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Toll-Like Receptor 2 Is Required for Inflammatory Responses to *Francisella tularensis* LVS

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Francisella tularensis*, a gram-negative bacterium, is the etiologic agent of tularemia and has recently been classified as a category A bioterrorism agent. Infections with *F. tularensis* result in an inflammatory response that plays an important role in the pathogenesis of the disease; however, the cellular mechanisms mediating this response have not been completely elucidated. In the present study, we determined the role of Toll-like receptors (TLRs) in mediating inflammatory responses to *F. tularensis* LVS, and the role of NF-κB in regulating these responses. Stimulation of bone marrow-derived dendritic cells from C57BL/6 wild-type (wt) and TLR4$^{-/-}$ but not TLR2$^{-/-}$ mice, with live *F. tularensis* LVS elicited a dose-dependent increase in the production of tumor necrosis factor alpha. *F. tularensis* LVS also induced in a dose-dependent manner an up-regulation in the expression of the costimulatory molecules CD80 and CD86 and of CD40 and the major histocompatibility complex class II molecules on dendritic cells from wt and TLR4$^{-/-}$ but not TLR2$^{-/-}$ mice. TLR6, not TLR1, was shown to be involved in mediating the inflammatory response to *F. tularensis* LVS, indicating that the functional heterodimer is TLR2/TLR6. Stimulation of dendritic cells with *F. tularensis* resulted in the activation of NF-κB, which resulted in a differential effect on the production of pro- and anti-inflammatory cytokines. Taken together, our results demonstrate the role of TLR2/TLR6 in the host's inflammatory response to *F. tularensis* LVS in vitro and the regulatory function of NF-κB in modulating the inflammatory response.

Francisella tularensis is a facultative, intracellular, gram-negative bacterium and the etiologic agent of tularemia in humans and other mammals. There are two subspecies of *F. tularensis* of major clinical importance, type A and type B, with type A being more virulent in humans (67). *F. tularensis* can infect humans through the skin, mucous membranes, gastrointestinal tract, and lungs, and is transmitted by various means, including bites from ticks, deerflies, or mosquitoes; ingestion of contaminated food or water; and inhalation of infectious aerosols (15, 22). Due to its various routes of infection and means of dissemination, *F. tularensis* has been considered a potential biological weapon for over 50 years and is currently classified by the Centers for Disease Control and Prevention as a category A bioterrorism agent (reviewed in references 33 and 45). Early efforts to develop a vaccine against tularemia led to the derivation of an attenuated, live vaccine strain (LVS) from a type B strain of *F. tularensis* (19, 56). In general, LVS does not cause disease in humans; however, it causes a fulminant infection in mice that is similar to human tularemia (26). Therefore, the mouse has been used as an experimental model to study the pathogenesis of tularemia, methods for inducing protective immunity, and the immune mechanisms involved in the interactions between the host and *F. tularensis* (13, 14, 17, 20, 25, 28, 30, 47, 58).

In recent years, the importance of the innate immune system and its critical relationship with adaptive immunity has become evident (reviewed in reference 2). Central to innate immunity are the Toll-like receptors (TLRs) that recognize distinct microbial products such as lipopolysaccharide (LPS) by TLR4 (49), flagellin by TLR5 (31), and double-stranded RNA by TLR3 (3). Furthermore, studies have shown that deficiencies in TLRs result in increased susceptibility to infections, e.g., TLR2$^{-/-}$ mice are susceptible to infection with gram-positive bacteria such as *Streptococcus pneumoniae* (18) and *Staphylococcus aureus* (64), whereas TLR11$^{-/-}$ mice are susceptible to infections with uropathogenic bacteria (73). Recognition of microbial components by TLRs leads to the activation of a variety of signal transduction pathways that mediate the resulting specific immune response (44, 52, 57, 59, 65, 69). Despite sequence homology among TLRs, the cytoplasmic tails of TLRs are not functionally equivalent, and thus TLR stimulation can induce the activation of specific subsets of genes (16, 26, 32, 51). TLRs are expressed primarily on antigen-presenting cells such as macrophages and dendritic cells. Recognition of microbial products by TLRs expressed on antigen-presenting cells leads to activation of NF-κB, mitogen-activated protein kinases, production of cytokines, and the induction of costimulatory molecules (43, 44, 51, 57). Activation of dendritic cells by microbes or microbial products can also cause the up-regulation of CD80, CD86, CD40, and class II major histocompatibility complex (MHC) molecules (10, 53, 54). This ability of TLRs to influence the production of immunoregulatory cytokines and to modulate expression of costimulatory molecules indicates their critical role in linking the innate and adaptive immune systems.

Studies employing TLR-deficient mice have been most relevant to delineate the role of specific TLRs in mediating im-

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munity to various microbial infections (65). Authors of previous studies using LPS-unresponsive (TLR4-defective) C3H/HeJ mice have reported that these mice were more susceptible to subcutaneous (41) or intravenous (5) challenge with *F. tularensis* than wild-type mice. However, other studies have reported that C3H/HeJ mice are slightly more resistant to intraperitoneal challenge (25) or of similar susceptibility to aerosol challenge (11) with *F. tularensis* LVS than wild-type mice. Chen et al. (12) also reported that the susceptibility of C3H/HeJ mice to intradermal challenge with *F. tularensis* LVS was dependent on the dose administered. Despite these contradictory findings on the role of TLR4 in the host response to *F. tularensis* challenge, other studies suggest the importance of the MyD88 (an adapter protein that is critical for cytokine production by all TLRs except TLR3) pathway in mediating susceptibility to sublethal infection by *F. tularensis* LVS (20). Although these studies implicate an involvement of TLRs, it is presently unclear as to which TLR may be involved in the host responses to *F. tularensis*.

