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Invited Review

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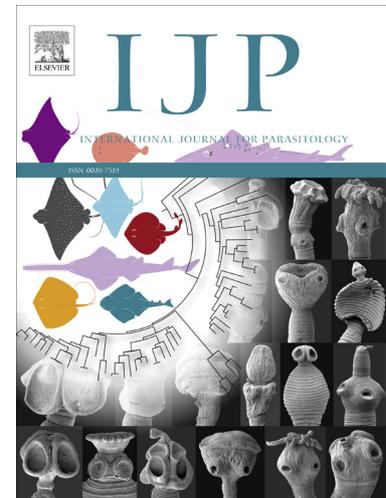
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Invited Review

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

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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 parasitocidal activity. The parasite has developed several escape mechanisms to slow down these protective mechanisms. Recent development of several three-dimensional (3D) 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 *C. 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.

Keywords: *Cryptosporidium*, Epithelial cell, Mononuclear phagocytes, Innate immunity, Immunostimulation

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 diarrhea, 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 diarrhea caused by infection with *Cryptosporidium* spp. (Putignani and Menichella, 2010) is becoming increasingly recognized 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 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. characterized 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 localized in Europe, especially in the UK (Caccio, 2005). Nitazoxanide, the only effective drug to treat cryptosporidiosis in humans, improves the resolution of diarrhea, 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 licensed 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- κ B-inducing kinase), leads to defective activation of both canonical and non-canonical NF- κ B signaling 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 recognize 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 signaling 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- κ B) signaling 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., 2013) (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 induces the polarized secretion of CXCL8 and CXCL1, which participate in the recruitment of neutrophils (Laurent et al., 1997). In the *C. parvum* neonatal mouse model, we showed that a broad range of chemokines belonging to CXC and CC chemokine families (CCL2, CCL5, CXCL10, CXCL9, etc.) are produced during infection, favoring the recruitment of diverse immune cell types (Lacroix-Lamande et al., 2002; Auray et al., 2007; Lantier et al., 2013). Immune cell recruitment and retention in infected intestine are crucial in the mechanism of protection against *C. parvum*, as revealed by increased susceptibility in mouse models deficient for chemokine receptors or

adhesion molecules, despite redundancy of recruitment mechanisms (Mancassola et al., 2004; Lacroix-Lamande et al., 2008; Lantier et al., 2013). Among the chemokines produced by intestinal ECs during infection, CXCL10 plays a major role in the IFN- γ dependent protective immune response of neonatal mice by recruiting dendritic cells (DCs) (Lantier et al., 2013) (see section 3.1). Consistent with these observations, such chemokine production was also detected in intestinal ECs in AIDS patients with active cryptosporidiosis (Wang et al., 2007).

Intestinal ECs produce many antimicrobial peptides (AMPs) as part of the innate immune response to control pathogens. AMPs are small polypeptides (< 100 amino acids) that possess antimicrobial and immunomodulatory properties and are evolutionarily conserved effectors of the innate immune system (Wehkamp et al., 2007). Many synthetic peptides can exert lytic activity in vitro against *C. parvum* sporozoites (Arrowood et al., 1991; Giacometti et al., 1999). Cationic peptides such as cathelicidin LL-37, β -defensins 1 and 2, and phospholipases, can neutralize *C. parvum* by mechanisms predominantly mediated through parasitocidal activity (Giacometti et al., 1999; Carryn et al., 2012). It was shown in an elegant in vitro study that AMPs such as β -defensin 2 and LL-37 are associated with exosomes produced by cholangiocytes after activation of TLR4/IKK2 signaling and downregulation of microRNA *Let-7* resulting in subsequent upregulation of SNAP23, a protein involved in vesicular exocytosis. After SNAP23-dependent luminal release, these exosomes bind to the surface of free stages of *C. parvum* and exert their direct anti-parasitic activity (Chen et al., 2005).

Cellular miRNAs have emerged as ubiquitous regulators of gene expression related to cell responses. Chen and colleagues have extensively studied their contribution in host antimicrobial immunity and EC defense (for a review, see Ming et al. 2017).

2.2. Escape mechanisms developed by *Cryptosporidium*

Host cell apoptosis is an intrinsic immune defense strategy that allows the elimination of intracellular pathogens such as *C. parvum* (Chen et al., 1999; Ojcius et al., 1999; McCole et al., 2000). However, extensive studies suggest that *C. parvum* has developed multiple strategies to limit apoptosis to facilitate its growth and maturation early after EC infection (Fig. 1C). *Cryptosporidium parvum* induces apoptosis of infected and surrounding uninfected cells by a

mechanism involving Fas and Fas-L molecules (Chen et al., 1999). Within a few hours, the parasite activates NF- κ B which, in turn, activates anti-apoptotic mechanisms (McCole et al., 2000). During later stages of infection, anti-apoptotic factors disappear and the host cell dies (McCole et al., 2000; Liu et al., 2008; Liu et al., 2009). To limit apoptosis, *C. parvum* induces the production of the anti-apoptotic factors BCL-2 and survivin (Mele et al., 2004; Liu et al., 2008). Moreover, *C. parvum* induces the production of osteoprotegerin (OPG) (Castellanos-Gonzalez et al., 2008), a member of the tumor necrosis factor (TNF) receptor superfamily that lacks signaling elements of the receptor and functions as a decoy receptor. Two ligands of OPG are TNF-related apoptosis-inducing ligand (TRAIL) and TNF-related activation-induced cytokine (TRANCE), both of which can induce EC apoptosis. By binding TRAIL, OPG prevents TRAIL-induced apoptosis of *C. parvum*-infected cells (Castellanos-Gonzalez et al., 2008).

The pro-inflammatory cytokine IFN γ is the key cytokine that controls *C. parvum* infection. Its neutralization or genetic depletion renders mice much more susceptible to infection (Theodos et al., 1997; Lacroix et al., 2001). Replication of the parasite in ECs is inhibited by IFN γ , by a mechanism that involves intracellular Fe $^{2+}$ deprivation (Pollok et al., 2001). However, in the mouse intestinal EC line CMT-93, IFN γ only reduces the infection by 40-60% and never completely blocks replication of the parasite. STAT1 is the predominant and essential transcription factor in interferon signaling. In enterocytes, *C. parvum* was shown to interfere with IFN γ -dependent gene expression involving STAT-1 signalling such as indoleamine 2, 3 dioxygenase (IDO) (Choudhry et al., 2009). IDO is an enzyme that catabolizes tryptophan required for the growth of the parasite. Hence, *C. parvum* restores the availability of tryptophan for its growth by inhibiting STAT-1-induced IDO expression (Fig. 1B) (Choudhry et al., 2009)

