

Campylobacter jejuni-Induced Activation of Dendritic Cells Involves Cooperative Signaling through Toll-Like Receptor 4 (TLR4)-MyD88 and TLR4-TRIF Axes[∇]

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Received 23 December 2008/Returned for modification 28 January 2009/Accepted 25 March 2009

Campylobacter jejuni is an important cause of human enteritis and has been linked to the development of autoimmune diseases. Recently we showed that infection of murine dendritic cells (DCs) with *C. jejuni* resulted in DC activation and induction of *Campylobacter*-specific Th1-effector responses. Toll-like receptor (TLR) signaling through myeloid differentiation factor 88 (MyD88) and/or Toll-interleukin 1 (IL-1) receptor domain-containing adaptor-inducing beta interferon (IFN- β) (TRIF) is critical in inducing immunity against pathogens. In this study, we investigated the role of TLR2, TLR4, MyD88, and TRIF signaling in *C. jejuni*-induced inflammatory activation of DCs. DC upregulation of major histocompatibility complex class II and costimulatory molecules after *C. jejuni* challenge was profoundly impaired by TLR2, TLR4, MyD88, and TRIF deficiencies. Similarly, *C. jejuni*-induced secretion of IL-12, IL-6, and tumor necrosis factor alpha was significantly inhibited in TLR2^{-/-}, TLR4^{-/-}, MyD88^{-/-}, and TRIF^{-/-} DCs compared to that in wild-type DCs; however, the magnitude of inhibition was greater in MyD88^{-/-}, TRIF^{-/-}, and TLR4^{-/-} DCs than in TLR2^{-/-} DCs. Furthermore, *C. jejuni* induced interferon regulatory factor 3 phosphorylation and IFN- β secretion by DCs in a TLR4-TRIF-dependent fashion, further demonstrating activation of this pathway by *C. jejuni*. Importantly, TLR2, TLR4, MyD88, and TRIF deficiencies all markedly impaired the Th1-priming ability of *C. jejuni*-infected DCs. Thus, our results show that cooperative signaling through the TLR4-MyD88 and TLR4-TRIF axes represents a novel mechanism mediating *C. jejuni*-induced inflammatory responses of DCs. To our knowledge, such a mechanism has not been demonstrated previously for an intact bacterium.

Campylobacter jejuni is an important human pathogen that causes food-borne diarrheal illness worldwide (6). *C. jejuni* is also one of the main causative agents of traveler's diarrhea (11). The most common presentation of *C. jejuni* infection is self-limiting enteritis characterized by mild watery to bloody diarrhea, while extraintestinal, severe, and persistent diseases, including septicemia and meningitis, can occur in immunocompromised patients. Most importantly, autoimmune neuropathies, including Guillain-Barré syndrome, have been associated with antecedent *C. jejuni* infections (46).

It is evident that *C. jejuni* infection results in various disease outcomes, although the immunological contribution to this phenomenon is poorly understood. We have recently shown that C57BL/6 interleukin 10^{+/+} (IL-10^{+/+}) and congenic IL-10^{-/-} mice can be used as *C. jejuni* colonization and enteritis models, respectively (25). Immunohistochemical examination of *C. jejuni*-infected intestinal tissues in our mouse models (25, 26) and from human patients (45) revealed that *C. jejuni* colonizes epithelial crypts of the intestine and invades the lamina propria. Dendritic cells (DCs) are the primary cell type in-

involved in immunosurveillance of mucosal tissues and play an instructive role in the development of appropriate T-cell responses to pathogens (27). DCs underlying the epithelial barrier are likely to encounter *C. jejuni* either in the lamina propria, as it transcytoses the barrier, or intraluminally when DCs extend their processes across the epithelium to sample the lumen (32). We found that following *C. jejuni* infection, DCs from C57BL/6 mice undergo maturation, secrete IL-12 and proinflammatory cytokines, and induce *Campylobacter*-specific Th1 effector-cell responses (38). However, the molecular mechanisms underlying the DC-mediated induction of adaptive immune responses to *C. jejuni* are not known.

DCs express a broad repertoire of Toll-like receptors (TLRs) for sensing pathogens and regulating expression of critical signals—including costimulatory molecules and cytokines—in response to pathogens, thereby controlling the development of adaptive immune responses (21). TLR2 and TLR4 have been shown to be critical for host innate and acquired immune responses to a variety of bacterial pathogens (17, 44). Furthermore, a relatively higher proportion of DCs in the colonic lamina propria express TLR4 (28). Previous studies have shown that *C. jejuni* possesses predicted ligands for TLR2 and TLR4, such as surface lipoprotein (JlpA) and lipooligosaccharide (LOS), respectively (24, 30). Moreover, the role of TLR2 and TLR4 in the recognition of *C. jejuni* becomes more important considering that TLR5, another crucial TLR that

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[∇] Published ahead of print on 30 March 2009.

recognizes bacterial flagellin, has been shown to play a very limited role in *C. jejuni* detection (2). Finally, *C. jejuni* DNA that can signal through TLR9 was found to be dispensable for eliciting IL-8 secretion from intestinal epithelial cells (IEC) (51).

Following ligand binding, TLRs initiate intracellular signaling cascades through cytoplasmic adaptor molecules, which ultimately result in activation of various transcription factors, including NF- κ B (1). MyD88 is an important adaptor, mediating signals from most TLRs. Several lines of evidence suggest a critical role for MyD88 signaling in host resistance to microbial infections (31). It has recently been demonstrated that MyD88 signaling is essential for IL-8 production by cultured IEC after infection with *C. jejuni* (51). Another adaptor molecule, TRIF, is known to mediate MyD88-independent signaling from TLR4 and TLR3 in response to lipopolysaccharide (LPS) and double-stranded RNA, respectively (1), but its contribution to immune responses against bacterial pathogens is just beginning to be understood. Recent studies have shown that TRIF is essential for the pulmonary host defense against gram-negative pathogens, such as *Pseudomonas aeruginosa* and *Escherichia coli* (23, 36). However, the contribution of MyD88 and TRIF signaling to DC responses against enteric pathogens, such as *C. jejuni*, has not been investigated.