The purpose of the present study was to determine the role of TLR1, TLR2, TLR4, and TLR6 in host responses to *F. tularensis*. Evidence is provided that the induction of inflammatory cytokines and the up-regulation of CD80, CD86, CD40, and MHC class II molecules on murine bone marrow-derived dendritic cells following incubation with *F. tularensis* LVS was contingent upon the presence of TLR2 but not TLR4. Cell activation by live *F. tularensis* LVS infection was through the TLR2 and TLR6 but not TLR2 and TLR1 heterodimer. Furthermore, cellular activation through TLR2 mediated phosphorylation of NF-κB p65 (Ser276) and the processing of phosphorylated p105. Finally, inhibition of NF-κB p50 or p65 resulted in a differential effect on the levels of pro- and anti-inflammatory cytokine production in response to *F. tularensis* LVS infection.

MATERIALS AND METHODS

Reagents. Biotin-conjugated anti-CD11c monoclonal antibody (MAb) and phycoerythrin-labeled or fluorescein isothiocyanate-labeled CD80 or CD86 or CD40 or class II MHC MAbs were obtained from eBioscience (San Diego, Calif.). Antibodies against NF-κB p65, phospho-NF-κB p65 (Ser276) and phospho–NF-κB p105 (Ser933) and the horseradish peroxidase (HRP)-linked anti-rabbit immunoglobulin G antibody were obtained from Cell Signaling Technology (Beverly, Mass.). The NF-κB p50 inhibitor SN50 and its inactive analog SN50 M were obtained from Calbiochem Biosciences Inc. (La Jolla, Calif.). The NF-κB p65 inhibitor PTD-p65 and the control peptide were obtained from Imgenex (San Diego, Calif.). Levels of tumor necrosis factor alpha (TNF-α), interleukin 12p40 (IL-12p40), IL-6, IL-10, and transforming growth factor β1 (TGF-β1) present in cell-free culture supernatants were determined using cytokine ELISA kits purchased from eBioscience and R&D Systems (Minneapolis, Minn.). *Escherichia coli* K235 LPS (32, 42) and *N*-palmitoyl-*S*-[2,3-bis(palmitoyloxy)-2*R*,*S*)-propyl]–cysteinyl-(*S*)-seryl-(*S*)-lysyl-(*S*)-lysyl-(*S*)-lysyl-(*S*)-lysine trihydrochloride (Pam3CSK4; InvivoGen, San Diego, Calif.) were used as positive control agonists for TLR4 and TLR2, respectively.

Bacteria. *F. tularensis* LVS (ATCC 29684; American Type Culture Collection, Rockville, Md.), a gift from Karen Elkins (Division of Bacterial and Parasitic Products, CBER/FDA, Bethesda, Md.), was grown as described previously (21, 24, 25). Initially, frozen stocks were prepared from bacteria grown to mid-log phase in Mueller-Hinton broth (BD Biosciences, Sparks, Md.) supplemented with 2% IsoVitaleX Enrichment (BD Biosciences), 0.1% glucose, 63 mM $CaCl_2$, 53 mM $MgCl_2$, and 34 mM ferric pyrophosphate. For each experiment, a frozen stock was thawed and streaked onto Mueller-Hinton II agar supplemented with 1% bovine hemoglobin (BD Biosciences) and 1% IsoVitaleX Enrichment. The bacteria were grown for 2 to 3 days at 37°C in 5% CO_2. A single colony was then used to inoculate supplemented Mueller-Hinton broth, and the culture was grown to late log phase for 16 to 18 h at 37°C with shaking at 100 rpm. The bacteria were harvested by centrifugation, and the pellet was suspended in phosphate-buffered saline (PBS). The number of bacteria in the suspension was determined by reading the optical density at 660 nm and by extrapolating from a standard curve and was confirmed by plating serial dilutions on supplemented Mueller-Hinton II agar and enumerating the number of colonies following incubation for 2 to 3 days at 37°C in 5% CO_2. The results are expressed using the numbers of bacteria estimated by spectrometry that were added to the cultures, which were similar to the actual numbers of CFU that were confirmed by plating.

Mice. C57BL/6 wild-type (wt), $TLR1^{-/-}$, $TLR2^{-/-}$, $TLR4^{-/-}$, and $TLR6^{-/-}$ mice were bred and maintained within an environmentally controlled, pathogen-free animal facility at the University of Alabama at Birmingham. The original $TLR^{-/-}$ breeding pairs were obtained under a materials transfer agreement from Shikuo Akira (Osaka University, Osaka, Japan) via Douglas Golenbock (University of Massachusetts Medical School, Worcester, Mass.). Mice were 8 to 12 weeks of age when used in the studies. All studies were performed according to the National Institutes of Health guidelines, and protocols were approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee.

Generation of dendritic cells. Bone marrow cells were collected as initially described (40). Briefly, the rear limbs of each mouse femur were dissected, and the epiphyses were removed from each end of the femurs using scissors to expose the bone marrow. Using a 5-ml syringe with a 22-gauge needle, 15 ml of ice-cold Hanks' balanced salt solution was used to aspirate the bone marrow out of the femur into polystyrene petri dish. The dendritic cells were generated by culturing the bone marrow-derived cells in the presence of 20 ng/ml rGM-CSF (Atlanta Biologicals, Atlanta, Ga.) for 10 days in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 20 mM HEPES, 1 mM sodium pyruvate, 50 U/ml penicillin, and 50 μg/ml streptomycin (RPMI 1640 complete medium) in a humidified 5% CO_2 incubator at 37°C (40). The resulting nonadherent dendritic cells were harvested after 10 days and the purity of the dendritic cell population was determined by flow cytometry analysis of $CD11c^+$ cells by using a FACScalibur (BD Biosciences, San Jose, Calif.). This procedure resulted in >80% $CD11c^+$ cells.

