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Human immune responses in cryptosporidiosis

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Abstract

Immune responses play a critical role in protection from, and resolution of, cryptosporidiosis. However, the nature of these responses, particularly in humans, is not completely understood. Both innate and adaptive immune responses are important. Innate immune responses may be mediated by Toll-like receptor pathways, antimicrobial peptides, prostaglandins, mannose-binding lectin, cytokines and chemokines. Cell-mediated responses, particularly those involving $CD4^+$ T cells and IFN-γ play a dominant role. Mucosal antibody responses may also be involved. Proteins mediating attachment and invasion may serve as putative protective antigens. Further knowledge of human immune responses in cryptosporidiosis is essential in order to develop targeted prophylactic and therapeutic interventions. This review focuses on recent advances and future prospects in the understanding of human immune responses to *Cryptosporidium* infection.

Keywords

antibody; cell-mediated immunity; chemokine; cryptosporidiosis; *Cryptosporidium*; cytokine; diarrhea; humoral immunity; parasite; T cell

Cryptosporidium is an apicomplexan protozoan parasite that causes diarrheal disease in humans worldwide. The biology, pathogenesis, epidemiology and clinical features of *Cryptosporidium* and cryptosporidiosis have been comprehensively described in several excellent reviews within the past 10 years [1-12] and are briefly summarized here.

The immune status of the host plays a critical role in determining susceptibility to infection with this parasite as well as the outcome and severity of cryptosporidiosis. In immunocompetent hosts infection is often asymptomatic or mild to moderate and self-limited (reviewed in [11]). However, in immunodeficient hosts such as patients with HIV/AIDS, congenital immunodeficiencies and transplant recipients, infection can result in persistent, debilitating and possibly fatal diarrhea and wasting (reviewed in [10,11]). In areas where cryptosporidiosis is endemic, most symptomatic infections occur in early childhood and in the immunodeficient [4]. Although *Cryptosporidium* primarily infects the distal small

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intestine, in severely immunodeficient patients this parasite can infect extraintestinal sites such as the lungs, biliary tract and pancreas (reviewed in [11]).

Transmission of the parasite occurs via the fecal–oral route, either by ingestion of contaminated water or food or by person-to-person or animal-to-human transmission (reviewed in [11,12]). A major mode of transmission is via contaminated water supplies, often resulting in widespread outbreaks (reviewed in [12]). While cryptosporidiosis can be endemic in developing countries, several epidemic waterborne outbreaks have also been reported in developed countries. The potential for intentional contamination of water supplies has led to inclusion of *Cryptosporidium* as a Category B priority pathogen for biodefense [13].

There are two major species of *Cryptosporidium* that infect humans. The zoonotic species *Cryptosporidium parvum* infects animals as well as humans whereas the anthroponotic species *Cryptosporidium hominis* primarily infects humans (reviewed in [8]). Other species that have occasionally been reported to infect humans include *Cryptosporidium felis*, *Cryptosporidium meleagridis*, *Cryptosporidium muris*, *Cryptosporidium wrairi*, *Cryptosporidium saurophilum*, *Cryptosporidium baileyi*, *Cryptosporidium andersoni*, *Cryptosporidium serpentis* and *Cryptosporidium nasorum* (reviewed in [8]).

After ingestion of as few as nine oocysts (reviewed in [2]), sporozoites are released from excysted oocysts into the intestinal lumen and invade epithelial cells, particularly in the terminal ileum [3,11]. The parasite then undergoes replication via asexual and sexual cycles within a parasitophorus vacuole that is located in the intestinal epithelial cell membrane in an intracellular yet extracytoplasmic location. Following sexual reproduction, large numbers of both thick- and thin-walled oocysts are released. Thin-walled oocysts excyst within the lumen to infect other epithelial cells resulting in an autoinfection cycle, while thick-walled oocysts are excreted in the feces [11]. Infection of the gut epithelium may result in villus flattening, which causes malabsorption and diarrhea [11]. In addition, there may be a secretory component to the diarrhea which may be due to increased substance P [14] or prostaglandin production [15], and disruption of the intestinal epithelium, which can inhibit NaCl absorption. The parasite may promote apoptosis in adjacent epithelial cells while inhibiting apoptosis in the infected cells, thereby facilitating prolonged survival of the parasite [16]. In immunocompetent persons, *Cryptosporidium* was reported to account for up to 6.1% of diarrheal disease worldwide (reviewed in [2]). However, these studies used stool microscopy for detection of infection, whereas recent studies using PCR for detection suggest that the number of infections may actually be higher [17]. The incubation period to establish infection can range from 1 to 2 weeks (reviewed in [10]). Most patients with symptomatic infection present with acute watery diarrhea that lasts for a few days to 2 weeks, but can be persistent and last for up to 5 weeks. Other accompanying symptoms may include nausea, vomiting, anorexia, abdominal cramping, fever and weight loss.

