Selective Activation of the p38 MAPK Pathway by Synthetic Monophosphoryl Lipid A*

Caglar Cekic1,9, Carolyn R. Casella5, Chelsea A. Eaves13,18, Atsushi Matsuzawa9, Hidenori Ichijo4, and Thomas C. Mitchell151

From the 1Department of Microbiology and Immunology and 3Institute for Cellular Therapeutics, University of Louisville School of Medicine, Louisville, Kentucky 40202 and the 5Laboratory of Cell Signaling, Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo 113-0033, Japan

TLR4 stimulation by lipopolysaccharide can cause both MAL/MyD88- and TRAM/TRIF (Toll IL-1 receptor domain-containing adaptor-inducing IFNβ)-dependent signaling events. Monophosphoryl lipid A (MPLA), a low toxicity derivative of endotox lipopolysaccharide, enhances antibodies responses, T cell expansion, and recall responses against antigens without causing excessive inflammatory side effects. Previously, we proposed that TRIF-biased activation of TLR4 by MPLA is responsible for its reduced toxicity while retaining potent adjuvant effects. However, some TRIF-associated genes, such as MCP-1, are only weakly expressed, and some MyD88-associated inflammatory and anti-inflammatory cytokines, such as tumor necrosis factor α and interleukin-10, are strongly activated after MPLA stimulation despite weak NF-κB but strong IRF3 activation. We now report that synthetic derivatives of MPLA retained TRIF bias as compared with synthetic diphosphoryl lipid A, indicating a change in a single phospholipid group is sufficient for TRIF-biased TLR4 stimulation. We extend our previous observations by showing that sMLA induces strong p38 MAPK but weak JNK activation, resulting in high IP-10 (interferon-inducible protein 10), tumor necrosis factor α, and interleukin-10 but low MCP-1 transcript levels. Results of this study identify a novel biochemical mechanism for regulation of sMLA-induced gene expression.

Numerous agonists of the immune system Toll-like receptor (TLR)2 family are under investigation as possible monotherapeutic agents and/or additives to vaccine preparations to improve protective immunity. However, strong inflammatory responses that can be caused by these compounds may limit their use in humans, especially in the context of prophylactic immunization of healthy individuals. One exception is the TLR4 agonist monophosphoryl lipid A (MPLA), a low toxicity derivative of lipopolysaccharide (LPS) that enhances T cell priming and antibody responses against antigens without causing excessive inflammatory side effects (1–4). We reported previously that the beneficial, immunostimulatory activities of MPLA are correlated with its ability to stimulate TLR4 preferentially through one of its two major signaling pathways, the TRIF adapter pathway (3). Gene expression and signaling events known to require the remaining adapter MyD88 were generally weak, suggesting a mechanism whereby TLR4 is able to trigger outcomes favorable for adaptive immunity without always driving proinflammatory outcomes. Understanding the mechanism(s) by which TLR4 and its co-receptor, MD-2, can be stimulated to signal in this TRIF-biased manner remains an important, unmet goal.

Upon exposure to its natural ligand MD-2/LPS, TLR4 first activates classical IkB kinases (IKKα/β) and MAPKs downstream of TRAF6 through MyD88 and its co-adaptor MAL at the plasma membrane (5–7). These events are followed by the internalization of TLR4 and activation of the TRAM/TRIF pathway, leading to activation of not only IKKα/β and MAPKs through TRAF6 but also of two non-classical IkB kinases, IKKe and TBK1 (TANK-binding kinase 1), through TRAF3 (6, 8–10). The IKKα/β and MAPK signaling pathways downstream of MyD88 and TRIF activate transcription factors such as NF-κB and AP-1, which are commonly associated with expression of inflammatory gene products (11). Activation of TBK1 and IKKe downstream of TRIF causes IRF3 (interferon regulatory factor 3) activation, which is required for transcription of IFNβ and of type I interferon-inducible genes (12).

MyD88- and TRIF-dependent pathways have overlapping functions that can cause harmful inflammatory effects as well as potent enhancement of adaptive immunity. This makes TLR4 an important therapeutic target because it is the only TLR known to engage both signaling pathways. One of the signaling pathways activated by both TRIF and MyD88 involves the MAPK family of signaling kinases (11). Growing evidence indicates that different MAPKs play different roles in mediating expression of MyD88-associated versus TRIF-associated genes at either transcriptional levels via activation of transcription factors or at post-transcriptional levels via mRNA transcript stabilization (13–19). We and others (1, 3, 20–22) noted previously that MPLA increased levels of not only TRIF-associated transcripts, such as IFIT1 (interferon-induced protein with tettratricopeptide repeats) and IFIT2, but also significant levels of NF-κB-associated IL-10, TNFa, IL-12, and co-stimulatory molecules, despite comparatively weak NF-κB activation. These
patterns suggest that specific members of the MAPK family may play a role in determining how MPLA-induced gene expression differs from that of LPS.

