Rickettsiae Stimulate Dendritic Cells through Toll-Like Receptor 4, Leading to Enhanced NK Cell Activation In Vivo

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Adoptive transfer of Toll-like receptor (TLR) 4–stimulated dendritic cells (DCs) induces protective immunity against an ordinarily lethal rickettsial challenge, but the mechanism underlying this protection remains elusive. Therefore, we sought to determine the importance of TLR4 in early immunity to rickettsiae in vivo, particularly that conferred by TLR4-stimulated DCs. Rickettsial growth proceeded logarithmically in mice lacking TLR4 function, whereas in TLR4-competent mice rickettsial growth manifested a lag phase early, suggesting that TLR4 may initiate innate rickettsial immunity. TLR4-competent mice produced significant amounts of interferon (IFN)–γ on day 1 of Rickettsia conorii infection, which was associated with significant expansion of the population of activated NK cells. Moreover, NK cells from TLR4-competent mice produced significantly higher levels of IFN-γ and had greater cytotoxic activity than did those from TLR4-deficient mice. Last, adoptive transfer of rickettsiae-exposed, TLR4-stimulated DCs activated NK cells in vivo. Together, these data reveal an important role for DCs in recognizing rickettsiae through TLR4 and inducing early antirickettsial immunity.

Much of the attention that has been paid to the induction of innate immunity to pathogens by dendritic cells (DCs) has focused on the role played by Toll-like receptors (TLRs). DCs directly trigger NK cell function in vivo, and TLR-matured DCs can activate NK cells in vivo via cell-cell contact and cytokine production [1]. TLR ligation leads to DC production of interleukin (IL)–12, and IL-2 is produced by DCs after TLR4-mediated lipopolysaccharide (LPS) stimulation [2, 3]. Moreover, these DC-derived cytokines may enhance NK cell activity in vivo [4, 5]. Previously, we demonstrated that stimulation of DCs with either LPS or rickettsiae leads to kinetically similar IL-2 production in vitro, suggesting that rickettsiae may ligate TLR4. The importance was further supported by the observation that TLR4-stimulated DCs partially protect mice from lethal Rickettsia conorii challenge [6].

Owing to the obligately intracellular lifestyle of rickettsiae, cytotoxic T lymphocytes and production of nitric oxide (NO) after interferon (IFN)–γ and tumor necrosis factor (TNF)–α activation of endothelium are critical in immunity [7, 8]. However, we do not fully understand the role played by innate immunity in protection against rickettsiae. Early mobilization of NK cells may play a role in protection against rickettsial and other bacterial diseases. NK cell–derived IFN-γ production appears to play an important role in Shigella flexneri infection [9]. Rickettsial antigens have been shown to increase human NK cell cytotoxicity in vitro, and depletion of NK cells increases susceptibility to rickettsiae in mice [10, 11]. The spotted fever group rickettsiae, Rickettsia africae, and the related organism Wolbachia can induce cellular activation through TLR4 and TLR2, for which the natural ligands are LPS and peptidoglycan, respectively [12, 13]. Nevertheless, the early triggers of NK cell–mediated innate immunity to rickettsiae, particularly the significance of TLR ligation, have not been evaluated.
Stimulation of DCs with LPS causes production of IL-12p40 in vitro and, on transfer, NK cell mobilization in draining lymph nodes. LPS-stimulated DCs also induce NK cell proliferation in vivo [14]. TLR9 is important in NK cell activation. Mice unable to signal through TLR9 have reduced NK cell–derived IFN-γ, and TLR9 expression in DCs is important in inducing this activity [15, 16]. TLR9 ligation in DCs directly induces NK cell activity in vivo [17], and IL-12 is required for the in vivo activation of NK cells. Therefore, although TLR ligation in DCs may lead to NK cell activation in vivo, the importance of TLR4 ligation in DC-induced NK cell activation has not been conclusively demonstrated.

