Published in final edited form as:

Pathog Dis. 2014 February ; 70(1): 17–27. doi:10.1111/2049-632X.12078.

Interaction of *Cryptosporidium parvum* with mouse dendritic cells leads to their activation and parasite transportation to mesenteric lymph nodes

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Abstract

Dendritic cells (DCs) are the antigen presenting cells capable of activating naïve T cells. Although CD4⁺ T cells are crucial for *Cryptosporidium parvum* clearance, little is known about the role of DCs in the immune response to this parasite. In this study the interaction between mouse DCs and *C. parvum* was investigated both *in vitro* and *in vivo*. For *in vitro* experiments, mouse bone marrow-derived dendritic cells (BMDCs) derived from wild type C57B1/6 or MyD88^{−/−} or C3H/HeJ mice and DC cell line DC2.4 were pulsed with *C. parvum*. Active invasion of parasites was demonstrated by parasite co-localization with host cell membranes and actin-plaque formation at the site of attachment. DC activation induced by the parasite invasion was demonstrated by upregulation of costimulatory molecules CD40, CD80, and CD86, as well as inflammatory cytokines IL-12, TNF- α , and IL-6. BMDCs derived from MyD88^{−/−} and C3H/HeJ mice failed to produce IL-12 in response to *C. parvum*, suggesting the importance of TLR-dependent signaling pathway specially presence of a functional TLR4 pathway, for *C. parvum*-induced cytokine production. *In vivo* experiments showed that both parasite antigens and live parasites were transported to mice mesenteric lymph nodes. All together, these data suggest that DCs play a key role in host immune responses to *C. parvum* and pathogenesis of the disease.

Keywords

Cryptosporidium parvum; dendritic cell; mesenteric lymph node

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No conflict of interest.

Introduction

Cryptosporidium parvum is an intracellular protozoan parasite that infects the epithelial cells of the gastrointestinal tract of mammals, including humans, and it is considered to be a major cause of diarrhea worldwide (Lopez-Velez, 1995). Transmission occurs by fecal-oral route, and the contamination of water supplies represents a major cause of cryptosporidiosis outbreaks. Unlike immunocompetent adults in whom cryptosporidiosis is usually self-limited to two weeks, immunocompromised people, such as malnourished children and those afflicted with AIDS, are susceptible to chronic cryptosporidiosis that can be a life-threatening disease characterized by voluminous diarrhea (Lopez-Velez, 1995; Tzipori and Ward, 2002). Despite extensive studies and multiple medications tested, no effective therapy is currently available (Tzipori, 1998). Studies in animal models have provided important insights into the host immune responses towards *C. parvum* and revealed that those responses play a critical role in the control of cryptosporidiosis. Indeed, infection with *C. parvum* elicits a cell-mediated immune response that is essential for the clearance of these organisms (Chen et al., 1993; McDonald, 2000).

Since their description as immune cells (Steinman and Cohn, 1973), dendritic cells (DCs) have provided a new understanding of the immune responses to pathogens (Banchereau and Steinman, 1998). DCs play a central role in the generation of intestinal cell-mediated immunity because of their ability to acquire, process, and present antigens to T cells (Reis e Sousa, 2001). DCs are heterogenic and widely distributed in tissues and organs. In the mouse intestine there are several subsets of DCs with distinctive phenotypes (Reis e Sousa, 2001). It has been shown that a subset of lamina propria DCs (CD11c⁺ and CD11b⁺) have dendrites that directly protrude into the intestinal lumen, crossing epithelial barriers to sample microbial antigens (Niess et al., 2005). The binding of a ligand to its DC-specific receptor leads to DC activation, which is characterized by upregulation of co-stimulatory molecules, cytokines, and chemokines (Mowat, 2003). The activated DCs present antigens to naïve T cells, triggering an antigen-specific immune response (Banchereau and Steinman, 1998). While T cell-mediated immunity appears to be essential in the control of *Cryptosporidium* infection, the mechanisms that elicit these immune responses are unclear. In this study, we investigated the mechanisms by which DCs acquire *Cryptosporidium* antigens and whether this interaction leads to their activation. Additionally, we investigated whether parasite antigens are transported to mesenteric lymph nodes (MLNs) where mucosal DCs activate naïve T cells. For these experiments, cells from mouse bone marrow-derived DC line DC2.4 and BMDCs were pulsed with *C. parvum* to investigate DC infection and activation. Also, wild-type C57BL/6 mice were orally infected with *C. parvum* oocysts to determine whether parasite antigens are transported to MLNs. We found that not only can DCs take up and process parasite antigens, but that *C. parvum* can also actively invade and infect DCs. Moreover, this interaction led to DC activation as determined by the overexpression of several co-stimulatory molecules and cytokines in both DC2.4 and BMDC cells. The latter also provided data to suggest that *C. parvum* induced proinflammatory cytokine production via TLR-dependent signaling pathways. We found that both parasite antigens and live parasites were transported to MLNs. Taken together,

these results suggest that DCs may play an important role in both innate and adaptive immune responses to *Cryptosporidium*.