Characterizing the biological activities of various structures related to that of LPS is the subject of current efforts to achieve less inflammatory yet potent immunostimulatory activity (1). Our earlier study (3) focused on a comparison of LPS and MPLA, which had both been derived from the cell wall of Salmonella minnesota Re595 and consisted of mixtures of acylated diglucosamine structures that had varying numbers of acyl side chains, from three to seven. Use of these heterogeneous mixtures allowed us to study the closest available approximation to the clinical grade form of MPLA, MPL™ adjuvant but prevented a more detailed understanding of how differential engagement of TLR4 occurs. For example, structural acyl chain heterogeneity may cause activation of receptors or molecules other than TLR4, such as TLR2 or members of the inflammasome-activating nucleotide-binding oligomerization domain-like receptor family. Although it has been shown elsewhere that repurification of commercially available LPS structures prevents nonspecific TLR2 and inflammasome activation, even ultrapure LPS or MPLA preparations (but not synthetic lipid A) have been reported to activate both TLR2 and TLR4 in the presence of the lipid A-binding molecule CD14 (22, 23). Furthermore, the “active” form of MPLA has several structural differences compared with LPS. These differences include length of the oligosaccharide side chains, lack of two of three phosphate groups, as well as the differences in acyl chain number and length, which made correlation of outcomes with a single structural element impossible. To determine whether alteration of a single phosphate group can cause TRIF-biased signaling, we therefore performed extensive comparisons of the signaling activities of synthetic monophosphate lipid A (sMLA) and diphosphate lipid A (sDLA). Due to reagent availability, it was necessary to make this comparison in the context of an Escherichia coli-type lipid A structure, which differs from that of S. minnesota lipid A at one secondary acyl chain.

We now report that homogeneous preparations of synthetic E. coli MPLA (sMLA) largely retained their TRIF bias as compared with E. coli sDLA, indicating that a change in a single phosphoryl lipid A (sDLA) derived from the lipid A structures of E. coli LPS were purchased from Invivogen and Peptides International, respectively. Paired batches of sMLA and sDLA were prepared simultaneously by dissolving in dimethyl sulfoxide (DMSO) with gentle vortexing until particles were no longer visible; stocks were then aliquoted in small, disposable vials and stored at −80 °C. Thawed aliquots of sMLA or sDLA were used once and were never refrozen or reused. Granulocyte macrophage colony-stimulating factor was purchased from R&D Systems. Single strand cDNA synthesis enzymes were obtained from Invitrogen. Enzyme Mix 2×, containing SYBR Green dye for quantitative real-time PCR, was purchased from Applied Biosystems. Inhibitors of p38 (SB202190) and JNK (SP600125) were obtained from Calbiochem and Sigma, respectively. All primary antibodies used for immunoblotting experiments in this study were purchased from Cell Signaling Technology, except for antibodies against total JNK, TAK1, IRF3, and β-actin (Santa Cruz Biotechnology), and total ASK1 (apoptosis-regulating signal kinase 1) (Abcam, Inc.). Phospho-ASK1 (Thr-845) was described in Ref. 24. Horseradish peroxidase-conjugated anti-rabbit, anti-mouse, and anti-goat secondary antibodies were from Jackson ImmunoResearch Laboratories.

Generation of Bone Marrow-derived Dendritic Cells—Bone marrow-derived dendritic cells (BM-DCs) were prepared according to a protocol modified from that of Lutz et al. (25). Briefly, femurs and tibiae were collected and flushed with sterile Hank’s balanced salt solution twice. The resulting bone marrow cells were resuspended in R10F (RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 2 mm l-glutamine, 1 mm sodium pyruvate, 50 units/ml penicillin, and 50 μg/ml streptomycin) plus 50 μM 2-mercaptoethanol and 5 ng/ml granulocyte macrophage colony-stimulating factor. 2 × 10^6 cells per bacteriological culture plate were cultured for 10 days, with feeding on days 3 and 8 by adding 10 ml fresh medium and on day 6 by replacing one-half of the culture medium. Nonadherent cells were collected on day 10 and verified to be between 85 and 95% CD11b+ /CD11c+ /MHCII+/CD80+/CD86low/Gr1−/CD4+/CD8−/B220−/CD19− by flow cytometry before use in experiments.

