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MyD88-dependent pathway is essential for the innate immunity to *Enterocytozoon bienersi*

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Abstract

Enterocytozoon bienersi is clinically the most significant microsporidian parasite associated with persistent diarrhea, wasting and cholangitis in 30-50% of individuals with HIV/AIDS, as well as in malnourished children and in recipients of immunosuppressive therapy. However, the host immune responses to *E. bienersi* have not been investigated until recently due to lack of sources of spores, cell culture system, and animal models. In this study, we purified spores from heavily infected human or monkey feces by serial salt-Percoll-sucrose-iodixanol centrifugation and the purity of spores was confirmed by FACS and scanning electron microscopy. Exposure of dendritic cells to *E. bienersi* spores induced up-regulation of the surface markers and production of pro-inflammatory cytokines. The cytokine production was independent of Toll-like receptor 4, but MyD88-dependent, since dendritic cells from MyD88 knockout mice failed to secrete these pro-inflammatory cytokines, whereas dendritic cells from C3H/HeJ (a Toll-like receptor 4 mutant) were activated by *E. bienersi* and secreted these cytokines. Furthermore, MyD88 deficient mice were susceptible to *E. bienersi* infection, in contrast to wild type mice which resisted the infection. Collectively the data demonstrate innate recognition of *E. bienersi* by dendritic cells and the importance of MyD88-dependent signaling in resisting infection in a murine challenge model.

Keywords

Dendritic cells; *Enterocytozoon bienersi*; innate immunity; MyD88; TLR

Introduction

Enterocytozoon bienersi, a member of the Microsporidia group previously classified as protozoa, is now considered a fungus (25). The Microsporidia are obligate intracellular organisms and true eukaryotes containing a nucleus with a nuclear envelope, but lack mitochondria (42). The parasite life cycle involves a proliferating merogonic stage followed by a sporogonic stage that results in the formation of infective and environmentally resistant

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spores (4). *E. bienersi*, the clinically most important Microsporidia, has emerged as a serious human enteric opportunistic pathogen associated with HIV/AIDS (10,11,23,41) and in patients receiving immunosuppressive therapy (22), in whom it can lead to chronic diarrhea and wasting (13,27). *E. bienersi* is responsible for 30-50% of cases of chronic diarrhea in individuals with HIV/AIDS (11). Drug treatment against *E. bienersi* infection is only partly effective (19).

The host innate and adaptive immunity against *E. bienersi* remains unstudied because of a lack of reagents, sources of fungal material, *in vitro* culture system, and heavily infected animal models (18,40). Consequently, the understanding of the immune response to *E. bienersi* is based on observations of other human microsporidia such as *Encephalitozoon spp.* and *E. cuniculi* in particular. Only limited published information about cell-mediated immunity in microsporidiosis is available (12,31), and some reports suggest that CD8⁺ T cells are important for protection (6,26,28). The role and nature of the innate immunity against *E. bienersi* and other microsporidial infections remain unknown. Our recently published studies demonstrated the importance of IFN- γ in the initial resistance to *E. bienersi* infection in rodent models (18).

Dendritic cells (DCs) as antigen presenting cells (APCs) show a remarkable potency in initiating a host defense against a variety of pathogens. Parasite induced DC activation is important for the host innate and adaptive response to their invaders (34). DCs recognize and respond to a variety of microbial stimuli through their unique repertoire of pattern recognition receptors (PRRs) (24). An important class of PRRs are the Toll-like receptors (TLR), a family of conserved transmembrane cellular receptors that bear a homology to IL-1 receptor type 1 (IL-1RI) (14). The common signal pathways used by IL-1RI and most TLRs involve the recruitment of an adapter protein, myeloid differentiation factor 88 (MyD88) (38). MyD88, in turn, activates downstream signaling pathway and results in the production of proinflammatory cytokines and chemokines. Pathogen-derived molecule stimulation may lead to the upregulation of costimulatory molecules that are critical for the priming of T cells. Whether MyD88/TLR signaling pathway plays any role in *E. bienersi* infection is unknown.

