. parvum infection promotes luminal release of epithelial exosomes and exosomal shuttling of antimicrobial peptides from the epithelium. By direct binding to the C. parvum surface, exosomal vesicles reveal anti-C. parvum activity. Activation of TLR4 signaling in epithelial cells after LPS stimulation also increases exosomal release and exosome-associated anti-C. parvum activity. Therefore, we speculate that TLR4-mediated exosome release may be relevant to innate mucosal immunity in general, representing a new target for therapeutic intervention for infectious diseases at the mucosal surface.

**Author Summary**

Exosomes are secreted membranous nanovesicles produced by a variety of cells. Exosomes shuttle various molecules to transfer them to neighboring or distant cells, and have been implicated as mediators in cell-cell communications to modulate physiological and pathological procedures. Here, we report that luminal release of exosomal vesicles is an important component of Toll-like receptor 4 (TLR4)-associated gastrointestinal epithelial defense against infection by Cryptosporidium parvum, an obligate intracellular protozoan that infects gastrointestinal epithelial cells. Activation of TLR4 signaling in host epithelial cells following C. parvum infection promotes luminal release of epithelial exosomes and exosomal shuttling of antimicrobial peptides from the epithelium. By direct binding to the C. parvum surface, exosomal vesicles reveal anti-C. parvum activity. Activation of TLR4 signaling in epithelial cells after LPS stimulation also increases exosomal release and exosome-associated anti-C. parvum activity. Therefore, we speculate that TLR4-mediated exosome release may be relevant to innate mucosal immunity in general, representing a new target for therapeutic intervention for infectious diseases at the mucosal surface.

C. parvum infection stimulates host epithelial monolayers to release apical exosomes through activation of TLR4 signaling, with the involvement of activation of the IKK2/SNAP23 exocytic sorting and let-7 miRNA-mediated gene regulation. Released exosomes shuttle several antimicrobial peptides, can bind to the C. parvum sporozoite surface, and display anti-C. parvum activity in vitro and ex vivo. Our results demonstrate, for the first time, that luminal release of exosomes is an important component of TLR4-associated epithelial immune reactions against C. parvum infection.

**Results**

C. parvum Infection Increases Luminal Release of Exosomes from the Host Epithelium through Activating the TLR4/IKK2 Signaling Pathway

Non-malignant human biliary epithelial cells, H69 cells, were grown to confluence on Percoll inserts to form monolayers; we detected a few exosome-like microvesicles in the apical side under electron microscopy (EM) (Figure 1A). These microvesicles were cup-shaped, with a diameter size about 40–100 nm, typical for exosomes. When H69 monolayers were exposed to infection by C. parvum, abundant exosome-like microvesicles were found in the apical region by the epithelium (Figure 1A). These microvesicles were morphologically similar to the exosome-like microvesicles found in non-infected monolayers. Interestingly, MVs were detected in the cytoplasm of infected cells. These MVs usually contained several exosome-like microvesicles that were morphologically similar to those released to the apical region (Figure 1A). Using a well-established ultracentrifugation approach [29], we isolated and purified these exosome-like microvesicles from the apical supernatants of the H69 monolayers 24 h after exposure to C. parvum infection. These purified microvesicles displayed cup-shape vesicular characteristics of exosomes, with a diameter size of 40–100 nm under scanning EM. Immunogold staining revealed that these exosome-like microvesicles were positive for exosome markers CD63 and ICAM-1 (Figure 1A), and, thus, they were subsequently referred to as exosomes. Interestingly, these apical exosomes released from H69 monolayers following infection were positive for MHC I and MHC II (Figure S1). We then quantified the release of apical exosomes from H69 monolayers at various time points after exposure to C. parvum. A time-dependent increase of apical exosomes was identified using multiple approaches, including Nanoparticle Tracking Analysis (NTA) [30,31] (Figure 1B and Figure S1), EM (Figure 1B), and Western blot for CD63 (Figure S1).

Having determined the increment of apical exosome release from cell monolayers following C. parvum infection, we then tested the potential underlying mechanisms. Previous studies demonstrated that several intracellular signaling pathways are activated in host epithelial cells following infection, including the TLR4/IKK/IκBα and PI-3K pathways [28,32]. Transfection of epithelial cells with the TLR4-dominant mutant (TLR4-DN) or treatment of cells with the IκK2 inhibitor SC-514 [33] significantly inhibited C. parvum-induced exosome release (Figure 1C). In contrast, an inhibitor of PI-3K showed no effects. Similarly, exposure of H69 monolayers to LPS (a potent TLR4 ligand) increased release of apical exosomes (Figure S1). Release of apical exosomes induced by LPS stimulation or C. parvum infection was also noted in cultured mouse biliary epithelial monolayers (603B monolayers) and intestinal epithelial monolayers (IEC4.1 monolayers) (Figure S1).