Flow cytometry analysis. Dendritic cells derived from wt, $TLR2^{-/-}$, and $TLR4^{-/-}$ mice were cultured in 96-well tissue culture plates (1×10^6/ml) containing RPMI 1640 complete medium at 37°C in 5% CO_2. Freshly grown *F. tularensis* LVS bacteria were added to wells at different multiplicities of infection (MOI). Following incubation for 20 h, cells were harvested, suspended in fluorescence-activated cell sorting (FACS) buffer, and then stained with biotin-conjugated anti-CD11c MAb together with phycoerythrin-labeled CD80 or CD40 and fluorescein isothiocyanate-labeled CD86 or MHC class II MAbs for 30 min on ice. The cells were washed three times and then stained with streptavidin-peridinin chlorophyll protein (BD Biosciences) for 30 min on ice. The cells were washed twice and resuspended in 500 μl of FACS buffer and immediately analyzed by flow cytometry using a FACSCalibur (BD Biosciences).

Cytokine analysis. Dendritic cells derived from wt and $TLR^{-/-}$ mice were cultured in 96-well tissue culture plates (1×10^6/ml) containing RPMI 1640 complete medium at 37°C in 5% CO_2. Freshly grown *F. tularensis* LVS bacteria were added to wells at different MOI. Following incubation for 20 h, culture supernatants were harvested and the levels of TNF-α, IL-12p40, IL-6, IL-10, and TGF-β were determined by enzyme-linked immunosorbent assay (ELISA) according to the manufacturers' instructions (eBioscience and R&D Systems). In some experiments, dendritic cells (1×10^6/ml) were pretreated with or without inhibitors to p65 or p50 for 2 h and then incubated with or without *F. tularensis* (10^7 bacteria/ml; MOI = 10) for 20 h. Levels of TNF-α and IL-10 in the culture supernatants were determined by ELISA.

Preparation of cell extracts. Dendritic cells derived from wt and $TLR^{-/-}$ mice were cultured in 24-well tissue culture plates (1×10^6 cells/well) and incubated with *F. tularensis* (10^7 bacteria; MOI = 10) for 0, 30, or 60 min. For the preparation of whole-cell lysates, cells were washed with PBS and then lysed on ice for 10 min in radioimmunoprecipitation assay lysis buffer (Upstate, Lake Placid, N.Y.) freshly supplemented with 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, 1 mM NaF, and 1 μg/ml of protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, Ind.). The whole-cell lysates were transferred to tubes and incubated on ice for an additional 20 min, and then following centrifugation, the supernatants were collected. To prepare cytoplasmic and nuclear extracts, a nuclear extract kit (Active Motif, Carlsbad, Calif.) was used according to the manufacturer's protocol. Specifically, cultured dendritic cells were washed with PBS/phosphatase inhibitors and harvested by centrifugation. The cell pellet was then suspended in hypotonic buffer and incubated on ice for 15 min. Detergent was then added and cells were mixed vigorously before centrifugation. The cytoplasmic extract was collected, and the nuclear pellet was sus-

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pended in complete lysis buffer and rotated at 150 rpm on ice for 30 min. The nuclear fraction was collected after centrifugation at 14,000 rpm for 10 min at 4°C.

Western blot analysis. Equivalent amounts of protein samples from cell extracts were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 10% Tris-HCl gel (Bio-Rad Laboratories, Hercules, Calif.). Protein was electrotransferred to immobilon-P transfer membranes (Millipore, Bedford, Mass.) and probed with specific antibodies against the phosphorylated form of NF-κB p65 (Ser276) and NF-κB p105. Detection of activation was carried out using HRP-linked anti-rabbit IgG, followed by ECL Western blotting detection reagents (Amersham Biosciences UK Limited, Little Chalfont Buckinghamshire, United Kingdom). In some experiments, the same blot was used for total p38 detection after striping of the previous antibody with Restore Western blot stripping buffer (Pierce). Densitometer scans of the blots were performed using the AlphaImager 2000 documentation and analysis system (Alpha Innotech, San Leandro, Calif.).

NF-κB DNA binding assay. NF-κB activation in the nuclear extracts was quantified by TransAM NF-κB assay kit (Active Motif) based on an ELISA principle. Specifically, an immobilized oligonucleotide containing the NF-κB consensus site (5′-GGGACTTTCC-3′) was bound to 96-well microtiter plates. The active form of NF-κB present in nuclear extracts that specifically binds to this oligonucleotide was detected using a primary antibody that recognizes an epitope on p65 that is accessible only when NF-κB is activated and bound to its target DNA. Following the addition of an HRP-conjugated secondary antibody, the plates were read on an automated plate reader and the level of nuclear NF-κB p65 was expressed as the absorbance at 450 nm.

Statistical analysis. Statistical significance between groups was evaluated by analysis of variance and the Tukey multiple-comparisons test using the InStat program (Graphpad Software, San Diego, Calif.). Differences between groups were considered significant at the level at which *P* was <0.05.

