

Role of Toll-Like Receptor 4 in Macrophage Activation and Tolerance during *Salmonella enterica* Serovar Typhimurium Infection

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Toll-like receptors (TLRs) play an important role in the innate immune response, particularly in the initial interaction between the infecting microorganism and phagocytic cells, such as macrophages. We investigated the role of TLR4 during infection of primary murine peritoneal macrophages with *Salmonella enterica* serovar Typhimurium. We found that macrophages from the C3H/HeJ mouse strain, which carries a functionally inactive Tlr4 gene, exhibit marked impairment of tumor necrosis factor alpha (TNF- α) secretion in response to *S. enterica* serovar Typhimurium infection. However, activation of extracellular growth factor-regulated kinase and NF- κ B signaling pathways was relatively unaffected, as was increased expression of TNF- α mRNA. Furthermore, macrophage tolerance, which is associated with increased expression of the NF- κ B p50 and p52 subunits, was induced by *S. enterica* serovar Typhimurium even in the absence of functional TLR4. These results indicate that during infection of macrophages by *S. enterica* serovar Typhimurium, TLR4 signals are required at a posttranscriptional step to maximize secretion of TNF- α . Signals delivered by pattern recognition receptors other than TLR4 are sufficient for the increased expression of the TNF- α transcript and at least some genes associated with macrophage tolerance.

Serovars of *Salmonella enterica* subspecies I cause two types of clinical problems in humans, both of which are important public health concerns. *S. enterica* serovar Typhimurium is a leading agent of food poisoning, an acute, generally self-limiting disease characterized by intestinal inflammation and diarrhea (42). *S. enterica* serovar Typhi causes typhoid, a systemic febrile illness that is responsible for significant morbidity and mortality worldwide, particularly in developing countries (21). *S. enterica* serovar Typhimurium also causes a typhoid-like disease in wild-type mice, and much of what we know about the host-pathogen interactions involved in *Salmonella* infections has been derived from studies with the mouse model. Like *S. enterica* serovar Typhi in human typhoid, *S. enterica* serovar Typhimurium invades the intestinal epithelium in the mouse and is then taken up by phagocytic cells in the gut-associated lymphoid tissue. From that site, the organism disseminates systemically and survives intracellularly for extended periods within splenic macrophages and hepatic Kupffer cells (45, 55). The interaction between *S. enterica* serovar Typhimurium and these macrophage populations results in activation of protective mechanisms, such as the production of reactive oxygen and nitrogen species, and increased expression of a number of proinflammatory genes, such as those encoding tumor necrosis factor alpha (TNF- α), interleukin-1, and interleukin-18 (4, 30, 50). Together with the development of adaptive immunity, these innate immune responses result in the generation of granulomatous foci that help to confine and control the infection.

A key aspect of the interaction between *Salmonella* and host

macrophages is the activation of mammalian pattern recognition receptors (PRRs) that recognize conserved microbial molecules, often referred to as pathogen-associated molecular patterns (16). The best-studied of the mammalian PRRs are the surface proteins of the Toll-like receptor (TLR) family, and currently there are 10 known members of this family (33). TLR2, in a heterodimeric complex with either TLR1 or TLR6, responds to bacterial peptidoglycan (PG) and lipopeptides, while TLR4, TLR5, and TLR9 recognize lipopolysaccharide (LPS), flagellin, and hypomethylated CpG-rich DNA, respectively. In general, TLR4 has been considered to be the major receptor for gram-negative bacteria, while the recognition of gram-positive bacteria has been ascribed to TLR2 (48). TLRs are connected via the adaptor protein MyD88 to a signal transduction machinery that is utilized by all members of the family (33). Not surprisingly, they induce similar patterns of gene expression, including those involved in microbicidal and inflammatory responses. However, it is becoming increasingly clear that certain signaling mechanisms are specific to individual TLRs (20, 54), with the result that different patterns of gene expression are induced by different TLR agonists (8, 18, 23, 43, 49).

Prolonged interactions between microbial components and macrophages can lead to a state of subsequent hyporesponsiveness to the same stimulus or a related stimulus, a phenomenon known as tolerance. The mechanisms underlying the induction of macrophage tolerance have been the subject of intense study, particularly with respect to TLR ligands. Some of the mechanisms that have been implicated in this process include down-regulation of TLR4 (37); decreased expression of postreceptor signaling molecules, such as the interleukin-1 receptor-associated kinase (IRAK) (32); alterations in the association between TLR5 and downstream signaling molecules (34); increased expression of proteins that block TLR signal

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transduction, such as IRAK-M, the macrophage-specific, kinase-deficient member of the IRAK family (27), and SOCS-1 (suppressor of cytokine signaling 1) (26, 36); and, finally, up-regulation of the p50 and p52 subunits of the NF- κ B transcription factor (24, 51). Although the exact physiologic significance of macrophage tolerance is not clear, it may be important in limiting the extent and duration of inflammatory responses. In keeping with this idea, disruption of tolerance by genetically engineered mutations results in exaggerated *in vitro* and *in vivo* inflammatory responses to bacteria or bacterial products (3, 26, 27, 36). Furthermore, in mice with a disruption of the NF- κ B p50 gene, oral infection with *Helicobacter hepaticus* results in a chronic inflammatory bowel disease, illustrating the importance of macrophage tolerance to normal immune homeostasis in the intestine (12).

Most studies of TLR-dependent macrophage activation and tolerance have been carried out with purified microbial components, and less is known about the role of the TLRs in these processes during the course of infection by whole bacteria, which express multiple PRR ligands. In particular, the role of PRRs other than TLR4 has been given relatively little attention in the context of the response to gram-negative bacteria, such as salmonellae. In order to understand and ultimately manipulate the *Salmonella*-macrophage interaction, it is necessary to know which responses to the organisms are in fact caused by activation of TLR4 and which are attributable to activation of receptors other than TLR4. To elucidate the role of TLR4 and other PRRs in macrophage responses to salmonellae, we examined the interaction between *S. enterica* serovar Typhimurium and primary peritoneal macrophages from either the C3H/HeOuJ mouse strain (wild type) or the C3H/HeJ strain, which carries a naturally occurring missense mutation in the TLR4 cytoplasmic domain that prevents signaling through the receptor (40). We report here that the production of TNF- α , a key cytokine involved in inflammatory and protective responses to salmonellae (30), is significantly compromised at a posttranscriptional step in macrophages that lack functional TLR4. However, activation of proximal signaling pathways and induction of genes associated with tolerance still occur in these cells, indicating that TLR4-independent mechanisms are operating.

