SELECTIVE ACTIVATION OF p38 MAPK PATHWAY BY SYNTHETIC MONOPHOSPHORYL LIPID A

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TLR4 stimulation by lipopolysaccharide (LPS) can cause both MAL/MyD88- and TRAM/TRIF-dependent signaling events. Monophosphoryl Lipid A (MPLA), a low toxicity derivative of endotoxic LPS, enhances antibody 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 MCP1 are only weakly expressed and some MyD88-associated inflammatory and anti-inflammatory cytokines such as TNFα and IL-10 are strongly activated after MPLA stimulation despite weak NF-κB but strong IRF3 activation. We now report that synthetic derivatives of MPLA (sMLA) retained TRIF-bias as compared to synthetic diphosphoryl Lipid A (sDLA), indicating a change in a single phosphoryl 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, TNFα and IL-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) receptor 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; 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 pro-inflammatory outcomes (3). 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 IκB Kinases (IKKα/β) and MAPKs downstream of TRAF6 through MyD88 and its co-adapter 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 IκB-kinases, IKKε and TANK-binding kinase 1 (TBK1) 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 IKKε downstream of TRIF causes interferon regulatory factor 3 (IRF3) 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 vs 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 noted previously that MPLA increased levels of not only TRIF-associated transcripts such as IFIT1 and IFT1 but also significant levels of NFκB-associated IL-10, TNFα, IL-12 and co-stimulatory molecules, despite comparatively weak NF-κB activation (1,3,20-22). 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 activity (1). Our earlier study (3) focused on a inflammatory yet potent immunostimulatory activity (1). Growing evidence indicates that different MAPKs play different roles in mediating expression of MyD88 vs 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 noted previously that MPLA increased levels of not only TRIF-associated transcripts such as IFIT1 and IFT1 but also significant levels of NFκB-associated IL-10, TNFα, IL-12 and co-stimulatory molecules, despite comparatively weak NF-κB activation (1,3,20-22). 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.

We now report that homogeneous preparations of synthetic E. coli MPLA (sMLA) largely retained their TRIF-bias as compared to E. coli sDLA, indicating that a change in a single phosphoryl group is sufficient to bring about TRIF-biased activation of TLR4. Examination of signaling activity downstream of TLR4 revealed that sMLA strongly activated p38 but not JNK MAPK activity, which was associated with optimal IP-10, TNFα, and IL10 but weak MCP-1 and COX2 expression. Both MyD88 and TRIF were found to play non-redundant roles in p38 activation after sMLA stimulation, indicating that sMLA-induced signaling is not completely devoid of MyD88 involvement. Results of this study thus identify a novel biochemical mechanism for TLR4-dependent patterns of gene expression by monophosphorylated Lipid A that can be exploited to improve the safety and efficacy of immunostimulatory agents.

Experimental Procedure

Mice and Reagents: C57BL/6 mice and TRIF<sup>−/−</sup> mutant mice were purchased from The Jackson Laboratory. MyD88<sup>−/−</sup> mice were a gift from Shizuo Akira (via Ross Kedl, University of Colorado Health Sciences Center). All mice were kept in a specific pathogen-free animal facility at the University of Louisville and experiments were performed under supervision of its Institutional Animal Care and Use Committee. Synthetic versions of monophosphoryl lipid A (sMLA) and diphosphoryl 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 DMSO with gentle vortexing until particles were no longer visible; stocks were then aliquoted in small, disposable volumes and stored at -80°C. thawed aliquots of sMLA or sDLA were used once and never re-frozen or re-used. GM-CSF was
purchased from R&D Systems. Single strand cDNA synthesis enzymes were obtained from Invitrogen. Enzyme mix 2X, 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 (Abcam Inc.). Phospho-ASK1 (thr845) was described in (24). HRP-conjugated anti-rabbit, anti-mouse and anti-goat secondary antibodies were from Jackson ImmunoResearch Inc.