Measurement of Cytokine Production—Splenocytes (1 × 10^6) were preincubated for 2 h in 96-well flat bottom plates at 37 °C prior to stimulation with different concentrations of sMLA or sDLA. Supernatants were collected after overnight incubation at 37 °C and tested for IP-10 (R&D Systems) and IFNγ (BD Biosciences Pharmingen) using enzyme-linked immunosorbent assay kits according to the manufacturer’s instructions.

Quantitative Real-time PCR—TLR4 agonist-induced changes in steady-state mRNA levels, which reflect both transcriptional activity and transcript stability, were measured in quantitative PCR (qPCR) assays. BM-DCs (1 × 10^6) were rested for 2 h in polystyrene tubes (12 × 75 mm) at 37 °C and then treated with 100 ng/ml sMLA or sDLA (diluted in R10F). In all experiments, DMSO was used as vehicle control. Cells were lysed 1, 2, or 3 h.
after activation in guanidine thiocyanate buffer. For p38 or JNK inhibition experiments, BM-DCs were preincubated for 30 min with 10 μM SB202190 (p38 inhibitor) or 10 μM SP600125 (JNK inhibitor) and then treated with the indicated concentrations of sMLA or sDLA for 1 h. Total RNA was isolated using the RNeasy mini kit (Qiagen), and cDNA was synthesized using the SuperScript III Platinum Two-Step quantitative reverse transcription-PCR kit (Invitrogen). Quantitative reverse transcription-PCR was performed using the Applied Biosystems 7500 Fast system and Power SYBR Green RT-PCR Master Mix. QuantiTect primers (Qiagen) were used for all qPCR assays except for primers used to measure β-actin mRNA (forward: TGGAATCCTGTGGCATCCATGAAAC; reverse: TAAAA-CGCAGCTCAGTAACAGTCCG), which were purchased from Sigma-Genosys. Expression of each target gene was normalized to β-actin and fold expression over vehicle control was calculated using the 2^−ΔΔC_T method (26).

Immunoblotting—BM-DCs (2–3 × 10^6) were rested for 2 h in polystyrene tubes at 37 °C and then exposed to sMLA or sDLA. In all experiments, DMSO was used as vehicle control. At the indicated time points, cells were centrifuged in ice-cold Hank’s balanced salt solution containing 50 mM NaF and then lysed in radioimmune precipitation assay buffer containing Complete mini protease inhibitor mixture tablets (Roche Applied Science), phosphatase inhibitor mixture (Sigma), and 250 nM okadaic acid (Sigma). Protein concentrations in the resulting lysates were measured using BCA assay (Pierce) and then mixed with 5× SDS sample buffer (1× final concentration). Lysates containing equal amounts of protein were loaded onto 8–10% SDS-PAGE gels for electrophoresis, after which resolved proteins were transferred onto nitrocellulose membranes (GE Healthcare) and blocked for 1 h with 5% nonfat dry milk or with 5% bovine serum albumin for some immunoblots. Primary antibodies were dissolved in 5% bovine serum albumin except for β-actin and IRF3 total antibodies, which were dissolved in 5% nonfat dry milk and incubated with the blocked membranes overnight at 4 °C. After exposure to horseradish peroxidase-conjugated anti-rabbit, antimouse, or anti-goat secondary antibodies for 1 h in nonfat dry milk, bands were visualized using the ECL Plus detection system (GE Healthcare), and band intensities were analyzed with Quantity One software (Bio-Rad version 4.6.6).