To test C. parvum-induced exosome release in vivo, we applied a mouse model of biliary cryptosporidiosis through gallbladder injection of C. parvum oocysts into wild-type and TLR4-deficient...
mice [34]. Consistent with our previous results [35], a higher biliary infection burden was found in TLR4-deficient mice compared with the wild-type animals (Figure 2A). Intriguingly, abundant exosomes were detected in the lumen of the biliary tract from the wild-type animals following infection (Figure 2B and 2C). Only a few exosomes were detected in the biliary lumen in the infected TLR4-deficient mice. Taken together, these data suggest that *C. parvum* infection increases luminal release of exosomes from the biliary epithelium, probably through TLR4/IKK2-mediated activation of the MVB exocytotic pathway.

**C. parvum**-induced Release of Apical Exosomes from Biliary Epithelial Cells Involves Rab- and SNAP23-mediated MVB Exocytosis

To test the contribution of MVB-associated exocytosis in *C. parvum*-induced apical exosome release, we performed experiments using confocal analysis on *C. parvum*-infected H69 cells. Using CD63 as the marker for MVBs [2], we detected an increase of MVBs in the cytoplasm of infected cells following infection (Figure 3A). The CD63-positive staining showed patchy distribution in the cytoplasm, with a diameter from 300 to 1,000 nm, consistent with characteristics of MVBs in cells as shown by EM in Figure 1A. In addition, these CD63-positive vesicles in the cytoplasm were overlaying with exosomes labeled with N-(Lissamine) rhodamine B sulfonyl diocoyphosphatidylethanolamine [N-Rh-PE] [36] (Figure 3B), suggesting that they were MVBs. Immunostaining revealed positive reactions to Rab11, Rab27b, and SNAP23 co-localized to the CD63-positive MVBs (Figure 3C–3E). Of note, accumulation of SNAP23 was obviously around the MVBs in infected cells (Figure 3E), which was further confirmed in cells overexpressing SNAP23 (Figure S2). Interestingly, *C. parvum*-induced SNAP23 localization around MVBs was not detected in H69 cells stably expressing TLR4-DN (Figure 3F).

To further investigate the role of SNAP23 in *C. parvum*-induced exosome release, H69 cells were first treated with an siRNA to SNAP23, followed by exposure to *C. parvum* infection for 24 h. SNAP23 siRNA treatment resulted in a significant decrease in exosome release from infected H69 monolayers (Figure 4A). H69 cells were infected by *C. parvum* for various time points, followed by measurement of total SNAP23 by Western blot and phosphorylated SNAP23 by immunoprecipitation (IP). Increased levels of total SNAP23 (Figure 4B) and phosphorylated SNAP23 (Figure 4C) were detected in infected cells. *C. parvum*-induced SNAP23 expression and phosphorylation were not detected in H69 cells stably expressing TLR4-DN (Figure 4F). Induction of SNAP23 total protein was detected in mouse biliary epithelial...
603B cells infected by C. parvum (Figure S3). Upregulation of SNAP23 and enhanced phosphorylation of SNAP23 were also detected in H69 cells following LPS stimulation (Figure S3). Previous studies demonstrated that SNAP23 is a substrate for IKK2, and phosphorylation of SNAP23 at Ser120 and Ser95 by IKK2 stimulates fusion of intracellular vesicles to the cell membrane, promoting exocytosis [37]. Indeed, a direct association between IKK2 and SNAP23 was evident from IP analysis (Figure 4D). Increased interaction between IKK2 and SNAP23 was detected in infected cells (Figure 4D). Such observations, however, do not prove that an IKK2-SNAP23 association is occurring in MVBs or exosomes or that it is just relevant to these

Figure 2. TLR4-dependent luminal release of exosomes during C. parvum biliary infection in mice. (A) and (B) Luminal release of biliary exosomes during biliary cryptosporidiosis in the wild type and TLR4-deficient mice. C. parvum oocysts were injected into the gallbladder of wild-type or TLR4-deficient mice, and liver tissues were collected one week post-injection. Quantitative analysis detected a higher infection burden and a lower amount of luminal exosome content in TLR4-deficient mice. (C) Abundant exosome-like microvesicles were observed in the luminal region of the wild-type mice one week post-infection, compared with that in the TLR4-deficient mice by transmission EM (b and d are higher magnifications in a and c, respectively). Scale bars = 1 μm; * p<0.05 ANOVA versus the wild-type.

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Figure 3. Colocalization of Rab11, Rab27, and SNAP23 to MVBs in epithelial cells following C. parvum infection. (A) and (B) Visualization of MVBs by CD63 staining and N-Rh-PE labeling. H69 cells were exposed to C. parvum infection for 12 h and stained with CD63 antibody. CD63 staining showed typical patchy intracellular distribution of MVBs in infected cells (arrows in A). Pre-incubation of cells with N-Rh-PE to label MVBs followed by C. parvum infection and CD63 staining reveal overlay of CD63 staining with the labeling of newly-formed MVBs (arrows in B). (C) and (D) Co-localization of Rab11 and Rab27b with MVBs in infected cells (indicated by arrows). H69 cells were exposed to C. parvum infection for 12 h and co-stained with antibodies to CD63 and Rab11 or Rab27b. (E) and (F) TLR4-dependent co-localization of SNAP23 to MVBs in infected cells. Cells were exposed to C. parvum for 12 h and co-stained with antibodies to CD63 and SNAP23. C. parvum-induced co-localization of SNAP23 to the MVBs was detected in H69 cells (arrows in E), but not in cells stably expressing TLR4-DN (F). Scale bars = 5 μm.