Based on the accumulating evidence on the critical role of TLR signaling in microbial infections and on the presence of potential TLR2 and TLR4 agonists in *C. jejuni*, we hypothesized that *C. jejuni*-induced activation of bone marrow-derived DCs (BM-DCs) is mediated by TLR2 and TLR4 and that MyD88 and/or TRIF signaling is necessary for this activation. In this study, we show that maturation, IL-12 secretion, and Th1-priming ability of *C. jejuni*-infected BM-DCs depend on TLR2 signaling through MyD88 and TLR4 signaling through both MyD88 and TRIF adaptor molecules. To our knowledge, this is the first report demonstrating cooperation between MyD88-dependent and -independent arms of the TLR4 pathway in mediating immune responses against an intact bacterial pathogen. We also delineate the type I interferon induction occurring in response to *C. jejuni* and signaling mechanisms involved in this pathway.

MATERIALS AND METHODS

Mice. C57BL/6J, TLR2^{-/-} mice on the C57BL/6 background, and C3H/HeJ (TLR4^{-/-}) mice purchased from the Jackson Laboratory were bred and maintained as described previously (3, 25). C3H/HeOJ (TLR4^{+/+}) mice were purchased from the Jackson Laboratory. MyD88^{-/-} and TRIF^{-/-} mice on the C57BL/6 background were kindly provided by Shizuo Akira. Mice were used at 8 to 13 weeks of age. Experiments using DCs from C57BL/6 genetic background mice (wild type [WT], TLR2^{-/-}, MyD88^{-/-}, and TRIF^{-/-}) were conducted concurrently and separately from experiments using DCs from C3H/HeOJ TLR4^{+/+} and C3H/HeJ TLR4^{-/-} mice. Animal protocols were approved by the Michigan State University Institutional Animal Care & Use Committee and conformed to NIH guidelines.

Bacterial inoculum preparation. *C. jejuni* 11168, originally isolated from the feces of a diarrheic patient, was obtained from the American Type Culture Collection (Manassas, VA). The *C. jejuni* 11168 inoculum was prepared as described previously (38). In all experiments described in this study, BM-DCs were infected with *C. jejuni* at a multiplicity of infection of 100 bacteria per BM-DC, which was previously determined to be the optimal dose (38).

Generation of BM-DCs and assessment of maturation and cytokine secretion by *C. jejuni*-infected BM-DCs. BM-DCs were generated from WT, TLR2^{-/-}, TLR4^{-/-}, MyD88^{-/-}, and TRIF^{-/-} mice as described previously (38). Briefly, 2.5 × 10⁶ bone marrow cells from femurs and tibiae were cultured in bacterio-

logical-grade petri dishes in 10 ml of R10 medium (RPMI 1640 medium Dutch modification [Sigma-Aldrich, St. Louis, MO] supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin [Invitrogen, Grand Island, NY], and 20 ng/ml murine granulocyte-macrophage colony-stimulating factor [Peprotech, Rocky Hill, NJ]). DCs were collected and used on day 9. BM-DCs diluted at a density of 2 × 10⁵ or 3 × 10⁵ cells/ml of R10 medium without antibiotics were placed in a 6-well plate for maturation experiments or a 24-well plate for cytokine experiments and were treated with *C. jejuni*, medium alone (negative control), or 0.1 μg/ml *Salmonella enterica* serovar Typhimurium LPS (positive control; Sigma-Aldrich). One h after infection, gentamicin (250 μg/ml) was added to all wells to kill extracellular bacteria. At 24 h postinfection (p.i.), DCs were stained with fluoro-chrome-conjugated antibodies specific for cell surface markers, such as major histocompatibility complex class II (MHC-II), CD80, CD86, and CD40 and analyzed by flow cytometry as described previously (38). For cytokine analysis, culture supernatants were collected at 24 h p.i. and stored in aliquots at -80°C until further analysis. Previous studies showed that 24 h p.i. is the optimal time point to analyze cytokine responses from *C. jejuni*-infected DCs (38).

TLR2 assay. HEK293 cells that are stably transfected with human TLR2, CD14, and a NF- κ B-inducible reporter system, secreted alkaline phosphatase (HEK-Blue-2 cells; InvivoGen, San Diego, CA), were cultured according to the manufacturer's instructions. HEK-Blue-2 cells in 96-well cell culture plates (5 × 10⁴ cells/well) were treated with medium alone, *C. jejuni*, or 100 ng/ml Pam3CSK4 (synthetic lipoprotein; InvivoGen). After 1.5 h of infection, gentamicin (250 μg/ml) was added to all wells, and at 23 h p.i., 160 μl of supernatant was added to 40 μl of QUANTI-Blue (InvivoGen) to detect secreted alkaline phosphatase in the supernatant. After 30 min of incubation, plates were read at 630 nm using a Bio-Tek EL-800 Universal plate reader (Bio-Tek Instruments, Winooski, VT).

ELISA. Cytokines in culture supernatants were measured using Ready-SET-Go! enzyme-linked immunosorbent assay (ELISA) sets (eBioscience, San Diego, CA) according to the manufacturer's instructions. Assay detection limits (in pg/ml) were as follows: for IL-12, 15; for IL-6, 4; for tumor necrosis factor alpha (TNF- α), 8; for gamma interferon (IFN- γ), 0.8.

Beta interferon (IFN- β) ELISA was performed as described previously (48) with modifications. Nunc-Immuno MaxiSorp 96-well plates (Nalge Nunc International, Rochester, NY) were coated overnight at 4°C with 100 μl/well of 1 μg/ml anti-IFN- β monoclonal antibody (7F-D3; Abcam, Cambridge, MA), followed by blocking for 1 h with assay diluent (eBioscience, San Diego, CA) and addition of 100 μl/well of IFN- β standard (PBL Interferon Source, Piscataway, NJ) or samples. After overnight incubation at 4°C and washing, 100 μl/well of 40 U/ml rabbit anti-IFN- β polyclonal antibody (PBL Interferon Source) was added. After 1 h of incubation at room temperature and washing, 100 μl of a 1:10,000 dilution of 0.8 mg/ml goat anti-rabbit immunoglobulin G-horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA) was added per well and incubated for 1 h at room temperature. Plates were washed, and 100 μl of 3,3',5,5'-tetramethylbenzidine (eBioscience, San Diego, CA) was added per well. After 15 min, 100 μl of 2N sulfuric acid was added per well to stop the reaction, and the plate was read at 450 nm using a Bio-Tek EL-800 Universal plate reader (Bio-Tek Instruments, Winooski, VT). The sensitivity of the assay was 7 pg/ml.

