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Invited Review

Innate immune responses play a key role in controlling infection of the intestinal epithelium by *Cryptosporidium*

Fabrice Laurent *, Sonia Lacroix-Lamandé *

UMR1282 Infectiologie et Santé Publique, INRA Centre Val de Loire, Université François Rabelais de Tours, 37380 Nouzilly, France

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ABSTRACT

Cryptosporidium infection leads to acute diarrhea worldwide. The development of cryptosporidiosis is closely related to the immune status of its host, affecting primarily young ruminants, infants, and immunocompromised individuals. In recent years, several studies have improved our knowledge on the immune mechanisms responsible for the control of the acute phase of the infection and have highlighted the importance of innate immunity. The parasite develops in the apical side of intestinal epithelial cells, giving these cells a central role, as they are both the exclusive host cell for replication of the parasite and participate in the protective immune response. Epithelial cells signal the infection by producing chemokines, attracting immune cells to the infected area. They also actively participate in host defense by inducing apoptosis and releasing antimicrobial peptides, free or incorporated into luminal exosomes, with parasiticidal activity. The parasite has developed several escape mechanisms to slow down these protective mechanisms. Recent development of several three-dimensional culture models and the ability to genetically manipulate *Cryptosporidium* will greatly help to further investigate host-pathogen interactions and identify virulence factors. Intestinal epithelial cells require the help of immune cells to clear the infection. Intestinal dendritic cells, well known for their ability to induce and orchestrate adaptive immunity, play a key role in controlling the very early steps of *Cryptosporidium parvum* infection by acting as immunological sentinels and active effectors. However, inflammatory monocytes, which are quickly and massively recruited to the infected mucosa, seem to participate in the loss of epithelial integrity. In addition to new promising chemotherapies, we must consider stimulating the innate immunity of neonates to strengthen their ability to control *Cryptosporidium* development. The microbiota plays a fundamental role in the development of intestinal immunity and may be considered to be a third actor in host-pathogen interactions. There is an urgent need to reduce the incidence of this yet poorly controlled disease in the populations of developing countries, and decrease economic losses due to infected livestock.

1. Cryptosporidiosis is a major threat for infants and young ruminants

Cryptosporidiosis is a disease caused by the protozoan parasite *Cryptosporidium*. This parasite is considered to be minimally invasive and completes its life cycle in intestinal epithelial cells (ECs). The main clinical symptom is diarrhoea, which often results in dehydration. Cryptosporidiosis primarily affects young ruminants, infants, and immunocompromised individuals. Major sources of infection are contaminated water and contact with infected humans or animals.

Cryptosporidiosis is among the waterborne diseases under European Union surveillance at the European Centre for Disease Prevention and Control (ECDC). It is a notifiable disease in several countries including Sweden (PHAS), France (ANSES), and the UK (PHE). However, the major impact of human cryptosporidiosis worldwide is in developing countries, where diarrhoea caused by infection with *Cryptosporidium* spp. (Putignani and Menichella, 2010) is becoming increasingly recognised as a major contributor to morbidity and mortality in children. This has been supported by two recent publications in The Lancet (Kotloff et al., 2013) and The Lancet Global Health (Platts-Mills et al., 2015), based on "Global Enteric Multicenter study" (GEMS) and "The Etiology, Risk Factors, and Interactions of Enteric Infections and Malnutrition and the Consequences for Child Health and Development Study" (MAL-ED) studies, respectively. Susceptibility to the disease is further

* Corresponding authors.
E-mail addresses: fabrice.laurent@inra.fr (F. Laurent), sonia.lamande@inra.fr (S. Lacroix-Lamandé).

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increased in malnourished and immunocompromised children such as those suffering from HIV infection (Guerrant et al., 2008) or solid organ transplant recipients (Bonatti et al., 2012; Bhadauria et al., 2015; Lanternier et al., 2017). Of the 31 *Cryptosporidium* spp. characterised to date, *Cryptosporidium hominis* and *Cryptosporidium parvum* are by far the most common species reported in humans worldwide, followed by *Cryptosporidium meleagridis* and *Cryptosporidium ubiquitum* (Caccio and Chalmers, 2016). Epidemiological studies have shown that the geographical distributions of *Cryptosporidium* spp. vary around the world. *Cryptosporidium hominis* is more prevalent in North and South America, Australia and Africa, while *C. parvum* is more localised in Europe, especially in the UK (Caccio, 2005). Nitazoxanide, the only effective drug to treat cryptosporidiosis in humans, improves the resolution of diarrhoea, parasitological eradication, and mortality in HIV-seronegative, but not HIV-seropositive, children (Amadi et al., 2002). The Bill and Melinda Gates Foundation has recently supported several calls to accelerate the development of new therapies for childhood *Cryptosporidium* infection. Bumped-kinase inhibitors of *C. parvum* calcium-dependent protein kinase 1 (CpCDPK1) and phosphatidylinositol-4-OH kinase (PI(4)K) inhibitors are promising new drug candidates for cryptosporidiosis treatment (Arnold et al., 2017; Hulverson et al., 2017; Manjunatha et al., 2017) that warrant further preclinical evaluation. As stated by Dr. B. Striepen in his editorial in Nature in 2013, "It is time to tackle cryptosporidiosis", the second leading cause of diarrheal disease in sub-Saharan Africa and southern Asia (Striepen, 2013).

