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Sublethal Infection of C57BL/6 Mice with *Salmonella enterica* Serovar Typhimurium Leads to an Increase in Levels of Toll-Like Receptor 1 (TLR1), TLR2, and TLR9 mRNA as Well as a Decrease in Levels of TLR6 mRNA in Infected Organs

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Sublethal infection of C57BL/6 mice with *Salmonella enterica* serovar Typhimurium M525P initiates a strong inflammatory response. We measured organ expression of mRNA for Toll-like receptors and their associated signaling molecules during *S. enterica* serovar Typhimurium infection. During infection, the Toll-lie receptor 1 (TLR1), TLR2, and TLR9 mRNA levels increased, while TLR6 mRNA expression decreased.

Cellular invasion by *Salmonella* triggers responses that dictate the outcome of infection. In sublethal infections the host controls the initial exponential bacterial growth in the liver and spleen. This plateau phase limits the primary infection and depends upon the release of proinflammatory mediators leading to granuloma formation (8, 9, 11). This process requires Toll-like receptor 4 (TLR4) activation (20, 22). The detailed mechanisms involved in plateau phase formation by the host during *Salmonella* infection are largely unknown.

Salmonellae possess many structures that act as pathogen-associated molecular patterns to signal bacterial presence to the host (for example, lipopolysaccharide, lipoproteins, flagellin, peptidoglycan, and bacterial DNA). These ligands bind to specialized pathogen-associated molecular pattern receptors, such as TLRs, that signal the cell to induce a response (21). TLR4, in association with the proteins MD2 and CD14, binds lipopolysaccharide (14, 18); TLR2 recognizes bacterial lipoproteins and lipoteichoic acid (16, 18), probably in cooperation with TLR6 and/or TLR1 (1, 13, 19); TLR5 responds to bacterial flagellin (3, 5, 17); and TLR9 is activated by bacterial DNA (detecting unmethylated CpG motifs) (6). Activation of TLRs recruits adapter proteins, such as MyD88 and TIRAP, to activate signaling pathways that induce proinflammatory proteins, such as cytokines (e.g., tumor necrosis factor alpha [TNF-α]), and inducible enzymes (e.g., inducible nitric oxide synthase [iNOS]).

Here we infected C57BL/6 mice with *Salmonella enterica* serovar Typhimurium M525P (10), a strain whose growth is controlled in these mice, leading to plateau formation in the spleen and liver. We analyzed expression of mRNA for TLR1, TLR2, TLR4, TLR5, TLR9, the adapter molecules MyD88 and TIRAP, the accessory protein MD2, and the proinflammatory proteins iNOS and TNF-α during a 14-day sublethal *Salmonella* infection in C57BL/6 mice.

Sublethal infection of C57BL/6 mice with *S. enterica* serovar Typhimurium M525P induces the inflammatory mediators TNF-α and iNOS. Six- to eight-week-old C57BL/6 mice (Harlan Olac Laboratories) were inoculated with 10^3 CFU of *S. enterica* serovar Typhimurium M525P (10) in the tail vein. RNA was isolated, and bacterial counts were obtained for the spleen and liver (20).

The bacterial counts initially increased at a rate of approximately 0.4 log per day. From day 4 onward a plateau in the bacterial growth curve occurred (Fig. 1A). The spleen and liver levels of TNF-α and iNOS mRNA, as measured by real-time reverse transcriptase PCR (20) with the primers shown in Table 1 (standard curve data are shown in Table 2), increased over the course of the infection (Fig. 1B and C). The basal levels of TNF-α mRNA were high in both organs (Fig. 1B and 2), which probably allowed rapid translation of mRNA into TNF-α protein after infection. Macrophage iNOS expression increases during *Salmonella* infection (2), probably because the reactive nitrogen intermediates produced are bactericidal (10). We saw significant sustained increases in the iNOS mRNA level from day 4 in the liver and from day 7 in the spleen (Fig. 1C).

