Role of Toll-Like Receptor 9 in *Legionella pneumophila*-Induced Interleukin-12 p40 Production in Bone Marrow-Derived Dendritic Cells and Macrophages from Permissive and Nonpermissive Mice

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The progression of *Legionella pneumophila* infection in macrophages is controlled by the Lgn1 gene locus, which expresses the nonpermissive phenotype in cells from BALB/c mice but the permissive phenotype in cells from A/J mice. Activation of dendritic cells and macrophages by *L. pneumophila* is mediated by the pathogen recognition receptor Toll-like receptor 2 (TLR2); furthermore, *Legionella* induces innate and adaptive immune cytokines by the MyD88-dependent pathway. TLR9 is coupled to MyD88 and mediates the production of interleukin-12 (IL-12) in dendritic cells infected with other facultatively intracellular pathogens. In the current study, *L. pneumophila* growth in dendritic cells from BALB/c and A/J mice was examined along with the role of TLR9 in the induction of IL-12 in these cells. Dendritic cells from both strains were nonpermissive for *L. pneumophila* intracellular growth, suggesting that the products of the Lgn1 gene locus that control intracellular growth in macrophages do not control the growth of *Legionella* in dendritic cells. In addition, chloroquine treatment suppressed IL-12 p40 production in response to *Legionella* treatment in dendritic cells and macrophages from BALB/c and A/J mice. Furthermore, the TLR9 inhibitor ODN2088 suppressed the *Legionella*-induced IL-12 production in dendritic cells from both mouse strains. These results suggest that *L. pneumophila* is similar to other intracellular bacteria in that it stimulates the production of immune-transitioning cytokines, such as IL-12, through activation of TLR9 and that this receptor provides a common mechanism for sensing these types of microbes and inducing innate and adaptive immunity.

*MATERIALS AND METHODS*

Mice. Female BALB/c (National Cancer Institute-Harlan, Frederick, MD) and A/J (Jackson Laboratory, Bar Harbor, ME) mice were used at 8 to 12 weeks.
of age. The mice were housed and cared for in the animal facility of University of South Florida Health Sciences Center, which is fully accredited by the American Association for Accreditation of Laboratory Animal Care.

**Bacteria.** Legionella pneumophila M124, a serogroup 1 isolate from a case of Legionnaires’ disease (Tampa General Hospital, Tampa, FL), was grown on buffered charcoal yeast extract (BD-Difco, Sparks, MD) plates for 48 h from a passage 3 stock stored at −80°C. For _L. pneumophila_ infection, DCs and macrophages were centrifuged, resuspended in medium (0.5 ml), and infected at a 10:1 ratio (bacteria to cells) for 30 min at 37°C in a CO2 incubator. Following the 30 min of uptake, cells were washed twice to remove noninternalized bacteria.

**Bone marrow-derived dendritic cells and macrophages.** The leg bones were removed, cleaned, and sterilized. The bone marrow was flushed from femurs and tibias by use of a syringe containing culture medium. For DC isolation, the bone marrow cells were washed and cultured overnight with RPMI 1640 medium (Sigma, St. Louis, MO) containing 10% bovine growth serum (HyClone, Logan, UT) plus granulocyte-macrophage colony-stimulating factor (GM-CSF [5 ng/ml]; BD Pharrnigen, San Diego, CA). Antibiotic-antimycotic solution, and 2-mercaptoethanol (0.05%; Sigma). On the following day, nonadherent cells were discarded, fresh GM-CSF (10 ng/ml)-containing medium was added to the adherent population, and the cells were cultured for an additional 7 to 9 days. Bone marrow-derived macrophages were generated using L929 conditioned (30%) medium containing M-CSF in place of GM-CSF, following a previously described protocol (9). These macrophages were examined by flow cytometric analysis and found to be >95% positive for CD11b, >85% positive for F4/80, and <5% positive for CD11c (data not shown).

**RT-PCR.** RNAs were extracted with TRI reagent (Sigma) from 3-h-infected DCs as previously described (35), and DNA was removed with a DNA-free kit from Ambion (Austin, TX). Reverse transcription (RT) of RNAs was performed with Superscript reverse transcriptase (Promega, San Diego, CA) and the cDNA products were PCR amplified as previously described, using a MasterGradient thermocycler (Eppendorf, Westbury, NY) (40). The gene products were amplified using Tag DNA polymerase from Takara Mirus Bio Corporations (Madison, WI) and primer pairs specific for β-actin, IL-12 p40 (40), and TLR9 (10). PCR products for β-actin and IL-12 p40 were amplified by duplex reactions, i.e., both sets of primers were run in the same reaction mix. All products were visualized with ethidium bromide in a 2% agarose gel. The mRNA in infected DCs, as measured by RT-PCR (Fig. 3).