To determine the significance of TLR4 ligation in DCs in the context of rickettsial infection, we used C3H/HeJ mice that are defective in TLR4 signaling and unresponsive to LPS—hereafter, TLR4(LPS-d) mice—because of a single amino acid change in the cytoplasmic portion of TLR4 [18–20]. Previously, we demonstrated that TLR4(LPS-d) mice, because of their impaired adaptive immune responses, were more susceptible to lethal R. conorii infection than genetically related C3H/HeN mice, which have functional TLR4 responses [21]. Here, we further show that rickettsial growth in TLR4(LPS-d) mice proceeds logarithmically early in infection, whereas rickettsial growth experiences a lag phase in mice with TLR4 function, implying that TLR4(LPS-d) mice have impaired innate responses to rickettsiae. Additionally, we demonstrate that TLR4(LPS-d) mice have significantly lower levels of activated NK cells and serum IFN-γ. Last, we demonstrate that rickettsiae stimulate DCs through TLR4 and that transfer of TLR4-stimulated DCs induces NK cell activity in vivo. Therefore, TLR4 ligation in DCs is important in augmenting NK cell activity in vivo.

METHODS

Mice and infections. Male C3H/HeN (H-2k) mice (Harlan Sprague Dawley) and C3H/HeJ (H-2k) mice (Jackson Laboratories) between the ages of 6 and 12 weeks were housed under animal biosafety level 3, specific pathogen–free conditions according to a protocol approved by the Institutional Animal Care and Use Committee at the University of Texas Medical Branch. Mice were infected intravenously with $8 \times 10^3$ pfu, which was $\sim 1$ LD$_{50}$ in C3H/HeN mice [6, 21].

Rickettsia. R. conorii (Malish 7 strain; ATCC VR 613) was propagated in our laboratory in Vero cells from a 10% yolk sac stock ($1 \times 10^6$ pfu/150-cm$^2$ flask), as described elsewhere [6]. Rickettsiae were then purified by discontinuous density gradient centrifugation in Renografin [22]. Viable rickettsiae were collected in sucrose-phosphate-glutamate (SPG) buffer and stored at $\sim 80^\circ$C until use. Before use, rickettsiae were quantified by plaque assay.

Brain microvascular endothelial cell isolation. Isolation of mouse brain endothelial cells was adapted from protocols reported elsewhere [23, 24]. Briefly, whole brains from male C3H/HeN or C3H/HeJ mice were removed aseptically and briefly soaked with 70% ethanol to render leptomeningeal vessels non-viable. The fresh brains were stored in ice-cold Dulbecco’s modified Eagle medium (DMEM)/F12 medium containing 2% fetal calf serum (FCS) before homogenization. Brains were homogenized using a glass Dounce homogenizer and centrifuged. The resulting pellet was resuspended in 15% dextran (molecular weight, 70 kDa) and centrifuged for 10 min at 11,400 g and 4°C to remove myelin-containing cells. The pellet was washed once more in DMEM/F12 medium with 2% fetal bovine serum and incubated at 37°C for 1.5 h with constant agitation in medium containing 1 mg/mL collagenase/dispase, 10 U/mL DNAse I, and 0.147 mg/mL Na$_2$-tosyl-l-lysine chloromethyl ketone. After digestion, the crude microvessels were washed in medium and plated on rat-tail collagen–coated plates in growth medium containing DMEM/F12 medium, 10% FCS, 10% normal horse serum, 100 μg/mL endothelial cell growth supplement (Biomedical Technologies), 100 μg/mL heparin, and 3 μg/mL puromycin. After 3 days of incubation, the puromycin was removed from the culture medium, and the cultures then consisted of pure brain endothelial cells. The cells were maintained at 37°C in 5% CO$_2$.