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 (HBSS) twice. The resulting bone marrow cells were resuspended in R10F (RPMI 1640 medium containing 10% heat-inactivated FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 U/ml penicillin, 50 μg/ml streptomycin) plus 50 μM 2-mercaptoethanol and 5 ng/ml GM-CSF. 2x10^6 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. Non-adherent 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 (1x10^6) were pre-incubated for 2h 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 ELISA 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 Q-PCR assays. BM-DCs (1x10^6) were rested for 2h in polystyrene tubes (12x75 mm) at 37°C and then treated with 100ng/ml sMLA or sDLA (diluted in R10F). In all experiments DMSO was used as vehicle control. Cells were lysed 1, 2 or 3 hrs after activation in guanidine thiocyanate buffer. For p38 or JNK inhibition experiments BM-DCs were preincubated for 30m with 10μM SB202190 (p38 inhibitor) or 10μM SP600125 (JNK inhibitor) and then treated with the indicated concentrations of sMLA or sDLA for 1h. Total RNA was isolated using the RNeasy Mini Kit (Qiagen), and cDNA was synthesized using the SuperScript III Platinum Two-Step qRT-PCR kit (Invitrogen Life Technologies). Quantitative RT-PCR (qPCR) was performed using the Applied Biosystems 7500 Fast system and Power SYBR-Green RT-PCR mastermix. QuantiTect Primers (Qiagen) were used for all qPCR assays except for primers used to measure β-actin mRNA (Fwd: TGGGAATCTCTG TGGCATCCATGAAAC; Rev: TAAAACGCAGCTCAGTAACAGTCCG), 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^ΔΔCt method (26).

Immunoblotting: BM-DCs (2-3x10^6) 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 HBSS containing 50μM NaF and then lysed in RIPA lysis buffer containing Complete Mini protease inhibitor cocktail tablets (Roche), phosphatase inhibitor cocktail (Sigma-Aldrich) and 250nM Okadaic acid (Sigma-Aldrich). Protein concentrations in the resulting lysates were measured using the BCA assay (Pierce Biotechnology) and then mixed with 5X SDS-sample buffer (1X 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 1h with 5% non-fat dry milk (NFDM) or with 5% bovine serum albumin (BSA) for some immunoblots. Primary antibodies were dissolved in 5% bovine serum albumin (BSA) except for β-actin and IRF3 total antibodies, which were dissolved in 5% NFDM and incubated with the blocked membranes overnight at 4°C. After exposure to HRP-conjugated anti-rabbit, anti-mouse, or anti-goat secondary antibodies for an hour in NFDM, bands were visualized using the ECLplus 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 vs. treatment) analysis of variance (ANOVA) and post-hoc Tukey’s tests were performed to determine the significance of the differences between sDLA- vs. 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 phospho-protein 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.