Another immune evasion strategy developed by the parasite is to reduce the production of the antimicrobial peptides mBD-1 and HBD-1 by ECs via an as yet unknown mechanism (Zaalouk et al., 2004) (Fig. 1B). We recently demonstrated that the chemokine CCL20 can exert antimicrobial activity against *C. parvum* sporozoites (Guesdon et al., 2015). *Cryptosporidium parvum* can circumvent this protection during infection in neonatal mice by downregulating CCL20 production independently of the IFN γ -response and the presence of microbiota. This

downregulation of CCL20 was associated with increased expression of miR21 induced by *C. parvum* infection (Guesdon et al., 2015). In a recent study, *C. parvum* was shown to deliver parasite transcripts into the nucleus of infected ECs that modulate gene transcription (Wang et al., 2017a). Among modulated host genes, LRP5 and SLC7A8 are down-regulated by epigenetic mechanisms (Wang et al., 2017b). The proteins encoded by these genes promote cellular proliferation and are involved in amino-acid transport, respectively. Their downregulation induced by infection could therefore be a strategy to favour parasite multiplication (Fig. 1C).

2.3. New methods to investigate *Cryptosporidium*-intestinal EC interactions

The understanding of host-parasite interactions in the intestines of humans and ruminants has long been limited by the lack of methods for long-term culture of primary intestinal ECs (PECs). Most studies were therefore performed with transformed cell lines. In addition, these cell lines do not readily support *Cryptosporidium* infection and the parasite undergoes incomplete replication (Karanis and Aldeyarbi, 2011). Extensive efforts have been made to develop new methods to overcome these limitations. Human PECs have been successfully propagated in vitro and can persist for at least 60 days, supporting *C. parvum* infection (Castellanos-Gonzalez et al., 2013). Another elegant model has been established with 3D cultures of HCT-8 cells grown in hollow fibers that mimic the gut by delivering nutrients and oxygen from the basal layer upwards (Morada et al., 2016). Infectivity of oocysts from a 6 week culture produced with this method was confirmed in a dexamethasone immunosuppressed mouse model. A similar human 3D model, using silk protein as the scaffolding biomaterial and supporting long term culture (> 17 days), is now also available (DeCicco RePass et al., 2017). The development of human and mouse enteroids, functional 3D-cultured intestinal epithelial units that recapitulate integral aspects of the intestine, is also a major technological advance (Zachos et al., 2016). Indeed, enteroids contain the multiple intestinal EC types that constitute the intestinal epithelium, namely enterocytes and goblet, enteroendocrine, Paneth and stem cells. Derived from mice, those have been recently used as models for *C. parvum* infections (Zhang et al., 2016). Finally, microfluidic 'Gut-on-a-Chip' technology, which exposes cultured cells to physiological peristalsis-like motions and liquid flow, can be used to induce human Caco-2 cells

to spontaneously undergo robust morphogenesis of 3D intestinal villi (Huh et al., 2013; Kim and Ingber, 2013). These physiologically relevant models that better mimic gut complexity will undoubtedly help to further decipher *Cryptosporidium*-intestinal EC interactions.

3. Mononuclear phagocytes are key innate immune cells during the acute phase of infection and promoters of adaptive immunity

3.1. Mononuclear phagocytes in a steady state and their mobilization to the intestine during *C. parvum* infection

Mononuclear phagocytes consist of monocytes, macrophages and DCs. The nature of the markers used to identify various populations and subsets have regularly evolved over time. In particular, the CD11c marker initially thought to be specific for DCs must be used in combination with several other markers to differentiate DCs from intestinal macrophages, which also express intermediate to high levels of CD11c. In addition, the phenotype of these cells changes during intestinal protective immune responses or inflammation, making it more difficult to accurately identify these cells. In mice, the situation has recently been clarified using the marker CD64 to identify monocytes/macrophages (Persson et al., 2013; Bain and Mowat, 2014) among the CD11c⁺MHCII⁺ cells. Macrophages are now considered to be CD11c⁺MHCII⁺CD64⁺F4/80⁺CX3CR1^{hi/int} and conventional DCs (cDCs) CD11c⁺MHCII⁺CD64⁻CD103⁺ or CD11c⁺MHCII⁺CD64⁻CD103⁻.

CCR2⁺ monocytes regularly enter the lamina propria where they differentiate into macrophages in response to environmental factors. During *C. parvum* infection of neonatal mice, we observed a 60-fold increase in the number of Ly6c^{hi} inflammatory monocytes by a CCR2-dependent mechanism at 6 days p.i. Interestingly, these cells were not only located in the lamina propria of infected neonates, but also in the subepithelium, in close contact with infected enterocytes. CCL2 is strongly upregulated (50 to 100-fold) in infected intestinal ECs at the peak of infection (Auray et al., 2007), as are many other chemokines, and may participate in inflammatory monocyte recruitment. However, in addition to impaired recruitment via CCR2, CCR2^{-/-} mice exhibit impaired egress of Ly6c⁺ monocytes from the bone marrow (Egan et al., 2009; Platt et al., 2010; Willenborg et al., 2012). The absence of recruitment of Ly6c⁺ cells during

C. parvum infection in $CCR2^{-/-}$ neonatal mice may therefore result from the cumulative effect of these two mechanisms. According to the monocyte-macrophage differentiation model in the intestinal lamina propria at steady state, $CCR2^{+}$ monocytes downregulate Ly6c expression and progressively gain expression of CX3CR1 to become fully differentiated macrophages (Bain et al., 2014; Bain and Mowat, 2014). Neonatal mice have few $CX3CR1^{int}$ expressing macrophages in their intestine, but their number gradually increases until weaning (F. Laurent, unpublished data). In the intestine, macrophages are first established before birth from precursors originating in the yolk sac or fetal liver (Bain et al., 2014). These embryo-derived cells are replaced in the gut shortly after birth by cells derived from $Ly6C^{+}$ blood monocytes. Under inflammatory conditions, $CX3CR1^{int}$ cells accumulate in large numbers and display enhanced pro-inflammatory characteristics (Bain and Mowat, 2014). We have observed the accumulation of a large number of $CX3CR1^{int}$ cells in the lamina propria of neonates at the peak of *C. parvum* infection (de Sablet et al., 2016), in accordance with this finding.

It is now well established that intestinal cDCs originate from pre-cDCs present in the blood. Neonatal mice possess fewer $CD103^{+}$ DCs in the intestinal lamina propria than weaned and adult mice (Lantier et al., 2013). During *C. parvum* infection, these cells are recruited to the site of infection and, once activated, quickly migrate towards mesenteric lymph nodes (MLNs). We used both transgenic animals and oral inoculation of recombinant chemokine to show that CXCL10, an IFN γ -inducible chemokine produced by intestinal ECs in response to *C. parvum* infection, is important for the recruitment of $CD103^{+}$ DCs via its interaction with cell surface CXCR3 (Lantier et al., 2013). The basal level of CXCL9 and CXCL10 in neonatal intestinal ECs at steady state was much lower than in adults (Lantier et al., 2013). This may result from lower basal levels of IFN γ in the neonatal mucosa at homeostasis (Lantier et al., 2013), contributing to the paucity of resident DCs in the intestine of neonates.