NK cell cytotoxicity assay. Cytotoxicity was measured using the Live/Dead cell–mediated cytotoxicity kit for animal cells (Molecular Probes), and the percentage of cytotoxicity was assessed by flow cytometry, as described elsewhere [25]. Density gradient–enriched lymphocytes were mixed with DiOC$_{18}^+$-labeled target cells at effector-to-target cell ratios of 50:1, 25:1, and 12.5:1 in duplicate. Propidium iodide (PI) was added to culture medium to allow for the determination of Yac-1 cell death after incubation for 3 h at 37°C in 5% CO$_2$. The percentage of cytotoxicity was assessed by flow cytometry after collection of 3000 DiOC$_{18}^+$ events. Lysed (PI$^+$ and DiOC$_{18}^+$) and viable (DiOC$_{18}^+$ and PI$^-$) Yac-1 cells were identified by their dual- or single-positive staining. Total cytotoxicity was defined as the experimental percentage cytotoxicity minus the background percentage cytotoxicity observed in control Yac-1 cultures.

NK cell activation assay and determination of IFN-γ production. To quantify NK cell–derived IFN-γ production, splenocytes ($3 \times 10^6$) were cocultured with $1 \times 10^6$ Yac-1 cells in 24-well plates in 2 mL of complete RPMI 1640 medium for 18 h at 37°C. GolgiStop (BD Pharmingen) was added, followed by a 6-h incubation. The percentages of IFN-γ–positive NK cells were determined by flow cytometry.

Adoptive transfer of DCs. Bone marrow–derived DCs were stimulated with heat-killed R. conorii (MOI of 5), LPS (50 ng/mL), or SPG buffer diluted in complete Iscove’s modified Dulbecco’s medium (mock infected), as described elsewhere [6]. Cells were harvested 24 h after stimulation, washed 3 times in PBS, and resuspended at a concentration of $5 \times 10^6$ cells/mL. Mice were injected with a DC suspension ($5 \times 10^5$ cells/mouse)
Figure 1. Blunting of early rickettsial proliferation in vivo through a Toll-like receptor (TLR) 4–dependent mechanism. To determine the influence that TLR4 has on the kinetics of rickettsial growth in vivo, mice were infected and rickettsial titers were determined by quantitative polymerase chain reaction (PCR) analysis in brain (A) and lung (B) tissues. Data represent means ± SDs for 3 mice per time point. Primary brain microvascular endothelial cells (BMECs) obtained from TLR4(LPS-d) and TLR4-competent mice were infected in vitro with Renografin-purified *Rickettsia conorii*, and rickettsial titers were quantified by real-time PCR (C). After *R. conorii* infection, chemokine and cytokine production was determined by Bio-Plex assay (D). IL, interleukin; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; ND, not detected; TNF, tumor necrosis factor.

**Flow cytometry.** Phenotypic analysis of cells was accomplished by staining cell suspensions in fluorescence-activated cell sorter buffer, as described elsewhere [6]. The intensity of fluorescence was measured on a FACSCalibur flow cytometer (BD Biosciences) and analyzed using CellQuest (version 5.2.1; BD Biosciences) or FCS Express (version 3; De Novo Software) software.

**Bio-Plex assay for cytokine production.** Serum cytokine levels were quantified using the Bio-Plex system, a bead-based array for simultaneous detection of up to 23 individual cytokines (Bio-Rad). Cytokine concentrations were determined in duplicate wells. Data were collected and evaluated on a Bio-Plex analyzer and associated software.