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A decreased luciferase activity was detected in cells transfected with SNAP23 or SNAP23 siRNA, followed by exposure to *C. parvum* infection for 24 h. Exosomes released into the apical supernatants were isolated and quantified.

miRNAs of the *let-7* Family Target SNAP23, and Downregulation of *let-7* miRNAs Is Associated with *C. parvum*-induced Exosome Release from Epithelial Cells

miRNAs, such as the *let-7* family members, have been implicated in the regulated exocytotic process [38–40]. Interestingly, several members of the *let-7* miRNAs, including *let-7i*, *let-7d*, *let-7f*, *let-7e*, and *let-7g*, showed significant downregulation in H69 cells following *C. parvum* infection by array analysis (Figure 5A), consistent with our previous studies on H69 cells [27,28]. Decreased expression of *let-7i* and *let-7g* was further confirmed by Northern blot and real-time PCR analysis in H69 and 603B cells following *C. parvum* infection (Figure 5A). Consistent with results from our previous studies on LPS-suppressed expression of *let-7* miRNAs in epithelial cells [41], we detected a decreased expression level of *miR-98* in H69 cells following LPS stimulation (Figure S3). Using *in silico* database analysis, we found that there is conserved complementarity between SNAP23 3’UTR and miRNAs of the *let-7* family (Figure 5A). To test the potential targeting of SNAP23 mRNA by *let-7* miRNAs, we generated pMIR-REPORT luciferase constructs containing the SNAP23 3’UTR with the putative *let-7* binding site (Figure 5B). In addition, constructs with the CCGC to GGAG for human and ACCT to TGGA for mouse mutation at the putative binding sites were also generated as controls. We then transfected H69 cells with these reporter constructs, followed by assessment of luciferase activity 24 h after transfection. As shown in Figure 5C, a significant decrease of luciferase activity was detected in cells transfected with the SNAP23 3’UTR construct containing the potential binding site compared with mutant control vector. No change in luciferase activity was observed in cells transfected with the mutant SNAP23 3’UTR construct, suggesting endogenous translational repression of the construct with the SNAP23 3’UTR. Anti-miRs (anti-miR miRNA inhibitors) and miRNA precursors are chemically modified RNA molecules designed to specifically inhibit and mimic, respectively, endogenous miRNAs [42]. Accordingly, anti-*let-7i* markedly increased SNAP23 3’UTR-associated luciferase reporter translation (Figure 5C). In contrast, the miR-98 precursor significantly decreased the luciferase activity (Figure 5C). Of note, anti-*let-7i* and miR-98 precursor only caused a modest alteration in the luciferase activity, probably due to the targeting of SNAP23 3’UTR by other members of the *let-7* miRNA family in the cells. Similar results were also confirmed in 603B cells (Figure 5C).

To test whether miRNA-mediated suppression of SNAP23 is directly relevant to SNAP23 expression in biliary epithelial cells, we treated H69 cells with miR-98 precursor or anti-*let-7i* and then measured SNAP23 protein level (treated for 48 h) by Western blot or mRNA level (treated for 24 h) by real-time PCR. Treatment of cells with the anti-*let-7i* caused a significant increase in SNAP23 protein content (Figure 6A). Conversely, a decrease in SNAP23 protein level was detected in cells after treatment with the miR-98 precursor (Figure 6A). No significant changes in SNAP23 mRNA level were detected in cells after treatment with miR-98 precursor or anti-*let-7i* (Figure 6A). No significant changes in the phosphorylated SNAP23 level were detected in cells after treatment with the anti-*let-7i* (Figure 6B). These data suggest that *let-7* family miRNAs target SNAP23 3’UTR, resulting in posttranscriptional suppression through translation suppression.

To test the impact of *let-7* miRNAs on *C. parvum*-induced SNAP23 upregulation in H69 cells, we treated cells with the...
mir-98 precursor for 48 h and then exposed them to C. parvum for 24 h, followed by Western blot for SNAP23. The miR-98 precursor diminished C. parvum-induced SNAP23 expression (Figure 6C). Moreover, the miR-98 precursor partially blocked C. parvum-induced exosome release in H69 cells (Figure 6D). Interestingly, treatment of cells with the anti-let-7i showed no significant effects on C. parvum-induced exosome release (Figure 6D), presumably because of the low level of let-7i in the infected cells as shown in Figure 5A.