Material and Methods

Mice and cells

Wild-type C57BL/6 and C3H/HeJ mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA). MyD88^{-/-} mice were provided by Douglas Golenbock (University of Massachusetts Medical Center, Worcester, MA). Mouse bone marrow-derived DCs (BMDCs) were generated as previously described (Inaba et al., 1992) by culture in GM-CSF and IL-4, followed by positive immunomagnetic selection using CD11c antibody-coated microbeads (Miltenyi Biotec, Auburn, CA, USA). The selected BMDCs were >98% CD11c⁺ DCs by flow cytometry analysis. These DCs were CD11c⁺ and B220⁻, expressed high levels of CD11b and MHC class II, and expressed moderate levels of CD80 and CD86. Bone marrow-derived DC cell line DC2.4 (Shen et al., 1997) was provided by Kenneth Rock (University of Massachusetts Medical School, Worcester, MA, USA). Cells were cultured in RPMI-1640 medium containing 2 mM L-glutamine, 100 U/ml penicillin G, 50 µg/ml streptomycin sulfate, 50 µM β-mercaptoethanol and 10% fetal bovine serum unless otherwise indicated. Human ileocecal adenocarcinoma HCT-8 cell line (ATCC CCL-224) was maintained in RPMI-1640 medium containing 10% fetal bovine serum and grown in 25 mm² to 80% confluent monolayers in a 5% CO₂ humidified incubator prior to infection. All experiments were performed in accordance with guidelines of the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School and the recommendations in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences, 1996).

Parasites and infection

The Iowa isolate of *C. parvum* was purchased from Bunch Grass Farm, Deary, ID, USA. *C. parvum* isolate MD (Okhuysen et al., 2002) was propagated in our laboratory. For excystation, oocysts were treated with 10% sodium hypochlorite for 7 minutes on ice. After two washes in phosphate buffered saline (PBS), oocysts were resuspended in 0.8% sodium taurocholate (NaTc; Sigma, St. Louis, MO, USA) and incubated at 37°C for 30 minutes. Oocyst preparations with an excystation rate greater than 80% were used in this study. Excysted oocysts (1–2 × 10⁶) were added directly to cell monolayers in 6-well plates. Two hours after infection, cells were profusely rinsed and fresh medium was added. Heat-killed parasites were generated by incubating the same number of oocysts at 65°C for 30 min. In some cases, bleached oocysts were directly added to cells in the presence of 0.05% NaTc for 24 hours. *Enterocytozoon bienersi* spores were purified from fresh stools of infected adult humans or Rhesus macaques as describe elsewhere (Sheoran et al., 2005).

Infection of mice with *C. parvum* was carried out following review and protocol approval by the Institutional Animal Care and Use Committee of Tufts University. Two hours prior to infection, wild-type C57BL/6 mice received an intraperitoneal injection of 1 mg of anti-INF-γ monoclonal antibody XMG-6 (Castellaneta et al., 2004). Successful *C. parvum* infection

was determined by detection of oocyst shedding three times per week by microscopic observation of 30 high-power fields of a Kinyoun carbol fuchsin-stained fecal smear from each infected animal. Age- and genetic background-matched C3H/HeJ and MyD88^{-/-} mice were orally inoculated with 10⁶ *C. parvum* oocysts or 10⁶ *E. bieneusi* spores (in 10 µl of PBS). *E. bieneusi* spore shedding was monitored three times a week using an indirect immunofluorescent assay (Sheoran et al., 2005).

CFSE staining

Carboxyfluorescein diacetate, succinimidyl ester (CFSE) (Invitrogen, Carlsbad, CA, USA) staining of *C. parvum* has been described previously (Feng et al., 2006). Briefly, oocysts were suspended in 0.8% sodium taurocholate in PBS and incubated at 37°C for 15 minutes, followed by incubation in 10 µM of CFSE for 15 minutes. Sporozoites were washed 3 times with ice-cold PBS prior to inoculation.

