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*J Immunol* 2009; 183:8110-8118; Prepublished online 18 November 2009; doi: 10.4049/jimmunol.0901031

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The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Signaling Crosstalk during Sequential TLR4 and TLR9 Activation Amplifies the Inflammatory Response of Mouse Macrophages

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The TLR family of pattern recognition receptors is largely responsible for mediating the activation of macrophages by pathogens. Because macrophages may encounter multiple TLR ligands during an infection, signaling crosstalk between TLR pathways is likely to be important for the tailoring of inflammatory reactions to pathogens. Here, we show that rather than inducing tolerance, LPS pretreatment primed the inflammatory response (e.g., TNF production) of mouse bone marrow-derived macrophages (BMM) to the TLR9 ligand, CpG DNA. The priming effects of LPS, which correlated with enhanced Erk1/2, JNK, and p38 MAPK activation, appeared to be mediated via both c-Fms-dependent and -independent mechanisms. LPS pretreatment and inhibition of the M-CSF receptor, c-Fms, with GW2580 had comparable effects on CpG DNA-induced Erk1/2 and p38 MAPK activation. However, c-Fms inhibition did not enhance CpG DNA-induced JNK activation; instead, the levels of TNF produced were significantly lower than those from LPS-primed BMM. Thus, the priming effects of LPS on TLR9 responses appear to be largely mediated via the c-Fms-independent potentiation of JNK activity. Indeed, inhibition of JNK abrogated the enhanced production of TNF by LPS-pretreated BMM. The c-Fms-independent priming effects of LPS are unlikely to be a consequence of the inhibitory constraints of M-CSF signaling on TLR9 expression being relieved by LPS; instead, LPS may exert its priming effects via signaling molecules downstream of TLR9. In summary, our findings highlight the importance of signaling crosstalk between TLRs, as well as between TLRs and c-Fms, in regulating the inflammatory reaction to pathogens. The Journal of Immunology, 2009, 183: 8110–8118.
and IL-6) production was reported to be primed by substimulatory doses of LPS (23–26), leading to the suggestion that, rather than simply inducing tolerance, LPS was capable of reprogramming the inflammatory response of macrophages (24, 25). Thus, both the type and concentration of TLR ligand(s) encountered by macrophages appear to be important in dictating the nature of subsequent inflammatory responses.

Various mechanisms appear to contribute to the induction of tolerance to TLR ligands. These include down-regulation of IL-1R-associated kinase (IRAK-1), IRAK-4, and Mal expression levels (14, 15, 17, 27–30) and up-regulation of the TLR inhibitory molecules, IRAK-M and MyD88s (31, 32). However, our understanding of the molecular basis of priming is limited. Here, we report that LPS pretreatment primes macrophages for heightened CpG DNA responses (e.g., TNF and IL-6 production). The priming effects of LPS correlated with amplified CpG DNA-induced MAPK activation. Finally, we provide evidence that the priming effects of LPS involve the potentiation of JNK activation and, in the case of bone marrow-derived macrophages, suppression of M-CSF signaling.

Materials and Methods

Reagents

Cell culture medium and supplements, FCS, SuperScript III reverse transcriptase, and precast 10% SDS-PAGE gels were from Invitrogen. Recombinant human M-CSF was a gift from Chiron. Ultrapure LPS (Escherichia coli 0111:B4) was from InvivoGen, while the mouse type B phosphorothioate backbone modified CpG DNA oligonucleotide 1668 (5'-TCCATGACGTTCCTGATGCT-3') was from GeneWorks. The anti-IRAK-1, anti-Erk2, and anti-c-Fms Abs were obtained from Santa Cruz Biotechnology. The anti-IVBa, anti-phospho-Erk1/2, anti-phospho-JNK, anti-JNK, anti-phospho-p38 MAPK, and anti-p38 MAPK Abs were from Cell Signaling Technology, while the anti-photophorytrosine (4G10) Ab was from UBI. Complete protease inhibitors were supplied by Roche Biochemicals. Chloroquine was from Sigma-Aldrich, while GW2580 (c-Fms inhibitor) and SP600126 (JNK inhibitor) were purchased from Merck.

