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# MyD88-Dependent SHIP1 Regulates Proinflammatory Signaling Pathways in Dendritic Cells after Monophosphoryl Lipid A Stimulation of TLR4

Caglar Cekic,<sup>\*,†,1</sup> Carolyn R. Casella,<sup>†</sup> Duygu Sag,<sup>\*,1</sup> Frann Antignano,<sup>‡,2</sup> Joseph Kolb,<sup>\*,†</sup> Jill Suttles,<sup>\*</sup> Michael R. Hughes,<sup>§</sup> Gerald Krystal,<sup>‡</sup> and Thomas C. Mitchell<sup>\*,†</sup>

We previously showed that monophosphoryl lipid A (MLA) activates TLR4 in dendritic cells (DCs) in a Toll/IL-1R domain-containing adaptor inducing IFN- $\beta$  (TRIF)-biased manner: MLA produced from *Salmonella minnesota* Re595 induced signaling events and expression of gene products that were primarily TRIF dependent, whereas MyD88-dependent signaling was impaired. Moreover, when tested in TRIF-intact/MyD88-deficient DCs, synthetic MLA of the *Escherichia coli* chemotype (sMLA) showed the same activity as its diphosphoryl, inflammatory counterpart (synthetic diphosphoryl lipid A), indicating that TRIF-mediated signaling is fully induced by sMLA. Unexpectedly, we found that the transcript level of one proinflammatory cytokine was increased in sMLA-treated cells by MyD88 deficiency to the higher level induced by synthetic diphosphoryl lipid A, which suggested MyD88 may paradoxically help restrain proinflammatory signaling by TRIF-biased sMLA. In this article, we demonstrate that sMLA induces MyD88 recruitment to TLR4 and activates the anti-inflammatory lipid phosphatase SHIP1 in a MyD88-dependent manner. At the same time, MyD88-dependent signaling activity at the level of IL-1R-associated kinase 1 is markedly reduced. Increased SHIP1 activity is associated with reductions in sMLA-induced I $\kappa$ B kinase  $\alpha/\beta$  and IFN regulatory factor 3 activation and with restrained expression of their downstream targets, endothelin-1 and IFN- $\beta$ , respectively. Results of this study identify a pattern that is desirable in the context of vaccine adjuvant design: TRIF-biased sMLA can stimulate partial MyD88 activity, with MyD88-dependent SHIP1 helping to reduce proinflammatory signaling in DCs. *The Journal of Immunology*, 2011, 186: 3858–3865.

**T**oll-like receptor 4 activation on APCs enhances immune responses to Ag and augments the effectiveness of vaccines (1–3). The natural TLR4 agonist, LPS, causes strong inflammatory responses and is not safe for clinical use. Therefore, structural derivatives or mimetics of its endotoxic portion, diphosphoryl lipid A, have been tested for safe and potent

adjuvanticity (2, 4). This led to the discovery of monophosphoryl lipid A (MLA), a low-toxicity LPS derivative that potentiates adaptive immune responses without causing strong inflammation (2, 5). The clinical version of MLA made from *Salmonella minnesota* strain Re595 (MPL adjuvant) is already in use in several vaccine formulations (3, 4). Nevertheless, full understanding of the mechanism(s) that enable potent but safe adjuvanticity after TLR4 stimulation has not yet been achieved.

Dendritic cells (DCs) are the main APCs involved in initial Ag-specific immune responses (6). TLR4 activation induces DC maturation, which involves upregulation of MHC class II (MHC-II) and costimulatory molecules, and secretion of immunomodulatory cytokines such as type I IFNs (7). Unlike other TLRs discovered to date, only TLR4 makes use of both MyD88 and Toll/IL-1R domain-containing adaptor inducing IFN- $\beta$  (TRIF) signaling adaptors (8, 9). Signaling cascades downstream of these adaptors lead to the activation of NF- $\kappa$ B and MAPKs, which are required for inflammatory cytokine secretion and expression of costimulatory molecules (10). TRIF also causes the activation of a different signaling pathway, leading to phosphorylation of IFN regulatory factor 3 (IRF3) and its translocation to the nucleus, which is required for expression of type I IFNs and other immunomodulatory cytokines with inflammatory potential (10, 11). We have previously shown that TRIF-biased activation of TLR4 by MLA may explain its potent adjuvant properties because TRIF-dependent gene expression is associated with T cell priming and other adaptive immune responses (12). However, dual activation of the MyD88 and TRIF pathways induce maximal DC maturation after LPS stimulation (7), indicating that TRIF-“biased” rather than fully TRIF-“selective” signaling via TLR4 may be desirable.

<sup>\*</sup>Department of Microbiology and Immunology, University of Louisville School of Medicine, Louisville, KY 40202; <sup>†</sup>Institute for Cellular Therapeutics, University of Louisville School of Medicine, Louisville, KY 40202; <sup>‡</sup>Terry Fox Laboratory, British Columbia Cancer Research Center, British Columbia Cancer Agency, Vancouver, British Columbia, Canada V5Z 1L3; and <sup>§</sup>The Biomedical Research Centre, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z3

<sup>1</sup>Current address: La Jolla Institute for Allergy and Immunology, Division of Inflammation Biology, La Jolla, CA.

<sup>2</sup>Current address: The Biomedical Research Centre, University of British Columbia, Vancouver, British Columbia, Canada.

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Address correspondence and reprint requests to Dr. Thomas C. Mitchell, Institute for Cellular Therapeutics, Donald Baxter Research Building, 570 South Preston Street, Room 410, Louisville, KY 40202. E-mail address: tom.mitchell@louisville.edu

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Abbreviations used in this article: BMDC, bone marrow-derived dendritic cell; BMDM, bone marrow-derived macrophage; IKK, I $\kappa$ B kinase; IRAK1, IL-1R-associated kinase 1; IRF3, IFN regulatory factor 3; MHC-II, MHC class II; miR-155, microRNA-155; MLA, monophosphoryl lipid A; NFD, nonfat dry milk; QRT-PCR, quantitative real-time PCR; sDLA, synthetic diphosphoryl lipid A; sMLA, synthetic MLA; TAK1, TGF- $\beta$ -activated kinase 1; TRIF, Toll/IL-1R domain-containing adaptor inducing IFN- $\beta$ .