Flow cytometry

Flow cytometry was performed using FACSCalibur and CellQuest software (BD Biosciences, Mountain View, CA, USA) with fluorophore-conjugated Abs to IL-6 (MP5-20F3), IL-12 (C15.6), TNF-α (MP6-XT3), I-A^b (AF6-120.1), CD11c (HL3), CD40 (3/23), CD80 (16-10A1), CD11b (CBRM1/5), CD8α (53-6.7), B220 (RA3-6B2), and CD86 (GL1) from BD Pharmingen (San Diego, CA, USA). Isotype control antibodies were used as negative controls. *C. parvum* parasites were stained with mouse monoclonal antibody JF4 (generated in our laboratory) against *C. parvum* sporozoites followed by Alexa 488-conjugated goat anti-mouse antibody (Invitrogen). For external staining, 2 × 10⁵ cells per microtiter well were washed with FACS buffer (PBS containing 2% heat-inactivated fetal bovine serum and 0.1% sodium azide) and incubated with an Fc receptor-blocking antibody (BD Pharmingen) for 5 minutes, and then incubated with saturating amounts of monoclonal antibodies for 30 minutes at 4°C. For intracellular cytokine staining, cells were treated with 20 µM brefeldin A for the last 12 hours of culture or as indicated, resuspended in 50 µL FACS buffer, and permeabilized using the Caltag Laboratories (Burlingame, CA, USA) Fix and Perm kit according to the manufacturer's protocol. Saturating amounts of fluorochrome-conjugated antibodies were added in the permeabilization buffer, and cells were incubated at room temperature for 15 minutes. Cells were washed and resuspended in FACS buffer with 1% formaldehyde for analysis.

Toll-like receptor *in vitro* screening

TLRs involved in interacting with *C. parvum* were assessed by testing NF-κB activation in HEK293 cells expressing a given TLR. The cells were transfected with pNiFty-SEAP (Invivogen, CA, USA), which carries a gene for secretory alkaline phosphatase (SEAP) under the NF-κB promoter. All cells were cultured under standard conditions in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum, 10 U/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, and 1 mM pyruvate. Antibiotics zeocin (400 µg/ml) and G418 (500 µg/ml) were supplemented in the medium. The amount of SEAP secreted in culture media was determined by SEAP reporter assay (Invivogen, CA, USA). As positive control ligands for TLR2-9 we used heat-killed *Listeria monocytogenes* at 10⁸

cells/ml (TLR2), Poly (I:C) at 1 µg/ml (TLR3), *Escherichia coli* K12 lipopolysaccharide (LPS) at 100 ng/ml (TLR4), *Salmonella typhimurium* flagellin at 1 µg/ml (TLR5), loxoribine at 1 mM (TLR7), ssRNA40 at 5 µg/ml (TLR8), and CpG ODN 2006 at 1 µg/ml (TLR9).

Confocal microscopy

BMDCs or DC2.4 cells were directly grown on 12-mm coverslips overnight to reach 80% confluence. Cells were pulsed with *C. parvum* oocysts (5 oocysts/cell) and cultured for 16 or 24 hours before harvesting. To stain DC F-actin or lipids, cells were fixed with 4% paraformaldehyde for 10 min at room temperature followed by permeabilization using 0.5% Triton X-100 in PBS containing 1% BSA. Excess aldehyde groups were quenched with 10 mM ethanolamine in PBS for 5 minutes, and cells were stained with Alexa 568-phalloidin (Invitrogen) for 10 minutes at room temperature. In some cases, we used a fluorescent lipophilic probe (CM-Dil) that avidly binds to the cell membrane. *C. parvum* parasites were stained with mouse monoclonal antibody JF13 (generated in this lab) against *C. parvum* sporozoites followed by Alexa 488-conjugated goat anti-mouse antibody (Invitrogen). After 3 rinses, coverslips were mounted on slides using Prolong Gold antifade (Invitrogen), and slides were examined using a Leica TCS SP2 laser-scanning microscope (Leica Microsystems Inc., Bannockburn, IL, USA).

Electron microscopy

DCs treated with *C. parvum* for 1 or 2 hours were prepared for scanning and transmission electron microscopy (SEM and TEM, respectively). For TEM, cell pellets were fixed by glutaraldehyde (2.5%, v/v) for 3 hours and post-fixed with 1% OsO₄ in PBS. The cells were dehydrated in a graded series of ethanol and embedded in spurr resin (a low-viscosity epoxy resin embedding medium). Samples were then thin-sectioned, stained, and mounted on a grid. The fixed samples were observed with a transmission electron microscope (JEM-1010, JEOL, Japan). For SEM, DCs grown on coverslips were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) overnight at 4–8°C, post-fixed in 1.5% OsO₄, dehydrated in ethanol, and then sputter coated with gold-palladium. The samples were observed with a Hitachi 450 scanning electron microscope.