Cryptosporidiosis is a common cause of parasitic diarrhea in patients with HIV/AIDS and was reported to occur in up to 24% of these patients in the era before highly active antiretroviral therapy (HAART) was available (reviewed in [2]). In patients with HIV/AIDS the severity of the disease varies, depending on the degree of immunosuppression, as reflected by $CD4^+$ counts. In patients with relatively high $CD4^+$ counts (>180 cells/mm^3) the infection may be asymptomatic or result in mild diarrhea. However, patients with $CD4^+$ counts of less than 50 cells/mm^3 can develop persistent or intractable diarrhea, thus emphasizing the importance of the host immune response in controlling the disease (reviewed in [4]).

Cryptosporidium is a major cause of diarrhea in children in developing countries, particularly in those under 2 years of age. Up to 12% of diarrheal disease in children in these countries may be caused by this parasite [11]. In developing countries, cryptosporidiosis is more common, and its consequences are more severe in malnourished than well-nourished children, possibly because of impaired T-cell responses [18]. Cryptosporidiosis in early childhood can lead to growth faltering and impaired cognitive development, as well as further malnutrition which can put these children at higher risk for recurrent diarrheal diseases. A recent investigation suggests a possible genetic predisposition to cryptosporidiosis in Bangladeshi children with the *B*15* HLA I allele and the *DQB1*0301* HLA II allele [19].

In developed areas of the world, the prevalence of cryptosporidiosis in patients with HIV/AIDS has declined with implementation of strategies to improve water supplies and the institution of HAART. However, the emergence of drug-resistant HIV variants and failure or discontinuation of HAART is associated with the re-emergence of *Cryptosporidium* infection [20,21]. Furthermore, HAART is not widely available or affordable to patients in most developing countries. Currently available therapy for cryptosporidiosis is suboptimal. Although over 100 therapeutic agents have been evaluated for anticryptosporidial efficacy, nitazoxanide is the only drug that has been approved by the US FDA for treatment of cryptosporidiosis in immunocompetent persons in the USA. However, this drug is not effective in immunocompromised individuals [22,23]. In patients with HIV/AIDS, the mainstay of treatment is immune reconstitution with HAART, which results in clearance or abrogation of infection. There is no vaccine available for cryptosporidiosis.

The persistent threat posed by *Cryptosporidium*, the paucity of effective chemotherapeutic agents and lack of a vaccine underscores the importance of continuing efforts to identify specific target antigens and better understand host immune responses to the parasite in order to develop effective immune-based prophylactic and/or therapeutic strategies.

Immune response

Although the outcome and severity of infection is critically dependent on the immune status of the host, the nature of the immune response in cryptosporidiosis, particularly in humans, is poorly understood. Much of what is known about immune responses to *Cryptosporidium* is based on studies done in animals, particularly in mice (reviewed in [24,25]). However, there have been very few studies on immune responses in humans. Most studies in humans have focused on systemic antibody responses with a few addressing cell-mediated responses. Other than fecal antibody responses, there have been no studies on mucosal immune responses in humans. Such studies are challenging to perform in humans since they would involve invasive tissue sampling. Thus, most investigations have been done in tissue culture models utilizing human cell lines, or on peripheral blood mononuclear cells (PBMCs). *In vitro* studies in cancer-derived human cell lines and *ex vivo* studies on peripheral blood mononuclear cells have limitations since they may not accurately reflect the intricate dynamics of the mucosal immune system *in vivo* [26]. In this review we have focused mainly on studies of immune responses in humans and have also included *in vitro* studies in human cell lines.

Innate immune responses

Recent studies in humans as well as *in vitro* in human cell lines suggest that specific innate immune responses may play a role in resistance to cryptosporidiosis. Studies done in humans are summarized in Table 1.

Toll-like receptor-mediated pathways—Toll-like receptors (TLRs) are a family of conserved molecules that play an important role in mediating resistance to a wide array of pathogens by recognizing specific pathogen-associated molecular patterns (PAMPs) [27,28]. Most TLRs signal by recruiting MyD88, an adaptor protein which is involved in stimulation of several pathways of the innate immune system, including the IL-1 receptor, IL-18 receptor and TLR pathways [29,30]. Recognition of PAMPs by TLRs and subsequent recruitment of MyD88 activate a cascade of kinases, resulting in the nuclear translocation of NF-κB, the expression of proinflammatory cytokine genes and the initiation of host immune responses. Recent studies have shown that *C. parvum* induced recruitment of TLR2 and TLR4 to the site of infection leading to activation of downstream effectors in a human cholangiocyte model *in vitro* [31]. Knockdown of MyD88, TLR2 and TLR4 expression using dominant negative mutant and siRNA approaches resulted in inhibition of downstream signaling pathways and increased *C. parvum* infection in cells in which MyD88 expression was decreased. TLR4 expression in this model appears to be regulated by the miRNA let-7. *C. parvum* infection resulted in MyD88 and NF-κB-dependent suppression of let-7 and consequent upregulation of TLR4 in these cells [32].