Statistical Analysis and Sample Normalization—Two factor (time or dose versus treatment) analysis of variance (ANOVA) and post hoc Tukey’s tests were performed to determine the significance of the differences between sDLA-induced versus sMLA-induced cellular effects on gene expression and signaling. p < 0.05 was considered to indicate statistically significant differences between treatment groups. All qPCR data were normalized to β-actin, and phosphoprotein band intensities were normalized to total protein levels. In some cases, β-actin normalization was used for phospho-IRF3 due to failure of recognition by total antibody after stripping. Signals from DMSO alone-treated cells were used as reference points for calculation of fold increases in expression and intensities.
A Single Phosphate Difference Is Responsible for TRIF-biased TLR4 Stimulation—Because biological preparations of LPS and MPLA are heterogeneous and have several structural differences from one another, we compared synthetic MPLA (sMLA) and synthetic diphosphoryl lipid A (sDLA) to test whether TRIF bias is attributable to a single phosphate difference between lipid A structures rather than acylation, heterogeneity, or other structural differences. Therefore, we stimulated splenocytes with different concentrations of sMLA versus sDLA and checked the secreted levels of IP-10 (TRIF-associated) and IFNγ/H9253 (MyD88-associated) from culture supernatants after overnight incubation. As shown in Fig. 1A, sMLA and sDLA are equally potent at inducing IP-10 protein secretion, whereas sMLA-induced IFNγ secretion is weaker as compared with sDLA. To test if TRIF-biased signaling outcomes also occur in more homogenous cell populations, we next tested dendritic cells, which are relevant due to their primary role in antigen presentation and adjuvant activity. We performed time course and dose-response experiments using BM-DCs and measured transcript levels of TRIF-associated versus MyD88-associated genes with qPCR 1–3 h after exposure to agonist. sMLA appeared to be as efficient as sDLA at increasing transcript levels of TRIF-associated gene products (IP-10, IFIT1, and IFIT2) while only weakly affecting those of MyD88-associated inflammatory gene products (COX-2, serpine-1, and endothelin-1) (Fig. 1, B and C).

TRIF-associated gene expression requires the activation of IRF3 through the TRIF/TRAF3 pathway, whereas both MyD88 and TRIF activate the NF-κB pathway to induce maximal inflammatory cytokine expression (6, 9, 11). Thus, we hypothesized that TRIF-biased gene expression by sMLA will be associated with strong IRF3 activation through TRIF but with weak stimulation of the NF-κB pathway. As can be seen in Fig. 2A, sMLA induced slightly reduced but nevertheless strong IRF3 phosphoactivation, which was completely TRIF-dependent because no activity was detectable with either agonist in TRIF-deficient cells (Fig. 2A, right). Both IKK and its downstream
target NF-κB (p65) were only weakly phosphoactivated after sMLA stimulation (Fig. 2, B and C). Thus, a change in a single phosphate was sufficient to shift TLR4 activation from driving balanced MyD88 and TRIF contributions to a state that is biased to the TRIF pathway.

Contributions of MyD88 and TRIF to the Expression of Indicator Genes—In our earlier results defining biological monophosphoryl lipid A (MPLA) as a TRIF-biased agonist of TLR4, not all TRIF-dependent genes (such as MCP-1) were induced to the same levels as observed with LPS. Furthermore, not all MyD88-associated genes were weakly activated after MPLA stimulation (3). To better understand adaptor component requirements for TRIF and/or MyD88-associated gene expression, we exposed BM-DCs from wild-type (WT), or MyD88-deficient (MyD88KO), or TRIF-deficient (Lps2) mice to either sMLA or sDLA and measured IP-10, MCP-1, or COX-2 transcript levels using qPCR. Importantly, for MyD88KO cells, in which TLR4 signaling is forced to occur through the TRIF adaptor pathway, sMLA and sDLA gave identical patterns indicating that the TRIF-stimulating activity of sMLA was fully functional as compared with sDLA (Fig. 3, A–C, right panels). The IP-10 pattern in WT versus mutant cells (Fig. 3A) indicates IP-10 expression was primarily a function of TRIF and that sMLA had full activity with respect to its induction (Fig. 3A). MCP-1 transcript levels were also strictly dependent on TRIF, but comparison of the activity of sDLA (open symbols) in WT (Fig. 3B, left) versus MyD88KO (Fig. 3B, far right) cells indicated that MyD88 plays a role in maximizing and maintaining MCP-1 transcript levels in sDLA-stimulated cells. Notably, MCP-1 transcript levels were low in sMLA-stimulated WT cells, which is consistent with weak stimulation of MyD88. Finally, COX-2 transcript levels indicated an important role for MyD88 (Fig. 3C, as expected, but also an unanticipated contribution by TRIF (Fig. 3C, center). Overall, these results suggested that sMLA retains full potency with respect to TRIF-stimulating activity, whereas weak induction of MCP-1 and COX-2 was attributable to the weak stimulation of MyD88 by sMLA because these genes require MyD88 as well as TRIF for optimal expression.

