Optimal CD8 T-Cell Response against Encephalitozoon cuniculi Is Mediated by Toll-Like Receptor 4 Upregulation by Dendritic Cells^v

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CD8^+ T-cell immunity has been shown to play an important role in the protective immune response against Encephalitozoon cuniculi. Although earlier studies suggest that dendritic cells (DC) are important for the induction of this response, the factors responsible for initiation of the dendritic cell response against this pathogen have not been evaluated. In the current study, we demonstrate that E. cuniculi infection causes strong Toll-like receptor 4 (TLR4)-dependent dendritic cell activation and a blockade of this molecule reduces the ability of DC to prime an antigen-specific CD8^+ T-cell response. Pretreatment of DC with anti-TLR4 antibody causes a defect in both in vitro and in vivo CD8^+ T-cell priming. These findings, for the first time, emphasize the contribution of TLR4 in the induction of CD8^+ T-cell immunity against E. cuniculi infection.

Microsporidia are small obligate intracellular parasites that, until recently, were thought to be protozoans; however, evidence now suggests that they are related to fungi (15, 17). Microsporidia can infect a vast number of species of vertebrates and invertebrates; of the 150 genera of microsporidia, however, only 7 have been found to infect humans (13). Severe infections have been reported predominantly for immunocompromised patients, such as patients with HIV and organ transplant recipients (2, 7, 23, 37). Acute infections have also been reported in travelers and the elderly (26, 27), and there is evidence of colonization of healthy, nonsymptomatic patients (34).

Due to the prevalence of opportunistic microsporidian infections associated with the HIV-AIDS pandemic, recent research has focused on the host’s immune response to these pathogens. Early animal studies showed that cellular immunity was necessary to protect SCID mice from a lethal Encephalitozoon cuniculi challenge. Moreover, depletion of CD8^+ T cells caused mice to succumb to intraperitoneal (i.p.) E. cuniculi infection (21), and previous studies in our laboratory have shown that cytotoxic lymphocytes play a major role in protection against this effect (20, 21).

Recent reports from our laboratory have demonstrated that dendritic cells (DC) play an important role in the priming of the immune response against E. cuniculi (31, 32). T cells incubated with E. cuniculi-pulsed DC exhibited antigen-specific characteristics, specifically gamma interferon (IFN-γ) production, cytotoxicity, and proliferation (31, 32). In order to mount an immune response against a foreign pathogen, DC must first recognize the pathogen to initiate an appropriate response. One key method of recognition is through Toll-like receptors (TLRs), which were first discovered in Drosophila in response to infection with fungal pathogens (24). However, specific TLR molecules involved in DC activation during E. cuniculi infection have not been identified previously. We evaluated the upregulation of specific molecules involved in activation of the DC response after E. cuniculi infection. Different TLR molecules were tested, and TLR4 expression was found to be essential for induction of the optimal CD8^+ T-cell response by these cells.

MATERIALS AND METHODS

Mice. Six- to 8-week-old C57BL/6 mice were purchased from the National Cancer Institute (Frederick, MD). The animals were housed under Institutional Animal Care and Use Committee-approved conditions at the Animal Research Facility at The George Washington University (Washington, DC).

Parasites and infection. A rabbit isolate of E. cuniculi (genotype II), kindly provided by L. Weiss (Albert Einstein College of Medicine, Bronx, NY), was used throughout this study. The parasites were maintained by continuous passage in rabbit kidney (RK-13) cells, obtained from the American Type Culture Collection (Manassas, VA). The RK-13 cells were maintained in RPMI 1640 containing 10% fetal calf serum (FCS) (HyClone Laboratories, Logan, UT). Mice were infected via the intraperitoneal (i.p.) route with 2 × 10^6 spores/mouse. In vitro stimulation was performed using irradiated parasites (220 krad).

TLR expression by dendritic cells. Expression of TLR2, -4, and -9 by dendritic cells was assessed on various days postinfection (p.i.) (2, 4, and 6 p.i.) by performing a phenotypic analysis. Briefly, spleens were harvested, and this was followed by enzymatic (collagenase D and DNase I) and mechanical, disruption, allowing for DC separation. The cell suspension was labeled for CD11c, NK1.1, CD19, and TLR2 (eBioscience, San Diego CA) or TLR4 (BD Bioscience, San Jose CA) expression. Intracellular TLR9 expression was determined after permeabilization and fixation with FoxP3 staining buffer (eBioscience) and intracellular staining with anti-TLR9 antibody (eBioscience). Cells were acquired with a FACScalibur (BD Biosciences) and were analyzed with FlowJo (Tree Star, Inc., Ashland, OR).