Levels of TLR1, TLR2, and TLR9 mRNA increase and the level of TLR6 mRNA decreases during infection with *S. enterica* serovar Typhimurium. Measurement of mRNA levels with the primers shown in Table 1 (standard curve data are shown in Table 2) showed that in the liver the level of TLR2 transiently increased during the plateau phase (Fig. 3B), and the levels of TLR1 and TLR9 also increased during infection (Fig. 3A and C). Expression of TLR6 mRNA was reduced from day 7 onward (Fig. 3D), while the expression of TLR4, TLR5, MD2, MyD88, and TIRAP/Mal mRNA did not change in response to sublethal *Salmonella* infection (data not shown). We were unable to determine whether TLR mRNA expression correlates with cell surface protein expression in the liver because fluorescence-activated cell sorting analysis in this tissue is not practical, but in murine bone marrow-derived macro-

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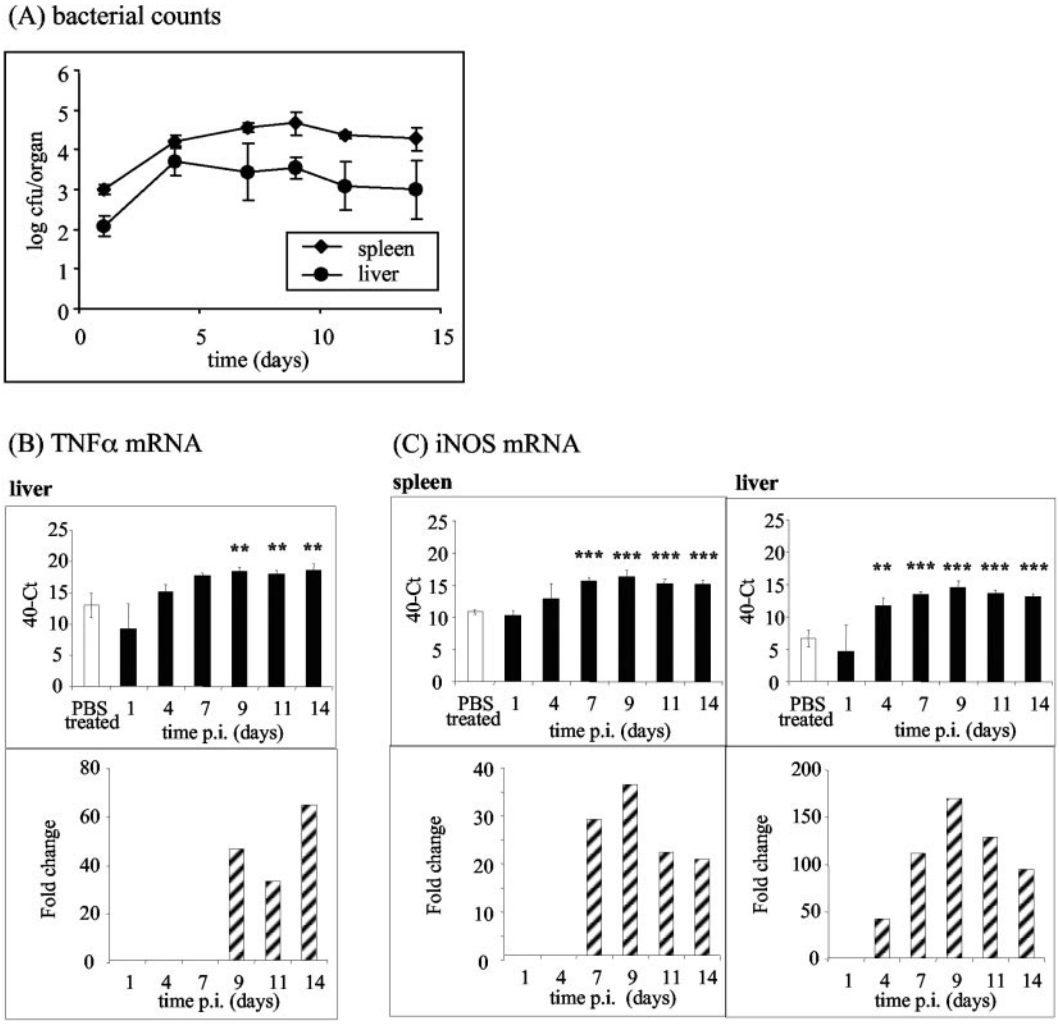


