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Toll-Like Receptor 2-Mediated Signaling Requirements for *Francisella tularensis* Live Vaccine Strain Infection of Murine Macrophages[▽]

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Francisella tularensis*, an aerobic, non-spore-forming, gram-negative coccobacillus, is the causative agent of tularemia. We reported previously that *F. tularensis* live vaccine strain (LVS) elicited strong, dose-dependent NF-κB reporter activity in Toll-like receptor 2 (TLR2)-expressing HEK293T cells and proinflammatory gene expression in primary murine macrophages. Herein, we report that *F. tularensis* LVS-induced murine macrophage proinflammatory cytokine gene and protein expression are overwhelmingly TLR2 dependent, as evidenced by the abrogated responses of TLR2$^{-/-}$ macrophages. *F. tularensis* LVS infection also increased expression of TLR2 both in vitro, in mouse macrophages, and in vivo, in livers from *F. tularensis* LVS-infected mice. Colocalization of intracellular *F. tularensis* LVS, TLR2, and MyD88 was visualized by confocal microscopy. Signaling was abrogated if the *F. tularensis* LVS organisms were heat or formalin killed or treated with chloramphenicol, indicating that the TLR2 agonist activity is dependent on new bacterial protein synthesis. *F. tularensis* LVS replicates in macrophages; however, bacterial replication was not required for TLR2 signaling because LVSΔ*guaA*, an *F. tularensis* LVS guanine auxotroph that fails to replicate in the absence of exogenous guanine, activated NF-κB in TLR2-transfected HEK293T cells and induced cytokine expression in wild-type macrophages comparably to wild-type *F. tularensis* LVS. Collectively, these data indicate that the primary macrophage response to *F. tularensis* LVS is overwhelmingly TLR2 dependent, requires de novo bacterial protein synthesis, and is independent of intracellular *F. tularensis* replication.

The facultative intracellular gram-negative coccobacillus *Francisella tularensis* is the etiologic agent of the potentially fatal infectious disease tularemia (reviewed in references 11, 16, and 54). There are three subspecies of *F. tularensis*: *F. tularensis* subsp. *mediaasiatica*, *F. tularensis* subsp. *holarctica*, and *F. tularensis* subsp. *tularensis*, but only the latter two subspecies are a significant cause of human infection. Tularemia is transmitted through insect bites, handling infected animals, ingestion of contaminated food or water, or inhalation of contaminated air. The size of the infectious dose, the symptoms associated with infection, and the severity of illness are highly dependent upon the route of inoculation. For example, disease symptoms are elicited by ingestion of ~10^8 *F. tularensis* organisms, but inhalation of only ~25 organisms elicits symptoms (48, 49). The incidence of tularemia, previously called "rabbit fever" or "deer fly fever," has steadily declined in the United States since 1950; between 1990 and 2000, 1,368 cases of tularemia were reported to the Centers for Disease Control and Prevention. However, interest in *F. tularensis* has surged in recent years due to its potential use as a biological weapon. The Centers for Disease Control and Prevention has classified *F. tularensis* as a category A agent due to its ability to be disseminated via the aerosol route, its extremely low infectious dose, and its potential to cause severe morbidity and mortality. As untreated tularemia can have a mortality rate of >30% (4, 11, 16, 43, 54), most of the basic research into the pathogenesis of *F. tularensis* has employed the attenuated live vaccine strain (LVS) (reviewed in references 14 and 54). *F. tularensis* LVS was developed in the former Soviet Union in the 1940s by repeated passage of *F. tularensis* subsp. *holarctica* on agar plates and subsequently through mice (13). While the molecular basis for its attenuation in humans is presently under investigation, *F. tularensis* LVS is virulent in mice when introduced by some routes, causing an infection that resembles human tularemia (14).

The host defense against pathogens involves both innate and adaptive immunity. For many years, the innate immune response was considered to consist primarily of phagocytosis and intracellular killing of bacteria, while the adaptive immune response provided pathogen-specific immunity. It has since become clear that the innate immune response provides pathogen-specific recognition and ultimately shapes the adaptive immune response. Toll-like receptors (TLRs), type I transmembrane receptor proteins, are a family of pattern recognition receptors (PRRs) that function as sentinels of the innate immune system and recognize conserved microbial structures

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referred to as pathogen-associated molecular patterns (PAMPs) (40). Various bacterial PAMPs are recognized by distinct TLRs. For example, TLR2 recognizes gram-positive lipopeptides as well as some nonenterobacterial species of lipopolysaccharide (LPS) (17, 29, 50, 60), while TLR4 has enterobacterial LPS as its prototype agonist (44). TLR5 recognizes bacterial flagellin (27), and TLR9 recognizes unmethylated CpG DNA (28). The failure to recognize and respond to microbial structures by TLRs can lead to increased susceptibility to infection. For example, TLR2-null mice show greatly enhanced susceptibility to infection with *Streptococcus pneumoniae* (12) and *Staphylococcus aureus* (52). Lack of TLR4 expression leads to increased susceptibility to gram-negative infections (10, 25, 41, 62) and chronic infection due to impaired elimination of *Mycobacterium tuberculosis* (1). Infection of mice with *F. tularensis* LVS results in a pronounced in vivo inflammatory response (8, 19, 23, 24, 51). We previously reported exposure of primary murine macrophages to *F. tularensis* LVS, and infection of mice with *F. tularensis* LVS led to production of proinflammatory cytokine mRNA and protein (8). We also established that *F. tularensis* LVS activation of NF-κB is dependent upon TLR2 expression in both HEK293T cells that overexpress TLR2 and in murine dendritic cells (8, 30). Therefore, we sought to determine the extent to which the proinflammatory response of *F. tularensis* LVS-infected macrophages is TLR2 dependent, as well as to identify additional signaling requirements for the *F. tularensis* LVS-induced proinflammatory cytokine response in macrophages. In this study, we report that the proinflammatory response to *F. tularensis* LVS is overwhelmingly TLR2 dependent and requires *F. tularensis* LVS protein synthesis to initiate signaling by TLR2; however, intracellular replication of *F. tularensis* LVS is not required. In addition, *F. tularensis* LVS dramatically increases expression of TLR2 both in vitro and in vivo, suggesting that *F. tularensis* LVS may amplify its capacity to elicit a strong proinflammatory response by increasing expression of this key signaling receptor. Collectively, these data provide important new insights into the molecular mechanisms by which *F. tularensis* induces early inflammatory responses in the host.

MATERIALS AND METHODS

Mice. Wild-type (WT; C57BL/6J) and TLR2$^{-/-}$ (B6.129-Tlr2<tm1Kir>/J) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were inoculated intraperitoneally (i.p.) with saline or ~40,000 CFU of *F. tularensis* LVS, >500 50% lethal doses (20). This high infectious dose was used in our previous study of protection afforded by pretreatment with *F. tularensis* LVS LPS and was chosen to demonstrate the remarkable efficacy of the pretreatment regimen (8). In this study, we again used this same challenge dose so that our results would be comparable between studies. At the indicated times after inoculation, mice were sacrificed and livers collected. Organs were snap-frozen in an ethanol-dry ice bath and stored at −80°C for subsequent RNA extraction.