These *in vitro* studies were performed in human cholangiocytes, which are not the primary site of infection. However, there have been no studies reported on the role of TLR-mediated pathways in *C. parvum* infection in intestinal epithelial cells or other immune cells that the parasite or its antigens may come in contact with, such as dendritic cells and macrophages. Although TLR ligands for *Cryptosporidium* have not been identified, the parasite expresses surface-associated glycosylinositol phospholipids [33] and glycosylphosphatidylinositol anchors [34], which have been implicated as putative TLR ligands in other apicomplexan parasites such as *Plasmodium* [35].

Antimicrobial peptides—With improved understanding of mucosal defense it is evident that the intestinal mucosa not only serves as a physical barrier, but plays an active and important role in innate defenses. Antimicrobial peptides (AMPs) are one of the components of the intestinal mucosal barrier. AMPs are small polypeptides (<100 amino acids) that have antimicrobial and immunomodulatory properties and are evolutionarily conserved effectors of the innate immune system [36]. AMPs that are expressed and secreted by human intestinal epithelial cells include α- and β-defensins and cathelicidins. There are six known human β-defensins (HBDs), of which HBD-1 is constitutively expressed and HBD-2, -3 and -4 are expressed during infection and/or inflammation.

In vitro studies in human intestinal epithelial cell models have shown that *C. parvum* infection initially downregulates HBD-1 production, perhaps thus facilitating parasite survival [37]. However, infection also induces expression and secretion of HBD-2, which has antimicrobial activity against the parasite *in vitro* and may play a role in recruitment of T cells and dendritic cells *in vivo*, eventually facilitating clearance of the parasite. *C. parvum* has also been shown to upregulate HBD-2 expression via TLR2- and TLR4-mediated signaling and NF-κB activation in infected human cholangiocytes [31].

Mannose-binding lectin—Mannose-binding lectin (MBL) is another highly conserved component of the innate immune system [38]. It is a collagenous lectin found in serum that binds to specific carbohydrate residues on a variety of infectious organisms including *Cryptosporidium* [39]. Upon binding, MBL activates the lectin complement pathway in an antibody-dependent manner via mannose-binding lectin-associated serine proteases (MASPs), thereby promoting opsonization and phagocytosis. MBL activation is thought to result in formation of a membrane attack complex that may diminish parasite viability in addition to promoting opsonization and phagocytosis [39]. In a study of AIDS patients with cryptosporidiosis, patients homozygous for structural mutations in the *mbl2* gene and

consequent low serum MBL levels were more susceptible to infection with *Cryptosporidium* [39]. Low MBL levels may also be related to malnutrition or protein losses in the gut [39]. A study of Haitian children with cryptosporidiosis, many of whom were malnourished, reported that children with MBL deficiency (MBL ≤ 70 ng/ml) were more likely to be infected with the parasite [40]. However, this study did not investigate polymorphisms in the *mbl2* gene in these children. A recent study in Bangladeshi pre-school children reported that MBL deficiency and *mbl2* polymorphisms were strongly associated with *Cryptosporidium* infection, particularly in those with repeated infections [41]. Further studies are needed to better understand the implications of malnutrition, MBL deficiency, the role of *mbl2* polymorphisms and risk of subsequent infections.

Chemokines—Chemokines are a family of small 8–10-kDa protein molecules that are produced by epithelial cells. They exert their effects by interacting with G-protein-linked transmembrane chemokine receptors and function as chemoattractants for inflammatory cells by inducing chemotaxis and activating leukocytes. They are divided into two major groups, CC chemokines with adjacent cysteine residues, and CXC chemokines with cysteine groups separated by an amino acid. The CC chemokine ligand (CCL)-5 is a potent chemoattractant that is upregulated in a human intestinal epithelial cell model of *C. parvum* infection [42]. A study of *C. parvum* infection in human intestinal epithelial cells and intestinal xenografts reported increased expression of the CXC chemokine ligand (CXCL)-8 (or IL-8) and growth-regulated oncogene (GRO)-α in infected cells [43].