**Flow cytometry analysis.** TLR9 and Legionella pneumophila infection. Figure 1B shows that these cells were also over 95% positive for CD11b and 62% positive for CD11c, as determined by flow cytometry, and that the CD11c cells were approximately 90% positive for TLR9, as determined by specific binding of the TLR9 ligand ODIN1826-FITC (Fig. 1C and D). Macrophages from BALB/c mice were >95% positive for the F4/80 marker and were approximately 90% positive for TLR9, as determined by specific ligand binding (Fig. 1E and F). Similar results were obtained with DC- and macrophage-enriched populations isolated from A/J mice: the mean fluorescence intensities (± standard errors of the means [SEM]) of the two populations were 454.7 ± 62 and 672.3 ± 83, respectively, for mice treated with ODIN1826-FITC and 122.3 ± 24 and 139.3 ± 30, respectively, for mice treated with ODIN1826-FITC and the ODN 2088 inhibitor. The fluorescence intensity was significantly decreased by TLR9 inhibitor treatment.

**In vitro cultures.** The cells were incubated in 5% fetal calf serum (HyClone)–RPMI plus 2-mercaptoethanol at a concentration of 10% cells/ml unless stated otherwise. For chloroquine treatment, the drug (100 μM; MP Biomedicals, Aurora, OH) was added for 30 min prior to stimulation with ODIN1826 (25 μg/ml; InvivoGen), Escherichia coli O111:B4 LPS (100 ng/ml; Sigma), or formalin-killed _L. pneumophila_ cells (10% bacteria/ml) or, as previously stated, to infection with _L. pneumophila_ at a 1:1 ratio. In some experiments, ODIN2086 (5 to 10 μM; InvivoGen), an inhibitor of TLR9 (41), was added 0.5 to 3 h prior to ODIN1826 or formalin-killed _L. pneumophila_ or prior to infection with _L. pneumophila_. All supernatants were collected at 24 h of culture and analyzed for IL-12 p40/70 by an enzyme-linked immunosorbent assay (ELISA).

**Cytokine ELISA.** Supernatants and standards were tested for IL-12 p40 activity by sandwich ELISAs using antibody pairs from BD-Pharmingen (San Diego, CA) following previously described protocols (29). The plates were read at 450 nm on an Elx800 microplate reader (Molecular Devices, Menlo Park, CA), and units were calculated from the standard curve performed for each plate. The low-end sensitivity was 100 pg/ml for IL-12 p40.

**CFU determination.** DCs and macrophage cultures were infected with _L. pneumophila_ at a 1:10 ratio (bacteria to cells) for 30 min at 37°C and washed to remove noninternalized bacteria. Uptake by all cell groups was similar, ranging from 1 × 10^6 to 3 × 10^4 CFU per culture. The infected cultures were incubated for 0 to 48 h and centrifuged prior to being lysed with 0.2% saponin. The lysates were diluted and incubated on buffered charcoal yeast extract plates. Bacterial CFU were determined on an AutoCount counter (Dynatech Labs, Chantilly, VA).

**Statistical analysis.** Data were analyzed by one-way analysis of variance with Dunnett’s test for comparing groups, using SigmaStat (Jandel Scientific, San Rafael, CA).

**RESULTS AND DISCUSSION.**

DCs are known to be important in sensing and responding to microbial products and are vital for induction of innate and adaptive immunity (24). DCs have been reported to respond to TLR9 ligands with the production of IL-12, and this production is augmented by costimulation of the cells with ligands for other TLRs, such as LPS (32). To characterize the expression of TLR9 in DCs and macrophages from BALB/c and A/J mice, bone marrow cells were cultured with GM-CSF- or M-CSF-containing L929 supernatant and analyzed for various markers by flow cytometry and RT-PCR. Figure 1A shows a robust expression of TLR9 mRNA by DCs from BALB/c mice which was not attenuated by _L. pneumophila_ infection. Figure 1B shows that these cells were also over 95% positive for CD11b and 62% positive for CD11c, as determined by flow cytometry, and that the CD11c cells were approximately 90% positive for TLR9, as determined by specific binding of the TLR9 ligand ODIN1826-FITC (Fig. 1C and D). Macrophages from BALB/c mice were >95% positive for the F4/80 marker and were approximately 90% positive for TLR9, as determined by specific ligand binding (Fig. 1E and F). Similar results were obtained with DC- and macrophage-enriched populations isolated from A/J mice: the mean fluorescence intensities (± standard errors of the means [SEM]) of the two populations were 454.7 ± 62 and 672.3 ± 83, respectively, for mice treated with ODIN1826-FITC and 122.3 ± 24 and 139.3 ± 30, respectively, for mice treated with ODIN1826-FITC and the ODN 2088 inhibitor. The fluorescence intensity was significantly decreased by TLR9 inhibitor treatment.