### Released Apical Exosomes Display Anti-C. parvum Activity through Binding to the C. parvum Surface

To investigate the potential effects of released apical exosomes on C. parvum, we incubated freshly excysted C. parvum sporozoites with a serial dilution of exosomes isolated from H69 monolayers after exposure to C. parvum for 24 h. Incubation of C. parvum sporozoites with isolated exosomes resulted in a decrease in C. parvum viability in a dose-dependent manner by the viability assay (Figure 7A). Sporozoites incubated with exosomes isolated from non-infected monolayers showed a modest decrease in C. parvum viability (Figure 7A). Exosomes isolated from the basolateral side of infected H69 monolayers, or apical exosomes from infected H69 cells expressing TLR4-DN, showed no significant effects on C. parvum viability (Figure 7A). A decrease in C. parvum viability was also detected after incubation with apical exosomes isolated from infected H69 monolayers following LPS stimulation (Figure S3). We then incubated the same number of C. parvum sporozoites with apical exosomes isolated from infected H69 monolayers for 2 h; after extensive washing, these sporozoites were added to H69 cells for infection. A decreased infectivity to host cells for C. parvum sporozoites after pre-incubation with exosomes was detected, compared with the sporozoites after incubation with culture medium, as assessed by real-time PCR (Figure 7B) and immunostaining (Figure 7C). Incubation with exosomes from the apical side of non-infected H69 monolayers, or infected cells stably expressing TLR4-DN and MyD88-DN, showed no inhibitory effects on C. parvum sporozoite infectivity (Figure 7B and 7C). A decreased infectivity of C. parvum sporozoites to intestinal IEC4.1 cells after pre-incubation with exosomes was also detected by real-time PCR (Figure S4).

Notably, binding of exosomes to the C. parvum sporozoite surface after incubation with apical exosomes was detected by scanning EM. In H69 cell cultures after exposure to C. parvum for 2 h, we observed binding of exosomes to the parasite surface (Figure 8A). Some of the sporozoites with several exosomes binding to their surface were dying (showing high electrical density, as indicated by the asterisk in Figure 8A). To further confirm the direct binding of exosomes to the C. parvum sporozoite surface, we first labeled exosomes with the N-Rh-PE as previously reported [36]. Labeled exosomes were then incubated with freshly excysted C. parvum sporozoites, followed by observation under confocal microscopy. Fluorescent activity was detected in these sporozoites incubated with the labeled exosomes (Figure 8B). No detectable fluorescent activity was observed in the sporozoites incubated with the non-labeled exosomes.

Direct interactions between the Gal/GalNAc-containing glycoproteins on the epithelial cell apical membrane surface and Gal/GalNAc-ligand molecules on the C. parvum sporozoite surface have been implicated to mediate the attachment of C. parvum sporozoites to host cells [25]. The Gal/GalNAc-specific lectin PNA can markedly decrease the infection [43,44]. We found that isolated apical exosomes carry Gal/GalNAc molecules (recognized by PNA), displaying positive reactions to PNA blotting at the epithelial apical membrane (Figure 8C). Interestingly, blotting of whole lysates of C. parvum sporozoites after pre-incubation with isolated exosomes revealed a positive reaction to exosome marker.
Moreover, pre-incubation of isolated exosomes with PNA (followed by extensive washing and ultracentrifugation to clear out unbound/free PNA) abolished the inhibitory effects of exosomes on *C. parvum* viability (Figure 8E). The above data indicate that apical exosomes released from *C. parvum*-infected biliary epithelium possess anti-*C. parvum* capacity. Interestingly, exosomes isolated from *C. parvum*-infected H69 monolayers showed no detectable inhibitory effects on the viability of the K12 strain *E. coli* after incubation *in vitro* (Figure 8E).

**Apical Exosomes Released from *C. parvum*-infected Biliary Epithelium Carry Antimicrobial Peptides, a Process that Is Mediated by TLR4 Signaling**

Production of antimicrobial peptides, such as beta-defensins and cathelicidins, is a major element for TLR-mediated epithelial anti-*C. parvum* defense [19,25,45]. Increasing amounts of human beta-defensin 2 (HBD2) and cathelicidin-37 (LL-37) were detected by ELISA in apical exosomes isolated from *C. parvum*-infected H69 monolayers, compared with those isolated from non-infected cells (Figure 9A). In contrast, no increase in HBD2 and LL-37 contents was detected in the isolated exosomes from infected H69 cells stably expressing TLR4-DN (Figure 9A). Exosomal shuttling of HBD2 and LL-37 was further confirmed by immunogold staining, using the apical exosomes isolated from *C. parvum*-infected H69 monolayers (Figure 9B and 9C). Unfortunately, we have been unable to achieve efficient exosome preparations to demonstrate that the antimicrobial peptides in the exosomes are biochemically active. Therefore, to elucidate whether exosomal shuttle of HBD2 and LL-37 accounts for the anti-*C. parvum* activity of these isolated exosomes from *C. parvum*-infected cells, we performed the gain-and-loss-of-function experiments through overexpression or knockdown of HBD2 and LL-37 in H69 cells. These cells were then infected with *C. parvum* for 24 h, and the released exosomes were isolated and then incubated with freshly excysted *C. parvum* sporozoites. Of note, pre-incubation of sporozoites with the exosomes isolated from cells overexpressing HBD2 and LL-37 showed a further decrease in infectivity (Figure 9D). In contrast, incubation with exosomes isolated from *C. parvum*-infected H69 cells with knockdown of HBD2 and LL-37 showed no significant effects on the infectivity of *C. parvum* sporozoites (Figure 9E and 9F). These data suggest that antimicrobial peptides...
HBD2 and LL-37 in apical exosomes that are released from epithelial cells contribute to mucosal immune defense in response to C. parvum infection.