3.2. Functional studies

The functional role of DCs and macrophages during cryptosporidiosis has long remained unexplored. Using a conditional mouse model that allows transient and specific depletion of $CD11c^{+}$ cells ($CD11c^{-DTR}$), we showed, to our knowledge for the first time, the critical importance of these cells in controlling the acute phase of *C. parvum* infection in neonates and

adults (Lantier et al., 2013). Indeed, in the absence of these cells, neonatal mice cannot control the infection before replenishment of intestinal CD11c⁺ cells differentiated from blood precursors. Moreover, adult mice, which are strongly resistant to the infection, were heavily infected if CD11c⁺ cells were depleted before inoculation of the parasites. This emphasizes the critical role of resident CD11c⁺ cells in immune surveillance and highlights their key role in controlling the very early steps of *C. parvum* infection by acting as immunological sentinels and active effectors. However, as previously stated, CD11c is expressed by macrophages and DCs in the lamina propria. Diphtheria toxin (DT) administration to CD11c⁻DTR neonatal mice resulted in 85-90% depletion of CD11c⁺CD103⁺ DCs (Lantier et al., 2013) and approximately 70% of CD11c⁺CD64⁺CX3CR1^{int/hi} macrophages (F. Laurent, unpublished data). We demonstrated the role of DCs during protection from infection by first amplifying the low number of resident DCs in the neonatal intestine to mimic the situation in adults. Repeated injections of FLT3-L, a key hematopoietic factor for the differentiation of CD103⁺ DCs, resulted in a large, specific increase in the number of resident DCs in the lamina propria of neonates and significantly improved resistance to subsequent *C. parvum* challenge (Lantier et al., 2013). In adult mice, adoptive transfer of stimulated bone marrow derived DCs in DT-treated CD11c⁻DTR mice significantly lowered parasite load (Bedi et al., 2014). During *C. parvum* infection in neonates, the two subsets of CD103⁺ intestinal DCs (CD11b⁺ and CD11b⁻) produce IL12 and IFN γ . Neonatal and adult mice deficient for these cytokines are particularly susceptible to *C. parvum* infection. We further deciphered the role of DC subsets using mice deficient for the transcription factor Batf3, which resulted in a large reduction in the number of CD11b⁻CD103⁺ DCs. This deficiency rendered adult mice susceptible to *C. parvum* infection (F. Laurent, unpublished data). This result confirms the key role of CD103⁺ DCs, especially CD103⁺CD11b⁻ DCs, in the innate mechanism of protection. These innate immune properties of DCs may be more prominent in newborns, which rely heavily on innate responses before the establishment of adaptive immunity. The mechanism by which DCs positively influence innate responses against *C. parvum* in intestinal EC may be greater than their ability to promptly produce cytokines such as IL12 and IFN γ . The exact mechanism is yet to be elucidated. This DC subset, often called DC1, is also critical for immune control of acute infection with *Toxoplasma gondii* and *Leishmania major*

(Mashayekhi et al., 2011; Ashok et al., 2014). CD103⁺CD11b⁻ DCs appear therefore to represent an important cell subset for the control of intracellular parasites in general.

Defining the role of macrophages in a whole animal model requires examination of the effect of depleting these cells from the complex cellular environment. The first experiments to examine the protective role of macrophages were performed using clodronate. This drug induces irreversible damage and cell death by apoptosis upon delivery into phagocytic cells using liposomes as vehicles. Although the nature of cells depleted was not investigated in the study, the authors stated that macrophage depletion in infected Rag2^{-/-}γc^{-/-} mice, which are deficient for T and B lymphocytes and natural killer (NK) cells, allowed rapid growth following infection, with no increase in IFNγ expression (Choudhry et al., 2012). This suggests that macrophages may be another source of IFNγ during infection, in the absence of T and NK cells. Another publication suggested the cooperation of macrophages and neutrophils in which the authors used a similar adult mouse model, including an additional deficiency of PMNs (SCIDbgMN mice). The SCIDbgMN mice died within 16 days of *C. parvum* infection, whereas 100% of SCIDbg mice exposed to the parasite survived (Takeuchi et al., 2008). The use of mouse models with multiple deficiencies makes it difficult to arrive at a definitive conclusion. More specific depletion models will be required before fully confirming the role of macrophages during cryptosporidiosis.

We recently investigated the susceptibility of neonatal CCR2^{-/-} mice to *C. parvum*. There were no significant differences in parasite load in this mouse model with respect to wildtype mice, despite the fact that there were few inflammatory monocytes in the intestine of the infected neonates (de Sablet et al., 2016). This result does not favor a major role of inflammatory monocytes in the mechanism of protection. However, we recently observed a significant increase in the number of CD11b⁺CD103⁺ DCs in these neonatal mice, reflecting a putative compensatory mechanism that may mask the positive role of inflammatory monocytes in the clearance of the parasite (F. Laurent, unpublished data). During *C. parvum* infection of neonates, intestinal permeability to soluble substances such as FITC-Dextran strongly increases. Intestinal permeability to FITC-Dextran was strongly reduced in infected CCR2^{-/-} neonates, despite similar levels of infection to that of wildtype mice (de Sablet et al., 2016). This suggests

that these cells may contribute to the loss of barrier function during cryptosporidiosis. We further observed that Ly6c⁺ inflammatory monocytes produced large amounts of TNF α and were the unique producers of IL1 β , among other macrophage subsets, during neonatal infection. These two cytokines destabilize tight junctions by binding to their respective receptors on the basolateral side of enterocytes. The downstream mechanism involves the canonical NF- κ B pathway and subsequent myosin light chain kinase (MLCK or MYLK) synthesis, leading to cytoskeletal contraction and tight junction opening (Al-Sadi et al., 2016). As expected, TNF α or IL1 β administration during *C. parvum* infection aggravated intestinal permeability (de Sablet et al., 2016). The available data suggest a deleterious role for inflammatory monocytes during *C. parvum* infection by altering intestinal permeability, a mechanism often associated with diarrhea (Fig. 2).