TLR2, -4, and -9 messages were detected by real-time PCR according to standard protocol in our laboratory (45). Splenic DC were isolated according to a previously described protocol (45). Briefly, spleens were harvested as described above. A cell suspension was then labeled with anti-CD11c biotin-conjugated antibodies (eBioscience) and positively selected by magnetic purification using the manufacturer’s protocol (Stem Cell Technology, Vancouver, British Columbia, Canada). Positively selected cells were then labeled, and CD11c^-CD19^-NK1.1^- DC were purified using a cell sorter (FACSaria; BD Biosciences). RNA was isolated with an RNaseasy kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions and reverse transcribed with Moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen, Carlsbad, CA). Quantitative
real-time PCR was carried out using a MyIQ single-color real-time PCR detection system (Bio-Rad, Hercules CA) and each of the following primers at a concentration of 200 nM: 5mTLR2 (5'-GCCACCATTTTCCAGACT-3'), 3mTLR2 (5'-ACCTGCTGTGTTACACGCT-3'), 5mTLR4 (5'-GTCAGAGACATTGACA GAA-3'), 3mTLR4 (5'-GGCTCTCTGGCCGCTGG-3'), 5mTLR9 (5'-ACCTGAG CACCGCTCTTCA-3'), 3mTLR9 (5'-AGATATGATCGCGCAGGAA-3'), 5m-actin (5'-AGAGGGAATGCTGCTGAGC-3'), and 3m-b-actin (5'-CAATT AGTGATGACCTGGCCGT-3'). Amplification was performed with SYBR GreenER qPCR Supermix (Invitrogen) for TLR2 and TLR4 or with iQ SYBR Green Supermix (Bio-Rad) for TLR9 and consisted of 40 cycles of 95°C for 15 s and 60.5°C for 1 min, followed by melt curve analysis. A baseline value of 1.0 was established using DC from uninfected mice.

**Detection of intracellular IL-12.** To assess interleukin-12 (IL-12) production, splenic DC were isolated as described above. DC were plated at a concentration of 5 × 10^5 DC/well and incubated with monensin (BD Biosciences) overnight. On the following day, cells were stained for surface markers using anti-CD11c, anti-NK1.1, and anti-CD19 antibodies. The cells were then fixed and permeabilized using a Cytofix/Cytoperm kit (BD Biosciences). Cells were then labeled with anti-IFN-γ, and incubated with irradiated E. cuniculi and -KI67 antibodies. Cells were acquired with a FACSCalibur and were analyzed with FlowJo software.

**Immunoprecipitation.** Splenic dendritic cells from naive and day 4 infected mice were isolated as described above. Purified DC were then incubated with lysis buffer (20 mM Tris-HCl, 137 mM NaCl, 10% glycerol, 1% NP-40, 2 mM EDTA) for 20 min on ice. Supernatants were collected following centrifugation at 12,000 rpm for 10 min and incubated with myeloid differentiating factor 88 (MyD88)-F(ab')-1 antibody (Santa Cruz Biotechnology). The immunoprecipitated complex was separated on a precast 4 to 15% Tris-HCl gel and transferred to a polyvinylidene difluoride membrane (Bio-Rad). After overnight incubation with 5% dry milk in TBST (Tris-NaCl with 0.05% Tween 20), membranes were incubated with anti-mouse TLR4 antibody (1:4,000; Bio-science) for 1 h and then with rabbit IgG peroxidase-conjugated secondary antibody (1:160,000; Sigma Aldrich, St. Louis, MO). The hybridized bands were visualized by chemiluminescence using an ECL kit (Pierce).

**Expression of costimulatory molecules.** DC were purified as described above and plated at a concentration of 5 × 10^5 cells/well in a 96-well plate. The cells were then incubated with mouse IgG2a isotype control antibody (eBioscience) or rat IgG2a isotype control antibody (eBioscience) for 1.5 h before intracellular IL-12 staining (B and C). Experiments were performed twice, and the data are representative of the results of one experiment.