Macrophages

The use of mice in this study was approved by the Melbourne Health Animal Ethics Committee. Bone marrow-derived macrophages (BMM) were obtained by culturing bone marrow cells from 6- to 8-wk-old female C57BL/6 mice in DMEM supplemented with 5000 U/ml recombinant M-CSF, 10% FCS, 100 U/ml Penicillin, 100 µg/ml streptomycin, and 2 mM GlutaMax-1 for 3 days at 37°C in a humidified atmosphere of 5% CO2. Nonadherent macrophage precursors were collected and seeded in the required format; they were then cultured for an additional 3–4 days in the presence of M-CSF (5000 U/ml) until a homogeneous population of adherent macrophages was obtained. RAW 264.7 cells were cultured in DMEM supplemented with 10% FCS, 100 U/ml Penicillin, 100 µg/ml streptomycin, and 2 mM GlutaMax-1.

Stimulation of macrophages with TLR ligands

BMM and RAW 264.7 cells were treated for 2 or 12 h with the indicated concentrations of LPS in the presence of 5000 U/ml M-CSF (in the case of BMM, unless indicated otherwise). The cells were then washed twice with serum-free medium and incubated for 1 h in complete medium (containing 5000 U/ml M-CSF in the case of BMM, unless indicated otherwise) before being stimulated with either CpG DNA (CpG oligonucleotide 1668) or LPS. The cell supernatants were harvested for measurement of TNF and IL-6 cytokine levels; cells were retained for the isolation of total RNA (for quantitative real-time PCR analysis) and preparation of cell lysates (for Western blot analysis).

Measurement of cytokine levels by ELISA

TNF and IL-6 levels in cell culture supernatants were quantified by ELISA using mouse OptEIA enzyme immunoassay kits (BD Pharmingen) according to the manufacturer’s instructions.

Quantitative real-time PCR analysis

Total RNA was isolated from macrophages using RNAeasy Mini kits (Qia-gen) and then reverse-transcribed using SuperScript III reverse transcriptase. Quantitative real-time PCR was performed using an ABI Prism 7900HT sequence detection system and predeveloped TaqMan probe/primer combinations for TNF, TLR9, and 18S rRNA (Applied Biosystems). Relative mRNA levels were then calculated by transforming the threshold cycle numbers using the ∆∆Ct and relative value method as described by the manufacturer.

Cell lysis and Western blot analysis

Following washing with ice-cold PBS, cells were scraped into Nonidet P-40 lysis buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 10% glycerol, 1 mM sodium orthovanadate, 10 mM NaF, 10 mM β-glycerophosphate, and Complete protease inhibitors) and incubated on ice for 30 min. The lysates were then clarified by centrifugation at 13,000 × g for 10 min at 4°C, and the protein concentrations of the cell lysates were measured using a Bio-Rad protein assay kit. Lysates were subjected to electrophoresis on 10% SDS-PAGE gels followed by Western blotting according to standard techniques (33). Immunoreactive bands were visualized using ECL reagents (Amersham Biosciences) and exposure to x-ray film (Fuji). Films were scanned using either a GS710 or GS800 calibrated imaging densitometer (Bio-Rad), and the resulting images were exported as TIFF files. If membranes needed to be reprobed with additional Abs, they were incubated at 55°C for 30 min in stripping buffer (50 mM Tris-HCl (pH 7.0), 2% SDS, 100 mM 2-ME) before probing with primary Ab.

Flow cytometric analysis of c-Fms cell surface levels

BMM were stimulated with LPS as indicated; they were then harvested and nonspecific binding sites were blocked using CD16/CD32 (Fc receptor) block, followed by incubation with an anti-c-Fms mAb (AFS98) or an appropriate isotype control Ab. Cell surface expression was detected using a FACSCalibur (BD Biosciences) and analyzed using CellQuest (BD Biosciences).