Discussion

One of the major findings of this study is that activation of TLR4 signaling increases luminal release of exosomes from the biliary epithelium during C. parvum infection. Whereas a basal level of exosomal luminal release exists in cultured biliary epithelial monolayers and in the murine biliary tract, a TLR4-dependent increase in luminal release of epithelial exosomes was detected following C. parvum infection. Release of exosomes to the extracellular environment involves fusion of MVBs with the plasma membrane. Intracellular trafficking and fusion of compartments classically require small GTPases of the Rab family [2]. Further supporting this concept and consistent with results from previous reports on induced exosome release [17], we detected a significant co-localization of Rab11 and Rab27b with MVBs in the cytoplasm of epithelial cells following C. parvum infection. Besides Rab11 and Rab27b, we identified that SNAP23 may regulate the fusion of MVBs with the plasma membrane required for exosome release. C. parvum infection increases the protein content of total SNAP23 and enhances phosphorylation of SNAP23 in infected cells. Intriguingly, activation of TLR4 may contribute to both events: TLR4 signaling increases SNAP23 protein expression through modulation of let-7-mediated gene regulation, and stimulates SNAP23 phosphorylation through activation of IKK2. We previously demonstrated TLR4/NF-κB-dependent downregulation of let-7 family miRNAs in C. parvum-infected biliary epithelial cells [27,41]. Targeting of the 3’UTR of SNAP23 by let-7 family miRNAs resulted in translational suppression. Functional manipulation of let-7i caused reciprocal alterations in cellular SNAP23 protein content. Indeed, overexpression of let-7i miRNA members attenuated C. parvum-induced SNAP23 expression and exosome release from the biliary epithelium. In addition, C. parvum-induced phosphorylation of SNAP23 is dependent on TLR4 signaling. Knockdown of TLR4 blocked C. parvum-induced phosphorylation of SNAP23 in infected cells and, consequently, exosome release into the supernatants. Our data were supported by a recent report showing that SNAP23 is a substrate for IKK2 [37]. Phosphorylation of SNAP23 at Ser120 and Ser95 by IKK2 stimulates fusion of intracellular vesicles to the cell membrane, promoting exocytosis [37]. The next challenge will be to examine in greater depth the involvement of TLR4 signaling in C. parvum-induced co-localization of Rab11 and Rab27b with MVBs, and to determine the magnitudes of miRNA-mediated SNAP23 expression versus its phosphorylation in TLR4-regulated exosome release.

Another key point from this study is that released exosomes shuttle antimicrobial peptides and display anti-C. parvum capacity, thus contributing to mucosal anti-C. parvum defense. It has been reported that antimicrobial peptides, such as HBD2 and LL-37, are present in breast milk and provide protection for maternal breast tissue and the developing digestive tracts of newborns [46]. Additionally, antimicrobial peptides were identified in cervical-vaginal fluid, suggesting their involvement in extracellular immunology [47]. Notably, exosomes have been reported in

Figure 7. Apical exosomes released from C. parvum-infected biliary epithelium display anti-C. parvum activity. (A) Effects of incubation with isolated exosomes from the biliary epithelium on C. parvum viability. Exosomes were isolated from the basolateral or apical supernatants of H69 monolayers of non-infected control, cells of C. parvum infection for 24 h, and cells stably expressing TLR4-DN after infection for 24 h. Freshly excysted C. parvum sporozoites were incubated with isolated exosomes (as indicated) for 2 h, and parasite viability was assessed by propidium iodide staining. (B) and (C) Effects of incubation with isolated apical exosomes from the biliary epithelium on the infectivity of C. parvum sporozoites. The same number of freshly excysted C. parvum sporozoites was incubated with exosomes isolated from H69 or 603B monolayers for 2 h and then added to H69 cells for 2 h. Parasite infection burden was measured by real-time PCR (B) and fluorescence microscopy (C). Pre-incubation of isolated exosomes abolished their inhibitory effects on C. parvum infectivity. C. parvum parasites were stained in green using a specific antibody and cell nuclei in blue by DAPI. Scale bars = 5 μm; *, p<0.05 ANOVA versus medium control. doi:10.1371/journal.ppat.1003261.g007
breast milk and cervical-vaginal fluid [1,2]. The anti-C. parvum activity of HBD2 was previously reported in intestinal and biliary cryptosporidiosis [19,45]. Here, we identified shuttling of both HBD2 and LL-37 in the apical exosomes released from H69 monolayers. An increase in HBD2 and LL-37 content was detected in the apical exosomes from infected cells, and inhibition of TLR4 signaling decreased their exosomal content. Because these exosomes display a protein profile different from the whole cell lysate, targeting of cytoplasmic proteins to the MVBs within epithelial cells, such as HBD2 and LL-37, must be controlled through highly selective and regulated mechanisms. However, how TLR4 signaling regulates targeting of antimicrobial peptides to MVBs in epithelial cells remains largely unknown. One additional unanswered question is whether shuttling of MHC proteins in the apical exosomes contributes to antigen presentation.