Peripheral blood mononuclear cells of malnourished Haitian children with cryptosporidiosis expressed higher levels of CXCL-8 upon *ex vivo* stimulation with *C. parvum* [44]. This cytokine was also detectable in stool samples of some Brazilian and Haitian children with cryptosporidiosis [45,46]. In addition, the CXC chemokine CXCL-10 (IFN-γ inducible protein 10), which recruits IFN-γ-producing T cells, was expressed in higher levels in jejunal biopsies of AIDS patients with cryptosporidiosis compared with uninfected control AIDS patients or normals [47]. Additional studies are required to confirm the role of specific chemokines in human cryptosporidiosis.

Cytokines—Cytokines are proteins that play a key role in modulation of both innate and adaptive immune responses and are discussed in this section as well as in that on adaptive immune responses. Studies in mice have established that IFN-γ is a major player not only in cell-mediated immunity, but in early innate immune responses as well [24,25,48]. However, the role of IFN-γ in human infections is not as clear. *In vitro* studies using the human intestinal cell lines, Caco-2 and HT-29, have demonstrated that IFN-γ directly prevents the parasite from invading host cells, most likely by activation of the JAK/STAT signaling pathway following binding to IFN-γ receptors on intestinal epithelial cells, as well as by modification of intracellular Fe^{2+} concentrations [49]. A recent study suggested that *C. parvum* infection may downregulate IFN-γ by suppression of STAT1-α signaling, thus facilitating invasion [50].

A case of persistent cryptosporidiosis has been described in a child with primary IFN-γ deficiency [51], suggesting that this cytokine is also important in human infections. In a study of experimentally infected humans, jejunal biopsies from previously uninfected (seronegative) individuals demonstrated no mucosal IFN-γ production, whereas those from most of the seropositive individuals did [52]. In the absence of IFN-γ production, seronegative individuals expressed IL-15, suggesting that other cytokines may play a role as well. Those volunteers that expressed higher levels of IL-15 in their jejunal mucosa had symptomatic infection but shed fewer oocysts than seronegative volunteers that did not express IL-15 [53]. Subsequently, it was demonstrated that IL-15 activates natural killer cells and γδ-T cells and may assist in recruiting other cells, which leads to clearance of the

parasite [54]. IL-4 was also expressed in the jejunal mucosa from seropositive volunteers, but there was no association with presence of symptoms or oocyst shedding. Jejunal biopsies from some experimentally infected human volunteers also expressed TGF-β [55]. Again, additional studies are essential to define the role of specific cytokines in innate and consequently adaptive immune responses.

Prostaglandins & substance P—Prostaglandins are potent lipid molecules that exert their effects via a wide array of receptors. In a study of human volunteers experimentally infected with *C. parvum*, it was shown that jejunal biopsies expressed TNF-α and IL-1β, which can stimulate prostaglandin production, but there was no association with symptoms [56]. *C. parvum* infection of human intestinal epithelial cells *in vitro* resulted in activation of prostaglandin H synthase 2 expression and increased production of prostaglandin (PG)-E2 and -F2-α [15]. Although prostaglandins may contribute to the pathogenesis of secretory diarrhea by altering chloride uptake and fluid secretion, they may also upregulate mucin production from epithelial cells, which can protect the host intestinal mucosa from being infected with *C. parvum* by interfering with attachment of the parasite. In addition, prostaglandins may stimulate HBD production and downregulate expression of inflammatory cytokines. Substance P is a neuropeptide that is located in the GI tract as well [57]. It can cause chloride ion secretion and play a role in secretory diarrhea. Jejunal biopsies from AIDS patients with cryptosporidiosis showed increased expression of substance P mRNA and protein in patients with diarrheal symptoms [14]. However, the exact roles of prostaglandins and substance P need to be further defined.

Adaptive immune responses

Cell-mediated immunity & T-cell responses—The crucial role of cell-mediated immune responses in protection from, and resolution of, cryptosporidiosis has been well established in both murine models and human studies [25,58,59]. Recent studies in humans are summarized in Table 2. The increased susceptibility of AIDS patients to *Cryptosporidium* infection and resolution of cryptosporidiosis following immune reconstitution underscores the importance of $CD4^+$ T cells [60,61]. Patients with $CD4^+$ counts less than 50 cells/mm^3 are more likely to have a fulminant form of the disease, while those with $CD4^+$ counts of 180 cells/mm^3 or more tend to have less severe self-limited disease (reviewed in [4]). $CD4^+$ cell responses are mediated in large part by the cytokine IFN-γ [25]. PBMCs from infected humans proliferate in response to recombinant and crude preparations of *Cryptosporidium* antigens [62-65]. The response is MHC II-dependent and is characterized by increased production of IFN-γ [62,63]. T-cell clones derived from PBMCs of *Cryptosporidium*-exposed patients stimulated with native and recombinant antigens were predominantly $CD4^+$ TCR-α/β $CD45RO^+$ (memory phenotype), and were characterized by hyper-production of IFN-γ. Some of the T-cell clones exhibited a Th0 phenotype, secreting IL-4, IL-5 or IL-10 in addition to IFN-γ [64]. In a recent study, in which whole blood from seropositive and seronegative human volunteers was stimulated *ex vivo* with recombinant *C. hominis* gp15 antigen, both $CD4^+$ and $CD8^+$ cells from seropositive donors produced greater amounts of IFN-γ than those from seronegative donors [66]. In a human volunteer study of adults experimentally infected with *Cryptosporidium*, mucosal IFN-γ production correlated significantly with the presence of pre-existing anti-*Cryptosporidium* antibodies, and reduction in oocyst shedding, suggesting that prior exposure to *Cryptosporidium* may be important in developing protective IFN-γ-mediated memory responses in subsequent infections [52]. The role of $CD8^+$ T cells in protection from or clearance of infection is not clear. However, in the human study referred to above, both $CD4^+$ and $CD8^+$ T cells secreted IFN-γ in response to *ex vivo* stimulation with recombinant gp15 [66].