In this study, we demonstrated that purified *E. bienersi* spores were capable of activating DCs by increasing the expression of costimulatory molecules and cytokine production. DC activation appeared to be MyD88 signaling-dependent but was independent of TLR4. We demonstrated that unlike wild type C57BL/6, MyD88 knockout mice were susceptible to *E. bienersi* infection.

Materials and Methods

Experimental animals

Six- to eight-week-old C57BL/6 and C3H/HeJ mice were obtained from the Jackson Laboratory (Bar Harbor, ME), and MyD88^{-/-} mice (1) from the University of Massachusetts Medical School (Worcester, MA). MyD88^{-/-} mice have been back bred onto a C57BL/6 background for 11-12 generations. Animals were housed at the animal facility of Tufts University Cummings School of Veterinary Medicine in accordance with the standards of the American Association of Laboratory Animal Care and Tufts University's Animal Care and Use Committee.

Purification of *E. bienersi* spores

E. bienersi spores were purified from fresh stools of infected adult humans or rhesus macaques (33,45). Briefly, the feces were homogenized and serially filtered through American standard sieves (pore sizes, 425, 180, 100 and 63 μ m; Newark Wire Cloth

Company, Newark, NJ). The spores were pelleted at 3200×g for 40 minutes and washed 4 times with distilled water (3200×g, 20 min). The pellet was mixed with saturated sodium chloride and centrifuged at 1000×g for 15 min. The middle layer was collected and washed before re-suspended in PBS. The sample was further purified using Percoll density gradient centrifugation, 30-60% (W/W) sucrose gradient centrifugation, and 10-50% (W/W) OptiPrep (Sigma, St. Louis, MO) gradient centrifugation. The purified spores were stored at 4 °C and subjected to purity identification.

Scanning electron microscopy (SEM)

Purified spores were fixed overnight in 2% glutaraldehyde in 0.1 M cacodylate buffer, rinsed several times in buffer and post fixed in osmium tetroxide (OsO₄) for 1 hour at 4°C. The fixed spores were applied to poly-L-lysine coated cover slips (0.1% for 10 minutes at RT). The cover slips were then washed in distilled water for 5 minutes followed by dehydration in a graded serial of ethanol. The samples were critical point dried with CO₂ and sputter coated with gold palladium, viewed and photographed with a JSM 6320F scanning electron microscope.

Flow cytometry analysis of the purity of spores

Purified spores (1×10⁶ in 50 µl of PBS) were incubated with monoclonal antibody 8E2 (45) followed by AlexaFluor-488 labeled goat anti-mouse IgG (Invitrogen, Carlsbad, California) staining. For carboxy-fluorescein diacetate succinimidyl ester (CFSE) (Invitrogen) staining (15), spores were incubated for 15 minutes in PBS containing 10 µM of CFSE. After extensive wash, cells were fixed with 2% paraformaldehyde in PBS and then analyzed using FACSCalibur and CellQuest software (BD Biosciences, Mountain View, California).

Dendritic cells and spore preparation

Mouse bone marrow-derived DCs (BMDCs) were generated by culturing bone marrow cells in a medium containing mouse recombinant GM-CSF and IL-4 (16,17), followed by positive immunomagnetic separation using CD11c antibody-coated microbeads (Miltenyi Biotec, Auburn, CA). The selected BMDCs were >98% CD11c⁺ DC by flow cytometry analysis. These DCs are CD11c⁺ and B220⁻, expressing high levels of CD11b and MHC class II and moderate levels of CD80 and CD86. Bone marrow-derived DC cell line DC2.4 (32) was provided by Kenneth Rock (University of Massachusetts Medical School, Worcester, MA). Cells were cultured in RPMI-1640 medium containing 2 mM L-glutamine, 100 units/ml penicillin, 50 µg/ml streptomycin, 50 µM β-mercaptoethanol and 10% fetal bovine serum unless otherwise indicated. *E. bieneusi* spores were directly added to DC culture and incubated for 24 hours. For heat treated *E. bieneusi*, spores in PBS were incubated at 65 °C for 30 min.