A requirement for TLR9 activation is endosomal maturation and/or acidification, which is inhibited by chloroquine pretreatment (39, 46). To see if _L. pneumophila_ was stimulating IL-12 production through TLR9, DCs (Fig. 2A) and macrophages (Fig. 2B) from A/J mice were stimulated with formalin-killed _L. pneumophila_ (kLp), _E. coli_ LPS, or ODIN1826 or were infected with virulent _L. pneumophila_ (Lp) in either the presence or absence of chloroquine. The results show that chloroquine attenuated ODIN1826 stimulation of the IL-12 p40 level (Fig. 2A and B), as previously reported, and also suppressed IL-12 p40 in response to _L. pneumophila_ cells (killed or living) (Fig. 2A and B), suggesting that the bacteria are activated through TLR9. The effect of LPS, as previously reported, was not suppressed by chloroquine. Similar results with chloroquine were obtained with DCs from BALB/c mice (Fig. 2C). To ensure that chloroquine treatment was not affecting the uptake and intracellular survival of _L. pneumophila_, CFU were measured in the DC cultures from both mouse strains and in macrophages from A/J mice. As reported by others (5, 33, 45), macrophages from A/J mice supported the replication of _L. pneumophila_, as evidenced by an increase in the number of CFU (Fig. 2D); however, intracellular growth was not seen in DCs from both mouse strains, and furthermore, chloroquine treatment had no effect on the number of CFU (Fig. 2D). It should also be noted that the uptake of _L. pneumophila_ by all groups of cells was similar, ranging from 10^3 to 3 × 10^4 CFU. In addition to IL-12 p40 protein, chloroquine also suppressed the mRNA in infected DCs, as measured by RT-PCR (Fig. 3).

In addition to suppression by chloroquine, the activation of TLR9 can also be assessed by treatment with the ligand inhibi-
itor ODN2088 (41). As expected, pretreatment (0.5 to 3 h) with the inhibitor completely attenuated IL-12 p40 production in response to the TLR9 ligand ODN1826 in cells from both BALB/c and A/J mice (Fig. 4A and D). It is interesting that ODN2088 pretreatment also significantly inhibited cytokine production in response to stimulation with either living or killed *L. pneumophila* cells (Fig. 4B to F), suggesting a role for TLR9. Although cells from A/J mice (Fig. 4D and E) appeared to produce more IL-12 p40 in response to infection, cells from both strains were equally inhibited by ODN2088. Interestingly, attenuation by the inhibitor was lower at higher concentrations of *L. pneumophila*, suggesting that moieties other than TLR9 are involved in the response.

