GLYCAN TRIGGERS OF LIFE CYCLE DEVELOPMENT IN THE APICOMPLEXAN PARASITE *CRYPTOSPORIDIUM*

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DOCTOR OF PHILOSOPHY

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ABSTRACT

Cryptosporidium is an apicomplexan parasite that causes the diarrheal disease cryptosporidiosis, an infection that can become chronic and life threating in immunocompromised and malnourished individuals. Development of novel therapeutic interventions is critical as current treatments are entirely ineffective in treating cryptosporidiosis in populations at the greatest risk for disease. Repeated cycling of host cell invasion and replication by sporozoites results in the rapid amplification of parasite numbers and the pathology associated with the disease. Little is known regarding the factors that promote the switch from invasion to replication of *Cryptosporidium*, or the mechanisms underlying this change, but identification of replication triggers could provide potential targets for drugs designed to prevent cryptosporidiosis. The focus of this dissertation was to identify potential triggers and the mechanisms underlying the transition from invasive sporozoite to replicative trophozoites in *Cryptosporidium*. We demonstrate glycoproteins secreted by host cells promote the transition from invasion to replication in *Cryptosporidium*, and free Gal-GalNAc triggers nuclear division in linear and rounded sporozoites. The proportion of rounded and multinucleated cells in response to the host secretions differed between *C. parvum* and *C. hominis*, the two species of greatest concern to human health, with *C. parvum* sporozoites progressing towards rounding and replication faster. We demonstrated the use of glycomimetic polymers for studying *Cryptosporidium* biology. Slides imprinted with increasing densities of Gal and GalNAc had the greatest proportion of trophozoite development. Gal and GalNAc glycomimetic polymers were able to cause parasite replication; however the effect was less than what was observed in the presence of secreted host glycoproteins and free Gal-GalNAc. We also characterized a rhomboid protease (ROM) in *Cryptosporidium* which we name CpROM2,

providing information regarding expression and localization of the protease during *C. parvum* development. Using inhibitors specific for ROM activity we show ROMs play a critical role in excystation, but are also required for the transition from invasion to replication in *C. parvum*. These findings have strengthened our understanding of how *Cryptosporidium* transitions from invasion to replication by identifying host glycoproteins and Gal-GalNAc as triggers for trophozoite development via a ROM driven mechanism.

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LIST OF ABBREVIATIONS

p.i. ....Post infection.

p.e. ....Post excystation.

ROM ....Rhomboid protease.

TgROM ....*Toxoplasma gondii* rhomboid protease.

CpROM ....*Cryptosporidium parvum* rhomboid protease.

TRAP-C1 ....Thrombospondin-related adhesive protein of *Cryptosporidium*-1.

GENERAL INTRODUCTION AND RESEARCH OBJECTIVES

Introduction

Every year there are a reported 1.7 billion cases of diarrheal diseases around the world [1]. Diarrheal diseases are one of the most common reasons for hospital visits among low and middle income individuals and people in developing countries. Children are one of the largest demographics affected with almost one million children under the age of five, and 72% of infant and toddler deaths within the first two years of life are attributable to diarrheal disease [1, 2].

The leading non-viral contributor to the global burden of diarrheal disease is the apicomplexan *Cryptosporidium,* a waterborne protozoan parasite that causes cryptosporidiosis [1-3]. Included in the World Health Organization's Neglected Disease Initiative, *Cryptosporidium* infections continue to be a problem in the United States and worldwide [4]. Even with modern surveillance and diagnostic techniques infections go largely unreported with estimates of only 1% of cases being diagnosed in the United States [5]. Poverty stricken and developing countries are disproportionately affected with nearly 20% of childhood cases of diarrhea caused by the parasite [6]. Although the disease is typically self-limiting, cryptosporidiosis can become chronic and life threatening in immunocompromised populations [7-9]. There is a critical need for novel drug treatments to prevent disease as current treatments are ineffective in immunocompromised patients [10, 11].

Much of what is known regarding *Cryptosporidium* biology has been generated from studies involving experimental infections in animals and *in vitro* cell culture experiments[12-15]. This work has been critical in identifying the factors that regulate attachment and invasion, giving a more complete understanding of the life cycle of *Cryptosporidium* [16-19]. Infections occur when the environmentally stable oocyst stage is ingested via fecal-oral route. Transmission

primarily comes from contaminated food or water sources [20, 21]. Upon reaching the intestinal epithelium sporozoites are released from the oocyst which subsequently invade the host tissue [22]. Sporozoites have an apical complex which consists of micronemes and rhoptries which sequester proteins that are eventually released to the parasite surface to engage with the host cell [23-26]. Receptor-ligand interactions between the host and parasite initiate extensive host cell actin remodeling, facilitating the formation of an intracellular, but extracytoplasmic vacuole [18, 27, 28]. Repeated cycling between invasion and replication results in rapid amplification of parasite numbers and the pathology associated with cryptosporidiosis [28, 29].

The transition to replication from a mode of attachment and invasion is not well understood in *Cryptosporidium*, but the unique location and extracytoplasmic niche adopted by the parasite suggests triggers for replication are located at the surface of the host cell [28, 30]. Understanding how host factors contribute to this transition and the mechanisms underlying this change in the parasite remain a gap in our knowledge of *Cryptosporidium* biology.
Bridging these gaps has relied heavily on studies using the model apicomplexans *Toxoplasma* and *Plasmodium* [31-33]. Engagement and processing of host factors by *Toxoplasma* and *Plasmodium* proteases have been implicated not only as triggers, but also a potential mechanism used for the transition from invasion to replication [34-38].

Genetic recalcitrance, coupled with the inability to purify individual stages and propagate the parasite *in vitro* has limited the pursuit in answering these important questions in *Cryptosporidium*. However, an increased focus of *Cryptosporidium* has led to new insights into the biology of the parasite. Stark differences in biology, invasion strategy and the extracellular development have led to a reassessment of the classification of *Cryptosporidium* within the phylum apicomplexan, but have also identified novel targets that can be used for vaccine and

drug development [30]. Identify the triggers for replication and the mechanisms governing this transition offer a means of inhibiting or slowing the progression of the parasite life cycle which would significantly dampen the damage associated with cryptosporidiosis.

Aims of the dissertation project

The long-term goals of this project are to identify triggers that promote the transition to replication in *Cryptosporidium* and the mechanisms underlying this change. This knowledge can then be used to develop novel, effective treatments for the prevention of cryptosporidiosis. The specific objective of the work presented in this dissertation was to determine the role of host glycoproteins and sporozoite rhomboid proteases in promoting and initiating parasite replication. The central hypothesis is that rounding and replication of *Cryptosporidium* at the host surface is triggered by glycoproteins expressed by the host cell, and is governed by the rhomboid machinery used during motility and invasion.

The specific aims of this dissertation are:

To identify and characterize the extent to which glycoproteins serve as triggers for Cryptosporidium replication. The working hypothesis is that *Cryptosporidium* sporozoites bind host glycoproteins and Gal and GalNAc glycans induce parasites differentiation into rounded, replicative stages.

To determine the extent to which rhomboid proteases contribute to the excystation and the transition to a replicative Cryptosporidium trophozoite. The working hypothesis is that inhibition of rhomboid proteases will inhibit excystation and the proportion of replicative *Cryptosporidium* trophozoites after exposure to triggers for replication.

Organization of the dissertation

Chapter 1 serves as an introduction and reviews the literature pertinent to the content of the thesis. This comprehensive overview addresses topics concerning the history, biology, epidemiology and disease associated with *Cryptosporidium*.

The experimental work completed is included in Chapters 2 through 5, and is written and prepared in manuscript format for journal submission. The Chapters therefore read as separate documents including an abstract, introduction, method, results, discussion and reference section written specifically for each Chapter, with some overlap in the sections and references used.

The final Chapter is written as a general discussion to provide future directions and perspective to the work outlined in Chapters 2 through 5.

Overview of *Cryptosporidium* and cryptosporidiosis

Cryptosporidium, an apicomplexan parasite discovered by Ernest Edward Tyzzer in 1907 [39], infects all major vertebrate classes and causes the disease cryptosporidiosis. Tyzzer originally described *C. muris*, a gastric species and *C. parvum*, an intestinal species of *Cryptosporidium* in the house mouse, classifying the parasite as a coccidian based on observations of the life cycle [40, 41]. However, he also recognized that the localization of *Cryptosporidium* to the epithelial cell surface and the presence of extracellular stages in the lumen of the mouse showed similarities to gregarines, an extracellular parasite of invertebrates [41]. To date, thirty-one valid species have been described through molecular, biological and morphological data, but over 70 genotypes have been identified. Of the species identified *C. hominis* and *C. parvum* continue to be the largest contributors to human cryptosporidiosis worldwide [42, 43] and are of the greatest concern to public health; however, other species of *Cryptosporidium* can cause human disease (Table 1).

Cryptosporidium is a waterborne parasite typically spread by fecal contaminated water sources [44]. Disease is caused by the ingestion of the environmental oocyst stage. The infectious dose is small, ranging between 10 and 30 oocysts [45, 46]. The oocyst is environmentally stable, resistant to desiccation, freezing, pH, and chlorination, making the spread the swimming pools or even acidic foods possible [47-51]. Common disinfectants such as bleach and ethanol are ineffective, but 6% hydrogen peroxide and UV radiation has proven effective at reducing *Cryptosporidium* oocyst infectivity [52, 53]. Oocysts can be difficult to remove from water or food supplies due to the small size (4-6 µm), but the use of water filtration significantly lowers the incidence of disease [54, 55]. The major symptom in patients diagnosed with cryptosporidiosis is watery diarrhea, but this can be accompanied by several other maladies, including abdominal cramps, weight loss, anorexia, fatigue, nausea, vomiting, and a fever [56].

Cryptosporidiosis has been reported on all continents, with variation in the incidence of disease [78-80]. Of the 199 outbreaks caused by the waterborne transmission of protozoan parasites from 2004 to 2010, the largest contributor to disease was *Cryptosporidium* (60.3%) [21]. A majority of cases were reported in Australia, North America and Europe with the incidence of cryptosporidiosis varying from 0.6 to 20% based on location [21, 81]. From 2001-2010 *Cryptosporidium* was the leading cause of waterborne disease in the United States, associated with 0.4 to 1 % of reported cases of diarrhea. The yearly cases of cryptosporidiosis in the United States and around the world are likely underreported, however, due to asymptomatic cases, immunocompetent individuals not seeking medical attention, or a lack of testing for the parasite [82-84]. In the United States alone, there are anywhere between 165,380–826,900 estimated cases annually, whereas only 8,269 cases were reported to the CDC [85]. Based on seroprevalence however, approximately 30% of the US population has been exposed to the parasite [86]. The

largest outbreak of cryptosporidiosis in the US occurred in Milwaukee, WI in 1993. More than a quarter of the population of Milwaukee (over 400,000 individuals) were affected. In total, the outbreak was estimated to cost $96.2 million, including $31.7 million in medical costs with the source of the outbreak traced back to the city water supply [87].

Table 1. *Cryptosporidium* species causing human cryptosporidiosis.

***Cryptosporidium* species**	**Animal reservoir**
C. meleagridis [57]	Birds
C. cuniculus [58]	Rabbits
C. canis [59]	Dogs
C. felis [59]	Cats
C. andersoni [60]	Cattle
C. bayleyi [61]	Birds
C. bovis [62]	Calves
C. fayeri [63]	Kangaroos
C. muris [64]	Mouse
C. scrofarum [65]	Pigs
C. suis [60]	Pigs
C. tyzzeri [66]	Mouse
C. ubiquitum [67]	Lambs, rodents, primates
C. viatorum [68]	Not defined
C. fragile [69]	Toads
C. galli [70]	Birds
C. macropodum [71]	Marsupials
C. molnari [72]	Fish
C. rynae [73]	Buffalo
C. scophthalmi [74]	Fish
C. serpentis [75]	Lizards and snakes
C. varanii [75]	Lizards and snakes
C. wrairi [76]	Guinea pigs
C. xiaoi [77]	Lambs and sheep

Adapted from Cacciò, S.M. & Widmer, G. *Cryptosporidium: parasite and disease*. (Springer Science & Business Media, 2013).

Based on its worldwide prevalence and its ability to spread rapidly in the environment, particularly through contaminated water sources, *Cryptosporidium* is regarded as an important pathogen from a public health perspective. The impact of cryptosporidiosis on children and immunocompromised individuals is a particular concern. Children can exhibit stunted cognitive and physical development because of the malnutrition associated with diarrheal disease [88]. Children and immunocompromised individuals can also be chronically infected with the parasite, and in extreme cases the infection can become fatal [89]. A study in Italy found HIV/AIDS patients were almost 3 times more likely to become infected than immunocompetent individuals. Of patients tested, 72% of those who experienced prolonged diarrhea (>15days) died [90]. *Cryptosporidium* was identified in approximately 27% of HIV/AIDS patients studied in Ethiopia, of which *C. parvum* was the dominant species identified (65% of the cases) [91]. While in the UK, of 128 HIV/AIDS patients studied, 60% were classified as chronically infected with *Cryptosporidium* [92].

To date, the most common treatment for *Cryptosporidium* infections is a drug course of nitazoxanide, paromycin and azithromycin; however, the medication regimen can have negative side effects and is not effective for all patients [93]. Nitazoxanide is entirely ineffective in immunocompromised patients [94]. Treatments involving the use of inhibitors to prevent enzymatic activity governing the mannitol cycle, oocyst formation, GMP synthesis and DNA replication/repair have been targeted for potential disease prevention with limited success [95-98]. In all, over 200 chemotherapeutic agents have been tested for potential use in treating cryptosporidiosis, especially in immunocompromised populations [99-101]. Unfortunately adequate drug therapies or vaccine candidates for disease prevention in humans have yet to be identified.

In animal models, calves immunized with recombinant *Cryptosporidium* protein promoted immunity, and the immunity could be transferred to calves via bovine colostrum [102]. Remission of disease in a child as well as in AIDS patients infected with the parasite has been reported after treatment with hyperimmune bovine colostrum [103, 104]. Monoclonal antibodies targeting antigens present on the surface of *Cryptosporidium* sporozoites have shown some protective ability in mice, but not to the same degree as immune bovine colostral whey [105, 106]. Recombinant vaccines utilizing a 15kDa antigen (Cp15) have been used to immunize pregnant goats with successful transfer of immunity to offspring [107, 108]. The efficacy of a Cp15 has also been shown in a mouse model; however, in malnourished mice the cell-mediated response to the antigen is blunted indicating malnutrition plays a critical role in the development of protective immunity [109]. Vaccination of mice using a heterologous prime-boost regimen involving Cp15, profiling and apyrase fused to a vector containing Cytolysin A in *Salmonella* induced a humoral response, implicating these targets as potential vaccine candidates [110] . Other antigens have been investigated for use in a vaccine including Cp23, Muc4 and Muc5, glycoproteins expressed at the surface of the parasite [110, 111].

Epidemiology of *Cryptosporidium*

First identified as a human pathogen in 1976, *Cryptosporidium* came to prominence in the 1980s and 1990s as a cause of severe diarrheal disease and death in people with HIV/AIDS [40, 112]. Patients diagnosed as HIV positive were found to be disproportionally affected by *Cryptosporidium*, with 24% of cases diarrhea were attributable to cryptosporidiosis [112].

Outbreaks of cryptosporidiosis tend to be localized to North America, Australia and Europe [113]. The prevalence of *Cryptosporidium* infections in North America is between 1 and 3% but higher, 5-10%, in developing countries. *Cryptosporidium* is one of the most common causes

of waterborne illness in the United States, with an estimated 750,000 cases annually [5]. In one study, *C. parvum* was identified to have caused half of the 325 water-associated outbreaks of protozoan diseases, with the remainder caused by *Giardia duodenalis* (40.6%), *Entamoeba histolytica* (2.8%), *Cyclospora cayetanensis* (1.8%), *Toxoplasma gondii (*0.9%), *Cystisospora belli* (0.9%), *Blastocystis hominis* (0.6%), *Balantidium coli* (0.3%), and free living amoeba (0.3%) [114].

Serological testing indicates approximately 20% of the US population has experienced cryptosporidiosis by young adulthood [115]. By age one, 90% of children in areas of Brazil are identified as seropositive and 65% of children ages 8-10 living in rural areas of China are seropositive [116, 117]. The prevalence and relative ease with which *Cryptosporidium* is spread in the environment by water sources makes cryptosporidiosis a major concern for human health [49, 78, 115, 118-124]. Proper sanitation and access to clean water through proper water treatment systems has reduced the occurrence waterborne outbreaks in developed countries, but the disease remains a problem[44, 125, 126]. Populations in developing countries at risk for severe or chronic cases of cryptosporidiosis include the elderly, immunocompromised, expecting mothers, children and toddlers [47,81, 92, 93]. In the developing world, *Cryptosporidium* is the leading non-viral cause of diarrheal disease associated deaths in children under the age of 24-months [1].

Life cycle of *Cryptosporidium*

The life cycle of *Cryptosporidium* has been reported in a limited number of species with differences in the life cycle observed across *Cryptosporidium* species [12, 127, 128]. The *Cryptosporidium* life cycle has been most intensively studied in the zoonotic species *C. parvum* (Figure 1).

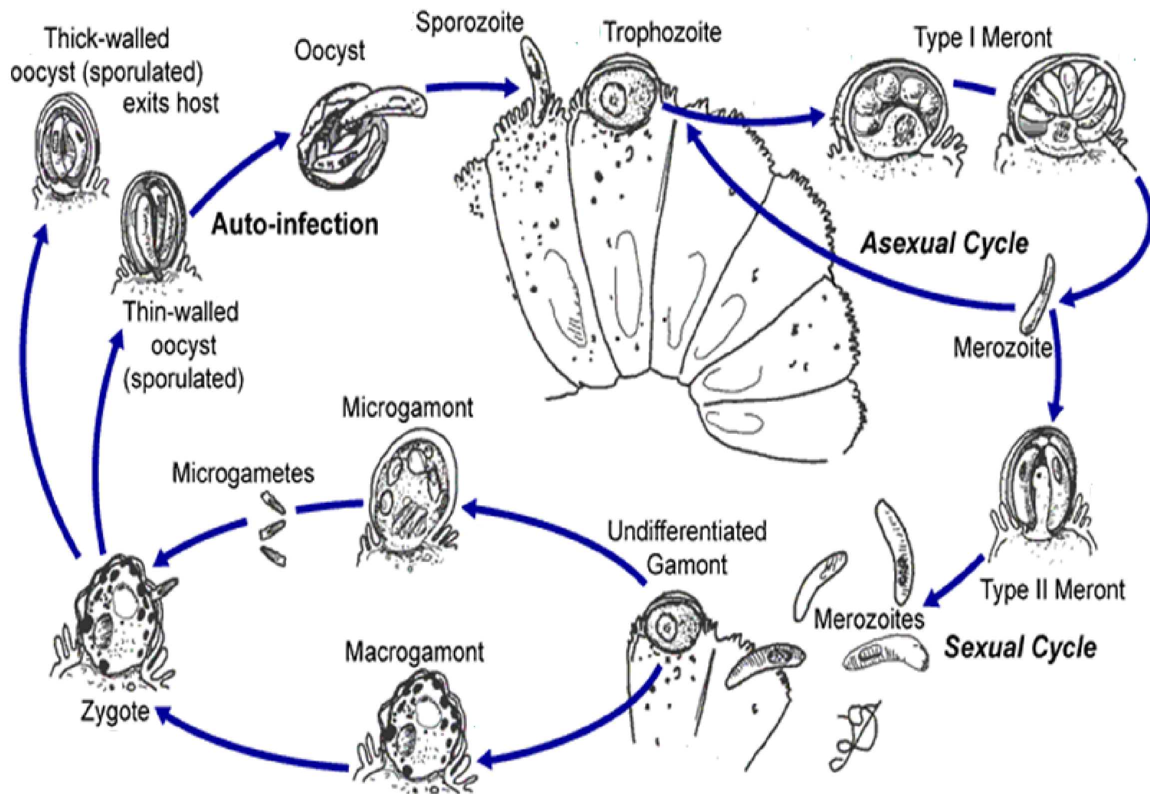

Figure 1. Life cycle of *Cryptosporidium parvum*.
Source: Center for Disease control and Prevention, USA.

The life cycle of *C. parvum* begins with the ingestion of an environmentally resistant oocyst by a suitable host. Each sporulated oocyst averages 4-6 µm in diameter and contains four infective sporozoites [129]. Oocysts release sporozoites which infect many different sites along the intestinal tract but are most commonly found in the middle and lower small intestine in humans [22]. Sporozoites invade host intestinal epithelial cells, forming an intracellular but extracytoplasmic parasitophorous vacuole where they transform into trophozoites (feeding stage) and ultimately Type I meronts (asexual replication stage). Asexual replication occurs by schizogony, also known as merogony, where multiple rounds of nuclear replication and fission happen, followed by segmentation of the cytoplasm giving rise to eight merozoites. Newly

formed merozoites are structurally similar to sporozoites and are released from meronts to invade other cells.

Cryptosporidium can cycle through asexual replication multiple times, resulting in a huge increase in parasite numbers. For reasons that are as yet unknown, some merozoites develop into Type II meronts, which produce four merozoites that are committed to the sexual phase of development (gametogony). Microgametes that lack a flagella penetrate and fertilize a macrogamont [129] to form a zygote, which undergoes sporogony to produce an oocyst with four sporozoites and no sporocyst. Some oocysts are thin walled and are thought to participate in autoinfection [127], while others are thick walled and are shed in the feces.

Cryptosporidium genomes

To date, the genomes of seven *Cryptosporidium* species have been characterized: *C. andersoni*, *C. baileyi*, *C. hominis*, *C. meleagridis*, *C. muris*, *C. parvum* and *C. ubiquitum* [130]. The *Cryptosporidium* genome is very compact and has few introns in comparison to other apicomplexans (approximately 5%) [130-132]. Although *C. parvum* and *C. hominis* have distinctly different host ranges they share 99% nucleotide sequence identity, each having a genome of approximately 9.2 Mb spanning over a total of eight chromosomes [130, 132-136]. Therefore, differences in the biology and host range of these parasites is likely due to variability in gene expression at the molecular level, and polymorphisms in crucial protein coding regions of the genome [137, 138]. Comparatively speaking, the genome of these two species of apicomplexans is relatively small. Related apicomplexans *Toxoplasma* and *Plasmodium* have genomes the size of 80 Mb and 23Mb respectively [132, 136].

Molecular and microscopic analysis shows that *Cryptosporidium* does not have an apicoplast, a vestigial plastid present in most apicomplexans [131, 133, 139, 140]. Other apicomplexans

that lack an apicoplast include: colpodellids (predatory flagellates) and the gregarines (parasites of invertebrates) [131, 141-143]. Phylogenic analysis shows the gregarines and *Cryptosporidium* are early-branching apicomplexans and form a sister group [139, 144].

Cryptosporidium lacks de novo synthesis of many essential biomolecules including fatty acids, some amino acids, nucleosides and pyrimidines [145]. The parasite encodes for nucleotide salvage enzymes, as well as highly streamed scavenging pathways [146-149]. Transporters are also used for the uptake of sugars, amino acids and nucleosides from the environment [150]. *C. parvum* and *C. hominis* lack a TCA cycle which leads to oxidative phosphorylation because there is no mitochondrial genome [130, 137]. Energy generated by the parasite is therefore largely dependent on repeated cycles of glycolysis.

General apicomplexan motility and invasion

Knowledge of the motility and invasive machinery used by *Cryptosporidium* is lacking, providing an incomplete understanding of motility and invasion. The presence of an apical complex is a key feature of apicomplexans which consists of a conoid, micronemes and rhoptry organelle(s). These organelles are essential for motility and invasion [24]. The micronemes and rhoptry are storage vesicles for proteins that are eventually secreted to the parasite surface. The parasite has an inner membrane complex (IMC) that lies subjacent to the plasma membrane of sporozoites and is subject to competitive inhibition and neutralization by antibodies [28].

Cryptosporidium sporozoites use a gliding motility which is dependent on substrate binding [28]. Gliding motility appears to be conserved among apicomplexans with the mechanisms underlying the motility extensively investigated using *T. gondii.* Motility of *T. gondii* zoites is achieved through a glideosome, which is a complex that includes an actomyosin motor between parasite membrane and the IMC [151]. Adhesins and proteins released by micronemes to the

anterior surface of the parasite which subsequently bind host receptor proteins [152, 153]. Bound host proteins are translocated to the posterior pole of the zoite which propels the parasite forward [151, 154, 155]. Deposition of parasite protein has been observed on the host cell during gliding motility in the form of trails indicating proteolytic cleavage of surface parasite proteins[156 157].