Flow cytometry to measure DC surface markers and cytokine production

Flow cytometry was performed using FACSCalibur and CellQuest software (BD Biosciences) with fluorochrome-conjugated antibodies to IL-6 (MP5-20F3), IL-12 (C15.6), TNF-α (MP6-XT3), CD11c (HL3), CD40 (3/23), and CD86 (GL1) (all from BD PharMingen, San Diego, CA). 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. Cells were harvested and incubated in 50 µL Fix/Perm buffer (BD PharMingen) at room temperature for 15 minutes. Saturated amounts of fluorochrome-conjugated antibodies were added in the permeabilization buffer (BD

PharMingen) and cells were incubated at room temperature for 15 minutes. Cells were washed and resuspended in FACS buffer with 1% formaldehyde for analysis.

Cytokine array assay

DCs were treated with medium, LPS (1 µg/ml), or *E. bieneusi* (10 spores/cell) for 24 hours. Cell supernatants were collected and a variety of cytokines and chemokines in DC culture supernatants were measured using cytokine array (RayBiotech, Inc., Norcross, GA) according to the manufacture's instructions. After developing the membranes, the densitometric analysis of individual dots was performed using SigmaGel software.

E. bieneusi in vivo infection

Age- and genetic background-matched 6 week-old wild type C56BL/6 and MyD88^{-/-} mice were orally inoculated with 10^{5~6} *E. bieneusi* spores (in 10 µl of PBS) per mouse. Mouse feces were collected three times a week and spore shedding was monitored using an indirect immuno-fluorescent assay (35).

Results

Purification of *E. bieneusi* spores

Using the same technique as previously described (23), spores were purified from human and from rhesus macaque feces, resulting in 99.5% pure spores as determined by scanning electron microscopy (SEM) (Figure 1). The purity of spores was confirmed by microbial culture with various agar plates (45) and was further confirmed by FACS analysis after antibody staining (data not shown). Under a low magnification (×3,500) of SEM, no bacteria were detected, except the spores measuring 1×1.5 µm (Figure. 1). FACS histogram also showed single peaks for both CFSE-labeled and monoclonal antibody stained spores (data not shown).

E. bieneusi treated BMDC up-regulates the expression of co-stimulatory molecules

DCs can be activated by pathogen-derived molecules and the activation is characterized by the upregulation of MHC and costimulatory molecules, and the production of cytokines and chemokines (3,30). To test the hypothesis that mouse BMDC can be activated with *E. bieneusi*, cells were treated with live or heat-treated *E. bieneusi* for 24 hours prior to harvesting. Cells were stained with fluorochrome-conjugated antibodies against surface markers. FACS analysis showed that *E. bieneusi* treatment enhanced DC expression of surface molecule CD40, with a mean fluorescent intensity (MFI) value of 15.2 and 15.04 for live and heat-treated *E. bieneusi* spores respectively, as compared with medium treatment (with MFI value as 4.73) (Figure 2A). *E. bieneusi* treatment also enhanced costimulatory molecule CD86 (B7.2) expression (Figure 2B). Live *E. bieneusi* spores stimulated greater CD86 expression on DCs (MFI = 554) than of heat-treated spores (MFI = 406). The MFI of both treatments however was much higher than that of medium only (MFI = 277) (Figure 2B). *E. bieneusi* treatment had similar effects on the upregulation of DC costimulatory molecules like LPS, a known DC-stimulatory ligand (Figure 2A and 2B).