The Importance of p38 MAPK Activation for sMLA-induced Gene Expression—It has been shown that p38 and JNK MAPKs can modulate the activation of both MyD88- and TRIF-associated genes (11, 14, 15, 27). By using specific inhibitors of these enzymes (Fig. 4A), we next tested if sMLA differentially requires one MAPK pathway over another to induce potent IP-10 but weak MCP-1 and COX-2 expression. We preincubated BM-DCs with the inhibitors of either p38 or JNK pathway before activating with sDLA and tested specificity of their inhibitory activity by measuring phosphoactivation of c-Jun and MAPKAPK2 as direct downstream targets of JNK and p38, respectively. As can be seen in Fig. 4A, preincubation of BM-DCs with SP600125 (JNK inhibitor) diminished phosphoactivation of c-Jun but not MAPKAPK2, whereas SB202190 (p38 inhibitor) preincubation prevented only phosphoactivation of MAPKAPK2 but not c-Jun, confirming specific activity of these inhibitors for their corresponding targets. To test involvement of p38 and JNK MAPKs in IP-10, MCP-1, and COX-2 expression, we preincubated BM-DCs with the inhibitors of these kinases or with DMSO as control before exposing to either sMLA or sDLA and performing qPCR. We plotted the percent decrease in transcript levels after inhibitor + TLR4 ago.

FIGURE 3. Adaptor contribution to sMLA-induced indicator gene expression. A, BM-DCs from WT, MyD88–/–, or TRIFlps2/lps2 mice were treated with 100 ng/ml sMLA versus sDLA for the indicated time periods. qPCR was performed to detect fold increases in transcript levels of IP-10 (A), MCP-1 (B), and COX-2 (C). Results are mean values ± S.E. calculated from at least three independent experiments with three replicates in each. *, p < 0.05; n.s., not statistically significant by two-factor ANOVA and post hoc Tukey’s test.
nist treatment group relative to DMSO + TLR4 agonist treatment as a measure of percent inhibition of sMLA- or sDLA-induced mRNA expression. (Fig. 4B shows a sample of this calculation.) Preincubation with the inhibitor of p38 but not JNK caused a significant inhibition of IP-10 expression after either sMLA or sDLA treatment as compared with DMSO preincubated control group (Fig. 4B, black bars). MCP-1 RNA expression, on the other hand, was strongly dependent on JNK activation because JNK but not p38 inhibition caused significant decreases in MCP-1 transcript levels (Fig. 4C, white bars), indicating that sMLA can be a strong inducer of the p38 but not the JNK pathway. This pattern might explain potent IP-10 but not MCP-1 mRNA expression. Finally, COX-2 mRNA expression was found to depend partially on p38 (50% inhibition of transcript levels by p38 inhibitor; Fig. 4D, white bars) and almost entirely on JNK activation (90% inhibition by JNK inhibitor; Fig. 4D, white bars), suggesting low COX-2 transcript levels after exposure to sMLA may result from weak activation of the JNK pathway.

In our previous study, we found that MPLA and LPS had unexpectedly similar potencies in induction of proinflammatory TNFα and anti-inflammatory IL-10 (3). Several studies have implicated the p38 pathway as being required for optimal expression of both of these cytokines (27, 28). Therefore, we...
sMLA Selectively Activates the p38 MAPK Pathway—Because p38 but not JNK inhibitors reduced the expression of genes that were strongly induced by sMLA (IP-10, TNFα, and IL-10) and because a majority of the genes that were weakly induced by sMLA (MCP-1 and COX-2) largely depend on JNK activation, we hypothesized that sMLA would induce the p38 MAPK pathway strongly and the JNK MAPK pathway weakly when compared with sDLA. To test this hypothesis, we activated BM-DCs with sMLA or sDLA for different time intervals and performed immunoblotting to detect phosphoactivation of p38 and JNK MAPKs. As shown in Fig. 6B (center), sMLA induced significantly lower phosphoactivation of JNK than sDLA. However, there was only a slight and statistically insignificant reduction in early but not in late p38 phosphoactivation after sMLA stimulation (Fig. 6A, center). To further test activation of the p38 and JNK pathways, we measured phosphoactivation of MAPKAPK2 and c-Jun as direct downstream targets of p38 and JNK, respectively. sMLA and sDLA induced similar phosphoactivation of MAPKAP2 (Fig. 6A, right). However, c-Jun phosphorylation was weaker after sMLA stimulation (Fig. 6B, right), indicating the p38 but not JNK pathway was fully active after sMLA stimulation.

Activation of MAPKs (p38 and JNK) and the NF-κB pathway requires phosphoactivation of TAK1 (29). As can be seen in Fig. 6B (left), sMLA only weakly phosphoactivated TAK1, suggesting that other pathways may be strongly activated by sMLA to selectively activate p38 but not JNK. Recent studies in BM-DCs and splenocytes have revealed the existence of an alternative pathway involving TRAF6 and ASK1, which was required for optimal p38 phosphoactivation after LPS stimulation (13, 24). We found that sMLA-induced ASK1 phosphoactivation was stronger than that of sDLA (Fig. 6A, left) suggesting ASK1 or another unknown pathway can be triggered by sMLA to selectively activate the p38 pathway rather than the JNK and NF-κB pathways. Overall, our findings suggest that the lack of a single phosphate on lipid A structure results in strong activation of the p38 but not the JNK pathway.