Motility and invasion are tightly connected processes in *T. gondii*, as the molecular machinery required for motility is also used during invasion [153-155, 158, 159]. Invasion is an active process for *T. gondii* where apical orientation of *T. gondii* zoites results in the formation of a tight junction, also known as the moving junction (MJ) with the host cell [160]. The MJ is a tight connection between the parasite and host membrane facilitated by interactions between apical membrane antigen 1 (TgAMA1) on the parasite surface and the rhoptry neck protein (RON2), a parasite protein that is translocated to the host cell membrane [161]. The actomyosin powered movement of TgAMA1 in an anterior to posterior direction along the zoite surface propels the parasite through the host cell membrane and into a parasitophorous vacuole [161, 162]. Cleavage and release of TgAMA1 during invasion is facilitated by intermembrane rhomboid proteases expressed by the parasite [38, 163].

Rhomboid proteases

Transmembrane adhesins are cleaved by a family of intramembrane rhomboid proteases (ROMs) releasing the extracellular domain [164]. Cleavage of adhesins by rhomboids has been hypothesized to: 1.Facilitate motility and invasion by propelling the parasite forward during the translocation of surface adhesins from the anterior to the posterior, 2. Prevent the accumulation of surface proteins at the cell surface in order to evade an immune response, and 3. Allow for proper orientation of the parasite during invasion [34]. The active site of the rhomboid protease – a serine-histidine catalytic dyad that cleaves peptide bonds – is embedded in the membrane [34, 165].

All apicomplexan genomes sequenced to date have genes encoding rhomboid proteases [37, 166, 167]. Conventional nomenclature of ROMs in apicomplexans involves using the initials of the genus and species of the parasite, and designated number based on phylogenetic relationship to ROMs present in *T. gondii* and *P. falciparum* [167]. Phylogenetic trees constructed using ROM sequences from apicomplexans reveals that *C. parvum* encodes homologs of three of the six *T. gondii* ROMs, One homolog clustering with TgROM1 and 2, and two CpROMs that cluster with TgROM4 and TgROM5 respectively [167]. A fourth *Cryptosporidium* ROM was identified in *C. muris*, CmROM6, but not in *C. parvum,* showing the diversity of ROMs in the genus *Cryptosporidium* [168].

A comparison of ROM homologs in *T. gondii* (TgROMs) and *P. falciparum* (PfROMs) reveal similarities in localization and function of ROMs that cluster together in phylogenies [34, 37]. Although the function of ROM1 is unknown, in both *T. gondii* and *P. falciparum* the protease localizes to the micronemes or mononemes (microneme like storage vesicles) (PfROM1) [169, 170]. ROM2 has been identified in only *Neospora caninum* and *T. gondii*, localizing to the Golgi apparatus, and like ROM1, the function of ROM2 remains unknown [171]. CpROM4, TgROM4 and PfROM4 can be found throughout the plasma membrane [152, 171, 172]. Conditional knockouts of TgROM4 demonstrated an essential role in the cleavage of TgMIC2, TgAMA1 and TgMIC8 – all adhesins associated with motility and invasion [35, 152, 165, 173]. Critically, TgROM4 cleaves AMA1, releasing its cytoplasmic domain and triggering the transition from an invasive to a replicative stage [38]. CpROM4 can cleave GP900, a mucin-like glycoprotein that mediates attachment and invasion of host cells, and the thrombospondin related adhesive protein protein 10 (CpTSP10) [23, 172]. Thrombospondin proteins (TSPs) localize to micronemes and are necessary for motility and invasion [153, 174]. CpROM5 and TgROM5 are also found in the plasma

membrane, and CpROM5 can also be found in the oocyst wall [171, 172]. CpROM5 can cleave the transmembrane adhesive proteins CpTSP6, CpTSP8, CpTSP9, CpTSP10, TRAPC-1 and GP900, suggesting and major role in motility and invasion [172]. ROM6 localizes to the mitochondrial membrane and is the only protease that has homologs outside of the phylum apicomplexa [168]. Although extensive work has been done on the role of ROMs in *Toxoplasma* and *Plasmodium* little is known about how ROMs govern cellular processes in *Cryptosporidium*.

***Cryptosporidium* invasion and development**

Although *Cryptosporidium* uses a similar mechanism of gliding motility to other apicomplexans, application of the *T. gondii* model to understand *Cryptosporidium* biology has its limitations. This is clear when comparing the process of invasion, and the extensive remodeling of the host cell cytoskeleton during a *Cryptosporidium* infection, a process not observed in other apicomplexans [27, 157, 175-177].

Cryptosporidium is unusual in that it lacks homologs for AMA1 or RON2 [130, 132]. Also, *Cryptosporidium* zoites appear to not require the actomyosin motor, or a MJ to enter the host cell [157]. Cellular entry of *Cryptosporidium* is dependent on a phosphatidylinositol 3-kinase (PI-3K)/Cdc42 pathway that facilitates extensive host cell remodeling [178, 179]. Polymerization of host actin filaments causes a build-up of actin at the cell-parasite interface, accompanied by an increase in host cell volume via glucose uptake and water influx [27]. This facilitates membrane protrusion, driven by actin polymerization and the formation of the extracytoplasmic parasitophorous vacuole [28]. Production of Protease Activated Receptor-2 (PAR_2) also increases in response to *Cryptosporidium* which aids in host membrane remodeling and increases host mucus production [180].

During invasion, *Cryptosporidium* causes host activation of acid-sphingomyelinase, an enzyme important for sphingolipid-enriched membrane microdomain (SEM) aggregation and accumulation, which helps tp promote host cell remodeling [18, 27]. Disruption of SEM aggregation reduces attachment and invasion of *Cryptosporidium* sporozoites, and reduces the accumulation of Gal-GalNAc glycoproteins at the site of infection [18]. Experiments using Caco-2A cells showed maturation of intestinal cells leads to differences in the presentation, amount and arrangement of the glycoconjugates expressed on the on the host cell surface, reducing sporozoite infectivity [181]. Diminished levels of available glycoconjugates presented on the host cell surface and site of invasion is thought to negatively impact lectin-mediated sporozoite attachment to the host cells [182].

In further contrast to related apicomplexans, a defining feature of *Cryptosporidium* is the development of a unique intracellular but extracytoplasmic niche at the host cell surface for the duration of the life cycle [28]. Using an epimerite-like structure, the parasite forms a channel between the host cytoplasm and the developing trophozoite [175, 183]. The channel allows the parasite to draw nutrients from the host cell while remaining epicellular [184]. Like many gregarines, which lack intracellular life cycle stages, specialized structures such as mucrons, protomerites, and an epimerite facilitate attachment and nutrient uptake [185, 186]. Through the process of myzocytosis, also known as cellular vampirism, gregarines use these structures to feed on the contents of the host cell cytoplasm [187, 188]. This is in stark contrast to related apicomplexans like *Toxoplasma* and *Plasmodium* which both actively invade host cells and mature within the host cell cytoplasm [32, 189]. Consequently, the *Cryptosporidium* classification was recently re-evaluated, and it was decided that it should be reassigned to a new class, the Gregarinomorphea, with a specific subclass of Cryptogregaria [20, 30]. This reclassification is

defined by an organism that lacks an apicoplast, is epicellular and has a gregarine like feeder organelle [141].

In contrast to the host cell, little is known about the changes that take place in the *Cryptosporidium* sporozoite during invasion. One of the key unanswered questions is what triggers the parasite to switch from a motile, banana-shaped, invasive sporozoite to a rounded, replicating trophozoite. The trigger for replication appears to be independent of attachment or invasion of host cells as rounded, extracellular *Cryptosporidium* sporozoites have been observed in cell culture [190]. Studies on *C. parvum* and *C. hominis* in axenic culture have revealed the capacity for replication in the absence of host cells [191-193]. Evidence for extracellular development include changes in morphology and increses in DNA levels [128, 194]. Differerent extracellular stages of *Cryptosporidium* have been observed to pair, in a process known as syzygy, and this may be a precursor of merogony [184, 195-198]. Moreover, *Cryptosporidium* can use the epimerite-like structure to actively acquire nutrients from the external environment if not engaged with a host [199, 200]

The unique invasion strategy of *Cryptosporidium* positions the parasite between the extracellular gregarines and the intracellular parasites *Toxoplasma* and *Plasmodium*, sharing similarities in motility and nutrient uptake, but differing in the invasion process [184, 197]. Critically, the use of a feeder organelle, the epicellular location during an infection, and the ability to replicate without a host cell suggests that the triggers for the transition from invasion to replication are located externally to the host cytoplasm.

Mucin biology

The intestinal epithelium is responsible for the absorption of nutrients and water, and is also the primary line of defense against intestinal pathogens [201]. As part of the immune defense,

intestinal epithelial cells produce antimicrobial molecules and secrete a mucus layer [202]. Ranging from 300 to 700 μm in thickness, the mucus layer is comprised primarily of secreted, gel forming mucins [203, 204]. Secreted mucins are found at many epithelial surfaces, including the gastrointestinal, urinary and respiratory tracts, providing a protective barrier between the host cell surface and the native microbiota or invading pathogens [205-207].

Mucins are glycoproteins that are extensively O-glycosylated, with a common core N-acetyl-galactosamine (GalNAc) residue from which the glycan chain is extended [208]. The common core GalNAc, known as the T_n antigen, is α-linked to either a serine or threonine residue within the protein backbone. A hallmark of mucins is the presence of serine/threonine rich regions, referred to as variable number tandem repeats (VNTR). Overall the amino acid composition of mucins heavily favors serine, proline and threonine [209]. VNTR regions can have hundreds of O-GalNAc glycans added due to the abundance of acceptor sites for O-glycosylation. Addition of O-glycans within VNTRs cause clustering of the glycans and can give the mucin a "bottle brush" appearance. Due to the amount of glycans added to the protein backbone, O-glycans can make up to 80% of the molecular weight of the mucin [210]. Although O-glycosylation occurs at serine and threonine residues on mucins, the defined positions at which glycans are added is not known [209, 211].

O-glycosylation and addition of the T_n antigen to the mucin is dependent on the transfer of GalNAc from UDP-GalNAc to either serine or threonine through a polypeptode-N-acetyl-galactosaminyltransferase (ppGalNAcT) [212, 213]. Although 21 known ppGalNAcTs bind UDP-GalNAc, there are differences in the protein substrates that each one recognizes, with some preferentially adding to threonine, but not serine and vice versa [214, 215]. ppGalNAcTs have a lectin-like domain used to bind GalNAc glycans previously added to the mucin and *in vitro*

studies have shown further addition of GalNAc is influenced by existing O-glycosylation [212, 216]. Present within the Golgi, expression of ppGalNAcTs varies depending on tissue type [217]. The localization of the enzyme within the Golgi appears to play a role in determining the types of O-glycans produced by the cell, with ppGalNAcTs localized in late compartments of the Golgi producing shorter O-glycans, further contributing to the heterogeneity and diversity in the mucins produced by the cell [217]. Comparatively, in a strand of DNA, three nucleic acids can make six unique sequences, whereas three glycans on a mucin can provide over 1000 different arrangements.

The process of glycosylation, including the addition of glycans and the extension of glycan chains is poorly understood. Unlike the transcription and translation of DNA, there is no template for glycan addition. There are a total of 8 core O-GalNAc glycan structures decorating mucins [218], each building from the T_n antigen (Table 2). The type of core depends on the location, function, cell type and glycotransferase(s) present [212, 219, 220]. Core 1 is found in nearly all cell types and cells lacking core 1 synthesis have a higher expression of T_n and sialyl-T_n antigens [221]. Core 2 O-glycans are also found in many tissues and synthesis is correlated with immune system stimulation and tumor progression [221, 222]. Core 3 appears to be exclusive to epithelia of the gastrointestinal and respiratory tracts. Core 4 is predicated on core 3 synthesis. Core 3 is absent in cell culture of colonic cells and minimally expressed in colonic tumors [222]. Core 5 is found in colonic tissues and colonic adenocarcinoma; however the enzymes required for Core 5-8 synthesis have yet to be identified [218, 221]. In tumor cells, mucins lack the same glycosylation patterns as normal cells. Mucins from cancer cells are often decorated with only the T_n antigen, suggesting extension beyond the core GalNAc is blocked [222]. Another common

feature in cancer cells is the presence of sialyl-T_n antigen which is a sialic acid residue linked to GalNAc at the C-6 position, preventing the addition of other sugars [223].

Table 2. O-glycan core structures of mucins.

Core	Structure
Tn antigen	GalNAcαSer/Thr
Sialyl-Tn antigen	Siaα2-6GalNAcαSer/Thr
Core 1 or T antigen	Galβ1-3GalNAcαSer/Thr
Core 2	GlcNAcβ1-6(Galβ1-3)GalNAcαSer/Thr
Core 3	GlcNAcβ1-3GalNAcαSer/Thr
Core 4	GlcNAcβ1-6(GlcNAcβ1-3)GalNAcαSer/Thr
Core 5	GalNAcα1-3GalNAcαSer/Thr
Core 6	GlcNAcβ1-6GalNAcαSer/Thr
Core 7	GalNAcα1-6GalNAcαSer/Thr
Core 8	Galα1-3GalNAcαSer/Thr

From Brockhausen I, Schachter H, Stanley P. Essentials of Glycobiology. 2nd edition. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 2009.

In addition to being a physical barrier to invading pathogens, mucins function in intercellular recognition by serving as ligands for cell to cell interactions and signaling [224, 225]. Terminal glycans decorating mucins are biologically significant as these are the primary ligands targeted by other cells for signaling and attachment [225, 226]. As a consequence, terminal glycan decorations present at the host cell surface become targets for pathogen binding [227]. Pathogens have evolved to engage and bind mucins on the surface of host cells, mediating pathogen-host attachment [228, 229]. For example, terminal GlcNAc and galactose residues on respiratory epithelial cells are targeted by *Streptococcus pneumoniae* [230]. *Cryptosporidium* uses mucin-like glycoproteins and Gal-GalNAc specific lectins and to bind host mucins, facilitating motility, attachment and invasion [16, 17, 19, 23].

Cryptosporidium adhesins

A total of 31 mucin like-proteins are encoded by the *Cryptosporidium* genome and these function in attachment and invasion of host cells [133]. Genes encoding seven of these glycoproteins cluster on chromosome two [231], indicating the potential for co-expression and related functions [232]. CpMuc4 and CpMuc5 are antigenic surface mucins that are expressed throughout *Cryptosporidium* development, and inhibition studies have revealed that CpMuc4 is necessary for establishing an infection *in vitro* [231, 233]. CSL and P23 are glycoproteins that facilitate attachment, and P23 is shed from the surface of motile sporozoites [156]. Neutralization antibodies against CSL and P23 demonstrated the importance of these two glycoproteins in the processes of attachment and motility [156, 234-236].

A large mucin-like glycoprotein, GP900, localizes to the oocyst wall, tethering sporozoites to the oocyst wall, micronemes, and the surface of zoites [237, 238]. Gal-GalNAc decorations on GP900 have been implicated in host cell interactions that are necessary for attachment and invasion [23, 238]. A precursor protein, GP60, is proteolytically cleaved by the protease CpSUB1 to produce the catalytically active GP40/15, which is found in the oocyst wall and at the surface of sporozoites [237, 239, 240], contributing to attachment and invasion [16, 240]. GP40/15 is heavily o-glycosylated, similar to GP900 [240-242]. Lectins that specifically target the T and T_n antigens present on surface glycoproteins like GP900 and GP40/15 have been shown to inhibit sporozoite attachment to and invasion of host cells *in vitro* [240, 243].

Although mucins and glycoproteins aid in attachment and invasion, sporozoites pretreated with purified mucins have been shown to inhibit *Cryptosporidium* sporozoite infectivity. Bovine submaxillary mucin (BSM) lowered invasion of primary bovine cells and HCT-8 cells by *C. parvum*, but not entry of HCT-8 or primary human cells by *C. hominis*. This

effect was also observed after a pretreatment with free Gal-GalNAc, indicating *Cryptosporidium* sporozoite lectins specific for the T antigen (Gal-GalNAc) also mediate attachment and invasion of host tissues [17, 244].

CpClec, a C-type lectin with a mucin-like glycoprotein domain is found at the apical region of sporozoites, has been shown to bind Gal-GalNAc residues on host cells [245, 246]. *Cryptosporidium* lectin P30 has specificity for Gal-GalNAc glycans [16]. P30 is associated with GP900 and GP40/15 at the surface of sporozoites, forming an adhesive complex that plays a major role in attachment, motility and invasion [16, 23, 25, 178, 247]. The multivalent binding properties of P30 and shared sequence homology between GP900 and host MUC2 and MUC5 suggest P30 may enhance parasite attachment through the formation of complex lattice structures [23, 248-250]. Lectin-glycoprotein lattices play key roles in cell to cell signaling and induction of an immune response following host-pathogen interactions [251]. In the case of *Cryptosporidium*, the lattice structures formed as a result of lectin-Gal-GalNAc binding could provide the signal necessary for the intiation of replication.

Host immune response

Cryptosporidiosis is self-limiting and can be asymptomatic in hosts with a fully developed and competent immune system [252, 253]. Infections are most frequently localized to the intestine, but have also been reported in the lungs, eyes, ears, pancreas, biliary tract, and stomach [254-257]. The prepatent period varies between 2 and 10 days, with the infection typically lasting from 7 to 15 days [93, 258]. Cryptosporidiosis can become chronic and life-threatening in children, who have an underdeveloped immune system, and adults who are immunocompromised [259]. In children, cryptosporidiosis can severely impact cognitive function, growth, and physical development [260]. While it is clear that a competent and fully developed immune response is

necessary to clear cryptosporidiosis, a detailed understanding of the immune response to an infection by *Cryptosporidium* is lacking [261, 262].

In humans and cattle, *C. parvum* causes increased expression of the Toll-like receptors TLR2 and TLR4 on the surface of host cells [263, 264]. *Cryptosporidium* has glycoinositol phospholipids and glycosylphosphatidylinositol anchors which in *Plasmodium* have been shown to serve as potential ligands for TLR signaling [265-268]. TLRs are important in the recruitment of MyD88, which increases expression of pro-inflammatory cytokines via the transcription factor NF-κB [269-271]. A study showed knockdown of MyD88 expression using siRNA resulted in increased infectivity and also demonstrated the capacity for *C. parvum* to modulate the host immune response by dampening miRNA let-7 via an MyD88/NF-κB dependent pathway [272].

The mucus layer also contains antimicrobial peptides, including α- and β defensins and cathelicidins [273]. *Cryptosporidium* downregulates the constitutively expressed human-β-defensin-1 (HBD-1), but induces expression of HBD-2, indicating that the parasite modulates the host immune response to enhance survival [274].

Mannose-binding lectins (MBLs) present in host serum can bind *Cryptosporidium* and are an important activator for the lectin complement pathway of the innate immune system. MBL-associated serine proteases (MASPs) are activated by an antibody dependent process causing opsonization and formation of a membrane attack complex (MAC) while also promoting phagocytosis [275]. Immunocompromised patients with low serum MBLs or mutations in MBLs were more susceptible to *Cryptosporidium* infections [276]. Children in Haiti and Bangladesh with serum MBL deficiencies were also more likely to suffer from cryptosporidiosis [277, 278].

Human jejunal biopsies from cryptosporidial infections revealed increased expression of TNF-α and IL-1β, cytokines that stimulate prostaglandin synthesis [279]. Increased levels of

substance P, a neural peptide found in the GI tract which causes chloride ion secretion and potentially diarrhea, were observed in AIDS patients infected with *Cryptosporidium* [280, 281]. Infections with *C. parvum* also resulted in higher prostaglandin H synthase levels, which correlated with higher levels of prostaglandin and greater diarrheal output [282]. Production of prostaglandins may also cause HBD production, downregulating inflammatory cytokine synthesis and upregulating mucin synthesis [280]. Increased secretion of mucins can disrupt attachment of *Cryptosporidium*, providing a physical barrier and a display of mock epitopes for the removal of the parasite during mucus turnover [229, 283, 284].

IFN-γ is important for innate and adaptive immunity to *Cryptosporidium* in mice [285-287]. The role of IFN-γ in humans is less clear; however, a case of persistent cryptosporidiosis was reported in a child deficient in IFN-γ production, suggesting the importance of IFN-γ in clearing the parasite [288]. In experimental human infections, those who were seropositive for *Cryptosporidium* had increased IFN-γ production. Those who were seronegative showed no increases in IFN-γ, but had increased IL-15 expression that was associated with lower oocyst output [289-291]. IL-15 activates NK-cells, γδ-T-cells and immune cell recruitment to the site of infection for parasite removal [290]. In some experimentally infected humans TNF-β was also found [292]. Studies involving Caco-2 and HT-29 cancer cell lines indicate IFN-γ prevents invasion by stimulating the JAK/STAT pathway [293]. Further investigation revealed *Cryptosporidium* lowers host cell IFN-γ production, demonstrating the capacity for immune system evasion via JAK/STAT pathway suppression [294].

Epithelial cells produce chemokines which activate leukocytes and function as chemoattractants for inflammatory cells to the site of infection. Jejunal biopsies from AIDS patients with cryptosporidiosis showed greater expression of CXCL-10, a chemokine that

recruits T-cells, potentially inducing the JAK/STAT pathway [295]. IL-8 and CCL-5 are upregulated in human intestinal epithelial cells and intestinal xenographs infected with *C. parvum*, [296, 297]. IL-8 was found in fecal samples, and elevated levels of the chemokine were observed in peripheral blood harvested from malnourished children with cryptosporidiosis [298-300].

Increased susceptibility to severe cryptosporidiosis in immunocompromised individuals underscores the importance of cell mediated immunity and the T-cell response in clearing the infection. The incidence of cryptosporidiosis increases in immunocompromised individuals who have CD4+ lymphocyte counts that are below 200 cells/µl [135]. Persistent, reoccurring diarrhea was observed in 87% of patients with CD4+ counts below 180 cells/ml [301]. In an *ex vivo* analysis, CD4+ and CD8+ cells from seropositive donors exposed to *C. hominis* recombinant GP15 had marked increases in IFN-γ production in comparison to seronegative individuals [302]. The CD4+, MHC II dependent immune response, is mediated largely by IFN-γ [286]. Reduction in oocyst shedding and the presence of anti-*Cryptosporidium* antibodies correlated with mucosal IFN-γ production in human subjects experimentally infected with *Cryptosporidium* [289].

Deficiencies in CD4+ T-cell responses to cryptosporidial infections have been associated with less effective humoral immunity. Microarrays exposed to sera from seropositive humans showed the presence of both IgM and IgG antibodies [303]. Humans with deficiencies in IgM and IgA production have increased susceptibility to cryptosporidiosis [112, 304]. Patients in Bangladesh were found to have IgM, IgA, and IgG antibodies against Cp23, and had a shorter disease period [305]. Mucosal immunity plays a critical role in clearing an infection, with fecal IgA present during active cryptosporidiosis [306]. Pre-existing antibodies based on serological analysis of human volunteers suggests partial protection against subsequent infections; however, whether protection

is mediated by the antibodies or, more generally, cell-mediated immunological memory, is not known [286, 307-309].

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PAPER 1. GLYCOPROTEINS AND FREE GAL/GALNAC CAUSE *CRYPTOSPORIDIUM* TO SWITCH FROM AN INVASIVE SPOROZOITE TO A REPLICATIVE TROPHOZOITE[1]

Abstract

The apicomplexan parasite *Cryptosporidium* causes cryptosporidiosis, a diarrheal disease that can become chronic and life threatening immunocompromised and malnourished people. There is no effective drug treatment for those most at risk of severe cryptosporidiosis. The disease pathology is due to a repeated cycle of host cell invasion and parasite replication that amplifies parasite numbers and destroys the intestinal epithelium. This study aimed to better understand the *Cryptosporidium* replication cycle by identifying molecules that trigger the switch from invasive sporozoite to replicative trophozoite. Our approach was to treat sporozoites of *C. parvum* and *C. hominis*, the species causing most human cryptosporidiosis, with various media under axenic conditions and examine for rounding and nuclear division as markers of trophozoite development and replication, respectively. Fetal bovine serum (FBS) had a concentration dependent effect on trophozoite development in both species. Trophozoite development in *C. parvum*, but not *C. hominis*, was enhanced when RPMI supplemented with 10% FBS (RPMI-FBS) was conditioned by HCT-8 cells for 3 h. The effect of non-conditioned and HCT-8 conditioned RPMI-FBS on trophozoite development was abrogated by proteinase k and sodium metaperiodate pretreatment, indicating a glycoprotein trigger. *C. parvum* and *C.*

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hominis trophozoite development also was triggered by free Gal/GalNAc in a concentration dependent manner. *C. parvum* replication was greatest following treatments with free Gal/GalNAc, followed by conditioned RPMI-FBS and non-conditioned RPMI-FBS ($P<0.05$). *C. hominis* replication was significantly less than that in *C. parvum* for all treatments ($P<0.05$), and was greatest at the highest tested concentration of Gal/GalNAc (1 mM).