FIG. 1. Induction of TNF-α and iNOS in the spleen and liver during sublethal infection with *S. enterica* serovar Typhimurium M525P. (A) C57BL/6 mice were intravenously infected with 10^3 CFU of *S. enterica* serovar Typhimurium M525P per mouse. At each time postinfection, spleens and livers of four mice were divided, and samples were immediately snap frozen in liquid nitrogen for RNA isolation or homogenized for determination of viable bacterial counts. (B and C) Quantification of TNF-α (B) and iNOS (C) mRNA in spleens and livers from C57BL/6 mice at various times postinfection after intravenous inoculation of 10^3 CFU of *S. enterica* serovar Typhimurium M525P. Cycle threshold (Ct) values are expressed subtracted from 40 (the negative endpoint), and higher values represent higher levels of mRNA. These mRNA levels were standardized to the 18S rRNA levels in spleens and livers from infected and control mice. The data for times at which there is a significant difference between mock-infected and *Salmonella*-infected organs are also expressed as fold changes in mRNA levels compared to the mock-infected control (striped bars), calculated as follows: $2^{[(40 - \text{Ct for infected mice}) - (40 - \text{Ct for control mice})]}$. Two asterisks indicate that the *P* value is <0.01, and three asterisks indicate that the *P* value is <0.001. PBS, phosphate-buffered saline; p.i., postinfection.

phages (12, 15) the TLR2 mRNA and protein levels both increased in response to infection (Fig. 4). It is likely that the macrophage response reflects what happens in an infected liver.

In contrast to our observations with C3H/HeN mice (the level of TLR4 mRNA decreased on day 1 and then increased to uninfected control levels [20]), in C57BL/6 mice the level of TLR4 mRNA was unchanged throughout the 14-day experiment. This could have been due to the use of different *Salmonella* strains or to differences between the mouse strains (for example, C3H/HeN mice are *Nramp*$^+$, while C57BL/6 mice are *Nramp*).

The levels of both TLR2 and TLR1 mRNA increased during infection, suggesting that more TLR2-TLR1 heterodimers may form during infection. In contrast, TLR2-TLR6 formation is probably reduced, since the TLR6 mRNA levels decreased in the spleen and stayed at basal levels in the liver over the course of the *Salmonella* infection (Fig. 3D). The TLR2-TLR6 complex recognizes primarily gram-positive bacteria and mycoplasmas (4, 7, 13, 19) and probably plays no role during gram-negative infections. Low levels of TLR6 might therefore make more TLR2 available to form TLR2-TLR1 heterodimers.

Increased TLR1, TLR2, and TLR9 expression can be only partially explained by an influx of macrophages and polymorphonuclear leukocytes into the organs, since the levels of some macrophage mRNA, such as MD2 mRNA, remain unchanged (data not shown). This correlates with our previous data which showed that the numbers of macrophages increase two- to