**Real-time polymerase chain reaction (PCR) quantitation of rickettsial loads.** To determine bacterial loads, the brain, lungs, and spleen were harvested on days 1, 3, and 5 after infection, and 1-mm³ pieces of tissue were stored at −20°C until processing. For in vitro rickettsial quantitation, brain microvascular endothelial cells were collected 24, 48, and 72 h after infection and stored at −20°C until processing. Tissues were homogenized, and DNA was purified using the DNeasy Tissue Kit (Qiagen). Plasmids containing rickettsial gltA and murine Actb (actin, beta) PCR products were constructed using the TOPO 2.1 and TOPO 4 cloning kits, respectively. Rickettsial gltA was amplified using forward primer CS-5 (5′-GAGAGAAAT-TATATCCAAATGTTGAT-3′) and reverse primer CS-6 (5′-AGGGTCTTCGTGCATTTCTT-3′). Murine Actb was amplified using forward primer 5′-AGAGGGAAATCGTGCGTGAC-3′ and reverse primer 5′-CAATAGTGATGACCTGGCCGT-3′. Real-time PCR was performed using SYBR Green SuperMix, 1 µL of DNA, and primers (0.2 µmol/L) on an iCycler real-time PCR apparatus (Bio-Rad). Standard curves were generated using plasmids containing cloned PCR products. All PCRs were performed using the following protocol: 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, 50°C for 15 s, and 60°C for 15 s. Data are expressed as the average copy number of rickettsial gltA per 10,000 copies of murine Actb.

**Statistical analyses.** Data are expressed as means ± SEs or SDs, and the significance of differences between 2 series of results were determined using Student’s unpaired *t* test. Differences were considered significant at *P* < .05.

**RESULTS**

Logarithmic rate of rickettsial growth increase in mice with deficient TLR4 function. To examine the effect of TLR4 ligation and innate immunity on rickettsial infection, we determined the kinetics of rickettsial infection in vivo. After intravenous inoculation, increases in rickettsial titers occurred logarithmically in the brain and lungs of TLR4(LPS-d) mice, whereas mice with functional TLR4 responses controlled the initial rickettsial proliferation during the first 3 days, resulting in a lag phase of rickettsial growth early in infection (figure 1A and
1B). These data suggest that defective TLR4 signaling significantly delays initiation of immunity to rickettsiae.

Independence of enhanced resistance to rickettsial disease with respect to rickettsial growth and NO production in endothelial cells. The kinetic differences in rickettsial proliferation in vivo could be attributed to TLR4-mediated activation of innate immune responses or to TLR4 ligation in the vascular endothelium (main rickettsial target cells) that leads directly to an endothelial antirickettsial effect. To determine whether TLR4 ligation in vascular endothelium plays a role in rickettsial proliferation, we cultured primary brain microvascular endothelial cells in vitro to determine the rate of proliferation of rickettsiae in the absence of immune pressure. As shown in figure 1C, bacteria replicated logarithmically with comparable growth rates in endothelium obtained from TLR4(lps-d) and TLR4-competent mice, although at 24 h after infection TLR4-competent mice had significantly greater rickettsial loads than did TLR4(lps-d) mice (P < .05). These data argue that growth differences in vivo are due to early initiation of innate immunity that required cells other than endothelium.

NO production is an important innate effector response against rickettsiae in endothelium. Although TLR4 ligation is important in inducing NO production in macrophages, the role played by TLR4 ligation after rickettsial infection is unknown. To address this issue, we used R. conorii–infected primary endothelial cells obtained from TLR4(lps-d) and TLR4-competent mice. We did not observe significant differences in NO production, as judged by nitrite concentrations in supernatants after 48 h of infection in vitro (data not shown). Although R. conorii did not stimulate significant NO production in endothelium through a TLR4-dependent mechanism, we did observe that several cytokines and chemokines were produced in endothelium by a TLR4-dependent mechanism after rickettsial infection. Supernatants obtained from primary TLR4-competent but not TLR4(lps-d) brain microvascular endothelial cells 48 h after infection with R. conorii contained markedly elevated levels of IL-1α, monocyte chemoattractant protein 1, macrophage inflammatory protein 1α, RANTES, and TNF-α (figure 1D). Although TLR4 ligation does not play a direct role in limiting rickettsial growth, TLR4-dependent production of cytokines and chemokines in endothelium may cooperate with other immune effectors to induce antirickettsial immunity in vivo.