Humoral immunity & antibody responses—While the critical role of T-cell-mediated responses in the control of cryptosporidiosis is undisputed, the specific role of humoral immunity is still unclear. Invasive stages of the parasite, including sporozoites and merozoites, when present in the intestinal lumen are the most likely stages to be targeted by specific antibodies, which may function by blocking attachment and invasion of these stages into host cells.

In humans, the increased susceptibility of patients with primary immunodeficiencies, such as X-linked hyper-IgM syndrome, and selective IgA deficiency to cryptosporidiosis [2,67,68] suggest that humoral immunity may play a role. However, many of these individuals may have defects in both B- and T-cell responses. A possible role for antibodies is also supported by the partial efficacy of some hyperimmune bovine colostrum preparations (derived from cows immunized with *C. parvum*) in cryptosporidial infections in healthy human volunteers as well as in AIDS patients (reviewed in [25]). Sera from AIDS patients recognized fewer cryptosporidial antigens when compared with non-AIDS patients, suggesting that deficient $CD4^+$ T-cell responses may result in ineffective antibody responses as well [69].

A number of studies have reported the presence of *Cryptosporidium*- specific serum IgG, IgM and IgA, and fecal IgA antibodies following cryptosporidial infection in humans (reviewed in [25]). Many of these have been seroprevalence studies that have reported a wide range of seropositivity depending on age, geographic location, living and environmental conditions. Recent studies of antibody responses to crude or specific native or recombinant *C. parvum* antigens in humans with cryptosporidiosis are summarized in Table 3. A study of mucosal antibody responses in experimentally infected human volunteers found that anticryptosporidial fecal IgA was present in active infection [70]. Studies in human volunteers and serological surveys suggest that individuals with pre-existing antibodies to *Cryptosporidium* may be partially protected and experience less diarrheal symptoms upon subsequent challenge [71-74]. However, it remains to be determined whether antibody responses are themselves protective or whether they are merely markers of a protective cell-mediated response [25].

Cryptosporidium antigens—In order to develop targeted immune-based interventions such as vaccines, or passive immunotherapy for cryptosporidiosis, it is essential to identify and characterize specific antigens that mediate attachment and invasion of the parasite into host cells and which may therefore serve as putative targets for these interventions. Like other apicomplexan parasites, the apical region of *Cryptosporidium* sporozoites and merozoites contains specialized secretory organelles (rhoptries, micronemes and dense granules) collectively known as the apical complex. These organelles secrete and successively exocytose proteins, which facilitate attachment, gliding motility, invasion and parasitophorus vacuole formation. Surface proteins of invasive stages may also mediate attachment. Surface and apical complex proteins, which are believed to mediate attachment and invasion of *Cryptosporidium*, include circumsporozoite-like protein (CSL), gp900, p23/27, TRAP C1 and other TRAPs, gp40/45, cp47, gp15/ cp17, Cp2, Cpa 135, Muc4 and Muc5 [3,9,75]. Many of these are mucins or mucin-like glycoproteins. Sequencing of the *Cryptosporidium* genome has facilitated identification of some of these proteins [76,77]. However, progress in establishing their functional role has been hampered by the inability to propagate *C. parvum in vitro* and to genetically manipulate the parasite [9]. Characteristics of these proteins have been recently reviewed or published [3,9,75,78,79]. Here we focus on those proteins which have been shown to induce immune responses in humans.