Peritoneal macrophages were isolated from mice 4 days after i.p. injection of sterile 3% thioglycolate and cultured as described previously (8). Macrophages were plated in 6-well (4×10^6 cells/well) or 24-well (1×10^6 cells/well) tissue culture plates (Corning, Inc., Corning, NY). After overnight incubation, cells were washed with phosphate-buffered saline (PBS) to remove nonadherent cells. Cells were cultured in antibiotic-free medium for 24 h prior to and during all experiments. Treatments were carried out in duplicate or triplicate. Cytokine concentrations in culture supernatants were measured by enzyme-linked immunosorbent assay by the Cytokine Core Laboratory (University of Maryland, Baltimore). All animal experiments were conducted with Institutional Animal Care and Use Committee approval.

Reagents. The synthetic lipopeptide *S*-[2,3-bis(palmitoyloxy)-(2-*RS*)-propyl]-*N*-palmitoyl-(*R*)-Cys-(*S*)-Ser-Lys4-OH, trihydrochloride (Pam3Cys) was purchased from EMC Microcollections (Tuebingen, Germany). Chloramphenicol (2,2-dichloro-N-[2-hydroxy-1-(hydroxymethyl)-2-(4-nitrophenyl)ethyl]-acetamide) (CAP) was purchased from Calbiochem-EMD Biosciences, Inc. (Darmstadt, Germany). CAP was solubilized in 100% ethanol and was used at a concentration of 20 μg/ml in all assays. Bacteria were incubated in CAP-containing medium for 15 min prior to the addition of the bacteria to the cell cultures.

Bacteria. Frozen aliquots of *F. tularensis* LVS (ATCC 29684; American Type Culture Collection, Manassas, VA) were prepared and stored as previously described (15). Frozen stocks of *Escherichia coli* strain DH5α were purchased (Invitrogen, Carlsbad, CA). A guanine auxotrophic mutant, LVSΔ*guaA*, was constructed in *F. tularensis* LVS by deleting the *guaA* locus, the gene that encodes guanine monophosphate synthase, a critical enzyme in the purine biosynthetic pathway. Briefly, the entire *guaA* gene was replaced using a two-step allelic exchange strategy. *F. tularensis* LVS was electroporated with pFT695, a suicide plasmid developed for use in *F. tularensis* in which the entire *guaA* gene was replaced with a kanamycin resistance cassette and the *sacB* gene was included for counterselection. Kanamycin-resistant colonies were selected, and a second recombination event was prompted by growing a cointegrant strain in 10% sucrose to select for deletion of the *guaA* gene. The deletion of *guaA* was confirmed by PCR (47; A. Santiago, unpublished data; A. Santiago, M. M. Levine, and E. M. Barry, patent pending). Guanine auxotrophy was confirmed by measuring growth in Mueller-Hinton (MH) broth. While the parental *F. tularensis* LVS strain grew equally well in MH broth, without or with guanine supplementation, the LVSΔ*guaA* mutant only grew in guanine-supplemented medium. Intracellular growth of the wild-type *F. tularensis* LVS parental strain and LVSΔ*guaA* mutant was compared in J774 cells, a mouse macrophage cell line. While uptake of both strains was equivalent at time zero, there was a greater-than-4-log difference in the recovery of *F. tularensis* LVS versus LVSΔ*guaA* after 24 h. The LVSΔ*guaA* mutant was grown on MH agar plates supplemented with guanine and suspended in PBS prior to use in all in vitro assays. Viable bacteria were quantified by plating serial dilutions on MH agar plates, and maintenance of the *guaA* deletion was ensured by comparing growth of bacteria on plates that lacked or contained guanine.

F. tularensis LVS organisms were formalin killed by incubation in 4% formalin–PBS for 45 min in a 37°C water bath. After the incubation, residual aldehydes were quenched with 10 mM glycine in PBS. The bacteria were subsequently washed three times in 1× PBS and then resuspended in tissue culture medium. *F. tularensis* LVS was heat treated by incubation in a boiling water bath for 10 min. Lack of viability of formalin- or heat-treated organisms was confirmed by a lack of growth on MH agar plates in each experiment.

Real-time PCR. Total RNA extraction from macrophage cultures and homogenized organs, as well as real-time PCR, was carried out as described previously (8). Relative levels of mRNA for specific genes are reported as relative gene expression, as described previously (8). The primer sets used in these studies were detailed in our previous report (8).

Plasmid constructs and reporter assay. Transient transfections of HEK293T cells with TLR2 and reporter constructs were detailed in our previous report (39). Briefly, 2×10^5 HEK293T cells were seeded into each well of 12-well Costar plates (Corning, Inc.). After an overnight incubation, cells were transfected with SuperFect transfection reagent (QIAGEN Inc., Valencia, CA) for 3 h with 0.5 μg pELAM-Luc, 0.1 μg pCMV1-β-gal, 0.3 μg of pFLAG-TLR2 (kindly provided by Douglas T. Golenbock, University of Massachusetts Medical School, Worcester), and 0.6 μg of pCDNA3 (Invitrogen, Carlsbad, CA). After an overnight recovery, cells were stimulated with Pam3Cys, *F. tularensis* LVS, or LVSΔ*guaA* for 24 h. Relative luciferase activity was calculated by normalizing each sample's luciferase activity with the β-galactosidase activity measured within the same sample.

Confocal microscopy. Approximately 1×10^5 RAW 264.7 cells, a murine macrophage-like cell line, or primary thioglycolate-elicited macrophages from either WT C57BL/6J or TLR2$^{-/-}$ mice were plated into each chamber of glass-bottom, eight-well chamber slides (Nunc International, Naperville, IL). RAW 264.7 cells were incubated for 5 h, while primary cells were allowed to incubate overnight to ensure adherence of macrophages to the slide prior to infection. Cells were infected with *F. tularensis* LVS or *E. coli* at a multiplicity of infection (MOI) of ~1 to 5 at 37°C for 1 h. Bacterial contact with cells was facilitated by centrifuging the slides in a clinical centrifuge after addition of the bacteria. Bacteria were labeled prior to infection with 5 μM Celltrace calcein violet (Molecular Probes, Carlsbad, CA) according to the manufacturer's instructions. Lysosomes were labeled with 50 nM Lysotracker red (Molecular Probes) for 5 min at 37°C at the end of the infection. After 1 h of coincubation with bacteria, cells were washed with PBS and then fixed with 4% *p*-formaldehyde for 12 min

at room temperature. Cells were blocked and permeabilized for 30 min with PBS, 1% bovine serum albumin, 1% normal donkey serum, 0.3% Triton X-100 at room temperature. TLR2 was visualized by immunofluorescence using polyclonal antibody against the N terminus of TLR2 (Imgenex, San Diego, CA), followed by Cy2-conjugated donkey anti-rabbit immunoglobulin G (IgG; Jackson ImmunoResearch Laboratories, West Grove, PA). TLR2$^{-/-}$ macrophages did not stain with anti-TLR2 antibody, indicating the specificity of this antibody (data not shown). MyD88 was detected using polyclonal antibody raised against recombinant mouse MyD88 (amino acids 1 to 296; R&D Systems, Minneapolis, MN), followed by secondary and tertiary staining with biotin-conjugated donkey anti-goat IgG and Cy5-conjugated streptavidin, respectively (Jackson ImmunoResearch Laboratories). Coverslips were mounted on slides using a DABCO-based antifading fluorescent mounting medium and viewed with an Olympus FluoView 500 confocal microscope (60×, numerical aperture 1.4 objective) fitted with standard excitation and emission filters for the visualization of Celltrace violet, Cy2, Cy3, and Cy5.