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Indeed, we recently reported that synthetic MLA (sMLA) corresponding to an *Escherichia coli* lipid A structure retains the ability to activate the p38 MAPK signaling pathway in DCs through combined contributions by MyD88 and TRIF (13), which suggested that desirable sMLA-induced cellular responses in DCs include MyD88-dependent activities. Interestingly, our subsequent observations showed that although MyD88 is required for sustained inflammatory gene expression, MyD88 deficiency significantly increased early expression of several cytokines in DCs and macrophages after sMLA stimulation (13). Therefore, in addition to contributing to p38 MAPK activation, MyD88 may also play an important role in limiting TLR4-associated proinflammatory signaling in response to sMLA stimulation. These unexpected findings highlight the complex nature of MyD88 involvement in sMLA-induced APC responses; we therefore focused in this study on mechanisms by which "proinflammatory" MyD88 may paradoxically reduce inflammatory outcomes when TLR4 signaling is activated by MLA structures.

SHIP1 is an important negative regulator of innate immune cell activities such as proliferation, survival, and proinflammatory cytokine production (14–16). For example, SHIP1-deficient macrophages are hyperresponsive to LPS and do not undergo endotoxin tolerance, a state of transient unresponsiveness to subsequent LPS stimulation (17). SHIP1 deficiency also causes mast cell hyperplasia, increased cytokine production, and allergic inflammation (14). SHIP1 activity has been reported to be regulated at the protein level in response to various inflammatory and anti-inflammatory stimuli (18–20). In addition, phosphorylation at Tyr1020 has been shown to be required for SHIP1 activity (21) and may be essential for the translocation of SHIP1 from the cytosol to the plasma membrane where it dephosphorylates phosphatidylinositol 3,4,5-trisphosphate to phosphatidylinositol 3,4-bisphosphate, and therefore counteracts PI3K activity. However, both phosphatase-dependent and -independent mechanisms have been proposed for SHIP1-dependent suppression of cytokine production (14, 20). Although the biological functions of SHIP1 are well studied in macrophages and mast cells, little is known concerning SHIP1 activation after TLR4 stimulation in DCs, especially in the context of low-toxicity TLR4 stimulation by MLA. Moreover, because LPS-induced SHIP1 upregulation in macrophages has been shown to be MyD88 dependent (20), it raises the possibility that MyD88 might limit cytokine expression in sMLA-stimulated DCs via upregulation of SHIP1 activity.

In this study, we investigated whether SHIP1 plays an important role downstream of TLR4 signaling in DCs after exposure to proinflammatory synthetic diphosphoryl lipid A (sDLA) or less inflammatory sMLA. We found that phosphorylated and total SHIP1 levels increased after TLR4 stimulation, and that sMLA sustained increased SHIP1 levels in an MyD88-dependent manner, which resulted in reduced proinflammatory cytokine expression. Overall, SHIP1 was found to be a potential regulator of DC activity after TLR4 stimulation, especially after less proinflammatory stimulation by sMLA.

## Materials and Methods

### Mice and reagents

C57BL/6 mice and TRIF<sup>lps2/lps2</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). SHIP heterozygotes, derived from an F2 generation of C57BL/6 × 129Sv mice, were bred to generate SHIP<sup>+/+</sup> and SHIP<sup>-/-</sup> littermates. 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. sMLA and sDLA derived from the lipid A structures of *E. coli* LPS were purchased from Invivogen and Peptides

International, respectively. Both compounds were dissolved in DMSO by gently vortexing until complete solubilization; stocks of each were always prepared at the same time to minimize differences in activity that might be affected by reagent preparation. Dissolved compounds were aliquoted in small volumes for single use and stored at -80°C. M-CSF and GM-CSF were 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 (QRT-PCR) was purchased from Applied Biosystems. All phospho-specific Abs and TLR4 Ab used for immunoblotting experiments in this study were purchased from Cell Signaling Technology, and all nonphospho-specific and  $\beta$ -actin Abs were from Santa Cruz Biotechnology, except for the MyD88-specific Ab, which was from eBioscience. HRP-conjugated anti-rabbit and anti-goat secondary Abs were from Jackson ImmunoResearch.

### Generation of bone marrow-derived DCs and macrophages

Bone marrow-derived DCs (BMDCs) were prepared according to a protocol modified from that of Lutz et al. (22). In brief, femurs and tibiae from 8- to 12-wk-old mice were collected and flushed with sterile 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  $\mu$ g/ml streptomycin) plus 50  $\mu$ M 2-ME and 5 ng/ml GM-CSF. A total of  $2 \times 10^6$  cells per bacteriological culture plate were cultured for 10 d, with feeding on days 3 and 8 by adding 10 ml fresh medium, and on day 6 by replacing half of the culture medium. Nonadherent cells were collected on day 10 and verified to be at least 85–95% CD11b<sup>+</sup>/CD11c<sup>+</sup>/MHC-II<sup>+</sup>/CD86<sup>low</sup>/Gr1<sup>-</sup>/CD4<sup>-</sup>/CD8<sup>-</sup>/B220<sup>-</sup>/CD19<sup>-</sup> by flow cytometry before use in experiments.

Bone marrow-derived macrophages (BMDMs) were prepared according to a protocol modified from Sag et al. (23). In brief, bone marrow cells obtained as described earlier were cultured overnight in standard tissue culture plates in the presence of 10 ng/ml M-CSF. Nonadherent cells from this initial culture were then transferred to low-attachment six-well plates (Corning Life Sciences) in 4 ml R5F containing 30% L929 conditioned medium and 10 ng/ml M-CSF for 7 d, adding 1.5 ml fresh medium on days 3 and 5. Cells were verified to be at least 90–98% CD11b<sup>+</sup>/CD11c<sup>-</sup>/MHC-II<sup>+</sup>/CD80<sup>-</sup>/CD86<sup>-</sup>/Gr1<sup>-</sup>/CD4<sup>-</sup>/CD8<sup>-</sup>/B220<sup>-</sup>/CD19<sup>-</sup>/F4/80<sup>+</sup> by flow cytometry before use in experiments.

The human monocytic cell line THP-1 was purchased from American Type Culture Collection (TIB 202) and cultured in RPMI 1640 medium (Life Technologies A10491) containing 4.5 g/L D-glucose, 1.5 g/L sodium bicarbonate, 1 mM sodium pyruvate, 10 mM HEPES, 300 mg/L L-glutamine, with added 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 50  $\mu$ M 2-ME, and 10% FBS. The cells were cultured at a density of  $2\text{--}6 \times 10^5$ /ml at 37°C in a 5% CO<sub>2</sub> incubator. Cells were used between weeks 6 and 11 of culture.

### QRT-PCR

BMDCs ( $1 \times 10^6$ ) 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. 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). QRT-PCR was performed using the Applied Biosystems 7500 Fast system or CFX96 Real-Time PCR Detection System and Power SYBR Green RT-PCR mastermix. QuantiTect Primers (Qiagen) were used for all QRT-PCR assays except for primers used to measure  $\beta$ -actin mRNA (forward: 5'-TGGAACTCCTGTGGCATCCATGAAAC-3'; reverse: 5'-TAAA-ACGCAGCTCAGTAACAGTCCG-3'), which were purchased from Sigma-Genosys. Expression of each target gene was normalized to  $\beta$ -actin, and fold expression over vehicle control was calculated using the  $2^{-\Delta\Delta C_t}$  method (24).