3.3. Immunostimulation

The importance of CD11c⁺ cells in the process of protection against *C. parvum* infection prompted us to investigate an immunostimulation strategy to strengthen neonatal immunity. It consisted of favoring the recruitment of innate immune cells, important for the process of protection, and activating those to promote the release of protective pro-inflammatory mediators. Targeting PRRs such as TLRs expressed by CD11c⁺ phagocytes with natural or synthetic ligands, appeared to be an attractive strategy. Moreover, although neonates exhibit limited adaptive immunity, we and others have shown in several animal models susceptible to *C. parvum* infection (goat kids, lambs, piglets, neonatal mice) that they strongly respond to TLR-ligand stimulation by releasing large amounts of IL-12 (Tourais-Esteves et al., 2008; Zhao et al., 2008; Lacroix-Lamande et al., 2009; Ferret-Bernard et al., 2010; Ferret-Bernard et al., 2011; Auray et al., 2013). Our work with TLR-ligands as immunostimulants was initiated using synthetic CpG-ODN, which mimics bacterial DNA and binds to TLR9. A single oral administration of CpG-ODN1668 the day before or after *C. parvum* infection resulted in a strong decrease in intestinal parasite load of 80%–95%, 6 days p.i. (Barrier et al., 2006). This treatment induced rapid upregulation of inflammatory cytokines and chemokines (IFN γ , IL12p40, IL1 β , IL-6, CCL7) and the protection was dependent on TLR9 and IFN γ . TLR signaling occurs via two main pathways involving binding of major adaptors (MyD88, TRIF) to the intracellular domain of TLRs,

leading to the expression of specific sets of cytokines and antimicrobial peptides through activation of the transcriptional factors NF- κ B, AP-1 and IRF-3. The rapid reduction in *C. parvum* burden after Poly(I:C) administration to neonatal mice was shown to be dependent on CD11c⁺ cells and TLR3/TRIF signaling (Lantier et al., 2014). We initially thought that MyD88 activation (ex via TLR9 signaling) and activation of the TRIF pathway independently led to control of the parasite via the expression of common effectors. This was challenged by the loss of induced protection after Poly(I:C) administration in MyD88^{-/-} neonatal mice, and the requirement of additional MyD88 signals from the commensal flora via TLR5 (Lantier et al., 2014). The mechanism of protection was IL-12p40 and type 1 interferon receptor (IFNAR)-dependent. This unexpected finding demonstrated that commensal flora in neonatal mice synergize with Poly(I:C) to elicit protective intestinal immunity (Lacroix-Lamande et al., 2014). Of note, CpG-ODN, which signals through MyD88, does not require these additional signals from the microbiota. The precise mechanism by which the MyD88 pathway leads to the control of cryptosporidiosis is under investigation in our laboratory.

It has long been known that intestinal infections lead to malnutrition and that malnutrition worsens intestinal infections (Guerrant et al., 2008). In a mouse model of malnutrition, in which 21-day-old wild-type mice were fed a low protein diet (isocaloric chow containing 2% protein) before infection and during the entire experiment after infection, mice receiving daily gavage of CpG-ODN1668 had less body weight loss, and a slightly decreased parasite burden in the ileum (Costa et al., 2012). Further work by the same laboratory showed that CpG ODN-1668 can partially attenuate the severity of *C. parvum* infection during protein malnutrition by partially restoring the TH1 type immune response (Bartelt et al., 2016).

Natural molecules with immunostimulatory properties may therefore provide a safe and acceptable way to strengthen neonatal immunity against *Cryptosporidium*, as well as possibly other neonatal enteric pathogens, even in undernourished children.

3.4. Triggering of adaptive immunity

Innate immunity plays a key role in controlling the early acute phase of the infection (Barakat et al., 2009b; Korbel et al., 2011), but studies performed with T-cell deficient mouse

models (SCID (McDonald and Bancroft, 1994), Rag2^{-/-} (Barakat et al., 2009a), CD3ε^{-/-} (Lantier et al., 2013)) confirm the role of adaptive immunity in clearance of the parasite. Triggering adaptive immunity during enteric infections requires local antigen sampling by “immature” antigen presenting cells and migration of DCs via lymph towards the MLN, where they present antigens to T cells.

Migration of intestinal DCs to MLN upon *C. parvum* infection is restricted to the ileum, where the parasite develops, whereas strong inflammatory signals provided by oral administration of TLR ligands induce rapid mobilization of all intestinal DCs to the draining lymph nodes (F. Laurent, unpublished data). A pioneer study by Ponnuraj and Hayward (Ponnuraj and Hayward, 2001) revealed that only parasite antigens, not live parasites, reached the MLN during infection. These results were challenged by a recent study by Perez-Cordon, revealing that live *C. parvum* are found in CD11c⁺ cells in the MLN, shown by the presence of parasite mRNA (Perez-Cordon et al., 2014). Whether this results from parasite engulfment by phagocytic cells in the M cell pocket of Peyer's patches and/or the capture of early apoptotic intestinal ECs containing schizonts, and/or direct parasite capture in the lumen is yet unknown. The first possibility is supported by an old electron microscopy study showing that *Cryptosporidium* may be found deep within the cytoplasm of M cells, overlying Peyer's patches, but also associated with phagocytes subjacent to such M cells, both intact and partially digested (Marcial and Madara, 1986). DCs, but not macrophages, migrate to the draining lymph nodes under conventional conditions. If macrophages capture free developmental stages of *C. parvum*, they transfer the intact or degraded parasite to migratory DCs, which subsequently prime naïve T cells in MLN. Peyer's patches are intestinal lymphoid tissues programmed to develop during fetal life following a three-step model of Peyer's patch (PP) development (Eberl and Lochner, 2009), but their size increases substantially in rodents during maturation after birth. In sheep and cattle, the ileal Peyer's patch (IPP), which extends 1-2 m along the terminal small intestine, is a primary lymphoid organ for B-cell development and undergoes accelerated involution, such that the follicles have almost completely disappeared by 3-4 months of age (Yasuda et al., 2006). In young lambs and calves, the IPP is an area heavily infected by *C. parvum* (Fig. 3). Thus, the frequency of capture by phagocytic cells in Peyer's Patches may vary depending on age and

the species considered. The second possibility is that CD103⁺ DCs, which continuously patrol among enterocytes (Farache et al., 2013) and sample apoptotic intestinal ECs at steady state for the induction of tolerance, also capture infected intestinal ECs, thus indirectly acquiring *C. parvum*. A third mechanism has also been described following 2-photon microscopy. It shows luminal capture of microbiota and pathogens by CD103⁺ DCs via dendrite extensions, but there is currently no evidence to indicate whether this mechanism occurs during *C. parvum* infection (Farache et al., 2013). Transgenic *Cryptosporidium*-expressing fluorescent proteins will help in evaluating the respective importance of these non-mutually exclusive mechanisms in monogastric species and ruminants.