To quantify the extent to which lysosomes colocalized with *F. tularensis* or *E. coli* in the phagosome, we randomly selected 10 well-isolated organisms per strain (organisms in contact with each other would have distorted the measurements, and these were rejected from the selection) from multiple images derived from separate experiments. The distance between the center position (centroid) of bacteria (*F. tularensis* LVS or *E. coli*) and the centroid of the lysosomes was calculated by measuring an intensity-position profile along the axis of greatest separation for microscopic images of *F. tularensis* LVS or *E. coli* and their associated lysosomes using Adobe Photoshop (Adobe, San Jose, CA). All profile intensities were normalized by setting the highest intensity to 1.0. The distance between the centroids of the bacterial and lysosomal peaks was then measured as described elsewhere (46).

FACS analysis. Peritoneal macrophages from C57BL/6 WT mice were cultured without or with *F. tularensis* LVS (MOI, ~10) for 16, 24, or 48 h. Cells were then harvested, washed, and suspended in fluorescence-activated cell sorting (FACS) buffer and stained with phycoerythrin-labeled anti-mouse TLR2 or TLR4 antibody or mouse IgG1 isotype control antibody (eBiocience, San Diego, CA) for 30 min on ice. The cells were washed, suspended in FACS buffer, and immediately analyzed using a FACSCalibur apparatus (BD Biosciences, Sunnyvale, CA) as described previously (30).

Statistics. Results were analyzed using a one-way analysis of variance with repeated measures, followed by a Tukey's post hoc test for multiple paired comparisons. Data analysis was performed using the GraphPad PRISM 4 program for Windows (GraphPad Software, Inc., San Diego, CA). The Mann-Whitney rank sum test was used for centroid analysis.

RESULTS

TLR2 is required for *F. tularensis* LVS stimulation of gene expression in murine macrophages. We previously reported that *F. tularensis* LVS specifically activates an NF-κB luciferase reporter in HEK293T cells transfected with a vector encoding human TLR2, but not with other TLR expression vectors (8). This finding was supported by Katz et al., who showed that live *F. tularensis* LVS elicited a dose-dependent increase in tumor necrosis factor alpha (TNF-α) secretion and upregulation of CD80, CD86, CD40, and major histocompatibility complex class II molecules in bone marrow-derived dendritic cells (DC) from WT (C57BL/6) and TLR4$^{-/-}$, but not TLR2$^{-/-}$, mice (30). Katz et al. further showed that TLR6$^{-/-}$ DC also fail to respond to *F. tularensis* LVS infection, indicating that TLR2/6 is the functional heterodimer that responds to *F. tularensis* LVS in DC (30).

Since *F. tularensis* replicates intracellularly in macrophages, we first sought to confirm and extend these findings of TLR2 dependence in murine macrophages. To this end, thioglycolate-elicited macrophages derived from WT or TLR2$^{-/-}$ mice were stimulated with live *F. tularensis* LVS (MOI, 5) for 0 to 24 h, and proinflammatory gene expression and cytokine production were measured. *F. tularensis* LVS infection of macrophages derived from WT mice yielded robust induction of cytokine gene expression. As illustrated in Fig. 1A, quantitative real-time PCR revealed three general patterns of cytokine mRNA expression in macrophages derived from wild-type animals: expression that peaked prior to 8 h (e.g., interleukin-1β [IL-1β], TNF-α, KC), between 8 and 12 h (e.g., IL-12 p35 [data not shown] and IL-12 p40), and after 12 h (e.g., beta interferon [IFN-β], IFN-γ, inducible nitric oxide synthase [iNOS], IP-10 [not shown], and RANTES). In contrast, macrophages derived from TLR2$^{-/-}$ mice exhibited baseline mRNA expression of these same genes after exposure to *F. tularensis* LVS throughout the entire time course (Fig. 1A).

Supernatants collected from WT macrophages infected with *F. tularensis* LVS contained significantly increased concentrations of IFN-γ, IL-1β, IL-12 p40, KC, RANTES, and TNF-α, in contrast to supernatants collected from TLR2$^{-/-}$ macrophages, in which levels of IFN-γ, IL-1β, IL-12 p40, and TNF-α were below the limit of detection and only very low levels of the chemokines KC and RANTES were detected (Fig. 1B). These findings support the conclusion that *F. tularensis* LVS-induced macrophage production of cytokines is overwhelmingly TLR2 dependent.

Colocalization of *F. tularensis* LVS in phagosomes with TLR2 and MyD88. Our in vitro data strongly suggest that *F. tularensis* LVS-induced macrophage proinflammatory gene and protein expression is TLR2 dependent. Therefore, to confirm visually the interaction between *F. tularensis* LVS and the TLR2 signaling complex, four-color confocal imaging was used to detect fluorescently labeled bacteria (white), lysosomes (red), TLR2 (green), and MyD88 (blue) concurrently. Figure 2A illustrates a group of four macrophages that have been infected with *F. tularensis* LVS, and Fig. 2B is an enlarged region that includes three organisms within a single macrophage. Within 1 h of infection, *F. tularensis* LVS (white) can be observed in close proximity to lysosomes, TLR2, and MyD88 (Fig. 2B). Figures 2C to E and H to J represent the identical region shown in Fig. 2B but with only one or two colors displayed per panel, thereby enabling visualization of the individual components singly or together. An arrow is positioned identically on each panel as a reference point.

Consistent with previous reports that *F. tularensis* blocks phagosome fusion with lysosomes (7, 22), we observed that the lysotracker dye was most often positioned at one end of the *F. tularensis* LVS, rather than being evenly distributed around the bacterium (Fig. 2C, D, and E). In contrast, when *E. coli* was similarly labeled and exposed to macrophages, the lysotracker was more uniformly distributed around the phagocytosed bacterium (data not shown). To quantify this observation, we utilized an approach that compares the distance between the center of images derived from a single organism and its lysosomes. If there were no significant difference in their relative positions, the distance between the two image centers would not be significant; however, if the lysosomes are displaced relative to the center of the organism, a significant difference would be detected (46). These data are displayed in representative centroid plots (Fig. 2F and G). While the centroid-to-centroid distance between individual *F. tularensis* LVS organisms and their corresponding lysosomes (Fig. 2F) was found to be significantly different ($n = 10$; $P \leq 0.001$), there was no significant difference between the centroid distances of *E. coli*