Immunohistochemistry

For immunohistochemistry, MLNs from both *C. parvum*-infected and uninfected mice treated with PBS were isolated after 2 weeks and placed in 10% formalin. Sections from paraffin-embedded MLNs (5 µm) were stained using an immunohistochemistry kit (X BioGenex, CA, USA) according to the manufacturer's protocol. Rabbit polysera against *C. parvum* (generated in our laboratory and diluted at 1:10,000) was used as primary antibody.

Reverse transcriptase PCR (RT-PCR) and quantitative PCR (qPCR)

To study overexpression of *C. parvum* heat shock protein 105 (Hsp105), approximately 5 × 10⁶ oocysts in PBS were treated with heat shock at 42°C for 1, 3, or 5 hours in a water bath. *C. parvum*-infected HCT-8 cells were cultured for 12 or 24 hours and then subjected to heat shock at 42°C for 1 hour.

MLNs (3–5 mg of tissue) were removed from mice after 2 weeks and placed into prewarmed sterile physiological saline solution (0.85% NaCl) at 37°C and then treated with heat shock at 42°C for 1 hour in a water bath. Total RNA from oocysts, infected HCT-8 cells, and MLNs was extracted with Trizol (Invitrogen). Synthesis of cDNA was performed as describe elsewhere (Widmer et al., 1999). As a control for contaminant genomic DNA present during RNA purification, a replicate of each sample was subjected to the same extraction protocol but without adding reverse transcriptase. A fragment of the Hsp70 gene was amplified (590 bp) using a primer pair (5'-AGCAATCCTCTGCCGTACAGG-3' and 5'-AAGAGCATCCTTGATCTTCT-3'). Additionally, a 144 bp fragment of the *C. parvum* Hsp105/110 gene identified using the database Cryptodb.org (Heiges et al., 2006) was amplified by using a primer pair (5' CAGGAGGCAGCCTTCGTAAC 3' and 5' TCCAGCTTGCTTTAGCGCAG 3'). β -tubulin was also included in this study as a control of RNA isolation as this gene possesses an intron facilitating the differentiation between PCR products originating from mRNA or from co-purifying genomic DNA. 18S rRNA was chosen for housekeeping gene control. Amplifications were performed with *Taq* DNA polymerase, GoTaq® Green Master Mix (Promega). Additionally, we carried out quantitative PCR qPCR) for Hsp105 from MLNs of *C. parvum*-infected mice heat shock-treated at 42°C for 1 hour or incubated at 37°C for 1 hour. Fast SYBR® Green Master Mix (Applied Biosystems) was used to amplify the cDNA samples with the StepOnePlus™ Real-Time PCR equipment (Applied Biosystems) according to the manufacturer's protocol. All the data acquisition, dissociation curves, and data analysis were performed by the StepOnePlus software. Relative quantification of gene expression was carried out using the comparative C_T method (Getting Started Guide for Relative Standard Curve and Comparative C_T method, Applied Biosystems). Negative controls were included, and the relative target expression was normalized against the *C. parvum* 18S rRNA housekeeping gene. To confirm accuracy and reproducibility the experiment was performed in duplicate.

Results and Discussion

Despite the amount of data available regarding the immune responses to *C. parvum*, little is known about the role of DCs in cryptosporidiosis defense. Since *C. parvum* is capable of triggering a cell-mediated immune response where CD4+ T cells play a key role in parasite clearance (Chen, 1993; McDonald, 2000), we investigated the interaction between DCs and the parasites because of the DCs' ability to acquire, process, and present antigens to active naïve T cells as well as to initiate a cell-mediated immune response. Recently, it was shown that lamina propria DCs have dendrites that protrude into the intestinal lumen, crossing the epithelial barrier to sample microbial antigens (Niess et al., 2005). Therefore, we hypothesized that the intestinal DCs may take up parasite antigens, transporting them to MLNs to trigger T cell responses. We began studying the interaction between DCs and *C. parvum in vitro* using primary bone marrow-derived DCs and the DC cell line DC2.4 (Shen et al., 1997). We used CFSE labeling to track parasites on DCs. CFSE has been widely used for tracking the development and division of transplanted cells (Lyons et al., 2000; Kaech et al., 2001, Stoll et al., 2002). It has also been used to investigate the proliferation of *Leishmania spp.* (Kamau et al., 2000) and to track initial infections of *Listeria monocytogenes* both *in vitro* and *in vivo* (Feng et al., 2005). More recently, this technique