Recently, Sheoran et al. (2012) investigated cross-protection between *cryptosporidium* spp. and demonstrated that infection with *C. hominis* provided incomplete protection of gnotobiotic piglets against *C. parvum* challenge whereas the animals were fully protected against homologous challenge (Sheoran et al., 2012). In contrast, human volunteers re-challenged with the same *C. parvum* isolate 1 year after recovery exhibited a similar time of onset and duration of clinical illness to that observed after primary challenge (Okhuysen et al., 1998). Lukin et al. (2000) have shown that eradication of *C. parvum* infection can be obtained in mice by antigen-nonspecific CD4(+) T-cell effectors (ovalbumin-specific T cells) (Lukin et al., 2000). This intriguing observation adds another level of complexity. The mechanism of elimination of infected enterocytes that do not efficiently present antigens to conventional specific T-cells is still unknown. The fact that DCs induce T-cell-mediated immunity during *Cryptosporidium* infection, favoring clearance of the parasite, is undeniable. However, the mechanism of elimination of infected enterocytes via MHC presentation and T cell killing (???) killing), is still a matter of debate. In vitro, CD8⁺ T-cells can induce intestinal EC death via recognition of unconventional MHC-class I molecules expressed during the stress of *C. parvum* infection (Dann et al., 2005). This is a much more likely mechanism, but as unconventional MHC-class I molecules do not bind peptides, this mechanism does not explain the protection obtained via homologous *Cryptosporidium* challenge. This important issue requires further investigation as it will need to be resolved with respect to vaccine development dedicated to young children.

4. *Cryptosporidium* and microbiota, friends or foes?

Microbiota play a fundamental role in the development of intestinal immunity and maintenance of homeostasis (Round and Mazmanian, 2009). Interactions of enteric pathogens with their host and pathogenesis have to be investigated in light of the additional interactions with the vast microbial communities present in the intestine. This broader view of studying host-pathogen interactions is called the Pathobiome concept (Vayssier-Taussat et al., 2014). Any changes in microbiota composition can confer resistance to, or promote infection by, pathogens and conversely, infection by enteric pathogens can alter microbiota composition. A recent study by Ras et al. (2015), using immunosuppressed mice, demonstrated that the gut microbiota is affected by infection with *C. parvum* regardless of the isolate used to infect mice (Ras et al., 2015). This perturbation appeared to be reversible after mice cleared the infection. The impact of the microbiota on *C. parvum* development was investigated in a few relatively old studies. Adult mice are much more resistant than neonatal mice to *C. parvum* infection. The difference between adult and neonate microbiota was suspected to be involved in this difference in susceptibility. Antibiotic-treated adult mice were still resistant to *C. parvum* infection, whereas germ-free adult mice could be moderately infected (Harp et al., 1988). In our laboratory, experimental infection of germ-free neonatal mice and neonatal mice born to antibiotic-treated mothers unexpectedly revealed no significant difference in terms of parasite load and kinetics of elimination relative to that of conventional neonatal mice (S. Lacroix-Lamandé, unpublished data). The presence of microbiota was shown to delay the onset of *C. parvum* oocyst excretion by several weeks in adult SCID mice, which do not develop a specific immune response but which are relatively resistant to *C. parvum* infection (Harp et al., 1992). Therefore, microbiota can improve resistance to infection under certain circumstances in adult mice, probably via a mechanism involving innate immune responses. Recent data obtained in humans and mouse models suggest that, among the complex microbiota, specific phyla appear to provide positive or negative signals to modulate the immune response to *Cryptosporidium* infection. Indole producing bacteria, or indole alone, can affect the ability of *Cryptosporidium* to establish an infection (Chappell et al., 2016). Hence, it has been suggested that pre-existing indole levels in the gut can be used as a biomarker of susceptibility to *Cryptosporidium* infection in humans.

Recently Gorla et al (2014), in seeking new Inosine-5'-monophosphate dehydrogenase (IMPDH) inhibitors against *C. parvum*, identified compound A110 which specifically altered the microbiota, characterized by an increase in the proportion of Verrucomicrobiota and a decrease in Bacteroidetes (Gorla et al., 2014). Despite the ability of A110 to inhibit *C. parvum* IMPDH activity in vitro, the altered microbiota resulting from oral administration of the compound to infected IL12-deficient mice was associated with increased growth of the parasite.

Moreover, many studies have reported that supplementation with probiotics can have beneficial effects and decrease the intensity and/or duration of oocyst shedding, but does not prevent infection. For example, a delay in oocyst production was observed in SCID mice colonized with a mixture of anaerobic bacteria (Harp et al., 1992). Feeding of *Lactobacilli* was shown to enhance clearance in adult immunosuppressed mice (Alak et al., 1997; Alak et al., 1999). In humans, consumption of *Lactobacilli* was associated with the resolution of diarrhea and oocyst shedding in a young celiac patient with persistent cryptosporidiosis within 10 days of probiotic initiation (Pickerd and Tuthill, 2004). However, in some studies, no positive effect was observed when *Lactobacilli* were fed to infants (Salazar-Lindo et al., 2004) or children with cryptosporidiosis (Sindhu et al., 2014). Overall, these data show that although the microbiota is dispensable for *Cryptosporidium* development, it can affect disease outcome under certain circumstances.

5. Conclusion and future perspectives

The use of transgenic animals has led to new insights on protective immune responses to *C. parvum* infection in mice and such studies must continue. However, crucial knowledge is still lacking concerning the mechanisms governing protection and those responsible for the pathophysiology of infections in humans and large animals. Although studies in ruminants face obvious economic constraints for experimentation and broader genetic variability, major efforts must be made to transpose and validate the results gained with animal models in these target species. This includes basic immunology and identification of species specificities to provide adapted solutions for each species. New 3D intestinal models will undoubtedly advance

investigations on *Cryptosporidium*-EC interactions in humans and cattle. Recent progress in technologies such as high throughput approaches to investigate immune responses and drug screening, and the ability to genetically modulate *Cryptosporidium*, makes efficient control of cryptosporidiosis feasible in the short to medium term.