RESULTS

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 vs. sDLA and checked the secreted levels of IP-10 (TRIF-associated) and IFNγ (MyD88-associated) from culture supernatants after overnight incubation. As shown in Figure 1A, sMLA and sDLA are equally potent at inducing IP-10 protein secretion while sMLA-induced IFNγ secretion is weaker as compared to 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 bone marrow-derived dendritic cells (BM-DCs) and measured transcript levels of TRIF- vs. MyD88-associated genes with quantitative real-time PCR (QPCR) 1-3h after exposure to agonist. sMLA appeared to be as efficient as sDLA at increasing transcript levels of TRIF-associated genes (IP-10, IFIT1 and IFIT2) while only weakly affecting those of MyD88-associated inflammatory genes (COX-2, Serpine-1, Endothelin-1) (Figure 1B-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 NF-κB pathway. As can be seen in Figure 2A, sMLA induced slightly reduced but nevertheless strong IRF3 phospho-activation, which was completely TRIF-dependent because no activity was detectable with either agonist in TRIF-deficient cells (Figure 2A, right). Both IKK and its downstream target NF-κB (p65) were only weakly phospho-activated after sMLA stimulation (Figure 2B-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 more precisely understand adaptor component requirements for TRIF and/or MyD88-associated gene expression we exposed BM-DCs from wild type (WT), or MyD88- (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 sMLA’s TRIF-stimulating activity was fully functional as compared to sDLA (Figure 3A-C, right panels). The IP10 pattern in WT vs mutant cells (Figure 3A) indicates IP10 expression was primarily a function of TRIF and that sMLA had full activity with respect to its induction (Figure 3A). MCP-1 transcripts were also strictly dependent on TRIF, but comparison of sDLA’s activity (open symbols) in WT (Figure 3B, left) vs. MyD88KO (Figure3B, 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 (Figure 3C, right), as expected, but also an unanticipated contribution by TRIF (Figure 3C, center). Overall these results suggested that sMLA retains full potency with respect to TRIF-stimulating activity while weak induction of MCP-1 and COX-2 was attributable to sMLA’s weak stimulation of MyD88 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 (Figure 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 pre-incubated BM-DCs with the inhibitors of either p38 or JNK pathway before activating with sDLA and tested specificity of their inhibitory activity by measuring phospho-activation of c-Jun and MAPKAPK2 as direct downstream targets of JNK and p38, respectively. As can be seen in Figure 4A pre-incubation of BM-DCs with SP600125 (JNK inhibitor) diminished phospho-activation of c-Jun but not MAPKAPK2 whereas SB202190 (p38 inhibitor) pre-incubation prevented only phospho-activation 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 IP10, MCP1 and COX2 expression we pre-incubated 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 agonist treatment group relative to DMSO+TLR4 agonist treatment as a measure of percent inhibition of sMLA- or sDLA-induced mRNA expression (Figure 4B shows a sample of this calculation). Pre-incubation with the inhibitor of p38, but not JNK caused a significant inhibition of IP10 expression after either sMLA or sDLA treatment as compared to DMSO pre-incubated control group (Figure 4B, black bars). MCP1 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 (Figure 4C, white bars), indicating that sMLA can be a strong inducer of the p38 but not the JNK pathway. This pattern might explain potent IP10 but not MCP1 mRNA expression. Finally, COX-2 mRNA expression was found to depend partially on p38 (50% inhibition of transcript levels by p38 inhibitor, Figure 4D, white bars) and almost entirely on JNK activation (90% inhibition by JNK inhibitor, Figure 4D, white bars), suggesting late COX2 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 pro-inflammatory 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 tested if sMLA could induce equivalent mRNA expression of these cytokines as compared to sDLA and if p38 is required for this activity. As can be seen in Figure 5A-B, sMLA and sDLA also revealed similar potency with respect to increasing TNFα and IL-10 transcript levels. Pre-incubation of BM-DCs with the p38 but not the JNK inhibitor caused a significant reduction in sMLA-induced transcript levels of either TNFα or IL-10 (Figure 5C-D, respectively), confirming the central role of p38 MAPK for expression of multiple gene products after sMLA stimulation.

sMLA selectively activates the p38 MAPK pathway. Because p38 but not JNK inhibitors reduced the expression of genes that were strongly induced by sMLA (IP10, TNFα and IL10) and because a majority of the genes that were weakly induced by sMLA (MCP1 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 to sDLA. To test this hypothesis we activated BM-DCs with sMLA or sDLA for different time intervals and performed immunoblotting to detect phospho-activation of p38 and JNK MAPKs. As shown in Figure 6B (center) sMLA induced significantly lower phospho-activation of JNK than sDLA did. However, there was only a slight and statistically insignificant reduction in early but not in late p38 phospho-activation after sMLA stimulation (Figure 6A, center). To further test activation of the p38 and JNK pathways we measured phospho-activation of MAPKAPK2 and c-Jun as direct downstream targets of p38 and JNK, respectively. sMLA and sDLA induced similar phospho-
activation of MAPKAPK2 (Figure 6A, right). However, c-jun phosphorylation was weaker after sMLA stimulation (Figure 6B, right), indicating the p38 but not JNK pathway was fully active after sMLA stimulation.