Introduction

The apicomplexan parasite *Cryptosporidium* infects all major vertebrate groups and causes the diarrheal disease cryptosporidiosis. Human cryptosporidiosis, caused primarily by *Cryptosporidium parvum* and *Cryptosporidium hominis,* is associated with severe illness and toddler mortality in developing countries [1] and chronic, life-threatening disease in immunodeficient and malnourished adults [2-4]. New drugs to treat cryptosporidiosis are urgently needed because current drugs lack efficacy, particularly in those with a compromised immune system [5]. One aspect of the infection with potential for drug development is the repeating cycle of host cell invasion and parasite replication that rapidly and massively amplifies *Cryptosporidium* numbers in the intestinal epithelium, resulting in the pathology that defines cryptosporidiosis. The task of identifying inhibitors of this cycle will be helped by a better understanding of how *Cryptosporidium* transitions from an invasive to a replicative mode.

Current knowledge of apicomplexan invasion and replication mechanisms is derived primarily from studies on *Toxoplasma gondii*. During invasion, the anterior surface of the *T. gondii* zoite forms a junction with the host cell membrane through the interaction of rhoptry neck proteins (RONs), which are exported from the parasite to the host cell membrane, and the parasite-associated apical membrane antigen 1 (AMA1) [6, 7]. Powered by an actomyosin motor, the zoite moves the junction posteriorly along its surface, moving it through the host cell

membrane and into a cytoplasmic vacuole [8]. AMA1 is cleaved by an intramembrane protease as the parasite enters the vacuole [9], and the cleaved cytoplasmic tail may trigger parasite replication [10], although this remains in some doubt [11, 12].

In contrast to *T. gondii*, *Cryptosporidium* invasion is a passive process that does not to require actomyosin motility machinery [13] and results in an extracytoplasmic rather than cytoplasmic parasitophorous vacuole. In studies on *C. parvum*, it has been shown that sporozoite surface lectins bind to Gal/GalNAc ligands on host cells [14, 15]. Aggregation of Gal/GalNAc glycoproteins in the host cell membrane at the sporozoite attachment site initiates a signaling cascade that culminates in actin-dependent membrane protrusion and encapsulation of the sporozoite in an extracytoplasmic vacuole [16, 17]. It is not known what causes the invasive *Cryptosporidium* sporozoite to transform into a replicative trophozoite, but its unusual invasion mechanism and unique extracytoplasmic niche in the host cell suggests a different trigger to that in *T. gondii*.

Cryptosporidium is more closely related to the gregarines, invertebrate parasites that frequently develop extracellular trophozoites, than to *T. gondii* and other apicomplexans that parasitize vertebrates [18-20]. Consistent with its gregarine affinity, *Cryptosporidium* can occasionally develop extracellular trophozoites in cell culture [21], and trophozoites and other life cycle stages have been observed even in the absence of host cells [22-27]. We reasoned that the trigger of trophozoite development under axenic conditions would be similar to that in host cells, and that axenic culture would provide a simple model to identify the trigger. Using this approach, here we show that banana-shaped *Cryptosporidium* sporozoites become rounded and undergo nuclear division, hallmarks of a transformation to replicative trophozoites, when

triggered by free Gal/GalNAc and by glycoproteins in fetal bovine serum (FBS) and the secretome of HCT-8 cells.

Materials and methods

Source and excystation of Cryptosporidium oocysts. Mouse passaged *C. parvum* Iowa isolate was purchased from Waterborne Inc. (New Orleans, LA). *C. hominis* TU502 was propagated in a gnotobiotic pig at Tufts University [28]. Oocysts of *C. parvum* and *C. hominis* were treated with 10% sodium hypochlorite for 10 min at 4°C, washed twice with PBS, and excysted for 1 h at 37°C and 5% CO_2 in RPMI-1640 supplemented with 0.8% sodium taurocholate (Sigma-Aldrich, St. Louis, MO).

Preparation of RPMI with 10% fetal bovine serum (RPMI-FBS) and HCT-8 conditioned RPMI-FBS. To prepare RPMI-FBS, RPMI-1640 was supplemented with 10% Opti-MEM, 10% FBS, antibiotic/antimycotic solution, 1 mM sodium pyruvate, 50 mM glucose, 0.2 mM ascorbic acid, 2.3 µM folic acid, 29 µM 4-aminobenzoic acid, and 8.4 µM calcium pantothenate. RPMI-FBS was conditioned by incubation with confluent HCT-8 cells for 3 h at 37°C and 5% CO_2. Non-conditioned and HCT-8 conditioned RPMI-FBS were stored at 37°C and 5% CO_2 until use.

Treatment of sporozoites with non-conditioned and HCT-8 conditioned RPMI-FBS. For all treatments, excysted sporozoites of *C. parvum* and *C. hominis* were washed once with PBS, resuspended in 200 µL of media, and incubated at 37°C and 5% CO_2 for 0, 3, 4, 5, or 6 h. Treatments consisted of non-conditioned and conditioned RPMI-FBS; RPMI supplemented with 0, 5, 10, or 20% FBS; and non-conditioned and conditioned RPMI-FBS that was pretreated with proteinase k or sodium metaperiodate. Proteinase k (5 mg/ml) was added to RPMI-FBS and conditioned RPMI-FBS and incubated at 55°C overnight to digest proteins [29]. Proteinase k was subsequently inactivated by incubation at 90°C. Heat inactivated proteinase k was added to non-

conditioned and conditioned RPMI-FBS as a control. To oxidize glycans, an equal volume of 100 mM sodium metaperiodate (pH 7.2) was incubated with RPMI-FBS or conditioned RPMI-FBS overnight at 4°C in the dark. Excess sodium metaperiodate was removed by adding an equal volume of 260 mM glycerol. As a control, glycerol was added directly to non-conditioned or conditioned RPMI-FBS without sodium metaperiodate pretreatment.

Following treatments, zoites were spun onto a glass slide at 2,000 *g* for 5 min using a Shandon CytoSpin 2 (Thermo-Scientific, Waltham, MA), fixed in 100% MeOH for 15 min, and washed twice with PBS for 3 min. Fixed zoites were blocked with 1% BSA for 20 min at room temperature, washed with PBS, and labeled with FITC- or TRITC-SporoGlo (Waterborne Inc. New Orleans, LA). Slides were mounted and the numbers of banana-shaped and rounded zoites were determined under differential interference contrast (DIC) and fluorescence illumination in each of twenty, randomly selected high-powered fields using an Olympus BX61 microscope.

Viability assessment. The viability of *C. parvum* and *C. hominis* zoites was determined following exposure to non-conditioned and conditioned RPMI-FBS for 3, 4, 5, 6, 7, 8, and 12 h. Zoites were incubated with 0.04 mM fluorescein diacetate (FDA) and 7 µM propidium iodide (PI) in PBS for 3 min at room temperature [30]. A wet mount of stained zoites was prepared and the number of green fluorescing (FDA positive; viable) and red fluorescing (PI positive; non-viable) zoites was determined under fluorescence illumination in each of twenty, randomly selected high-powered fields using an Olympus BX61 microscope.

Treatment of sporozoites with Gal/GalNAc. Sporozoites of *C. parvum* and *C. hominis* were treated with 0 or 10 µM Gal/GalNAc (Sigma-Aldrich) in PBS, non-conditioned RPMI-FBS, or conditioned RPMI-FBS at 37°C and 5% CO_2 for 1 or 7 h. In a separate experiment, sporozoites of *C. parvum* and *C. hominis* were incubated with 0 µM, 10 µM, 100 µM, or 1 mM

Gal/GalNAc in PBS at 37°C and 5% CO_2 for 7 h. Slides were stained as described previously, with additional staining of nuclei using 300 nM 4', 6-diamidino-2-phenylindole (DAPI). The numbers of banana-shaped and rounded zoites and the proportion of zoites with two or more nuclei were determined under differential interference contrast (DIC) and fluorescence illumination in each of twenty, randomly selected high-powered fields using an Olympus BX61 microscope.

Statistical analyses. The proportion of rounded, viable, or multinucleate zoites in each of 20 high-powered fields was used to calculate mean and standard deviation values. Differences between treatments were determined at the 5% level of significance using the Student's *t*-test with the Holm-Šídák correction for multiple comparisons [31]. Statistical analyses were carried out using Prism 7 (Graphpad, La Jolla, CA).

Results

Non-conditioned and HCT-8 conditioned RPMI-FBS cause C. parvum and C. hominis sporozoites to become rounded. For both species, banana-shaped sporozoites (mean length to width ratio: 2.96 ± 0.54 µm) became rounded (mean length to width ratio: 1.14 ± 0.14 µm) following treatments in non-conditioned and HCT-8 conditioned RPMI-FBS (Figure 3). In non-conditioned RPMI-FBS, a greater proportion of *C. hominis* than *C. parvum* were rounded after 3, 4, and 5 h ($P<0.05$), but there was no significant difference between the species after 6 h. Conditioning of RPMI-FBS with HCT-8 cells increased the proportion of *C. parvum* sporozoites that became rounded, particularly at early time points: 63.4 ± 4.1% and 94.2 ± 4.5% of *C. parvum* were rounded after 3 and 4 h treatments, respectively, in conditioned RPMI-FBS, compared to 6.9 ± 1.9% and 31.1 ± 2.1%, respectively, in non-conditioned RPMI-FBS.

Compared to *C. parvum*, conditioning of RPMI-FBS had little effect on *C. hominis* rounding, particularly at early time points.

The proportions of *C. parvum* and *C. hominis* that were rounded increased with the concentration of FBS in RPMI (Figure 3). When FBS was excluded, only 12.2 ± 2.0% and 14.1 ± 2.7% of *C. parvum* and *C. hominis*, respectively, were rounded after 6 h, compared to 95.8 ± 5.2% and 91.9 ± 8.4%, respectively, in RPMI with 20% FBS.

Collectively, these data show that molecules in FBS cause banana-shaped sporozoites of *C. parvum* and *C. hominis* to become rounded, and that molecules secreted by HCT-8 cells increase rounding in *C. parvum*.

C. parvum and C. hominis remain viable in non-conditioned and HCT-8 conditioned RPMI-FBS. Some studies on axenic *Cryptosporidium* development have used the change in sporozoite morphology from banana-shaped to rounded to indicate trophozoite development [23-25], while others have reported that rounding results from a loss of sporozoite viability [32, 33]. To assess the likelihood that rounding is degenerative rather than developmental, viability was examined after treatment of *C. parvum* and *C. hominis* sporozoites with non-conditioned or conditioned RPMI-FBS for periods ranging from 3 to 12 h (Figure 4). In conditioned RPMI-FBS, 100% of *C. parvum* zoites were viable after 6 h, decreasing to 91.2 ± 4.7% after 12 h. In non-conditioned RPMI-FBS, 98.1 ± 3.7% and 88.9 ± 4.2% of *C. parvum* were viable after 6 and 12 h, respectively. *C. hominis* was less viable than *C. parvum* at time points between 5 and 12 h in conditioned RPMI-FBS and between 5 and 7 h in non-conditioned RPMI-FBS ($P<0.05$). In conditioned RPMI-FBS, the viability of *C. hominis* sporozoites decreased from 96.3 ± 5.7% after 3 h to 89.4 ± 5.1% after 6 h and 84.0 ± 5.7% after 12 h. Similar reductions in *C. hominis* viability were observed in non-conditioned RPMI-FBS. These relatively minor reductions in viability,

particularly during the initial 6 h period when most cells became rounded, are evidence that rounding is not associated with a loss of viability. We therefore concluded that rounding was indicative of trophozoite development rather than mortality.

Development of rounded trophozoites is triggered by glycoproteins in non-conditioned and HCT-8 conditioned RPMI-FBS. To determine if glycoproteins in non-conditioned and HCT-8 conditioned RPMI-FBS trigger trophozoite development, we pretreated these media with proteinase k and periodate to digest proteins and oxidize glycans, respectively (Figure 5). After 6 h in conditioned RPMI-FBS that had been pretreated with proteinase k, 39.0 ± 2.1% and 42.1 ± 2.7% of *C. parvum* and *C. hominis*, respectively, were rounded, compared to 99.1 ± 4.8% and 92.7 ± 5.6%, respectively, in conditioned RPMI-FBS without pretreatment. Similarly, 43.2 ± 3.8% and 38.0 ± 3.4% of *C. parvum* and *C. hominis*, respectively, were rounded after 6 h in proteinase k pretreated non-conditioned RPMI-FBS, compared to 70.8 ± 3.7% and 78.3 ± 5.3%, respectively, without pretreatment. The addition of heat-inactivated proteinase k to non-conditioned and conditioned RPMI-FBS had no effect on trophozoite development (data not shown). Periodate oxidation of glycans had a more dramatic effect on trophozoite development: after 6 h, 3.2 ± 1.0% and 8.9 ± 1.7% of *C. parvum* and *C. hominis*, respectively, were rounded in periodate pretreated conditioned RPMI-FBS. Similarly, 1.9 ± 0.7% and 6.4 ± 2.7%, of *C. parvum* and *C. hominis*, respectively, were rounded in non-conditioned RPMI-FBS pretreated with periodate. The addition of glycerol to non-conditioned and conditioned RPMI-FBS without periodate pretreatment had not effect on trophozoite development (data not shown). These data are evidence that the trigger of trophozoite development in RPMI-FBS and conditioned RPMI-FBS is a glycoprotein.

Free Gal/GalNAc triggers trophozoite development and nuclear division in C. parvum and C. hominis. We next examined the effect of free Gal/GalNAc on trophozoite development in *C. parvum* and *C. hominis*. Sporozoites were incubated for 1 and 7 h at 37°C in PBS, non-conditioned RPMI-FBS, and conditioned RPMI-FBS, with and without 10 μM Gal/GalNAc (Figure 6). A similar proportion of *C. parvum* zoites became rounded after 1 h in conditioned RPMI-FBS with (19.1 ± 2.4%) and without (19.1 ± 2.3%) Gal/GalNAc. For all other 1 h treatments, the inclusion of Gal/GalNAc caused a greater proportion of sporozoites to become rounded ($P<0.05$), but the proportion was generally less than 5%. The proportion of rounded *C. parvum* and *C. hominis* zoites approached 100% in non-conditioned and conditioned RPMI-FBS after 7 h, whether or not Gal/GalNAc was included. However, a greater proportion of *C. parvum* and *C. hominis* were rounded after 7 h in PBS with Gal/GalNAc (81.9 ± 5.7% and 61.9 ± 4.4%, respectively) than without Gal/GalNAc (7.4 ± 1.1% and 4.4 ± 1.1%, respectively).

DAPI staining showed some zoites to have two or more nuclei after 7 h treatments, indicating that nuclear division had taken place (Figure 7). Most multinucleate zoites were rounded (Figure 7B), but banana-shaped zoites also were observed (Figure 7C). Similar proportions of *C. parvum* zoites were multinucleate when Gal/GalNAc was included in PBS (39.2 ± 4.0%), non-conditioned RPMI-FBS (41.8 ± 3.8%), and conditioned RPMI-FBS (36.2 ± 4.8%). When Gal/GalNAc was excluded, the proportion of multinucleate *C. parvum* zoites was greatest in conditioned RPMI-FBS (12.0 ± 2.4%), followed by non-conditioned RPMI-FBS (5.0 ± 1.9%), and PBS (0.39 ± 0.39%) ($P<0.05$). The proportion of multinucleate *C. parvum* zoites was significantly greater in all treatments that included Gal/GalNAc ($P<0.05$).

Compared to *C. parvum*, the proportions of multinucleate *C. hominis* zoites were smaller for all treatments ($P<0.05$). Multinucleate zoites were not detected in PBS without 10 μM

Gal/GalNAc, and the proportion of multinucleate zoites was only 1.2 ± 0.5% when 10 µM Gal/GalNAc was included. The proportions of multinucleate *C. hominis* zoites in non-conditioned and conditioned RPMI-FBS, with and without Gal/GalNAc, were similar to that in PBS with Gal/GalNAc ($P>0.05$).

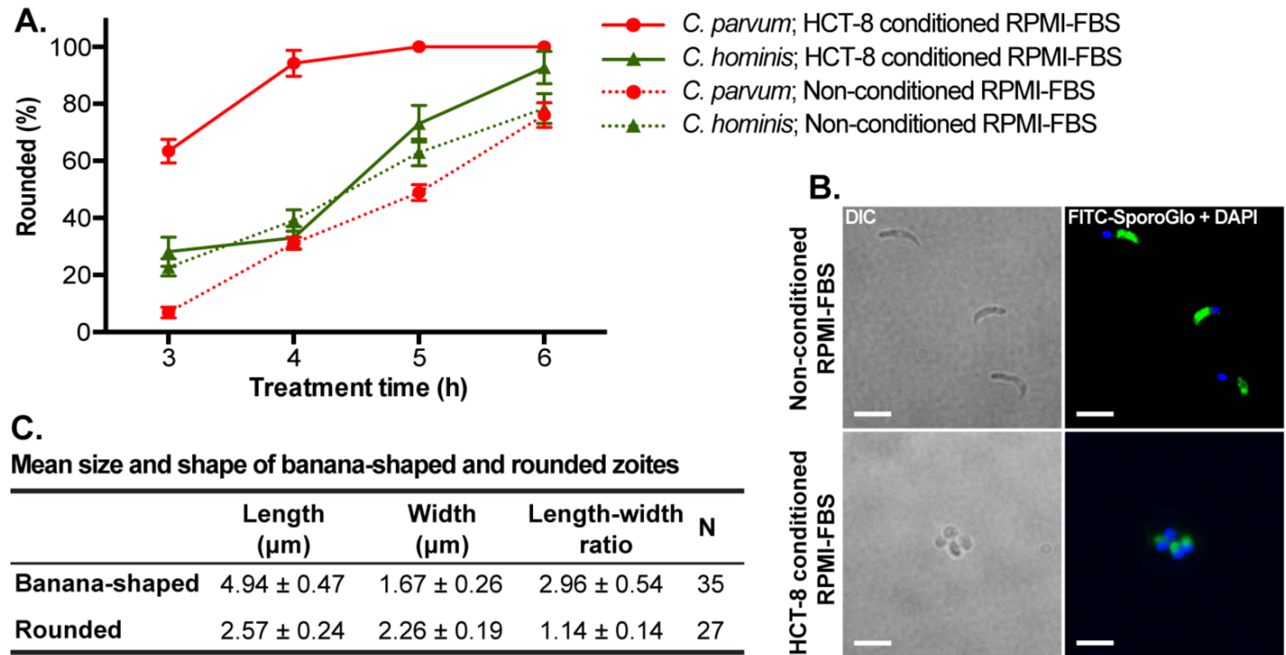
To determine if trophozoite development and nuclear division are dependent on the Gal/GalNAc concentration, we treated *C. parvum* and *C. hominis* with 0 µM, 10 µM, 100 µM, and 1 mM Gal/GalNAc in PBS (Figure 8). Increasing the Gal/GalNAc concentration caused a greater proportion of *C. parvum* and *C. hominis* zoites to become rounded ($P<0.05$). The proportion of *C. hominis* zoites that were multinucleate also increased from 0.9 ± 0.4% at 10 µM Gal/GalNAc to 12.7 ± 3.1% at 1 mM Gal/GalNAc ($P<0.05$). The proportion of multinucleate *C. parvum* zoites did not vary with Gal/GalNAc concentration.

Discussion

Apicomplexans alternate between invasion and replication during development, and efficient switching between these modes contributes to rapid parasite amplification. The switch to replication must be precisely timed because it involves major physiological changes that could compromise invasion if initiated prematurely. Here we have shown that the switch from invasive sporozoite to replicative trophozoite in *Cryptosporidium* is triggered by glycoproteins in FBS and the secretome of HCT-8 cells and by free Gal/GalNAc.

FBS triggered trophozoite development in *C. parvum* and *C. hominis* in a concentration dependent manner, with a transformation rate of approximately 80% in 6 h in 10% FBS. This is more rapid than previously reported in 1% FBS, where trophozoites of *C. parvum* and *C. hominis* were not observed before 24 h [22, 23, 25]. FBS similarly caused trophozoite development in the related apicomplexan *Plasmodium berghei* [34], a species that also develops intracellular

trophozoites in vivo. However, the transformation rate of *P. berghei* trophozoites in 10% FBS (13% in 24 h) was considerably lower than that in the present study.



Mean size and shape of banana-shaped and rounded zoites

	Length (µm)	Width (µm)	Length-width ratio	N
Banana-shaped	4.94 ± 0.47	1.67 ± 0.26	2.96 ± 0.54	35
Rounded	2.57 ± 0.24	2.26 ± 0.19	1.14 ± 0.14	27

Figure 2. Sporozoites of *C. parvum* and *C. hominis* become rounded following treatment in RPMI supplemented with 10% FBS (RPMI-FBS), and the rate of *C. parvum* rounding increases when RPMI-FBS is conditioned by HCT-8 cells.
[A] *C. parvum* and *C. hominis* sporozoites were treated with RPMI-FBS and HCT-8 conditioned RPMI-FBS (conditioned at 37°C and 5% CO_2 for 3 h.) [B] *C. parvum* zoites under DIC and fluorescence illumination following 3 h in non-conditioned RPMI-FBS (top panels) and HCT-8 conditioned RPMI-FBS (bottom panels). Note the banana-shaped zoites in the non-conditioned RPMI-FBS and the rounded zoites following treatment with HCT-8 conditioned RPMI-FBS. Scale bar is 5 µm. [C] Mean length, width, and length-to-width ratio of banana-shaped and rounded zoites.

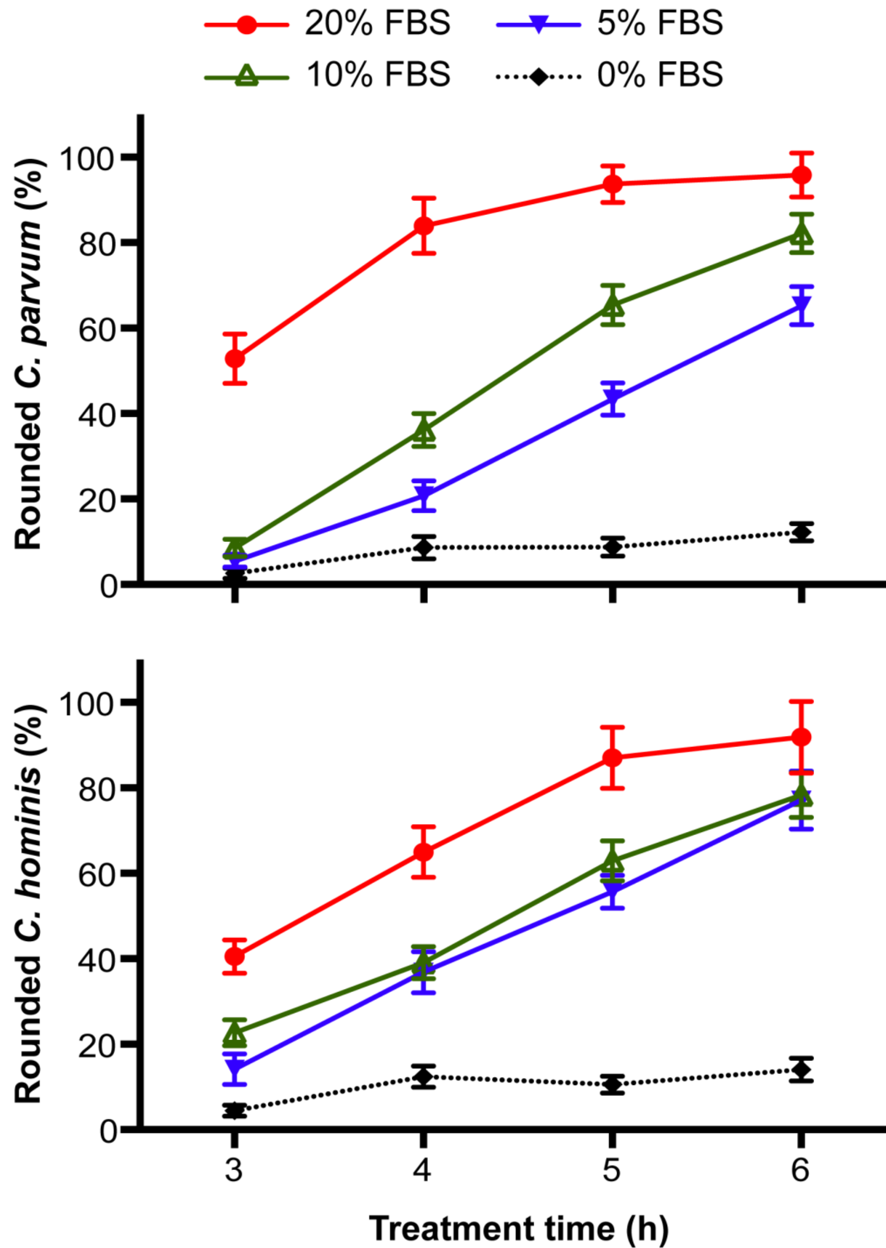


Figure 3. FBS causes rounding of sporozoites in a concentration dependent manner. Sporozoites of *C. parvum* and *C. hominis* were treated at 37°C and 5% CO_2 with RPMI supplemented with 0, 5, 10, or 20% FBS.