Q-PCR. Total RNA was extracted at 2.5 and 5 h posttreatment using an RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. cDNA was synthesized from 1 μg of total RNA using the iScript Select cDNA synthesis kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Quantitative real-time PCR (Q-PCR) for IFN- β (10) and β -actin (34) was performed as described previously in a final volume of 25 μl containing 12.5 μl of 2× iQ SYBR green supermix (Bio-Rad), 2 μl of 1:5 dilutions of cDNA, and 0.3 or 0.625 μl of primers (0.25 μM) in a Bio-Rad iQ5 iCycler. The change in expression was calculated according to the method of Pfaffl (35). Cycle threshold values of IFN- β were normalized to that of β -actin. Transcript levels (threshold cycle values) of β -actin were very similar between cells treated with medium alone and *C. jejuni*-infected cells.

Western blotting. BM-DCs were lysed in lysis buffer (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl) containing 1% Triton X-100 with phosphatase and protease inhibitors. The protein concentration in the lysate was determined using the bicinchoninic acid protein assay kit (Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions. Equivalent amounts of protein samples were run on 10% polyacrylamide gels and transferred onto nitrocellulose membranes. Blots were then probed with anti-IRF-3 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-phospho-IRF-3 (Ser396; 4D4G; Cell Signaling Technology, Inc. Danvers, MA), or antitubulin (Sigma-Aldrich) antibodies. Blots were washed and subsequently probed with fluorescently labeled anti-mouse (Rockland Immunochemicals, Inc., Gilbertsville, PA) or anti-rabbit (In-

vitrogen) secondary antibodies. Blots were scanned using a LI-COR Odyssey scanner (LI-COR Biotechnology, Lincoln, NE).

DC-CD4⁺ T-cell coculture. DC-CD4⁺ T-cell coculture experiments were performed as described previously with minor modifications (38). Briefly, highly enriched populations of CD4⁺ T cells ($\geq 93\%$; data not shown) were purified from spleens of WT mice by negative selection using the BD IMag mouse CD4 T-lymphocyte enrichment set-DM (BD Pharmingen), which contains the following antibodies: anti-mouse CD8a (clone 53-6.7); anti-mouse CD11b (clone M1/70); anti-mouse CD45R/B220 (clone RA3-6B2); anti-mouse CD49b (clone HM α 2); and anti-mouse TER-119/erythroid cells (clone TER-119). BM-DCs seeded in 24-well plates (10^5 cells/well) were treated with either *C. jejuni* or medium alone. One h after infection, gentamicin (250 μ g/ml) was added to all wells. At 24 h p.i., CD4⁺ T cells were added to DCs at a DC-to-T-cell ratio of 1:6. After coculturing for 72 h, supernatants were collected for measurement of IFN- γ .

Statistical analysis. One-way or two-way analysis of variance followed by the Holm-Sidak correction for multiple comparisons was performed using SigmaStat 3.1 (Systat Software, San Jose, CA). *P* values of ≤ 0.05 were considered significant.

RESULTS

***C. jejuni*-induced BM-DC maturation and IL-12 production are dependent on TLR2 and TLR4 signaling.** To determine the role of TLR2 and TLR4 in mediating DC maturation and cytokine secretion in response to *C. jejuni*, BM-DCs cultured from WT, TLR2^{-/-}, and TLR4^{-/-} mice were infected with *C. jejuni* and responses were assessed by flow cytometry and ELISA, respectively, at 24 h p.i. As we have shown previously, infection with *C. jejuni* induced significant ($P \leq 0.05$) upregulation of surface expression of maturation markers in WT DCs (38). In contrast, the mean fluorescence intensities—indicating cell surface expression levels—of the MHC-II, CD40, CD80, and CD86 markers and the proportion of cells expressing these markers were significantly ($P \leq 0.05$) lower in TLR4^{-/-} DCs than in WT DCs following *C. jejuni* challenge (Fig. 1A and B). TLR2 deficiency also significantly ($P \leq 0.05$) reduced the upregulation of MHC-II, CD80, and CD86 subsequent to *C. jejuni* challenge (Fig. 1C and D). However, the *C. jejuni*-induced increases in CD40 expression levels and in the percentage of cells with CD40 on their surface were comparable between TLR2^{-/-} DCs and WT DCs, in contrast to that observed in TLR4^{-/-} DCs. These results suggest a selective role for TLR2 in *C. jejuni*-induced upregulation of costimulatory surface proteins. We also observed that *Salmonella* LPS-induced responses were reduced in TLR2^{-/-} DCs relative to those in WT cells. This may be due to the contamination of this particular commercial preparation of LPS with certain TLR2 ligands from bacteria (1, 16) or LPS itself signaling through TLR2, besides the expected TLR4, as reported previously (43).

We also found that TNF- α and IL-6 secretory responses (referred to as secretion in the rest of this article), were significantly ($P \leq 0.05$) impaired by severalfold in TLR4^{-/-} DCs 24 h following *C. jejuni* infection (Fig. 2A). In contrast, *C. jejuni*-induced TNF- α and IL-6 production was only minimally reduced in TLR2^{-/-} DCs compared to that in WT DCs (Fig. 2B). Most importantly, *C. jejuni*-induced IL-12 secretion was completely abolished in TLR4^{-/-} DCs but was only halved in TLR2^{-/-} DCs (Fig. 2A and B). Overall, these data clearly show that the contributions of TLR2 and TLR4 signaling to maturation of DCs after *C. jejuni* infection were comparable and TLR4 plays a major role in relation to TLR2 in inducing cytokine responses.

Since TLR2 seemed to play a less-significant role than TLR4

in mediating DC cytokine responses to *C. jejuni*, we sought to confirm the role of TLR2 in *C. jejuni* infection further. We used the HEK-Blue-2 cell line, which is a HEK293 cell line stably transfected with human TLR2, CD14, and an NF- κ B-inducible reporter system (secreted alkaline phosphatase), to assess the ability of *C. jejuni* to activate NF- κ B via TLR2. This system allows specific assessment of TLR2 stimulation by monitoring NF- κ B activation through the level of secreted alkaline phosphatase in the culture supernatant. In this system, infection with *C. jejuni* induced more than a 12-fold increase in NF- κ B activation over that observed with medium-treated controls (Fig. 2C). Furthermore, *C. jejuni*-induced NF- κ B activation was comparable to that observed with the positive control, Pam3CSK4. These data also provide evidence that *C. jejuni* activates signaling through TLR2.