Both TRIF and MyD88 Signaling Are Involved in sMLA-induced p38 Activation—Because activation of p38 can occur downstream of both MyD88 and TRIF and because each adaptor has a sequential contribution to IKKα/β and other MAPK signaling pathways, we next asked whether sMLA requires both adaptors or whether one of the adaptor molecules is differentially required. As shown in Fig. 7, p38 phosphoactivation occurred with different kinetics in the absence of MyD88 or TRIF after sMLA stimulation, indicating both adaptors are involved in sMLA-induced p38 activation in a sequential manner (MyD88-early, TRIF-late). In the absence of TRIF, sDLA caused significantly higher p38 activation as compared with sMLA, suggesting a very strong activation of the TLR4/MyD88 pathway by endotoxic sDLA but not by low toxicity sMLA, which instead required TRIF for much of its activity.

DISCUSSION

TLR4 activation can cause both MAL/MyD88-dependent and TRAM/TRIF-dependent signaling events. In our previous work (3), we reported that a low toxicity derivative of LPS, MPLA, activates genes that are associated primarily with the TRIF branch of the signaling pathway of TLR4 (3). However, there were a number of issues related to this finding that required additional investigation. 1) Both MPLA and LPS are actually heterogeneous mixtures of lipid A structures with variable numbers of acyl side chains, which may have different
effects on different kinds of cells; 2) not all TRIF-dependent genes, such as MCP-1, were fully active and not all inflammatory genes, such as TNFα, were weakly induced when comparing M PLA to LPS; and 3) it was not clear that the same pattern would be observed in dendritic cells, the antigen-presenting cell most responsible for T cell priming. In this study, we extended our observations by showing that TRIF-biased gene expression in BM-DC is attributable to a single phosphate difference because it was observed in experiments performed with homogeneous, synthetic MLA and DLA structures. Furthermore, it was found that sMLA induces strong p38 MAPK but weak JNK activation, resulting in strong IP-10, TNFα, and IL-10 but weak MCP-1 mRNA expression. A summary of our findings is depicted in Fig. 8.

Previous studies by our group and others showed that MLA induces significant levels of TNFα, IL-10, IL-12, and co-stimulatory molecules as compared with LPS (3, 20), suggesting that addition to weak IKKi/β or JNK signaling, some other important regulator of inflammatory responses can be activated by sMLA (17). Here, we report that sMLA was found to be a potent inducer of p38 activity. As one of the central regulators of inflammatory responses, p38 can activate a variety of transcription factors and kinase substrates such as NF-IL6 and MAPKAPK2 to increase gene expression through both transcription and mRNA stabilization, respectively (16, 18, 19, 30, 31). Although p38 appears to be phosphoactivated to the same levels in sMLA-stimulated cells as in sDLA-stimulated cells (Fig. 7), our qPCR measurements of transcript levels do not allow us to distinguish between effects on promoter activity versus mRNA stability. This point is particularly relevant to assays of MCP-1 transcript levels, which seemed to show a MyD88-dependent effect on duration of transcript abundance (Fig. 3). Hence, the interesting possibility that the pattern of gene induction of sMLA is affected by differential effects on mRNA transcript stability, whether through p38 or other mediators, remains to be tested. Nevertheless, the fact that both MyD88 and TRIF play non-redundant roles in the robust phosphoactivation of p38 by sMLA emphasizes the point that signaling initiated by this low toxicity TLR4 agonist is not devoid of MyD88 involvement.

Because it is known as a potent inflammatory inducer, p38 inhibition has been targeted for treatment of inflammatory diseases (32, 33). However, direct inhibition of p38 was only partially successful in these attempts, perhaps due to the contribution to expression by p38 of anti-inflammatory IL-10 and to its feedback activation of phosphatases, which limit prolonged NF-κB activation (32, 33). Therefore, in the context of vaccine development, robust activation of the p38 pathway and not
other MAPKs, such as JNK, may permit critically important contributions to the potent but safe adjuvanticity of MPLA. Because p38 also stimulates IRF3 binding to DNA, p38 activation enables expression of TRIF-dependent anti-viral genes as well as pro- and anti-inflammatory genes downstream of TLR4 (13, 14, 34). Our findings confirm the functional outcomes of these observations because p38 inhibition reduced not only MyD88-associated TNFα, IL-10, and COX-2 but also TRIF-dependent IP-10 expression after sMLA stimulation.