### Immunoblotting and immunoprecipitation

BMDCs ( $2\text{--}3 \times 10^6$ ) were rested for 2 h in 12 × 75 mm polystyrene tubes at 37°C and then exposed to sMLA or sDLA. In all experiments, DMSO was used as vehicle control. After the indicated time points, cells were centrifuged in ice-cold HBSS containing 50  $\mu$ M NaF and then lysed in radioimmunoprecipitation assay lysis buffer containing Complete Mini protease inhibitor mixture tablets (Roche), phosphatase inhibitor mixture (Sigma-Aldrich), and 250 nM okadaic acid (Sigma-Aldrich). The resulting lysates were tested for their protein concentrations using the BCA assay (Pierce Biotechnology) and then mixed with 5X SDS sample buffer (1× final concentration). Lysates containing equal amounts of protein were loaded onto 10% SDS-PAGE gels for electrophoresis, after which resolved proteins were transferred onto

nitrocellulose membranes (GE Healthcare) and blocked with 5% nonfat dry milk (NFDM) for 1 h. Primary Abs were dissolved in 5% BSA except for  $\beta$ -actin and IRF3 total Abs, which were dissolved in 5% NFDM and incubated with the blocked membranes overnight at 4°C. After exposure to HRP-conjugated anti-rabbit or anti-goat secondary Abs for 1 h in NFDM, bands were visualized using the ECL detection system (GE healthcare), and band intensities were analyzed with Quantity One Software (Bio-Rad version 4.6.6).

THP-1 cells ( $1.5\text{--}3 \times 10^6$ ) were rested for 2 h at 37°C in  $12 \times 75$  mm polystyrene tubes. The cells were then exposed to 100 ng/ml sMLA, sDLA, or the vehicle control DMSO. At the indicated times, the cells were washed in 4°C HBSS containing 50  $\mu$ M NaF and lysed in radioimmunoprecipitation assay buffer containing Complete Mini protease inhibitor mixture tablets (Roche), phosphatase inhibitor mixture (Sigma-Aldrich), and 250 nM okadaic acid (Sigma-Aldrich). Equal amounts of protein, as determined by the Pierce Biotechnology BCA assay, were loaded on 8% SDS-PAGE gels and subsequently immunoblotted for pSHIP1 (Cell Signaling 3941), SHIP1 (Cell Signaling 2728), and  $\beta$ -actin (Santa Cruz Biotechnology sc1616). Bands were visualized by autoradiography or the FUJI LAS 4000. Measurements of band intensities were analyzed with either Quantity One Software (Bio-Rad version 4.6.6) or Multi Gauge software (FUJIFILM).

Immunoprecipitation of MyD88 was performed as described previously (25). In brief,  $3\text{--}6 \times 10^6$  BMDCs were lysed in 1% digitonin lysis buffer. After incubating with primary Abs overnight, beads conjugated to anti-rabbit secondary Abs were added to the lysates and incubated for another 2–4 h at 4°C. Beads were collected by a brief centrifugation, and immunoprecipitated proteins were released by suspension in 2X sample buffer before resolving by SDS-PAGE and immunoblotting.

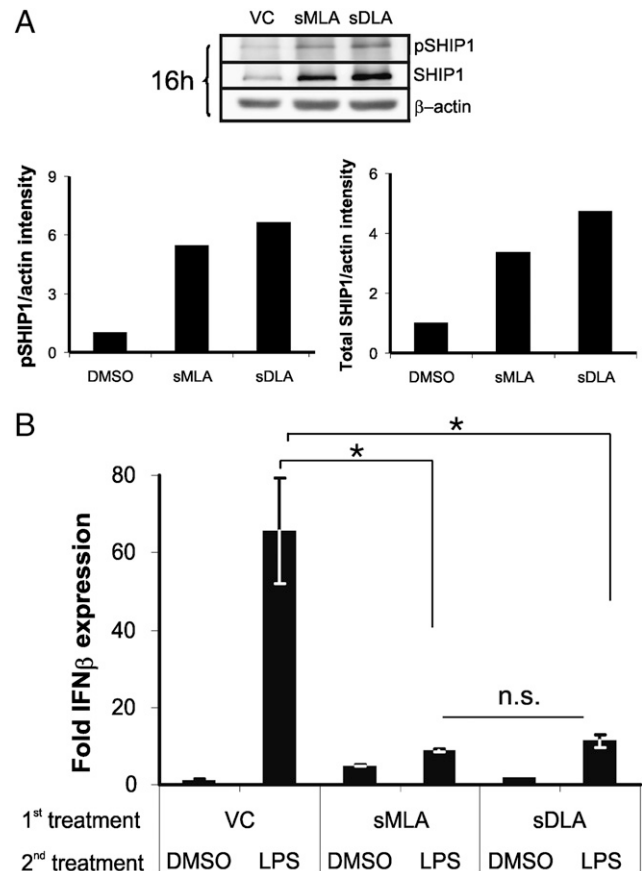
#### Statistical Analysis and sample normalization

Two-factor (time versus treatment) ANOVA and post hoc Tukey's tests were performed to determine the significance of the differences between sDLA- versus sMLA-induced cellular effects on gene expression and signaling. A  $p$  value  $<0.05$  was considered to indicate statistically significant differences between treatment groups. All QRT-PCR data were normalized to  $\beta$ -actin, and phosphoprotein band intensities were normalized to total protein levels for IRF3 and I $\kappa$ B kinase (IKK) $\alpha/\beta$ , and to  $\beta$ -actin for SHIP1. Lysates from DMSO alone-treated cells were used as reference point for calculation of fold expression and intensities.