Maturation and cytokine responses of *C. jejuni*-infected BM-DCs depend on both MyD88 and TRIF signaling. The finding that TLR4 recognition of *C. jejuni* is necessary for inflammatory activation of DCs suggests a role for MyD88 and/or TRIF cytoplasmic adaptors in this process, because TLR4 has been shown to utilize both of these adaptors for signal transduction (1, 49). To test this hypothesis, BM-DCs cultured from WT, MyD88^{-/-}, and TRIF^{-/-} mice were infected with *C. jejuni* and immune responses assessed at 24 h p.i. Experiments using BM-DCs from C57BL/6 genetic background mice (WT, TLR2^{-/-}, MyD88^{-/-}, and TRIF^{-/-}) were conducted concurrently, and data from these experiments were analyzed together. However, data from C57BL/6 WT and TLR2^{-/-} mice are presented with data from C3H/HeOuJ TLR4^{+/+} and C3H/HeJ TLR4^{-/-} mice for ease of comparison of results for TLR2 and TLR4. Therefore, results for BM-DCs from C57BL/6 WT mice are presented in Fig. 1, 2, and 3. Consistent with our findings for *C. jejuni*-infected TLR4^{-/-} DCs, MyD88 and TRIF deficiencies markedly decreased or abolished DC responses to *C. jejuni*. Importantly, both MyD88 and TRIF were required for optimal maturation and cytokine responses (Fig. 3A and B). We observed a profound reduction in *C. jejuni*-induced increases in the expression levels of MHC-II, CD40, CD80, and CD86 in MyD88^{-/-} and TRIF^{-/-} DCs (Fig. 3A). Similarly, MyD88 and TRIF deficiencies markedly reduced the proportion of DCs expressing MHC-II, CD40, and CD86 in response to *C. jejuni* challenge (Fig. 3A). Additionally, TNF- α and IL-6 secretion in response to *C. jejuni* was significantly ($P \leq 0.05$) reduced in MyD88^{-/-} and TRIF^{-/-} DCs (Fig. 3B). Most importantly, IL-12 production was completely abolished in infected MyD88^{-/-} and TRIF^{-/-} DCs. Collectively, these findings demonstrate that the MyD88 and TRIF signaling pathways are nonredundant and cooperate in *C. jejuni* infection to induce activation of DCs.

***C. jejuni*-induced TLR4 \rightarrow TRIF signaling leads to IRF-3 activation and IFN- β secretion in BM-DCs.** Signal transduction from TLR4 can occur through both MyD88 and TRIF adaptors (18), but only the TRIF arm of the pathway has been shown to mediate IFN- β production via the activation of the transcription factors IRF-3 and/or IRF-7 (9, 50). Since TLR4-TRIF signaling is activated by *C. jejuni*, we hypothesized that *C. jejuni* induces downstream IRF-3 activation and IFN- β production in DCs in a TLR4- and TRIF- but not MyD88-dependent manner. To test this hypothesis, we performed Western blots to analyze phosphorylation of IRF-3 1 h after *C. jejuni*