RESULTS

Role of TLR2 and TLR4 in mediating proinflammatory cytokine production in response to *F. tularensis* LVS. Dendritic cells derived from wt and $TLR^{-/-}$ mice were cocultured with or without various numbers of *F. tularensis* LVS and then the levels of cytokines of the culture supernatants were assessed by ELISA. *F. tularensis* induced TNF-α production by dendritic cells from wt and $TLR4^{-/-}$ but not $TLR2^{-/-}$ mice (Fig. 1). The induction of TNF-α production by *F. tularensis* was dose dependent, and optimal induction was observed with an MOI of 100. The levels of TNF-α produced after *F. tularensis* stimulation, increased ~10-fold over baseline with cells derived from wt and $TLR4^{-/-}$ mice. These results indicate that *F. tularensis* LVS signals through TLR2 for the induction of inflammatory cytokines.

Since *F. tularensis* LVS induced the production of the proinflammatory cytokine TNF-α, we next investigated its ability to induce the production of other inflammatory cytokines. Dendritic cells derived from the bone marrow of wt mice were cocultured with *F. tularensis* for 20 h and then the culture supernatants were assessed for levels of IL-12p40, IL-6, IL-10, and TGF-β. The dose-dependent increase in IL-6 and IL-10 (data not shown) was similar to that seen with TNF-α, with peak inductions of IL-6 (27.2 ± 1.7 ng/ml) and IL-10 (7.2 ± 0.3 ng/ml) in the supernatants of cultures incubated with *F. tularensis* LVS at an MOI of 100. In the case of IL-12p40, the peak induction (46.9 ± 4.9 ng/ml) was with an MOI of 10. An increase in TGF-β production was seen only in cultures infected with *F. tularensis* LVS at MOI of 1 (477 pg/ml) and 10 (231 pg/ml). These results indicate that *F. tularensis* LVS induces the production of both pro- and anti-inflammatory cytokines by murine bone marrow-derived dendritic cells.

Effect of *F. tularensis* on the expression of costimulatory molecules. To determine the effect of *F. tularensis* on CD80 and CD86 expression on dendritic cells and the role of TLRs in the response, dendritic cells derived from wt, $TLR2^{-/-}$, and $TLR4^{-/-}$ mice were cocultured in the presence or absence of various MOI of *F. tularensis* LVS and then analyzed by FACS. *F. tularensis* induced up-regulation of both CD80 and CD86 on dendritic cells derived from wt and $TLR4^{-/-}$ but not $TLR2^{-/-}$ mice (Fig. 2). The intensity of CD80 and CD86 expression increased in a dose-dependent manner up to an MOI of 100. A two- to threefold increase in the mean fluorescence intensity (MFI) over the baseline of CD80 and CD86 expression was seen on dendritic cells derived from wt and $TLR4^{-/-}$ mice following incubation with 1×10^8 *F. tularensis* LVS/ml of culture (MOI = 100). Furthermore, the increase in MFI of CD86 expression was significantly higher ($P < 0.05$) than that of CD80. These results indicate that *F. tularensis* LVS also stimulates the up-regulation of costimulatory molecules via TLR2.

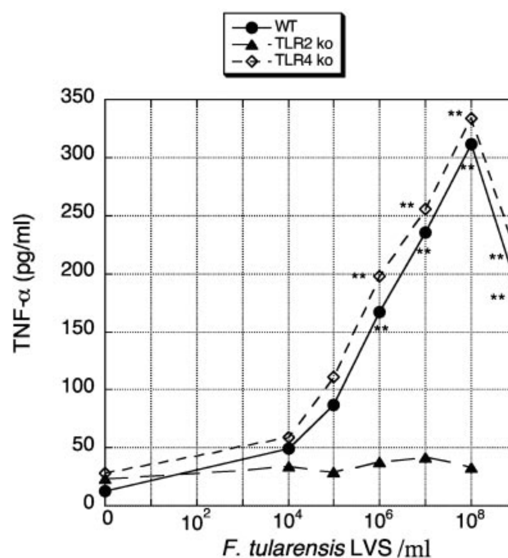

FIG. 1. Role of TLR2 and TLR4 in mediating the production of proinflammatory cytokines in response to *F. tularensis* LVS. Dendritic cells (1×10^6/ml) derived from wt, $TLR2^{-/-}$, and $TLR4^{-/-}$ mice were cocultured in the absence or presence of various numbers of *F. tularensis* LVS/ml for 20 h. Levels of TNF-α in culture supernatants were determined by ELISA. Data are the means of five separate experiments. ** indicates significant differences between nonstimulated and *F. tularensis*-stimulated dendritic cells at a *P* of <0.01. The levels (mean ± standard deviation) of TNF-α in supernatants of dendritic-cell cultures derived from wt, $TLR2^{-/-}$, and $TLR4^{-/-}$ mice and incubated with *E. coli* LPS (1 μg/ml) for 20 h were 1,355 ± 216 pg/ml, 751 ± 3 pg/ml, and 2 ± 0 pg/ml, respectively. Unstimulated control cultures contained <5 pg/ml. ko, knockout.