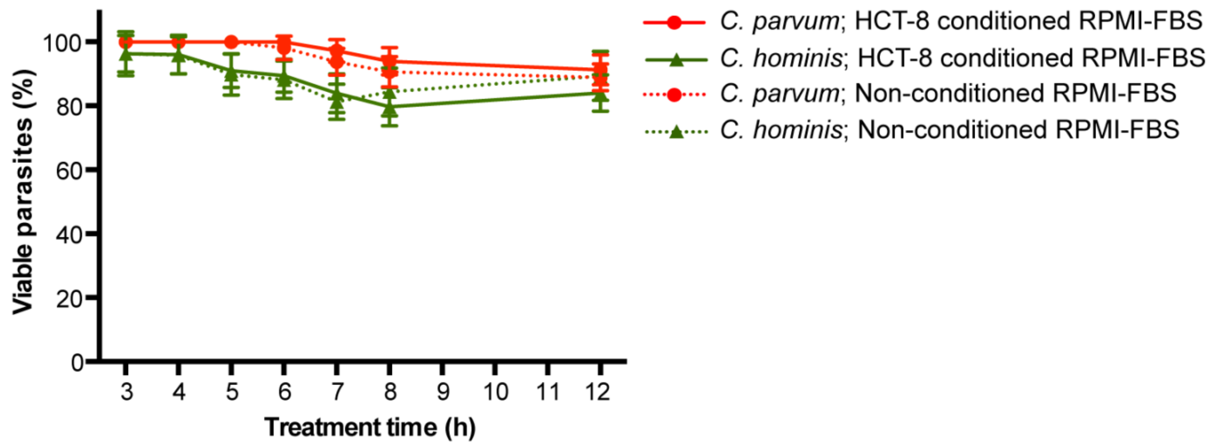


Figure 4. Rounding is not associated with a loss of viability.
The viability of *C. parvum* and *C. hominis* zoites was determined using fluorescein diacetate-propidium iodide staining following treatment with non-conditioned or HCT-8 conditioned RPMI-FBS at 37°C and 5% CO_2 for periods ranging from 3 to 12 h.

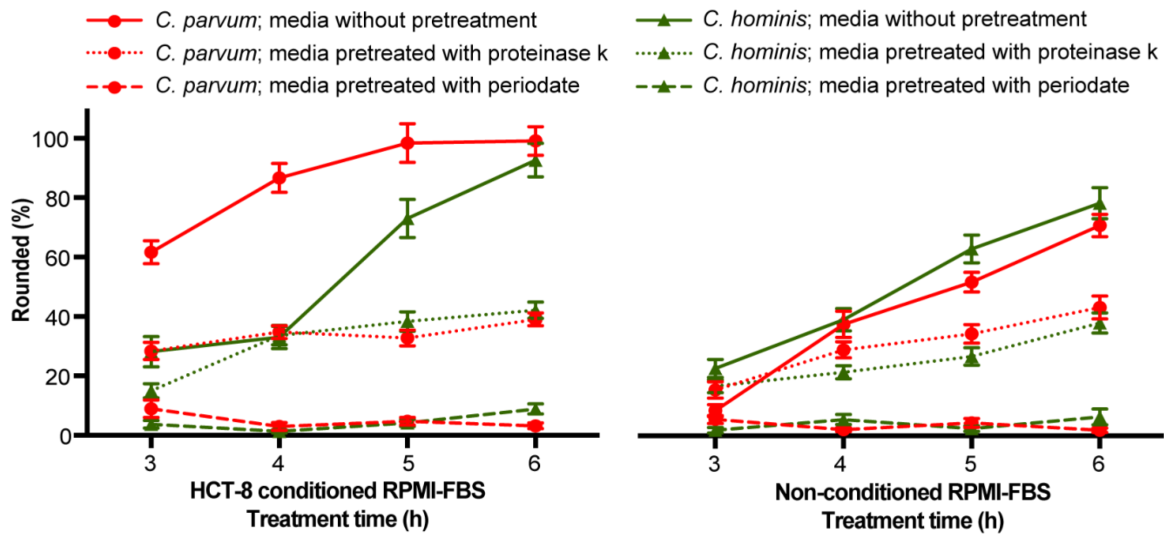


Figure 5. A glycoprotein in non-conditioned and HCT-8 conditioned RPMI-FBS causes rounding.
Sporozoites of *C. parvum* and *C. hominis* were treated with non-conditioned and HCT-8 conditioned RPMI-FBS without pretreatment, following pretreatment with proteinase k, or following pretreatment with sodium metaperiodate.

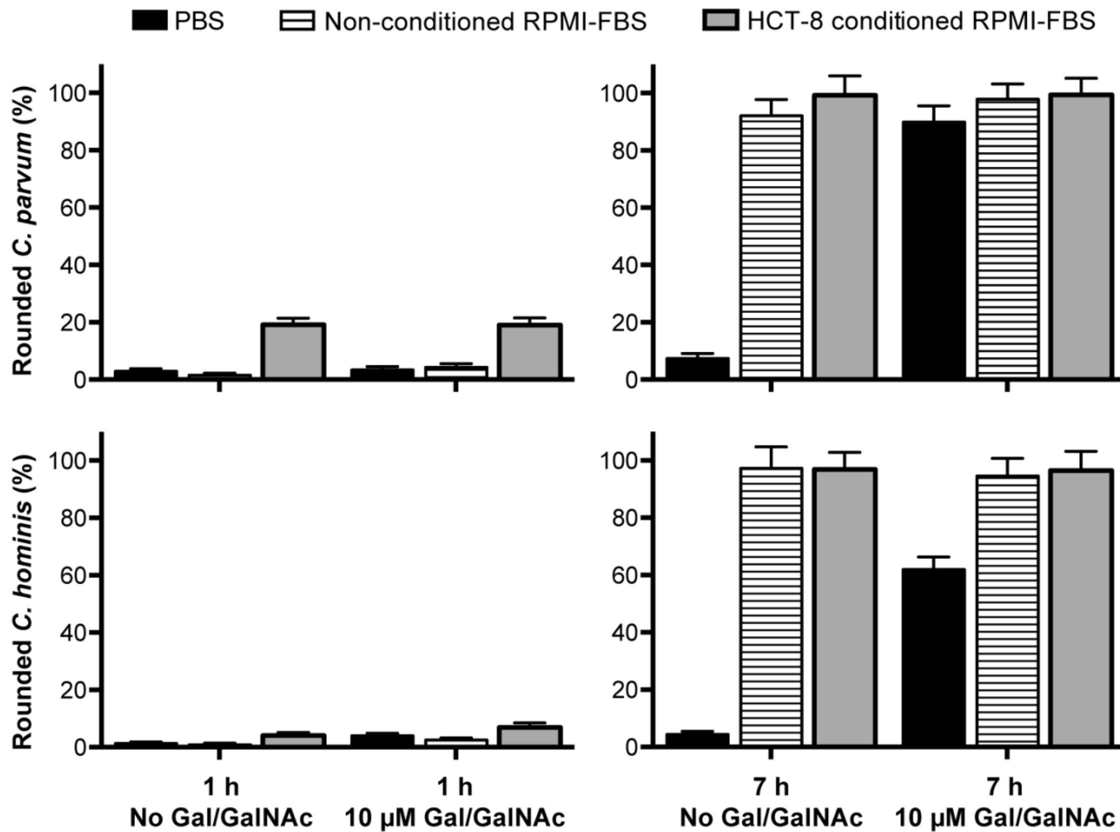


Figure 6. Treatment with 10 µM Gal/GalNAc causes rounding of *C. parvum* and *C. hominis* sporozoites.
Sporozoites of *C. parvum* and *C. hominis* were treated for 1 or 7 h with PBS, non-conditioned RPMI-FBS, or HCT-8 conditioned RPMI-FBS, with and without 10 µm Gal/GalNAc.

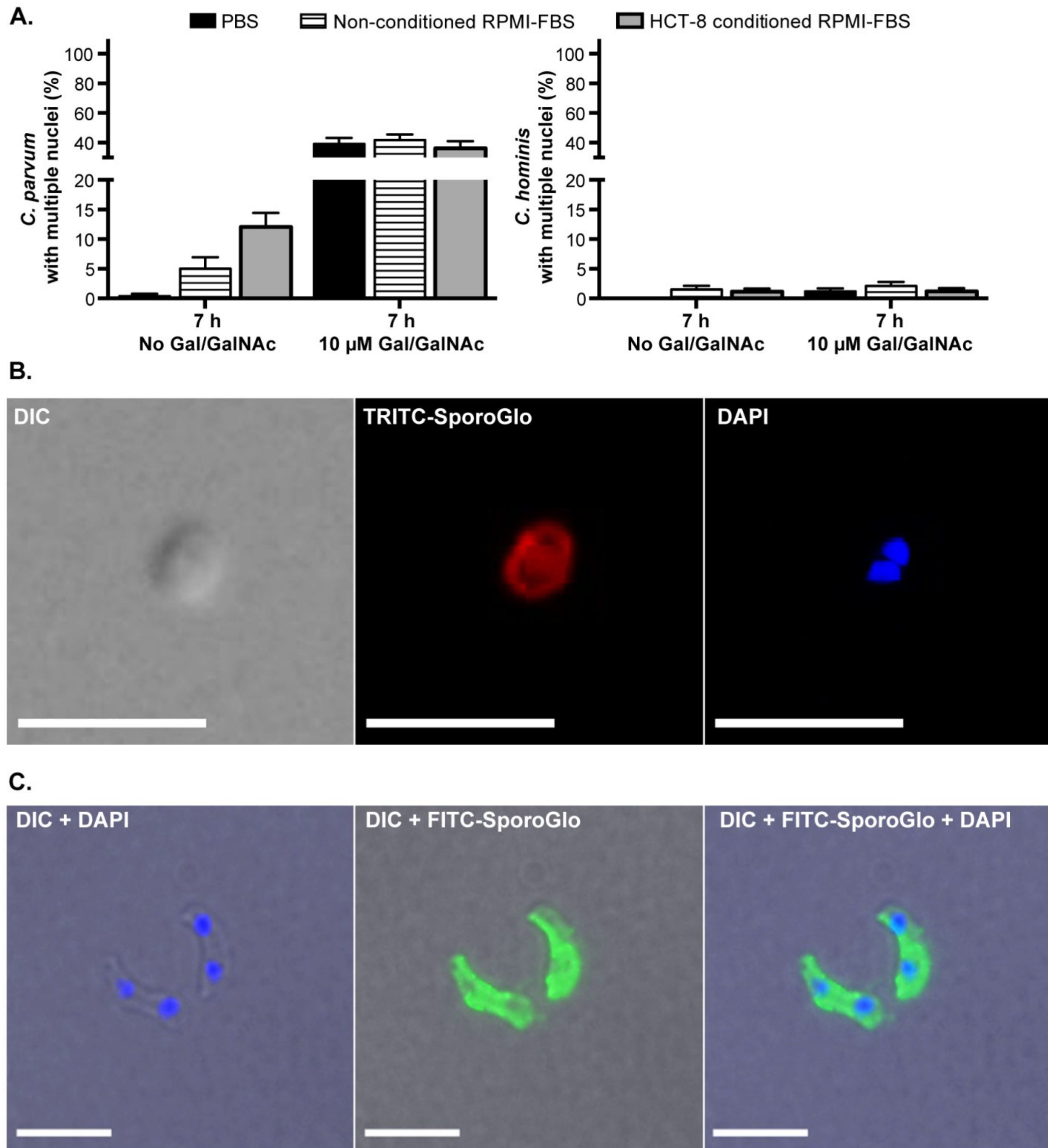


Figure 7. Treatment with 10 µM Gal/GalNAc causes nuclear division.
[A] Sporozoites of *C. parvum* and *C. hominis* were treated for 7 h with PBS, non-conditioned RPMI-FBS, or HCT-8 conditioned RPMI-FBS, with and without 10 µm Gal/GalNAc. [B] Rounded *C. parvum* zoite with two nuclei. [C] Banana-shaped *C. parvum* zoite with two nuclei. Scale bar is 5 µm.

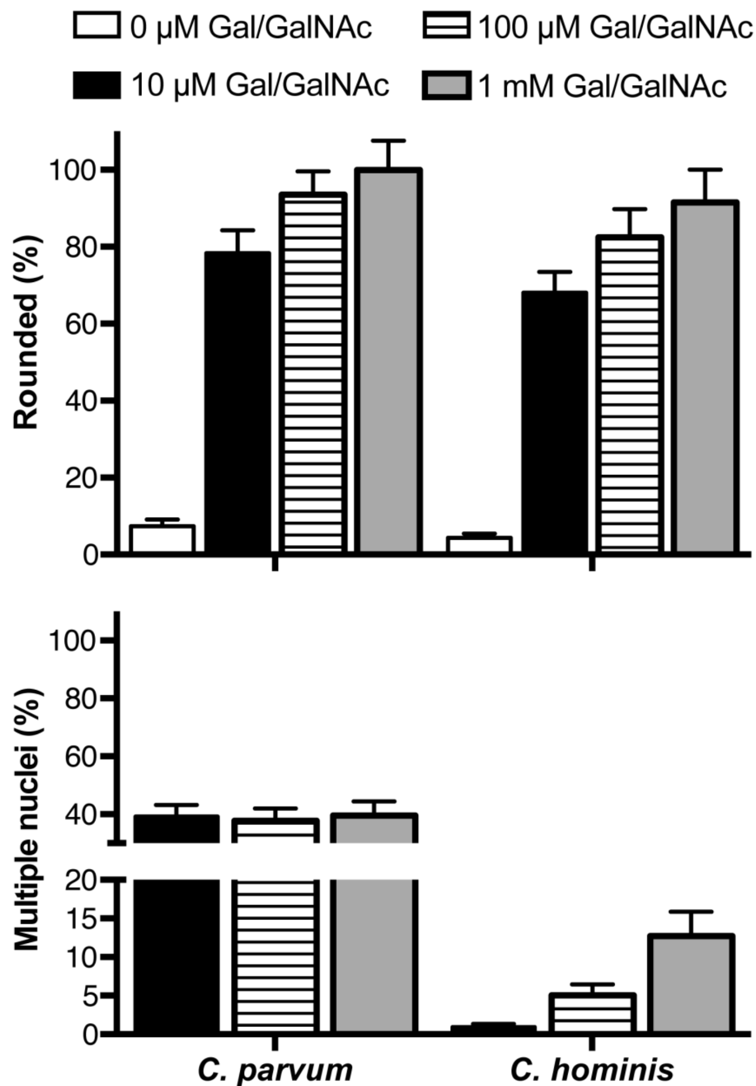


Figure 8. The proportions of rounded *C. parvum* and *C. hominis* zoites and the proportion of multinucleate *C. hominis* zoites increase with increasing Gal/GalNAc concentration. Sporozoites of *C. parvum* and *C. hominis* were treated for 7 h with 0 μM, 10 μM, 100 μM, and 1 mM Gal/GalNAc in PBS.

A report that extracellular *Cryptosporidium* trophozoites can develop in the supernatant from cultured HCT-8 cells [21] prompted us to examine the effect of the HCT-8 cell secretome on trophozoite development. The conditioning step to obtain the secretome required FBS for HCT-8 cell growth, so we were unable to determine whether the secretome can trigger trophozoite development independently of FBS. However, by comparing the effects of non-conditioned and

conditioned RPMI-FBS we found that the HCT-8 cell secretome significantly enhances trophozoite development in *C. parvum* but has little effect on *C. hominis*. More rapid trophozoite development in *C. parvum* should result in more rapid replication and a more proliferative infection. Consistent with this, a study by [35] showed that *C. parvum* is more proliferative than *C. hominis* in HCT-8 cells, infecting about a third more cells. If the rate of trophozoite development is an important factor in determining the success of an infection, the specificity of the trophozoite trigger could contribute to the host specificity of *Cryptosporidium* species.

The reduction in trophozoite development following pretreatment of non-conditioned and conditioned RPMI-FBS with proteinase k and periodate was evidence of a glycoprotein trigger. In particular, trophozoite development was almost completely prevented by periodate pretreatment, demonstrating that glycans are critical for trigger activity. We therefore asked if the glycan Gal/GalNAc that is targeted by *C. parvum* sporozoites during attachment to and invasion of host cells [14, 15] also triggers trophozoite development. We found that free Gal/GalNAc triggered not only trophozoite development but also nuclear division in *C. parvum*, indicating that the parasite had transitioned to a replicative mode. While non-conditioned and conditioned RPMI-FBS also triggered replication, which was not detected in our earlier experiments because DAPI staining was not included, these media were less effective triggers than free Gal/GalNAc. A possible explanation for this finding is that the Gal/GalNAc epitopes in FBS and the HCT-8 cell secretome were insufficient in number and/or density to meet the threshold to trigger replication. Gal/GalNAc, which is generally covered by other glycan molecules in mucin glycoproteins, is uncovered and reactive in carcinoma cells such as HCT-8 [36], which could explain the greater replication observed when RPMI-FBS was conditioned by HCT-8 cells. Glycoproteins with a sufficient number and density of Gal/GalNAc epitopes to

trigger replication may be more abundant in the host cell membrane than the secretome, which would increase the likelihood of differentiation coinciding with attachment and invasion.

C. hominis was less responsive than *C. parvum* to the Gal/GalNAc replication trigger, with only about 1% of zoites showing evidence of nuclear division after 7 h in 10 µM Gal/GalNAc. This is consistent with previous work showing that sporozoite pretreatment with 10 µM Gal/GalNAc inhibits infection of HCT-8 cells by *C. parvum* but not *C. hominis* [37], and suggests that *C. hominis* may not use Gal/GalNAc to infect HCT-8 cells [37] or to trigger differentiation. Alternatively, *C. hominis* may have a different threshold for Gal/GalNAc mediated attachment and differentiation than *C. parvum*. This is supported by the finding that trophozoite development and replication in *C. hominis* increased with the increasing Gal/GalNAc concentration.

In summary, we have identified triggers of the developmental transformation from an invasive to replicative form in *C. parvum* and *C. hominis*. The ability to induce differentiation outside the host cell opens new opportunities to study regulatory pathways driving the complex developmental changes in the life cycle of *Cryptosporidium* parasites.

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PAPER 2. FURTHER CHARACTERIZATION OF GLYCOPROTEIN AND FREE GAL-GALNAC EFFECTS ON *CRYPTOSPORIDIUM* DEVELOPMENT[2]

Abstract

Cryptosporidium is an apicomplexan parasite that causes the diarrheal disease known as cryptosporidiosis. A disease that can become chronic and life threatening, the pathology of cryptosporidiosis is defined by repeated cycling between host invasion and replication leading to damage of intestinal epithelium. Our previous work outlined host glycoproteins and Gal-GalNAc glycans as triggers for the transition from invasion to replication in *Cryptosporidium*. The work presented here is designed to address some of the gaps in knowledge that were not covered in our previously publication. We began by examining if rounded sporozoites are apoptotic, and if media from infected host cells promote more sporozoite rounding. We followed this by determining if rounded sporozoites are still infective. Using rounding as a marker for differentiation, we found sporozoite rounding was an active process and not the result of a degenerative phenotype. When compared to a control group, rounded sporozoites have lowered rate of infectivity indicating invasion is primarily host driven. Sporozoites treated with media isolated from active infections at 3 h had higher proportions of rounded cells when compared to media isolated from 24 h infections and non-infected HCT-8 cells.

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[2] The material in this chapter was co-authored by Adam Edwinson and Dr. John M. McEvoy. Adam Edwinson had the primary responsibility for collecting data, analysis, interpretation, and writing of this manuscript. John McEvoy served as the proofreader.

Introduction

Cryptosporidiosis, caused by the protozoan parasite *Cryptosporidium,* is a leading cause of infant death from diarrheal disease in developing countries [1, 2] and a cause of chronic diarrheal disease in immunocompromised populations [3-5]. There is no vaccine and drug treatments are ineffective in those who are most vulnerable to severe disease[6].

In previous work, we presented evidence that secreted host cell glycoproteins and free Gal-GalNAc cause *Cryptosporidium* to switch from an invasive sporozoite to a replicative trophozoite independently of host cells [7]. We used rounding and nuclear division as markers of trophozoite development. This was predicated on the knowledge that during an infection sporozoites invade cells and become rounded trophozoites that undergo multiple nuclear divisions prior to cytokinesis. Here, we present further evidence to support the conclusion that secreted host cell glycoproteins and free Gal-GalNAc trigger trophozoite development, trophozoite formation results in reduced infectivity, and we show that secretion of the glycoprotein trigger is increased in the early stages of an infection.

Materials and methods

Cryptosporidium oocyst source and excystation. Mouse passaged Iowa isolate of *C. parvum* was purchased from Waterborne Inc. (New Orleans, LA). Oocysts were treated with a 10% sodium hypochlorite solution for 10 min at 4°C. After washing three times with PBS, oocysts were resuspended in RPMI-1640 supplemented with 0.8% sodium taurocholate (Sigma-Aldrich, St. Louis, MO), held at 37°C, 5% CO_2 for 1 h.

Assessment of apoptosis in sporozoites exposed to the trophozoite trigger. In our previous work, we used vital dye staining to show that exposure to the trophozoite trigger did not cause a reduction in viability, leading us to conclude that rounding was not a degenerative process.

However, vital dye staining cannot differentiate apoptotic and viable cells because the outer membrane of apoptotic cells remains intact and impermeable to propidium iodide. Protozoans are capable of apoptosis and in the related apicomplexan *T. gondii*, apoptotic zoites take on a rounded morphology [8]. Therefore, we tested for apoptosis in *C. parvum* following treatment with host cell secreted molecules.

Sporozoites were treated with RPMI, conditioned RPMI, H_2O_2, or PBS for 6 h at 37°C and 5% CO_2. H_2O_2 and PBS were included as positive and negative controls, respectively. RPMI media was prepared by supplementing RPMI-1640 with 10% Opti-MEM, 10% FBS, antibiotic/antimycotic solution, 1 mM sodium pyruvate, 50 mM glucose, 0.2 mM ascorbic acid, 2.3 μM folic acid, 29 μM 4-aminobenzoic acid, and 8.4 μM calcium pantothenate. RPMI conditioned by HCT-8 cells (conditioned RPMI) was prepared as described previously [7].

Following treatment, sporozoites were sedimented for 1 min at 20,000 g and washed with ice cold PBS. Apoptosis was assessed using the Alexa Fluor 488 Annexin V/Dead Cell Apoptosis Kit (ThermoScientific, Waltman MA) in accordance with the manufacturer's instructions. Viable (no labeling/faint green), apoptotic (bright green), and dead (green and red fluorescence) sporozoites were counted using DIC and fluorescence microscopy using an Olympus BX61 epifluorescence microscope.

Infectivity of parasites following treatment with the trophozoite trigger. In contrast to *Toxoplasma* and *Plasmodium*, which actively invade cells using their actomyosin motility machinery, *Cryptosporidium* appears to be passively encapsulated by host cells. A potential consequence of this passive invasion strategy is that the parasite can switch to a replicative trophozoite before entering the host cell. The objective of this experiment was to determine the extent to which *C. parvum* can invade cells following the switch from an invasive to a replicative

mode. Sporozoites excysted from one million oocysts were treated for 6 h with conditioned RPMI, prepared as described above. This treatment has been shown previously cause 100% of sporozoites to become rounded. Untreated sporozoites were included as a positive control and a fixed monolayer of HCT-8 cells served as a staining control. The ability of parasites to attach to and invade HCT-8 cells was determined using a modified invasion assay [9]. Parasites from the treatment and control groups were washed with PBS and added to HCT-8 monolayers in RPMI. After a 2 h incubation at 37°C and 5% CO_2, cells were fixed in 4% formaldehyde for 15 min, blocked with 1% BSA, labeled with FITC-conjugated SporoGlo (Waterborne Inc. New Orleans, LA) for 1 h at 37°C, permeabilized with 1% Triton X for 15 min at room temperature, and labeled with TRITC-SporoGlo for 1 h at 37°C. Proportions of attached (FITC and TRITC labeled) and invaded (TRITC labeled) parasites were determined by examining 20 high powered fields using an Olympus BX61 epifluorescence microscope.