## Results

### Increased phospho-SHIP1 and total SHIP1 levels in BMDCs after overnight exposure to TLR4 agonists

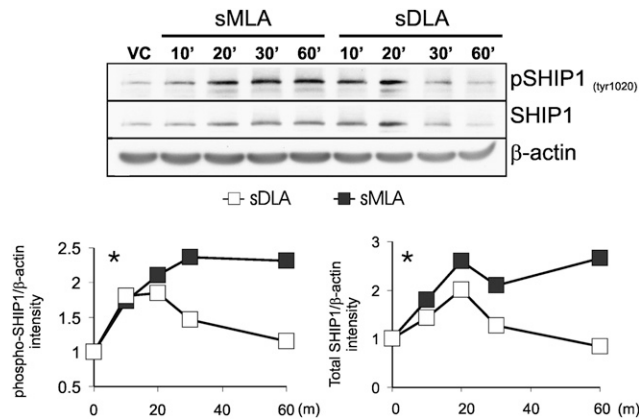
Overnight exposure of macrophages to LPS causes an increase in SHIP1 levels, which is correlated with establishment of endotoxin tolerance to subsequent LPS challenge (20). To find out whether SHIP1 is also increased in DCs after low-toxicity TLR4 stimulation by sMLA, we exposed a homogenous population of BMDCs to the synthetic TLR4 agonists sMLA or sDLA, which differ in structure by one phosphoryl group. As shown in Fig. 1A, both total and phospho-SHIP1 levels increased in BMDCs after overnight exposure to both high-toxicity sDLA and low-toxicity sMLA, indicating that SHIP1 may be a part of the TLR4 signaling cascade in DCs. To test whether increases in total and/or phospho-SHIP1 levels predicted reduced responsiveness to subsequent LPS challenge, we preincubated BMDCs for 4 h with sMLA, sDLA, or DMSO as vehicle control, and then washed the cells and incubated them in fresh medium for 12 h before exposing them to either DMSO or LPS. As shown in Fig. 1B, preincubating the cells with either sMLA or sDLA almost completely prevented subsequent IFN- $\beta$  expression on LPS stimulation, suggesting that the phosphorylated and total SHIP1 levels induced 16 h posttreatment explained the establishment of endotoxin tolerance to the same extent with sMLA as with sDLA in DCs. Interestingly, the increase in SHIP1 was similar in response to low- and high-toxicity agonists of TLR4 and induced tolerance to the same extent.



**FIGURE 1.** Equal potency of sMLA and sDLA for induction of increased phospho-SHIP1 and total SHIP1 levels after overnight exposure. *A*, Wild type BMDCs cultured from C57BL/6 mice were treated with 100 ng/ml sMLA or sDLA for 16 h. Western blotting was performed to detect changes in pSHIP1 (Tyr1020) and SHIP1 levels. (The blot shown is representative of two independent experiments.) *B*, BMDCs were pretreated with 100 ng/ml sMLA or sDLA for 4 h, washed, and incubated in fresh medium for another 12 h before exposure to either 1  $\mu$ g/ml LPS or DMSO. QRT-PCR was performed to detect fold increases in transcription of IFN- $\beta$ . DMSO alone was used as vehicle control, with the results representing average values from two independent experiments, each performed in triplicate. \* $p < 0.05$ , not statistically significant (n.s.) by ANOVA.

### Early changes in SHIP1 activation correlate with increased dependence on SHIP1 for decreased proinflammatory signaling by sMLA in DCs

Although previous studies have shown that SHIP1 levels start to increase in macrophages after 8 h, it is not known whether phospho-SHIP1 levels increase rapidly after TLR4 stimulation in BMDCs (17). Therefore, we exposed BMDCs to sMLA, sDLA, or DMSO as vehicle control and performed immunoblotting to detect changes in phospho-SHIP1 and total SHIP1 levels. Phospho-SHIP1 and total SHIP1 levels rapidly increased in BMDCs after sMLA or sDLA stimulation (10–20 min; Fig. 2). sDLA-induced phospho-SHIP1 and total SHIP1 levels started to decrease within 30 min and returned to basal levels by 60 min (Fig. 2). However, sMLA-induced phospho-SHIP1 and total SHIP1 levels remained increased for at least 60 min after stimulation (Fig. 2) in most experiments ( $n = 12$  independent repetitions); hence very early activation of SHIP1 was a hallmark of TLR4 stimulation by these agonists in BMDCs. In some experiments, the differences observed between sMLA- and sDLA-treated cells were small, with variability especially likely to occur after sDLA treatment (see Supplementary Table I for individual values in each experiment).



**FIGURE 2.** Early increase in pSHIP1 levels is more sustained after sMLA stimulation. BMDCs were treated with 100 ng/ml sMLA or sDLA for the indicated time periods. Immunoblotting was performed to detect changes in phosphorylation of SHIP1 (Tyr1020), as well as total SHIP1 levels. DMSO alone was used as vehicle control. The plotted values were averaged from  $n = 12$  independent experiments;  $*p < 0.05$ , as calculated by two-factor ANOVA and post hoc Tukey's test. The blot shown is from an individual experiment representative of the average values.

The unexpected finding that sMLA was more likely than sDLA to sustain this early activation of SHIP1 has important implications for its reduced proinflammatory signaling activity, but confirming that SHIP1 plays a role required further experiments with SHIP1-deficient cells.

To test whether SHIP1 might limit sMLA-induced inflammatory responses, we exposed wild type and SHIP1-deficient BMDCs to either sMLA or sDLA. To detect differences in MyD88 versus TRIF-mediated signaling, we tested IKK $\alpha/\beta$  and IRF3 activity by measuring changes in their phosphorylation status, as well as the mRNA levels of their downstream target genes (endothelin-1 and IFN- $\beta$ , respectively) at early time points. Within 15–30 min of stimulation, sMLA caused significantly stronger IKK $\alpha/\beta$  and IRF3 phosphorylation in SHIP1 $^{-/-}$  than in WT BMDCs (Fig. 3A, 3B). This pattern was associated with significantly increased endothelin-1 and IFN- $\beta$  transcript levels 1 h after sMLA stimulation of SHIP1-deficient cells (Fig. 3C, 3D). SHIP1 deficiency did not significantly increase sDLA-induced IRF3 or IKK $\alpha/\beta$  phosphorylation or mRNA levels of their downstream targets, endothelin-1 and IFN- $\beta$ , in this time frame, which may be partly because of strong activation of proinflammatory pathways by sDLA that overwhelm the suppressive activity of rapid SHIP1 activation.