E. bieneusi treatment enhances DC production of pro-inflammatory cytokines

We next examined whether *E. bieneusi* treatment induced the production of pro-inflammatory cytokines by mouse DCs. Figure 3A shows that *E. bieneusi* derived from both human and monkey induced IL-6 production by mouse dendritic cell line DC2.4, with 6.8% and 4.7% cells positively stained with PE-conjugated anti-IL-6 antibody. The positive control LPS treatment induced 4.3% cells to produce IL-6, whereas no IL-6 production in medium treated cells was observed (Figure 3A). *E. bieneusi* treatment also induced the

production of pro-inflammatory cytokines by BMDC from C57BL/6 mice (Figure 3B). Both heat-treated and live *E. bieneusi* stimulated a significant production of IL-12, IL-6, and TNF- α , as compared with control (Figure 3B). As expected, LPS treatment of BMDC also induced a significant upregulation of these cytokines (Figure 3B).

DC cytokine production induced by *E. bieneusi* is dependent on MyD88 signaling

DCs can be activated through pathogen-derived molecules, which bind to Toll-like receptors on DCs and trigger the downstream signaling (24). MyD88 is a common adaptor protein utilized by all of the known TLRs except the dsRNA receptor, TLR3, which plays a critical role in TLR signaling (36). To determine whether DC activation by *E. bieneusi* was MyD88 signaling dependent, BMDCs from MyD88^{-/-} mice were generated and treated with *E. bieneusi*. These DCs failed to produce either IL-6 or IL-12 when treated with *E. bieneusi* (Figure 4A) whereas *E. bieneusi* induced the production of both cytokines in DC-derived from C57BL/6 mice (Figure 3B). LPS did not induce the production of these cytokines by MyD88^{-/-} BMDCs either (Figure 4A), as LPS activates DCs through MyD88-dependent TLR4 signaling. Since *E. bieneusi* spores were purified from feces, we investigated whether DC activation was caused by possible contaminants such as LPS. BMDCs from C3H/HeJ mice were generated and treated with *E. bieneusi*. These mice lacked a functional TLR4 signaling and were non-responsive to LPS treatment. As expected, BMDCs from these mice failed to produce IL-6 or IL-12 after LPS treatment (Figure 4B). However, *E. bieneusi* was capable of inducing C3H/HeJ BMDCs to produce IL-6 and IL-12 (Figure 4B) to the similar level as C57BL/6 mouse BMDCs (Figure 3B).

We further measured a variety of inflammatory cytokines/chemokines produced by BMDCs using mouse cytokine array assay. This assay allows the detection of cytokines/chemokines in the culture supernatant. Figure 5 shows that *E. bieneusi* treatment stimulated the production of several cytokines/chemokines such as IL-6, IL-12, KC (CXCL1), and LIX (CXCL5) by C3H/HeJ BMDCs, but not by MyD88^{-/-} ones. LPS failed to induce detectable production of these cytokines/chemokines (Figure 5). RANTES production was expected in LPS-treated MyD88^{-/-} BMDCs, but not in DCs from C3H/HeJ mice (20). It is noteworthy that *E. bieneusi*-treated MyD88^{-/-} BMDCs also secreted RANTES similar to those treated with LPS, but *E. bieneusi*-treated C3H/HeJ BMDCs produced a distinct cytokine/chemokine profile compared with LPS-treated BMDCs. This data suggested that *E. bieneusi* treatment activated DCs, not by possible contaminant LPS, but through MyD88-dependent signaling pathway that was largely independent of TLR4.

MyD88^{-/-} mice are susceptible to *E. bieneusi* infection

Wild type mice are resistant to *E. bieneusi* infection (18), which may be due to their innate immunity. Since MyD88-dependent signaling plays an important role in the innate defense against infections, we examined whether MyD88^{-/-} mice were susceptible to *E. bieneusi* infection. From three independent experiments, 5 out of 7; 3 out of 5 (10⁵ spores/mouse for inoculation) and 8 out of 8 (10⁶ spores/mouse) MyD88^{-/-} experimentally infected mice excreted *E. bieneusi* spores respectively, whereas none of control C57BL/6 mice in each experiment had detectable spores in their feces (Figure 6). The shedding in MyD88-deficient mice lasted for three to four weeks with detectable spores in feces. A higher inoculation dose resulted in all 8 mice shed spores and the shedding period lasted longer (four weeks) (Figure 6). These data demonstrated that unlike C57BL/6 mice, MyD88^{-/-} mice were susceptible to the initial infection with *E. bieneusi*, although the infection was eventually resolved by as a yet unknown mechanism.