Correlation between elevated serum IFN-γ levels in R. conorii–infected TLR4-competent mice and NK cell activation. The comparable growth rates of rickettsiae in TLR4-deficient and TLR4-competent endothelial cells in vitro led us to examine TLR4-mediated activation of innate immune responses and the production of IFN-γ and TNF-α in vivo. On day 1 after infection with R. conorii, we detected significantly higher serum concentrations of IFN-γ in TLR4-competent mice (figure 2A). However, levels of IL-12p40 and IL-12p70 were not significantly elevated, although levels of IL-17 were marginally increased (P = .056) (figure 2A).

Our laboratory has previously shown that early IFN-γ production is largely due to activated NK cells and that IFN-γ and TNF-α are important in limiting rickettsial growth [8, 11, 26]. To test whether increased serum levels of IFN-γ on day 1 after infection in TLR4-competent mice were due to increased NK cell proliferation and activation, we used flow cytometry to determine the percentages, absolute numbers, and activation status of splenic NK cells during the course of infection. Although there was no significant increase in splenic NK cells on day 1 after infection (data not shown), NK cells obtained from TLR4-competent mice were more activated than those obtained from TLR4(lps-d) mice, on the basis of CD69+ phenotype (figure 2B). On day 3 after infection, TLR4-competent mice showed significant increases in the total numbers and percentages of splenic NK cells, compared with TLR4-defective mice (figure 2C). These results were in agreement with serum IFN-γ levels, suggesting the production of IFN-γ by activated NK cells at early stages of infection in TLR4-competent mice.

To confirm the role of NK cells, we determined their ability to produce IFN-γ after stimulation with Yac-1 cells in vitro. NK cells from both TLR4-competent and TLR4(lps-d)–infected mice produced markedly more IFN-γ than did those from uninfected mice; however, NK cells obtained from TLR4-competent mice on day 3 after infection produced significantly more IFN-γ than did those from TLR4(lps-d) mice (figure 2D). Together, these data suggest that activated NK cells contribute to the increased serum IFN-γ levels in infected TLR4-competent mice.

Impaired NK cell cytotoxicity in mice with defective TLR4 signaling. On days 1 and 3 after infection, NK cells obtained from R. conorii–infected TLR4-competent mice had significantly greater Yac-1 cell cytotoxicity than did those obtained from TLR4-deficient mice (figure 3). These data imply a direct correlation between resistance to rickettsial infection and NK cell functions (IFN-γ production and cytotoxic activity). However, they do not rule out TLR4-independent mechanisms in the induction of NK cell cytotoxic activity, because R. conorii–infected mice had significantly greater NK cytotoxicity than did uninfected control mice, regardless of ability to signal through TLR4.

DC activation of NK cells in vivo through a TLR4-dependent mechanism. Given that TLR4-competent mice have significantly greater NK cell activity after R. conorii infection and that adoptive transfer of TLR4-stimulated DCs could protect mice from an ordinarily lethal challenge [6], we speculated that TLR4-mediated signaling in DCs could lead to NK cell activation in vivo. To test this hypothesis, we stimulated bone marrow–derived DCs from TLR4(lps-d) and TLR4-competent mice with R. conorii in vitro. At 24 h after stimulation DCs were injected into the footpads of TLR4-competent mice, and splenocytes were harvested 24 h after cell transfer to determine splenic NK cell activity via Yac-1 cell cytotoxicity. As shown in figure 4, DCs capable of signaling through TLR4 induced significantly greater levels of NK cell cytotoxicity after R.

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conorii infection than did the counterpart DCs derived from TLR4(LPS-d) mice. These data suggest that TLR4-dependent, DC-mediated NK cell activation occurs during R. conorii infection in vivo.