TABLE 1. Real-time quantitative reverse transcriptase PCR probes and primers

RNA target	Probe sequence (5′-3′)	Forward primer sequence (5′-3′)	Reverse primer sequence (5′-3′)
18 SrRNA	ACCGGCGCAAGACGCACCAG	CGCCGCTAGAGGTGAAATTCT	CATTCTTGGCAAATGCTTTCG
TLR1	TCAGCCTCAAGCATTTGGACCTCTCCT	TGATCTTGTGCCACCCAACA	GCAGGGCATCAAAGGCATTA
TLR2	CGTTTTTACCACCCGGATCCCTGTACT	AAGATGCGCTTCCTGAATTTG	TCCAGCGTCTGAGGAATGC
TLR4	CCTGGTGTAGCCATTGCTGCCAACA	CCTCTGCCTTCACTACAGAGACTTT	GTGGAAGCCTTCCTGGATGA
TLR5	TGGTAATATCTCCCTGTTCTTCAGACGGCA	AACTTGACTTGCTCATAGGTGTGATC	CAGCCTCGGAAAAAGGCTATC
TLR6	AGCCAAGACAGAAAACCCATCGTGGG	TCTGGGATAGCCTCTGCAACA	GGCGCAAACAAAGTGGAAAC
TLR9	CGACCATGCCCCCAATCCCTG	TGATGTGGGTGGGAATTGC	GGGACTTTTGGCCACATTCTAT
MD2	TCTTTTCGACGCTGCTTTCTCCCATATTG	GGAGATATTAAATCATGTTGCCATTTTATT	ACCACTGTTGCTTCTCAGATTCAG
MyD88	CCCTTGGTCGCGCTTAACGTGG	CTGGACTCCTTCATGTTCTCCAT	GATAGGCGGCGCCTCACT
TIRAP	TGCCGTGTGCTGCTCATCACTCC	TGCCAGGCACTGAGTCGTAGT	GGCCTGCAGCATCTGGTACT
TNF-α	TCAGCCTCTTCTCATTCCTGCTTGTGG	TCCAGGCGGTGCCTATGT	CGATCACCCCGAAGTTCAGT
iNOS	CTTCCGGGCAGCCTGTGAGACCT	CGCAGCTGGGCTGTACAA	TGATGTTTGCTTCGGACATCA

[a] Primers and probes were designed by using the Primer Express software program (PE Applied Biosystems). The probes were labeled with the fluorescent reporter dye 5-carboxyfluorescein at the 5′ end and with the quencher *N,N,N′,N′*-tetramethyl-6- carboxyrhodamine at the 3′ end.

TABLE 2. Standard curve data from a real-time quantitative reverse transcriptase PCR analysis of total RNA extracted from stimulated RAW cells, a macrophage like cell line[a]

Target	ΔR_g significance threshold[b]	Log dilutions	Range of C_t values[c]	R^{2d}	Slope
18S rRNA	0.2	10^{-3}–10^{-7}	7–26	0.997	3.7997
TLR1	0.02	10^{-1}–10^{-5}	20–36	0.9805	3.444
TLR2	0.04	10^{-1}–10^{-5}	18–36	0.9776	3.414
TLR4	0.04	10^{-1}–10^{-4}	24–37	0.9891	3.719
TLR5	0.02	10^{-1}–10^{-3}	29–38	0.9913	3.915
TLR6	0.02	10^{-1}–10^{-4}	23–34	0.9561	3.219
TLR9	0.03	10^{-1}–10^{-4}	24–34	0.9900	3.133
MD2	0.04	10^{-1}–10^{-4}	19–38	0.9927	3.583
MyD88		10^{-1}–10^{-5}	22–35	0.9829	3.106
TIRAP	0.03	10^{-1}–10^{-3}	23–31	0.9320	3.435
TNF-α	0.03	10^{-2}–10^{-6}	17–38	0.9861	4.3687
iNOS	0.03	10^{-1}–10^{-5}	18–35	0.9805	3.495

[a] To generate standard curves for the specific reactions for the various probes, total RNA extracted from stimulated RAW macrophages was serially diluted.
[b] ΔR_n is the change in the reporter dye level.
[c] C_t is the threshold cycle value, the cycle at which the change in the reporter dye level detected passes the ΔR_n.
[d] R^2 is the coefficient of regression. Regression analyses of the mean values of three or four replicate reverse transcriptase PCRs for the $\log_{10}$-diluted RNA were used.

fivefold in the spleen and liver over the first 7 days of *Salmonella* infection (20). In comparison, the increase in expression of TLR1, TLR2, and TLR9 mRNA was about 15-fold and the increase in expression of TNF-α mRNA was about 30-fold.

In the spleens and livers of mock-infected animals we detected mRNA for TNF-α, iNOS, and the TLR-associated proteins (Fig. 2). Constitutive expression of TLR mRNA allows the immune system to respond immediately to pathogens, and continuous challenges with small amounts of bacterial constituents may be required to keep the immune system alert to infection (21).