The incidence of cryptosporidiosis is high in domestic animals and, in particular, young ruminants (goat kids, lambs and calves), due to its high worldwide prevalence and limited available control strategies. Cryptosporidiosis remains the main cause of diarrheal enteric disease in calves in the UK (VIDA report, 2012) and France for both dairy and suckling animals (Naciri et al., 1999). The disease and economic impact on livestock are mainly due to the zoonotic species *C. parvum* (Ryan et al., 2014). Halofuginone lactate is the only licenced medication available for calves born in contaminated herds in a number of countries in Europe. However, to reach a good level of efficacy, preventive administration is recommended during the first 7 days of life of the animals, which complicates herd management for farmers. An infected animal has the potential to shed millions of oocysts (dissemination stage of the parasite), which are environmentally stable and resistant to many disinfectants (for a description of the parasite life cycle see Bouzid et al. (2013). These characteristics highlight the great potential of *C. parvum* to disseminate among susceptible hosts, as only a few dozen oocysts are required to trigger an infection (Zambriski et al., 2013). The costs for farmers include that of the treatment and veterinary intervention, reduced performance, and eventually death of the young animals by dehydration.

Cryptosporidiosis due to the zoonotic *Cryptosporidium* spp. must be considered as a "One Health" threat that requires coordinated actions to reduce its incidence in humans and livestock (Ryan et al., 2016). A better understanding of the mechanisms underlying disease and protection is crucial for the design and production of new therapies and vaccines. Studies on host responses to *Cryptosporidium* in humans face obvious major ethical constraints. However, a few studies on patients with congenital immunodeficiencies have led to some insight on the importance of innate immune mechanisms during cryptosporidiosis. Genetic analyses in humans revealed that a biallelic loss-of-function mutation in the MAP3K14 gene encoding NIK (NF-kβ-inducing kinase), leads to defective activation of both canonical and non-canonical NF-kβ signalling and recurrent infection with *Cryptosporidium* in patients. In addition, a polymorphism in the mannose-binding lectin (MBL)-2 gene was shown to contribute to deficient or low serum levels of MBL and was reported to increase susceptibility to cryptosporidiosis in children and HIV-infected adults (Kelly et al., 2000; Kirkpatrick et al., 2006; Carmolli et al., 2009). The only animal model that allows studies on the main *Cryptosporidium* spp. infecting human (*C. parvum*, *C. hominis*) is the gnotobiotic piglet model (Sheoran et al., 2012). However, studies on immune responses with piglets are now relatively old (Argenzio et al., 1993; Kandil et al., 1994; Argenzio and Rhoads, 1997; Gookin et al., 2004; Zadrozny et al., 2006). Information on immune mechanisms in ruminants is scarce due to the difficulty of performing mechanistic studies because of limited immunological tools and the large size of the animals. However, the first discovery of antimicrobial peptides produced by intestinal epithelial cells (ECs) during *Cryptosporidium* infection was obtained in young calves (Tarver et al., 1998). Yet the vast majority of insight on the immune response during cryptosporidiosis has been gained with mouse models using genetically engineered animals. It is thus important to consider all information from various species, keeping in mind immune specificities.

In recent years, several studies have highlighted the importance of innate immunity. In this review, we will focus on the major role of intestinal ECs and mononuclear phagocytes in the mechanism of protection, highlight the contribution of microbiota in immune responses, and raise the key questions that remain to be elucidated.

2. Intestinal ECs, from beginning to end

The parasite develops in the apical side of intestinal ECs without being more invasive. Systemic infection has never been described, but *Cryptosporidium* infection may extend to the biliary tract epithelium in patients with acquired immune deficiency syndrome (AIDS). Free parasite stages are released into the lumen at different steps during the life cycle of the parasite, where they can invade new host ECs. Intestinal ECs thus play a central role in both parasite replication and the protective immune response.

2.1. ECs as guardians of gut homeostasis

ECs are sentinel cells in the mucosa, equipped with several defense mechanisms to fight pathogens, such as the induction of apoptosis and production of antimicrobial peptides and chemokines for the recruitment of immune cells to the site of infection. ECs express a variety of pathogen pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) at the cell surface or in the endosome, and the nucleotide binding and oligomerization domain-like receptors in the cytosol. These PRRs can recognise pathogens or microbe-associated molecular patterns. A TLR4-mediated response was required for efficient eradication of the infection in a model of biliary cryptosporidiosis in which *C. parvum* was injected into the gallbladders of adult mice (Chen et al., 2005; O'Hara et al., 2011). In contrast, deficiency of both TLR4 and TLR2 did not result in higher parasite load in the intestine of neonatal mice in a natural model of infection (Lantier et al., 2014). Intestinal ECs and ECs of the bile duct, called cholangiocytes, seem to respond differently to the parasite. Indeed, persistent microbiota exposure leads enterocytes to quickly downregulate TLR signalling shortly after birth to avoid a chronic inflammatory response in the gut, possibly explaining the difference (Chassin and Hornef, 2011).

Invasion of enterocytes by *C. parvum* activates nuclear factor-kappa B (NF-κB) signalling and subsequent production of chemokines, attracting immune effector cells to the site of infection (Laurent et al., 1997; Chen et al., 2001; Lacroix-Lamande et al., 2002; Auray et al., 2007; Hu et al., 2014) (Fig. 1A). We were the first to show, in vitro and in vivo in a model of human intestinal xenografts in SCID mice, that *C. parvum* infection of intestinal ECs

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