Activation of MAPKs (p38 and JNK), and NF-κB pathway requires phospho-activation of TAK1 (29). As can be seen in Figure 6B (left) sMLA only weakly phospho-activated 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 phospho-activation after LPS stimulation (13,24). We found that sMLA-induced ASK1 phospho-activation was stronger than that of sDLA (Figure 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 Figure 6, p38 phospho-activation 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 to sMLA, suggesting a very strong activation of 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- and TRAM/TRIF-dependent signaling events. In our previous work we reported that a low toxicity derivative of LPS, MPLA, activates genes that are associated primarily with the TRIF branch of TLR4’s signaling pathway. 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 MPLA to LPS , and 3) it was not clear that the same pattern would be observed in dendritic cells, the APC 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 Figure 8.

Previous studies by our group and others showed that MPLA induces significant levels of TNFα, IL-10, IL-12 and co-stimulatory molecules as compared to LPS (3,20) suggesting that in addition to weak IKKα/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 phospho-activated to the same levels in sMLA- as in sDLA-stimulated cells (Figure 7), our QPCR measurements of transcript levels do not allow us to distinguish between effects on promoter activity vs. 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 (Figure 3). Hence, the interesting possibility that sMLA’s pattern of gene induction 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 phospho-activation 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 p38’s contribution to expression of anti-inflammatory IL10 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 MAPK’s 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, IL10 and COX-2 but also TRIF-dependent IP-10 expression after sMLA stimulation.

Despite its low toxicity MPL™ 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 APCs 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 IP10 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 de-phosphorylation 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 endotoxin tolerance (27,28). However, our immune system may be well adapted to this evasion mechanism because priming T cell responses through TLR4 (2-4), and immunity to certain mucosal pathogens (39,40) are largely MyD88-independent. Therefore, TRIF-biased stimulation of TLR4 along with selective activation of p38 by 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 immuno-modulatory agents based on lipid A structures.

REFERENCES


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The abbreviations used are: TRIF, Toll IL-1 receptor domain-containing adaptor-inducing IFNβ; COX-2, cyclooxygenase-2; IKK, inhibitory κB kinase; ASK1, Apoptosis-Regulating Signal Kinase 1; TBK1, TANK-binding kinase 1; BM-DC; bone marrow-derived dendritic cells; MPLA, Monophosphoryl Lipid A; sMLA, synthetic MPLA; sDLA, synthetic diphosphoryl Lipid A; IFIT, interferon-induced protein with tetratricopeptide repeats; IP-10, interferon-inducible protein.

FIGURE LEGENDS

Figure 1. TRIF-biased gene expression after sMLA stimulation. A. Splenocytes were treated with indicated concentrations of sMLA or sDLA for 18h. ELISA was performed to detect secreted IP-10 (TRIF-associated) or IFNγ (MyD88-associated) from culture supernatants. B. BM-DCs were treated with the indicated concentrations of sMLA or sDLA for 3hrs. Q-PCR was performed to detect fold-increases in mRNA expression of IP-10 and COX-2 as representative of TRIF vs. MyD88-associated genes, respectively. C. BM-DCs were treated with 100ng/mL sMLA vs sDLA for the indicated time periods. Q-PCR was performed to detect fold-increases in expression of TRIF-associated genes (IFIT1 and IFIT2) or MyD88-associated genes (Serpine-1 and Endothelin-1) DMSO alone was used as vehicle control. Results are mean values ± SEM calculated from 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.

Figure 2. Strong IRF3 but weak NF-κB activation by sMLA. BM-DCs were treated with 100ng/mL sMLA or sDLA for the indicated time periods. Western blotting was performed to detect activating phosphorylations of A. pIRF3 (ser396) in WT or TRIFlop2/lop2 cells, and B. IKKα/β (ser176/ser180) and p65 (ser536) in WT cells. DMSO alone for 10m was used as vehicle control (VC) and 0’ time point. The result is representative of three or more independent experiments. *P<0.05; n.s., not statistically significant by two-factor ANOVA and post-hoc Tukey’s test.