MATERIALS AND METHODS

Reagents. Standard laboratory chemicals were purchased from Sigma (St. Louis, Mo.) or from Fisher Scientific (Suwanee, Ga.). Highly purified LPS from *Salmonella* strain Minnesota R595 was obtained from List Biological Laboratories (Campbell, Calif.), and stock solutions were made in sterile water. *Staphylococcus aureus* PG was purchased from Fluka (Milwaukee, Wis.) and was dissolved in sterile water according to the manufacturer's instructions. Antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, Calif.) unless specified otherwise.

Preparation of peritoneal macrophages. The TLR4 mutant C3H/HeJ strain (40) and the corresponding wild-type C3H/HeOuJ strain of mice were purchased from Jackson Laboratories, Bar Harbor, Maine. Thioglycollate-elicited macrophages were prepared by using a standard protocol (13). In brief, the mice were injected intraperitoneally with 1.5 ml of sterile 3% Brewer's thioglycollate (Becton-Dickinson Microbiology Systems, Sparks, Md.) per mouse. On day 5 after injection, macrophages were harvested from the peritoneal cavity by lavaging with sterile phosphate-buffered saline. The macrophages were centrifuged at 400 \times g for 10 min, resuspended in Dulbecco's modified Eagle medium (Invitrogen Corporation, Carlsbad, Calif.) with 10% heat-inactivated fetal calf serum and penicillin-streptomycin, and distributed into the wells of 24-well plates. After 2 h of incubation at 37°C, nonadherent cells were washed away, which left wells with

80 to 90% confluent adherent macrophages behind (approximately 0.5×10^6 macrophages per well). The macrophages were rested overnight before stimulation. Before treatments were started or before *Salmonella* infection, the cells were washed and recultured in medium without antibiotics.

Bacterial culture and infections. *S. enterica* serovar Typhimurium strains (wild-type strains SL1344 [53] and SL3201; flagellin-deficient strain SL3201 *fljC/fljB*, isogenic with SL3201, kindly provided by Andrew Gewirtz, Emory University [15]; HflA-deficient strain VV341, isogenic with SL1344 [22]) were grown to the stationary phase in Luria-Bertani (LB) medium at 37°C with constant shaking. Wild-type *Bacillus subtilis* strain PY79 was kindly provided by David Rudner, Harvard University, and was grown similarly. The bacteria were pelleted by centrifuging at 13,000 \times g for 5 min, washed with sterile phosphate-buffered saline, and then resuspended in antibiotic-free medium at a density of 4×10^8 cells/ml. Twenty-five-microliter aliquots of each suspension (10^7 bacteria) were used to infect each well of macrophages (multiplicity of infection [MOI], 20:1). Heat-killed bacteria were prepared by placing a bacterial suspension in boiling water for 10 min. Bacterial culture supernatants were prepared from stationary-phase cultures of wild-type or flagellin-deficient *S. enterica* serovar Typhimurium. After centrifugation of the bacteria at 13,000 \times g for 5 min, the supernatants were filtered through a 0.2- μ m-pore-size syringe filter (Nalge, Rochester, N.Y.) and added to cell cultures at a final concentration of 5% (vol/vol).

ELISA for TNF- α . Duplicate wells containing peritoneal macrophages were treated or infected as described below for the individual experiments, and supernatants were collected after 2 h. The supernatants were centrifuged at 13,000 \times g for 5 min at 4°C to remove the bacteria, and 50- μ l aliquots were analyzed in triplicate by an enzyme-linked immunosorbent assay (ELISA) by using a standard protocol (6). In brief, TNF- α in the supernatant was captured with purified rat anti-mouse TNF- α (Pharmingen, San Diego, Calif.) fixed to an Immulon 2HB microtiter plate (ThermoLab Systems, Franklin, Mass.). The captured TNF- α was detected with biotinylated rat anti-mouse TNF- α (Pharmingen), followed by streptavidin-conjugated horseradish peroxidase and *o*-phenylene diamine substrate (the latter two reagents were obtained from Zymed Laboratories, San Francisco, Calif.). The intensity of the color reaction was measured by determining the absorbance at 405 nm with an Emax microplate reader (Molecular Devices, Sunnyvale, Calif.). TNF- α concentrations were calculated based on a standard curve that was generated by using purified mouse recombinant TNF- α (Pharmingen).

After the supernatants were removed, the macrophages were washed and lysed in 1% Triton X-100. The protein concentrations of the lysates were determined with a DC protein assay kit (Bio-Rad, Hercules, Calif.) by following the manufacturer's recommendations. The normalized amount of TNF- α was derived by dividing the amount of TNF- α produced in each well by the total amount of protein in the cell lysate from that well.

Western blotting. Macrophages were lysed in 1% Triton X-100 in a solution containing 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10 μ g of aprotinin per ml, 10 μ g of leupeptin per ml, and 2 mM phenylmethylsulfonyl fluoride. Aliquots of clarified cell lysate, normalized for total protein content, were separated on sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis gels and transferred to nitrocellulose by using a semidry blotting apparatus as previously described (5). The blots were blocked with 5% nonfat dry milk and then incubated overnight at 4°C with the relevant primary antibody (mouse anti-phosphorylated extracellular growth factor-regulated kinase [anti-phospho-ERK], mouse anti-I κ B α , goat anti-NF- κ B p50, or mouse anti-NF- κ B p52, all obtained from Santa Cruz Biotechnology). The bound primary antibodies were detected by enhanced chemiluminescence by using the appropriate horseradish peroxidase-conjugated anti-immunoglobulin antibodies (Zymed Laboratories) and the West Pico Super Signal substrate reagent (Pierce, Rockford, Ill.), as described in detail previously (5). After the appropriate autoradiographic exposures were made, the blots were stripped and reprobed with either rabbit anti-total ERK (in the case of the phospho-ERK blot) or goat anti-actin (in the case of the I κ B α , p50, and p52 blots), both obtained from Santa Cruz Biotechnology.

For quantitation of the Western blots, the autoradiographs were scanned and analyzed with NIH Image software to obtain the mean pixel density of each band. To normalize the phospho-ERK signal, the pixel density of the phospho-ERK band was divided by the pixel density of the corresponding total ERK band. Similarly, the I κ B α and the NF- κ B p50 and p52 bands were normalized by using the corresponding actin bands.

RT-PCR analysis. Total RNA was prepared from control or *Salmonella*-infected cells with the Trizol reagent (Invitrogen Corporation) by following the manufacturer's directions. Reverse transcription-PCR (RT-PCR) was carried out by using a GeneAmp kit (Roche, Nutley, N.J.) and following the protocol provided with the kit. In brief, 100-ng aliquots of total RNA were reverse transcribed at 42°C for 15 min with Moloney murine leukemia virus reverse

transcriptase after annealing to random hexamers. One-quarter of each RT reaction mixture was used for PCR amplification with *Taq* polymerase by using commercially available primers specific for murine TNF- α or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (R & D Systems, Minneapolis, Minn.). Thirty cycles of amplification were carried out, and each cycle consisted of denaturation at 95°C for 1 min, annealing at 54°C for 1 min, and extension at 72°C for 2 min. One-quarter of each amplification reaction mixture was electrophoresed on a 1.2% agarose gel and photographed under UV transillumination.