To determine whether SHIP1 deficiency causes more pronounced increases in early proinflammatory cytokine secretion in response to sMLA than sDLA in vivo, we injected equal amounts of sMLA or sDLA i.p. into either WT or SHIP1-deficient mice, collected plasma after 1 (early) or 6 h (late), and tested for several inflammatory cytokines and anti-inflammatory IL-10 by multiplex analysis. One hour after sMLA, but not after sDLA, treatment plasma concentrations of IL-6 reached significantly greater levels in SHIP1-deficient mice as compared with those of WT mice (Supplemental Fig. 1, top), which is consistent with the preferential enhancement of early SHIP1 activity by sMLA observed in our initial experiments (Fig. 2). In addition, we observed a tendency for increased plasma concentrations of TNF- $\alpha$  (Supplemental Fig. 1, top middle) or no change in MIP1 $\alpha$  (Supplemental Fig. 1, bottom middle) in SHIP1-deficient animals as compared with what is observed in WT animals after either sMLA or sDLA stimulation, suggesting that not all proinflammatory cytokines are

equally sensitive to SHIP1-mediated suppression. Interestingly, 1 h after sMLA and 6 h after sDLA treatment, plasma concentrations of IL-10 significantly increased in SHIP1-deficient mice (Supplemental Fig. 1D, bottom), showing a pattern similar to that of IL-6. SHIP1 counteracts PI3K activity, which is generally proinflammatory. However, SHIP1 is also required for DC maturation and optimal IL-12 secretion (26). PI3K activation is also known to increase IL-10 and to limit IL-12 secretion. Enhanced PI3K activity in SHIP1-deficient mice likely accounts for the increase in IL-10 secretion, as well as the unequal responses of different proinflammatory cytokines to SHIP1-mediated suppression (27). Overall, these results indicate that very early SHIP1 activation regulates sMLA-induced inflammatory signaling and proinflammatory cytokine expression both in vitro and in vivo. However, SHIP1-deficient mice are not healthy because of developmental mast cell hyperplasia and other proinflammatory effects (14), which complicates interpretation of the in vivo findings.

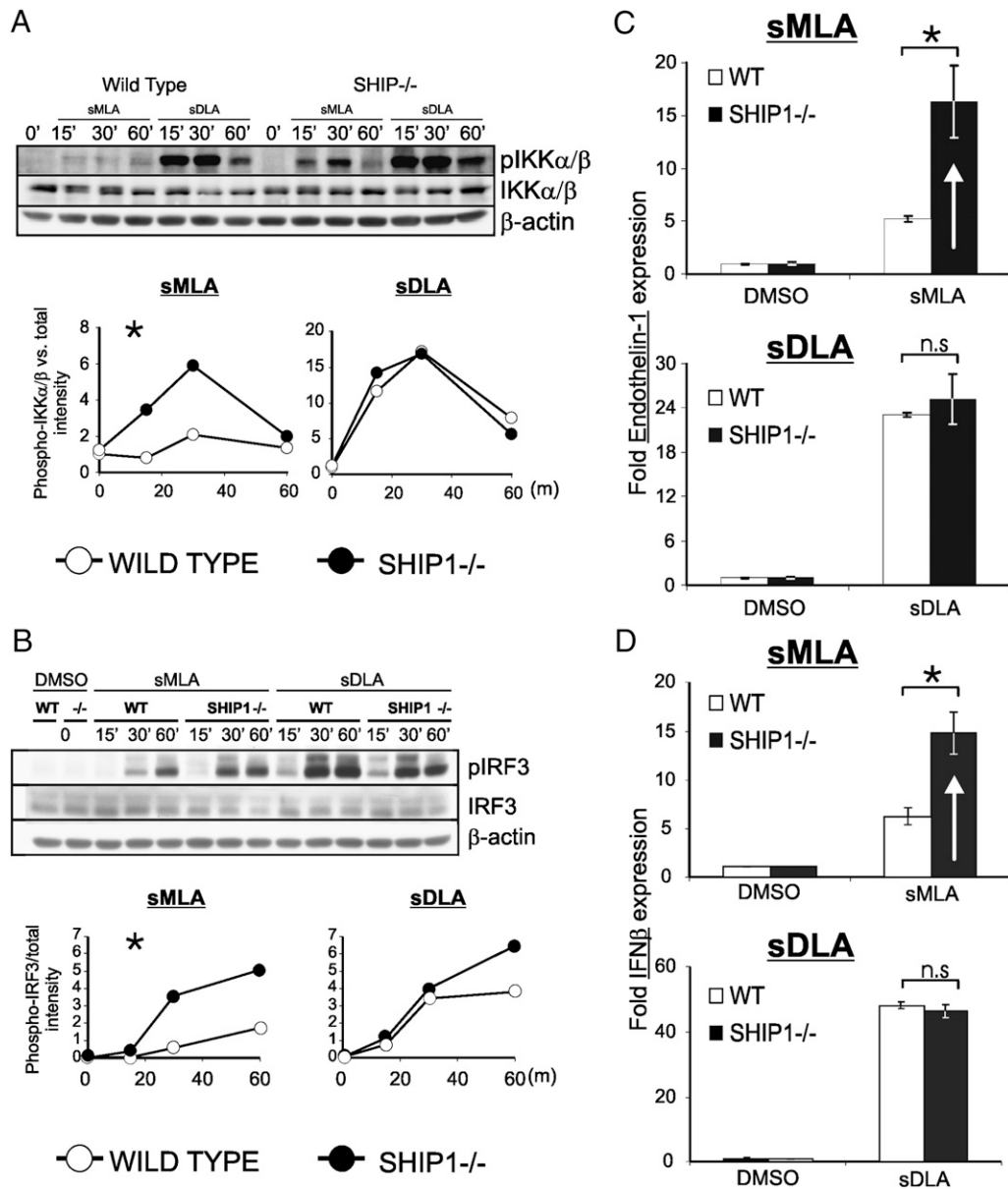
#### *Sustained, early SHIP1 activation by sMLA requires MyD88*

To reveal which adaptor is required for sustained increases in phospho-SHIP1 levels on sMLA stimulation in vitro, we exposed MyD88 $^{-/-}$  and TRIF<sup>lps2/lps2</sup> BMDCs to either sMLA or sDLA, using DMSO as vehicle control, and measured changes in phospho-SHIP1 levels by immunoblotting. sMLA failed to increase phospho-SHIP1 in MyD88 $^{-/-}$  cells, whereas in TRIF<sup>lps2/lps2</sup> BMDCs, phospho-SHIP1 levels increased after sMLA stimulation, indicating selective dependence on the MyD88-dependent pathway (Fig. 4). To test whether the MyD88-dependent increase in SHIP1 activity contributes to the lower proinflammatory profile of sMLA, we exposed wild type or MyD88 $^{-/-}$  BMDCs to either sMLA or sDLA and performed QRT-PCR to measure fold changes in endothelin-1 and IFN- $\beta$  mRNA levels. As shown in Fig. 5A, MyD88 deficiency significantly enhanced sMLA- but not sDLA-induced endothelin-1 and IFN- $\beta$  mRNA levels. This increase in sMLA-induced endothelin-1 and IFN- $\beta$  expression was also associated with concomitant increases in IRF3 phosphorylation in MyD88 $^{-/-}$  BMDCs (Fig. 5B). These results suggest that MyD88-dependent SHIP1 is activated by MLA, despite its consistently TRIF-biased stimulation of TLR4 (12, 13).