Anti-C. parvum activity of apical exosomes released from the epithelium may involve direct binding to the C. parvum surface. Morphologically, direct binding of exosomes to the C. parvum surface was evident by scanning and transmission EM observations. Confocal analysis with exosomal labeling confirmed the delivery of exosomal content to C. parvum sporozoites after incubation. It is not clear what mechanisms are responsible for this exosome binding/targeting. The infection process of epithelial cells by C. parvum is initiated by the attachment of the parasite to the plasma membrane of host epithelial cells [25]. Unidentified specific molecules on the surface of both epithelial cells and C. parvum sporozoites mediate this attachment process [43]. Gal/GalNAc epitopes of glycoproteins on the epithelial apical membrane and Gal/GalNAc-specific sporozoite surface lectins are involved in the mechanism(s) of C. parvum attachment to intestinal and biliary epithelial cells [43,44]. As expected, treatment of exosomes with Gal/GalNAc-specific PNA lectin diminished their anti-C. parvum activity, suggesting that these molecules may be involved, at least partially, in exosomal binding.
to the *C. parvum* sporozoite surface. The lifecycle of *C. parvum*, both in vitro and in vivo, has extracellular stages (i.e., sporozoites, merozoites, and microgametocytes) [25], and they are vulnerable to exosomal binding/targeting. Therefore, luminal release of exosomes from epithelial cells may represent an important element of TLR-mediated mucosal anti-*C. parvum* defense. Surprisingly, it appears that released exosomes have no significant effect on the viability of the K12 strain *E. coli*. One possibility is that the membrane structure of *E. coli* may not favor direct exosomal binding.

In addition, increased exosomal release and exosome-associated anti-*C. parvum* activity were detected in biliary epithelial cells after LPS stimulation. Therefore, TLR4-mediated exosome release may be relevant to innate mucosal immunity in general. Overall, our data suggest that activation of TLR4 signaling stimulates the biogenesis and luminal release of antimicrobial peptide-shuttling exosomes and contributes to gastrointestinal mucosal anti-*C. parvum* defense. Such a process may be explored for therapeutic intervention. Luminal release of exosomes may also mediate the function of distant cells along the gastrointestinal tract, or regulate the homeostasis of gut microbiota, through delivery signaling molecules.

### Materials and Methods

**Ethics Statement**

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health under the Assurance of Compliance Number A3348-01. All animal experiments were done in accordance with procedures (protocol number # 0868) approved by the Institutional Animal Care and Use Committee of the Creighton University School of Medicine. All surgery was performed under ketamine and xylazine anesthesia, and all efforts were made to minimize suffering.

**C. parvum and 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 [19]. H69 cells (a gift from Dr. D. Jefferson, Tufts University) are SV40-transformed normal human biliary epithelial cells originally derived from normal liver harvested for transplant [19,27]. 603B cells are immortalized normal mouse biliary epithelial cells (a gift from Dr. Y. Ueno, Tohoku University School of Medicine, Japan). Murine intestinal epithelial cell line (IEC4.1) was a kind gift from Dr. Pingchang Yang (McMaster University, Hamilton, Canada).

**Plasmids and Reagents**

TLR4-DN mutant was kindly provided by Dr. M. F. Smith (University of Virginia) and MyD88-DN was a gift from Prof. J. Tschopp (University of Lausanne, Switzerland). Cells stably
expressing TLR4-DN or MyD88-DN were established as previously reported [19]. Primers used to amplify the open reading frame of human SNAPP3 (NM_130738) were: 5’-GTCAATGGATTATTCCTCATCAGAGAAAT-3’ (forward) and 5’-TTAGGTCTGCAATGAGTTTCTTGTGCT-3’ (reverse). PCR products were cloned into pcDNA3.1-TOPO vector (Invitrogen). Primers used to amplify the open reading frame of human beta-defensin 2 (NM_004942) and cathelicidin-37 (NM_004943) were: 5’-CAAGCTTCTGAGGAAGCTTTGAGTATGAT-3’ (beta-defensin 2 forward) and 5’-CCGAATCTGGCCTTTTGGCAGCTTTG-3’ (beta-defensin 2 reverse) and 5’-CAAGCTTCTGGATATGGGACCATGAGGAAGACCCGAC-3’ (cathelicidin-37 forward) and 5’-CCGAATCCGGACTCTGCTTGGGTTAAGATTTCC-3’ (cathelicidin-37 reverse). PCR products were cloned into the HindIII and BamHI sites of the pEGPNN3 vector (Invitrogen). WGA and RNA latex kits were purchased from Sigma.