Real-time quantitative PCR analysis of TNF- α and GAPDH expression was carried out as described above, except that the reverse-transcribed RNA and primers were mixed with a SYBR Green master mixture (Applied Biosystems, Foster City, Calif.) instead of the usual PCR mixture components. Duplicate reaction mixtures were set up, and 40 cycles of amplification were carried out with an iCycler (Bio-Rad) by using the same conditions that were used for the standard PCR. A quantitative measure of the relative amounts of transcript was obtained based on the literature provided with the iCycler. In brief, SYBR Green is a double-stranded DNA-specific fluorescent dye, the fluorescence intensity of which is proportional to the amount of double-stranded DNA. The iCycler monitors the fluorescence of the reaction mixtures just before the denaturation step of each amplification cycle, and it records the cycle number at which the fluorescence crosses a specific threshold value in the exponential phase of amplification (the threshold cycle [C_t]). C_t is thus a measure of the quantity of the transcript of interest, and lower C_t values indicate larger starting quantities of transcript. The amount of TNF- α transcript was normalized to the amount of GAPDH transcript by subtracting the mean C_t value of the latter from the mean C_t value of the former (for each experimental condition). The difference between the normalized C_t values of the *Salmonella*-infected cells and the control, uninfected cells was plotted and used as a measure of the increase in TNF- α mRNA expression resulting from the infection. Because of the exponential nature of the PCR, a difference of n in C_t values represented a 2^n -fold difference in the transcript levels.

Statistical analysis. Statistical significance was determined by the Student t test, and a P value of <0.05 was considered significant.

RESULTS

Lack of a functional TLR4 significantly impairs TNF- α production during infection of macrophages by *Salmonella*. Infection of thioglycollate-elicited peritoneal macrophages from wild-type C3H/HeOuJ mice for 2 h with wild-type *S. enterica* serovar Typhimurium strain SL1344 at an MOI of 20 resulted in an approximately 100-fold increase in the amount of TNF- α secreted compared to the amount secreted by the control, uninfected cells, an effect that was similar to the effect observed after treatment with purified LPS (Fig. 1) (for control cells, 0.27 ± 0.44 pg/ μ g; for *Salmonella*-infected cells, 34.35 ± 5.51 pg/ μ g; for LPS-treated cells, 27.38 ± 6.00 pg/ μ g). In contrast, when macrophages from the C3H/HeJ strain, which has an inactivating point mutation in the TLR4 cytoplasmic domain (40), were used, the TNF- α responses to both LPS and *S. enterica* serovar Typhimurium were significantly lower than the corresponding C3H/HeOuJ responses (Fig. 1) (for control cells, 0.27 ± 0.42 pg/ μ g; for *Salmonella*-infected cells, 1.28 ± 1.01 pg/ μ g; for LPS-treated cells, 0.12 ± 0.12 pg/ μ g [$P < 0.001$ for the comparison of the responses to *S. enterica* serovar Typhimurium of C3H/HeOuJ and C3H/HeJ cells; $P < 0.0005$ for the comparison of the responses to LPS]). Treatment of the C3H/HeJ macrophages with the TLR2 ligand staphylococcal PG led to an approximately 35-fold increase in the amount of TNF- α secreted (Fig. 1) (for control cells, 0.27 ± 0.42 pg/ μ g; for PG-treated cells, 9.50 ± 3.68 pg/ μ g). This response indicated that the TLR2 signaling pathway was relatively preserved in the TLR4 mutant cells, even though the response was significantly reduced compared to the response of the C3H/HeOuJ macrophages to PG (20.19 ± 4.14 pg/ μ g; $P < 0.0025$). The response to the gram-positive bacterium *B. subtilis* was

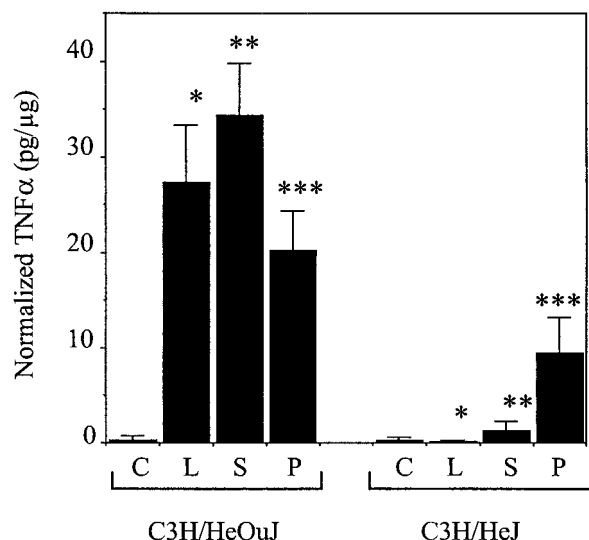


FIG. 1. Production of TNF- α by peritoneal macrophages from C3H/HeOuJ and C3H/HeJ mice. Macrophages were not stimulated (C), were stimulated with 100 ng of LPS per ml (L) or 25 μ g of PG per ml (P), or were infected with 10^7 wild-type *S. enterica* serovar Typhimurium strain SL1344 (S) for 2 h, and the supernatants were analyzed by ELISA for TNF- α . The amount of TNF- α produced was normalized to the protein content of the macrophage cell lysate. The data are the means of the results of at least four separate experiments, and the error bars indicate standard deviations. The P values for the differences between the responses of the two strains to LPS, *Salmonella*, and PG were <0.0005 (one asterisk), <0.001 (two asterisks), and <0.0025 (three asterisks), respectively.

similar to the response obtained with PG (data not shown). There was no increase in TNF- α production from the C3H/HeJ macrophages even when the dose of *Salmonella* used for infection was increased 10-fold or when the analysis was extended to 6 h after infection. These results demonstrate the importance of the LPS-TLR4 interaction in inducing TNF- α during *Salmonella* infection of macrophages. In contrast, when C3H/HeOuJ macrophages were infected with specific mutants that were either deficient in a functional *Salmonella* pathogenicity island 1 type III secretion system or lacked both flagellin subunit genes, no difference in TNF- α production was noted when the data were compared to the data for infection with wild-type *Salmonella*. Similarly, when we used a previously documented approach to inhibit TLR9 function (i.e., treatment of cells with chloroquin [1, 31]), we did not observe any effect on the amount of TNF- α produced by C3H/HeOuJ macrophages in response to wild-type *Salmonella* infection (data not shown).