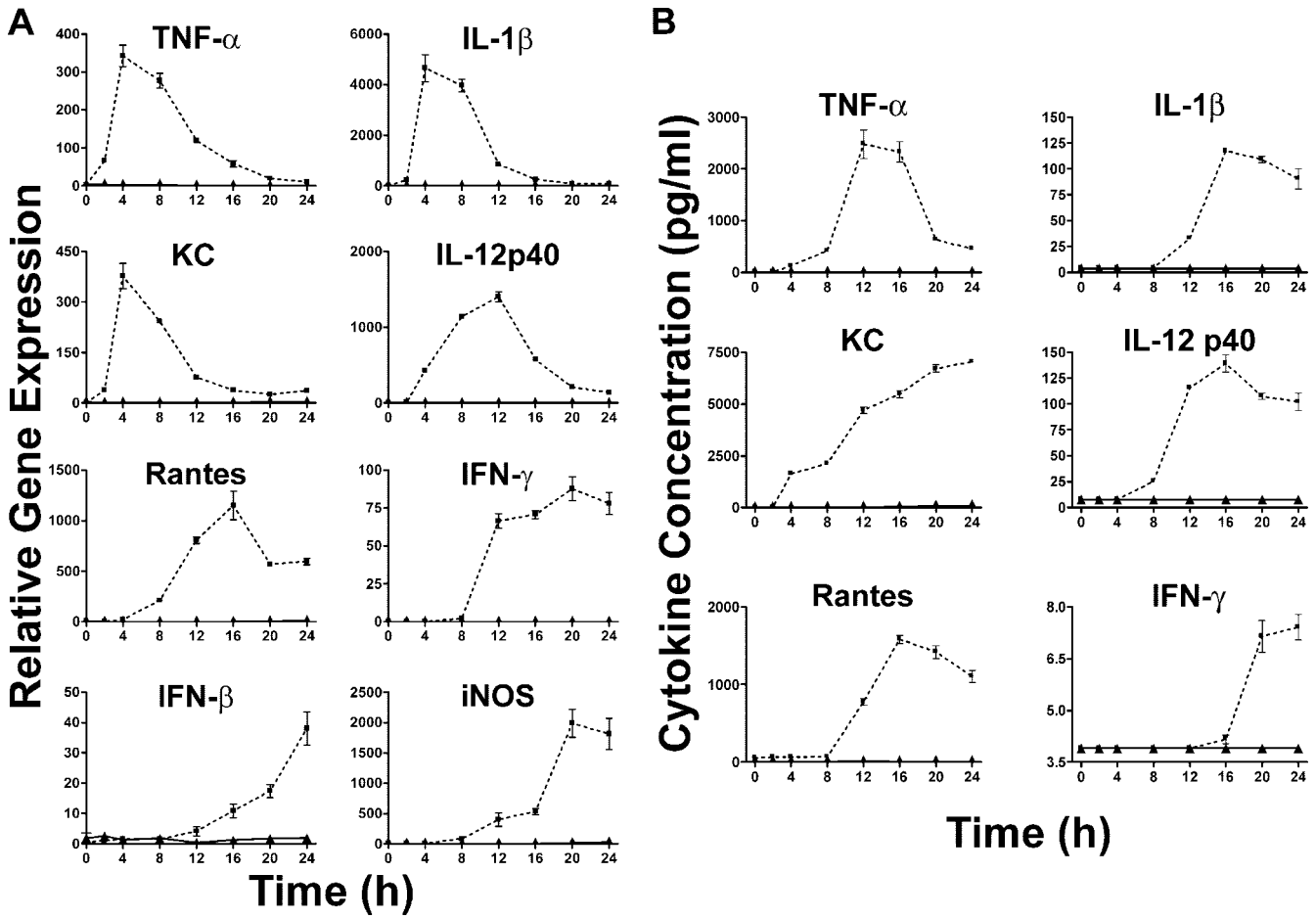

FIG. 1. Macrophage cytokine gene expression is overwhelmingly dependent upon TLR2. Peritoneal macrophages from either WT C57BL/6J (■, dotted lines) or TLR2$^{-/-}$ (▲, solid lines) mice were exposed to *F. tularensis* LVS (MOI, 5) for 0 to 24 h. At the indicated time points, supernatants were collected and total RNA extracted from the macrophage cultures. (A) cDNA was made from the extracted RNA and analyzed by real-time PCR. Gene expression is reported as relative gene expression compared to peritoneal macrophages exposed to medium only. (B) Supernatants were analyzed by enzyme-linked immunosorbent assay for the presence of various cytokines. Concentrations of TNF-α (7.8 pg/ml), IFN-γ (3.9 pg/ml), IL-12 p40 (7.8 pg/ml), and IL-1β (3.9 pg/ml) in supernatants from TLR2$^{-/-}$ macrophages were below the limit of detection, with assay limits of detection in parentheses. All treatments were performed in triplicate, and data are presented as means ± standard errors of the means. Data are representative of a single experiment ($n = 2$).

and its lysosome ($n = 10$), indicating that the center of the *E. coli* cell, but not the *F. tularensis* LVS cell, was coincident with the center of the lysosome (Fig. 2G).

Previous studies have demonstrated that uptake of yeast or bacteria into phagosomes is accompanied by TLR2-dependent signaling, as indicated by recruitment of the key adapter molecule, MyD88, to the cytoplasmic side of the phagosome membrane (2, 58). Although TLR2 and MyD88 were detectable throughout the macrophage, increased colocalization of these molecules with ingested *F. tularensis* LVS was observed (Fig. 2H to J). To permit visualization of the overlap of TLR2 and MyD88, regions of colocalization were pseudocolored yellow (Fig. 2J). The location of the bacteria in the images in Fig. 2H, I, and J are indicated with white, dotted circles, such that the white of the labeled *F. tularensis* LVS does not obscure the yellow pseudocolor. The strongest areas of colocalization of TLR2 and MyD88 are coincident with *F. tularensis* LVS (Fig. 2J); this is most clearly illustrated in Fig. 2J with the organism that is positioned furthest to the left. These confocal data, in conjunction with our genetic data (Fig. 1A and B) (8, 30), clearly indicate the intimate nature of the interaction between *F. tularensis* LVS and the TLR2 signaling complex.

TLR2 gene expression is increased both in vitro and in vivo after *F. tularensis* LVS infection. *F. tularensis* LVS-induced modulation of cytokine and chemokine gene expression in murine macrophages was clearly dependent upon TLR2 (Fig. 1); however, *F. tularensis* LVS infection of murine macrophages also impacted TLR2 expression. Exposure to *F. tularensis* LVS increased expression of TLR2 mRNA ~100-fold in WT macrophages (Fig. 3A). FACS analysis of primary murine macrophages confirmed that this increase in TLR2 mRNA was also observed at the level of macrophage surface expression of TLR2 (Fig. 3B). In contrast to TLR2 mRNA and protein, steady-state levels of TLR4 mRNA were transiently diminished in macrophage cultures by *F. tularensis* LVS infection (Fig. 3A); no change was seen in the level of TLR4 protein expression on macrophages, as determined by FACS analysis (data not shown). To determine if modulation of TLR2 mRNA