Statistics

Data are given as mean values ± SEM. Statistical significance was evaluated by Student’s t test where a p value of <0.05 was considered statistically significant.

Results

LPS primes CpG DNA-induced TNF and IL-6 production by BMM

Using mouse BMM, we examined the effects of LPS pretreatment on subsequent CpG DNA responses by measuring the production of the proinflammatory cytokines TNF and IL-6. Rather than inducing cross-tolerance LPS pretreatment resulted in the priming of CpG DNA responses (Fig. 1A). BMM that were treated for 2 h with LPS produced significantly more TNF when stimulated with CpG DNA in comparison to cells that had not been treated with LPS before CpG DNA stimulation. Moreover, the effects of LPS pretreatment on CpG DNA-induced TNF production were not simply due to the additive effects of LPS and CpG DNA. Notably, the priming effects of LPS were still evident 12 h post-LPS pretreatment. The effects of LPS on CpG DNA-induced TNF protein were also reflected at the level of TNF mRNA (Fig. 1B). Pretreatment of BMM with LPS for 2 or 12 h resulted in significantly greater levels of CpG DNA-induced TNF mRNA.

In contrast to the above results, BMM that had been pretreated with LPS for 2 h produced diminished levels of TNF protein when restimulated with LPS, while those pretreated for 12 h failed to produce detectable levels of TNF (Fig. 1C). The BMM also showed a significant reduction in LPS-induced TNF mRNA levels following 12 h of LPS pretreatment (Fig. 1D). Similar to LPS, CpG DNA-induced TNF protein and TNF mRNA were significantly lower in BMM that had been pre-exposed to CpG DNA for either 2 or 12 h when compared with untreated BMM (data not shown).
The priming effects of LPS on proinflammatory cytokine production by BMM were not restricted to TNF. CpG DNA-induced IL-6 production was also markedly enhanced following the pre-treatment of BMM with LPS (Fig. 1E). The priming effects of LPS were again greatest when assessed 2 h after LPS treatment, although the priming effects were still apparent at 12 h (Fig. 1E). BMM pretreated with either LPS or CpG DNA for 12 h became hyporesponsive to stimulation with the same TLR ligand, resulting in significantly reduced IL-6 production (Fig. 1F and data not shown).

LPS primes CpG DNA-induced signaling in BMM
To investigate the molecular basis for the priming effects elicited by LPS on CpG DNA responses in BMM, we examined the expression levels and activation states of several key TLR signaling molecules, including IRAK-1, IκBα, Erk1/2, JNK, and p38 MAPK. Stimulation of naive BMM with CpG DNA over a time course of 90 min resulted in the rapid degradation of IRAK-1 and IκBα; it also resulted in the activation of Erk1/2, JNK, and p38 MAPK (Fig. 2A). Similar observations were made when naive BMM were stimulated with LPS (Fig. 2B). In comparison to naive BMM, both the level and duration of TLR9 signaling was enhanced in BMM that had been pretreated with LPS for 2 or 12 h before their stimulation with CpG DNA (Fig. 2A). In contrast, TLR4 signaling was either greatly diminished or absent in BMM that had first been pretreated with LPS (Fig. 2B). Likewise, TLR9 signaling was greatly impaired or abolished in BMM that had been pretreated with CpG DNA (data not shown). Hence, tolerance to LPS and CpG DNA at the proinflammatory cytokine level is likely to be mediated by the suppression of TLR4 and TLR9 signaling pathways, respectively, while the priming of CpG DNA-induced TNF and IL-6 production by LPS appears to be mediated by amplified TLR9 signaling.