Effect of C. parvum infection on secretion of the trophozoite trigger. Previous work has shown that host cell glycoprotein expression increases during a *C. parvum* infection and aggregates at the site of attachment/invasion [10]. We therefore hypothesized secretion of the trophozoite trigger would increase during an infection. To test this hypothesis, we compared the effects of conditioned RPMI and infection conditioned RPMI on rounding of *C. parvum* sporozoites. RPMI conditioned by *C. parvum* infected HCT-8 cells (infection conditioned RPMI) was prepared by infecting HCT-8 cells grown to 90% confluency with *C. parvum* in RPMI media for 3 h and 24 h at 37°C and 5% CO_2. Non-conditioned, conditioned, and infection conditioned RPMI were stored at 37°C and 5% CO_2 until needed. After excystation, sporozoites were washed once with PBS and resuspended in 200 µL of treatment media (RPMI, conditioned RPMI, or infection conditioned RPMI) and incubated for 0-6 h at 37°C and 5% CO_2.

Statistical analyses. Statistical significance between treatments was determined using the Student's *t*-test with a Holm-Šídák correction for multiple comparisons [11] and a p-value < 0.05. Statistical analyses and graphing was done using Prism 6 (Graphpad, La Jolla, CA).

Results

Sporozoites treated with the trophozoite trigger are viable and not apoptotic. Sporozoites treated with PBS, RPMI, or conditioned RPMI were 94.8 ± 7.9%, 97.5 ± 8.3%, and 95.9 ± 7.8% viable respectively, and apoptosis was less than ≤ 2% for all treatments (Table 3). H_2O_2 treated sporozoites were not viable after treatment.

Treatment with trophozoite trigger reduces invasion of HCT-8 cells. After incubating with HCT-8 cells for 2 h, 70.0 ± 6.6% of observed parasites from the non-treated control had invaded, compared to 47.0 ± 4.7% of parasites treated with conditioned RPMI, and 46.6 ± 10.4% of parasites treated with conditioned RPMI and fixed with formaldehyde (Figure 9). None of the parasites invaded formaldehyde fixed HCT-8 cells, but some rounded parasites were found attached to the fixed monolayer (Figure 10).

C. parvum infection of HCT-8 cells alters secretion of the trophozoite trigger. We examined rounding in parasites treated with conditioned RPMI, conditioned RPMI from a 3 h infection, and conditioned RPMI from a 24 h infection. Conditioned RPMI from a 3 h infection was a more effective trigger of trophozoite development than conditioned media alone and conditioned media from a 24 h infection. After 3 h 89.5 ± 8.8% of sporozoites treated with conditioned RPMI from a 3 h infection had rounded. In contrast, parasites took 4 h to reach 81.6 ± 5.0% rounded in conditioned media alone and 6 h to reach 90.0 ± 8.7% rounded in conditioned media from a 24 h infection (Figure 11).

Table 3. Evaluation of apoptosis in *C. parvum* after sporozoite rounding.

	PBS	RPMI	Conditioned RPMI
Alive	94.8 ± 7.9%	97.5 ± 8.3%	95.9 ± 7.8%
Apoptotic	2.0 ± 0.7%	1.6 ± 0.6%	1.4 ± 0.7%

C. parvum sporozoites were treated with PBS, RPMI-1640 Media and HCT-8 conditioned RPMI-FBS for 6 h p.e. at 37°C and 5% CO_2. Parasites were then stained and the proportion of apoptotic cells was evaluated.

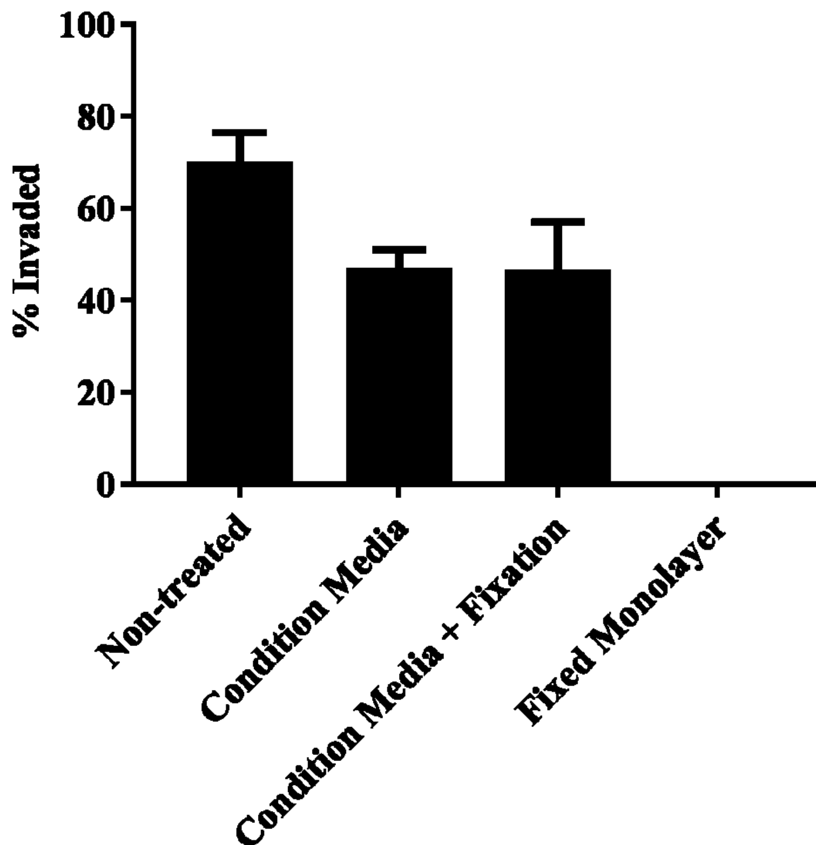

Figure 9. Rounded sporozoites are less invasive.
Sporozoites were treated with HCT-8 conditioned RPMI for 6 h post excystation and allowed to infect HCT-8 cells for 2 h. Infectivity of *C. parvum* sporozoites was determined using an attachment/invasion assay.

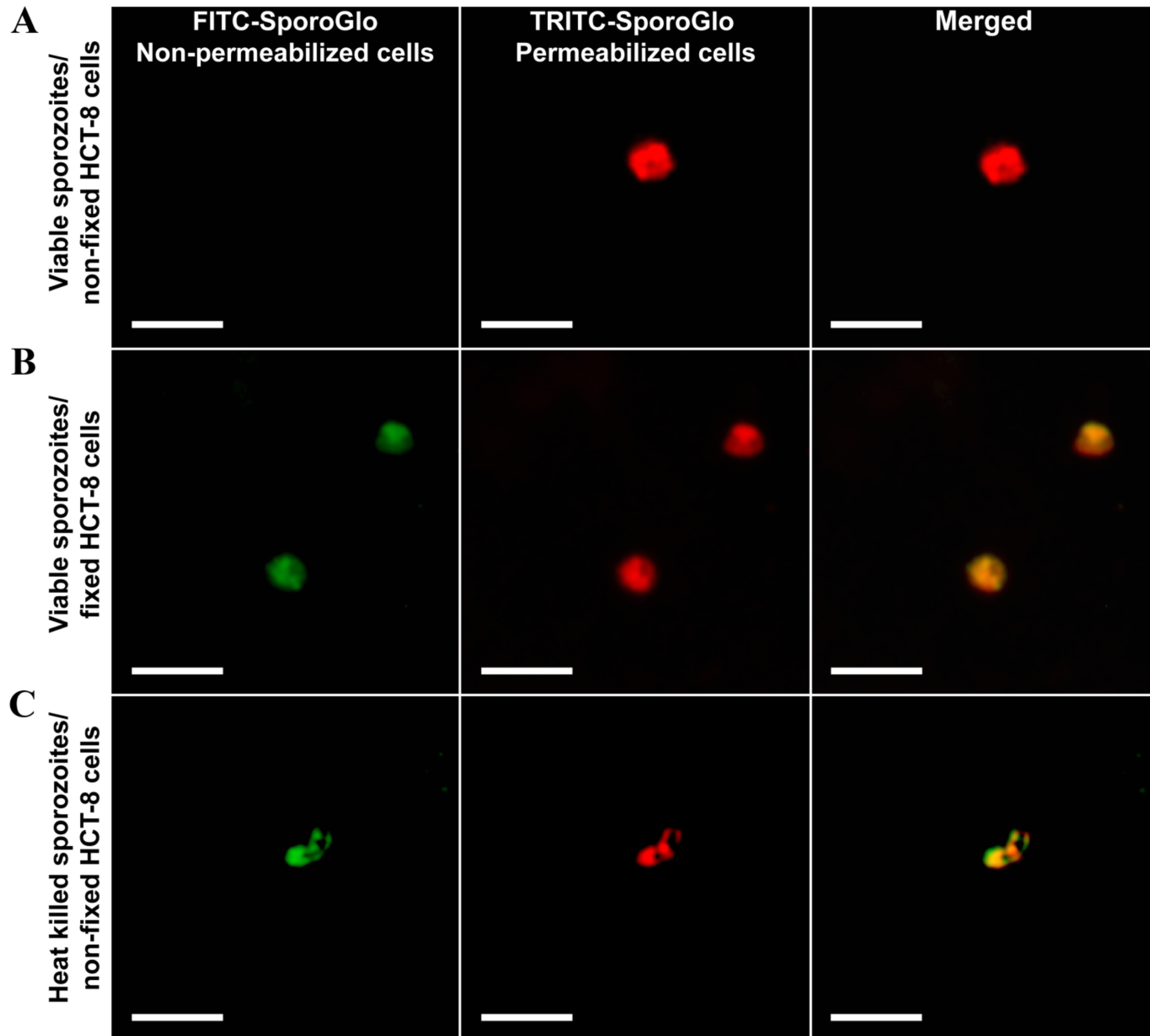

Figure 10. Sporozoite rounding at host cell surface occurs independent of invasion. Using an attachment/invasion assay, (A) invaded sporozoites were stained with TRITC. (B) Rounded, dual stained FITC and TRITC sporozoites were observed attached to the surface of fixed HCT-8 cells. (C) Heat-killed, non-invasive sporozoites included as a staining control. Scale bar 5 µm.

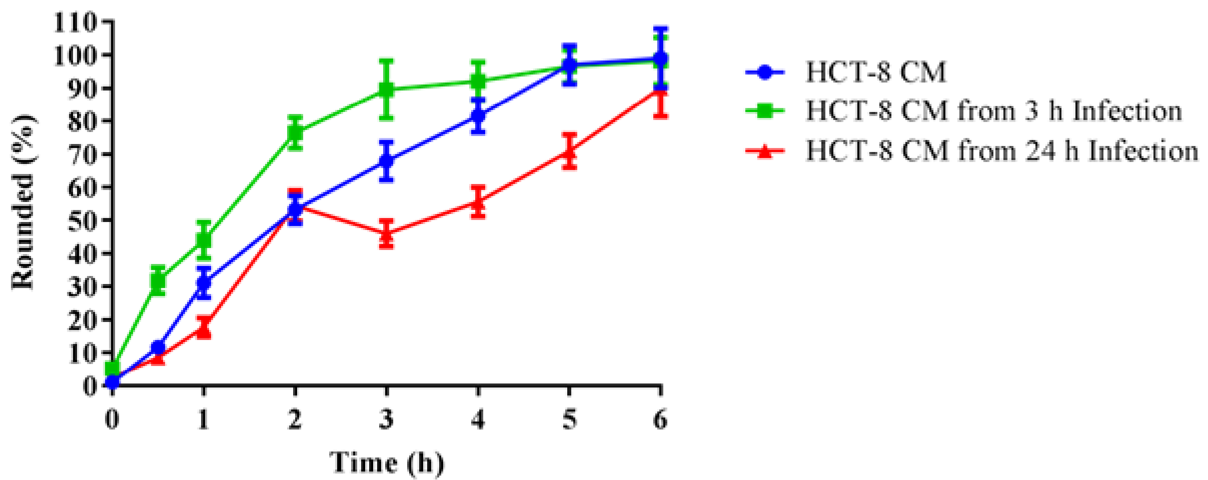

Figure 11. *C. parvum* sporozoites become rounded after treatment with infected HCT-8 conditioned RPMI-FBS media, and the rate of rounding increases in media isolated from early infections.
Sporozoites were treated with HCT-8 conditioned RPMI-FBS media and HCT-8 conditioned RPMI-FBS media isolated from 3 and 24 h infections at 37°C and 5% CO_2 over a period of 0-6 h post excystation.

Discussion

Glycoproteins and free Gal-GalNAc are important for attachment and invasion and serve as triggers for trophozoite development. Building on previous work from our group, this study has addressed critical questions about the *Cryptosporidium* switch from an invasive to a replicative form. Here we have shown rounding is not a result of apoptosis and that trophozoite formation results in lowered parasite infectivity. We also demonstrate rounded parasites are still infective and that trophozoite formation is enhanced by media from *C. parvum* infected HCT-8 cells.

In multicellular organisms, apoptosis is necessary for the removal of damaged or infected cells [12]. Apoptosis also has been reported in protozoans, although it is not clear how programed cell death is useful in single-celled organisms. Markers of apoptosis in the apicomplexans *Toxoplasma gondii* and *Plasmodium* include nucleus fragmentation and parasite rounding [8, 13, 14],

leading us to question whether *C. parvum* rounding following treatment with secreted host glycoproteins is also due to apoptosis. Although apoptosis has not been reported in *Cryptosporidium*, its genome encodes several apoptotic pathway proteins [15, 16]. To determine if rounding is the result of apoptosis we probed the outer membrane of rounded parasites for phosphatidylserine – apoptosis causes membrane inversion, exposing phosphatidylserine to the outer surface [17]. Our finding that rounded *C. parvum* parasites are viable and not apoptotic gives further evidence to support the conclusion that rounding is not a degenerative process.

Our investigation of the effect of the trophozoite trigger on invasion has revealed two important pieces of information. First, although invasion is primarily a host cell driven process, it appears that sporozoites do play some role. This is evident in the reduction in invasion following the switch from invasive sporozoite to replicative trophozoite. Second, because invasion is driven by host cells, trophozoites, and even inactive parasites, remain capable of invading host cells [18-20]. The observation that trophozoites can invade cells demonstrates that the switch to a replicative mode is independent of invasion, which may advantage the parasite during the early stages of infection by allowing it to commence replication before entering the cell. For this to be an effective strategy, proximity to the host cell is critical, as parasites rounding at a distance from the host cell are unlikely to successfully complete their life cycle. Extracellular trophozoites have been isolated from the supernatant of HCT-8 infected cells, and Paziewska-Harris et. al. showed an increase in the number of detached *Cryptosporidium* cells as infection of HCT-8 cells progressed [21, 22]. Ultimately for the parasite, there may be a fine line between success (rapid replication) and failure (rounding inappropriately and failing to complete its life cycle).

We observed that conditioned media from a 3 h infection was a more potent trigger of trophozoite development than media from a 24 h infection or non-infected cells, suggesting that

there is increased secretion of the glycoprotein effector early in an infection. One explanation for this finding is that host cells respond to infection by increasing mucin production to aid in pathogen removal. Pathogen engagement of TLR2 and TLR4 on host cell surface activates the transcription factor NF-κB, resulting in the upregulation of the pro-inflammatory cytokines IL8, TNFα and IL1-β, the induction of prostaglandin E_2 synthesis by the COX2 pathway, and increased mucin production (O-GalNAc glycoproteins) [23-25]. *C. parvum* can also modulate the host immune response by dampening NF-κB activation, thereby reducing the production of mucins [26-29]. This could explain the reduced potency of media from a 24 h infection in triggering trophozoite development.

In conclusion, we found rounded sporozoites were not apoptotic and remain infective after rounding, but are not as infective as non-treated parasites which confirms our previous findings that rounding is not a degenerative phenotype. This coupled with the observation that fixed rounded sporozoites are still infective is consistent with the observations that invasion is primarily a host cell driven process. Treatment with conditioned media from a 3 h infection resulted in greater trophozoite formation than conditioned media from non-infected and 24 h infected cells, suggesting an increase expression of potential replication triggers by host cells. More work must be done to identify the impact and role *Cryptosporidium* has on the expression of host glycoproteins during an infection, and the biological significance from both the host and parasite perspective. Our findings address some of the gaps in knowledge not covered in our previous work while further characterizing the effects of host glycoproteins on *Cryptosporidium* biology.

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PAPER 3. BOVINE SUBMAXILLARY MUCIN AND GLYCOMIMETIC POLYMERS DISPLAYING GAL AND GALNAC CAUSE *CRYPTOSPORIDIUM* TO SWITCH FROM AN INVASIVE SPOROZOITE TO A REPLICATIVE TROPHOZOITE.[3]

Abstract

Cryptosporidium is an apicomplexan parasite that causes cryptosporidiosis, a diarrheal disease that can become chronic and life threatening in malnourished and immunocompromised populations. Characterizing the environmental cues for *Cryptosporidium* life cycle development could reveal targets for the development of much needed drugs. The goal of the present study was to determine the extent to which Gal-GalNAc glycoproteins trigger *Cryptosporidium parvum* to switch from an invasive sporozoite to a replicative trophozoite. We quantified trophozoite development following treatment with suspensions of bovine submaxillary mucin (BSM; a glycoprotein rich in Gal-GalNAc), ovalbumin (a glycoprotein lacking Gal-GalNAc), and glycomimetic polymers displaying Gal, GalNAc, Glc, GlcNAc, Lac, LacNAc, or Rha at various concentrations. The same glycomimetic polymers were grafted onto glass slides at various glycan densities (glycan arrays) and examined for their effect on trophozoite development. Rounding of banana-shaped sporozoites and the presence of multiple nuclei, determined microscopically, were used as markers of trophozoite development. BSM and glycomimetic polymers displaying Gal or GalNAc were the most effective trophozoite triggers.

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[3] The material in this chapter was co-authored by Adam Edwinson and Dr. John M. McEvoy. Adam Edwinson had the primary responsibility for collecting data, analysis, interpretation, and writing of this manuscript. John McEvoy served as the proofreader.

Introduction

Cryptosporidiosis is a diarrheal disease caused by the apicomplexan parasite *Cryptosporidium*, a leading cause of diarrheal disease worldwide [1-3]. In developing countries, cryptosporidiosis in toddlers and infants can be severe and result in malnutrition, stunted growth and mortality [4-7]. Chronic, life-threatening infections also occur in immunocompromised populations [8, 9]. Current drug treatments are ineffective, particularly in immunocompromised patients, so there is a critical need for novel therapies [10, 11]. Characterizing the molecules that trigger the parasite switch from an invasive sporozoite to a replicative trophozoite can inform the development of novel drug treatments.

Cryptosporidium sporozoites invade host cells and switch to replicative trophozoites in an extracytoplasmic niche at the apical surface. This epicellular location and evidence that trophozoites can occasionally develop extracellularly [12-16] led us to hypothesize that the trigger of replication is a secreted molecule. Edwinson et. al. [17] showed that glycoproteins secreted by HCT-8 cells and free Gal-GalNAc cause sporozoites to become rounded and undergo nuclear division, indicating that they have transformed into replicative trophozoites. Gal-GalNAc is targeted by *Cryptosporidium* during attachment and invasion [18-20], and Gal-GalNAc glycoproteins aggregate at the site of attachment [21], initiating a signaling cascade in the host cell that results in parasite encapsulation within an extracytoplasmic vacuole [22, 23].

Secreted and membrane-associated O-GalNAc glycoproteins form the major part of the mucus that coats and protects the gut epithelium. Glycoproteins have complex glycosylation patterns (structure and spatial arrangements of glycans) that vary among species and tissue types and serve as receptors for mucosal pathogens such as *Cryptosporidium* [24]. In *Plasmodium* and

Toxoplasma the structure and presentation of host cell glycoproteins play a role in attachment, invasion and host specificity [25-27].

Determining the precise pattern of glycans targeted by *Cryptosporidium* and other pathogens is technically challenging due to the complexity of the glycosylation process [28]. Recently developed glycomimetic technology allows glycan structures to be arranged with nanoscale precision on a polymeric scaffold, mimicking native glycoproteins [29]. Glycomimetics and glycan arrays have been used to interrogate receptors for HIV, *T. gondii*, and *Trypanosoma cruzi* [30-32]. In *Cryptosporidium*, microarrays of synthetic glycopeptides have been used to study antigen targeting by the immune system during an infection [33]. Mice immunized with synthetic peptides decorated with *T. gondii* epitopes have enhanced survival when challenged with the parasite, demonstrating the potential broader application of glycomimetics in the development of novel vaccines that promote protective immunity [34].

In the present study, we use native glycoproteins and glycomimetics to determine the extent to which Gal and GalNAc decorated glycoproteins trigger trophozoite development in *Cryptosporidium*. We show that bovine submaxillary mucin (BSM), which has a high density of Gal-GalNAc, and glycomimetics displaying Gal or GalNAc are the most effective trophozoite triggers.

Materials and methods

Source and excystation of Cryptosporidium oocysts. Mouse passaged *C. parvum* Iowa isolate was purchased from Waterborne Inc. (New Orleans, LA). Oocysts were excysted by treating with a 10% sodium hypochlorite solution for 10 min at 4°C, washing twice in PBS, and incubating in RPMI-1640 supplemented with 0.8% sodium taurocholate (Sigma-Aldrich, St. Louis, MO) at 37°C and 5% CO_2 for 1 h.

Treatment of sporozoites in suspension. Sporozoites were treated at 37°C and 5% CO_2 in PBS containing 10 μM BSM, 10 μM ovalbumin (Sigma-Aldrich), or 10 μM glycomimetic polymer. The glycomimetic polymer displayed Galactose (Gal), N-Acetygalactosamine (GalNAc), Glucose (Glc), N-Acetyglucosamine (GlcNAc), Lactose (Lac), N-Acetylactosamine (LacNAc), 3'-sialyllactose (3'SialLac), or Rhamnose (Rha). Treatment times were 3, 4, 5, and 6 h for treatments with BSM or ovalbumin. Treatment times of 1, 3, 4, 5, 6, and 7 h were used for glycomimetic polymers. Following treatment, sporozoites were spun onto a glass slide using a Shandon Cytospin 2 (Thermo-Scientific, Waltman, MA) at 2,000 g for 5 min, fixed with 100% MeOH for 15 min, and washed twice with PBS for 3 min. Fixed parasites were blocked with 1% BSA at room temperature for 20 min, washed with PBS, and then labeled with TRITC-, or FITC-conjugated SporoGlo (Waterborne Inc. New Orleans, LA) for 1 h at 37°C. Nuclei were labeled with 300 nM 4', 6-diamidino-2-phenylindole (DAPI) for 30 min at room temperature and then examined using DIC and florescence microscopy with an Olympus BX61 microscope. The proportion of rounded parasites was determined by counting 200 observations using DIC and fluorescence microscopy on an Olympus BX61 microscope. The proportions of parasites with two or more nuclei were counted in 20 high powered fields using DIC and fluorescence microscopy.

Preparation and use of glycomimetic polymer arrays. Glycomimetic polymers were prepared using the protocol outlined by Huang et al. [35]. Glycomimetic polymer arrays were prepared using an approach described previously [36, 37], with some modifications. Glass slides (VWR, Radnor, PA) were washed while rocking using 30 min. intervals of ddH_2O, acetone and methanol, followed by five washes with ddH_2O. Slides were submerged in .05M NaOH for 1 h while rocked, and then rinsed four times with ddH_2O. Using an Allegra X-014R (Beckman

Coulter, Brea, CA) at 70xg for 5 min slides were spun dry, and baked in a vacuum oven, 1 h, 70 °C, -20 p.s.i. After cooling to room temperature in a desiccator, slides were silanized using a 98% toluene and 2% 3-(trimethoxysilyl) propyl methacrylate (vol/vol) solution (Alfa Aesar, Haverhill, MA), rocked for 2 h, rinsed three times with toluene, spin dried, and placed in a vacuum oven, (15min 70°C, -20 psi). Slides were activated with 0.5% (vol/vol) glutaraldehyde solution (Thermo Fisher, Waltham, MA), rocked for 30 min, rinsed with ddH_2O, spun dry and vacuum oven baked as before.