Discussion

E. bienewsi infection appears to induce clinical symptoms in the severely immunodeficient individuals, which indicates a crucial role of host immune defense against this pathogen. However, neither the components nor the mechanisms of immune defense against this particular organism have yet been investigated. In this first study on the immune response to *E. bienewsi*, we have demonstrated that this serious opportunistic pathogen is capable of activating DCs through a MyD88-dependent signaling pathway and MyD88^{-/-} mice are susceptible to *E. bienewsi* infection. These findings provide insight into the early mechanisms of host innate resistance to *E. bienewsi*, a useful knowledge to study specific aspects of the immune response to *E. bienewsi* infection and host/parasite interaction.

DCs are professional antigen presenting cells and play key roles in the host immune defense against pathogens (3). We have shown that the exposure of bone marrow derived DCs from C57BL/6 mice to *E. bienewsi* induces the upregulation of surface molecules CD40 and CD86. The interaction of CD40 and its ligand on B and T cells is important for their activation, whereas CD86-CD28 interaction provides costimulatory signal for the priming of naïve T cells (21). The exposure of DCs to *E. bienewsi* also induces the production of pro-inflammatory cytokines, such as IL-12, IL-6, and TNF- α , and some chemokines such as KC and LIX. The secretion of IL-12 stimulates NK cells and $\gamma\delta$ T cells to produce IFN- γ (5,34,39), and our previous study showed that IFN- γ was important in the initial resistance to *E. bienewsi* infection in rodent models (18). IL-6 relieves the suppressive effects of regulatory T cells (29). The role of TNF- α in the host resistance to parasitic infections is well documented (7,43). Therefore, our data suggests that the activation of DCs by *E. bienewsi* provide the initiation of innate resistance as well as shaped adaptive immunity against this infection.

The study of *E. bienewsi* interaction with the host immune system was until now greatly hampered due to the lack of sources of purified spores. The recent development of methods to concentrate and purify *E. bienewsi* spores from infected humans, monkeys or experimentally infected rodent feces (33,45) made such studies feasible. Propagation in rodents also helped establish animal models to investigate such interactions (18). SEM and FACS analysis showed that the purified spores displayed a single uniformed population. Since *E. bienewsi* spores were purified from feces, it was possible that the samples were contaminated with enteric bacterial endotoxin such as LPS, which is a potent activator of DCs through TLR4-dependent signaling. Although the function of the contaminated LPS can be ruled out by using BMDC derived from C3H/HeJ mice, it is uncertain that the RANTES production in BMDC from MyD88^{-/-} mice is triggered by *E. bienewsi* spores or contaminated LPS, which induces RANTES production via the TRIF-TRAM pathway (20). Our data has shown that *E. bienewsi* is capable of activating DCs derived from C3H/HeJ mice, which lack functional TLR4 signaling. This indicates that the activation of wild type DCs may be due to *E. bienewsi* but not LPS contaminant from feces.

MyD88 is an adaptor molecule essential for most TLRs as well as IL-1 and IL-18 signaling and plays a critical role in innate immunity (37). In the *in vitro* experiments, BMDCs from MyD88^{-/-} mice were shown to have defective pro-inflammatory cytokine responses after exposure to *E. bienewsi*. Further, unlike age- and genetic background-matched C57BL/6 mice, MyD88 knockout mice were susceptible to *E. bienewsi* infection. In addition to TLRs, the cytokine receptors IL-1R and IL-18R also signal through the MyD88 adaptor molecule and therefore are potentially involved in the MyD88-dependent host resistance to *E. bienewsi*. However, it is likely that these receptors do not play a major role, as a lack of IL-1 production after the DC exposure to *E. bienewsi* (data not shown). Therefore the additional receptors involved are likely to be TLRs. It is presently unclear which TLRs are involved in