CpG DNA continues to signal via TLR9 from endosomes following LPS pretreatment
LPS signaling through TLR4 takes place at the plasma membrane in macrophages, while CpG DNA signaling via TLR9 occurs within endosomal compartments (34, 35). In naive macrophages TLR9 resides in the endoplasmic reticulum, but following CpG DNA stimulation it translocates to endosomes where it encounters...
internalized CpG DNA (36). Given the priming effects of LPS on CpG DNA-induced signaling we wanted to establish whether CpG DNA continued to signal via TLR9 from endosomes following LPS pretreatment. A. TLR9-deficient and littermate control BMM or (B) wild-type BMM were treated with 100 ng/ml LPS for 2 or 12 h in the presence of M-CSF. The cells were washed with serum-free medium and then allowed to recover for 1 h in complete medium (containing M-CSF). A. The cells were stimulated with 0.5 μM CpG DNA for 30 min or (B) treated with vehicle (PBS) or 100 μM chloroquine for 30 min before being stimulated with 0.5 μM CpG DNA for 30 min. Cell lysates were subjected to Western blotting with anti-IRAK-1 (α-IRAK-1), anti-phospho-Erk1/2 (α-pErk1/2), anti-phospho-JNK (α-pJNK), and anti-phospho-p38 MAPK (α-p38) Abs. The blots were also probed with an anti-p38 MAPK Ab (α-p38) to confirm that equal amounts of cell protein had been loaded. The data are representative of three independent experiments.

FIGURE 3. CpG DNA continues to signal via TLR9 from endosomes following LPS pretreatment. A. TLR9-deficient and littermate control BMM or (B) wild-type BMM were treated with 100 ng/ml LPS for 2 or 12 h in the presence of M-CSF. The cells were washed with serum-free medium and then allowed to recover for 1 h in complete medium (containing M-CSF). A. The cells were stimulated with 0.5 μM CpG DNA for 30 min or (B) treated with vehicle (PBS) or 100 μM chloroquine for 30 min before being stimulated with 0.5 μM CpG DNA for 30 min. Cell lysates were subjected to Western blotting with anti-IRAK-1 (α-IRAK-1), anti-phospho-Erk1/2 (α-pErk1/2), anti-phospho-JNK (α-pJNK), and anti-phospho-p38 MAPK (α-p38) Abs. The blots were also probed with an anti-p38 MAPK Ab (α-p38) to confirm that equal amounts of cell protein had been loaded. The data are representative of three independent experiments.

or left untreated (Fig. 3B). These data imply that the priming effects of LPS on CpG DNA signaling are unlikely to be due to a change in TLR9 localization from endosomal compartments.