The best studied of these proteins is gp40/15 (also called GP60 or S60), a mucin-like glycoprotein [80-83]. It is encoded by a highly polymorphic gene *Cpgp40/15*, and is expressed as a precursor glycoprotein that is proteolytically processed [84,85] to yield

mature glycopeptides gp40 (also referred to as gp45) and gp15 (also referred to as Cp17/S16/15–17-kDa antigen), which remain noncovalently associated following cleavage [86]. In spite of the polymorphisms in the *Cpgp40/15* gene, the gp15 part of the molecule is relatively conserved. The C-terminal gp15 peptide is anchored to the membrane via a glycosylphosphatidylinositol linkage, localized to the surface of zoites, and is shed in trails during gliding motility [82,83,87,88]. gp15 is an immunodominant protein consistently recognized by sera from infected persons [81]. The presence of pre-existing serum antibodies to gp15 correlated with protection from diarrheal symptoms in infected adult humans [72,74]. In a longitudinal analysis of serological responses in a Peruvian cohort of children, IgG responses to gp15 were detected following infection and tended to be higher in older children, possibly indicating recurrent exposure [89]. In a case–control study of cryptosporidiosis in Bangladeshi children with diarrhea [90], cases had significantly higher IgM levels to gp15 following an episode of cryptosporidial diarrhea compared with controls [91]. In addition, the increase in IgG levels to gp15 after a 3-week follow-up period was significantly greater in cases as compared with controls. The N-terminal gp40 peptide is secreted [82,88] and localizes to the surface of the invasive stages in association with gp15 [92]. gp40 binds to human intestinal epithelial cells, and antibodies to gp40 inhibit *C. parvum* infection *in vitro* [82,88]. Studies in a birth cohort of children in a semi-urban slum area of south India where *C. hominis* subtype 1a infection predominates [93] indicated that IgG responses to this antigen occur following an episode of cryptosporidial diarrhea, and appear to be, at least in part, subtype specific [94]. Cp23 (also called p27 or 27-kDa antigen) is a surface protein expressed on the invasive stages of the parasite, is shed in trails during gliding motility [95] and is also predicted to display mucin-type *O*-glycosylation. Like gp15, Cp23 is an immunodominant protein and antibodies to it are frequently detected following *Cryptosporidium* infection [25]. In a study of experimentally infected human volunteers, those that had pre-existing serum IgG to the 27-kDa antigen excreted fewer oocysts compared with those that did not [72]. In a Peruvian cohort study, as was the case with the 17 kDa antigen, IgG levels to the 27-kDa antigen levels tended to be higher in older children [89]. A study of HIV-infected patients also showed that patients with higher antibody responses to the 27-kDa antigen tended to be asymptomatic [74]. In the case–control study of Bangladeshi children referred to above, there was a significant increase in IgG, IgA and IgM levels to p27 in children with cryptosporidial diarrhea compared with those with diarrhea due to other etiologies [96]. In addition, children with a shorter duration of diarrhea tended to have higher antibody levels to p27. p27 also induced cell-mediated immune responses in PBMCs of infected humans [57]. Although a few polymorphisms have been identified in the *p27* gene, this protein is relatively conserved among different isolates [97]. Neutralization-sensitive epitopes on this protein also appear to be conserved among isolates [95,98].

gp900 is a heavily glycosylated, high molecular weight glycoprotein that is localized to micronemes, secreted onto the surface of sporozoites and shed in trails during gliding motility [99,100]. It is a multidomain protein containing cysteine-rich, mucin-like and transmembrane domains [100]. Purified native gp900 binds to human intestinal epithelial cells and competitively inhibits *C. parvum* infection *in vitro* [100,101]. gp900 displays mucin-type *O*-linked glycosylation and *N*-acetylgalactosamine-containing glycotopes of this protein (which are also present on gp40), are the target of infection-neutralizing lectins as well as of a neutralizing monoclonal antibody [80]. A recombinant 40 kDa fragment of gp900 named surface antigen 40 (SA40) induced proliferative responses and IFN-γ secretion from PBMCs of *Cryptosporidium*-infected immunocompetent (but not immunocompromised) human volunteers [64].

Future perspective

It has been challenging to investigate immune responses, particularly mucosal cell-mediated responses to *Cryptosporidium*, in humans due to the invasive techniques required. In addition, characterization of putative protective antigens and their functional role has been hampered by the inability to propagate the parasite *in vitro* and to genetically manipulate it. Despite these impediments, there has been considerable advancement in our knowledge of human immune responses to *Cryptosporidium*. However, substantial gaps in understanding the nature of immune responses to this parasite still remain.

In future studies, the role of innate immune responses in human subjects could be further explored. The role of TLR-mediated pathways in human intestinal epithelial cells or dendritic cells in innate immune responses to *Cryptosporidium* could be investigated. The association of genetic alterations, such as polymorphisms in TLR genes and susceptibility to cryptosporidiosis in vulnerable populations could be evaluated. TLR ligands for *Cryptosporidium* that may serve as adjuvants, or other similar modulators of immune responses could be identified. Secretion of defensins and/or cathelicidins in response to infection could be studied by measuring fecal levels of these AMPs, as has been carried out in inflammatory bowel disease [102]. Investigation of the role of chemokines and cytokines in innate and adaptive responses in humans could be facilitated by the use of newer technologies that require only small volumes to quantify several cytokines in the same sample.