Functional TLR4 is not required for activation of macrophage signaling pathways by *Salmonella*. The impaired ability of C3H/HeJ macrophages to produce TNF- α in response to *Salmonella* infection could indicate either that there was exclusive activation of TLR4 during this process or that other PRRs were unable to induce high-level production of TNF- α . To evaluate these two possibilities, we examined signaling cascades that are known to be activated by all TLRs, viz., the ERK pathway and the NF- κ B pathway (33). Macrophages from either C3H/HeOuJ or C3H/HeJ mice were infected with a wild-type *Salmonella* strain for different periods, and Western blot-

ting of cell lysates was carried out to detect the phosphorylated (activated) form of the ERK protein or degradation of the I κ B α protein (as a marker of activation of the NF- κ B pathway). Figures 2A and B show that macrophages from the TLR4 mutant C3H/HeJ strain were defective in LPS-dependent activation of ERK when they were compared to the wild-type C3H/HeOuJ macrophages. As measured by the intensity of the phospho-ERK band (normalized to total ERK), the peak activation of ERK by LPS in the C3H/HeJ macrophages was only about 4% of that seen in the C3H/HeOuJ macrophages. In contrast, the activation of ERK in response to *Salmonella* infection was only slightly reduced by the TLR4 mutation (the peak activation in C3H/HeJ macrophages was about 86% of that in C3H/HeOuJ macrophages). Similarly, although activation of the NF- κ B pathway in response to LPS was very markedly impaired in C3H/HeJ macrophages (data not shown), the degradation of I κ B α in response to *Salmonella* infection was relatively unaffected in these cells (9.4% of the starting I κ B α level was present at 45 min in the C3H/HeJ macrophages, compared with 5.7% in the C3H/HeOuJ macrophages [Fig. 2C]). These observations are consistent with the idea that *Salmonella* activates TLR4-independent signaling pathways during infection of macrophages.

To further characterize TLR4-independent activation of signaling by *Salmonella*, we repeated the experiments described above using C3H/HeJ macrophages and either heat-killed bacteria or bacterial supernatants. As shown in Fig. 2D, treatment of the macrophages with heat-killed wild-type *S. enterica* serovar Typhimurium, as well as with culture supernatants of wild-type *S. enterica* serovar Typhimurium, resulted in robust activation of ERK. An equivalent volume of culture broth (LB broth) did not have this effect, nor did LPS at a concentration of 100 ng/ml. These results indicate that molecules that are present on viable and heat-killed bacteria or are released into the culture supernatant are able to activate signaling in macrophages independent of TLR4. Flagellin was not responsible for the activity, since culture supernatant from the flagellin-deficient strain *S. enterica* serovar Typhimurium SL3201 *fljC/fljB* activated ERK as well as, if not better than, supernatants from wild-type strain SL1344 (Fig. 2D) and SL3201 (data not shown).

Functional TLR4 is not required for induction of TNF- α mRNA in response to *Salmonella*. The data presented above indicate that PRRs other than TLR4 are activated during the course of *Salmonella* infection of macrophages. However, the signals resulting from such activation are insufficient for high-level production of secreted TNF- α . To determine at which level TNF- α production was blocked, we carried out a semi-quantitative RT-PCR analysis to detect TNF- α mRNA in C3H/HeOuJ and C3H/HeJ macrophages after infection with wild-type *S. enterica* serovar Typhimurium. Figure 3A shows the result of this analysis and indicates that in both the wild-type and TLR4 mutant cells, infection with *Salmonella* led to a clear increase in TNF- α mRNA. This observation was confirmed by a quantitative, real-time PCR analysis, which indicated that the levels of induction of TNF- α mRNA in response to *Salmonella* infection were similar in macrophages from the C3H/HeOuJ and C3H/HeJ strains and were about 60-fold higher than the control value for C3H/HeOuJ mice and about 75-fold higher than the control value for C3H/HeJ mice (Fig.

3B). Thus, increased expression of TNF- α mRNA in response to *Salmonella* infection is independent of a functional TLR4, suggesting that the requirement for TLR4 signals is at a post-transcriptional step in the biosynthesis of the cytokine.

Functional TLR4 is not required for induction of macrophage tolerance by *Salmonella*. The results described above indicate that although PRRs other than TLR4 are engaged during the course of *Salmonella* infection of macrophages, the signals activated are poor inducers of secreted TNF- α . To determine whether this TLR4-independent engagement of PRRs has functional consequences other than the production of TNF- α , we examined the induction of macrophage tolerance. C3H/HeJ macrophages were pretreated overnight with either heat-killed wild-type *Salmonella* strain SL1344 or culture supernatants of this strain. After the initial stimulus was washed away, the cells were stimulated with PG, and activation of ERK was analyzed. As shown in Fig. 4A, pretreatment of C3H/HeJ macrophages with either the heat-killed bacteria (upper panel) or with the culture supernatant (middle panel) resulted in significant inhibition of ERK activation by PG, similar to that achieved by pretreatment with PG itself (middle panel), whereas activation by the unrelated stimulus phorbol myristate acetate was not affected. Quantitation of the Western blots confirmed these findings (Fig. 4B). The inhibition of PG-dependent ERK activation by pretreatment with heat-killed *Salmonella* was similar in C3H/HeJ and C3H/HeOuJ macrophages (Fig. 4A, lower panel, and Fig. 4B), indicating that the phenomenon is TLR4 independent. As expected, pretreatment of the C3H/HeJ macrophages with LPS did not affect the response to subsequent treatment with PG (Fig. 4A, middle panel). These results indicate that there is specific, TLR4-independent tolerization of PG-induced signaling by prior exposure to *Salmonella* products.

To determine whether the TNF- α response to PG could also be tolerized by *Salmonella* in a TLR4-independent fashion, C3H/HeOuJ and C3H/HeJ macrophages were pretreated overnight with heat-killed bacteria and then stimulated the next morning with PG. As shown in Fig. 4C, pretreatment of the C3H/HeJ macrophages with heat-killed *Salmonella* resulted in an approximately 85% reduction in the amount of TNF- α produced compared with the amount obtained for cells that had not been pretreated ($P < 0.0005$), which was comparable to the approximately 95% reduction observed after pretreatment with PG itself. Pretreatment of C3H/HeJ macrophages with *Salmonella* culture supernatant had a similar inhibitory effect on the subsequent TNF- α response to PG (data not shown). Thus, in keeping with the data on ERK activation, tolerization of the PG-induced TNF- α response by *Salmonella* also does not require a functional TLR4. Interestingly, pretreatment of C3H/HeOuJ macrophages with heat-killed *Salmonella* did not lead to tolerization of the TNF- α response to subsequent treatment with PG and even increased the response to some extent (Fig. 4C). This significant difference ($P < 0.0005$) between the responses of the C3H/HeOuJ and C3H/HeJ macrophages is probably attributable to the fact that TLR4 signals can accentuate the subsequent response to TLR2 ligands rather than induce cross-tolerance (7).