**LPS priming of CpG DNA responses in BMM correlates with down-regulation of c-Fms surface expression**

The major growth factor for macrophages is M-CSF (or CSF-1) (38), which mediates its effects through a specific cell surface receptor, c-Fms (39). M-CSF has previously been shown to suppress responses to CpG DNA, potentially via its down-regulation of TLR9 gene expression (40). The reported ability of LPS to reduce c-Fms surface expression (41) could, therefore, potentially explain the priming effects of LPS on CpG DNA responses. Consequently, we first confirmed that LPS down-regulates c-Fms surface expression under our experimental conditions. As shown in Fig. 4A, flow cytometric analysis revealed a 5- to 10-fold decrease in c-Fms surface expression levels following the exposure of BMM to 100 ng/ml LPS for 2 or 12 h. Similar results were obtained by Western blot analysis, which showed a 5-fold decrease in the expression levels of c-Fms following LPS treatment (data not shown). The sensitivity of c-Fms surface expression to LPS was assessed by treating BMM with a range of LPS concentrations (10 pg/ml to 100 ng/ml) for 2 h. LPS had no apparent effect on c-Fms surface expression at concentrations of 10 pg/ml or 100 pg/ml; however, c-Fms levels started to decline when BMM were treated with 1 ng/ml LPS (Fig. 4B). Maximal down-regulation of c-Fms surface expression (~10-fold reduction) was achieved in response to 10–100 ng/ml LPS. The effects of different concentrations of LPS on CpG DNA responses largely mirrored those on c-Fms surface expression. Namely, pretreating BMM with LPS for 2 or 12 h resulted in reduced CpG DNA-induced TNF production. BMM were pretreated for 2 or 12 h with either the small molecule c-Fms inhibitor, GW2580 (42), or 100 ng/ml LPS before stimulation with CpG DNA. Notably, GW2580-pretreated BMM produced ~2.5-fold more TNF in response to CpG DNA stimulation than did BMM stimulated in the absence of GW2580 pretreatment (Fig. 5A). However, the effects of GW2580 pretreatment on CpG DNA-induced TNF production were not as great as those achieved when BMM were treated with LPS (Fig. 5A). The ability of GW2580 to inhibit c-Fms signaling was confirmed by demonstrating that M-CSF-induced tyrosine phosphorylation of cellular proteins, as well as Erk1/2 activation, was blocked in BMM that had been pretreated with GW2580 before stimulation (Fig. 5B). Therefore, the down-regulation of c-Fms surface expression, and hence loss of M-CSF signaling, by LPS may only partially account for the priming effects of LPS on CpG DNA responses in BMM. This conclusion is supported by our finding that LPS also primed CpG DNA-induced TNF production in BMM that had been deprived of M-CSF for 12 h before LPS pretreatment (Fig. 5C); LPS also primed TNF production in RAW 264.7 cells (Fig. 5D), an M-CSF-independent mouse macrophage cell line.
We next examined the effects of c-Fms inhibition (with GW2580) and LPS on TLR9 mRNA levels in BMM. As shown in Fig. 5E, TLR9 mRNA levels were ~2-fold higher in BMM that had been treated with GW2580 for 12 h in comparison to untreated cells. TLR9 mRNA levels were also elevated (2- to 3-fold) in BMM that had been deprived of M-CSF for 12 h (Fig. 5E). This latter finding is consistent with a prior report in which M-CSF was shown to repress TLR9 gene expression in BMM (40). In contrast to the effects of GW2580 (and M-CSF deprivation), LPS pretreatment partially suppressed TLR9 mRNA levels in BMM (Fig. 5E). Consequently, the c-Fms-dependent priming effects of LPS do not appear to be due to LPS relieving the inhibitory constraint of M-CSF signaling on TLR9 gene expression.

LPS pretreatment, but not c-Fms inhibition, amplifies CpG DNA-induced JNK activation

In view of the marked differences in the effects of LPS pretreatment and c-Fms inhibition (or M-CSF deprivation) on CpG DNA-induced TNF production in BMM, we directly compared the effects of LPS and c-Fms inhibition on TLR9 signaling. The activation of Erk1/2 by CpG DNA in BMM was affected to an equivalent degree, with LPS and GW2580 pretreatment both enhancing CpG DNA-induced Erk1/2 activation ~2.5-fold (Fig. 6A). Likewise, LPS and GW2580 were able to enhance CpG DNA-induced p38 MAPK activation to a similar extent (Fig. 6A). Notably, however, LPS pretreatment had a far greater effect on CpG DNA-induced JNK activation; this was even more pronounced than pretreatment with GW2580. In comparison to untreated BMM, CpG DNA-induced JNK activation was 4-fold higher in LPS-pretreated BMM, whereas pretreatment with GW2580 did not significantly enhance JNK activation in response to CpG DNA stimulation (Fig. 6A). Similar to the effects seen upon c-Fms inhibition with GW2580, BMM that had been deprived of M-CSF for 12 h showed greater CpG DNA-induced Erk1/2 and p38 MAPK activation than M-CSF-replete BMM (Fig. 6B). However, as with GW2580 pretreatment, M-CSF deprivation did not consistently enhance CpG DNA-induced JNK activation (Fig. 6B). Analysis of RAW 264.7 cells revealed that, like BMM, LPS pretreatment resulted in enhanced TLR9 signaling (Fig. 6C). Specifically, JNK remained activated for at least 90 min after CpG DNA stimulation in LPS-pretreated RAW 264.7 cells, whereas TNF production by LPS-pretreated BMM had been reduced. The data are representative of three independent experiments.