We next determined if *F. tularensis* also up-regulates the expression of CD40 and MHC class II and if the response is contingent upon the expression of TLRs. As shown in Fig. 3, *F. tularensis* induced an up-regulation in the expression of CD40 and MHC class II. The response was dependent on the expression of TLR2 but not of TLR4. Similar to the results observed with CD80 and CD86, the incubation of dendritic cells with *F. tularensis* resulted in a two- to threefold increase in the MFI of CD40 and MHC class II expression in a dose-dependent manner up to an MOI of 100. The increase in the MFI of CD40 expression on dendritic cells from wt mice following coculture with *F. tularensis* LVS at an MOI of 0.1 or 1 was higher than

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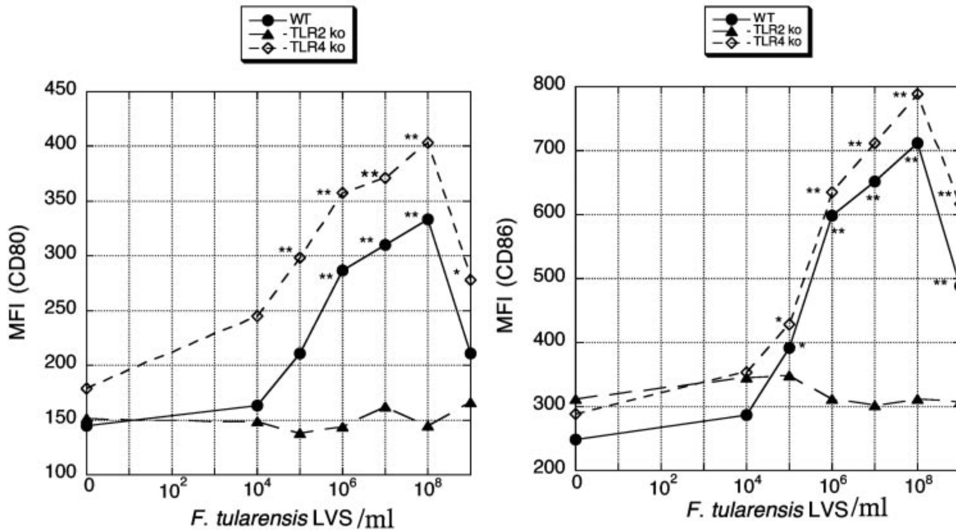

FIG. 2. Effect of *F. tularensis* LVS on the expression of the costimulatory molecules CD80 and CD86 on murine bone marrow-derived dendritic cells. Dendritic cells (1×10^6/ml) derived from wt, TLR2$^{-/-}$, and TLR4$^{-/-}$ mice were cocultured in the absence or presence of various numbers of *F. tularensis* LVS/ml for 20 h. Expression profiles represent the MFI of CD80 and CD86 expression on CD11c$^+$ cells. Data are the means of five separate experiments. * and ** indicate significant differences between nonstimulated and *F. tularensis*-stimulated dendritic cells at *P* values of <0.05 and <0.01, respectively. The MFI of CD80 and CD86 expression on CD11c$^+$ cells following incubation with *E. coli* LPS (1 μg/ml) for 20 h compared to unstimulated control cells were 98/41 and 36/9 (wt); 80/30 and 37/9 (TLR2$^{-/-}$); 36/27 and 12/8 (TLR4$^{-/-}$), respectively. ko, knockout.

that seen with cells derived from TLR4$^{-/-}$ mice. However, upon stimulation with a higher number of *F. tularensis* (MOI of 10 or 100), the MFI of CD40 expression was similar.

When dendritic cells were cocultured with *F. tularensis* at an MOI of >100, there was a marked decrease in the expression of the costimulatory molecules CD80 and CD86 (Fig. 2) and of CD40 and MHC class II (Fig. 3). These results suggest that coculturing *F. tularensis* LVS with dendritic cells at an MOI of 100 represented an optimal threshold for stimulation and that at a higher MOI, *F. tularensis* LVS may be toxic to the dendritic cells or cause apoptosis, as has been previously reported for a murine macrophage-like cell line (37). In the present study, we observed invasion of dendritic cells by *F. tularensis* LVS, and when dendritic-cell cultures were incubated with a high number of *F.*

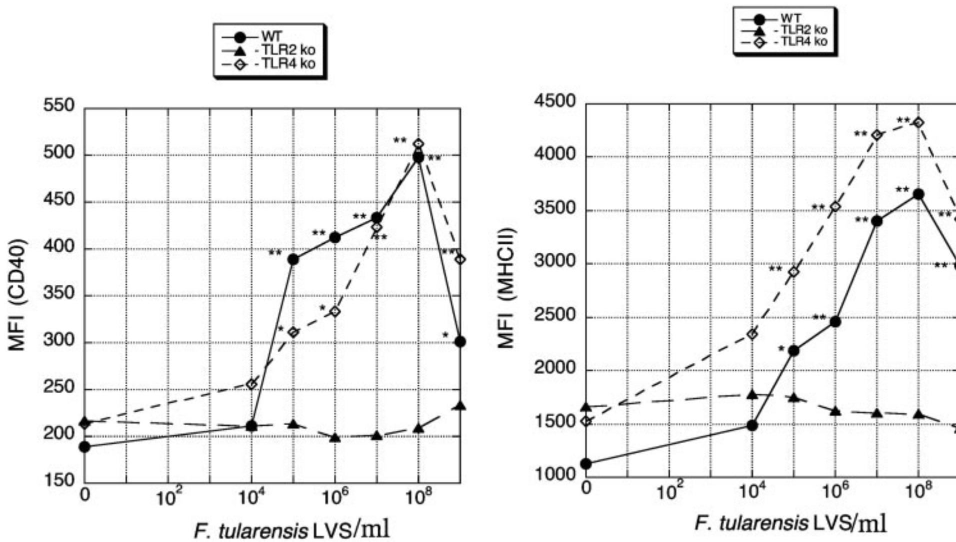

FIG. 3. Effect of *F. tularensis* LVS on the expression of CD40 and MHCII on murine bone marrow-derived dendritic cells. Dendritic cells (1×10^6/ml) derived from wt, TLR2$^{-/-}$ and TLR4$^{-/-}$ mice were cocultured in the absence or presence of various numbers of *F. tularensis* LVS/ml for 20 h. Expression profiles represent the MFI of CD40 and MHC class II (MHCII) expression on CD11c$^+$ cells. Data are the means of five separate experiments. * and ** indicate significant differences between nonstimulated and *F. tularensis*-stimulated dendritic cells at *P* values of <0.05 and <0.01, respectively. The MFI of CD40 and MHC class II expression on CD11c$^+$ cells following incubation with *E. coli* LPS (1 μg/ml) for 20 h compared to unstimulated control cells were 64/5 and 631/330 (wt); 52/3 and 354/192 (TLR2$^{-/-}$); 5/5 and 36/28 (TLR4$^{-/-}$), respectively. ko, knockout.