Various mechanisms have been proposed to explain the induction of macrophage tolerance, including increased expression of specific proteins, such as IRAK-M (27) and the

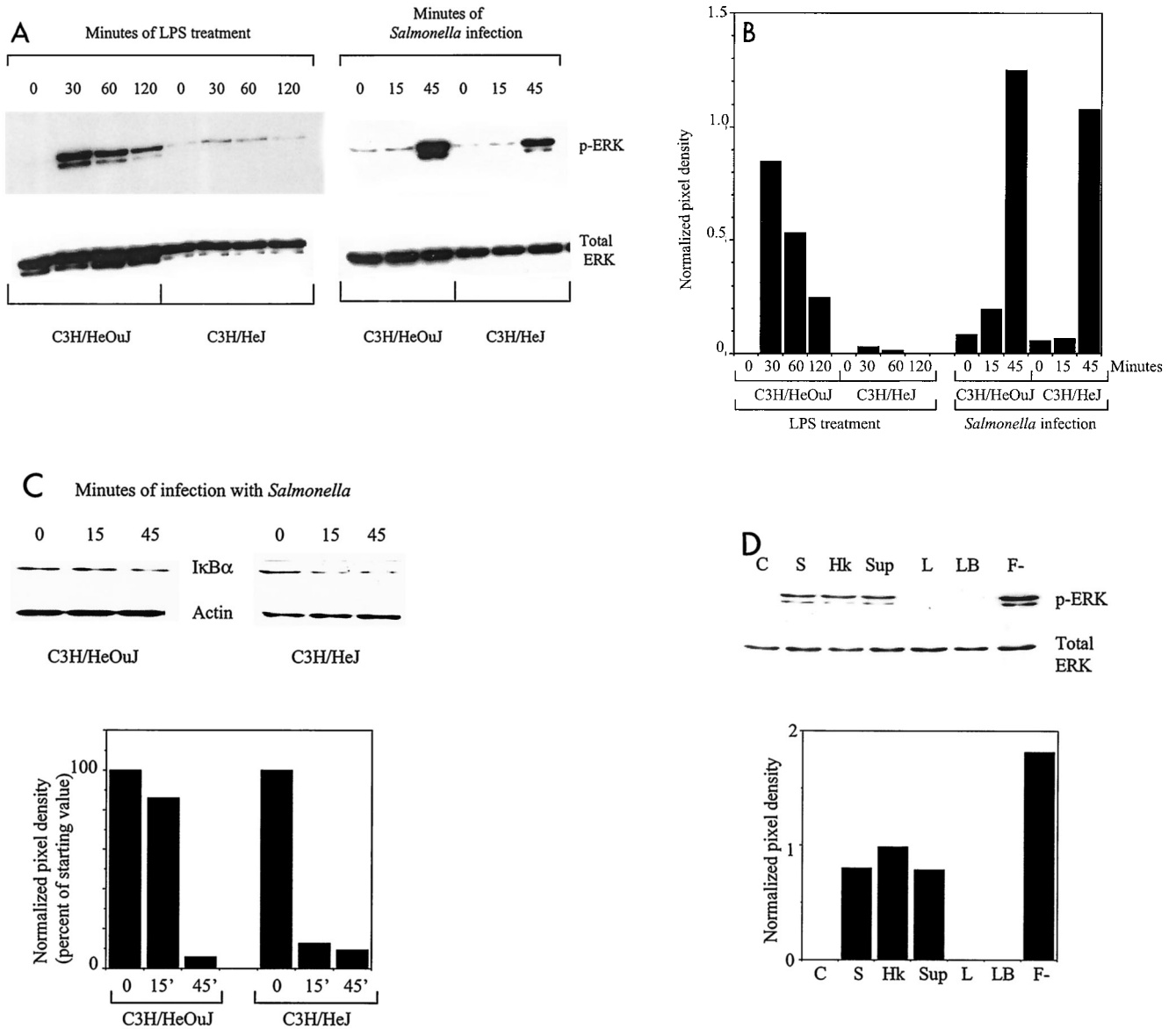


FIG. 2. (A) Activation of ERK in C3H/HeOuJ and C3H/HeJ peritoneal macrophages in response to LPS treatment or infection with wild-type *S. enterica* serovar Typhimurium. Macrophages were treated with 100 ng of LPS per ml or were infected with 10^7 SL1344 bacteria for the times indicated. Cell lysates were prepared and analyzed by Western blotting for the presence of phospho-ERK (p-ERK). The blots were then stripped and reprobed to detect total ERK. (B) Quantitation of the Western blot in panel A. The autoradiograph was scanned and analyzed by using NIH Image software. The mean pixel densities of the phospho-ERK bands (normalized to the pixel densities of the corresponding total ERK bands) are shown. (C) Degradation of IκBα in C3H/HeOuJ and C3H/HeJ peritoneal macrophages in response to infection with wild-type *S. enterica* serovar Typhimurium. Macrophages were infected with 10^7 SL1344 bacteria for the times indicated. Cell lysates were prepared and analyzed by Western blotting for the presence of IκBα. The blot was then stripped and reprobed for the presence of actin to confirm equivalent loading of lanes. Quantitation of the Western blot is shown at the bottom, and the data indicate the mean pixel densities of the IκBα bands (normalized to the pixel densities of the corresponding actin bands) expressed as percentages of the value at time zero for each strain. (D) Activation of ERK in C3H/HeJ peritoneal macrophages in response to *S. enterica* serovar Typhimurium or its products. Macrophages were left untreated (C) or were exposed to 10^7 wild-type *S. enterica* serovar Typhimurium strain SL1344 bacteria (S), 10^7 heat-killed SL1344 bacteria (Hk), a 5% (vol/vol) supernatant of a stationary culture of wild-type *S. enterica* serovar Typhimurium (Sup), a 5% (vol/vol) supernatant of a stationary culture of flagellin-deficient *S. enterica* serovar Typhimurium (F-), 5% (vol/vol) LB broth (LB), or 100 ng of LPS per ml (L) for 45 min. Cell lysates were prepared and analyzed by Western blotting for the presence of phospho-ERK. The blots were then stripped and reprobed to detect total ERK. Quantitation of the Western blot is shown at the bottom, and the data indicate the mean pixel densities of the phospho-ERK bands normalized to the pixel densities of the corresponding total ERK bands.