**JNK activity is required for enhanced CpG DNA-induced TNF production by LPS-pretreated BMM**

The finding that pretreatment of BMM with LPS and GW2580 had comparable effects on CpG DNA-induced activation of Erk1/2 and p38 MAPK but JNK activation was only enhanced in LPS-pretreated BMM suggested that LPS may largely prime the inflammatory response to CpG DNA by potentiating JNK activation. This idea was directly tested by treating LPS-pretreated BMM with a JNK inhibitor to block the CpG DNA-induced activation of JNK. As shown in Fig. 7A, SP600126 inhibited, in a concentration-dependent manner, the enhanced CpG DNA-induced production of TNF by LPS-pretreated BMM. The JNK inhibitor also suppressed TNF production by naive BMM. The dose-dependent effects of SP600126 on TNF production by LPS-pretreated BMM were found to correlate with the ability of the inhibitor to block the CpG DNA-induced activation of JNK (Fig. 7B).
Discussion

The activation of macrophages by TLR ligands triggers the release of proinflammatory cytokines (e.g., TNF and IL-6), which are central to the host response to infection. During an infection, macrophages are likely to encounter a number of different TLR ligands, potentially resulting in the sequential activation of TLRs. The types of TLR ligands encountered, as well as the timing of such encounters, may be important in dictating the nature of the inflammatory response elicited toward different pathogens. Indeed, signaling crosstalk between TLRs can result in fundamentally different responses: attenuation (i.e., tolerance) or the priming of inflammatory responses (12). Previous studies have largely explored the signaling crosstalk that gives rise to tolerance, while

FIGURE 5. Comparison of the effects of c-Fms inhibition with LPS pretreatment on TLR9 responses. A, BMM were treated with 5 μM GW2580 (gray bars) or 100 ng/ml LPS (black bars) for 2 or 12 h in the presence of M-CSF. The cells were washed with serum-free medium and then allowed to recover for 1 h in complete medium (containing M-CSF as well as GW2580 in the case of GW2580-treated BMM) before being stimulated with 0.5 μM CpG DNA for 2 h. TNF levels in culture supernatants were measured by ELISA. Combined data from three independent experiments are presented as the mean value ± SEM (*, p < 0.05). B, BMM were deprived of M-CSF for 12 h and then treated with 5 μM GW2580 for 30 min before stimulation with M-CSF for 5 min. Cell lysates were subjected to Western blotting with anti-phosphotyrosine (α-pY) and anti-phospho-Erk1/2 (α-pErk1/2) Abs. The blots were also probed with an anti-Erk2 Ab (α-Erk2) to confirm that equal amounts of cell protein had been loaded. C, RAW 264.7 cells were treated with 100 ng/ml LPS for 2 or 12 h. The cells were washed with serum-free medium and then allowed to recover for 1 h in complete medium (either containing or lacking M-CSF) before being stimulated with 0.5 μM CpG DNA for 2 h. TNF levels in culture supernatants were measured by ELISA. Representative data from three independent experiments are presented as the mean value ± SEM. D, RAW 264.7 cells were treated with 100 ng/ml LPS for 2 or 12 h. The cells were washed with serum-free medium and then allowed to recover for 1 h in complete medium before being stimulated with 0.5 μM CpG DNA for 2 h. TNF levels in culture supernatants were measured by ELISA. Representative data from two independent experiments are presented as the mean value ± SEM.