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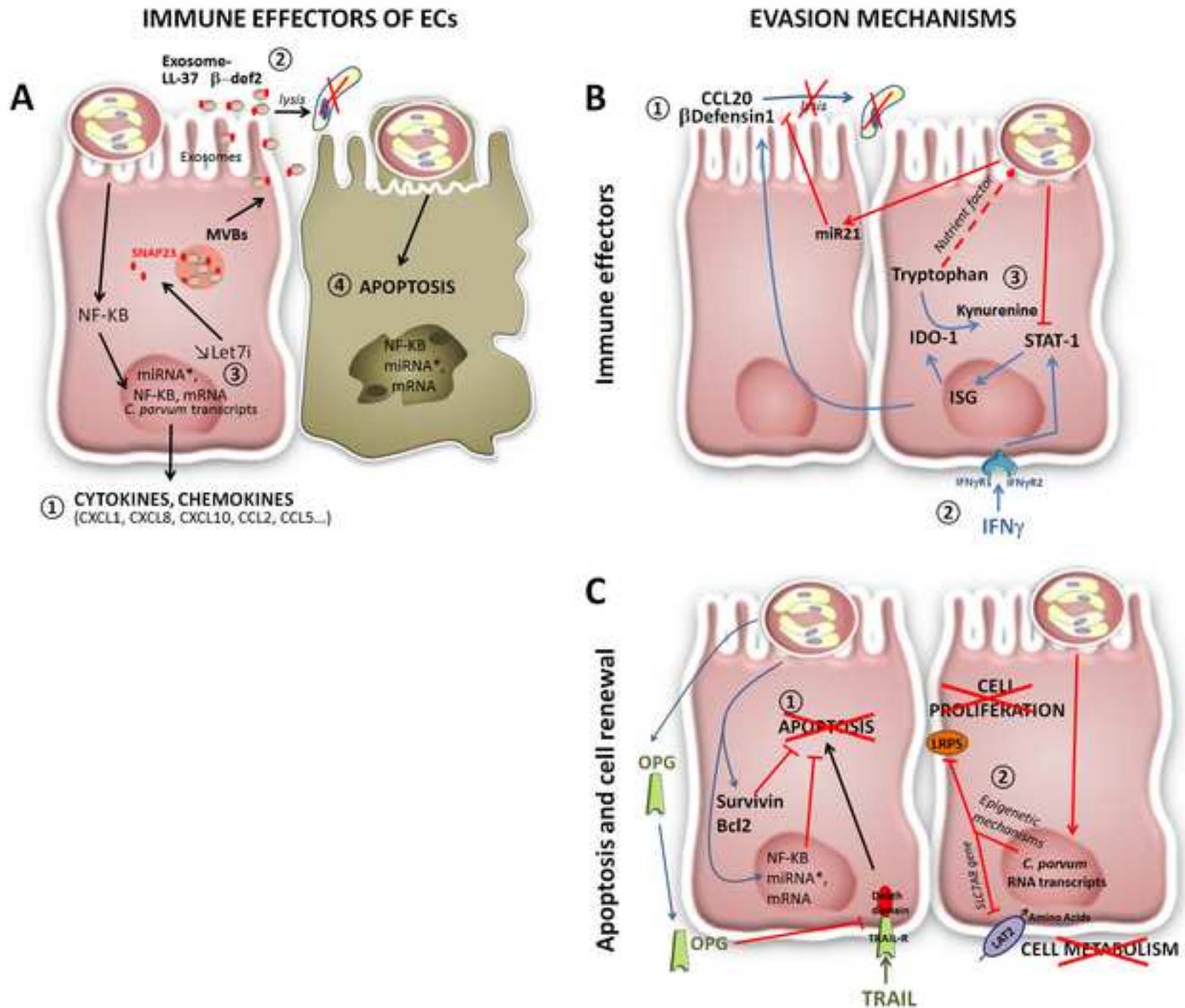
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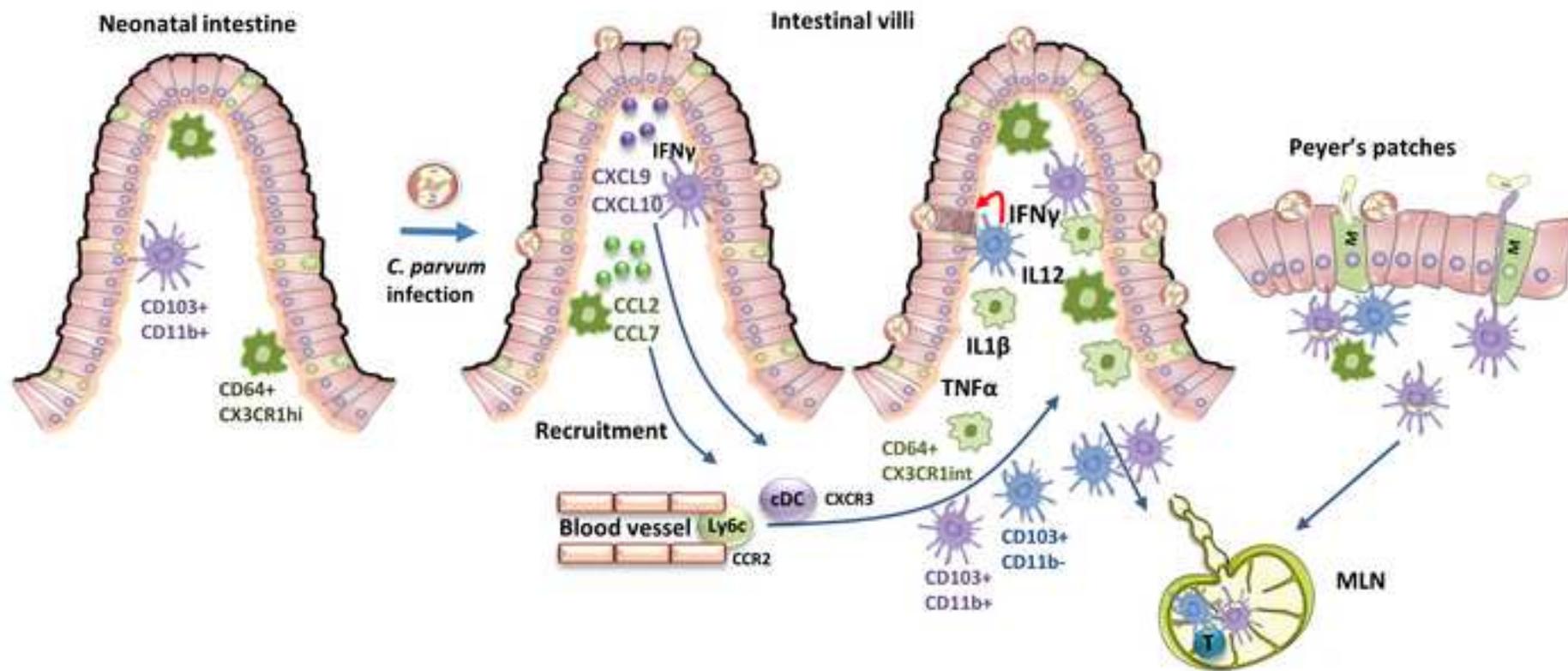
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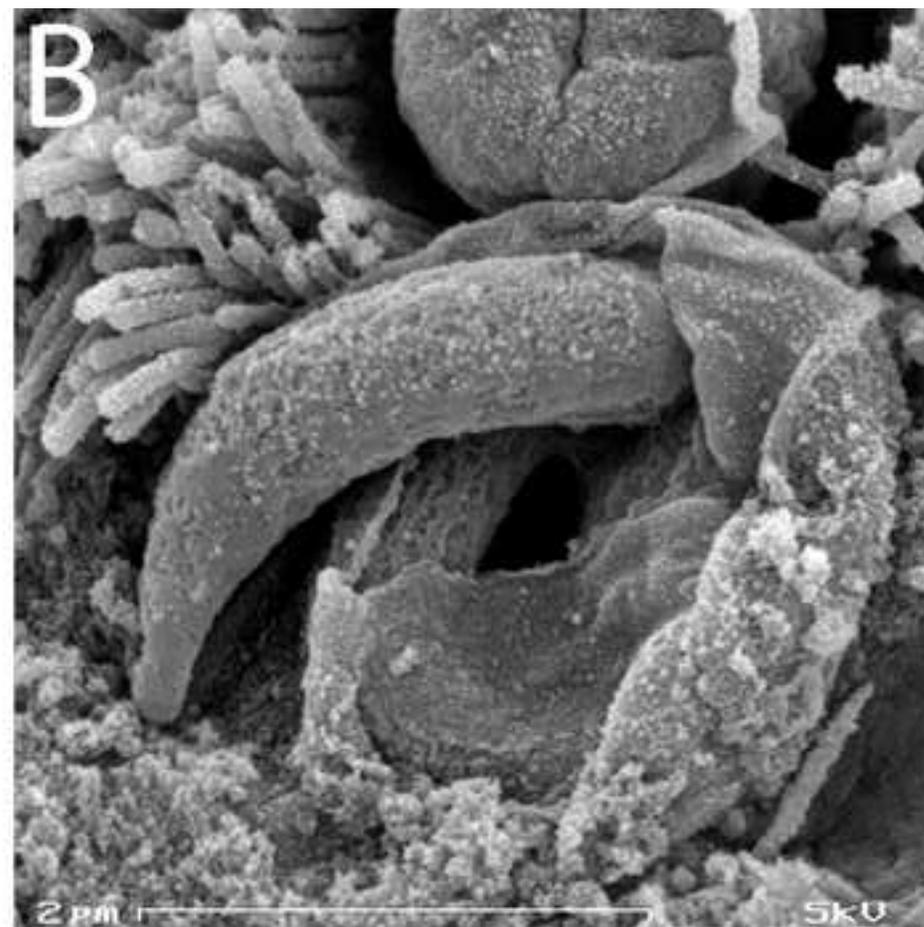
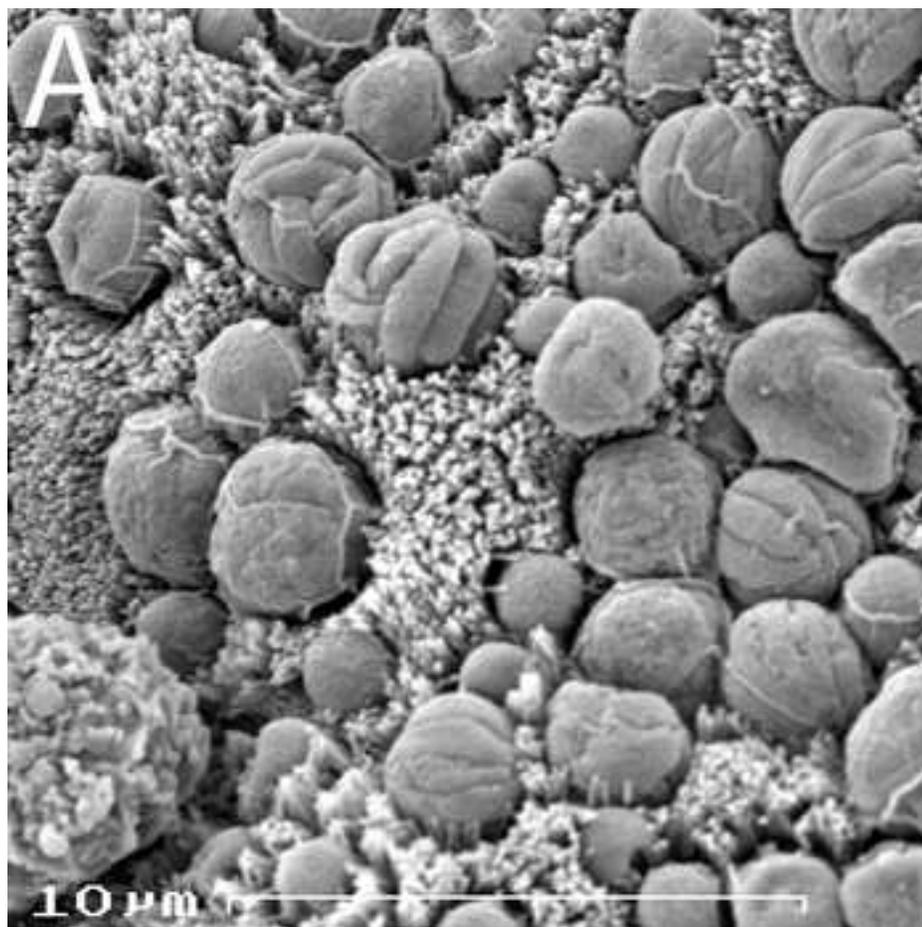
Fig. 1. Epithelial cell (EC) defense mechanisms against *Cryptosporidium parvum*, and evasion strategies developed by the parasite. (A) ECs respond to *C. parvum* infection by various defense mechanisms. They (i) produce cytokines and chemokines to attract immune cells to the site of infection ①, and (ii) release antimicrobial peptides (AMPs) that can kill free stages of the parasite. AMPs can be produced in a free form or associated with exosomes produced by infected cells ②. Most of these changes are mediated by miRNA expression (* for a review see Ming et al., 2017) ③; for example, the reduced expression of Let7i induced by infection favors the production of SNAP23. SNAP23 is a protein found on the multi-vesicular bodies (MVBs) in infected cells that stimulates exosome release. Host cell apoptosis is also a way for ECs to eliminate pathogens before they can fully differentiate ④. (B) *Cryptosporidium parvum* has developed strategies to subvert some innate immune effectors. For example, *C. parvum* reduces CCL20 expression known to exert antimicrobial activity against the parasite, by modulating miR21 expression ①. IFN γ produced during infection is essential for controlling the parasite and increases STAT-1 transcription factor levels in intestinal ECs, leading to the expression of many Interferon-stimulated genes (ISGs) including IDO1, an enzyme that catabolizes tryptophan, thus limiting parasite growth ②. To circumvent the effect of IFN γ , *C. parvum* is able to reduce STAT1 production and IDO1 expression by a yet unknown mechanism, thus restoring tryptophan availability in cells to allow parasite development ③. (C) *Cryptosporidium parvum* has developed strategies to interfere with apoptosis and epithelium renewal to provide it with enough time to complete its intracellular development. *Cryptosporidium parvum* infection in ECs induces survivin, Bcl2, NF κ B, and osteoprotegerin (OPG) production to prevent apoptosis ①. *Cryptosporidium parvum* is also able to deliver parasite transcripts into the nuclei of infected cells and suppresses host cell gene transcription involved in cell proliferation (LRP5) and amino-acid transport (SLC7A8 gene encoding LAT2 Transporter) by epigenetic modifications ②.