In summary, development of the plateau phase during sublethal *Salmonella* infection correlates with up-regulation of TLR1, TLR2, and TLR9 mRNA expression and down-regulation of TLR6 mRNA expression. This suggests that in addition to TLR4, the TLR2-TLR1 complex and TLR9 may play a role in controlling infection, particularly in the later stages when

FIG. 2. Basal mRNA expression in spleens and livers of mock-infected animals: comparison of basal levels of all molecules analyzed by real-time PCR in spleens and livers from mock-infected C57BL/6 mice. Cycle threshold (Ct) values are expressed subtracted from 40 (the negative endpoint); higher values represent higher levels of mRNA.

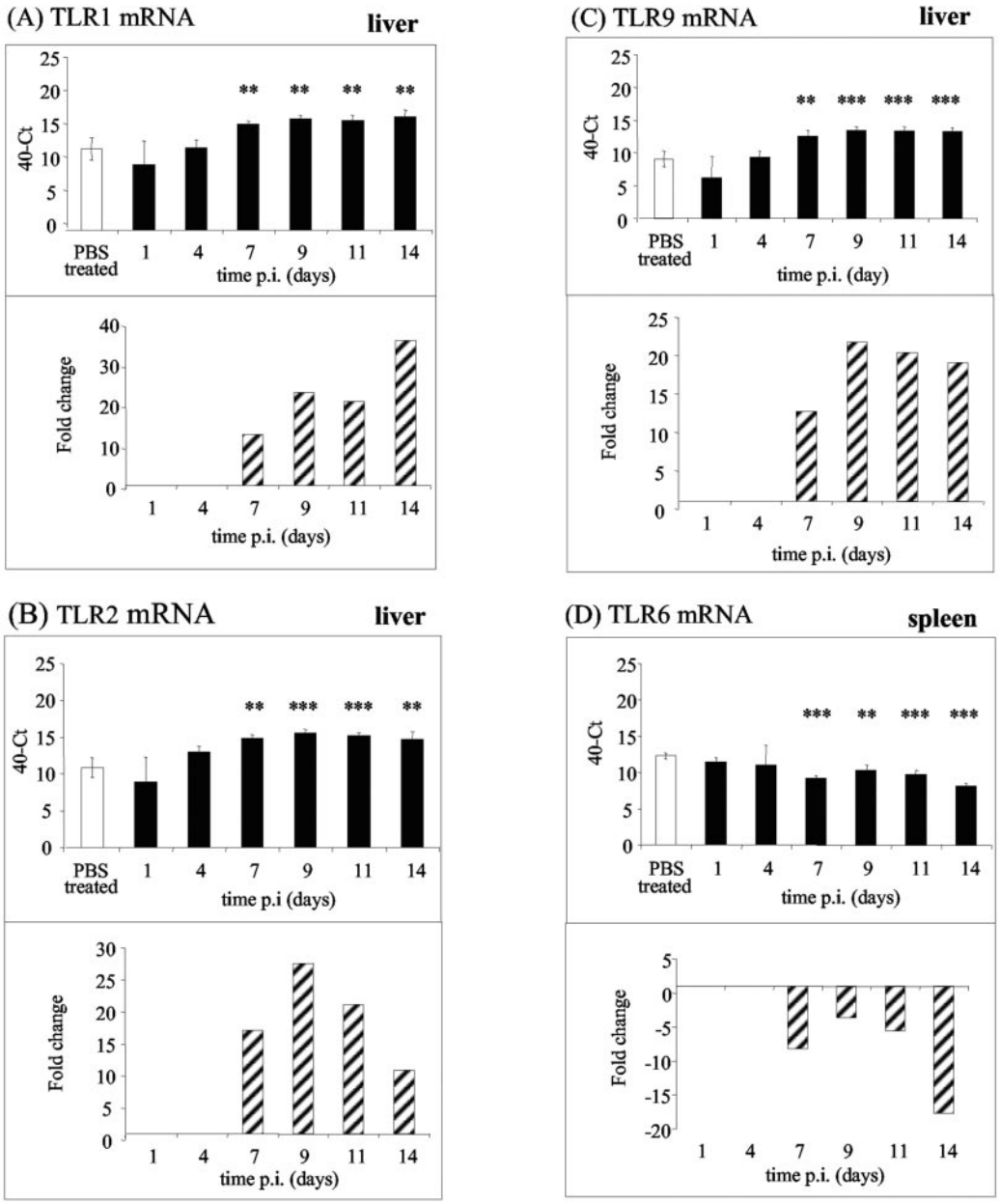
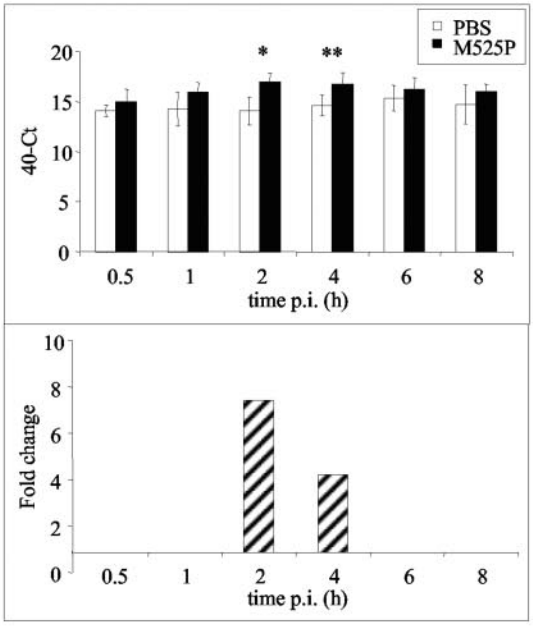