A

B

C

D

E

FIGURE 6. Comparison of the effects of LPS pretreatment with c-Fms inhibition on TLR9 signaling. A, BMM were treated with either 100 ng/ml LPS or 5 μM GW2580 for 2 or 12 h in the presence of M-CSF. The cells were washed with serum-free medium and then allowed to recover for 1 h in complete medium (containing M-CSF as well as GW2580 in the case of GW2580-treated BMM) before being stimulated with 0.5 μM CpG DNA for 30 min. Cell lysates were subjected to Western blotting with anti-phospho-Erk1/2 (α-pErk1/2), anti-phospho-JNK (α-pJNK), and anti-phospho-p38 MAPK (α-p38) Abs. The blots were also probed with an anti-p38 MAPK Ab (α-p38) to confirm that equal amounts of cell protein had been loaded. The data are representative of at least three independent experiments. B, BMM were either cultured in the presence of M-CSF or deprived of M-CSF for 12 h before being stimulated with 0.5 μM CpG DNA. Cell lysates were subjected to Western blotting with the indicated Abs. The data are representative of at least three independent experiments. C, RAW 264.7 cells were treated with 100 ng/ml LPS for 2 or 12 h. The cells were washed with serum-free medium and then allowed to recover for 1 h in complete medium before being stimulated with 0.5 μM CpG DNA. Cell lysates were subjected to Western blotting with the indicated Abs. The data are representative of two independent experiments.
little is understood about the mechanisms of priming. Here, we show that signaling crosstalk following LPS exposure can prime the inflammatory response of BMM to the TLR9 ligand, CpG DNA. The priming effects of LPS were not unique to BMM, as LPS pretreatment also potentiated CpG DNA-induced TNF production by RAW 264.7 cells. LPS appears to largely prime TLR9 responses in macrophages via effects on JNK; suppression of M-CSF signaling by LPS may also contribute to the priming effects in the case of BMM.

LPS signaling via TLR4 is mediated by four adaptor molecules including MyD88 (6), whereas TLR9 signaling is dependent exclusively on MyD88 (44, 45). In the induction of tolerance toward LPS a number of inhibitory molecules that block MyD88-dependent signaling, such as IRAK-M and MyD88s, are up-regulated (31, 32). The down-regulation of IRAK-1 and IRAK-4 expression levels also contributes to tolerance (14, 15, 17, 27, 29, 30). TLR9 signaling might therefore be expected to be blocked in BMM by preexposure to LPS. Given that we found this not to be the case, and that in fact LPS primed CpG DNA responses in both BMM and RAW 264.7 cells, it suggests that the levels of the inhibitory molecules induced by LPS (e.g., IRAK-M) were not sufficient, at least within the first few hours following LPS exposure, to block TLR9 signaling. Furthermore, although IRAK-1 was rapidly degraded in response to LPS, IRAK-2 was recently shown to function redundantly with IRAK-1 in TLR9 signaling (46). CpG DNA signaling via an alternative receptor, which signals independently of MyD88, following LPS pretreatment could also potentially explain the lack of cross-tolerance. However, our experiments with TLR9-deficient BMM and in macrophages inhibited for endosomal maturation demonstrated that CpG DNA-induced signaling following LPS exposure was still dependent on TLR9 expression and localization within the endosomal compartment. Why TLR4, but not TLR9, signaling was blocked by LPS preexposure is likely to be due to the rapid LPS-induced degradation of the critical TLR4 adaptor Mal (28), probably in concert with the down-regulation of IRAK-4 expression levels by LPS (14, 27). In contrast to TLR4, signaling through TLR9 is not dependent on Mal and is likely to be intact following LPS-pretreated Mal degradation (7, 28).

The priming effects of LPS on TLR9 responses appear to be explainable, at least in part, by LPS potentiating CpG DNA-induced JNK activation and, in the case of BMM specifically, by antagonizing M-CSF signaling. Pharmacologic inhibition of c-Fms with the small molecule inhibitor GW2580 enhanced CpG DNA-induced TNF production. However, the effects of GW2580 on TNF production were not as great as those achieved by LPS pretreatment. Moreover, while c-Fms inhibition potentiated the CpG DNA-induced activation of Erk1/2 and p38 MAPK, it did not enhance JNK activation; only LPS pretreatment enhanced the activation of JNK in response to CpG DNA stimulation. Given the role of JNK in regulating inflammatory cytokine production (47–50), the JNK-specific priming effects of LPS are likely to account for the higher levels of TNF produced by macrophages preexposed to LPS in comparison to those pretreated with GW2580. This conclusion is supported by the finding that pharmacologic inhibition of JNK abrogated the enhanced production of TNF by LPS pretreated BMM.