**DISCUSSION**

These data provide evidence that DCs recognize rickettsiae through TLR4 and promote NK cell activation in vivo (figure 5). Additionally, we have demonstrated, for the first time, that TLR4 stimulation is important both in the initiation of antirickettsial innate immunity (namely, the expansion of the NK cell population) and in the expansion of the Th1 cell population [21]. TLR4-ligated DCs induce recruitment of NK cells to draining lymph nodes [6, 14]; furthermore, this recruitment and the production of NK cell–derived IFN-γ in draining lymph nodes are important in augmenting the Th1 immune response [27–29]. Our results also argue strongly for an important role for TLR4-

![Figure 2](image-url)  
**Figure 2.** Inducement of significant activation of NK cells in vivo by rickettsiae through a Toll-like receptor (TLR) 4–dependent mechanism. Serum cytokine concentrations were determined in TLR4-competent and TLR4(LPS-d) mice on day 1 after infection with Rickettsia conorii (A). Splenocytes were obtained from rickettsiae-infected mice, and the percentages of activated NK cells (CD69+; DX5+, CD3−) (B) and total NK cells (C) were determined by flow cytometry. Splenocytes from TLR4(LPS-d) and TLR4-competent mice were obtained on day 3 after infection (D). Splenocytes from uninfected and R. conorii–infected mice were stimulated in vitro with Yac-1 cells, and IFN-γ production was assessed by flow cytometry. Bar graphs represent means ± SDs for 3 mice per time point. IFN, interferon; IL, interleukin. *P < .05.

![Figure 3](image-url)  
**Figure 3.** Significantly greater NK cell cytotoxicity in Toll-like receptor (TLR) 4–competent mice. Splenocytes were obtained from Rickettsia conorii–infected TLR4(LPS-d) or TLR4-competent mice or from mock-infected control mice. Splenocytes were cocultured with DiOC-labeled Yac-1 cells, and the percentage of killing was determined by flow cytometry. *P < .05.
induced innate immunity. After R. conorii infection in TLR4(LPS-d) mice, rickettsial growth proceeded immediately into the logarithmic phase, in contrast to the lag phase observed in mice possessing functional TLR4. However, we found that rickettsial proliferation rates and NO production were comparable in primary brain microvascular endothelial cells derived from TLR4(LPS-d) and TLR4-competent mice. These data therefore suggest that the synergistic action of IFN-γ and TNF-α in inducing rickettsicidal NO in endothelium may be more important than TLR4-mediated NO production in rickettsiae-infected endothelial cells.

Importantly, endothelial cells do produce significant levels of TLR4-dependent proinflammatory cytokines. Previous research had shown that rickettsiae-infected endothelial cells did not produce significant amounts of TNF-α. However, those studies were performed with human umbilical vein endothelial cells, which do not contain significant quantities of TLR4; therefore, our results suggest that endothelium may play a greater role in the immune response than previously thought. Of note, we have observed significant production of NO in cultured endothelium in response to Rickettsia rickettsii, via a TLR4-dependent mechanism (M.E.W. and J.M.J., data not shown). Thus, different rickettsial organisms may differentially ligate TLR, leading to differences in immune responses and pathogenesis. These findings are biologically important, because R. rickettsii is more pathogenic than R. conorii, with a significantly higher case fatality rate [30, 31].

It has been documented recently that TLR4 stimulation is also important in inducing NK cell activation in vivo [32]. Consistent with this report, we demonstrated that the quantity and percentage of NK cells in spleen were consistently lower in TLR4(LPS-d) mice than in TLR4-competent mice after R. conorii infection. Additionally, splenocytes from TLR4(LPS-d) mice had less NK cell cytotoxic activity than did those from TLR4-competent mice in vitro. Moreover, TLR4-competent mice also
had significantly higher serum levels of IFN-γ during early infection, and subsequent investigation demonstrated that NK cells in TLR4-competent mice produced significantly more IFN-γ after stimulation in vitro. Given these data, we suggest that this IFN-γ production is important in inducing early NO production in infected endothelial cells (figure 5). NO production is probably an important factor leading to the lag phase of rickettsial growth observed in TLR4-competent mice. In sum, the present study indicates that TLR4 ligation is an important step in limiting early rickettsial proliferation by activating NK cell cytotoxicity and IFN-γ production and that TLR4 signaling plays a significant role in both innate and adaptive protective immunity against R. conorii infection.

References