Ten milliliters of a 20:1:1 30% acrylamide stock solution was prepared consisting of 2.85g of Acrylamide (Sigma Aldrich, St. Louis, MO), 0.15g bisacrylamide (Promega, Madison, WI), and 0.15g allyl glycidyl ether (Thermo Fisher, Waltham, MA). A polymerization solution was prepared using 500 µL of the acrylamide stock solution, 985µL ddH_2O, 15µL 10% w/v ammonium persulfate solution (Thermo Fisher, Waltham, MA), and 0.6 µL of tetramethylethylenediamine (Millipore, Billerica, MA). 110µL of the polymerization solution is placed onto a dry, glutaraldehyde activated slide. A glass coverslip (Vineland, NJ) was added and the polymerization reaction went uninterrupted for 2 h. Slides are submerged into DMF for 15 minutes, and the coverslip is removed from the hydrogel using a razor.

Dibenzocyclooctyne-amine (ADIBO) (Sigma Aldrich, St. Louis, MO) functionalization was done in 75 mL of a 10 mM DIPEA (Sigma Aldrich, St. Louis, MO) and 1mM ADIBO DMF solution covered in foil and rocked overnight. Slides were placed in an ethanol-amine borate buffer for 12 hours (100 mM ethanol-amine (Spectrum, New Brunswick, NJ), 50 mM sodium tetraborate decahydrate (Alfa Aesar, Haverhill, MA)), then rinsed with ddH_2O while rocking for 48 hours, replacing ddH_2O daily. Slides were spun, followed by 15 minutes at 50°C, or until

completely dried. ADIBO functionalized acrylamide hydrogel slides were stored in a dry, air free 4°C environment until use.

Arrays were printed using the Arrayit SpotBot Extreme microarray printer. The printer was loaded with two 946MP15 pins, and printing was done at 65% humidity. Neoproteoglyca-printing solutions were prepared by diluting polymers into a 10% glycerol, 0.003% Triton x-100, PBS printing buffer. Solutions were printed in quadruplicate containing Gal, GalNAc, 3'SialLac, 6'SialLac, Lac and Rha at a 2-fold decrease in density ranging from 10 μM to 1.25 μM. After printing, arrays are allowed to dry overnight at room temp in a desiccator. The slides are then loaded into a slide rack, and repeatedly dunked in ddH_2O for 2 min, then washed three times for 15 min in PBS. Slides are then spin dried and stored at 4°C in a dry, air free environment until use.

Microarrays were blocked at room temperature for 30 min with 1% BSA. In PBS, 2 million excysted oocysts were added directly to each array. After 3, 4, 5, 6 h. post excystation, sporozoites were fixed with 4% formaldehyde for 15 min. Slides were blocked a second time with 1% BSA for 15 min. and stained as outlined in 2.3. DIC and fluorescent microscopy was used to visualize imprinted glycan spots. Each spot was divided into five sections and the proportion of rounded parasites was determined.

Statistical analyses. Twenty high-powered fields, and counts of sporozoites (n=200) in triplicate were used to calculate a mean and standard deviation values of the proportion of rounded, or multinucleated sporozoites. Statistical significance of differences between treatments was determined using the Student's *t*-test with and the Holm-Šídák correction for multiple comparisons [38] and a p-value < 0.05. Statistical analyses and graphing was carried out using Prism 6 (Graphpad, La Jolla, CA).

Results

BSM is a more effective trigger of trophozoite development than ovalbumin. Gal-GalNAc decorated BSM [39] caused greater parasite rounding than ovalbumin ($P<0.05$), an n-glycosylated glycoprotein that lacks Gal-GalNAc epitopes [40] (Figure 12). The effect of ovalbumin on rounding was similar to that of the PBS control. These data support the role of Gal-GalNAc as a trigger for sporozoite differentiation.

Glycomimetics decorated with Gal and GalNAc trigger the greatest amount of trophozoite development. Soluble glycomimetic polymers displaying Gal, GalNAc, Glc, GlcNAc, Lac, LacNAc, Rha and 3'SialLac were examined as triggers of trophozoite development. The proportion of rounded parasites increased over time in the presence of each tested glycan (Figure 13). Polymers displaying Gal and GalNAc caused a significantly greater proportion of rounded parasites across all time points, and after 6 h, 67.0 ± 4.6% and 70.2 ± 3.3% of parasites, respectively, were rounded, which was greater than BSM alone (62.2 ± 2.8%), but less than what is observed in HCT-8 conditioned media (99.0 ± 8.9%). The effect of rhamnose, a deoxy glycan not found in mucins, was not significantly different from that of PBS ($P>0.05$). These data are evidence that Gal and GalNAc contribute to rounding, but they are not the sole glycans that promote the phenotypic change.

Glycomimetic polymers displaying Gal or GalNAc are the most effective triggers of nuclear division. Treatment with glycomimetic polymers displaying Gal or GalNAc caused 11.3 ± 1.9% and 6.7 ± 1.6%, respectfully, of parasites to undergo nuclear division (Figure 14). Nuclear division also was observed following treatments with 3'sialylactose (3.7 ± 1.3%), lactose (1.8 ±0.9%) and N-acetyl-glucosamine (1.7 ± 0.8%). Rhamnose and the PBS control caused no nuclear division. Cells containing multiple nuclei were much lower than previously

reported and solubilized Gal-GalNAc remains the greatest trigger for nuclear replication, but these results are evidence that glycomimetic polymers can trigger replication in *C. parvum*.

Gycomimetic microarrays displaying the highest densities of Gal and GalNAc caused the greatest rounding. Glycomimetic polymers displaying Gal, GalNAc, 3'SialLac, 6'SialLac, Lac, or Rha were grafted onto glass slides at varying densities from 1.25 µM to 10 µM (Figure 15). Sporozoites were added to the slides in PBS and incubated for 3, 4, 5 or 6 h at 37°C. The proportion of rounded parasites increased with increasing glycan density. Spots imprinted with glycomimetics displaying Gal and GalNAc consistently caused greater rounding. The proportion of rounded parasites on 10 µM Gal and GalNAc spots increased from 51.0 ± 4.6% and 60.9 ± 3.8% at 3 h to 78.3 ± 6.4% and 84.2 ± 9.0% at 6 h, respectively (Figure 16). Incubation time appears to negate effects of glycan density as the proportion of rounded cells at 3 h on both the Gal and GalNAc at 10 µM (51.0 ± 4.6% and 60.9 ± 3.8%) was statistically similar to the rounding occurring on the 1.25 µM spots (52.8 ± 2.9% and 56.1 ± 5.6%) imprinted with the same sugars. Rounding was observed in the Rha sugar controls, but was never greater than 26%, and was significantly different than Gal and GalNAc mimetic polymers at all time points and concentrations ($P<0.05$).

Discussion

Recognition of host glycoproteins is critical for attachment and invasion by apicomplexans. Disrupting these interactions can slow or prevent parasite amplification and life cycle progression. Understanding the complexity of glycan receptors can be difficult due to host interference, or the inability to modify and isolate the intricate glycoproteins. Here we confirmed the importance of host glycoproteins and Gal-GalNAc in promoting life cycle changes in *C.*

parvum, and we have demonstrated that glycomimetic polymers can be used to study the effect of glycan structure and spatial presentation on parasite development.

Increased rounding was observed in sporozoites treated with BSM which is heavily decorated with Gal-GalNAc [39]. Ovalbumin, which lacks Gal-GalNAc glycans [40], did cause some rounding, although significantly less than BSM and similar to PBS. GlcNAc is the major glycan displayed on ovalbumin, and previous studies have shown a glycan which has shown some effect on attachment, but not to the degree Gal-GalNAc [18, 19, 41, 42].

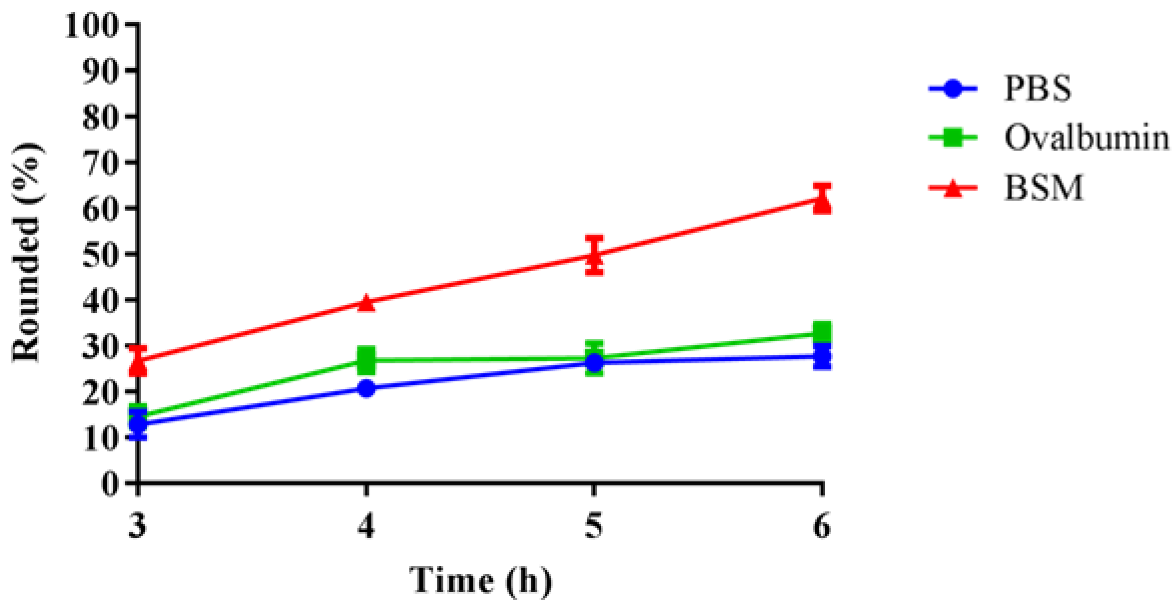

Figure 12. Treatment with Gal-GalNAc glycoproteins causes rounding of *C. parvum* sporozoites.
Sporozoites were treated with PBS, 10 µM Bovine Submaxillary Mucin (BSM) and 10 µM Ovalbumin in PBS 3-6 h post excystation.

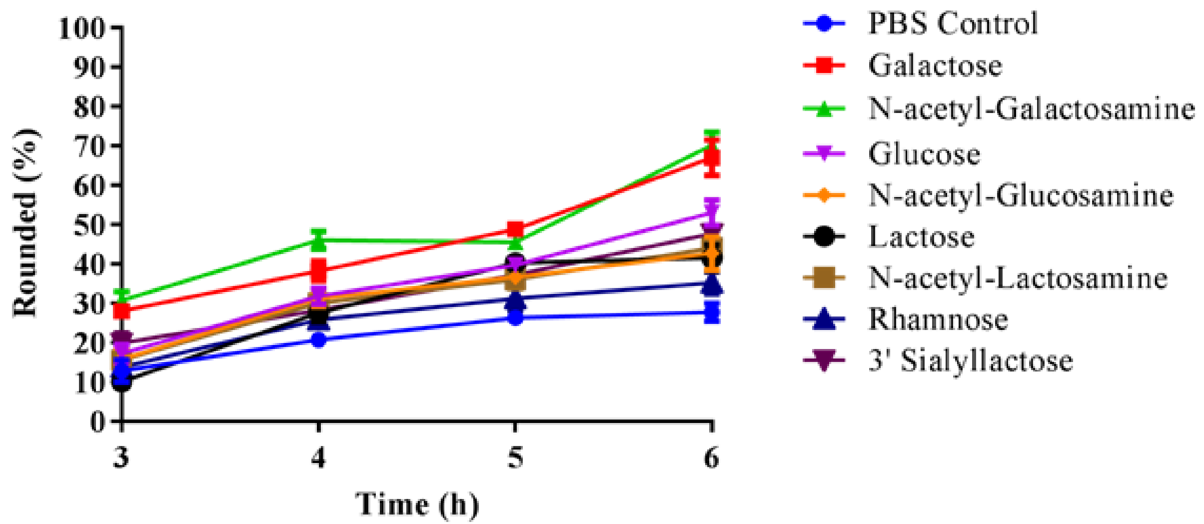

Figure 13. Synthetic glycoproteins cause *C. parvum* sporozoites to round.
Sporozoites were treated with 10 µM of synthetic glycoproteins suspended in PBS over a course of 3-6 h. PBS treatment served as a control.

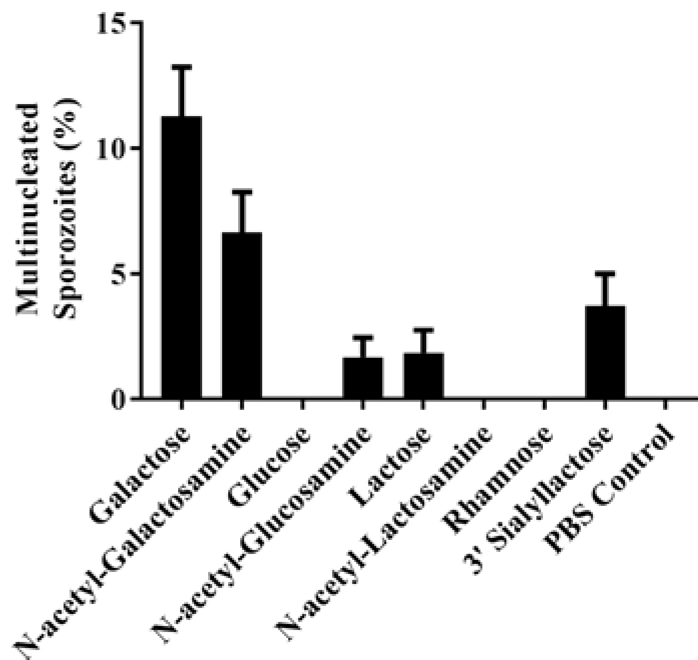

Figure 14. Soluble synthetic glycoproteins decorated with Gal and GlaNAc cause nuclear division in *C. parvum*.
Sporozoites were treated with each mimetic at a concentration of 10 µM over 7 h. PBS treatment served as a control and the number of nuclei were counted per sporozoite.

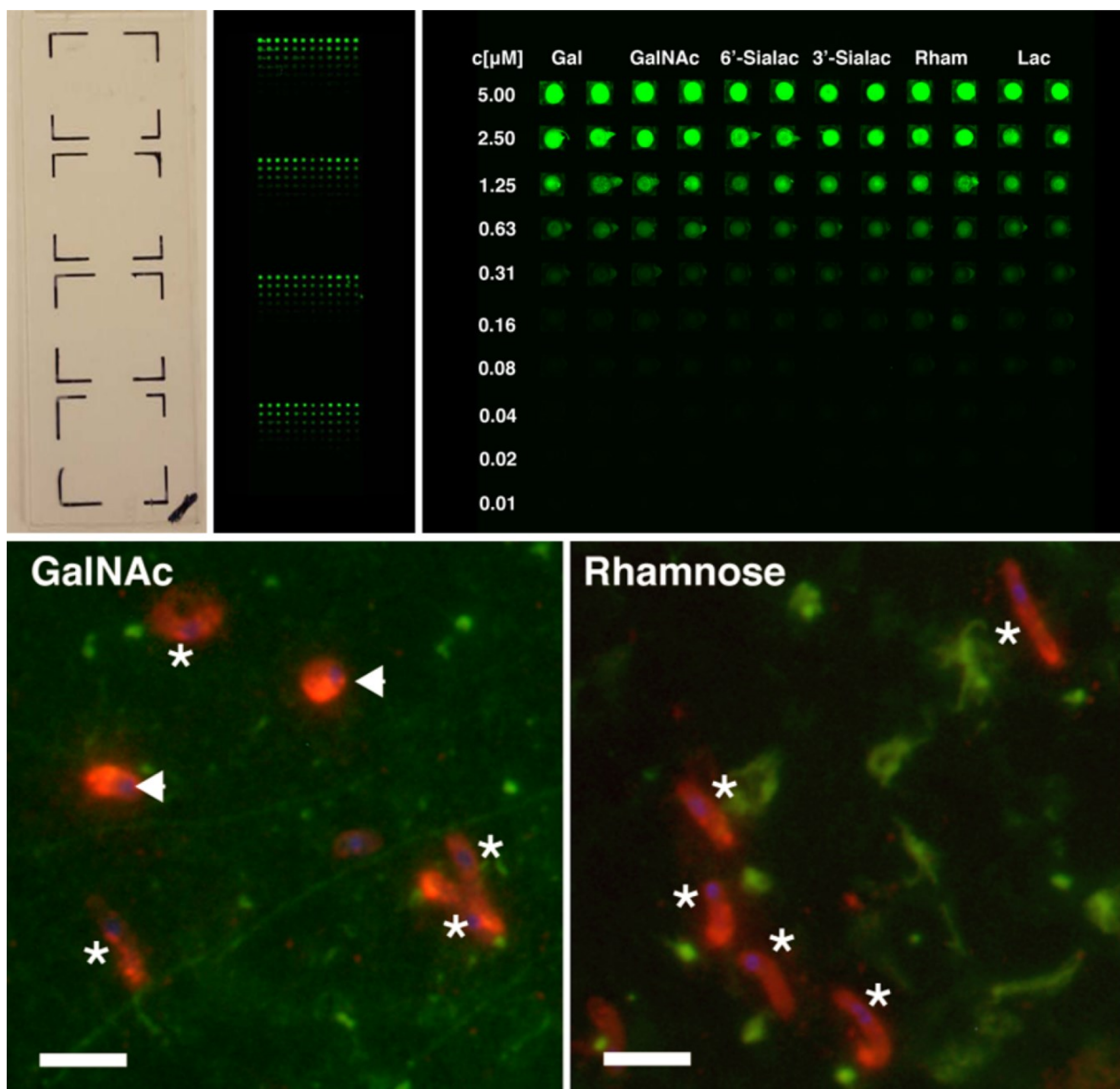

Figure 15. *C. parvum* on slides imprinted with glycomimetic polymers in an array format. *C. parvum* sporozoites were incubated on the slides imprinted with glycomimetics conjugated to FITC for spot identification. This is a representation of the imprinted array and the morphology of *C. parvum* parasites labeled with TRITC. FITC conjugated glycomimetic polymers indicate spot location Arrows indicate rounded while * denotes linear sporozoites. Scale bar = 5 µm.

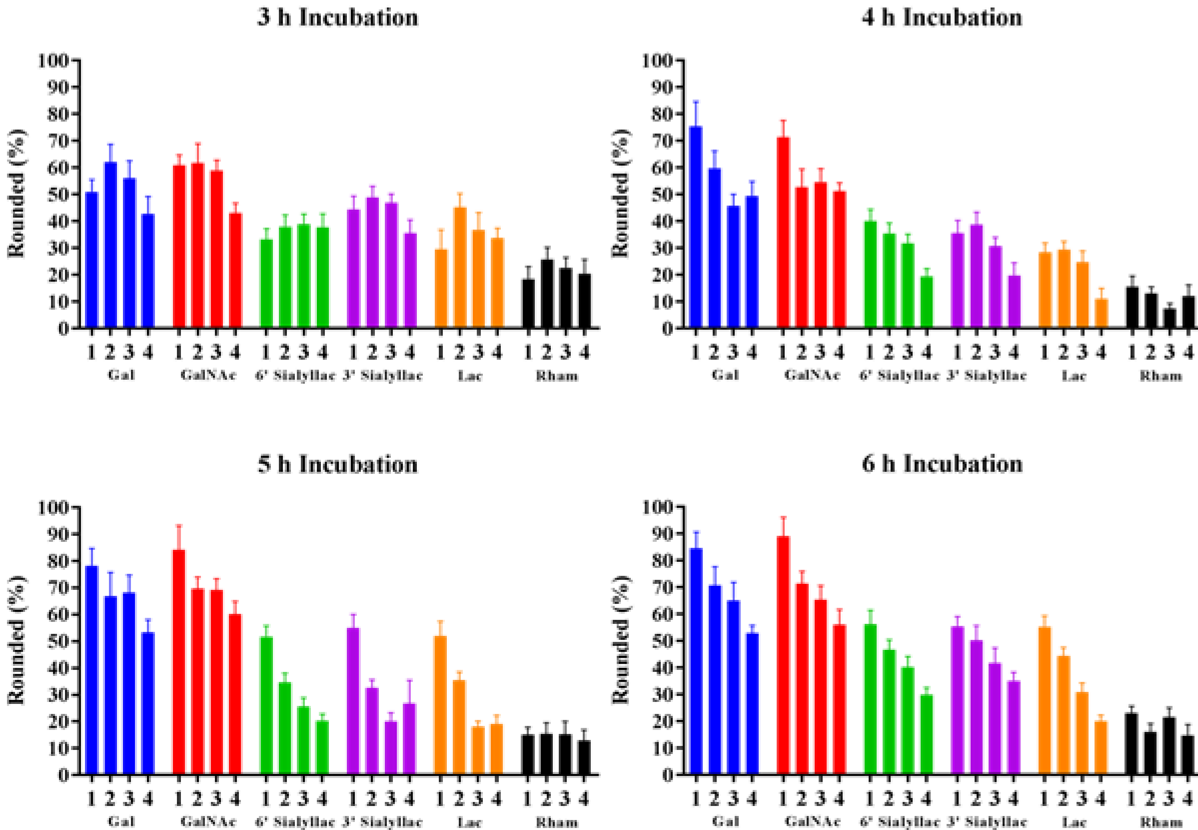

Figure 16. The proportions of rounded *C. parvum* increase with increasing density of Gal and GalNAc imprinted on a glycan microarray.
C. parvum sporozoites were added to a glycan microarray imprinted with (1) 10 µM, (2) 5 µM, (3) 2.5 µM and (4) 1.25 µM of Gal, GalNAc, 6'Siallac, 3'Siallac, Lac, and Rha for 3-6 h.

Heterogeneity of the host glycome can make it difficult to isolate manipulate and control glycan presentation. To better understand how the type and spatial arrangement of glycans trigger *C. parvum* differentiation we decided to use glycoprotein mimetics. Solubilized and imprinted glycomimetics containing Gal and GalNAc most effectively triggered rounding and nuclear division in *C. parvum*. This is consistent with previous work showing that immunodominant *Cryptosporidium* antigens selectively target the Tn antigen (GalNAcα-Ser/Thr-R) of host glycoproteins [43-45]. This could explain the capacity to round and begin replication in the presence of glycomimetics containing GalNAc. Our observation that parasites

were more likely to round when attached to higher density glycan spots is consistent with data showing that Gal-GalNAc glycoproteins accumulate at the attachment site. Although trophozoite development was triggered by BSM and synthetic glycopolymers displaying Gal or GalNAc, these triggers were less effective than conditioned media. HCT-8 cells have an elevated expression of Tn antigens which, with variation in glycan content and valency, could account for the observed difference [46].

In conclusion, we have shown that glycoproteins and glycomimetic polymers displaying Gal or GalNAc can trigger developmental changes in *C. parvum* similar to glycoproteins from the host secretome. Glycomimetic polymers provide novel and promising ways to investigate and develop treatments to prevent cryptosporidiosis. Further work must be done to achieve an accurate representation of glycoproteins from the host secretome, but this study does demonstrate the capacity for glycomimetic polymers to act as surrogates for native glycoproteins.

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PAPER 4. EVIDENCE THAT *CRYPTOSPORIDIUM* RHOMBOID PROTEASES ARE NECESSARY FOR EXCYSTATION AND TROPHOZOITE DEVELOPMENT.[4]

Abstract

Cryptosporidiosis is a diarrheal disease caused by the apicomplexan parasite *Cryptosporidium*, causing severe and life threatening disease in malnourished and immunocompromised individuals. The pathology defining cryptosporidiosis results from repeated cycling between invasive and replicative stages within a host. Identifying the mechanisms underlying the transition from invasion to replication is a potential avenue for targeted disease prevention. In related the related apicomplexans *Toxoplasma* and *Plasmodium* a family of intermembrane serine proteases known as rhomboid proteases (ROMs) are important for motility, invasion and have been implicated in promoting replication. The present work examined the expression of CpROM2, a previously uncharacterized rhomboid protease encoded for by *Cryptosporidium*, and the role of rhomboid proteases in excystation and trophozoite formation. CpROM2 localized sub apically and anterior to the nucleus in sporozoites and was identified during all stages of intracellular development, with peak expression 6 h post infection of HCT-8 cells. Treatment with isocoumarins, rhomboid specific inhibitors, reduced excystation and trophozoite formation in response to host glycoprotein triggers.