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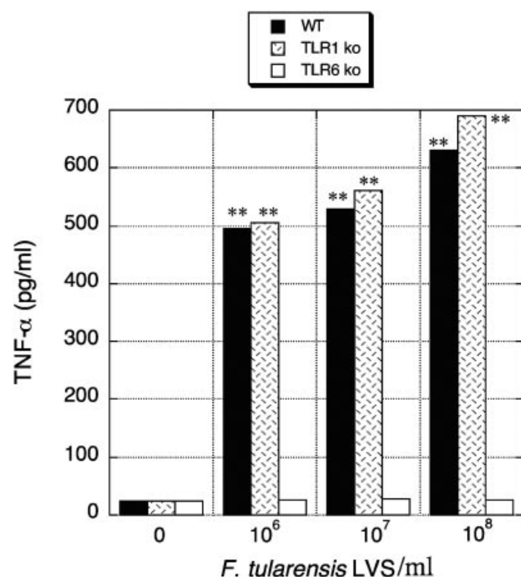

FIG. 4. Role of TLR1 or TLR6 in mediating TNF-α production in response to *F. tularensis* LVS. Dendritic cells (1×10^6/ml) derived from wt, TLR1$^{-/-}$, and TLR6$^{-/-}$ mice were cocultured in the absence or presence of various numbers of *F. tularensis* LVS/ml for 20 h. Levels of TNF-α in culture supernatants were determined by ELISA. Data are the means of five separate experiments. ** indicates significant differences between nonstimulated and *F. tularensis*-stimulated dendritic cells at a P of <0.01. The levels of TNF-α in supernatants of dendritic-cell cultures derived from wt, TLR1$^{-/-}$, and TLR6$^{-/-}$ and incubated with *E. coli* LPS (1 μg/ml) for 20 h were 1,355 pg/ml, 1,563 pg/ml, and 1,502 pg/ml, respectively. Unstimulated control cultures contained <5 pg/ml. ko, knockout.

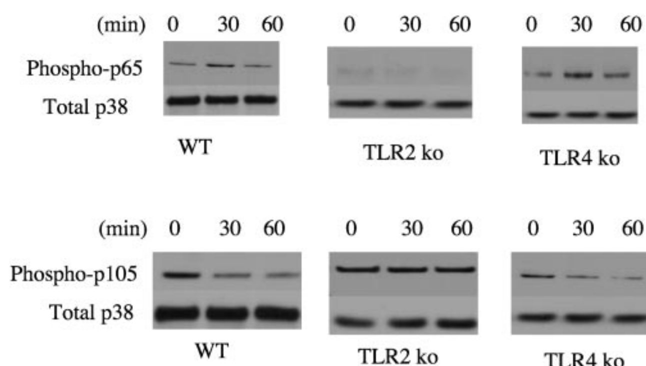

FIG. 5. Activation of NF-κB by *F. tularensis* LVS. Dendritic cells derived from wt, TLR2$^{-/-}$, and TLR4$^{-/-}$ mice were cocultured in the presence *F. tularensis* LVS (MOI = 10) for 0, 30, and 60 min. Equal amounts of whole-cell lysates were subjected to electrophoresis on a 10% Tris-HCl gel, and Western blots were performed using a specific antibody against the phosphorylated form of NF-κB p65 (Ser276) or NF-κB p105 (Ser933). Total p38 served as a protein loading control. Blots are representative of results obtained from five separate experiments. ko, knockout.

tularensis LVS (MOI = 1,000) for 24 h, 49% of the cell were apoptotic and 22% of the cells were dead, as determined by annexin V and propidium iodide labeling by using a Vybrant Apoptosis Assay kit (Molecular Probes, Eugene, Oreg.).

Role of TLR1 and TLR6 in responses to *F. tularensis* LVS infection. Since TLR2 functions as an obligate heterodimer with either TLR1 or TLR6 (reviewed in reference 63), we next determined the involvement of these TLRs in responses to *F. tularensis* LVS. Dendritic cells derived from wt, TLR1$^{-/-}$, and TLR6$^{-/-}$ mice were cocultured with *F. tularensis* LVS at various MOI, and the levels of TNF-α in culture supernatants were assessed. Similar levels of TNF-α were detected in supernatants of cell cultures derived from wt and TLR1$^{-/-}$ mice (Fig. 4). No induction of TNF-α was seen with dendritic cells derived from TLR6$^{-/-}$ mice. These results indicate a role for TLR6 but not TLR1 in the induction of TNF-α by dendritic cells stimulated with *F. tularensis* LVS. Taken together, the results further indicate that TLR2 acts in conjunction with TLR6 for responses to *F. tularensis* LVS.