The molecular and intracellular mechanisms surrounding *L. pneumophila* infection of macrophages have been studied extensively, but less is known concerning the mechanisms in infected DCs. Macrophages from A/J mice are permissive for *L. pneumophila* growth (43) because of low activity of the Naip5 (Birc1e) protein transcribed from the Lgn1 gene locus (11, 45). These proteins contain domains that interact with caspases and also contain Nod and LRR domains, which are important in sensing pathogen-associated antigens in the cytosol. Recently, it was shown that Birc1e proteins in the cytosol controlled the intracellular replication of *L. pneumophila* in macrophages through the activation of caspase-1 (IL-1β converting enzyme–protease-activating factor) (45). Others have shown that caspase-3 activation and cell death are important in controlling *L. pneumophila* growth in macrophages, although apoptosis was delayed following caspase-3 activation (1, 11, 13). TLR stimulation on macrophages also appears to be involved in resistance to *L. pneumophila* infection. For example, intracellular growth inhibition was dependent on TLR2 but not on TLR4 (2), and IL-12 and IL-10 production was attenuated in macrophages from TLR2-deficient mice. Furthermore, mice deficient in the TLR adapter protein MyD88 were more susceptible to *L. pneumophila* infection, and macrophages from these mice were deficient in IL-12, IL-6, and tumor necrosis factor alpha production following infection with *L. pneumophila* (5). Flagellin filaments by a TLR5-independent but Naip5-dependent mechanism were shown to induce defense against *L. pneum-
mophila replication in macrophages by activating caspase-1 and causing rapid cell death (38). This mouse macrophage resistance mechanism also required, in addition to the activation of caspase-1, a type IV secretion system and was postulated to contain features of pyroptosis and autophagy (31). From these studies, it is clear that various innate molecular mechanisms are activated in macrophages following \textit{L. pneumophila} infection, including Nod-LRR-sensing proteins, TLRs, caspase activation, and inflammatory cytokine production. Besides macrophages, DCs are also infected by \textit{L. pneumophila}. DCs from nonpermissive C57BL/6 mice were shown in culture to minimally support the growth of \textit{L. pneumophila} (2), and the growth was not affected in cells from TLR2- and TLR4-deficient mice. In the current report, we studied the growth of \textit{L. pneumophila} in bone marrow-derived DCs from both nonpermissive BALB/c mice and permissive A/J mice. Our results show that, unlike the case in macrophages, there is a gradual decline in the number of intracellular CFU in DCs from permissive as well as nonpermissive strains. Thus, it appears that the innate mechanisms that restrict \textit{L. pneumophila} growth in DCs are dependent upon factors other than the Naip5 proteins in that the diminished functioning of these proteins in A/J mice does not affect the overall restrictive capacity of the cell.

Cytokine production has also been studied in DC cultures exposed to \textit{L. pneumophila}. For example, cells from A/J mice cultured with formalin- or heat-killed \textit{L. pneumophila} cells produced more IL-12 p40, tumor necrosis factor alpha, and IL-6 than cells stimulated with living bacteria; furthermore, the stimulation of IL-12 p40 by heat-killed, but not formalin-killed, bacteria was shown to require, in part, TLR4 ligation (27).
Also, bone marrow DCs from TLR2-deficient mice but not from TLR4-deficient mice showed lowered responses to purified \textit{L. pneumophila} LPS as well as to viable or formalin-killed \textit{L. pneumophila} (7). These findings, coupled with our own preliminary results showing that DCs from TLR4-deficient mice display a robust IL-12 p40 response following infection with \textit{L. pneumophila} (data not shown), led us to speculate that TLRs other than TLR2 and TLR4 play a significant role in mediating cytokine production in response to living bacteria. Our results show that cells from both permissive and nonpermissive strains are potent producers of IL-12, with \textit{L. pneumophila} causing cytokine production in the ng/ml range. Furthermore, although the A/J cells appeared to be slightly more responsive in this regard, both groups of cells were equally inhibited by the TLR9 inhibitor, suggesting equivalent responsiveness to \textit{L. pneumophila} infection. Bone marrow macrophages from the two strains are also equivalent regarding IL-12 p40 production (data not shown), consistent with the view that some immune functions are independent of the mechanisms controlled by the Naip5 proteins.

Regarding the involvement of TLRs, our results with chloroquine and ODN2088 treatment strongly support a role for TLR9 ligation in the stimulation of IL-12 p40 by \textit{L. pneumophila}. This is also supported by previous findings linking the TLR9 adaptor protein MyD88 to \textit{L. pneumophila} resistance (5, 18). Although this is the first report showing an association between TLR9 and \textit{Legionella}, other facultatively intracellular bacteria have been shown to induce IL-12 through this receptor in DCs and macrophages. TLR9-deficient mice displayed defective IL-12 production in response to infection with \textit{Mycobacterium tuberculosis}, and a reported cooperation was observed between TLR9 and TLR2 in the overall cytokine response and resistance to infection (6). In addition, the DC production of IL-12 in response to treatment with heat-killed \textit{Brucella abortus} was shown to be dependent on TLR9 as well as the production of Th1 cytokines in whole-animal studies (23). In total, these results demonstrate that the induction of immunity by \textit{L. pneumophila} is mediated through a number of cellular receptor systems in macrophages and DCs. The array of bacterial antigens stimulates not only several different TLRs but also members of the Nod-LRR recognition system, resulting in restriction of bacterial growth, destruction of the host cell, and induction of cytokines important in innate and adaptive immunity.

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