Despite its low toxicity, MPLA adjuvant was shown to induce strong Th1 type immune responses, which is characterized by strong IgG2 antigen-specific immune responses and the induction of cytokines such as IL-12 and TNFα by antigen-presenting cells and IFNγ by NK cells and T cells (35, 36). Recent studies have shown that MCP-1 down-modulation correlates with concomitant increases in Th1 type cytokine expression and cellular responses (36). Thus, selective activation of p38 but not JNK and therefore strong TNFα and IP-10 but weak MCP-1 expression, may play an important role in the adjuvanticity of MPLA by contributing to low toxicity Th1 differentiation. This and other functional consequences of TRIF-biased cytokine expression are currently under investigation.

Alteration of lipid A structures such as hypophosphorylation is known to be used by pathogens as an immune evasion mechanism (37, 38). It is possible that active dephosphorylation of lipid A structures allows pathogens to escape MyD88-dependent initial inflammatory response and thus rapid clearance as well as to induce strong anti-inflammatory cytokine expression through p38 activation and TRIF-dependent NFκB and anti-inflammatory IL-10.
MPLA, may provide beneficial adjuvant properties, including induction of potent adaptive immune responses without causing excessive inflammation.

Overall, our study shows that sMLA-induced gene expression requires the TRIF and p38 MAPK pathways. This is the first study to define a TRIF-biased activation pattern of downstream TLR4 signaling events by a structurally well defined, synthetic lipid A molecule with a single phosphoryl group. These findings will help design better vaccine adjuvants and immunomodulatory agents based on lipid A structures.

Acknowledgments—We thank Dr. Paula M. Chilton and Dr. Duygu Sag for valuable comments and suggestions.