**RESULTS**

Splenic dendritic cell response following *E. cuniculi* infection. C57BL/6 mice were infected i.p. with 2 × 10^7 E. cuniculi spores. At different time points p.i. (days 2, 4, and 6), animals were sacrificed, spleens were harvested, and single-cell suspensions were prepared by using collagenase D and DNase I digestion. Cells were directly labeled for CD11c, CD19, and NK1.1 (A) or were incubated overnight with monensin before intracellular IL-12 staining (B and C). Experiments were performed twice, and the data are representative of the results of one experiment.

**Expression of costimulatory molecules.** DC were purified at a concentration of 5 × 10^5 cells/well and incubated with monensin (BD Biosciences) overnight. The next day, splenic TCRδ^-^ T cells were isolated from naive mice and added to the culture (5 × 10^5 cells/well). After 4 h of incubation, DC^-^ T-cell activation was examined using Ki67 and IFN-γ intracellular staining. Briefly, mouse DC^-^ T cells were added to the culture overnight according to the manufacturer’s protocol. Labeling of cells with anti-CD8a and anti-CD25 was followed by permeabilization using a Cytofix/Cytoperm kit (BD Biosciences). Cells were then labeled with anti-IFN-γ and anti-Ki67. Cells were acquired with a FACSCalibur and analyzed with FlowJo software.

**Adoptive transfer of *E. cuniculi*-pulsed DC.** DC were isolated, treated with anti-TLR4 antibody, and incubated with irradiated *E. cuniculi* as described above. The next day, splenic TCRδ^-^ T cells were isolated from naive mice and added to the culture (5 × 10^5 cells/well). After 4 h of incubation, DC^-^ T-cell activation was examined using Ki67 and IFN-γ intracellular staining. Briefly, mouse DC^-^ T cells were added to the culture overnight according to the manufacturer’s protocol. Labeling of cells with anti-CD8a and anti-CD25 was followed by permeabilization using a Cytofix/Cytoperm kit (BD Biosciences). Cells were then labeled with anti-IFN-γ and anti-Ki67. Cells were acquired with a FACSCalibur and analyzed with FlowJo software.

**Statistical analysis.** Statistical analysis of the data was performed using a two-tailed Student t test (33).
tion were noted (Fig. 2A). To further determine the TLR molecules involved in DC activation, the expression of these molecules by DC was measured by a flow cytometry assay. As shown in Fig. 2B, there was no change in the mean fluorescence intensity (MFI) for either TLR2 or TLR9 following infection. However, a significant increase in the TLR4 MFI at day 4 p.i. \((P \leq 0.0001)\) was noted. These observations suggest that splenic DC upregulate TLR4 in response to \(E. cuniculi\) infection.

**TLR4 expression is associated with MyD88.** Since activation of TLR4 by lipopolysaccharide (LPS) stimulation has been shown to act in both myeloid differentiating factor 88 (MyD88)-dependent and MyD88-independent pathways (12, 19), we sought to determine the involvement of MyD88 during \(E. cuniculi\) infection. Splenic DC were isolated at day 4 p.i., and the cell lysate was immunoprecipitated with anti-MyD88 antibody and the complex was then immunoblotted for TLR4 (C). Lane MW contains a protein ladder. Experiments were performed twice, and the data are representative of two experiments.

**In vitro TLR4 blockade reduces the expression of costimulatory molecules on DC.** Upregulation of costimulatory molecules and antigen presentation by DC are essential for induction of the optimal T-cell response (5). To further assess the role of TLR4 in the DC function, an *in vitro* blocking assay was performed. Splenic DC from naive mice were isolated and incubated with anti-TLR4 antibody or an isotype control antibody. Cells were stimulated overnight with irradiated \(E. cuniculi\) and stained for CD80, CD86, or MHC class II expression (A). Unstimulated cells are represented by the shaded histogram. The mean fluorescence intensity is presented next to each antibody treatment. IL-12 production by splenic DC was measured by intracellular staining after overnight treatment with monensin (B and C). Experiments were carried out twice, and the data are representative of one experiment.