was successfully used in our laboratory to quantitatively track *C. parvum* infection in cell culture (Feng et al., 2006). Our experiments showed that after pulsing DC2.4 cells with CFSE-labeled live or heat-killed (HK) *C. parvum* sporozoites, CFSE-positive DCs could be detected 2 hours post-infection. Flow cytometry analysis showed that 5%, 8.8%, and 19.1% of DCs were CFSE-positive when DCs were pulsed with live parasites at an oocyst-to-cell ratio of 1:1, 2:1, and 6:1 respectively (Fig. 1A). HK parasites could not invade cells actively but could bind to or be passively taken up by DCs. However, the percentages of DCs that were associated with HK parasites (2.8%, 3.2%, and 5% of CFSE-positive populations) were significantly lower compared to DCs incubated with live parasites at the same oocyst-to-cell ratios (Fig. 1A), suggesting that live *C. parvum* sporozoites actively invade mouse DCs. The histogram in Figure 1B shows that CFSE-positive cells R1 populations) were well separated from negative DC populations in both HK and live parasite groups.

We carried out further experiments to confirm whether *C. parvum* actively invades DCs. To test this, we performed confocal and electron microscopy analysis. By using electron microscopy we saw sporozoites attached to the surface of DCs 2 hours post-infection. At this initial invasion phase, the sporozoites were clearly attached to the surface of DCs, as revealed by SEM (Fig. 2A). *Cryptosporidium* sporozoites do not penetrate deeply into the host cells but rather stay in an intracellular, extracytosolic niche separated from the enterocyte cytosol by an electron dense band formed during the initial invasion by the deposition of host actin (Elliot et al., 2000). As shown by TEM, the initial infection of DCs induced an actin-dense plaque that separated the host cell from the parasite (Fig. 2B, arrow). In recent work using Caco-2 cells, we determined that the calcium-dependent thiol protease calpain is critical for regulating parasite-induced actin polymerization and subsequent invasion (Perez-Cordon et al., 2011). The active invasion of DCs by *C. parvum* was further confirmed by confocal microscopy. After infecting DC2.4 cells for 16 hours, we detected parasites colocalized with host cell membranes (Fig. 3D and F, arrow).

Parasite antigens diffused within the DC cytosol (Fig. 3G, arrowhead) suggesting that the parasites were taken up by DCs and degraded. Occasionally, we also saw internalization of whole *C. parvum* sporozoites by DCs (Fig. 3H). In order to be presented on MHC class II and to stimulate CD4⁺ helper T cells, antigens are taken up by phagocytosis or receptor-mediated endocytosis into endosomes where some proteolysis occurs (Rybicka et al., 2012). In either case, the interaction between *C. parvum* and DCs would lead to DC activation, which is critical for the induction of T cell activation (Guermomprez et al., 2002).

Next we examined the activation of DCs in response to *C. parvum* interaction. The interaction between DCs and a "danger signal" leads to DC activation, which is characterized by the upregulation of costimulatory molecules and the production of cytokines and chemokines (Re et al., 2004). In this study, we investigated both upregulation of costimulatory molecules and production of proinflammatory cytokines by flow cytometry. We found that the costimulatory molecules CD40, CD80, and CD86 were upregulated after 24 hours of exposure to *C. parvum* (Fig. 4A). One study showed that DCs in MLNs from *C. parvum*-infected neonatal mice were activated as determined by the overexpression of the costimulatory molecules CD40 and CD86 (Ponnuraj et al., 2001). CD40 is expressed by monocytes and DCs and is upregulated when DCs migrate to draining

lymph nodes in response to microbial challenge (Niess et al., 2005). Also, expression of one or both of the costimulatory molecules CD80 and CD86 on DCs is essential for effective activation of T lymphocytes and for IL-12 production (Hsieh et al., 1993). *C. parvum* also induced the production of proinflammatory cytokines, including IL-12, TNF- α , and IL-6, by mouse BMDCs (Fig. 4B). DC2.4 cells produced IL-12 and IL-6 upon treatment with the parasite, as well (Fig. 4C). The roles of proinflammatory cytokines have been extensively studied in *C. parvum* infection. Several studies have reported that IL-12 plays a critical role in the differentiation of Th1 cells and the subsequent production of IFN- γ , which is important for controlling *C. parvum* infection (Urban et al., 1996; Ehigiator et al., 2003; Pollock et al., 2001). TNF- α showed the highest upregulation among the cytokines studied (Fig. 4B). Previous studies demonstrated the importance of TNF- α in *C. parvum* infection by showing that the cytokine significantly inhibited *C. parvum* development in enterocyte cell lines (Lacroix et al., 2001; Lean et al., 2006). Our results on the production of proinflammatory cytokines by *C. parvum*-treated DCs agrees with a recent work reporting production of the cytokines interleukin-12 p70, IL-2, IL-1 β and IL-6 by *C. parvum*-treated mice and human DCs (Bedi & Mead., 2012).