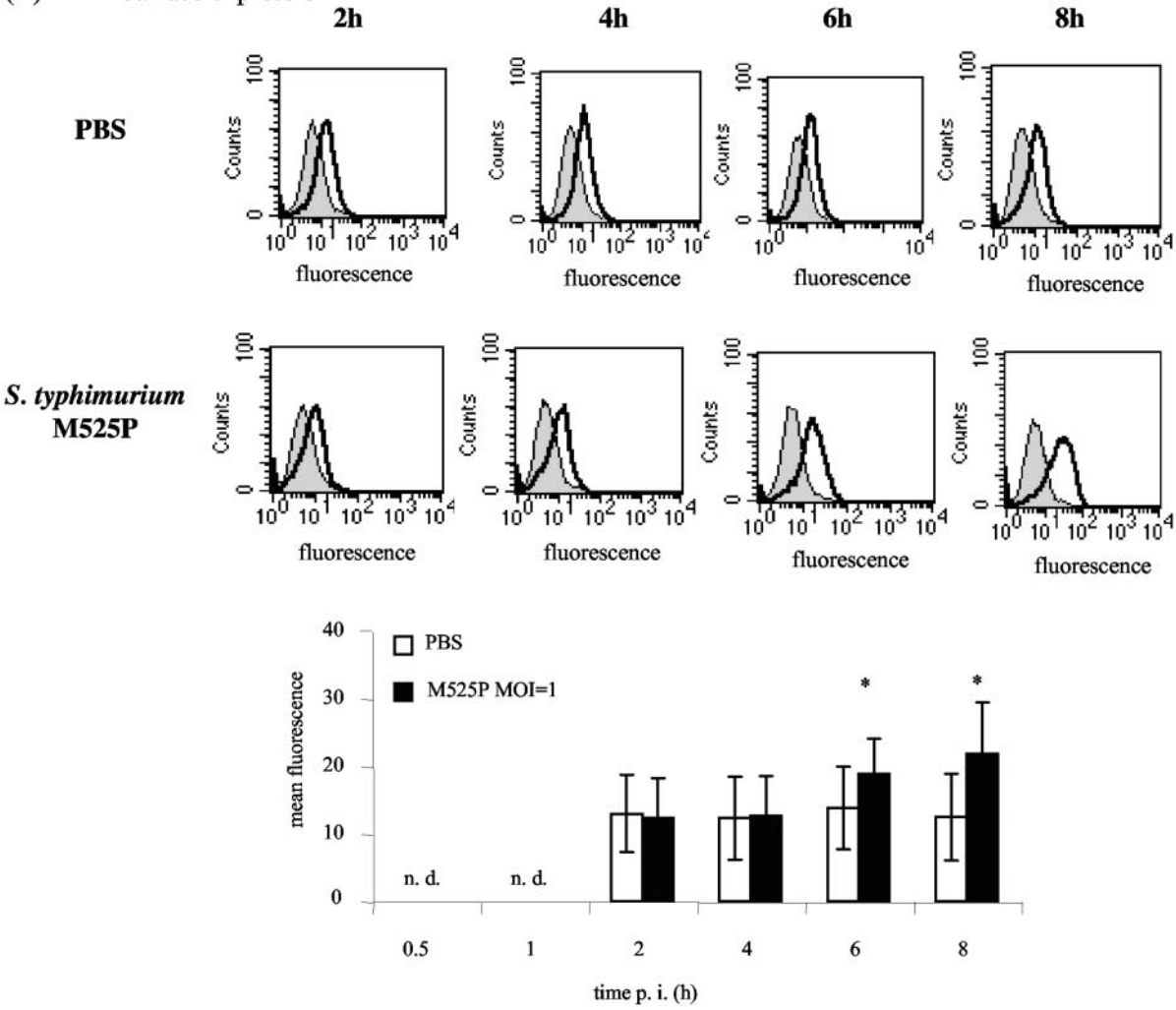
FIG. 3. Levels of TLR1, TLR2, and TLR9 mRNA increase in response to infection while TLR6 mRNA levels decrease during sublethal infection with *S. enterica* serovar Typhimurium M525P. TLR1 (A), TLR2 (B), TLR9 (C), and TLR6 (D) mRNA in spleens and livers from C57BL/6 mice were quantified at various times postinfection after intravenous inoculation of 10^3 CFU of *S. enterica* serovar Typhimurium M525P. Cycle threshold (Ct) values are expressed subtracted from 40 (the negative endpoint); higher values represent higher levels of mRNA. The mRNA levels were standardized to 18S rRNA levels in spleens and livers from infected and control mice. The data for times at which there is a significant difference between mock-infected and *Salmonella* infected organs are also expressed as fold changes in mRNA levels compared to the mock-infected control (striped bars), calculated as follows: $2^{[(40 - \text{Ct for infected mice}) - (40 - \text{Ct for control mice})]}$. Two asterisks indicate that the *P* value is <0.01, and three asterisks indicate that the *P* value is <0.001. PBS, phosphate-buffered saline; p.i., postinfection.