NF-κB transactivation in *F. tularensis* LVS-stimulated dendritic cells. The transcription factor NF-κB is involved in the regulation of many inflammatory cytokines, including TNF-α (reviewed in reference 27). The regulation of NF-κB can occur at multiple steps including the phosphorylation of NF-κB p65, and the processing of the p105 molecule that renders the NF-κB p50 subunit. One possible resulting interaction is a transcriptionally active, classical NF-κB p50/p65 heterodimer, which is one of the best characterized (reviewed in reference 27). Therefore, we next evaluated NF-κB activation in dendritic cells following incubation with *F. tularensis* LVS. Phosphorylation of NF-κB p65 (Ser276) was observed in dendritic cells derived from wt and TLR4$^{-/-}$ mice (Fig. 5). No p65 phosphorylation was observed in dendritic cells from TLR2$^{-/-}$ mice at all times tested. These results indicate that signaling through TLR2 mediated the transactivation of NF-κB upon stimulation with *F. tularensis* LVS. We next assessed NF-κB p105 degradation in dendritic cells following incubation with *F. tularensis* LVS. Degradation of p105 occurred within 30 min of incubating *F. tularensis* LVS with dendritic cells from wt or TLR4$^{-/-}$ mice (Fig. 5). No degradation of p105 occurred in dendritic cells from TLR2$^{-/-}$ mice. Taken together, our findings indicate that upon *F. tularensis* LVS stimulation of dendritic cells, NF-κB activation occurs involving the phosphorylation of p65 (Ser276) and the processing of p105.

Since it was shown that transactivation of NF-κB occurs by p65 phosphorylation and by the degradation of p105 following *F. tularensis* stimulation, we next determined how these molecules regulated cytokine production. Dendritic cells derived from wt mice were pretreated with the p50 or p65 inhibitor or the corresponding control and then stimulated for 20 h with *F. tularensis* LVS (MOI = 10). Culture supernatants were assayed for levels of TNF-α as a representative proinflammatory cytokine and of IL-10 as a representative anti-inflammatory cytokine. Supernatants from cultures of dendritic cells stimulated with *F. tularensis* LVS contained significant higher levels of TNF-α ($P < 0.01$) and IL-10 ($P < 0.05$) than supernatants from nonstimulated control cultures (Fig. 6). Pretreatment of cultures with the p50 inhibitor resulted in an abrogation in TNF-α production but a significant enhancement ($P < 0.01$) in IL-10 production when compared to untreated, *F. tularensis* LVS-stimulated cultures. In contrast, inhibition of p65 resulted in an abrogation in TNF-α production and no effect on IL-10 production compared to untreated, *F. tularensis* LVS-stimulated cultures.

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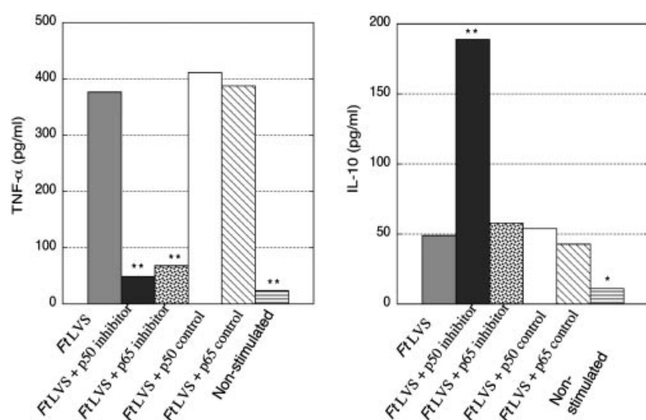

FIG. 6. Effect of NF-κB inhibition on cytokine production induced by *F. tularensis* LVS. Dendritic cells derived from wt mice were pretreated with SN50 (10 μM) or its inactive analog SN50 M (10 μM) or with PTD-p65 or its control peptide for 2 h and then stimulated with *F. tularensis* LVS (*Ft* LVS; MOI = 10) for 20 h. Levels of TNF-α and IL-10 in culture supernatants were determined by ELISA. Data are the mean of five separate experiments. Significant differences (*, $P < 0.05$; **, $P < 0.01$), compared to dendritic cells stimulated with *F. tularensis* LVS alone, were seen.

DISCUSSION

In the present study, we have shown that murine bone marrow-derived dendritic cells are stimulated by *F. tularensis* LVS in a TLR2-dependent manner. This was evidenced by the production of the proinflammatory cytokine TNF-α and the upregulated expression of MHC class II, CD80, CD86, and CD40. In addition, the nuclear translocation of NF-κB was also observed. Since these molecules are critical for both the innate immune response and the development of an antigen-specific adaptive immune response, their increased expression upon stimulation through TLR2 implies a link between this TLR and adaptive immunity (43, 44, 51, 57). *F. tularensis* LVS signaling through TLR2 and not TLR4 has also been alluded by Chen et al. (11, 12) in relation to their in vivo infection studies.