DCs recognize pathogens, including eukaryotic parasites, through Toll-like receptors (TLRs) that bind pathogenic ligands and initiate immune responses against infections (Abreu et al., 2005). A recent study has demonstrated that MyD88, a common adaptor protein for TLRs, is important for the initiation of an innate immune response against *C. parvum* infection in mice (Rogers et al., 2006). In order to study the role of MyD88 in DC cytokine expression, BMDCs derived from MyD88 $^{-/-}$ mice were pulsed with *C. parvum* sporozoites, and IL-12 expression levels in DCs were measured. We focused on IL-12 due to its key role in immune defense during *C. parvum* infection as stated previously.

BMDCs derived from MyD88 $^{-/-}$ mice failed to produce IL-12 in response to *C. parvum* treatment (Fig. 5A) suggesting that *C. parvum*-induced proinflammatory cytokine production occurs via the TLR-dependent signaling pathway. BMDCs from C3H/HeJ mice (a TLR4-defective strain) also failed to produce IL-12 after *C. parvum* treatment; however, approximately 10% of C3H/HeJ BMDCs showed positive staining with anti-IL-12 antibody when treated with another enteric pathogen *E. bieneusi* (Fig. 5B). Since C3H/HeJ mice lack a functional TLR4 signaling pathway, the data indicate that TLR4 signaling is important for *C. parvum*-induced proinflammatory cytokine production by DCs. This result is consistent with a report where TLR4 was critical for *C. parvum*-induced NF- κ B activation in human biliary epithelial cells (Chen et al., 2003). More recently, it has been shown that the production of Th-1 cytokines by *C. parvum*-treated DCs was MyD88 dependent which agrees with our finding (Bedi & Mead., 2012). This finding is further supported by our results from a TLR *in vitro* screening system to examine which TLRs are involved in the interaction with *C. parvum*. Both TLR2 and TLR4 recognize *C. parvum* antigens (Fig. 5C).

One of the key features of DCs is their ability to migrate. After activation, intestinal DCs migrate to MLNs to stimulate T cells. Because *C. parvum* can invade DCs *in vitro* leading to DC activation, we orally infected mice with *C. parvum* oocysts, harvested the MLNs, and determined whether DCs acquired parasite antigens by flow cytometry and immunohistochemistry analysis. Two weeks post-infection the percentage of parasite

antigen-positive cells in MLNs increased to 12% (red rectangle in Fig. 6A; box R1 denotes CD11c⁺ cells). In addition, about 1.4% of all cells in MLNs stained positively with JF13, of which half were CD11c⁺ DCs (Fig. 6B). Parasites were also detected in MLNs by immunohistochemistry using rabbit polyclonal antibodies. As shown in Figure 6C, parasite antigens (dark brown-stained cells (arrows) and spots (arrowhead)) were readily detected in MLNs among lymphocytes (light blue cells), as compared to PBS-treated mice which had no detectable parasite antigens (Fig. 6D). Therefore, *C. parvum* antigens may be acquired by lamina propria DCs via transepithelial dendrites and then transported to MLNs. Interestingly transepithelial dendrite formation requires the chemokine receptor CXCR1 on DCs found particularly in the terminal ileum, which is the preferred site of infection for *C. parvum* (Smith et al., 2005) and also where CX₃CL1 is abundantly expressed.