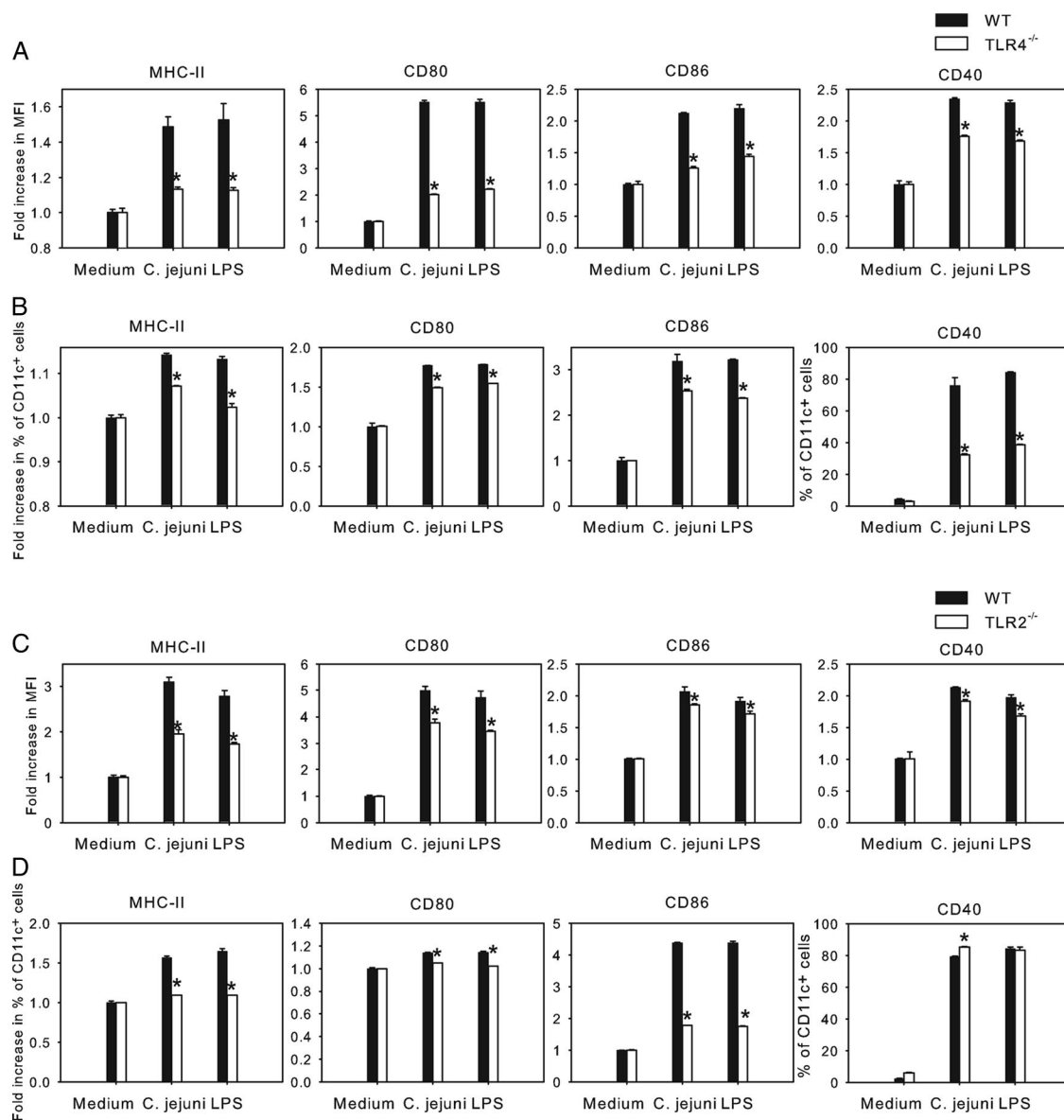


FIG. 1. TLR2 and TLR4 signaling is required for *C. jejuni*-induced maturation of BM-DCs. WT, TLR2^{-/-}, and TLR4^{-/-} BM-DCs were infected with *C. jejuni* or treated with *Salmonella* serovar Typhimurium LPS (0.1 μ g/ml) or medium alone. After 24 h, cells were immunostained and analyzed by flow cytometry. (A and C) *n*-fold increases in the mean fluorescence intensities (MFI) of the MHC-II, CD40, CD80, and CD86 markers on CD11c⁺ cells are shown. (B and D) *n*-fold increases in the percentages of CD11c⁺ cells expressing the MHC-II, CD80, and CD86 markers and the percentage of CD11c⁺ cells expressing CD40 are shown. The MFI and percentage of CD11c cells positive for markers in the medium-alone group in each DC type were considered the basal values, and the increase in these parameters in the other two treatment groups was expressed as an *n*-fold increase over the basal value. Asterisks indicate *P* values of ≤ 0.05 for WT versus TLR2^{-/-} or TLR4^{-/-} BM-DCs. Data are from three wells in an experiment and are expressed as means \pm SEM. A similar pattern of results was observed in the replicate experiment (data not shown).

challenge. *C. jejuni* infection markedly induced phosphorylation of IRF-3 in WT DCs and MyD88-deficient DCs (Fig. 4). In contrast, *C. jejuni*-induced IRF-3 phosphorylation was abolished in the absence of TLR4 and TRIF (Fig. 4). Surprisingly, total IRF-3 protein in BM-DCs increased markedly after infection with *C. jejuni* in a TLR4-, MyD88-, and TRIF-independent manner.

Next, IFN- β mRNA expression in *C. jejuni*-infected BM-DCs was assessed at 2.5 and 5 h p.i. by quantitative real-time PCR. At 2.5 h following *C. jejuni* infection, IFN- β mRNA

levels were upregulated approximately 280-fold over the basal levels observed in uninfected cells; this upregulation decreased to less than 100-fold over the basal levels at 5 h p.i. (Fig. 5A). This IFN- β mRNA expression was positively correlated with a significant induction of secreted IFN- β measured at 24 h after *C. jejuni* exposure (Fig. 5B). To determine the roles of TLR4, TRIF, and MyD88 in this response, we evaluated levels of secreted IFN- β in BM-DCs from mice deficient in these factors. Notably, TLR4^{-/-} and TRIF^{-/-} BM-DCs did not produce detectable amounts of IFN- β following *C. jejuni* infection

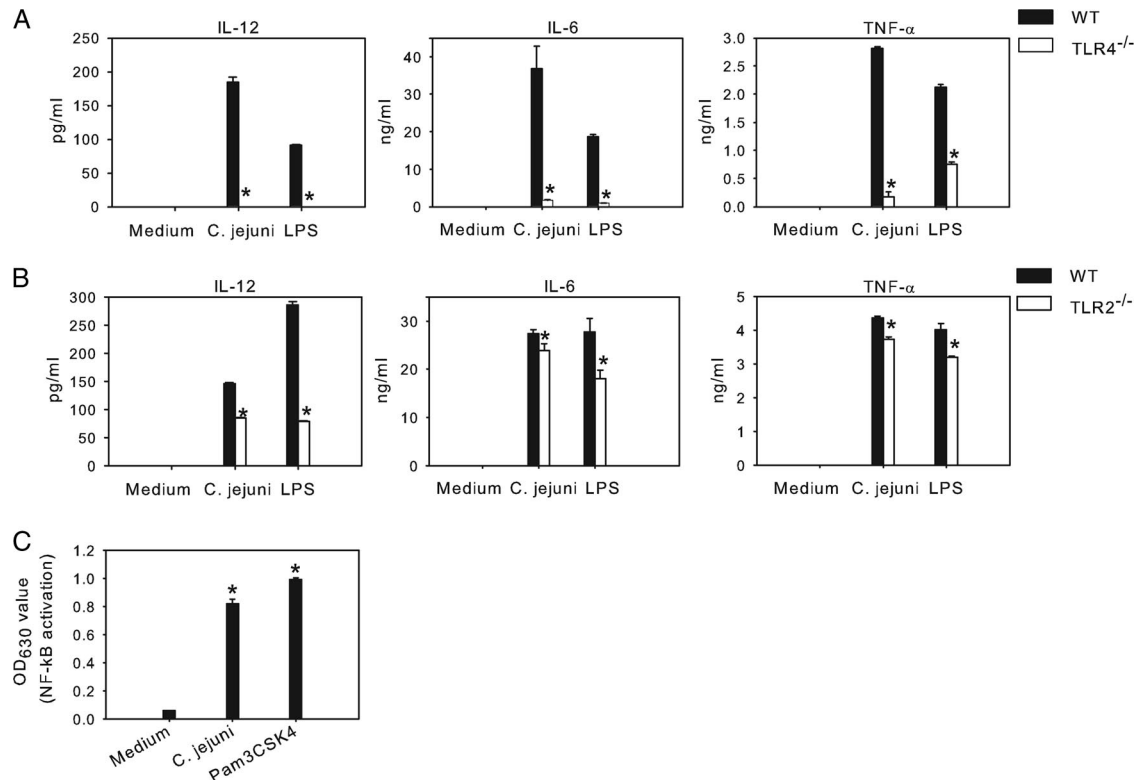


FIG. 2. *C. jejuni*-induced cytokine responses of BM-DCs are mainly dependent on TLR4 signaling. WT, TLR2^{-/-}, and TLR4^{-/-} BM-DCs were treated with *C. jejuni*, *Salmonella* serovar Typhimurium LPS (0.1 μg/ml), or medium alone. (A and B) Cytokine levels in the culture supernatant at 24 h p.i. were analyzed by ELISA. Asterisks indicate *P* values of ≤0.05 for WT versus TLR2^{-/-} or TLR4^{-/-} BM-DCs. (C) HEK-Blue-2 cells were treated with medium alone, *C. jejuni*, or Pam3CSK4 (100 ng/ml). After 23 h of incubation, the level of secreted alkaline phosphatase in the culture supernatant was quantified by incubating the supernatant with QUANTI-Blue and reading the plate at 630 nm (OD₆₃₀). Asterisks indicate *P* values of ≤0.05 for medium versus *C. jejuni* or Pam3CSK4. Data are from three wells in an experiment and are expressed as means ± SEM. A similar pattern of results was observed in the replicate experiment (data not shown).