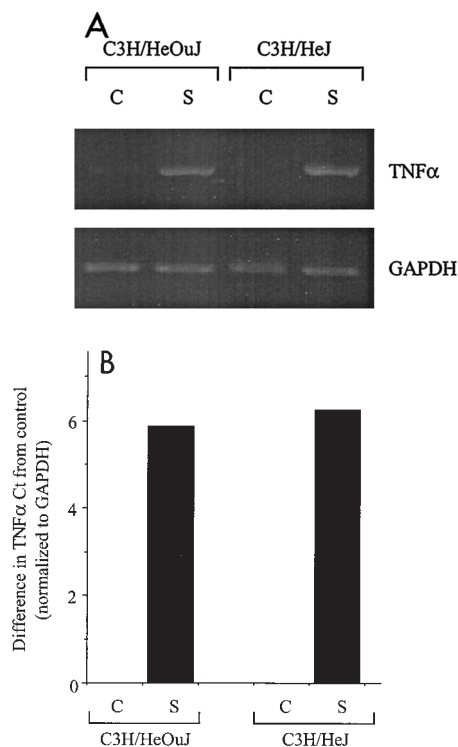


FIG. 3. (A) Induction of TNF- α mRNA in C3H/HeOuJ and C3H/HeJ macrophages by *Salmonella* infection: semiquantitative RT-PCR analysis. Macrophages were not infected (lanes C) or were infected for 2 h with 10^7 wild-type *S. enterica* serovar Typhimurium SL1344 bacteria (lanes S). Total RNA was prepared and used for the RT-PCR with primers specific for either TNF- α or the housekeeping transcript GAPDH. Aliquots of the PCR products were analyzed by agarose gel electrophoresis and were visualized by UV transillumination. (B) Induction of TNF- α mRNA in C3H/HeOuJ and C3H/HeJ macrophages by *Salmonella* infection: quantitative real-time PCR analysis. Macrophages were not infected (bars C) or were infected for 2 h with 10^7 wild-type *S. enterica* serovar Typhimurium SL1344 bacteria (bars S). Total RNA was prepared, reverse transcribed, and amplified with an iCycler by using primers specific for either TNF- α or GAPDH in the presence of SYBR Green. Normalized C_t values were derived as described in Materials and Methods and are expressed as the differences from control, uninfected cells.

NF- κ B p50 and p52 subunits (24, 51). To determine the role of the NF- κ B p50 and p52 proteins in the induction of tolerance by *Salmonella*, we examined the expression of these proteins after overnight incubation of wild-type and TLR4 mutant macrophages with either LPS or heat-killed *Salmonella*. As shown in Fig. 5, expression of both the NF- κ B p50 and p52 proteins was increased by LPS and heat-killed *Salmonella* in the wild-type C3H/HeOuJ macrophages. As expected, induction of these proteins by LPS was not observed in the TLR4 mutant C3H/HeJ macrophages. However, there was an appreciable (albeit attenuated) increase in the expression of both NF- κ B p50 and NF- κ B p52 in the C3H/HeJ cells in response to *Salmonella*, indicating that the induction was at least partially independent of TLR4. Thus, our results indicate that macrophage tolerance can be induced by *Salmonella* even in the absence of a functional TLR4 and that this TLR4-independent toleriza-

tion is associated with increased expression of NF- κ B p50 and p52.

DISCUSSION

The data presented in this paper indicate that both TLR4 and PRRs other than TLR4 are activated during infection of macrophages by *Salmonella*. Activation of TLR4 is crucial to high-level production of TNF- α ; in macrophages from the C3H/HeJ strain, which carries a mutated, functionally inactive Tlr4 gene (40), the amount of TNF- α protein detected in cell supernatants is markedly reduced (Fig. 1). Previously published work showed that expression of TNF- α is regulated at the level of transcription, mRNA stability, nucleocytoplasmic mRNA transport, translation, and secretion and that LPS-induced signals act at several of these steps (2, 9, 28, 29, 41, 47). Our results indicate that in the context of *Salmonella* infection, the major requirement for TLR4 signals is at a posttranscription step in TNF- α biosynthesis, since increased expression of the mRNA can be induced even in the absence of functional TLR4 (Fig. 3). Presumably, PRRs other than TLR4 are able to activate the signals necessary to increase the levels of the TNF- α transcript but are not sufficient for the subsequent steps involved in efficient translation and/or secretion of the protein. Consistent with this idea, we demonstrated that *Salmonella* activates at least two signaling pathways in macrophages independent of TLR4, the ERK pathway and the NF- κ B pathway (Fig. 2). Furthermore, this TLR4-independent signaling is sufficient for the induction of macrophage tolerance (Fig. 4) associated with increased NF- κ B p50 and p52 expression (Fig. 5). Based on our observations, we postulate a model of *Salmonella*-TLR interactions during macrophage infection (Fig. 6) in which both TLR4-dependent and TLR4-independent signals are activated. Both sets of signals are able to induce tolerance and to increase levels of the TNF- α mRNA, but the TLR4-dependent signal is essential for conversion of the TNF- α transcript into secreted protein. The precise nature of the TLR4-dependent signal necessary for high-level TNF- α expression remains to be identified. Recent studies have demonstrated that among the TLRs, TLR4 activates signaling mechanisms that are not activated by other members of the family, leading to patterns of gene expression that are unique to this receptor (8, 18, 23, 43, 49, 54). It is possible that these or similar TLR4-specific mechanisms function to increase TNF- α production.

The TLR4-independent induction of NF- κ B p50, NF- κ B p52, and tolerance clearly indicates that even in the case of gram-negative infection with relatively low numbers of organisms (the MOI was 20:1 in our experiments), ligands for PRRs other than TLR4 are available in amounts sufficient to have functional effects on macrophages. To generalize from these observations, it is likely that two types of genetic programs are activated as a result of the *Salmonella*-macrophage interaction; one of these, like the production of TNF- α , is TLR4 dependent, and the other, like the induction of tolerance, is TLR4 independent. This idea is in keeping with what is known about the in vivo response of the C3H/HeJ strain to *Salmonella* infection. This strain has increased susceptibility to infection with *Salmonella*, a trait that is associated with deficiencies in macrophage microbicidal activity and inflammatory cell re-