The capability of *C. parvum* to invade DCs is not unique, as other pathogens, including intracellular protozoan parasites such as *Leishmania spp.* and *Toxoplasma gondii*, have been reported to infect DCs (Prina et al., 2004; Persson et al., 2009). Some of these pathogens, such as *T. gondii*, which can initiate infection through intestinal mucosa, can also colonize distant tissues after being transported by DCs (Bierly et al., 2008; Courret et al., 2006). Because of these reports and our findings that parasite antigens can reach MLNs via DCs, we wanted to determine whether live *C. parvum* parasites could enter MLNs. Contrary to previous work showing that *C. parvum* β -tubulin mRNA was not detected in mouse MLNs (Ponnuraj et al., 2001), we identified *C. parvum* β -tubulin mRNA in MLNs (Fig. 7A). Our positive results may have been due to the isolation of MLNs from those mice showing the highest rate of infection as monitored by oocyst shedding, which may have increased the number of parasite-bearing DCs detected. However, the number of parasite-bearing DCs that can be detected in MLNs is quite low. As determined previously, we found that only 1.4% of cells in MLNs stained positively with JF13 (our anti-*C. parvum* mAb), and half of those were CD11c⁺ DCs (Fig. 6B). β -tubulin mRNA can be a useful marker for *C. parvum* viability due to its rapid decay in dead parasites. Additionally, the presence of an intron within this gene facilitates the differentiation between PCR products originating from mRNA or from co-purified genomic DNA (Widmer et al., 1999). To confirm that live *C. parvum* parasites could be detected in MLNs, we performed heat shock treatment experiments to induce heat shock protein mRNA. The transcript for heat shock protein 70 (*hsp70*) was chosen as a target for RT-PCR amplification because it is induced in some experimental organisms in response to heat shock. Although the *hsp70* RT-PCR proved to be a sensitive detection method (Stinear et al., 1996), the *hsp70* mRNA was not induced in oocysts exposed to heat. However, we found that *C. parvum* heat shock protein 105 (Hsp105) showed higher transcript expression in heat shocked oocysts and *in vitro* cultures (Fig. 7B), as well as in MLNs (Fig. 7C and 7D), compared to samples incubated at 37°C. The overexpression of this transcript would not be possible in dead parasites due to their impaired transcription machinery.

Several studies have reported both the presence and proliferation of *C. parvum* in respiratory tissues (Lopez-Velez et al., 1995; Dupont et al., 1996); however, the route leading to extraintestinal cryptosporidiosis remains unknown. Immunocompromised patients infected with *Cryptosporidium*, in whom the infection may become chronic and severe, may involve

extraintestinal cryptosporidiosis in locations such as the lung epithelium, gallbladder, pancreas, and biliary ducts. Several possible routes have been suggested to explain the invasion of extraintestinal tissues, including the inhalation of oocysts after vomiting or hematogenous spread. Although intestinal *Cryptosporidium* spp. are not usually invasive, oocysts have been found inside macrophages defective in phagocyte killing, suggesting spread via circulating phagocytes. This hypothesis is further supported by the detection of the parasite inside the lumen of submucosal blood vessels after postmortem examination (Gentile et al., 1987). The results obtained in our study lead us to suggest that *Cryptosporidium* might invade extraintestinal tissues in a way similar to *T. gondii*, via transport into MLNs by DCs after being taken up in the intestinal lumen or after DC infection by *Cryptosporidium* sporozoites.

In the present work we investigated the response of mouse DCs to *C. parvum* parasites. We determined that the interaction between DCs and *C. parvum* prompted three important events necessary for the initiation of adaptive immune responses: antigen uptake, DC activation, and transportation to MLNs. The data collected in this study suggest an important role of DCs in cell-mediated immune responses against *C. parvum*. However, further experiments are necessary to increase our knowledge of the role of DCs during cryptosporidiosis and the events involved in the adaptive immune responses to this parasite. Although we found live parasites in MLNs, further experiments, such as application of *in vivo* imaging methods, will be needed to confirm distant tissue colonization by the parasite through the lymphatic route.

Acknowledgments

This work was supported by National Institutes of Health grants R01AI071300, R01DK084509, and K01DK076549.

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A

B

Fig. 1. Invasion of DC2.4 cells by *C. parvum* sporozoites as determined by FACS analysis
 DC2.4 cell monolayers in 6-well plates were pulsed with CFSE-labeled heat-killed or live *C. parvum* sporozoites at the indicated oocyst-to-cell ratios. Cells were harvested two hours later and analyzed by FACS. (A) Percentages of CFSE-positive cells incubated with live or heat-killed parasites at the indicated oocyst-to-cell ratios. Data were analyzed by two-way ANOVA and Bonferroni post-hoc correction * Indicates $P < 0.05$ and ** $P < 0.001$. (B) FACS histogram of CFSE-positive cell population (R1) in both heat-killed (left) and live (right) parasites. Data are representative of three independent experiments.