Infection Models and Infection Assays
Models of human biliary cryptosporidiosis using H69 and 603B cells were employed as previously described [19,27,28,41,43]. Infection was done in a culture medium consisting of DMEM-F12, 100 U/ml penicillin, 100 μg/ml streptomycin, and freshly excysted sporozoites (from oocysts in a 3:1 ratio with host cells). All excysted sporozoite preparations were tested to exclude LPS contamination using the Luminex Ameboocyte Lysate (LAL) gel formation test [48]. Inactivated organisms (treated at 65°C for 30 min) were used for the non-infected controls and experiments were performed in triplicate.

For in vivo biliary infection, C57BL/6 wild-type mice and TLR4-deficient (C57BL/10ScNJ; Tlr4-/-/−/−/−/− genotype) mice were obtained from Jackson Laboratories. C.parvum oocysts were directly injected into the gallbladder of C57BL/6 or TLR4-deficient mice, as previously reported [32,33]. C. parvum infection and exosome release in the intrahepatic bile ducts were observed one week post-injection. Five animals from each group were sacrificed and livers were obtained for immunohistochemistry and electron microscopy.

Real-time PCR, immunofluorescence microscopy, and immunohistochemistry were used to assay C. parvum infection as previously reported [17,27,28,41,43]. Primers specific for C. parvum 18s ribosomal RNA (forward: 5’-TCTAGATGGAGGTGTTGCTTCTC-3’; and reverse: 5’-GCTCAACGCAAAGGAAGGCC-3’) were used to amplify the cDNA specific to the parasite. For immunofluorescence microscopy, cells were fixed with 2% paraformaldehyde and incubated with a polyclonal antibody against C. parvum [19], followed by anti-rabbit FITC-conjugated secondary antibody and co-stained with 4,6-diamidino-2-phenylindole (DAPI) (Molecular Probes). The liver tissues were stained with H&E, and parasite burden in the biliary tree was performed by counting all intracellular parasite stages in identified bile ducts, as previously reported [35].

Exosome Purification and Nanoparticle Tracking Analysis (NTA)
Supernatant medium from the apical reservoir of the Percoll inserts was collected at the indicated time points after infection. Medium was then harvested and centrifuged at 1000 rpm for 10 min to eliminate cells and again spun at 10,000 rpm for 30 min, followed by filtration through 0.22 μm filter to remove cell debris. Exosomes were pelleted by ultracentrifugation (Beckman Ti70 rotor) at 44,000 rpm for 70 min and precipitated using exosome precipitation solution (Exo-Quick; System Bioscience) following the manufacturer’s instructions. Exosomes were further quantified by the NTA analysis using a Nanosight (model NS500), as previously reported [30,31].

Immunofluorescence and Electron Microscopy (EM)
Cell cultures were fixed with 4% paraformaldehyde and incubated with antibodies (Santa Cruz) to SNAP23, CD63, Rab11, and Rab27, followed by treatment with Alexa Fluor 594–conjugated anti-rabbit secondary antibody (1:100; Invitrogen). Cell cultures were then mounted in Slow Fade anti-fade reagent with DAPI (Molecular Probes). Images were captured using an inverted fluorescence microscope TE2000-E (Nikon).

EM was performed as previously described [24]. Briefly, for transmission EM, exosome pellets were resuspended in 2.5% glutaraldehyde, embedded with a mixture of 4% uranyl acetate and 2% methylcellulose (1:9 ratio), and observed with a JEOL 1400 electron microscope (JEOL USA). For scanning EM, isolated exosomes were fixed immediately in 2.5% glutaraldehyde, dehydrated, dried in a critical point drying device, sputter coated, and examined with a Hitachi S-4700 microscope (Hitachi). For immunogold analysis, exosomes were fixed in 4% paraformaldehyde, blocked with 10% FCS-PBS for 20 min, and incubated overnight at 4°C with antibodies to CD63 (Santa Cruz), ICAM-1 (Santa Cruz), LL-37 (Hycult Biotech), or HBD2 (Alpha Diagnostic). After incubation with the secondary antibodies, samples were labeled with protein A-10-nm gold, embedded, and observed with a JEOL 1400 electron microscope. Transmission EM of mouse liver tissues was performed as previously reported [24].

siRNA, Anti-miRs, and miRNA Precursors
siRNAs to SNAP23, IL-37 and HBD2, and negative control oligos (Dharmacon) were used at a concentration of 10 nM and transfected with Lipofectamine RNAmax according to the manufacturer’s protocol (Invitrogen). Anti-miRs and miRNA precursors (Ambion) were used to manipulate miRNA function in cells, as previously reported [27,28,41].

Luciferase Reporter Constructs and Luciferase Assay
Complementary 39 bp for human and 33 bp for mouse DNA oligonucleotides containing the putative let-7 family miRNAs target site within the 3’UTR of human SNAP23 (Human-Sense: 5’-catGATGAATTCGTTTATCTCATTGAAGATAT-3’; Human-antisense: 5’-actTAATTCTGAGTATGAGGTAATGTTAATTATCGTGACTTG-3’; Mouse-Sense: 5’-catGATGAATTCGTTTATCTCATTGAAGATAT-3’; Mouse-antisense: 5’-actTAATTCTGAGTATGAGGTAATGTTAATTATCGTGACTTG-3’) were cloned into the multiple cloning site of the pMIR-REPORT Luciferase vector (Ambion). Another pMIR-REPORT Luciferase construct containing mutant 3’UTR (CCTG to GAGG for human; ACCT to TGGA for mouse) was also generated as a control. Transfection and assessment of luciferase activity were performed as previously reported [27,28,41].