Because macrophages are central regulators of inflammation, we next tested whether phospho-SHIP1 levels also rapidly increase in sMLA-treated macrophages in an MyD88-dependent manner. We stimulated wild type or MyD88 $^{-/-}$  BMDMs with sMLA, sDLA, or DMSO as vehicle control, and measured changes in phospho-SHIP1 levels by immunoblotting. sMLA increased phospho-SHIP1 levels in wild type BMDMs (Fig. 6A, left, black bars); however, there was no change in phospho-SHIP1 levels in MyD88 $^{-/-}$  cells (open bars). As shown in Fig. 6, decreased phospho-SHIP1 levels after sMLA stimulation in MyD88 $^{-/-}$  cells coincided with concomitant increases in phospho-IRF3, indicating that MyD88 may be required for increased SHIP1 activity in BMDMs, as well as BMDCs. Tests of SHIP1 activation in human monocytic THP-1 cells showed a pattern that was broadly similar to that of mouse cells: within 60 min, sMLA-stimulated cells showed greater levels of phospho-SHIP than sDLA-stimulated cells, whereas both TLR4 agonists increased it to equivalent levels 24 h later (Supplemental Fig. 2).

#### *sMLA can induce MyD88 recruitment but not IL-1R-associated kinase 1 disappearance*

We reported previously that synthetic *E. coli* MLA is a TRIF-biased agonist of TLR4 (13). However, the unexpected inhibitory role of MyD88 in sMLA-induced gene expression indi-



**FIGURE 3.** Increased cellular responses to sMLA in SHIP1<sup>-/-</sup> cells. BMDCs were treated with 100 ng/ml sMLA or sDLA for the indicated time periods. Immunoblotting was performed to detect activating phosphorylation of (A) IKKα/β (ser176/180) and (B) IRF3 (ser396), and QRT-PCR was performed to detect fold increases in transcript levels of (C) endothelin-1 and (D) IFN-β in WT (white bars and circles) or SHIP1<sup>-/-</sup> (black bars and circles) cells. The results are from two independent experiments. DMSO alone was used as vehicle control. \**p* < 0.05, not statistically significant (n.s.) by two-factor ANOVA and post hoc Tukey's test or ANOVA.

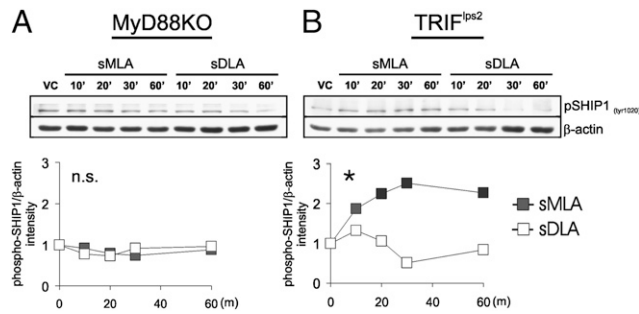
cates that sMLA may induce MyD88 recruitment to TLR4. To test this, we stimulated BMDCs with sMLA, sDLA, or DMSO for different periods, immunoprecipitated MyD88 from the cell lysates, and immunoblotted the precipitates for TLR4 to detect MyD88–TLR4 complexes. As shown in Fig. 7A, both sMLA and sDLA increased the extent to which TLR4 coprecipitated with MyD88, suggesting that sMLA can induce MyD88 recruitment to the TLR4 complex.

On TLR4 activation by LPS, IL-1R-associated kinase 1 (IRAK1) is polyubiquitinated, which results in its disappearance from immunoblots (28). This event is required to facilitate binding of NF-κB essential modulator to IRAK1, with subsequent activation of TGF-β-activated kinase 1 (TAK1) (29). Importantly, MyD88-dependent inflammatory signaling requires activation of TAK1 (6, 11), and we recently showed that sMLA only weakly activates TAK1 and its downstream targets (13), which suggests

that IRAK1 is not fully activated. To assess whether IRAK1 is differentially affected by sMLA and sDLA, we performed immunoblotting to determine whether MyD88 recruitment to the TLR4 complex is associated with IRAK1 disappearance after sMLA stimulation. As shown in Fig. 7B, sMLA failed to induce IRAK1 disappearance to the extent seen with sDLA, suggesting that sMLA fails to activate upstream mediators of IRAK1 polyubiquitination. Hence TLR4 stimulation by sMLA is characterized by apparently normal levels of MyD88 recruitment but abnormal IRAK1 use.

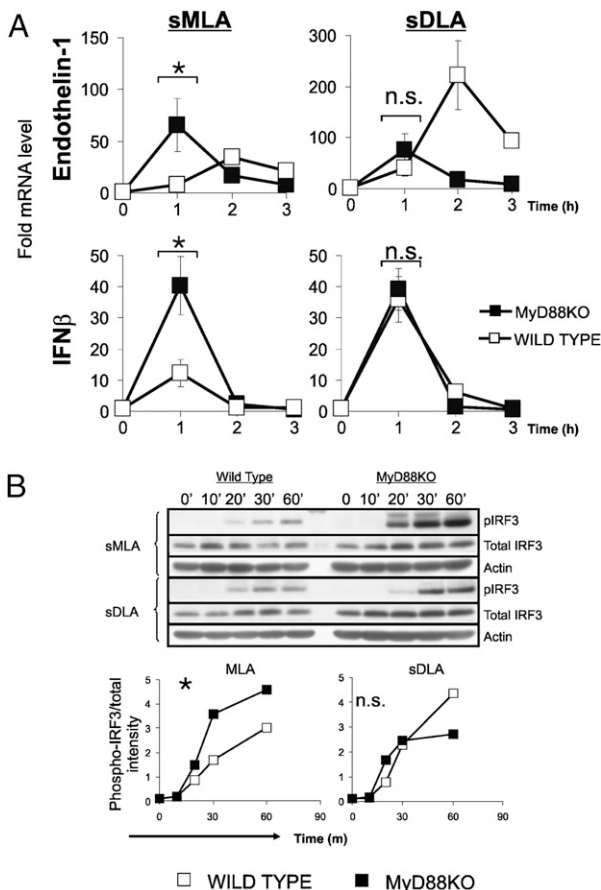
## Discussion

Although the immunostimulatory effects of MLA structures are well-known, relatively few studies address the precise signaling mechanisms by which effective immunostimulation is achieved without high levels of inflammation. Our previous work on this