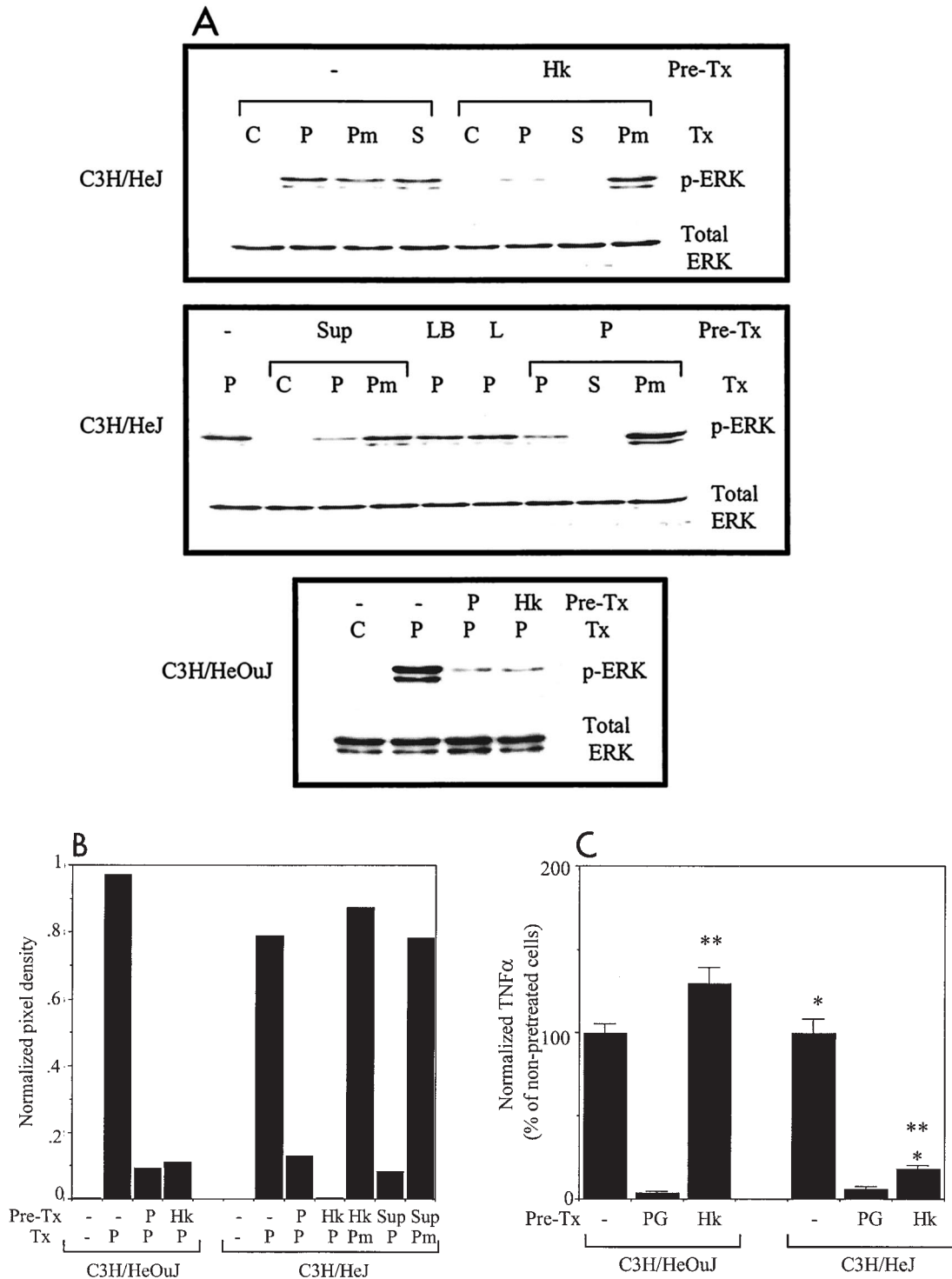


FIG. 4. (A) Pretreatment of C3H/HeJ peritoneal macrophages with *S. enterica* serovar Typhimurium products results in tolerization of ERK activation by PG. Macrophages were not pretreated (-) or were pretreated overnight with 10^7 heat-killed wild-type *S. enterica* serovar Typhimurium SL1344 bacteria (Hk), 5% (vol/vol) culture supernatant of SL1344 (Sup), 5% (vol/vol) LB broth (LB), 100 ng of LPS per ml (L), or 25 μ g of PG per ml (P). They were then left unstimulated (C) or were stimulated for 45 min with 10^7 live wild-type SL1344 bacteria (S), 25 μ g of PG per ml (P), or 1 μ g of phorbol myristate acetate per ml (Pm). Cell lysates were prepared and analyzed by Western blotting for the presence of phospho-ERK (p-ERK). Pre-Tx, pretreatment; Tx, treatment. (B) Quantitation of the Western blots shown in panel A. The autoradiographs were scanned and analyzed by using NIH Image software. The mean pixel densities of the phospho-ERK bands (normalized to the pixel densities of the corresponding total ERK bands) are shown. (C) Effect of pretreatment of peritoneal macrophages with heat-killed *S. enterica* serovar Typhimurium on subsequent TNF- α production in response to PG. Macrophages were not pretreated or were pretreated overnight with 25 μ g of PG per ml or with 10^7 heat-killed wild-type *S. enterica* serovar Typhimurium SL1344 bacteria. After the pretreatment stimulus was washed away, all groups of macrophages were stimulated for 2 h with 25 μ g of PG per ml, and the supernatants were analyzed by ELISA for TNF- α . The normalized amount of TNF- α was expressed as a percentage of the amount produced by the nonpretreated, PG-stimulated cells. The data are the means of the results of three separate experiments, and the error bars indicate standard deviations. The *P* values for the comparisons are <0.0005 (one asterisk) and <0.0005 (two asterisks).