Fig. 2. Initial infection of DCs by *C. parvum* sporozoites induces actin-dense plaque formation in the host DC

DCs treated with *C. parvum* sporozoites for 1 or 2 hours were prepared for scanning and transmission electron microscopy (SEM and TEM). (A) SEM image showing attachment of a *C. parvum* sporozoite to the surface of DCs during initial infection. (B) TEM image of DC2.4 cells infected with *C. parvum* for 1 hour. An actin-dense plaque separating the host DC from the parasite can be seen at the site of infection (arrow; scale bar 0.2 μm). Data are representative of three independent experiments.

Fig. 3. Confocal and transmission electron microscopy analysis of BMDC and DC2.4 cells pulsed with *C. parvum* sporozoites

For confocal microscopy, BMDCs or DC2.4 cells were pulsed with *C. parvum* oocysts and cultured for 16 or 24 hours before harvesting. For transmission electron microscopy, DCs were treated with *C. parvum* for 1 or 2 hrs. Labeling of F-actin using Alexa 568-phalloidin or lipid using CM-Dil dye was performed to stain DCs for confocal microscopy experiments. CM-Dil labeled DC2.4 cells were pulsed with *C. parvum* sporozoites for 16 hours. Parasites were stained with monoclonal antibody JF13 against *C. parvum* sporozoites followed by Alexa 488-conjugated antibody. Nuclei were stained with DAPI. The data shown are representative of three independent experiments.

Fig. 4. DCs upregulate costimulatory molecules and cytokines after exposure to *C. parvum*

DCs were exposed to *C. parvum* for 24 hours. Cells were harvested and stained with fluorophore-conjugated antibodies against the indicated surface molecules and cytokines and then analyzed by FACS. (A) FACS analysis of the upregulation of costimulatory molecules CD40, CD80, and CD86 in BMDCs after treatment with *C. parvum* for 24 hours. (B) FACS analysis of IL-12, TNF- α , and IL-16 production by BMDCs after treatment with *C. parvum* for 24 hours. (C) FACS analysis of IL-12 and IL-6 production by DC2.4 cells treated with *C. parvum* for 24 hours. Gate R3 indicates *C. parvum* positive DCs. Data are representative of three independent experiments.

Fig. 5. Cytokine production by DCs occurs via TLR-dependent signaling pathways
 BMDCs from MyD88^{-/-} (A) or C3H/HeJ (B) mice were treated with medium, LPS, *C. parvum* or *E. bieneusi* for 24 hours. Cells were harvested and analyzed by FACS analysis (C) HEK293 cells expressing human TLRs (hTLR2-9) were treated with medium, specific ligands as positive controls, or *C. parvum* for 24 hrs and TLR activation was measured by SEAP reporter assay as described in Materials and Methods. Data are representative of three independent experiments.

Fig. 6. *C. parvum* antigens are transported to mesenteric lymph nodes as assessed by flow cytometry and immunohistochemistry

Mice were inoculated orally with *C. parvum* oocysts. (A, B) MLNs were collected 2 weeks later, and cells were stained with fluorophore-conjugated antibodies to B220, CD11b, CD11c, or CD8α to determine the phenotype of the MLN cells. *C. parvum* was stained with the mouse monoclonal antibody JF13 directed against *C. parvum* sporozoites followed by Alexa 488-conjugated antibody. (C, D) Optical microscopy images of immunohistochemistry staining of MLNs from mice infected with *C. parvum* (C) or PBS

control (D) (Scale bars: 20 μm). *Cryptosporidium* positive cells were determined by staining with rabbit anti-cryptosporidium polyclonal antibodies. Data are representative of two independent experiments.

Fig. 7. Live *C. parvum* parasites reach mesenteric lymph nodes in orally infected mice

RT-PCR and qPCR were used to determine whether live *C. parvum* parasites reach MLNs in orally infected mice. (A) Detection of *C. parvum* β -tubulin mRNA by RT-PCR in MLNs from orally infected mice. Lane 2, uninfected mice. A fragment of 18S rRNA was used as PCR positive control (arrow). (B) Hsp-105 mRNA was overexpressed in both oocysts and in vitro cultures after heat shock. (C) Hsp-105 mRNA overexpression from in vivo experiments. Lanes 3 and 4, uninfected mice. Detection of Hsp-105 mRNA overexpression in ileum (*C. parvum* target tissue) was used as positive control (lane 6). (D) Detection of live parasites in MLNs by qPCR. Relative quantification of gene expression was carried out using the comparative C_T method. Negative controls were included, and the relative target expression was normalized against the *C. parvum* 18S rRNA housekeeping gene. Data are representative of three independent experiments.