Figure 3. Adaptor contribution to sMLA-induced indicator gene expression. A. BM-DCs from WT or MyD88-/− or TRIFlop2/lop2 mice were treated with 100ng/mL sMLA vs. sDLA for the indicated time periods. Q-PCR was performed to detect fold-increases in transcript levels of A. IP-10, B. MCP-1, and C. COX2. Results are mean values ± SEM 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.

Figure 4. Significant dependence on p38 for sMLA-induced gene expression. A. BM-DCs were preincubated with either DMSO or 10μM (p38 inhibitor) or 10μM SP600125 (JNK inhibitor) for 30m and then treated 100 ng/mL sDLA for 1h. Immunoblotting was performed to detect levels of phosphorylated c-jun and MAPKAPK2 to test specificity of SB202190 as p38 inhibitor and SP600125 as JNK inhibitor, respectively. B-D. BM-DCs were preincubated with either DMSO or 10μM SB202190 (p38 inhibitor) or 10μM SP600125 (JNK inhibitor) for 30m and then treated with the indicated concentrations of sMLA or sDLA for 1h. Q-PCR was performed to measure inhibitor activity of B. IP-10, C. MCP-1 and D. COX2. As shown for IP10, the percent inhibition of expression was calculated from observed decreases in mRNA levels upon addition of inhibitor. DMSO alone was used as vehicle control. Results are mean values ± SEM calculated from three independent experiments duplicates in each *P<0.05; n.s., not statistically significant by ANOVA.

Figure 5. Equivalent expression of TNFα and IL10 by sMLA requires p38 activity. BM-DCs were treated with 100ng/mL sMLA or sDLA for 1 and 2h. Q-PCR was performed to detect fold-increases in
expression of A. TNFα and B. IL10. BM-DCs were preincubated with either DMSO or 10μM SB202190 (p38 inhibitor) or 10μM SP600125 (JNK inhibitor) for 30m and then treated with 100ng/mL concentrations of sMLA or sDLA for 1h. Q-PCR was performed to detect fold expression of C. TNFα and D. IL10. DMSO alone was used as vehicle control. Results are mean values ± SEM calculated from three independent experiments duplicates in each *P<0.05; n.s., not statistically significant by ANOVA.

**Figure 6.** Effective phospho-activation of p38 but not JNK MAPKs by sMLA. BM-DCs were treated with 100ng/mL sMLA or sDLA for the indicated time periods. Western blotting was performed to detect activating phosphorylations of A. p38 (thr180/tyr182), its upstream kinase pASK1 (thr845), and its downstream target MAPKAPK2 (thr222). B. JNK1/2/3 (thr183/tyr185), its upstream kinase TAK1 and its downstream target c-Jun (ser63). DMSO alone for 10m was used as vehicle control (VC) and 0’ time point. The membranes were stripped and reprobed with antibodies against total proteins and β-actin as loading controls. Blots are representative of three or more independent experiments. Phosphorylated/total ratios of band intensities from all independent experiments were plotted and shown below the representative blots. *P<0.05; n.s., not statistically significant by two-factor ANOVA and post-hoc Tukey’s test.

**Figure 7.** Both MyD88 and TRIF contribute to sMLA-induced p38 phospho-activation. BM-DCs were treated with 100ng/mL sMLA or sDLA for the indicated time periods. Western blotting was performed to detect activating phosphorylation of p38(thr180/182) in MyD88KO BM-DCs (left) or TRIFlps2/lps2 BM-DCs (right). DMSO treatment for 10m was used as vehicle control (VC) and 0’ time point. The membranes were stripped and re-probed with antibodies against total proteins and β-actin as loading controls. Blots are representative of three or more independent experiments. Average phosphorylated/total ratios of band intensities from all independent experiments were plotted and shown below the representative blots. *P<0.05; n.s., not statistically significant by two-factor ANOVA and post-hoc Tukey’s test.