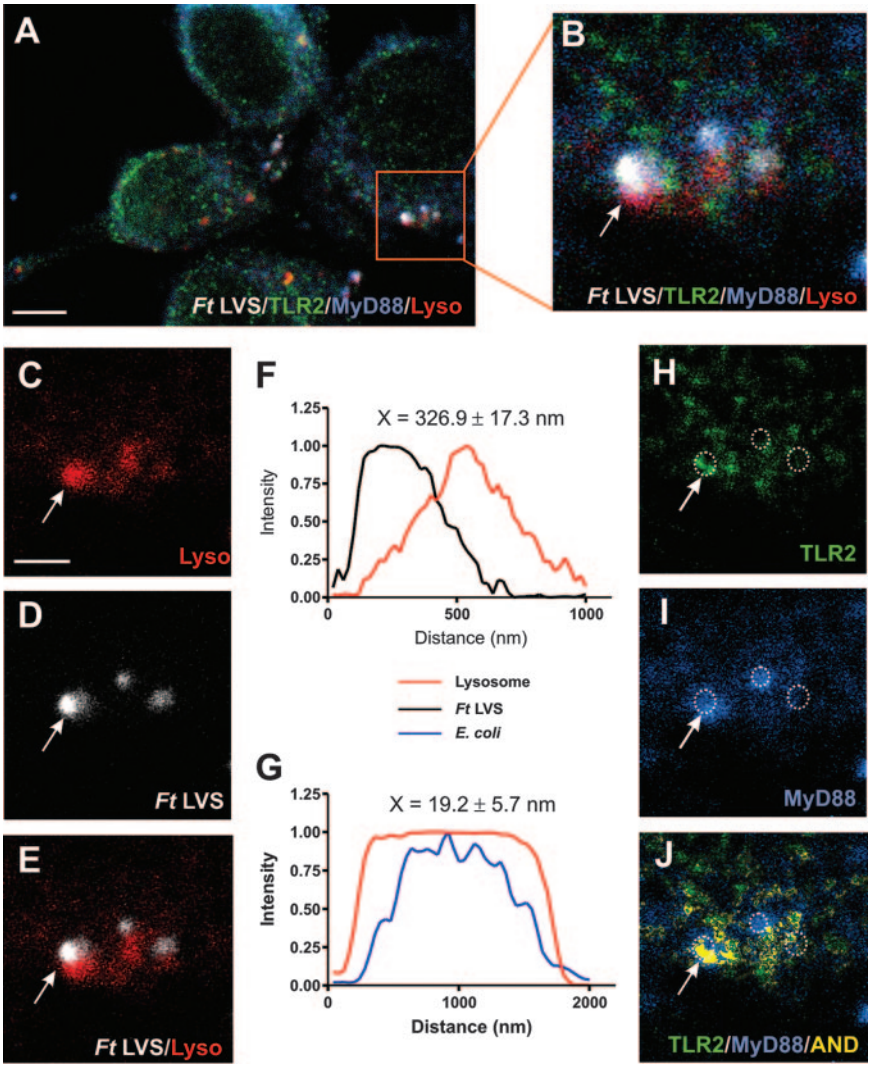

FIG. 2. Confocal imaging of *F. tularensis* LVS, TLR2, MyD88, and lysosomes in murine macrophages. Macrophages were stained for *F. tularensis* LVS (white), lysosomes (red), TLR2 (green), and MyD88 (blue). The image shows RAW 264.7 cells that have been exposed to fluorescently labeled, live *F. tularensis* LVS for 1 h (MOI, ~5). (A) In this image, four cells are seen interacting with *F. tularensis* LVS. TLR2 stains the RAW 264.7 cells primarily at the cell surface, while the signaling adapter MyD88 is more diffusely distributed within the cytoplasm. Lysosomes appear as discrete clusters within the cells. The boxed area in panel A has been enlarged in panel B for better visualization of the four-color labeling. Panels C, D, H, and I illustrate the individual staining patterns for lysosomes (C), *F. tularensis* LVS (D), TLR2 (H), and MyD88 (I). (E) Pairwise staining for *F. tularensis* LVS and lysosomes. (F and G) *F. tularensis* LVS (F) and *E. coli* (G) representative centroid plots for the distances between the centers of the bacteria and their respective lysosomes. The centroid-centroid distance between *F. tularensis* LVS (F) and lysosomes was significantly different ($n = 10$; $P \leq 0.001$, Mann-Whitney rank sum test), while the average distance between *E. coli* (G) and its corresponding lysosome was not. The positions of the three bacteria in panel D are depicted as dotted circles in panels H to J to show the positioning of TLR2 (H), MyD88 (I), and TLR2 and MyD88 (J) relative to *F. tularensis* LVS. To visualize the colocalization of TLR2 and MyD88, the overlap between MyD88 (blue) and TLR2 (green) has been pseudocolored yellow (J). Bars, 5 μm (A) or 2 μm (C); the scale bar shown in panel C also applies to panels D, E, and H to J.

was also a feature of *F. tularensis* LVS infection in vivo, mice were injected i.p. with ~40,000 CFU and sacrificed 1, 2, or 3 days later. TLR2 and TLR4 mRNA expression was analyzed in RNA extracted from livers of infected mice. As was observed in vitro, *F. tularensis* LVS infection increased TLR2 mRNA expression in a time-dependent fashion, while TLR4 mRNA expression was not significantly modulated (Fig. 3A). These data indicate that *F. tularensis* LVS is able to increase expression of the gene that encodes its own receptor.

***F. tularensis* LVS-induced TLR2 activation requires de novo protein synthesis by the bacterium.** Having previously established that *F. tularensis* LVS is able to activate NF-κB through TLR2 (8, 30) and that *F. tularensis* LVS LPS is not a TLR2 agonist (8), we sought to define further the TLR2 signaling requirements of *F. tularensis* LVS. When *F. tularensis* LVS were killed by heat treating (100°C for 10 min) or treatment with 4% formalin for 15 min, activation of the NF-κB luciferase reporter in TLR2-expressing HEK293T cells was reduced

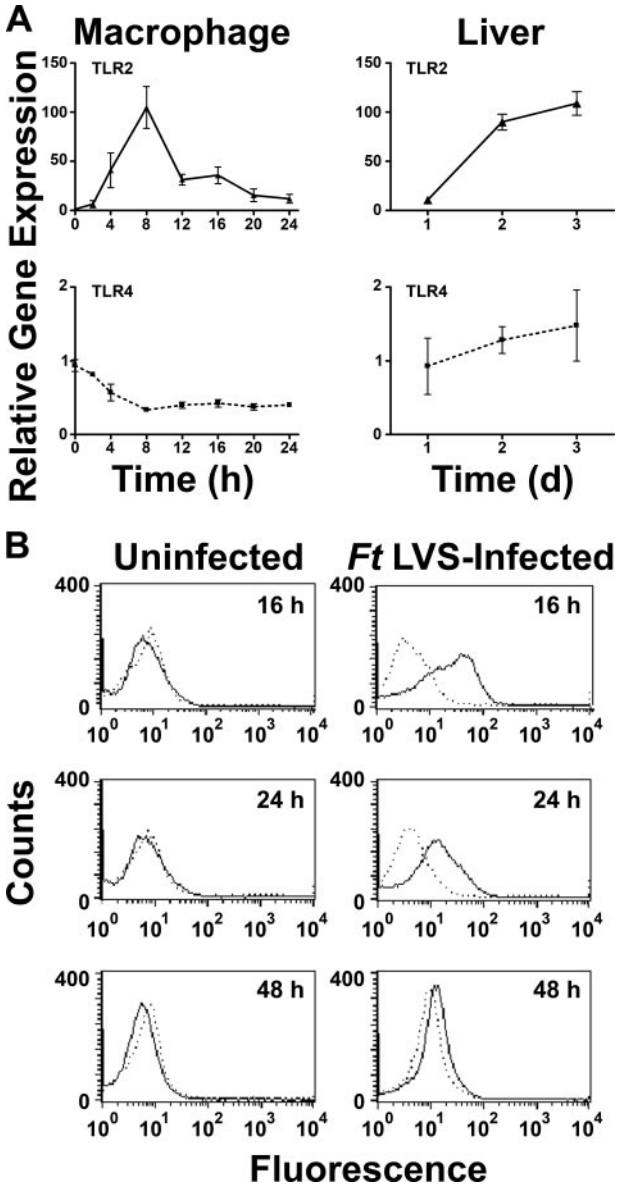

FIG. 3. *F. tularensis* LVS increases the expression of TLR2 both in vitro and in vivo. (A) Total RNA was extracted from peritoneal macrophages from C57BL/6J mice exposed to *F. tularensis* LVS (MOI, 5) for 0 to 24 h (left panel) or from the livers of C57BL/6 mice 24, 48, or 72 h after i.p. injection of 4×10^4 *F. tularensis* LVS (right panel). cDNA was made from the extracted RNA, and expression of TLR2 (▲, solid lines) and TLR4 (■, dashed lines) was analyzed by real-time PCR. Genes are expressed in terms of relative gene expression compared to levels present in peritoneal macrophages exposed only to medium or livers of mice injected with saline only. For the in vitro macrophage experiments (left panels), all treatments were performed in triplicate; the data shown are derived from a single representative experiment ($n = 5$). For the in vivo experiment (right panels), livers from five mice were examined at each time point. (B) FACS profiles of peritoneal macrophages coincubated without (left panels) or with (right panels) *F. tularensis* LVS (MOI, 10) for 16, 24, or 48 h. The peritoneal macrophages were stained with phycoerythrin-conjugated anti-mouse TLR2 (solid lines) or mouse IgG1 isotype control (dashed lines) antibody and were analyzed via FACScan.