Fig. 2. Mucosal innate responses to *Cryptosporidium parvum* - focus on mononuclear cells. Few resident macrophages and dendritic cells (DCs) are present in the neonatal mouse mucosa. After infection, the rapid release of chemokines by infected intestinal ECs and those responding to the first wave of IFN γ release by resident mononuclear cells allows the recruitment of multiple immune cell subsets to the lamina propria. Ly6c⁺CCR2⁺ inflammatory monocytes recruited by CCL2 and CCL7 quickly differentiate into CD64⁺CX3CR1^{int} macrophages, producing proinflammatory cytokines including IL1 β and TNF α , that decrease trans-epithelial resistance. CD103⁺ DCs are recruited by CXCL9 and CXCL10 (IFN γ -dependent chemokines) and produce high amounts of IL12 and IFN γ , favoring control of the parasite in infected intestinal ECs (red arrow) during the acute phase of the infection. CD103⁺CD11b⁻ DCs produce the largest amounts of IFN γ and therefore play a key role in the elimination process. Once activated, CD103⁺ DCs quickly migrate to the draining lymph node to activate specific T cells. *Cryptosporidium parvum* antigens and live parasites are also captured in specialized lymphoid tissues (Peyer's patches). Putative mechanisms of capture are represented (passage through M cells, capture by transepithelial dendrites). T-cell-mediated adaptive immunity results in the definitive clearance of the infection.