In order to identify and evaluate putative protective antigens as targets for vaccines or passive immunotherapy, longitudinal studies of cellular and humoral responses to these specific antigens in vulnerable cohorts of human populations are required. In the few studies that have been done to evaluate human responses to specific antigens, *Escherichia coli*-derived recombinant proteins have been used. However, unlike the native proteins, these are not glycosylated. Glycosylated epitopes of these proteins may be required for effective immune responses. Use of heterologous expression systems, such as the *Toxoplasma* system [103], to generate glycosylated recombinant antigens may be required to fully evaluate immune responses to mucin-like glycoproteins, which include most of the currently known *Cryptosporidium* adhesins. Development of robust systems for continuous propagation of *Cryptosporidium in vitro*, and for stable transfection, knockout or knockdown of *Cryptosporidium* genes as well as genomic and proteomics approaches will facilitate identification of new antigens and confirmation of their functional role. A better understanding of the nature and dynamics of secretory mucosal antibody responses, including measurement of fecal and salivary secretory IgA to specific antigens, using highly sensitive newer technology may give more insight into mucosal humoral immune responses.

Despite the numerous advances made in understanding immune responses to *Cryptosporidium*, a significant amount remains unknown. Given the paucity of effective therapeutics, the lack of a vaccine and the challenges in eradicating transmission of this parasite, it is imperative to develop more targeted prophylactic and therapeutic interventions to ameliorate the morbidity resulting from cryptosporidiosis, especially in children and in immunosuppressed populations.

Executive summary

Innate immune responses

Toll-like receptor-mediated pathways

- *In vitro* studies in human cholangiocytes demonstrate that activation of Toll-like receptor-2 and -4 responses leads to chemokine production via a MyD88 dependent NF-κB pathway.

Antimicrobial peptides

- *Cryptosporidium parvum* infection downregulates expression of the antimicrobial peptide human β-defensin (HBD)-1 and induces HBD-2 expression in human cell lines *in vitro*.

Mannose-binding lectin

- Low serum mannose-binding lectin (MBL) levels and *mbl2* polymorphisms are associated with increased susceptibility to cryptosporidiosis.

Chemokines

- Studies in human intestinal epithelial cells and jejunal biopsies show that CCL-5, CXCL-8 and CXCL-10 are upregulated in cryptosporidial infection and CXCL-8 is detectable in stool samples of infected humans.

Cytokines

- IFN-γ-independent pathways, including IL-15, play an important role in innate immune responses by activating natural killer cells.

Prostaglandins & substance P

- Cryptosporidial infection upregulates prostaglandin-E2 and -F2 production in human intestinal cells *in vitro*, which may contribute to antimicrobial peptide production, and downregulation of inflammatory cytokines.
- Substance P levels in jejunal biopsies from AIDS patients are higher in those with symptomatic cryptosporidiosis.

Adaptive immune responses

Cell-mediated immunity & T-cell responses

- T-cell immune responses play a critical role in resolution of cryptosporidial infection, predominantly via $CD4^+$ cells and IFN-γ-mediated pathways.
- IFN-γ may play a role in T-cell memory responses.

Humoral immunity & antibody responses

- Several studies have reported serum antibody responses to cryptosporidial antigens following infection.
- Human volunteer studies show that those with pre-existing anticryptosporidial antibodies are protected from diarrheal symptoms but not infection.

Cryptosporidium antigens

- A number of antigens, including gp900, p23/27, gp40 and gp15/17, that mediate *Cryptosporidium* host–parasite interactions induce antibody and or/cell-mediated immune responses in humans.

Future perspective

- Investigation of genetic polymorphisms in Toll-like receptors and MBL genes and susceptibility to cryptosporidiosis in vulnerable populations.
- Investigation of the role of antimicrobial peptides in infected humans.

- Investigation of systemic and mucosal cell-mediated and humoral responses to putative protective antigens in longitudinal birth cohort studies in vulnerable human populations such as malnourished children in developing countries.
- Further studies in humans to determine whether antibody responses are themselves protective, or if they are just markers of cellular immune responses.
- Investigation of immune responses to glycotopes of putative protective antigens.
- Use of genomic and proteomic approaches to identify additional putative protective antigens.
- Development of targeted prophylactic and therapeutic immune-based strategies.

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Table 1

Recent studies of innate immune responses to *Cryptosporidium* in humans.