REFERENCES

1. Alderson, M. R., McGowan, P., Baldridge, J. R., and Probst, P. (2006) J. Endotoxin Res. 12, 313–319
2. Thompson, B. S., Chilton, P. M., Ward, J. R., Evans, J. T., and Mitchell, T. C. (2005) J. Leukocyte Biol. 78, 1273–1280
3. Mata-Haro, V., Cekic, C., Martin, M., Chilton, P. M., Casella, C. R., and Martin, M. (2006) Exp. Hematol. 34, 219–229
4. Evans, J. T., Cluff, C. W., Johnson, D. A., Lacy, M. J., Persing, D. H., and Baldridge, J. R. (2003) Expert. Rev. Vaccines 2, 219–229
5. Kawai, T., Adachi, O., Ogawa, T., Takeda, K., and Akira, S. (1999) Immunity 11, 115–122
6. Kagan, J. C., Su, T., Horng, T., Chow, A., Akira, S., and Medzhitov, R. (2006) J. Immunol. 176, 3635–3641
7. Yamamoto, M., Sato, S., Hemmi, H., Sanjo, H., Uematsu, S., Kaisho, T., Hoshino, K., Takeuchi, O., Kobayashi, M., Fujita, T., Takeda, K., and Akira, S. (2002) Nature 420, 324–329
8. Husebye, H., Halaas, Ø., Stemmark, H., Tunheim, G., Sandanger, Ø., Bogen, B., Brech, A., Latz, E., and Espevik, T. (2006) EMBO J. 25, 683–692
9. Haste, K., Xu, X., George, P., Janssen, E., Tabeta, K., Kim, S. O., Goode, J., Lin, P., Mann, N., Mudd, S., Crozat, K., Sovath, S., Han, J., and Beutler, B. (2003) Nature 424, 743–748
10. Yamamoto, M., Sato, S., Hemi, H., Uematsu, S., Hoshino, K., Kaisho, T., Takeuchi, O., Takeda, K., and Akira, S. (2003) Nat. Immunol. 4, 1144–1150
11. Lee, M. S., and Kim, Y. J. (2007) Annu. Rev. Biochem. 76, 447–480
12. Kawai, T., Takeuchi, O., Fujita, T., Inoue, I., Mühlradt, P. F., Sato, S., Hoshino, K., and Akira, S. (2001) J. Immunol. 167, 5887–5894
13. Chiang, E., Dang, O., Anderson, K., Matsuizawa, A., Ichijo, H., and David, M. (2006) J. Immunol. 176, 5720–5724
14. Navarro, L., and David, M. (1999) J. Biol. Chem. 274, 35335–35338
15. Arndt, P. G., Suzuki, N., Avdi, N. J., Malcolm, K. C., and Worthen, G. S. (2004) J. Biol. Chem. 279, 10883–10891
16. Mestre, J. R., Mackrell, P. J., Rivadeneira, D. E., Stapleton, P. P., Tanabe, T., and Daly, J. M. (2001) J. Biol. Chem. 276, 3977–3982
17. Bohnenkamp, H. R., Papazisis, K. T., Burchell, J. M., and Taylor-Papadimitriou, J. (2007) Cell. Immunol. 247, 72–84
18. Horwood, N. J., Page, T. H., McDaid, J. P., Palmer, C. D., Campbell, J., Mahon, T., Brennan, F. M., Webster, D., and Foxwell, B. M. (2006) J. Immunol. 176, 3635–3641
19. Laza, M., Mahtani, K. R., Finch, A., Brewer, G., Saklatvala, J., and Clark, A. R. (2000) Mol. Cell. Biol. 20, 4265–4274
20. Salkowski, C. A., Detore, G. R., and Vogel, S. N. (1997) Infect. Immun. 65, 3239–3247
21. Okamoto, K., Kasawaki, K., Hanada, K., Miura, M., and Nishijima, M. (2006) J. Immunol. 176, 1203–1208
22. Martin, M., Micheale, S. M., and Katz, J. (2003) Infect. Immun. 71, 2498–2507
23. Spiller, S., Doret, S., Meng, G., Grabiec, A., Thomas, W., Hartung, T., Pfeffer, K., Hochrein, H., Brade, H., Bessler, W., Wagner, H., and Kirshning, C. J. (2007) J. Biol. Chem. 282, 13190–13198
24. Matsuizawa, A., Suegasa, K., Noguchi, T., Sadamitsu, C., Nishitoh, H., Nagai, S., Koyasu, S., Matsumoto, K., Takeda, K., and Ichijo, H. (2005) Nat. Immunol. 6, 587–592
25. Lutz, M. B., Kukutsch, N., Oligvieve, A. L., Rössner, S., Koch, F., Romani, N., and Schuler, G. (1999) J. Immunol. Methods 223, 77–92
26. Livak, K. J., and Schmittgen, T. D. (2001) Methods 25, 402–408
27. Salmon, R. A., Guo, X., Teh, H. S., and Schrader, J. W. (2001) Eur. J. Immunol. 31, 3218–3227
28. Foey, A. D., Parry, S. L., Williams, L. M., Feldmann, M., Foxwell, B. M., and Brennan, F. M. (1998) J. Immunol. 160, 920–928
29. Wang, C., Deng, L., Hong, M., Akkaraju, G. R., Inoue, J., and Chen, Z. J. (2001) Nature 412, 346–351
30. Thornton, T. M., Pedraza-Alva, G., Deng, B., Wood, C. D., Aronshtam, A., Clements, J. L., Sabio, G., Davis, R. J., Matthews, D. E., Doble, B., and Schuler, G. (1999) J. Immunol. 162, 4279–4288
31. Basak, C., Pathak, S. K., Bhattacharya, A., Mandal, D., Pathak, S., and Kundu, M. (2005) J. Biol. Chem. 280, 4279–4288
32. Cohen, P. (2009) Curr. Opin. Cell Biol. 21, 317–324
33. Rincón, M., and Davis, R. J. (2009) Immunol. Rev. 228, 212–224
34. Dang, O., Navarro, L., Anderson, K., and David, M. (2004) J. Immunol. 172, 747–751
35. Wheeler, A. W., Marshall, J. S., and Ulrich, J. T. (2001) Int. Arch. Allergy Immunol. 126, 135–139
36. Del, C. M., Michienzi, A., Masotti, A., Da, S. L., Bottazzio, G. F., Belardelli, F., and Gessani, S. (2009) Blood 114, 796–906
37. Dixon, D. R., and Darveau, R. P. (2005) J. Dent. Res. 84, 584–595
38. Tran, A. X., Whittimore, J. D., Wyrick, P. B., McGrath, S. C., Cotter, R. J., and Trent, M. S. (2006) J. Bacteriol. 188, 4531–4541
39. Park, S. M., Ko, H. J., Shim, D. H., Yang, J. Y., Park, Y. H., Curtiss, R., and Kweon, M. N. (2008) J. Immunol. 181, 6447–6455
40. Sukhumavasi, W., Egan, C. E., Warren, A. L., Taylor, G. A., Fox, B. A., Bizk, D. J., and Denkers, E. Y. (2008) J. Immunol. 181, 3464–3473