**Reduced IL-12 production by DC after anti-TLR4 treatment.** DC are known to be an important source of IL-12, a cytokine reported to play an important role in the immune response against \(E. cuniculi\) (35). Moreover, data shown in Fig. 1 demonstrate that there was an increase in the frequency of IL-12-producing DC in response to \(E. cuniculi\) infection. Next, we determined the effect of TLR4 blockade on IL-12 production by DC. Splenic DC were purified and treated with anti-mouse TLR4 antibody or an isotype control, as described measured by flow cytometry. As shown in Fig. 3A, blocking TLR4 prior to \(E. cuniculi\) stimulation prevented upregulation of all of the costimulatory molecules mentioned above. To ensure that the stimulation through TLR4 was not due to LPS contamination, polymyxin B (an antibiotic that binds to and neutralizes LPS) was added to DC cultures prior to the \(E. cuniculi\) pulse. Addition of polymyxin B to the cultures did not alter the expression of costimulatory molecules, ruling out the possibility that LPS was involved (data not shown). These results demonstrate that TLR4 blockade prevents upregulation of costimulatory molecules (CD80, CD86, and MHC class II) on DC, which most likely impairs their functions.

**FIG. 2.** TLR4 upregulation after \(E. cuniculi\) infection is MyD88 dependent. Splenic DC were isolated at day 4 p.i. and analyzed for TLR2, TLR4, and TLR9 expression. TLR mRNA expression was normalized using \(\beta\)-actin mRNA levels (A). Relative expression was measured using the mean for each group and the formula for the relative transcript level: \(2^{-\Delta \Delta C_T}\), where \(C_T\) is the cycle threshold. DC were labeled with CD11c, CD19, NK1.1, and either TLR2, TLR4, or TLR9 antibodies (B). Splenic DC were lysed and immunoprecipitated with anti-MyD88, and the complex was then immunoblotted for TLR4 (C). Lane MW contains a protein ladder. Experiments were performed twice, and the data are representative of two experiments.

**FIG. 3.** Costimulatory molecule expression and IL-12 production are TLR4 dependent. Splenic DC were purified from naïve mice, plated, and incubated with anti-TLR4 (αTLR4) or isotype control antibody. Cells were stimulated overnight with irradiated \(E. cuniculi\) and stained for CD80, CD86, or MHC class II expression (A). Unstimulated cells are represented by the shaded histogram. The mean fluorescence intensity is presented next to each antibody treatment. IL-12 production by splenic DC was measured by intracellular staining after overnight treatment with monensin (B and C). Experiments were carried out twice, and the data are representative of one experiment.
above. Cells were pulsed with *E. cuniculi* overnight, and IL-12 was detected by intracellular staining the next day. As shown in Fig. 3B, the frequency of IL-12+ DC was significantly reduced after anti-TLR4 treatment compared to the results for cells treated with the isotype control (P = 0.006).

**Inhibition of TLR4 signaling suppresses effective T-cell priming.** The data presented above demonstrate that TLR4 blockade affects the upregulation of costimulatory molecules known to be critical for T-cell priming (5). To study the downstream effect of this blockade, we determined if prior treatment with anti-TLR4 antibody inhibits the ability of DC to initiate an optimal CD8+ T-cell response against *E. cuniculi* infection. Purified DC were isolated and treated with anti-mouse TLR4 or isotype control antibody, as described above. Following overnight stimulation with *E. cuniculi* spores, T cells were added to the cultures, and 96 h later, the response was measured by measuring IFN-γ, CD25, and Ki67 expression. As expected, an increase in the frequency of CD25+ IFN-γ+ CD8+ T cells was observed when DC were incubated with the isotype control (Fig. 4A). Conversely, prior treatment with TLR4 antibody significantly reduced the ability of these cells to prime the CD8+ T-cell response compared to that of cells treated with the isotype control (P = 0.0085) (Fig. 4A). Similarly, there was no significant increase in the percentage of CD8+ T cells (CD25+ Ki67+) when DC were treated with antibody to TLR4. These observations suggest that this molecule is important for priming the CD8+ T-cell immunity against *E. cuniculi*.