FIG. 4. TLR2 mRNA and surface expression increase in bone marrow-derived macrophages in response to infection with *S. enterica* serovar Typhimurium M525P. TLR2 mRNA (A) and surface protein expression (B) were quantified by using bone marrow-derived macrophages from C57BL/6 mice at various times after infection with *S. enterica* serovar Typhimurium M525P at a multiplicity of infection of 1. (A) Cycle threshold (Ct) values are expressed subtracted from 40 (the negative endpoint); higher values represent higher levels of mRNA. The mRNA levels were standardized to 18S rRNA levels. The data from times at which there is a significant difference between control and *Salmonella*-infected bone marrow-derived macrophages are also expressed as fold changes in mRNA levels compared to the control (striped bars). (B) Flow cytometry analysis of *Salmonella*-infected and control bone marrow-derived macrophages after cell surface staining with anti-mouse TLR2 (thick line) or isotype control antibody (thin line). The graph at the bottom shows fluorescence values (means ± standard deviations of the means) for four independent experiments. One asterisk indicates that the *P* value is <0.05, and two asterisks indicate that the *P* value is <0.01. PBS, phosphate-buffered saline; p.i., postinfection; n. d., not determined.

(A) TLR2 mRNA



(B) TLR2 surface expression



the bacterial growth is suppressed, possibly at the adaptive phase of the immune response. Coordinate regulation of TLR receptor expression would then complement the proposed sequential activation of TLRs during *S. enterica* serovar Typhimurium infection (23).

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