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[4] The material in this chapter was co-authored by Adam Edwinson and Dr. John M. McEvoy. Adam Edwinson had the primary responsibility for collecting data, analysis, interpretation, and writing of this manuscript. John McEvoy served as the proofreader.

Introduction

The apicomplexan parasite *Cryptosporidium* causes the diarrheal disease cryptosporidiosis, which can be severe and life-threatening in children under five and those with a compromised immune system [1-3]. In developing countries, *Cryptosporidium* is the leading cause of death from diarrheal disease in toddlers and contributes to malnutrition and stunted growth [3-6]. There is no vaccine and current drug treatments lack efficacy in the most vulnerable group – the immunocompromised [7, 8]. Previous work from our group has shown that glycoproteins and Gal-GalNAc cause *Cryptosporidium* to switch from motile, invasive sporozoites to replicative trophozoites [9]. Understanding the mechanisms underpinning this developmental transition could inform the development of much-needed, novel drug treatments that disrupt parasite invasion and replication.

Motility and invasion are mechanistically similar processes in apicomplexans. Transmembrane adhesins secreted from micronemes to the anterior surface of zoites provide traction (by engaging host cell receptors) and force (by undergoing retrograde translocation along the zoite surface) [10]. The cytoplasmic tail of these adhesins links with filamentous actin, which, in turn, links to the parasite inner membrane complex via myosin A and moves in an anterior direction, displacing the actin bound adhesin in a posterior direction (retrograde translocation) [11]. Proteolytic shedding of adhesins from the zoite surface is essential for motility and host cell invasion, and rhomboid-family intramembrane serine proteases (ROMs) have been repeatedly identified as the responsible sheddases in *Toxoplasma* and *Plasmodium* [12-14].

T. gondii encodes six ROMs (TgROM1-6), four of which have homologs among the 8 ROMs encoded by *Plasmodium falciparum* (PfROM1, 3, 4, and 6). ROM1 localizes to micronemes in *T. gondii* and *Plasmodium* [15], while ROM2 localizes to the Golgi apparatus [16] and

ROM6 localizes to the mitochondrion [17]. Based on their specificities for microneme adhesins and localization to the plasma membrane of invasive zoites, the most likely sheddases are TgROM4 and TgROM5 in *T. gondii*, and PfROM4 in *P. falciparum* [12, 16, 18]. *Cryptosporidium* encodes three ROMs. Tabe [19] showed that two of these ROMs, which he named CpROM4 and CpROM5, localize to the membrane of sporozoites and cleave several transmembrane adhesins, including GP900, a mucin-like glycoprotein that forms a complex at the parasite surface with the Gal-GalNAc specific lectin P30 [20]. A third *Cryptosporidium* ROM, which appears to be a ROM1/2 homolog, remains uncharacterized.

Rhomboid proteases are necessary for parasite egress, motility, and apicomplexan invasion strategies, but have also been implicated in initiating replication [21, 22]. In *T. gondii,* TgROM4 cleaves AMA1 during invasion, releasing the cytoplasmic portion of AMA1. Accumulation of the AMA1 cytoplasmic domain triggers replicative development in the invading sporozoite [23-26]. The role rhomboids have during the transition from invasion to replication in *Cryptosporidium* has yet to be addressed, but the rhomboids encoded by the parasite act on complexes that bind Gal-GalNAc epitopes [19]. Engagement of host glycoproteins decorated with Gal and GalNAc is important for sporozoite attachment and invasion, but is also critical for triggering *Cryptosporidium* replication and differentiation [9, 27-29].

Processing of the host glycoprotein-parasite motility complex by rhomboid proteases may be important in driving *Cryptosporidium* sporozoite motility, but may also facilitate invasion and the switch to a replicative mode. Here we characterize a third *Cryptosporidium* rhomboid, which we name CpROM2, providing information regarding gene expression and localization of the protease throughout different stages in the parasite life cycle. Using specific inhibitors of ROM

activity, we show that ROMs play a role in excystation and the transition from invasion to replication in *Cryptosporidium*.

Materials and methods

Cryptosporidium oocyst source and excystation. Mouse passaged Iowa isolate of *C. parvum* was purchased from Waterborne Inc. (New Orleans, LA). Oocysts were excysted using a 10% sodium hypochlorite solution for 10 min at 4°C. Oocysts were resuspended in RPMI-1640 supplemented with 0.8% sodium taurocholate (Sigma-Aldrich, St. Louis, MO) after two PBS washes, held at 37°C, 5% CO_2 for 1 h.

Generation of polyclonal antibodies. A short peptide sequence of CpROM2 was produced *in silico* and used to immunize rabbits. Polyclonal antibodies from antisera were purified through sepharose columns using affinity chromatography.

Expression of the CpROM gene during in vitro infection. At a concentration of 10^6 cells/mL, human colorectal adenocarcinoma (HCT-8) cells were seeded in 6 well polystyrene plates (Lab-Tek™, Becton Dickinson, Franklin Lakes, NJ). Cells were grown in RPMI media (pH 7.4) supplemented with 10% fetal bovine serum (RPMI-FBS), 1 mM sodium pyruvate, 50 U/mL penicillin, 50 U/mL streptomycin, and 0.25 µg/mL amphotericin B (Sigma-Aldrich Co. St. Louis, MO). Cells were placed in an incubator held at 37°C, 5% CO_2 and grown to 80-90% confluency before use.

One million oocysts were treated with 10% (v/v) ice-cold sodium hypochlorite for 10 min at 4°C. After washing three times with PBS, oocysts were resuspended in infection media (RPMI-FBS with 10 mg/mL streptomycin, 25 µg/mL amphotericin B, 35 µg/mL ascorbic acid, 1.0 µg/mL folic acid, 4.0 µg/mL para-aminobenzoic acid, 2.0 µg/mL calcium pantheoate, 1 mM sodium pyruvate, and 50 mM glucose). Infections were stopped after 2, 6, 12, 24, 48, and 72 h

post infection using a Qiagen lysing buffer (600 µL/well, Qiagen, Hercules, CA) supplemented with 10 µL of 2-β-Mercaptoethanol (Sigma Aldrich, St. Louis, MO) for 15 min. Total RNA was subsequently extracted using a Qiagen RNeasy extraction kit (Qiagen), following the manufacturer instructions provided. RNA was eluted after purification using RNase-free water and quantified with a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE). 2 µg of RNA was then hybridized with random hexamers at 500 µg/mL (Promega, Madison, WI) and subsequently transcribed to cDNA using SMART MMLV Reverse Transcriptase (Promega).

The small subunit ribosomal RNA (SSU) COWP1 and CpROM2 were quantified using the TaqMan quantitative RT-PCR method, with SSU used as a normalizing gene and COWP1 used as a marker for sexual stage development. The following primer and probe sequences used were: SSU For: 5'-CAGCTTTAGACGGTAGGGTATTGG-3', Rev: 5'-CGAACCCTAATTCCCCGTTAC -3', Probe: 5'-CCGTGGCAATGAC-3' CpROM2: For: 5'-GGAAATCTCTTTTCAGTTGCCATT-3', Rev: 5'-CCTGAGGTGGATGCACCAA-3', Probe: 5'-AAGTAGCTGCGTTGTAGC-3' and COWP1: For: 5'-GAAACTGCGAACAACTCATTCG -3', Rev: 5'-CACCAGCCTGAGGTCTATAATATCC -3', Probe: 5'-TCACCACCAACGATGG-3'. Samples were run using an initial denaturation step of 94°C for 1 min, followed by 34 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 1 min. All infections were performed in duplicate, with mock infections using heat killed sporozoites (70°C for 1 h) as a negative control, and reactions lacking reverse transcriptase as a control for any genomic DNA.

Immunolocalization of CpROM2 in oocysts and sporozoites. Protein localization was done on untreated oocysts, oocysts treated with bleach (10% v/v ice-cold sodium hypochlorite for 10 min at 4°C) to remove the outer glycocalyx, and excysted sporozoites. Approximately 500

of intact, bleach treated, or excysted oocysts were air dried on a glass slide and fixed with equal volumes of ice-cold 100% methanol for 10 min. After washing with PBS, 4% formaldehyde was added for another 10 min. Parasites were then permeabilized with 1% Triton X-100 in 1% bovine serum albumin (BSA) for 15 min, followed by a 1% BSA blocking for 20 min. Slides were then stained overnight at 4°C with a 1% BSA solution containing polyclonal antibodies targeting CpROM2 at a 1:100 dilution and biotinylated *Vicia villosa* lectin (VVL) at a 1:2500 dilution (Vector Laboratories Inc., CA). VVL is designed to bind both intracellular and extracellular stages of the parasite by specifically targeting Gal-GalNAc residues. As a negative control, 1% BSA was used.

After three PBS washes, slides were probed with secondary antibodies (goat anti-rabbit IgG) conjugated to fluorescein isothiocyanate (FITC) at a 1:1000 dilution in 1% BSA for 1 h at 37°C (Life Technologies, NY). Slides were washed with PBS and stained with streptavidin (1:1000 dilution in 1% BSA, Vector Laboratories Inc., CA) conjugated to tetramethylrhodamine isothiocyanate (TRITC) to detect VVL binding. 300 nM 4', 6-diamidino-2-phenylindole (DAPI) was used to stain nuclei at 4°C for 15 min.

Slides were mounted with No-Fade™ mounting media (Waterborne™ Inc. New Orleans, LA) and examined by confocal microscopy using a Zeiss Observer Z.1 inverted microscope powered by a LSM700 Confocal Scanning Laser (Carl Zeiss Microscopy, Jena, Germany). Carl Zeiss ZEN2011 Software (Carl Zeiss Microscopy, Jena, Germany) was used to merge image captured at different wavelengths from a set of Z-stack images taken with an Axiocam MRc camera (Carl Zeiss Microscopy, Jena, Germany).

Immunolocalization of CpROM2 during in vitro infection. In an 8-well chamber slide (Becton Dickinson Labware, Franklin Lakes, NJ), HCT-8 cells were seeded at a concentration of

10^5 cells/mL and grown similarly to the gene expression experiments. Each well was infected with 100,000 oocysts and allowed to infect for 2, 6, 12, 24, 48, and 72 h. Infections were in duplicate with heat-inactivated oocysts as a control. Staining was done as described above with analysis done on using an Olympus BX61 microscope.

Inhibition of excystation and trophozoite formation. Oocysts were treated for 10 min at 4°C with a 10% sodium hypochlorite solution and washed with PBS. 100,000 oocysts were then added to RPMI-1640 with 0.8% sodium taurocholate (Sigma-Aldrich, St. Louis, MO) supplemented with isocoumarin (ROM specific inhibitor) 7-Amino-4-chloro-3-methoxy-1H-2-benzopyran (JLK6), 3,4-Dichloroisocoumarin (DCI), or the serine protease inhibitor Phenylmethylsulfonyl fluoride (PMSF), at concentrations of 25, 50 and 100 μM. Equal volumes of DMSO were added to RPMI-1640 with 0.8% sodium taurocholate as a negative control. To determine the effect of inhibitors on trophozoite formation, HCT-8 conditioned RPMI-FBS was prepared as described [9]. HCT-8 conditioned RPMI-FBS was supplemented with PBS, DMSO, JLK6, DCI, or PMSF at a concentration of 100 μM for 3, 4, 5 and 6 h post excystation. A Shandon Cytospin 2 (Thermo-Scientific, Waltman, MA) was used to spin sporozoites at 2,000 g for 5 min onto a glass slide after treatment. 100% MeOH for 15 min was used to fix the parasites, followed by a two 3 min PBS washes. A 1% BSA block was done at room temperature for 20 min. Slides were washed with PBS, and labeled with TRITC, or FITC conjugated SporoGlo™ (Waterborne™ Inc. New Orleans, LA) for 1 h at 37°C. 30 min incubation at room temperature with 300 nM DAPI solution was used to visualize nuclei using an Olympus BX61 microscope.

Statistical analyses. Counts of sporozoites (n=200) in triplicate and twenty high-powered fields were used to calculate means and standard deviation values of the proportion of rounded

sporozoites or excysted oocysts. For qPCR experiments, biological triplicates and technical replicates of gene expression were completed at each time point. Statistical significance of the differences observed within experiments was determined using a p-value < 0.05 using the Student's *t*-test and the Holm-Šídák correction for multiple comparisons [30]. Graphing and statistical calculations were done using Prism 6 (Graphpad, La Jolla, CA).

Results

CpROM2 is expressed during asexual development and excystation. Our goal was to determine the dynamics of CpROM2 gene expression during *C. parvum* development *in vitro*. We also examined the expression of COWP1, a marker for sexual stage development that shows peak expression at 48 h post infection [31, 32]. CpROM2 expression was greatest between 2 and 24 h post infection, with a peak expression at 6 h, indicating that CpROM2 is associated with asexual development (Figure 17).

CpROM2 localizes to the apical pole of sporozoites and is expressed during intracellular development. Using immunolocalization, CpROM2 was detected in sporozoites within non-excysted oocysts, but it was not detected in the oocyst wall. CpROM2 was present in excysted sporozoites at two locations – sub-apically and anterior to the nucleus (Figure 18).

We next examined the localization of CpROM2 during an *in vitro* infection of HCT-8 cells (Figure 19). CpROM2 was detected during all stages of development, including the intracellular stages at 48 and 72 h p.i, with greatest fluorescence intensity observed within developing meronts 12 h p.i. A distinct halo-like localization was observed in intracellular stages at 2 h p.i. (early developing trophozoites) and 24 h p.i.

CpROMs play a role during excystation and trophozoite development. We used the isocoumarins DCI and JLK-6 to specifically inhibit CpROMs and determine whether they

function during excystation and trophozoite development. PMSF, a serine protease inhibitor that does not inhibit CpROMs, was used as a control. DMSO, the solvent for protease inhibitors, was used as a negative control. DCI and JLK-6 significantly inhibited excystation at all concentrations (Figure 20). The greatest inhibition of excystation was observed at 50 μM with where only 62.5 ± 5.3% and 60.3 ± 7.3% of oocysts excysted following treatments with JLK-6 and DCI, respectfully (P<0.05). PMSF did not significantly affect excystation.

Trophozoite development (measured by sporozoite rounding) was inhibited by JLK-6, and to a lesser extent DCI (Figure 21). For all treatments, except JLK-6, the proportion of rounded parasites increased over time. The ranges of rounded cells was 22.0 ± 5.2% to 32.1 ± 5.2% for the JLK-6 treatment, compared to 63.6 ± 4.5% to 97.6 ± 6.8% for the PBS control, 38.7 ± 5.2% to 82.0 ± 8.2% for PMSF, and 32.5 ± 3.8% to 81.72 ± 7.3% for DCI.

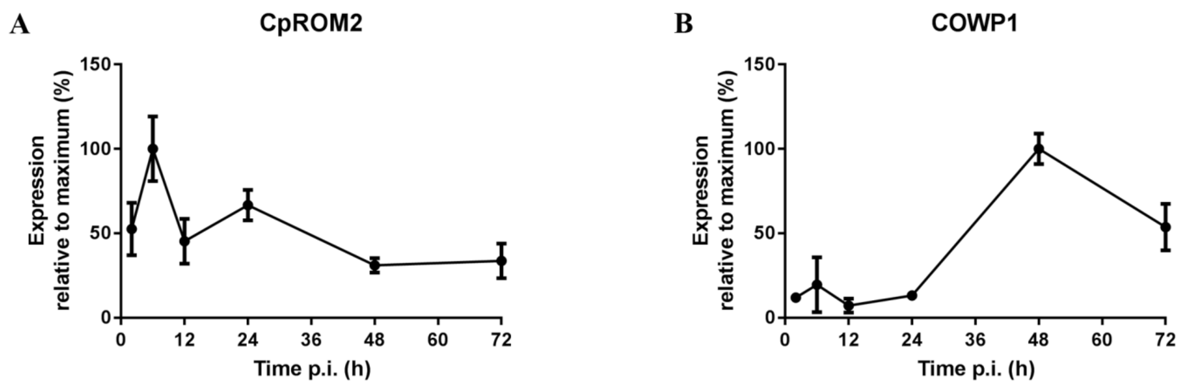

Figure 17. Expression of CpROM2 and COWP1 during an *in vitro* infection.
HCT-8 cells were infected with *C. parvum* sporozoites to study the temporal changes of CpROM2 (A) and COWP1 (B). mRNA was quantified via qRT-PCR and normalized against 18SrRNA. COWP1 is an oocyst wall protein used as a marker of sexual stage development, and was highest expressed 48 h p.i. CpROM2 was primarily expressed during early development and asexual stages with peak expression 6 h p.i.

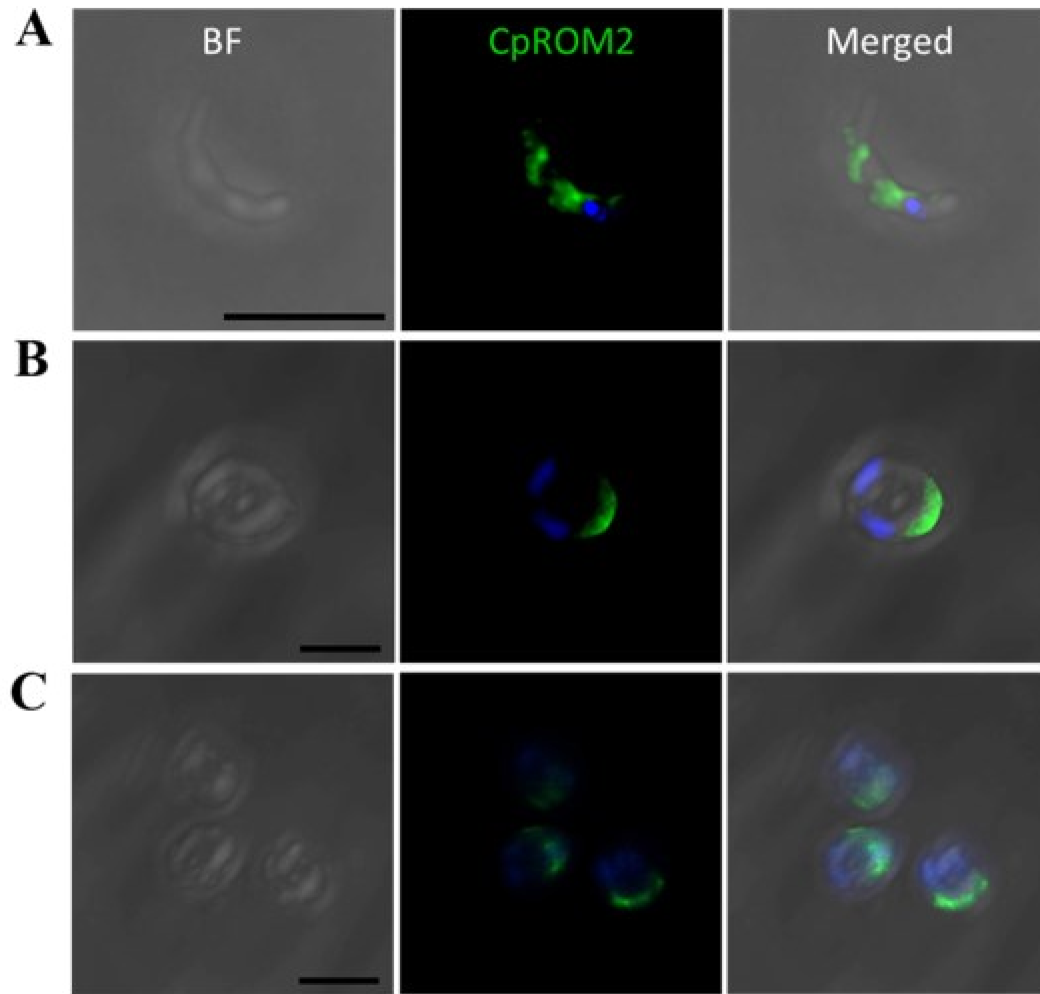

Figure 18. Immunolocalization of CpROM2 in *C. parvum* oocysts and sporozoites.
After fixation, intact oocysts, bleach treated oocysts and sporozoites were incubated with antibodies targeting CpROM2 that were conjugated to FITC (green). (A) CpROM2 was detected in sporozoites both sub-apically and anterior to the nucleus. CpROM2 was detected in sporozoites within oocysts, but was not found in the outer oocyst wall of intact oocysts (B), or the inner layer after bleach treatment (C). Images were captured using laser confocal scanning microscopy (LSM700) with Z-stacks processed using Imaris 8.4 imaging software. Scale bar is 5 µm.

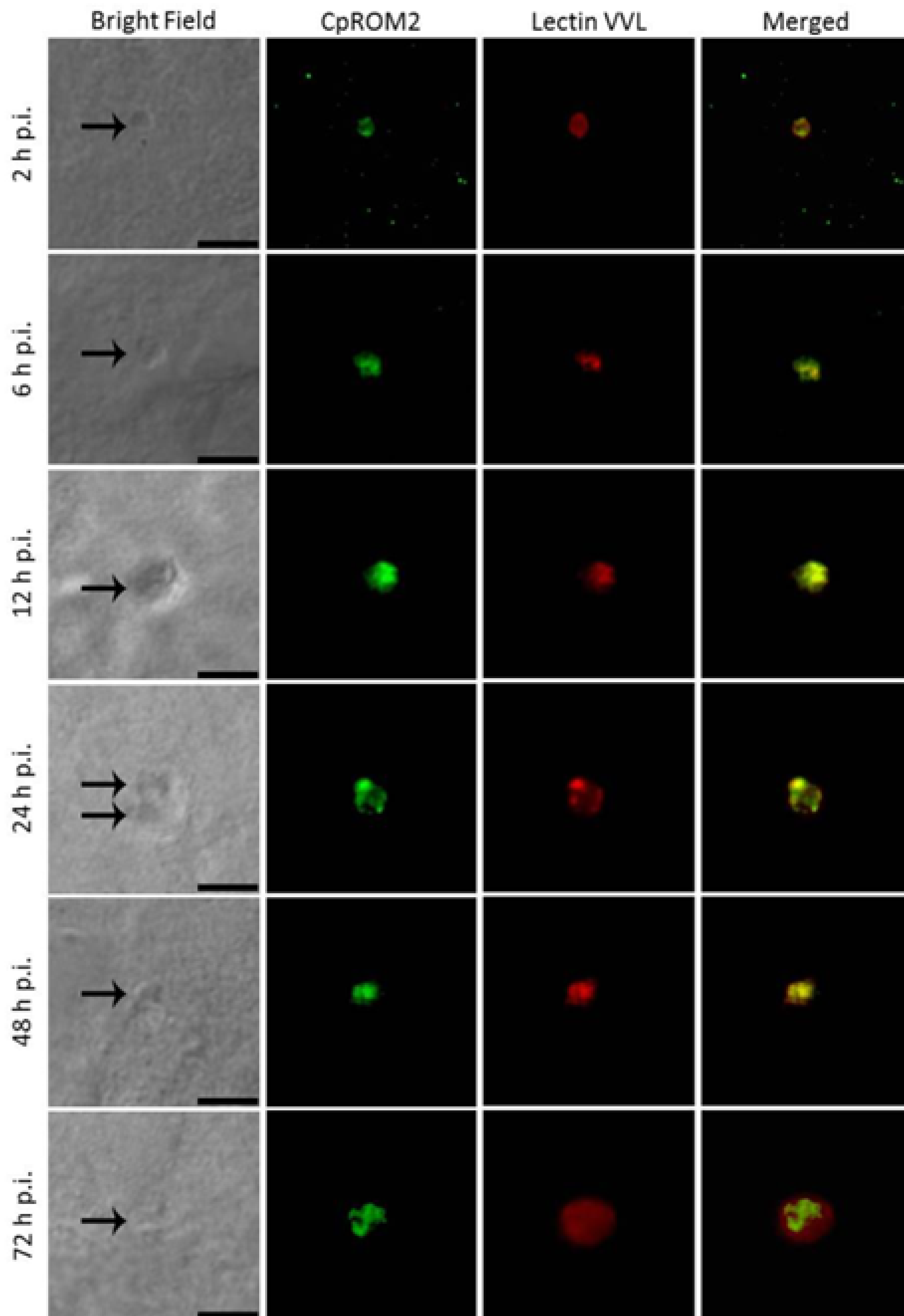

Figure 19. Intracellular localization of CpROM2 during a 2-72 h infection of HCT-8 cells. Infected cells were probed for CpROM2 localization (green) after fixation using the described methods. CpROM2 was seen in all intracellular stages with a distinct "halo-like" presentation at 2 and 24 h p.i. Lectin VVL binds to surface glycoproteins present during intracellular stages and is conjugated to Tetramethylrhodamine isothiocyanate (TRITC) and appears red. Images were captured using laser confocal scanning microscopy (LSM700) with Z-stacks processed using AxioVision 4.8 image software. Scale bar is 5 µm.