**Figure 8.** Summary of Trif-biased signaling and selective p38 activation by sMLA. Upon activating the TLR4 complex, sMLA only weakly induces downstream events known to be activated through the TRIF/TRAF6/TAK1 axis, such as activation of IKKα/β and JNK. These kinases are activated strongly by sDLA, which leads to increased activation of transcription factors required to activate COX2 and MCP1. On the other hand, sMLA treatment results in equivalent IRF3 activation downstream of TLR4/TRIF and p38 activation as compared to sDLA; this signaling results in potent activation of not only TRIF-associated IP10 but also pro-inflammatory TNFα and anti-inflammatory IL10.
FIGURE 1

A. Splenocytes

- **IP-10**: n.s.
- **IFN**

B. BM-DC

- **IP-10**: n.s.
- **COX-2**: * 

C. BM-DC

- **TRIF-dependent**
  - **IFIT-1**: n.s.
  - **IFIT-2**: n.s.

- **MyD88-TRIF co-dependent**
  - **Serpine-1**: * 
  - **Endothelin-1**: * 

Relative mRNA level vs. agonist (ng/mL)
FIGURE 2

A. WILD TYPE

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<th>sDLA</th>
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Time (min.)

sMLA (100ng/mL) sDLA (100ng/mL)

B. TRIF

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Time (min.)

sMLA (100ng/mL) sDLA (100ng/mL)

C. WILD TYPE

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<tr>
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Time (min.)

sMLA (100ng/mL) sDLA (100ng/mL)

D. TRIF

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Time (min.)

sMLA (100ng/mL) sDLA (100ng/mL)
FIGURE 3

A. Wild Type  TRIF<sup>lps2/lps2</sup>  MYD88KO

IP10  IP10  IP10

n.s.  n.s.  n.s.

Relative mRNA level

B. MCP1  MCP1  MCP1

Relative mRNA level

C. COX2  COX2  COX2

Relative mRNA level

Time (h)

- sDLA (100ng/mL)
- sMLA (100ng/mL)
FIGURE 4

A. 

B. 

C. 

D. 

FIGURE 4
FIGURE 5

A. **TNFα**

B. **IL-10**

C. **TNFα**

D. **IL-10**

* Indicates significant difference.

n.s. Indicates not significant.
FIGURE 6

A. ASK1  
B. TAK1  

MAPKAPK2  

pJNK (p54)  
pJNK (p46) (thr183/tyr185)  
JNK (p54)  
b-actin  

pp38 (thr180/tyr182)  
p38  
b-actin  

phospho c-Jun (Ser63)  
total c-Jun  
b-actin  

MAPKAPK2 (thr222)  
pMAPKAPK2  
b-actin  

p38  
b-actin  

sMLA (100ng/mL)  
sDLA (100ng/mL)
FIGURE 7

MyD88KO

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TRIF<sup>lps2/lps2</sup>

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<td>pp38</td>
<td>β-actin</td>
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</tr>
</tbody>
</table>

- **sMLA (100ng/mL)**
- **sDLA (100ng/mL)**

*MyD88KO and TRIF<sup>lps2/lps2</sup> experiments were conducted with different conditions and time points.

**Note:** The image contains a graph showing the levels of phospho-p38 and pp38 over time, with time points at 0, 10, 20, 30, and 60 minutes. The data is represented by different lines and symbols for sMLA and sDLA treatments.
FIGURE 8

MyD88 TRIF TRAM TLR4 MD-2 MyD88 TRIF TRAM TLR4 MD-2

MAPKAPK2

p38

NF-κB AP-1

IKKα/β

JNK

sMLA → LBP → CD14 → MyD88 → TLR4

Weak activation by sMLA

Strong activation by sMLA
Selective activation of the p38 MAPK pathway by synthetic monophosphoryl lipid A
Caglar Cekic, Carolyn R. Casella, Chelsea A. Eaves, Atsushi Matsuzawa, Hidenori Ichijo
and Thomas C. Mitchell

J. Biol. Chem. published online September 15, 2009

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