significantly (Fig. 4A). Bacterial killing was confirmed by lack of growth on MH plates. Pretreatment of *F. tularensis* LVS with CAP, an antibiotic that inhibits bacterial, but not mammalian, protein synthesis (61), also led to a significant reduction in *F. tularensis* LVS-mediated NF-κB activation (Fig. 4A). These findings were confirmed using a second antibiotic, streptomycin sulfate (data not shown). CAP had no impact on TLR2 stimulation by Pam3Cys (data not shown), indicating that the CAP does not exert a nonspecific inhibitory effect on TLR2-mediated signaling. Although CAP was solubilized in 100% ethanol, addition to HEK293/TLR2 cultures of an equivalent amount of ethanol alone had no impact on *F. tularensis* LVS-mediated activation of NF-κB (data not shown). Taken together, these data indicate the *F. tularensis* LVS-induced activation of TLR2 requires de novo protein synthesis by the bacterium. Heat or CAP treatment of *F. tularensis* LVS prior to stimulation of C57BL/6 macrophages resulted in markedly reduced expression of IL-1β (an early gene) and IFN-γ (a late gene) mRNA (Fig. 4B), as well as IL-12 p35, IL-12 p40, iNOS, KC, RANTES, and TNF-α mRNA (data not shown). Collectively, these data indicate that macrophage cytokine gene expression is also dependent on the ability of *F. tularensis* LVS to synthesize proteins.

TLR2-mediated signaling by *F. tularensis* LVS does not require bacterial replication. The *guaA* gene in *F. tularensis* encodes a critical enzyme in the guanine nucleotide biosynthetic pathway. Recent efforts to develop a vaccine for *F. tularensis* have led to the engineering of a Δ*guaA* mutant strain of *F. tularensis* LVS. The LVSΔ*guaA* mutant strain was created by deletion of the entire *guaA* gene and replacing it with a kanamycin resistance cassette; this mutant strain is both autotrophic for guanine and attenuated for virulence in mice (47; Santiago et al., unpublished). In the absence of exogenous guanine, the LVSΔ*guaA* mutant fails to grow in medium or replicate in the J774 macrophage cell line or in BALB/c mice (data not shown). Therefore, we utilized this auxotrophic mutant to determine if bacterial replication was necessary for activation of NF-κB or cytokine gene expression through TLR2. Regardless of whether exogenous guanine was present or not, the LVSΔ*guaA* strain activated NF-κB in HEK293T cells that overexpress TLR2 (Fig. 5A). This demonstrates that neither extracellular nor intracellular replication of *F. tularensis* LVS is required for signaling through TLR2. Similar findings were observed in primary murine macrophages infected with the LVSΔ*guaA* strain compared to WT *F. tularensis* LVS. Figure 5B illustrates that by 4 h after infection, TNF-α gene expression was induced comparably when macrophages were infected with WT or mutant LVSΔ*guaA*. By 16 h postinfection of macrophages, TNF-α mRNA levels had returned to baseline. Thus, in both HEK293T cells that overexpress TLR2 and in macrophages, bacterial replication is not required for TLR2-mediated signaling.

DISCUSSION

Recognition of bacteria by PRRs is a fundamental aspect of the innate immune response to pathogens. Impaired recognition can lead to severe illness and death. For example, mutations or TLR polymorphisms that affect the interaction of TLR with either agonists or signaling proteins have been associated