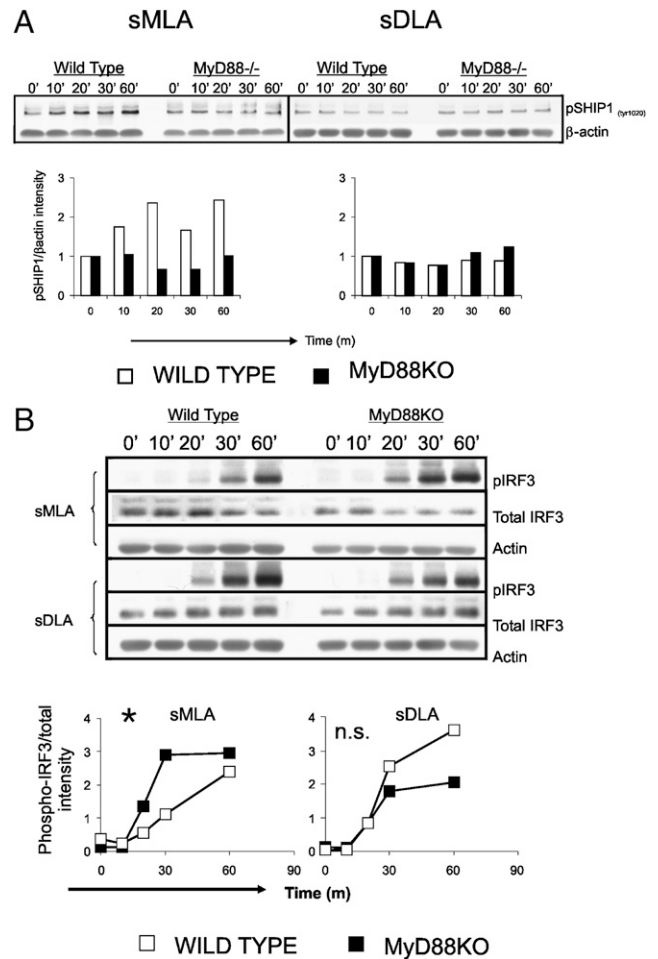


**FIGURE 4.** Sustained pSHIP1 levels after sMLA stimulation is MyD88 dependent. BMDCs were treated with 100 ng/ml sMLA or sDLA for the indicated time periods. Immunoblotting was performed to detect change in pSHIP1 (Tyr1020) levels in (A) MyD88<sup>-/-</sup> and (B) TRIF<sup>Ips2</sup> cells. DMSO alone was used as vehicle control. \**p* < 0.05, not statistically significant (n.s.) by two-factor ANOVA and post hoc Tukey’s test.

topic shows that TLR4 stimulation by MLA is associated with TRIF-biased signaling, in which TRIF-associated signaling events are largely intact, but MyD88-dependent outcomes are impaired (12, 13). In this study, we identify a novel intrinsic pattern in which low-toxicity sMLA and its inflammatory sDLA counterpart activate pathways branching from MyD88 that appear to limit



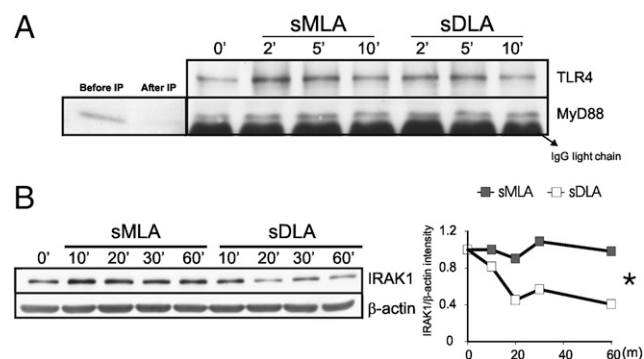
**FIGURE 5.** Increased cellular responses to sMLA in MyD88<sup>-/-</sup> cells. A, BMDCs were treated with 100 ng/ml sMLA or sDLA for the indicated time periods. QRT-PCR was performed to detect fold-increases in transcript levels of endothelin-1 (*n* = 2) and IFN-β (*n* = 5) in WT or MyD88<sup>-/-</sup> cells. B, BMDCs (*n* = 3) were treated with 100 ng/ml sMLA or sDLA for the indicated time periods. Western blotting was performed to detect activating phosphorylation of IRF3 (ser396). \**p* < 0.05, not statistically significant (n.s.) by two-factor ANOVA and post hoc Tukey’s test.



**FIGURE 6.** MyD88-dependent increase in phospho-SHIP1 levels in BMDMs after sMLA stimulation. A, BMDMs (*n* = 2) were treated with 100 ng/ml sMLA or sDLA for the indicated time intervals. Western blotting was performed to detect change in pSHIP1 (tyr1020) in WT and MyD88<sup>-/-</sup> cells. DMSO alone was used as vehicle control. B, BMDMs (*n* = 2) were treated with 100 ng/ml sMLA or sDLA for the indicated time periods. Western blotting was performed to detect activating phosphorylation of IRF3 (ser396). \**p* < 0.05, not statistically significant (n.s.) by two-factor ANOVA and post hoc Tukey’s test.

proinflammatory immunostimulation. Both sMLA and sDLA induced MyD88 recruitment to TLR4, but sMLA induced early MyD88-dependent SHIP1 phosphorylation/activation. In contrast, only sDLA treatment caused MyD88-dependent IRAK1 disappearance in immunoblotting assays, which has been shown to be associated with activating K-63 polyubiquitination, TAK1 activation (29, 30), and subsequent stimulation of NF-κB and MAPK signaling activity (6, 11). Hence sMLA’s TRIF-biased signaling is not devoid of MyD88 engagement; rather, the consequences of MyD88 engagement differ markedly between these nearly identical lipid A structures in a way that appears to more rapidly activate a lipid phosphatase, one of whose functions has been shown elsewhere to include prevention of endotoxic shock by contributing to endotoxin tolerance (17).

Recently, the structural basis of LPS recognition by the MD2-TLR4 complex was revealed through X-ray crystallographic methods (31). In this structure, five of six acyl chains of the lipid A portion of *E. coli* LPS fill a hydrophobic pocket within MD2. The remaining acyl chain and the head group phosphate residues of lipid A, together with hydrophobic surfaces of MD2, interact with both members of a TLR4 dimer. The 4’-phosphate group of



**FIGURE 7.** sMLA induces MyD88 recruitment to TLR4 but not IRAK1 disappearance. BMDCs were activated for the indicated periods with either sMLA or sDLA and lysed in digitonin lysis buffer. *A*, MyD88 in the lysates were immunoprecipitated using primary rabbit Abs and anti-rabbit Ab-conjugated agarose beads (16 h with primary rabbit + 4 h with the beads). Resulting precipitates were dissolved in 2X sample buffer and immunoblotted for TLR4 and MyD88. *B*, Western blotting was performed to detect IRAK1 disappearance. DMSO alone was used as vehicle control. \* $p < 0.05$  by ANOVA and post hoc Tukey's test as calculated from values obtained in five independent experiments.