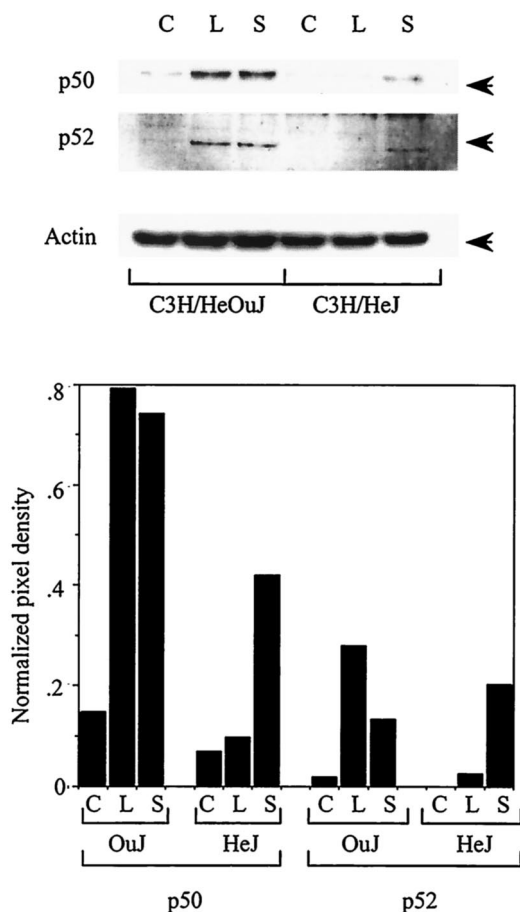


FIG. 5. Induction of genes associated with macrophage tolerance by *Salmonella*. Peritoneal macrophages from either C3H/HeOuJ or C3H/HeJ mice were left untreated (C) or were treated overnight with 100 ng of LPS per ml (L) or 10⁷ heat-killed wild-type *S. enterica* serovar Typhimurium SL1344 bacteria (S). Protein lysates were prepared the next morning and analyzed by Western blotting with antibodies to NF-κB p50, NF-κB p52, or actin. The arrows indicate the relevant bands. Quantitation of the Western blots is shown at the bottom, and the data indicate the mean pixel densities of the p50 or p52 bands normalized to the pixel densities of the corresponding actin bands.

cruitment (38, 39, 52). At the same time, *Salmonella*-dependent activation of macrophages for tumoricidal function and interleukin-6 production, *Salmonella*-induced dendritic cell maturation, and the development of anti-*Salmonella* adaptive immunity appear to be relatively unaffected in C3H/HeJ mice (10, 25, 44, 46, 56), indicating that there are TLR4-independent mechanisms. Our observations add macrophage tolerance to the list of functions that can be induced by *Salmonella* in a TLR4-independent manner and raise the possibility that tolerization in the context of compromised TLR4-dependent microbicidal and inflammatory mechanisms may contribute to the unusual susceptibility of the C3H/HeJ strain to *Salmonella* infection (38, 39).

It should be noted, however, that a deficiency of TLR4 function is not the only factor involved in the susceptibility of C3H/HeJ mice to *Salmonella*. LPS responsiveness has been clearly dissociated from *Salmonella* susceptibility in the C3H lineage (11), indicating that genes other than Tlr4 play a role

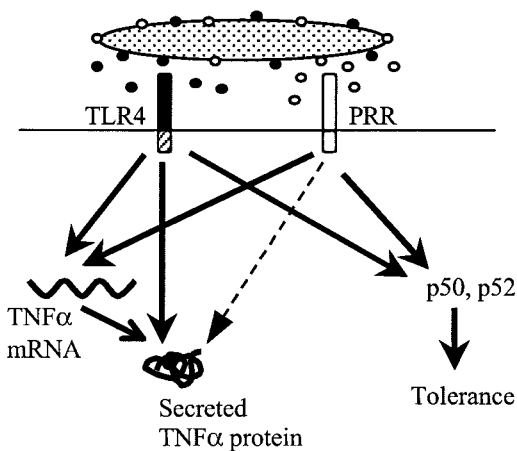


FIG. 6. Model for the role of TLR4 and other PRRs in the *Salmonella*-macrophage interaction. *Salmonella* activates proximal signaling pathways via TLR4 and other PRRs. TLR4 signals are essential at a posttranscriptional step for high-level expression of secreted TNF-α protein. Signals provided by other PRRs are sufficient for induction of the TNF-α transcript and at least some genes associated with tolerance but not for production of high levels of secreted TNF-α. The dashed arrow indicates the signal required for high-level secreted TNF-α production that PRRs other than TLR4 are ineffective at providing.

in the latter characteristic. Interestingly, we found that peritoneal macrophages from C3HeB/FeJ mice, which are LPS responsive but *Salmonella* susceptible (11), produced significantly less TNF-α than C3H/HeOuJ macrophages produced in response to *Salmonella* (for C3HeB/FeJ, 14.77 ± 0.82 pg/μg; for C3H/HeOuJ, 34.35 ± 5.51 pg/μg [*P* < 0.001]), whereas there was not a significant difference between the two strains in terms of the amount of TNF-α produced in response to LPS (for C3HeB/FeJ, 23.90 ± 3.19 pg/μg; for C3H/HeOuJ, 27.38 ± 6.00 pg/μg [*P* < 0.25]). Further work is required to determine whether this observation is relevant to the susceptibility of the C3HeB/FeJ strain to *Salmonella*, but the observation suggests that variations in genes other than Tlr4 within the C3H lineage may contribute to differences in the TNF-α response to salmonellae. As an additional note of caution, it should be mentioned that all the studies described here were carried out with thioglycollate-elicited macrophages because of the difficulty in conveniently obtaining the numbers of resident peritoneal macrophages required for biochemical analyses. Differences between the responses of thioglycollate-elicited peritoneal macrophages and the responses of resident peritoneal macrophages have been documented (14, 19, 35), and this fact needs to be kept in mind when our findings are interpreted.

The identity of the *Salmonella* molecule or molecules that activate macrophages independent of TLR4 is the subject of on-going studies. The fact that salmonellae can induce tolerance to PG in C3H/HeJ macrophages (Fig. 4) is consistent with the involvement of a TLR ligand. The relevant bacterial molecule is unlikely to be flagellin based on the fact that flagellin-deficient *Salmonella* mutants activated signaling in C3H/HeJ macrophages as well as the wild-type strain (Fig. 2D). Bacterial DNA is also not likely to be involved since pretreatment of macrophages with chloroquin to inhibit activation of TLR9 did not affect signaling or TNF-α production (data not shown). A

TLR2 ligand remains a reasonable candidate, and indeed, in preliminary experiments we detected TLR2 agonist activity in *Salmonella* culture supernatants (unpublished data). This activity may correspond to a previously described outer membrane lipoprotein released by several types of enterobacteria, including *Salmonella*, that was shown to activate C3H/HeJ macrophages to produce interleukin-6 (56). It may also be related to a recently identified PG-associated lipoprotein that is released by several gram-negative bacteria and that is able to activate C3H/HeJ macrophages (17). A membrane lipoprotein that is released in a soluble form would be consistent with our detection of TLR4-independent activity associated with both bacteria and bacterial supernatants. If the relevant *Salmonella* molecule does in fact engage TLR2, it is surprising that it is such a poor inducer of TNF- α production compared to PG, another TLR2 ligand (Fig. 1). This difference may be attributable to the amount of the molecule available for activation of TLR2. Alternatively, since it is known that different TLR2 ligands engage different TLR2 heterodimers (33), it is possible that PG activates one type of heterodimer, while the *Salmonella* molecule activates another. Future experiments will be directed at addressing some of these issues by identifying the *Salmonella* molecules involved in TLR4-independent activation of macrophages and clarifying their roles in pathogenesis.

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