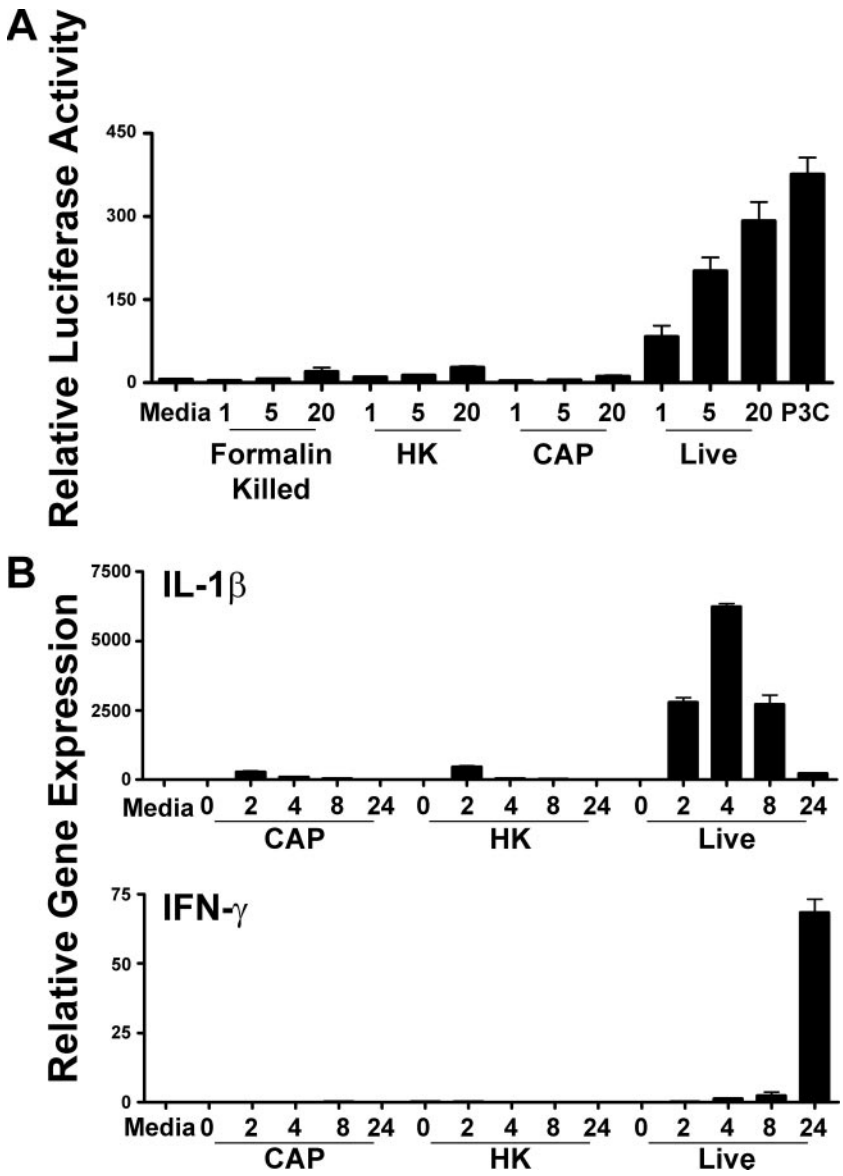

FIG. 4. *F. tularensis* LVS-induced signaling in TLR2-expressing HEK293T cells and murine macrophages is heat and formalin sensitive and requires de novo bacterial protein synthesis. (A) HEK293T cells transiently transfected with TLR2 were exposed to either live, heat-killed, or CAP-treated *F. tularensis* LVS at the indicated MOI or the TLR2 agonist Pam3Cys (P3C) for 24 h. After incubation, cells were lysed and relative luciferase activity was calculated by normalizing each sample's luciferase activity to the constitutive β-galactosidase activity measured within the same sample. Data are presented as means ± SEM from five individual experiments. (B) Peritoneal macrophages from C57BL/6J mice were exposed to *F. tularensis* LVS (MOI, 5) for 0 to 24 h. At the indicated time points, RNA was extracted from the macrophage cultures and cDNA was made from the extracted RNA and analyzed by real-time PCR. Relative gene expression was calculated as previously described (8). Data shown were derived from a single representative experiment ($n = 3$).

with greatly increased susceptibility to infection in humans (reviewed in reference 59). We demonstrated previously that *F. tularensis* LVS is specifically recognized by TLR2 in HEK293T/TLR2 transfectants and in murine DC (8, 30) and that *F. tularensis* LVS infection induces in mice or their macrophages a very strong proinflammatory response as measured at the level of gene and protein expression (8). Thus, our demonstration herein that signaling through TLR2 is an obligatory component of the early macrophage response to *F. tularensis* LVS infection (Fig. 1) represents a key step forward in unraveling the potent proinflammatory response induced by this bacterium.

F. tularensis is an intracellular pathogen that, once internalized, can survive within both phagocytic and nonphagocytic cells (54). Within macrophages, *F. tularensis* inhibits phagosome-lysosome fusion and ultimately escapes into the cytoplasm, where the organisms replicate (22, 32). As TLR2 is recruited to macrophage phagosomes (58), the initial engagement of TLR2 by *F. tularensis* LVS potentially allows for signaling both at the cell surface, where TLR2 is localized, as well

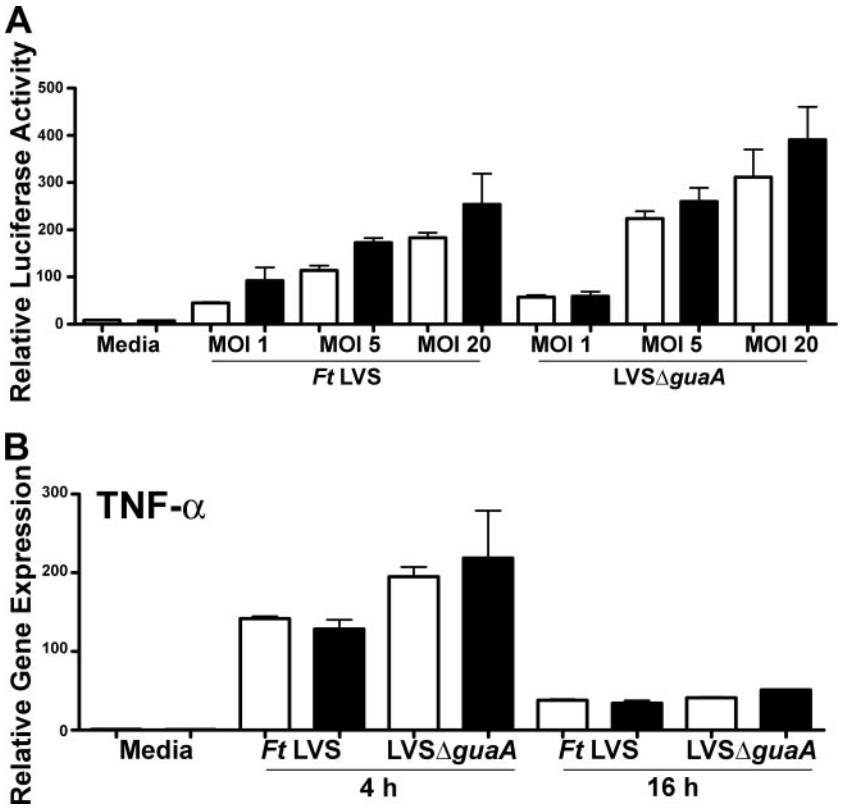

FIG. 5. *F. tularensis* LVS does not require replication to activate NF-κB in HEK293T cells transiently transfected with TLR2 or TNF-α expression in murine macrophages. (A) HEK293T cells transiently transfected with TLR2 were exposed to either live *F. tularensis* LVS or the auxotrophic *guaA* mutant (LVSΔ*guaA*) at the indicated MOI, or the TLR2 agonist P3C (1 μg/ml), for 24 h. Medium only (open bars) or exogenous guanine (closed bars) was added to the cultures concurrent with the addition of the bacteria. After infection, cells were lysed and relative luciferase activity was determined by normalizing each sample's luciferase activity with the constitutive β-galactosidase activity measured within the same sample. Data are presented as means ± standard errors of the means. Data shown are derived from a single representative experiment. (B) Thioglycolate-elicited peritoneal macrophages from C57BL/6J mice were stimulated with either *F. tularensis* LVS or LVSΔ*guaA* in the absence (open bars) or presence (closed bars) of exogenously added guanine. At the indicated time points, total RNA was extracted from the macrophage cultures and analyzed using real-time PCR. TNF-α gene expression is reported as relative gene expression above levels present in peritoneal macrophages exposed only to medium. The data presented are derived from a single representative experiment ($n = 3$).

as within the phagosome, as has been demonstrated for other organisms that express TLR2 PAMPs (2, 42, 58). Our confocal data support the hypothesis that once the *F. tularensis* LVS is taken up into the macrophage, MyD88 and TLR2 colocalize within close proximity of the phagosome membrane. The observation that the lysosomes fail to distribute themselves around the phagocytosed bacterium is consistent with the previous observations that *F. tularensis* blocks phagosome-lysozome fusion. These data suggest the intriguing possibility that intracellular signaling might be prolonged as a consequence of the organisms remaining in contact with the interior of the phagosome for a longer period of time. This notion is consistent with our observation that proinflammatory gene expression was observed for more than 24 h postinfection. We also demonstrate herein that *F. tularensis* LVS infection also greatly increased expression of TLR2 both in vitro in macrophage cultures and in vivo in livers of infected mice (Fig. 3A and B), and this was confirmed in vitro at the level of protein expression by FACS analysis (Fig. 3C). Thus, it is tempting to speculate that *F. tularensis* LVS also sustains the proinflammatory response, both by inhibiting phagosome-lysosome fusion (i.e., keeping the organism in contact with intraphagosomal TLR2 for a longer period of time) and, perhaps, by increasing TLR2 expression. At this juncture, we do not know the nature of the specific TLR2 agonist; however, our data showing that the *F. tularensis* LVS-induced TLR2 agonist activity was eliminated by chloramphenicol treatment of the organism indicate that the *F. tularensis* LVS TLR2 agonist is dependent upon bacterial protein synthesis in addition to being sensitive to high heat or formalin denaturation. Experiments to identify the biochemical nature of this PAMP are ongoing.