To date, 13 TLRs have been identified in mammalian cells, and most have been associated with recognition of specific-pathogen-associated molecular patterns (62). For instance, TLR4 characteristically recognizes enterobacterial LPS (49), TLR3 recognizes double-stranded RNA (3, 35), and TLR9 bacterial CpG DNA (6). Interestingly, TLR2 exhibits a broad ligand recognition, including lipoteichoic acid, lipoproteins, lipoarabinomannan, zymosan, certain glycolipids, nonenterobacterial LPS, and porins (1, 63). Since *F. tularensis* is a gram-negative bacterium, its LPS could be responsible for the observed cell activation. However, this is not likely due to its weak endotoxic properties, including its weak ability to induce the production of proinflammatory cytokines and mediators by murine macrophages and human monocytes in vitro (4, 55). Furthermore, a recent extensive study by Cole et al. (13a) has shown that the LPS from *F. tularensis* LVS only minimally stimulates primary murine macrophages or HEK293T cells transiently transfected with TLR4/MD2/CD14 but not with other TLRs, while infection with *F. tularensis* LVS bacteria activates NF-κB reporter activity via TLR2-expressing HEK293T cells. Prebeck et al. (50) demonstrated that activation of murine bone marrow-derived dendritic cells by the gram-negative pathogen *Chlamydia pneumoniae* was also through TLR2 and such was not due to the chlamydial endotoxin. The authors considered that the chlamydial component may be one of the heat shock proteins, especially hsp60. Studies with *F. tularensis* have demonstrated the importance of its heat shock proteins. For instance, individuals who have been infected with *F. tularensis* showed higher T-cell proliferative responses to chaperone-60, chaperone-10, and DnaK than individuals with no past history of tularemia or vaccination (23, 34). Thus, it is possible that chaperonins are the relevant stimulatory component of *F. tularensis* LVS that mediate signaling through TLR2. Ongoing studies in our laboratory are assessing this possibility.

The present investigation also showed that TLR6 in addition to TLR2 was involved in responses to *F. tularensis* LVS infection of dendritic cells. It is known that TLR2 responds to ligand as a heterodimer (46) with either TLR1 (71) or TLR6 (66) or perhaps with other TLRs or non-TLR molecules (70). Since TLR6 but not TLR1 was necessary for the induction of cell activation, our findings support the role of TLR2/TLR6 as the heterodimer mediating the activation of dendritic cells by *F. tularensis* LVS. Studies have demonstrated that the ligand for TLR2/TLR1 is made up of triacylated lipoproteins, whereas diacylated lipoproteins make up the ligand for the TLR2/TLR6 heterodimer (62, 63, 66, 71). At present, the specific components of *F. tularensis* LVS responsible for the induced responses have not been elucidated. It is possible that a 17-kDa lipoprotein that is conserved in *F. tularensis* (60) and immunogenic in mice (61) could serve as a TLR2 agonist. However, evidence to support this possibility is currently not available.

Although we observed the induction of TNF-α production by bone marrow-derived dendritic cells stimulated with *F. tularensis*, Telepnev et al. (68) showed no TNF-α production by the murine macrophage cell line J774A.1 following infection of with *F. tularensis* LVS. Furthermore, *F. tularensis* LVS also inhibited the ability of these cells to produce TNF-α in response to *Escherichia coli* LPS. An MOI of greater than 100 was needed to see the inhibitory effect. In the present studies, we observed only a reduction in the level of TNF-α production in cultures incubated with *F. tularensis* at an MOI of >100. The decrease in cell activation of cultures stimulated with 10^9 *F. tularensis* could be due to apoptosis. This possibility is based on studies by Lai et al. (37) who showed that *F. tularensis* LVS caused apoptosis of J774.A1 cells within 12 h when cocultured at an MOI of 500. Moreover, when an MOI of 50 was used, apoptosis was observed in 72 h, whereas with an MOI of 5, the apoptotic process took more than a week (37). In the present investigation, MOI of 10 and 100 did not cause a decrease in cell activation following a stimulating period of 24 h, similar to the findings of Lai et al. (37); however, at an MOI of 1,000 (10^9 *F. tularensis*), a decrease in cell activation and an increase in apoptosis was seen within the 24-h experimental period.

In addition to observing the induction of TNF-α production, we also observed the induction of IL-12p40, IL-6, IL-10, and TGF-β production in supernatants of dendritic-cell cultures infected with *F. tularensis* LVS for 20 h. These results differ from those of Bosio and Dow (9) who reported that *F. tularensis* LVS failed to induce the production of the inflammatory cytokines TNF-α, IL-6, and IL-10 by dendritic cells derived

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from the bone marrow of C57BL/6 mice. However, they did observe the induction of TGF-β production. One possible explanation for the observed differences between these studies could relate to the experimental conditions used. In the study by Bosio and Dow (9), dendritic cells were infected with *F. tularensis* LVS at an MOI of 50 and culture supernatants were assessed for cytokine levels after 48 h. In the present study, supernatants were analyzed for cytokine levels 20 h following infection of dendritic cells with *F. tularensis* LVS at various MOI. Further studies will be required to define the kinetics of cytokine production following infection with *F. tularensis*.

Finally, the observed inhibition of TNF-α production by both the p50 and p65 inhibitors supports the involvement of the p50/p65 heterodimer in the production of this proinflammatory cytokine (38; reviewed in reference 27). However, this does not appear to be the case for the production of IL-10. Guizani-Tabbane et al. (29) have reported the involvement of the p50/cRel NF-κB heterodimer in IL-10 secretion; however, other studies have indicated that NF-κB is not involved in IL-10 regulation (8, 36, 72). The cyclic-AMP-dependent pathway and cyclic AMP response elements on the promoter region seem to be required for IL-10 gene expression (48). Furthermore, STAT3, Sp1, and C/EBPβ and -δ also appear to be involved in IL-10 regulation (7, 39). Thus, the molecular regulation of the anti-inflammatory cytokine IL-10 gene appears to be fundamentally different from the regulation of proinflammatory cytokine genes, such as the TNF-α gene.

It is evident that *F. tularensis* is a most challenging pathogen and its aggressiveness and multiple routes of infection make it a serious threat to society. Much work is still needed to understand tularemia pathogenesis, the understanding of which is critical for the development of protective or preventive measures against such devastating disease.

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