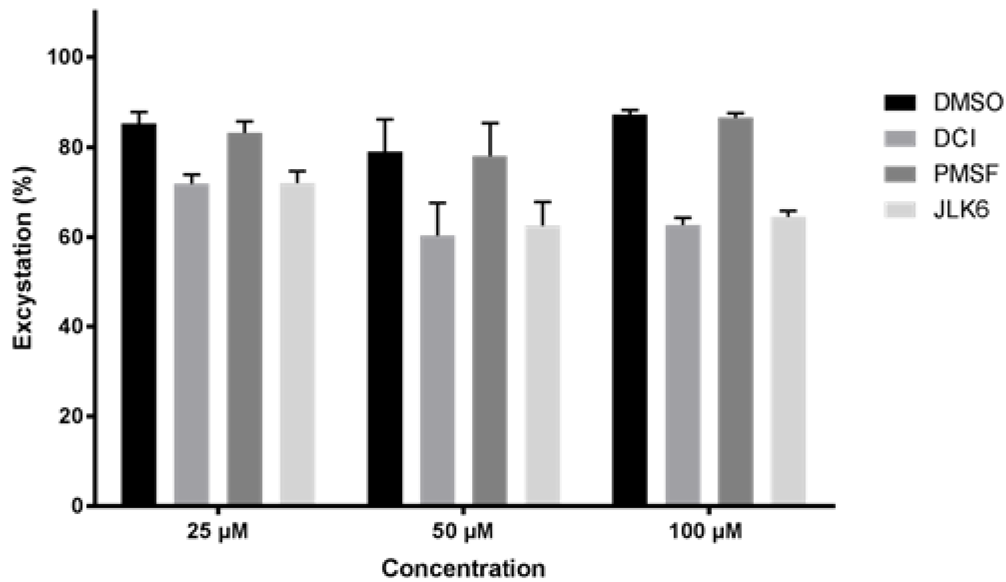

Figure 20. Excystation is lowered in the presence of serine protease inhibitors.
C. parvum oocysts were resuspended in RPMI-1640 supplemented with 0.8% sodium taurocholate and 25 μM, 50 μM and 100 μM of inhibitors. DMSO served as a control. After 1 h, the proportion of excysted oocysts was counted.

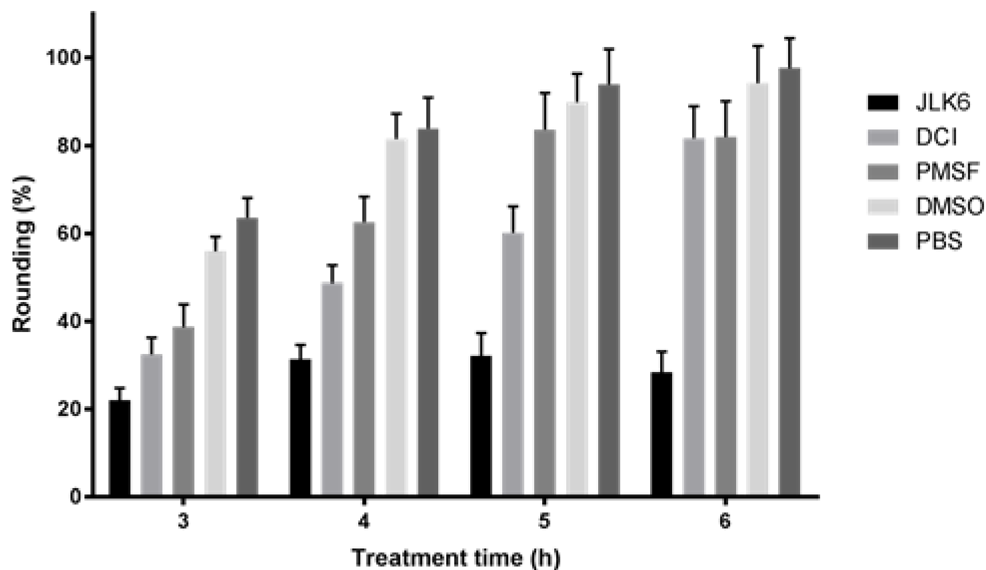

Figure 21. Treatment with rhomboid protease inhibitors decreases the proportion of rounded *C. parvum* sporozoites.
HCT-8 conditioned RPMI-FBS was supplemented with 100 μM of JLK6, DCI, PMSF, and DMSO. PBS served as a control. *C. parvum* sporozoites were exposed to the media for 3-6 h p.e., and the amount of rounded parasites was counted at each time point.

Discussion

ROMs are critical for motility and host cell invasion by the apicomplexans *Toxoplasma* and *Plasmodium* [22, 33]. However, little is known about the role of ROMs during *Cryptosporidium* development. Here we show CpROM2, a previously uncharacterized *Cryptosporidium* ROM, is expressed throughout intracellular development and localizes sub-apically within sporozoites. Using ROM specific inhibitors we also provide evidence that *Cryptosporidium* ROMs contribute to sporozoite excystation and trophozoite formation.

CpROM2 gene expression differed from that previously reported for CpROM4 and CpROM5 [19]. The highest CpROM2 expression was at 6 h p.i., compared to 24 h. p.i. for CpROM4 and CpROM5. TgROM2, the CpROM2 homolog in *Toxoplasma*, is almost exclusively expressed in oocysts and zoites (sporozoites and tachyzoites), similar to our observations of early CpROM2 expression in *Cryptosporidium* [12, 21].

The CpROM2 protein localized to areas immediately anterior to nucleus and sub-apically in sporozoites. TgROM2 is also found anterior to the nucleus in *T. gondii* zoites, in an area identified as the Golgi apparatus. It is not known where the *Cryptosporidium* Golgi apparatus is located, but the molecular machinery for Golgi function has been described [34, 35]. Although TgROM2 is exclusive to the Golgi apparatus, TgROM1 localizes to micronemes in the apical region of zoites, similar to the localization of CpROM2 in the present study [15]. Considering that CpROM2 is a ROM1/2 homolog, it is possible that it performs the functions of ROM1 and ROM2 in *Cryptosporidium*. The localization of CpROM2 is distinctly different than CpROM4, which localizes to the anterior and posterior poles, but shows some similarity to CpROM5, which is also found in the apical/sub-apical region [19].

The substrate specificity of CpROM2 is not known. However, in a heterologous assay, TgROM2 can cleave the *Cryptosporidium* thrombospondin-related adhesive protein TRAP-C1 and the mucin-like glycoprotein GP900, which function during excystation, attachment and motility of sporozoites [36-38]. The substrate specificity of the other *Cryptosporidium* ROMs is known. CpROM4 cleaves GP900 and the thrombospondin-related adhesive protein CpTSP10. CpROM5 cleaves GP900 and the thrombospondin-related adhesive proteins TRAP-C1, CpTSP6, CpTSP8, CpTSP9, and CpTSP10 [19].

Excystation and the transition from sporozoite to trophozoite were reduced in presence of the isocoumarins DCI and JLK-6, which are specific inhibitors of ROM activity. In contrast, the general serine protease inhibitor PMSF, which does not inhibit ROM activity, had no effect on life cycle development. Widmer et al. [39] similarly showed that DCI significantly reduced excystation and the infectivity of *C. parvum* in a MDBK infection model. DCI forms a covalent bond with serine in the catalytic dyad of rhomboid proteases; however, the bond is unstable and activity of the ROM can return [40]. In contrast to DCI, JLK-6 irreversibly inactivates ROMs by alkylating the active site [40-42]. This could explain why trophozoite formation was more effectively inhibited by JLK-6 than DCI. Collectively, these data are evidence that CpROMs play a significant role in excystation and trophozoite development.

In summary, we determined the gene expression and protein localization of CpROM2 during *Cryptosporidium* life cycle development, and we showed that CpROMs play a significant role during excystation and trophozoite development. This knowledge of the early stages of *Cryptosporidium* development can inform the development of novel therapeutics.

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GENERAL DISCUSSION

Apicomplexans are one of the largest phylum of intracellular parasites with more than 6000 named species and potentially over a million still unnamed [1]. Infecting millions each year, these parasites are a major cause of disease and death in animals and humans [2-4]. *Cryptosporidium* infects all major vertebrate groups, causing the intestinal disease cryptosporidiosis [5]. Infected children and toddlers suffer from malnutrition resulting in cognitive deficiencies, morbidity and even mortality [6-8]. Cryptosporidiosis is of particular concern in immunocompromised individuals, potentially becoming chronic and life threatening [9, 10]. There is an urgent need for novel therapeutics for the treatment of cryptosporidiosis because current strategies lack efficacy, especially in immunocompromised populations. Discovery of potential vaccine candidates or the development of new drug regimens is contingent on a comprehensive understanding of *Cryptosporidium* biology. This dissertation focused on the role glycoproteins and glycan decorations have in promoting *Cryptosporidium* replication with the core objective of identifying triggers and mechanisms underlying this change. The hypothesis tested was that *Cryptosporidium* binds host glycoproteins and the glycans decorating these glycoproteins are necessary for promoting the transition from invasion to replication.

Cryptosporidium sporozoites passively infect the intestinal epithelium of a host, remaining extracytoplasmic within a parasitophorous vacuole, for the duration of the life cycle [11]. This is in stark contrast to *Toxoplasma* and *Plasmodium,* which actively invade the host cell cytoplasm, forming an intracellular vacuole [12, 13]. The unique location of *Cryptosporidium* led us to investigate potential triggers located at the surface of a host cell, external to the host cell cytoplasm.

We identified glycoproteins of the host secretome of HCT-8 cells, Gal-GalNAc decorated mucins and free Gal-GalNAc glycans trigger *Cryptosporidium* differentiation into replicative stages. Glycoproteins and lectins, specifically GP900, GP40/15 and P30 form complexes at the surface of *Cryptosporidium* parasites that specifically target and engage Gal and GalNAc host glycoproteins [14]. Processing of bound host cell glycoproteins expressed at the surface of host cells helps facilitate attachment and motility [15-17]. Aggregation and clustering of Gal-GalNAc rich glycoproteins at the surface of host cells as a result of motility play a critical role in initiating, and driving the host directed uptake of the *Cryptosporidium* [18, 19]. We do not know the effect glycoprotein clustering has on *Cryptosporidium* sporozoites, but one potential explanation is the clustering of bound host glycoproteins provides the necessary signal for initiating *Cryptosporidium* replication. Therefore, glycoprotein clustering may serve two purposes for the parasite: provide the stimulus required for passive (host directed uptake) invasion of sporozoites, and triggering the switch from an invasive sporozoite to a replicative trophozoite.

Rounding in response to secreted host glycoproteins is a morphological change consistent with a switch to replication and may signal a committed step towards a replicative mode. As a consequence rounded sporozoites may not express the necessary molecular machinery for invasion. Extracellular trophozoites have been observed in cell culture and life cycle progression has been reported in axenic culture, but the infectivity of these parasites was never tested [20-22]. We found that trophozoite formation correlated with a loss of infectivity, and although invasion is primarily a host driven process, sporozoite have some role in invasion. While investigating the infectivity of trophozoites we made the observation that the transition to a replicative trophozoite occurs independent of invasion. Initiating replication prior to invasion may be advantageous to the parasite, devoting energy and resources to increasing parasite numbers while the host cell

encapsulates the parasite. Therefore, proximity to host cells is necessary during trophozoite formation is critical for parasite survival and success. Trophozoite formation must be tightly regulated as inappropriate rounding and replication away from the host cell surface could be detrimental. Turnover over the mucus layer lining intestinal cells may result in the removal of trophozoites formed away from the host cell surface [23, 24]. Rounding away from the host cell surface would also leave the parasite exposed to the intestinal environment and potential removal by the immune system.

Our observation that increasing concentrations of FBS, and as a consequence glycoprotein triggers, prompted us to look at the potency of the trophozoite trigger recovered from active infections of HCT-8 cells. We found trophozoite formation was enhanced during treatments with conditioned media from a 3 h infection (early infection) when compared to conditioned media from a 24 h infection (late infection) and non-infected HCT-8 cells. A potential explanation for this is that the pro-inflammatory response to *C. parvum* infections in HCT-8 cells leads to increased prostaglandin synthesis. Elevated Prostaglandin E_2 levels directly correlate to increased colonic mucin (O-GalNAc glycoprotein) production [25, 26]. Recent work found increased amounts of detached sporozoites as infection times progressed [27]. Increases in glycoprotein production would increase the availability of potential triggers for trophozoite formation, but can also help the host by aiding in pathogen removal. Lowered trophozoite formation in conditioned media from late infection may be a consequence of *C. parvum* dampening the host immune response. *C. parvum* has been shown to reduce host apoptosis in response to the parasite as well many of the cytokines involved in protective glycoprotein synthesis [28-31]. A delicate balance therefore must be struck by the parasite to successfully evade

the host immune response, while potentially maximizing the elevated expression of host glycoproteins for initiating invasion and replication.

Although we identified host glycoproteins and free Gal-GalNAc as triggers for trophozoite development we observed *C. parvum* progressed towards replication faster when compared to *C. hominis* Differences in the rate of trophozoite formation may indicate the trigger for replication is species specific. This is supported by the observation that Gal-GalNAc pre-treatment of *C. hominis* sporozoites does not result in a loss in the infectivity of HCT-8 cells [17]. *C. parvum* and *C. hominis* continue to be the two species of *Cryptosporidium* of greatest concern to human health and despite sharing a 99% genetic identity, the zoonotic *C. parvum* affects a wide range of mammal hosts while the anthroponotic *C. hominis* only infects humans [32-35]. Antigenic difference in surface adhesins required for attachment and motility may explain the differences in host ranges and infection patterns [36-38]. In *Plasmodium*, host specificity comes from the recognition of the proper host glycoproteins decorating the surface of erythrocytes [37, 39]. However, our observations of increasing concentrations of Gal-GalNAc triggering a greater proportion of *C. hominis* sporozoites to round and replicate supports Gal-GalNAc as a trigger, but demonstrates concentration of the trigger is critical. As a consequence, we hypothesize there may be a minimum density of Gal-GalNAc necessary to promote differentiation, or Gal-GalNAc may not be the trigger for *C. hominis*. An alternative hypothesis that replication and life cycle progression is slower in *C. hominis* is supported by evidence that *C. parvum* rapidly displaces *C. hominis* during infections [40].

Glycoproteins are important ligands for *Cryptosporidium* and mucosal pathogen binding [23, 36, 41]. With complexity in structure, arrangement, composition of amino acids and glycan moieties, characterizing these glycoproteins can be difficult, impeding our progress in

identifying the structures and residues that are targeted by invading pathogens. This is compounded when FBS and other media supplements are added to an already heterogeneous host secretome in order to support host cell growth in culture. In this work, we describe one of the first attempts at using glycomimetic polymers to study *Cryptosporidium* biology. These glycomimetics offer us the opportunity to study the role of glycoproteins and glycans and modify the presentation of these residues with nanoscale precision. This is a strategy that has been used in other apicomplexans with mimetics used to inhibit invasion of *Plasmodium* and provide protective immunity against *Toxoplasma* infections [42, 43].

The limited work that has been done in *Cryptosporidium* and glycomimetics has focused on understanding the immune response to synthetic *Cryptosporidium* epitopes [44-47]. We present here a demonstration on the potential use for glycomimetic polymers in *Cryptosporidium* research. A key outcome from this work is the use of glycan microarrays and the ability to control the density of imprinted glycomimetic polymers. We demonstrate glycomimetics decorated with Gal and GalNAc were the largest contributors to replication, and trophozoite development is triggered in a density dependent manner. This is consistent with our previous findings that free Gal-GalNAc trigged replication in *Cryptosporidium* sporozoites. The observation of increased trophozoite formation in the spots imprinted with higher densities of Gal and GalNAc could be explained by the observation that Gal-GalNAc glycoproteins aggregate at the site of attachment during invasion [16]. More work must be done to determine not only density effects, but also how variation and arrangement of glycans triggers the transition to replication. Trophozoite formation was still greater after treatment with HCT-8 conditioned media when compared to solubilized and imprinted glycomimetic polymers, so more work needs to be done to accurately represent the glycoproteins of the host secretome. This work provides

the framework for future experiments involving glycomimetic polymers, but analysis needs to be optimized for high throughput screening of potential glycomimetic candidates for disease prevention. Glycomimetic polymers could be constructed and designed as competitive inhibitors for *Cryptosporidium* attachment, or inducers of rounding away from the host cell surface. Preventing attachment or causing premature replication initiation could aid in parasite removal via the host immune system. At a minimum, these findings demonstrate the use of glycomimetic polymers as a means to investigate *Cryptosporidium* biology.

Proteolytic shedding of adhesins present on the sporozoite surface by a family of intermembrane serine proteases known as the rhomboids (ROMs) have been consistently identified as the major contributors to motility and invasion in apicomplexans, specifically *Toxoplasma* and *Plasmodium* [48-52]. The proteolytic activity of ROMs in *T. gondii* has even been implicated in promoting the transition from invasion to replication [53, 54]. Little is known about the role of ROMs in *Cryptosporidium* development, especially during the formation of replicative trophozoites. Three ROMs have been identified in *Cryptosporidium*, named CpROM4, CpROM5 and CpROM1/2, of which CpROM1/2 has yet to be characterized [55]. The CpROM2 homolog has only been identified in two other apicomplexans *T. gondii* and *Neospora caninum*, with expression predominantly found in sporozoites [56].

We found CpROM2 localized CpROM2 anterior to the nucleus and sub-apically in sporozoites, which is contrast to reports in *T. gondii* where ROM2 localizes primarily to the Golgi apparatus [56, 57]. We also determined CpROM2 co-localizes with TRAP-C1 and CpROM5. In *T. gondii* the ROM2 and ROM5 homologs have similar substrate specificity, and in heterologous assays, TgROM2 can cleave CpTRAP-C1 and CpGP900 substrates recognized by CpROM5 [56, 58] . Although the scope of this dissertation did not cover the substrate specificity of

CpROM2, the temporal expression and localization of CpROM2 was consistent with what has been reported for TRAP-C1 [55]. This observation implicates TRAP-C1 as a potential substrate and CpROM2 as a protease used during egress and early intracellular development. Intracellular localization of CpROM2 as opposed to membrane associated like CpROM4 and CpROM5 suggests CpROM2 may cleave the cytoplasmic domain of adhesins expressed at the parasite surface. Cleavage and the subsequent accumulation of the cytoplasmic domain of AMA1 have been shown to initiate replication in *T. gondii* [53]. Activity from CpROM2 and the other CpROMs may contribute to trophozoite development by providing the internal signal required for initiating replication pathways. Further work must be done to clarify the role of CpROM2 during the *Cryptosporidium* life cycle, including examining ROM2 substrate specificity and enzyme activity.

Another significant finding was that excystation trophozoite formation was inhibited in the presence of isocoumarins, inhibitors specific for ROMs. This information begins to clarify the role ROMs have in the *Cryptosporidium* life cycle. These findings are consistent with previous studies outlining the importance of serine proteases during *Cryptosporidium* excystation and invasion of host cells, but specifically identify ROMs as a primary player [59, 60]. The reduction in excystation suggests CpROM5, which has been identified in the oocyst wall, is a major be contributor to the release of sporozoites from oocysts [55]. Critically, treatment with isocoumarins revealed ROM activity is essential for trophozoite formation and initiating replication. The potential role of ROMs in motility and invasion of *Cryptosporidium*, coupled with our study identifying ROMs as necessary for trophozoite formation, suggests the transition from motility and invasion to replication uses similar ROM machinery. Moreover, it indicates

motility and replication are intimately connected and implicates ROM machinery/activity as the common thread.

Recommendations for future work

Use of glycomimetic polymers opens up new and innovative approaches to studying the ligands targeted by *Cryptosporidium.* As mentioned, optimization of microarray analysis must be investigated for high-throughput screening of potential ligands, especially if valency and heterogeneous synthetic peptides are investigated in the future. Though we have shown receptor specificities can be identified through mimetics it is important to expand on this and evaluate the impact type and density have during an infection. Clustering of sphingolipid-enriched membrane microdomains (SEMs) aggregate Gal-GalNAc decorated glycoproteins at the site of invasion. The increased density of Gal-GalNAc glycoproteins has been suggested as facilitating the intracellular signaling required for host-cell actin remodeling through a PI-3K/Cdc42 pathway [16, 18, 19, 61].

A potential hypothesis is that during motility, engagement of host ligands causes the aggregation Gal-GalNAc glycoproteins. To answer this question, the spatial arrangement of glycoproteins can be modified by grafting glycomimetics to the surface of host cells, while deglycosylating native proteins [62]. Deglycosylation of host cells and supplanting with glycomimetic polymers can be used to determine the density of Gal-GalNAc required for initiating replication at the host cell surface. We acknowledge we may not be able to address the question of how clustering of glycoproteins at the site of invasion promotes host-cell remodeling and invasion as this may be disrupted. However, we will gain valuable information about glycoprotein engagement and aggregation at the host cell surface. Determining the density of

Gal-GalNAc required for host-cell remodeling could be useful in the development of competitive inhibitors for the control of *Cryptosporidium* infections.

Our data also suggests during infections there is an increased expression of host-secreted glycoproteins. This could be a result of a directed response by the host cell. Elevated expression of TLRs and activation of the NF-κB signaling pathway are responsible for increased IL-1β, TNFα and IL-8 production. These pro-inflammatory cytokines could cause mucin turnover and production [63-66]. One hypothesis is that a proinflammatory response increases the production and secretion of intestinal mucins reducing the infectivity of *Cryptosporidium*. Elevated mucin production could explain the greater proportion of rounded parasites in conditioned media isolated from an active infection.

To answer these questions we can evaluate the total glycan content in non-conditioned, conditioned and infected conditioned media recovered to evaluate glycoprotein secretion. We can also directly measure the immune response to *Cryptosporidium* and evaluate the production of mucins at a transcriptional level. This approach will aid in interrogating the pathways either induced or controlled by *Cryptosporidium*. Information gleaned from this will lead to a better understanding of *Cryptosporidium* biology, but will also identify mechanisms of host immune response evasion and survival by parasites.

Finally it is important to further investigate the substrates recognized and role of CpROM2 in *Cryptosporidium*. Our data suggests CpROM2 co-localizes with TRAP-C1 and is expressed during invasion and intracellular development. Although we do not know the substrates CpROM2 acts on, homologs for ROM2 in *Toxoplasma* target similar substrates [58]. One potential hypothesis is that CpROM2 serves a redundant role during the *Cryptosporidium* life cycle. To test this, we could apply a CRISPR/Cas9 approach to modify the genome of

Cryptosporidium. CRISPR/Cas9 has only recently been reported as a potential strategy for genome manipulation in *Cryptosporidium*, but would allow us to examine the role of individual rhomboid proteases during the parasite life cycle [67]. This would lead to a more complete understanding of *Cryptosporidium* biology and clarify the roles of rhomboid proteases during development. We anticipate this could be used to exploiting targets that disrupt infections and the pathology of cryptosporidiosis.

Conclusion

The data presented in this work has identified host glycoproteins and free Gal-GalNAc as triggers for life cycle progression in *Cryptosporidium,* and have demonstrated the use of glycomimetic polymers in studying *Cryptosporidium* biology. We provide the framework for which glycomimetic polymers can be used in microarrays or in suspension, but more work needs to be done to optimize analysis, especially for high throughput screening of targets. Glycomimetic polymers have tremendous potential in identifying the host ligands bound by pathogens. Glycomimetic technologies can contribute to our overall understanding host-pathogen interactions while guiding the development of targeted drug interventions. We also characterized a third ROM encoded by *Cryptosporidium*, which we call CpROM2, while providing evidence that supports CpROMs having a significant role in excystation and trophozoite development. This information helps shed light on the role ROMs have during the *Cryptosporidium* life cycle and can help inform the development of novel therapeutics designed to inhibit *Cryptosporidium* replication, thereby preventing cryptosporidiosis.

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APPENDIX. PUBLICATIONS

Peer reviewed publications

1. Edwinson, A., Widmer, G. & McEvoy, J. Glycoproteins and Gal-GalNAc cause *Cryptosporidium* to switch from an invasive sporozoite to a replicative trophozoite. *International Journal for Parasitology* **46**, 67-74 (2016).
2. Rašková, V. *et al.* Human cryptosporidiosis caused by *Cryptosporidium tyzzeri* and *C. parvum* isolates presumably transmitted from wild mice. *Journal of clinical microbiology* 51, 360-362 (2013).