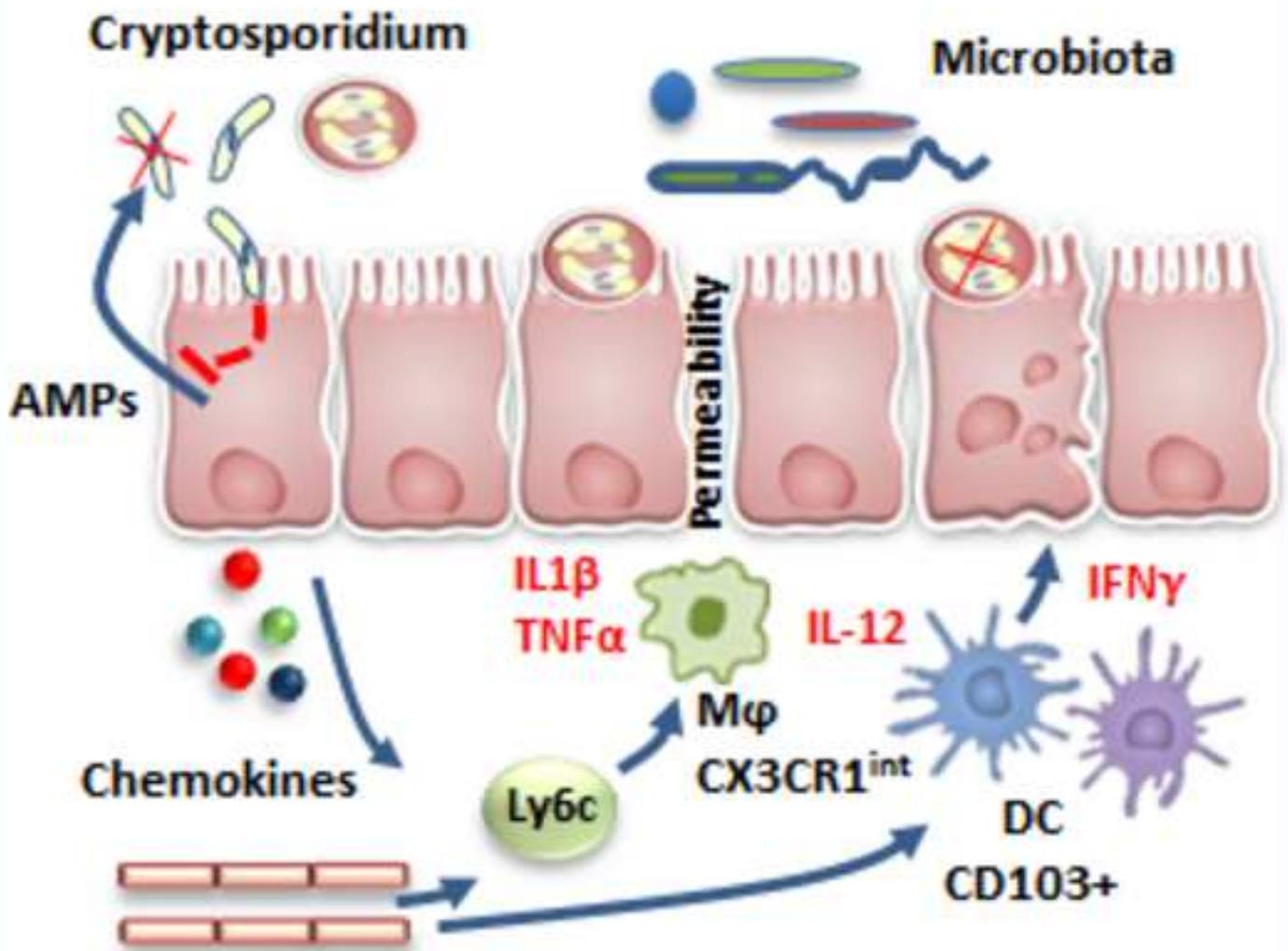
Fig. 3. Infected ileal Peyer's patch of a 1 week old lamb. Scanning electron micrograph of the ileal Peyer's patches of a young lamb infected by *Cryptosporidium parvum* (scale bar in μm). (A) The dome is covered by numerous developing parasites. (B) Merozoite emerging from an infected cell.







Innate immune response to *C. parvum*



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Highlights

- Intestinal epithelial cells are watchdogs and primary effector cells against *Cryptosporidium*
- New promising 3D models for long-term *Cryptosporidium* propagation are discussed
- CD103+ dendritic cells play a key role in controlling the acute phase of infection
- Inflammatory monocytes, recruited in large numbers, participate in loss of epithelial permeability
- Immunostimulation is a way to strengthen neonatal immunity and control *Cryptosporidium* development

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