(Fig. 5B). In contrast, IFN-β production was only marginally increased in MyD88^{-/-} BM-DCs after *C. jejuni* challenge compared to results for WT BM-DCs. Taken together, these findings demonstrate that the TLR4-TRIF signaling axis is necessary for *C. jejuni*-stimulated IRF-3 phosphorylation and IFN-β production by BM-DCs.

TLR2, TLR4, MyD88, and TRIF signaling pathways are all necessary for maximal Th1-cell priming by *C. jejuni*-infected BM-DCs. The role of the TLR2, TLR4, MyD88, and TRIF pathways in the induction of Th1-cell responses by *C. jejuni*-infected DCs was investigated using an in vitro DC-T-cell coculture system (38). Highly enriched CD4⁺ T cells from WT mice were cocultured with *C. jejuni*-treated WT, TLR2^{-/-}, TLR4^{-/-}, MyD88^{-/-}, and TRIF^{-/-} DCs; IFN-γ levels in the culture supernatants following 72 h of coculturing were assessed by ELISA. Consistent with our previous findings (38), WT DCs infected with *C. jejuni* induced high levels of IFN-γ from CD4⁺ T cells (Fig. 6). In contrast, there was a marked decrease in IFN-γ production from CD4⁺ T cells cocultured with *C. jejuni*-infected TLR4^{-/-}, MyD88^{-/-}, and TRIF^{-/-} DCs (Fig. 6). TLR2^{-/-} DCs infected with *C. jejuni* also induced significantly lower levels of IFN-γ relative to WT DCs infected with *C. jejuni*. These findings show that the TLR2, TLR4, MyD88, and TRIF signaling pathways all contribute to

maximal induction of Th1-cell responses by *C. jejuni*-infected DCs, although to various degrees.

DISCUSSION

The role of TLR signaling in eliciting host immune responses against the clinically significant enteric pathogen *C. jejuni* remains largely unknown. In this study, we investigated the contributions of the TLR2, TLR4, MyD88, and TRIF signaling pathways to DC recognition and responses to a known virulent strain of *C. jejuni*.

This study demonstrates for the first time that *C. jejuni*-induced phenotypic maturation of DCs is mediated by both TLR2 and TLR4 signaling whereas cytokine production by DCs in response to *C. jejuni* is predominantly dependent on TLR4. LOS of *C. jejuni* is the most likely candidate to stimulate TLR4 signaling in DCs since it possesses diphosphorylated hexa-acyl lipid A, which was reported to be essential for optimal stimulation of cellular responses (30). Indeed, *C. jejuni* LOS has been shown to trigger proinflammatory responses in cultured human DCs to an extent similar to that observed with live bacteria (20). Furthermore, previous studies have shown that *C. jejuni* evades TLR5 detection by having a modification in the TLR5 recognition site in its flagellin (2). Taken together

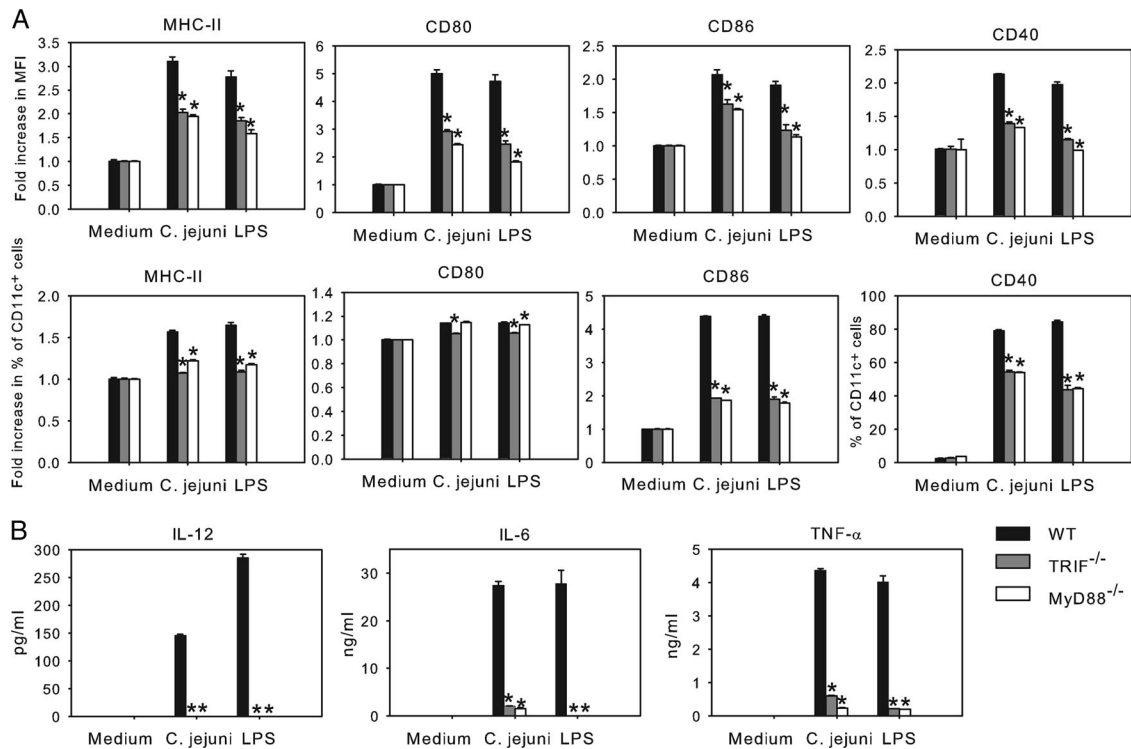


FIG. 3. MyD88 and TRIF signaling-dependent upregulation of surface markers and cytokine secretion by *C. jejuni*-infected BM-DCs. BM-DCs derived from WT, MyD88^{-/-}, and TRIF^{-/-} mice were treated with *C. jejuni*, *Salmonella* serovar Typhimurium LPS (0.1 μg/ml), or medium alone. After 24 h, surface expression of maturation markers (A) or secretion of cytokines (B) was analyzed by flow cytometry or ELISA, respectively. Data for BM-DCs from C57BL/6 WT mice are the same as in Fig. 1C and D and 2B. Asterisks indicate *P* values of ≤0.05 for WT versus TRIF^{-/-} or MyD88^{-/-} BM-DCs. Data are from three wells in an experiment and are expressed as means ± SEM. A similar pattern of results was observed in the replicate experiment (data not shown).

with these published findings, our data demonstrate that TLR4 is the major pattern recognition receptor involved in DC recognition of *C. jejuni*.