Accession Numbers
The mRNA sequence data for genes described in this study can be found in the NCBI under the following accession numbers: Homo sapiens CD63 (NM_001257389), Mus musculus CD63 (NM_001042580); Homo sapiens TLR4 (NM_001257389), Mus musculus TLR4 (NM_025817); Homo sapiens Rab27b (NM_004163), Mus musculus Rab27b (NM_030554); Homo sapiens MyD88 (NM_01172566), Mus musculus MyD88 (NM_010851); Homo sapiens IKK2 (NM_001190720), Mus musculus IKK2 (NM_001159774); Homo sapiens Rab11 (NM_014904), Mus musculus.
Supporting Information

Figure S1  Nanosight Tracking Analysis (NTA), Western blot for CD63 and MHC proteins, and release of apical exosomes from monolayers of different epithelial cell types following *C. parvum* infection or LPS stimulation. (A) Size and particle distribution plots of isolated apical exosomes from the non-infected and *C. parvum*-infected (12 h) H69 monolayers by NTA. Both plots show a peak size around 100 nm for these isolated exosomes. Of note, the concentration scores for exosomes from non-infected and infected monolayers are different. Insert images are the representative screenshots of isolated exosomes by NTA. Image size recorded by video is approximately 100 μm. (B) Increased exosome release to the apical region following *C. parvum* infection was also confirmed by Western blot for CD63. (C) Increased amounts of MHC I and MHC II were detected in both the apical and basolateral exosomes released from H69 monolayers following infection. (D) Release of apical exosomes from monolayers of different epithelial cell types following *C. parvum* infection or LPS stimulation. Cells were grown to form monolayers on the Percoll inserts and then exposed to *C. parvum* infection for 24 h or LPS stimulation for 12 h. Exosomes were isolated from the apical supernatants and assessed by Western blot for CD63 (upper panel) and NTA (lower panel). *, p<0.05 ANOVA versus the non-infected controls. (TIF)

Figure S2 Association of SNAP23 with MVBs in H69 cells overexpressing SNAP23. H69 cells were transfected with the SNAP23 plasmid and then exposed to *C. parvum* infection for 12 h. Cells were stained with CD63 antibody. Accumulation of SNAP23 around CD63-positive multi-vesicular structures was obvious as assessed by confocal microscopy. Scale bars = 5 μm. (TIF)

Figure S3 Expression of SNAP23 and phosphorylation of SNAP23 in 603B cells following *C. parvum* infection, and epithelial expression of miR-98 and anti-*C. parvum* activity of isolated exosomes from H69 monolayers after LPS stimulation. Cells were grown to form monolayers on the Percoll inserts and then exposed to *C. parvum* infection or LPS stimulation. SNAP23 expression and its phosphorylation were assessed by Western blot and IP analysis (A and B). Expression of miR-98 was quantified by real-time PCR (C), and viability of *C. parvum* sporozoites after incubation with isolated exosomes was measured by epifluorescence microscopy (D). *, p<0.05 ANOVA versus the medium control. (TIF)

Figure S4 Anti-*C. parvum* activity of isolated exosomes from IEC4.1 monolayers following *C. parvum* infection. Cells were grown to form monolayers on the Percoll inserts and then exposed *C. parvum* infection for 24 h. Released apical exosomes were collected and purified. These exosomes were then incubated with freshly excysted *C. parvum* sporozoites for 2 h, and after extensive washing, the sporozoites were added to IEC4.1 cells for infection. Parasite infection burden was measured by real-time PCR. *, p<0.05 ANOVA versus the medium control. (TIF)

Figure S5 Viability of *E. coli* K12 strain after incubation with isolated apical exosomes from *C. parvum*-infected H69 monolayers. K12, a laboratory strain of *E. coli* susceptible to all antimicrobial drug classes, was incubated with apical exosomes isolated from non-infected or *C. parvum*-infected H69 monolayers. OD_{600} measurements were taken every 60 min for a total of 5 h. Recombinant human LL-37 was used as the positive control and phosphate buffered saline was used as a negative control. These viability measurements were substantiated by performing plate counts collected at each time point. (TIF)

Text S1 Details of Western blot, real-time PCR, Northern blot, ELISAs, immunoprecipitation (IP), *C. parvum* viability assay, *E. coli* viability experiments, and statistical analysis of data. (DOC)

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Author Contributions

Conceived and designed the experiments: GH AYG ALR NDH XMC. Performed the experiments: GH AYG ALR BQH. Analyzed the data: GH

HDW GZ NFL. Wrote the paper: GH XMC.

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