In contrast to the results seen with peritoneal macrophages derived from TLR2$^{-/-}$ mice, macrophages from WT mice displayed robust up-regulation of various cytokines and chemokines. TNF-α levels peaked very rapidly after infecting macrophages with *F. tularensis* LVS and then decreased, as reported previously (56). This same pattern was also seen with IL-1β and KC. The early expression of TNF-α, IL-1β, and KC potentially acts to recruit neutrophils to the site of infection and activate the inflammatory response regionally. In contrast, mRNA expression of IL-12 p35 and IL-12 p40 peaked later, between 8 and 12 h postinfection, and the levels of IFN-β, IFN-γ, iNOS, IP-10, and RANTES mRNA rose throughout

much of the time course and peaked 12 h or later postinfection. To our knowledge, this represents the first large-scale temporal analysis of cytokine and chemokine expression in macrophages following infection by *F. tularensis* LVS. Induction of proinflammatory cytokine and chemokine gene expression achieved in macrophages upon infection with *F. tularensis* LVS is similar to that observed in macrophages stimulated with the potent proinflammatory agent *E. coli* LPS (8). Expression of TLR2 mRNA and protein was strongly increased by *F. tularensis* LVS infection (Fig. 3A and B). In contrast, *F. tularensis* LVS infection of murine macrophages led to a reduction in expression of TLR4 mRNA by approximately half that measured in uninfected macrophages (Fig. 3A). The observed decrease in TLR4 mRNA induced by infection of macrophage cell cultures with *F. tularensis* LVS was observed in vitro but could not be confirmed by FACS analysis due to very low expression. Nonetheless, it is possible that such a decrease may account for the previous observation that *F. tularensis* LVS-infected macrophages have a diminished response to treatment with the TLR4 agonist LPS (55, 56). Other TLR2 agonists, peptidoglycan (33) and porin of *Shigella dysenteriae* type 1 (45), as well as the TLR4 agonist LPS (18, 38), have previously been shown to up-regulate expression of TLR2 in murine macrophages. The TLR2 agonist *Borrelia burgdorferi* lipoprotein increases expression of TLR2 and TLR1 while decreasing the expression of TLR5 and flagellin-induced TLR5 signaling (3). Such pathogen-induced changes in TLR expression suggest a strategy by which pathogens alter host responsiveness to themselves or other pathogens.

Recently, a family of intracellular sensing PRRs, originally called CATERPILLERs (26) but now called nucleotide-binding oligomerization domain–leucine-rich repeats (NOD-LRRs) proteins and NACHT leucine-rich repeats proteins, has been identified (reviewed in reference 37). Much like the TLRs, these PRRs detect conserved pathogenic structures through leucine-rich regions, but unlike TLR-mediated detection, pathogen sensing occurs exclusively within the cytosol. Some of the members of this protein family can assemble to form a cytosolic complex of proteins referred to as an "inflammasome," which responds to intracellular bacteria by activating the serine protease caspase 1 (36). Once activated, caspase 1 cleaves biologically inactive 31-kDa pro-IL-1β, resulting in the generation of the mature, 17-kDa active form of IL-1β. Mariathasan et al. recently reported that caspase 1 is essential for *F. tularensis* LVS-induced secretion of the proinflammatory pyrogen IL-1β (35), and Gavrilin et al. (21) recently showed that IL-1β is only released from human $CD14^+$ monocytes if *Francisella novicida* escapes from the phagosome. We have demonstrated herein that macrophage IL-1β gene expression and protein secretion (Fig. 1) are wholly dependent upon TLR2, as $TLR2^{-/-}$ macrophages infected with *F. tularensis* LVS did not increase IL-1β mRNA and no IL-1β protein was detected in the supernatants of $TLR2^{-/-}$ macrophages (Fig. 1A and B). Taken together, these data suggest a two-step model of IL-1β secretion where TLR2 recognition of *F. tularensis* leads to production of pro-IL-1β while cleavage and secretion of the biologically active IL-1β is dependent upon later assembly and activation of the inflammasome, after the organism escapes from the phagosome.

Our data showing a key role for TLR2 in the recognition and proinflammatory response to *F. tularensis* LVS in macrophages would predict that TLR2 would also play an important role in the control of an in vivo infection. However, based on previously published data, the results have been mixed. $TLR2^{-/-}$ mice have been reported to be more susceptible to pulmonary infection after intranasal *F. tularensis* LVS inoculation than WT mice (34). Those authors reported a significantly higher *F. tularensis* bacterial burden (nearly 3 logs) was recovered from lungs of $TLR2^{-/-}$ mice compared to the bacterial recovery from WT mice (34). When bone marrow-derived macrophages derived from $TLR2^{-/-}$ or WT mice were infected with *F. tularensis* LVS at an MOI of 100, significantly more *F. tularensis* LVS was recovered from the $TLR2^{-/-}$ macrophages (34). Additionally, the $TLR2^{-/-}$ macrophages were more sensitive to *F. tularensis* LVS-induced cell death than their WT counterparts (34). Taken together, these data suggest that in the absence of TLR2, *F. tularensis* LVS-induced macrophage cytokine and chemokine expression is greatly mitigated, leading to uncontrolled intracellular *F. tularensis* LVS growth that, in turn, underlies enhanced *F. tularensis* LVS-induced eukaryotic cell death.

In contrast, Collazo et al. reported that $TLR2^{-/-}$ mice were only slightly more susceptible than WT animals to intradermal injection of *F. tularensis* LVS, while MyD88-deficient animals were extremely susceptible (9). This is significant, because TLR2 activation of NF-κB is highly dependent upon the adapter protein MyD88 (31, 53). These observations suggest that while TLR2 recognition of *F. tularensis* LVS is extremely important, additional MyD88-dependent pathways may be involved in control of *F. tularensis* LVS infection. Although IL-1 and IL-18 also utilize MyD88 for signaling, IL-18 knockout mice are not significantly more susceptible to intradermal *F. tularensis* LVS infection, and IL-1R knockout mice were, at best, slightly more susceptible (9). MyD88 functions as an adapter protein not only for TLR2, but also for TLR4, -5, -7, and -9. However, while TLR4 typically plays a major role in control of gram-negative infections, at best, it plays a minor role in controlling *F. tularensis* LVS infection (5, 6, 9).

In addition to the increased susceptibility of $TLR2^{-/-}$ mice to pulmonary infection, lung homogenates from infected $TLR2^{-/-}$ mice were reported to contain significantly lower, but detectable, levels of TNF-α and IL-6 and increased levels of IFN-γ and MCP-1 compared to infected WT mice (34). This suggests the possibility that additional, regional forms of pathogen recognition are involved, particularly in IFN-γ and MCP-1 synthesis. It is also possible that after *F. tularensis* LVS gains entry into cells and/or initiates intracellular signaling through TLR2, it replicates and moves into the cytosol, where other structural components of the organisms, e.g., muramyl dipeptide, DNA, RNA, or other proteins, can be readily sensed by intracytosolic or endosomally associated PRRs.

In conclusion, we have demonstrated a pivotal role for TLR2 in the macrophage recognition and response to *F. tularensis* LVS. In the absence of TLR2, recognition of *F. tularensis* LVS and cytokine and chemokine synthesis by macrophages are virtually eliminated. Taken together, these data represent a significant step forward in the understanding of the interaction of *F. tularensis* LVS and the innate immune system.

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