LPS interacts with MD2 and one member of the TLR4 dimer, whereas MD2 and the acyl chain exposed on its surface interact with the other member of the TLR4 dimer. Interestingly, among all of these structural components, the 1'-phosphate group of lipid A, which is missing from sMLA, was shown to bind to a positively charged cluster of lysines and an arginine from each TLR4 in the dimer, as well as to MD2. These observations suggest that the absence of the 1'-phosphate group may have profound effects on structural rearrangements and/or dimerization efficiency of TLR4/MD2 + TLR4/MD2 heterodimerized complexes. The existence of such structural rearrangements remains to be shown.

Previously, we argued that sMLA almost fully activates TRIF signaling because its effects on early gene expression were indistinguishable from those of sDLA when tested in BMDCs that could only signal through TRIF because they lacked MyD88 (13). In that study, we also showed that MyD88 is involved in sMLA-induced biochemical responses such as p38 activation and sustained COX-2 expression (13). These results suggested that sMLA must induce, or fail to disrupt, TLR4 dimerization because dimerization is required for overall TLR4 activity (32). Surprisingly, although sMLA induces MyD88 recruitment and activation of the anti-inflammatory mediator SHIP1, it fails to induce IRAK1 disappearance. In this article, we can speculate that the rigidity of the TLR4-MD2-lipid A complex that forms within the [TLR4/MD2]<sub>2</sub> superdimer may decrease in the absence of 1'-phosphate group, thereby allowing rapid MyD88 recruitment and subsequent internalization before important mediators of inflammation such as the E3 ubiquitin ligase known as "Pelle-associated protein" or Pellino can interact with TLR4-MyD88-IRAK1 complexes. That MyD88-dependent SHIP1 activation occurs in sMLA-treated cells early in this process is supported by our observation that SHIP1 phosphorylation was consistently observed within the first hour of treatment (Fig. 2), and that SHIP deficiency increased the strength of sMLA's activation of IKKα/β and IRF3 in the same time frame (Fig. 3A, 3B).

As a lipid phosphatase that counteracts the effects of PI3K, SHIP1 activity is known to reduce inflammatory cytokine secretion by NK cells, mast cells, and macrophages (14, 33, 34), although the mechanism by which it is activated rapidly by sMLA is not clear. Previous studies have revealed that SHIP1 levels can be increased by anti-inflammatory TGF-β and decreased by proinflammatory

TNF-α or by the Th2 cytokine IL-4 (19, 20). SHIP1 is a primary target of microRNA-155 (miR-155) (18, 19). It was observed that TNF-α can increase miR-155 levels and decrease SHIP1 levels in B cell leukemia cell lines (20). sDLA could theoretically keep SHIP1 levels low via rapid secretion of inflammatory mediators that increase miR-155 levels. Alternatively, sMLA may rapidly induce TGF-β presentation on the extracellular matrix to generate early SHIP1 activity. These possible scenarios are currently being tested.

An important aspect of SHIP1 biology is its recently reported role in enabling DC maturation (26). Although proinflammatory IL-6 and TNF-α expression was increased in SHIP1<sup>-/-</sup> DCs, consistent with SHIP1 playing an anti-inflammatory role in DCs, as well as in macrophages, SHIP1 was unexpectedly required for DC maturation at the levels of MHC-II expression, optimal expression of IL-12, and T cell expansion and differentiation to become Th1 effectors (26). Our *in vivo* tests showed that SHIP-deficient mice produced significantly more anti-inflammatory IL-10 1 h after sMLA and 6 h after sDLA stimulation (Supplemental Fig. 1, bottom), which may be responsible for limiting DC maturation into effective APCs. Therefore, sMLA's ability to activate SHIP1 via the TLR4/MyD88 signaling branch is likely to contribute to its activity as an immunomodulatory compound in ways that warrant further analysis.

PI3K activity is required for optimal expression of proinflammatory TNF-α and IL-6 after TLR4 stimulation (20). However, Martin et al. (27) showed that PI3K activity downstream of TLR4 leads to activation of Akt, which subsequently inhibits Glycogen synthase kinase 3β activity through Ser9 phosphorylation and causes the release of CREB to the nucleus, where it binds and activates the IL-10 promoter. Our findings agree with these observations because SHIP1 deficiency increased sMLA-induced proinflammatory TNF-α, IL-6, and anti-inflammatory IL-10 production *in vivo*. However, it should be noted that SHIP1-deficient mice are more prone to anaphylaxis and can have increased levels of TNF-α, IL-6, and IL-5 because of mast cell hyperplasia (14), suggesting that: 1) increased secretion of proinflammatory cytokines and IL-10 may result from independent mechanisms *in vivo*, 2) SHIP1 may play different roles in different cell subsets, and 3) definitive evaluation of SHIP1's role in immunomodulation and adjuvanticity by sMLA requires the generation of inducible or conditional knockout models of SHIP1 deficiency.

Because the MPL adjuvant used in human vaccines is derived by sequential acid and base hydrolysis of cultured *S. minnesota* Re595 LPS, it contains a mixture of hexa-acylated, penta-acylated, tetra-acylated, and triacylated species (35). Among these, hexa-acylated structures were determined to be more potent in human and rodent species as compared with other structures (35). Our preliminary studies show that hexa-acylated *E. coli* sMLA fails to function as a potent inducer of IFN-β expression, in contrast with MLA species derived from *S. minnesota* LPS formulations (data not shown), suggesting that penta-acylated, tetra-acylated, or triacylated MLA structures may activate other receptors such as TLR2 to reduce the inhibitory effect of SHIP1 on IFN-β expression through inactivation of the PI3K pathway. Alternatively, these structures may interfere with sustained SHIP1 activation by hexa-acylated derivatives. Also, we cannot exclude the possibility that the minor structural difference between hexa-acylated *S. minnesota* MLA and hexa-acylated *E. coli* sMLA, which consists of a change in one secondary acyl chain, may be responsible for this markedly different biological activity. Identifying the structural combinations responsible for optimal IFN-β expression is an important goal, given the potential role of type I IFN signaling in robust expansion and differentiation of memory T cells.



In conclusion, in this study, SHIP1 was activated in DCs rapidly after TLR4 stimulation by the low-toxicity sMLA, and contributed to low-toxicity TLR4 signaling by sMLA via some level of engagement of the MyD88 pathway. At the same time, proinflammatory IRAK-1 failed to participate fully in signaling downstream of MyD88. Results of this study may have important implications for the design of novel TLR4 agonists as candidate vaccine adjuvants by identifying a pattern of MLA-dependent signaling that correlates with low-toxicity stimulation of immune functions.

## Disclosures

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