To further establish the importance of TLR4 signaling in the priming of the CD8+ T-cell response, adoptive transfer studies were performed. Anti-TLR4 antibody-treated DC were pulsed with *E. cuniculi* spores *in vitro* and subsequently transferred to naïve syngeneic mice. At day 6 posttransfer, the recipient animals were sacrificed, and splenic CD8 T cells were evaluated for IFN-γ, CD25, and Ki67 expression. As shown in Fig. 4B, recipient animals that received anti-TLR4 antibody-treated DC exhibited reduced T-cell priming compared to control animals, which received control antibody-treated DC. A signifi-
cantly lower percentage of IFN-γ-producing cells was observed for the CD25⁺ CD8⁺ T-cell population of these animals \((P = 0.0083).\) Similarly, the frequency of proliferating CD25⁺ CD8⁺ T cells was significantly reduced in the mice that received anti-TLR4 antibody-treated DC \((P = 0.00647)\) (Fig. 4B). These findings further establish that TLR4 upregulation by DC is essential for the CD8⁺ T-cell response against \textit{E. cuniculi} infection.

**DISCUSSION**

Our results show that TLR4 plays a major role in activation of splenic DC during \textit{E. cuniculi} infection, which results in initiation of a protective immune response. While increases in TLR4 transcript and surface protein expression on splenic DC were observed, the expression of other important TLRs (TLR2 and TLR9) remained the same. The data presented in this paper demonstrate that TLR4 expressed by DC signals through the MyD88 adapter molecule and that \textit{in vitro} blockade of this receptor reduces the expression of MHC class II costimulatory molecules, as well as IL-12p40 production by these cells. This significantly decreases the ability of DC to prime an optimal T-cell response against \textit{E. cuniculi}. These results emphasize the role of the TLR4 molecule in initiating immunity against this pathogen.

TLR signaling on DC is involved in the immune responses to numerous pathogens \((18, 36, 39).\) Specifically, TLR4 has been demonstrated to be one of the molecules stimulated in several infectious diseases \((3, 6, 8–10).\) Additionally, it has been shown that TLR polymorphism increases susceptibility to certain diseases. For instance, Mockenhaupt et al. found that African children with a TLR4 polymorphism were more susceptible to severe malaria infections, which showed the importance of this molecule in initiation of the immune response against \textit{P. falciparum} \((28).\) Furthermore, it has been reported that a mutation in TLR4 increased the risk of invasive meningococcal disease in infants less than 12 months old \((10).\)

The TLR molecules involved in the priming of the T-cell response during \textit{E. cuniculi} infection have not been well described. This pathogen has been reported to induce a less robust immune response in TLR9⁻/⁻ mice following oral infection \((16).\) The altered immune response in these mutant animals was attributed to enhancement of the regulatory T-cell response by commensal DNA in the intestinal tissues and/or to the inability of the mice to generate optimal effector T-cell immunity \((16).\) Recent \textit{in vitro} studies have demonstrated that \textit{E. cuniculi} infection upregulates the TLR2 message in human monocye-derived macrophages \((11).\) However, no increase in the TLR4 message was observed in this study \((11).\) Although a role for TLR2 in activation of DC during \textit{E. cuniculi} was not observed in our study, it is possible that TLR2 may be involved in activation of other antigen-presenting cells, like macrophages, as reported in previous studies \((3).\) Alternatively, it is very likely that TLRs involved in the responses to microsporidian infections in human and mouse macrophages or DC are different.

In the present study, we demonstrated that TLR4 signaling is important for generation of robust CD8⁺ T-cell immunity against \textit{E. cuniculi}. Interestingly, TLR4 blockade reduces IL-12 production by \textit{E. cuniculi}-pulsed DC. A role for IL-12 in induction of short-lived effector CD8⁺ T cells against intracellular parasitic infection has been demonstrated \((40).\) Thus, reduced IL-12 production by DC due to TLR4 blockade may contribute to the inability of these cells to induce an optimal CD8⁺ T-cell response.

As \textit{E. cuniculi} and other microsporidia are an ever-increasing problem for immunocompromised patients, an effective vaccination against these pathogens would be advantageous. This study demonstrated that TLR4 is involved in the priming of CD8⁺ T cells, which have been shown to be critical for protection against \textit{E. cuniculi} infection \((21, 29, 30).\) The novel feature of our observations is that they demonstrate the importance of TLR4 in elicitation of CD8⁺ T-cell immunity against \textit{E. cuniculi} infection, which has been reported to be critical for protection of hosts against this pathogen. These findings suggest the need to target the TLR4 molecule for developing immunotherapeutic reagents against \textit{E. cuniculi} and other microsporidian species, such as \textit{Enteroctozytozoon bieneusi}, which cause severe complications in HIV-infected individuals.

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