Furthermore, it was recently reported that the maturation and cytokine responses of DCs derived from WT and TLR4^{-/-} mice to a TLR2 agonist (Pam3CSK4, a synthetic lipoprotein) were very similar (4). TLR9 ligand (CpG DNA)-induced responses of immune cells from TLR4^{-/-} mice were comparable to those of cells from WT mice (15, 40). All of this functional evidence indicates that signaling through other TLRs, such as TLR2 and TLR9, that are involved in bacterial recognition is

not altered in TLR4-deficient cells. Therefore, the defects observed in the responses of TLR4-deficient DCs in our experiments can be attributed to the lack of signaling through TLR4.

It is also clear from our results that in addition to TLR4 signaling, maximal expression of MHC-II and IL-12 after *C. jejuni* infection requires TLR2 signaling. This suggests that some of the DC responses to *C. jejuni* require cooperative signaling through TLR2 and TLR4. TLR2-dependent responses described here may be due to recognition of a surface exposed lipoprotein of *C. jejuni*, JlpA. JlpA of *C. jejuni* has been shown to induce activation of NF-κB and p38 mitogen-activated protein kinase in a HEp-2 epithelial cell line, suggesting that the host innate immune system can recognize JlpA and trigger inflammatory responses (24).

Our data, together with previous findings (47, 51), show that MyD88 signaling is necessary to initiate the *C. jejuni*-induced innate inflammatory responses noted in a variety of host cells, such as IEC and antigen-presenting cells. Importantly, the severe impairment of expression of critical signals—MHC-II, costimulatory molecules, and IL-12—in *C. jejuni*-infected MyD88^{-/-} DCs implies that MyD88 signaling plays an important role in the induction of anti-*C. jejuni* mucosal immunity. Similarly, MyD88 signaling has been shown to be indispensable for inducing DC proinflammatory responses and for mounting optimal adaptive responses in vivo to a related gastric pathogen, *Helicobacter pylori* (37).

In these studies, we also demonstrate for the first time a

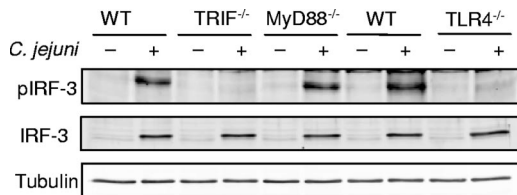


FIG. 4. Activation of IRF-3 phosphorylation by *C. jejuni*-induced TLR4-TRIF signaling. WT, TLR4^{-/-}, MyD88^{-/-}, and TRIF^{-/-} BM-DCs treated with *C. jejuni* or medium alone were lysed at 1 h p.i. The lysates were electrophoresed on 10% polyacrylamide gels and transferred to a nitrocellulose membrane. Blots were then probed with appropriate antibodies and scanned with a LI-COR Odyssey scanner. One experiment that is representative of two independent experiments is shown. A similar pattern of results was observed in the replicate experiment (data not shown).

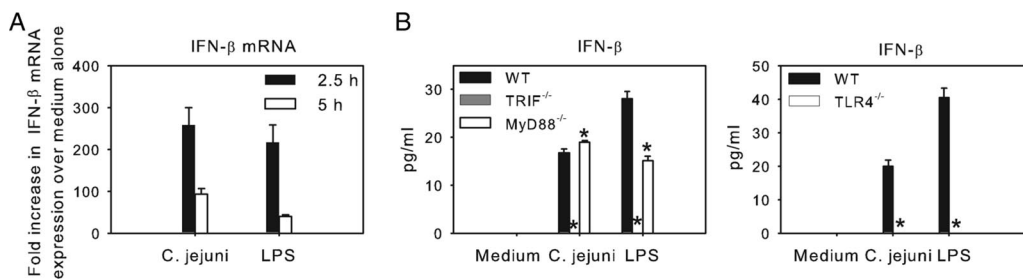


FIG. 5. IFN-β production by *C. jejuni*-infected BM-DCs. (A) IFN-β mRNA expression. Total RNA was extracted from BM-DCs treated with *C. jejuni*, *Salmonella* serovar Typhimurium LPS, or medium alone at 2.5 and 5 h posttreatment. IFN-β mRNA levels were quantified by Q-PCR. IFN-β mRNA levels in the *C. jejuni* and LPS treatment groups were expressed relative to that observed in the control group, treated with medium alone. (B) BM-DCs derived from WT, TLR4^{-/-}, MyD88^{-/-}, and TRIF^{-/-} mice were treated with *C. jejuni*, *Salmonella* serovar Typhimurium LPS (0.1 μg/ml), or medium alone. IFN-β levels in the culture supernatant at 24 h p.i. were analyzed by ELISA. Asterisks indicate *P* values of ≤0.05 for WT versus TRIF^{-/-}, MyD88^{-/-}, or TLR4^{-/-} BM-DCs. Data are from three wells in an experiment and are expressed as means ± SEM. A similar pattern of results was observed in the replicate experiment (data not shown).

functional role for the TRIF (MyD88-independent) signaling pathway during *C. jejuni* infection: DC responses following *C. jejuni* infection, including maturation and cytokine secretion, require TRIF signaling. The most significant finding is that this MyD88-independent pathway in DCs cooperates with the MyD88-dependent pathway to elicit optimal anti-*C. jejuni* immune responses. Consistent with our results, emerging evidence points to synergism between multiple TLR signaling pathways as a mechanism critical for ensuring efficient activation of host defense responses against pathogens (33). Although the exact mechanism underlying the cooperation between the MyD88 and TRIF signaling pathways is not known, it is possible that these two signaling pathways, when activated by *C. jejuni*, converge at the level of critical transcription factors, such as NF-κB and/or IRF-5, resulting in their enhanced and sustained activation and nuclear translocation. Supporting this notion, infection with *C. jejuni* has been shown to induce NF-κB activation in cultured human DCs (20) and IEC (51). Additionally, Fox et al. (12) previously demonstrated that NF-κB is essential for the development of Th1-associated antibody responses against *C. jejuni* in a murine model. However, further investigation is warranted to elucidate the exact mechanism operating in *C. jejuni* infections.