the host defense against microsporidiosis. In addition, both heat killed and live spores induced cytokine production to the same extent (Fig. 3B), which suggests that the cytokine production from DCs stimulated by spores is triggered through the interaction of surface or endosomal component of DCs with spores, but not through DC cytosolic receptors. Taken together, the data demonstrate that the activation of DCs by *E. bienersi* depends on MyD88 signaling, which may play an important role in the resistance to this microsporidial infection.

Protozoan parasite glycosylphosphatidylinositol anchors are capable of activating TLR2-mediated signaling (2,8). TLR9 has been reported to recognize malaria pigment hemozoin (9) and TLR11 is raised as an important receptor for Apicomplexan protozoa profilin (44). Because of the microsporidial unique taxonomic position and their wide range of hosts from invertebrate to vertebrate including humans, this study warrants further investigations on TLR(s) or other receptors engaged by *E. bienersi* for DC activation.

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Figure 1. Scanning electron microscopy

A representative view of the purified spores under scanning electron microscopy. Note the uniform of *E. bienersi* spores with size of $1 \times 1.5 \mu\text{m}$

Figure 2. *E. bieneusi* treatment induces DCs to upregulate the expression of surface molecules
 BMDCs from wild type C57BL/6 mice were treated with medium (gray line), LPS (1 μ g/ml) (green line), heat-treated (HT, blue line), or live *E. bieneusi* (red line) (10 spores/cell) for 24 hours. Cells were harvested and the expression of DC surface molecules CD40 (A) and CD86 (B) was determined using fluorochrome-conjugated antibody staining and FACS analysis.

Figure 3. *E. bieneusi* stimulates the production of pro-inflammatory cytokines by DCs
 (A) DC2.4 cells were treated with medium, LPS, *E. bieneusi* derived from human or monkey fecal samples for 24 hours. (B) BMDCs from wild type mice were treated with medium, LPS, HT, or live *E. bieneusi* for 24 hours. Brefeldin A (20 μ M) was added in the final 12 hours of incubation to block cytokine extracellular transport. Cells were harvested and intracellular cytokine staining was utilized to measure the production of the indicated cytokines. The data showed one representative from three independent experiments.

Figure 4. The cytokine production of DCs induced by *E. bieneusi* is MyD88-dependent but independent of TLR4 signaling pathway

BMDCs were generated from MyD88^{-/-} (A) or C3H/HeJ (B) mice and treated with medium, LPS, or *E. bieneusi* for 24 hours. Cells were added with Brefeldin A to block cytokine secretion in the last 12 hours of incubation. Cells were harvested and IL-12 and IL-6 production were measured using intracellular cytokine staining.

Figure 5. BMDCs from wild type or C3H/HeJ but not MyD88^{-/-} mice secrete various cytokines/chemokines after *E. bieneusi* treatment

DC cell culture supernatants were collected and cytokines/chemokines were detected using mouse cytokine array assay. The percentage ratios of densitometric value of each cytokine/chemokine over positive control (POS) were determined. Open bar represents medium treatment; grey bar shows LPS (1 µg/ml) treatment and black bar shows *E. bieneusi* treatment. WT, CH, or MD represents BMDCs from wild type, C3H/HeJ, or MyD88^{-/-} mice respectively.

Figure 6. MyD88^{-/-} mice are susceptible to *E. bienersi* infection

The same background of C57BL/6 or MyD88 knockout mice were inoculated orally with *E. bienersi* spores (10^6 spores / mouse) and spores in the feces were detected with an indirect immuno-fluorescent assay using anti-*E. bienersi* antibody. The fecal smear slides were prepared and the number of shed spores in 30 microscopic high power fields (HPF) was counted. The number represents an average count of spores per mouse in each group at one time point from three monitors per week.