Although the molecular basis for the priming of CpG DNA-induced JNK activation by LPS is unknown, it may involve the dual specificity phosphatases, MKP-1 and MKP-5 (50–52). MKP-1 plays a key role in attenuating JNK and p38 MAPK activity following their TLR-mediated activation (51, 52), while MKP-5 appears to selectively target activated JNK (50). Interestingly, LPS was recently reported to induce the acetylation of MKP-1, which results in the increased binding of MKP-1 to p38 MAPK (53). The JNK-specific priming effects of LPS could therefore potentially arise from the selective targeting of MKP-1 to p38 MAPK rather than JNK in LPS-treated macrophages. LPS-induced reactive species, possibly in concert with MKP-1 acetylation, could also contribute to the potentiation of CpG DNA-induced JNK activation through inhibition of MKP-1 and/or MKP-5 activity (54–56).

Down-regulation of c-Fms surface expression by LPS also appears to be involved in the priming of TLR9 responses in BMM. It has previously been reported that M-CSF can suppress CpG DNA-induced BMM activation through its down-regulation of TLR9 gene expression (40). The ability of LPS to reduce c-Fms surface expression (41), which we have confirmed here, led to the suggestion that LPS could potentially enhance CpG DNA signaling by indirectly bringing about an increase in TLR9 expression (40). Although pharmacologic inhibition of c-Fms up-regulated TLR9 mRNA levels in BMM, LPS exerted an inhibitory rather than a stimulatory affect on TLR9 expression. Thus, the priming effects that occur as a result of the LPS-induced down-regulation of c-Fms surface expression are unlikely to be a consequence of the inhibitory constraints of M-CSF signaling on TLR9 expression being relieved by LPS. Instead, LPS may release TLR9 from the inhibitory actions of M-CSF via effects on signaling molecules (e.g., Erk1/2 and p38 MAPK) downstream of TLR9.

In contrast to our findings here, LPS pretreatment was previously reported to suppress CpG DNA-induced TNF production (15, 19). A likely reason for this difference is that the induction of...
cross-tolerance in previous studies was tested after the macrophages had been pretreated with LPS for around 24 h (15, 19). We have found that the priming effects of LPS on TLR9 responses in BMM and RAW 264.7 cells appear to be generally restricted to M-CSF-pretreated RAW 264.7 cells (15). Thus, the timing (and order) of sequential TLR activation may be particularly important in determining the nature of inflammatory responses. Such a conclusion is consistent with earlier reports that proposed that, rather than simply inducing tolerance, LPS can reprogram the inflammatory response of macrophages (24, 25).

We have shown here that signaling crosstalk between TLR4 and TLR9, as well as between these TLRs and c-Fms, affects the inflammatory response of macrophages. Our data also suggest that M-CSF suppresses some CpG DNA-induced signaling responses (e.g., Erk1/2 and p38 MAPK activation) but not other responses (e.g., JNK activation). This divergence in the effects of M-CSF on the activation of key inflammatory signaling molecules by TLR9 may provide an additional means by which macrophages can tailor their response to pathogens. The activation of JNK following sequential TLR activation may be particularly important in this regard. By regulating inflammatory gene expression (57), augmented JNK activation may heighten the ensuing inflammatory response. However, because JNK can also regulate cell survival through its role in apoptosis (58), the level of JNK activation resulting from sequential TLR activation may determine whether activated macrophages subsequently undergo apoptosis. This could represent a host defense mechanism to prevent macrophages from becoming parasitized by intracellular pathogens. Indeed, some intracellular pathogens, such as M. tuberculosis, have evolved strategies, including the targeting of JNK, to subvert apoptotic signaling pathways (59, 60). In summary, our study further demonstrates the complex nature of the TLR signaling crosstalk that regulates the host inflammatory reaction to bacterial infection.

Acknowledgments

Bone marrow cells from TLR9-deficient and littermate control mice were provided by Dr. Matthew Sweet (University of Queensland, Australia). The assistance of Dr. Jason Lenzo (University of Melbourne) with flow cytometry is also acknowledged.

Disclosures

The authors have no financial conflicts of interest.

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