This study reveals that *C. jejuni* infection of DCs triggers IRF-3 phosphorylation and IFN-β secretion in a TLR4- and TRIF-dependent manner. These data are consistent with *C. jejuni*-mediated activation of the MyD88-independent pathway downstream of TLR4. Surprisingly, we noticed that total IRF-3 production was inducible by *C. jejuni* infection in BM-DCs independently of TLR signaling. This result is in contrast to previous reports showing IRF-3 expression was not inducible (5, 39). This discrepancy might be due to differences in the tissue/cell type or culture conditions used in this study. The kinetics of *C. jejuni*-triggered IFN-β mRNA expression is consistent with the previously reported responses following *E. coli* LPS stimulation (19). We expect that the TLR4-TRIF-dependent activation of IRF-3, shown in this study, is most likely to mediate IFN-β induction after *C. jejuni* infection. The role of type I interferons in bacterial infections is just beginning to be understood. IFN-β has been shown to act in an autocrine or paracrine manner through a positive-feedback mechanism involving IRF-7 to enhance maturation and cytokine responses of DCs (8, 14, 29). Particularly, *C. jejuni*-induced IFN-β could contribute to the substantial production of IL-12p70 by DCs following *C. jejuni* exposure (38), as has been shown with *E.*

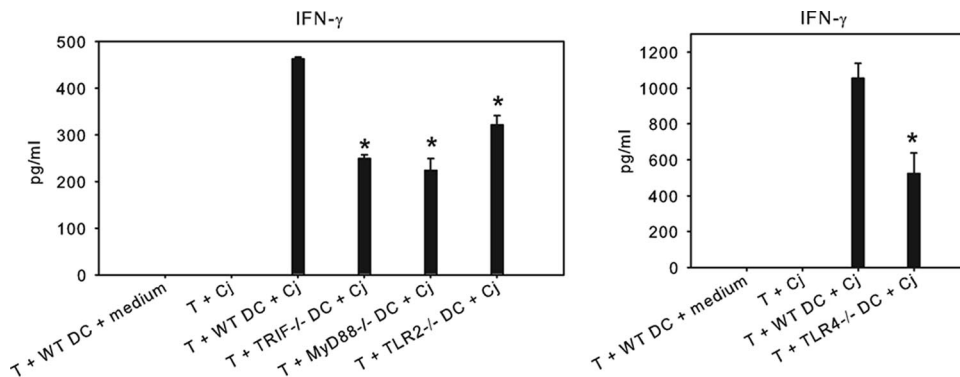


FIG. 6. TLR2, TLR4, MyD88, and TRIF signaling is necessary for maximal Th1 polarization by BM-DCs infected with *C. jejuni*. WT, TLR2^{-/-}, TLR4^{-/-}, MyD88^{-/-}, and TRIF^{-/-} BM-DCs were treated with *C. jejuni* (Cj) or medium alone, and CD4⁺ T cells (T) were added to each well at 24 h p.i. The IFN-γ level in the culture supernatant after 72 h of coculturing was analyzed by ELISA. Asterisks indicate *P* values of ≤0.05 for WT versus TLR2^{-/-}, TLR4^{-/-}, MyD88^{-/-}, or TRIF^{-/-} BM-DCs infected with *C. jejuni*. Data are from three wells in an experiment and are expressed as means ± SEM. A similar pattern of results was observed in the replicate experiment (data not shown).

coli LPS (14). Further investigations are under way to address the precise role of IFN- β in *C. jejuni* infections.

C. jejuni-treated TLR2^{-/-}, TLR4^{-/-}, MyD88^{-/-}, and TRIF^{-/-} DCs failed to induce maximal IFN- γ production from CD4⁺ T cells in an in vitro DC-T-cell coculture system. This is consistent with infection of MyD88-deficient mice, which, unlike WT mice, fail to clear intestinal colonization and control extraintestinal spread of *C. jejuni* (47). In light of these results, our data suggest that TLR2 signaling through MyD88 and TLR4 signaling through both the MyD88 and TRIF molecules play a significant role in the development of DC-mediated Th1-type mucosal immunity and resistance against *C. jejuni* infection.

It should also be noted that although *C. jejuni*-treated TLR4^{-/-}, MyD88^{-/-}, and TRIF^{-/-} DCs had no detectable production of IL-12—a critical instructive signal from DCs promoting IFN- γ -secreting Th1 cells—their ability to induce IFN- γ production from CD4⁺ T cells was preserved. These results show that *C. jejuni*-infected DCs can prime Th1 differentiation in an IL-12-independent mechanism for which TLR signaling is dispensable. Previous studies provide evidence for additional signaling mechanisms that are responsible for IL-12-independent priming of Th1 cells by DCs. These mechanisms include IL-18 (13), CD70 (42), and Delta 4 notch-like ligand (41) signaling. These findings suggest the possibility that such pathways may operate in *C. jejuni*-infected DCs. However, additional experiments are required to support this hypothesis.

Human and experimental animal model studies suggest that adaptive cellular immunity is required in successful defense against *C. jejuni* infection (7, 22). DCs are the key cell type involved in eliciting adaptive T-cell responses, and the expression of MHC-II and costimulatory molecules, along with concomitant cytokine (IL-12, TNF- α , and IL-6) signals, is highly critical for this function. This study demonstrates that MyD88-dependent and -independent (TRIF) pathways downstream of TLR4 cooperate in *C. jejuni* infection to mediate functional activation of DCs. Importantly, TLR2, TLR4, MyD88, and TRIF signaling all mediate maximal induction of *C. jejuni*-specific Th1-cell responses by DCs, suggesting that each has an important role in the development of anti-*C. jejuni* mucosal immunity. The findings described here provide novel insights into the contribution of TLR signaling to the host defense responses in *C. jejuni* infection and form a basis for further studies to dissect immunoregulation in *C. jejuni* infection and to aid in rational vaccine development approaches.

ACKNOWLEDGMENTS

We thank Jenna Gettings for breeding of mice, Jennifer Olmstead for technical assistance, Julia Bell for breeding of mice and critical review of the manuscript, and Louis King for discussions on flow cytometry. We thank Shizuo Akira for MyD88^{-/-} and TRIF^{-/-} mice.

This project was funded in part with federal funds from NIAID, NIH, Department of Health and Human Services, under contract no. NO1-AI-30058 and grant no. K26 RR023080-01. V. Rathinam was supported by funds from the Michigan State University College of Veterinary Medicine.

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