Type of study	Findings	Ref.
Mannose-binding lectin		
Cross-sectional study in AIDS patients in Zambia	Increased risk for cryptosporidiosis in patients homozygous for *mbl2* structural gene mutations	[39]
Case–control study of children in Haiti	Serum mannose-binding lectin deficiency was significantly associated with cryptosporidiosis	[40]
Prospective cohort study of children in Bangladesh	Serum mannose-binding lectin deficiency and *mbl2* polymorphisms were significantly associated with cryptosporidiosis	[41]
Chemokines and cytokines		
Study of experimentally infected human volunteers	TGF-β was expressed in jejunal biopsies of some volunteers shedding oocysts	[55]
Study of experimentally infected human volunteers	TNF-α and IL1-β were expressed in jejunal biopsies of some volunteers but were not associated with symptoms	[56]
Study of experimentally infected human volunteers	IL-15 was expressed in jejunal biopsies of volunteers who did not express IFN-γ and was associated with lower oocyst shedding. IL-4 expression was not associated with symptoms	[53]
Prospective triple cohort study in children in Haiti	Levels of IL-8, TNF-α, IL-13 and IL-4 but not IFN-γ were elevated in stool samples of cases with cryptosporidiosis	[46]
Study of experimentally infected human volunteers and children in Brazil	IL-8 was detectable in stool samples of some patients with cryptosporidiosis	[45]
Prospective case–control cohort study of children in Haiti	Peripheral blood mononuclear cells of children with cryptosporidiosis stimulated *ex vivo* with *Cryptosporidium* antigens expressed higher levels of CXCL-8 and TNF-α compared to controls	[44]
Case–control study in AIDS patients	Higher levels of CXCL-10 were detected in jejunal biopsies of AIDS patients with cryptosporidiosis compared with controls	[47]
Substance P		
Study of experimentally infected human volunteers and AIDS patients	Substance P mRNA and protein expression were higher in jejunal biopsies of symptomatic volunteers and AIDS patients compared with asymptomatic volunteers	[14]

Table 2

Recent studies of cell-mediated immune responses to *Cryptosporidium* in humans.

Type of study	Findings	Ref.
Study of immunocompetent individuals and AIDS patients	IFN-γ was produced from peripheral blood mononuclear cells stimulated *ex vivo* with crude cryptosporidial extract in patients with cryptosporidiosis	[63]
Study of experimentally infected human volunteers and AIDS patients	IFN-γ mRNA was detected in jejunal biopsies of volunteers after infection but not in AIDS patients with cryptosporidiosis. IFN-γ expression correlated significantly with the presence of anticryptosporidial antibodies and absence of oocyst shedding	[52]
Observational study in patients from Haiti	Patients with prior cryptosporidiosis displayed antibody- and cell-mediated responses to Cp23 and crude cryptosporidial antigens. Antibody responses were higher in those with T-cell responses	[65]
Study of immunocompetent patients	T-cell clones derived from peripheral blood mononuclear cells stimulated *ex vivo* with native and recombinant cryptosporidial antigens were TCR-$\alpha\beta^+$, $CD4^+$, $CD45RO^+$ and expressed either a Th1 (IFN-γ) or Th0 (IL-4 and IL-5) profile	[64]
Study of seropositive and seronegative human volunteers	$CD4^+$ and $CD8^+$ cells from seropositive donors produced greater amounts of IFN-γ than seronegative donors in response to *ex vivo* stimulation with recombinant gp15	[66]

Table 3

Recent studies of humoral immune responses to *Cryptosporidium* in humans.

Type of study	Findings	Ref.
Study of experimentally infected human volunteers	Volunteers with pre-exisiting anticryptosporidial antibodies had less diarrhea and oocyst shedding upon challenge with low doses of oocysts.	[71]
Study of experimentally infected human volunteers	Anticryptosporidial fecal IgA was detectable during active cryptosporidial infection.	[70]
Longitudinal study of Bedouin children in Israel	Serum anti-*Cryptosporidium parvum* IgG levels were high soon after birth, dropped by 6 months of age, and continuously increased until 23 months of age.	[104]
Case–control study of children with diarrhea in Bangladesh	Serum IgM levels to *C. parvum* antigens were higher in cases with *Cryptosporidum* infection than uninfected controls. Increase in IgG levels over 3 weeks was greater in cases compared with controls. Persistent diarrhea was associated with lower IgA and IgM levels.	[90]
Cross-sectional study of HIV-infected patients in Australia	Strong antibody responses to 27-kDa antigen were associated with reduced risk of diarrhea without weight loss.	[73]
Observational study of people served by ground and surface water sources	Strong antibody response to 15/17-kDa antigen were associated with reduced risk of diarrhea in users of surface water sources.	[74]
Longitudinal cohort study of children in Peru	Antibody responses to 15/17-kDa antigens were greater in older children, those with multiple infections and those with asymptomatic infection.	[89]