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ENDOTHELIAL CELL TOLERANCE TO LIPOPOLYSACCHARIDE CHALLENGE IS INDUCED BY MONOPHOSPHORYL LIPID A

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Abstract

Prior exposure to lipopolysaccharide (LPS) produces a reduced or “tolerant” inflammatory response to subsequent challenges with LPS, however the potent pro-inflammatory effects of LPS limit its clinical benefit. The adjuvant Monophosphoryl lipid A (MPLA) is a weak toll-like receptor 4 (TLR4) agonist that induces negligible inflammation but retains potent immunomodulatory properties. We postulated that pre-treatment with MPLA would inhibit the inflammatory response of endothelial cells to secondary LPS challenge. Human umbilical vein endothelial cells (HUVECs), were exposed to MPLA (10 µg/ml), LPS (100 ng/ml) or vehicle control. HUVECs were then washed and maintained in culture for 24 hours before being challenged with LPS (100 ng/ml). Supernatants were collected and examined for cytokine production in the presence or absence of siRNA inhibitors of critical TLR4 signaling proteins. Pretreatment with MPLA attenuated IL-6 production to secondary LPS challenge to a similar degree as LPS. The application of MyD88 siRNA dramatically reduced MPLA-induced tolerance while TRIF siRNA had no effect. The tolerant phenotype in endothelial cells was associated with reduced IKK, p38 and JNK phosphorylation and enhanced IRAK-M expression for LPS primed HUVECs, but less so in MPLA primed cells. Instead, MPLA-primed HUVECs demonstrated enhanced ERK phosphorylation. In contrast to leukocytes in which tolerance is largely TRIF-dependent, MyD88 signaling mediated endotoxin tolerance in endothelial cells. Most importantly, MPLA, a vaccine adjuvant with a wide therapeutic window, induced tolerance to LPS in endothelial cells.

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Declarations of Interest:

None

Author Contributions:

Ryan Stark, Benjamin Fensterheim, Fred Lamb and Edward Sherwood designed the experiments; Ryan Stark, Hyehun Choi and Stephen Koch performed the experiments; Ryan Stark, Hyehun Choi and Stephen Koch, Benjamin Fensterheim, Fred Lamb and Edward Sherwood analyzed the data; Ryan Stark, Hyehun Choi and Stephen Koch, Benjamin Fensterheim, Fred Lamb and Edward Sherwood wrote and revised the manuscript.

Keywords

endotoxin; tolerance; monophosphoryl lipid a; endothelial cells; toll-like receptor 4

Introduction

Endothelial dysfunction significantly contributes to the inflammatory response of a host to severe bacterial infections, also known as sepsis [1]. Dysfunction of the endothelium manifests itself as several pathological processes including capillary leak, altered vasomotor tone and microvascular thrombosis [2]. Despite the importance of endothelial-mediated inflammation in the pathology of sepsis, modulation of the inflammatory response of the endothelium remains elusive [2]. In leukocytes, prior challenge with lipopolysaccharide (LPS) renders the cells tolerant to subsequent exposures to LPS, a phenomenon known as endotoxin tolerance. Tolerance has been well described in both humans and other animals [4–6]. In each instance, providing a non-lethal dose of LPS to cells or a host confers protection against the inflammatory response to a subsequent LPS challenge. Numerous cytokines and chemokines, such as interleukin (IL)-6 and tumor necrosis factor (TNF) α , have been shown to have altered expression in models of endotoxin tolerance, with an overall shift from a pro-inflammatory response to an anti-inflammatory response [4, 7]. The attenuated cytokine response to LPS challenge has been observed in patients with severe infections and sepsis, and was initially thought to be a maladaptive response increasing susceptibility to secondary infections. However, this concept has been challenged recently with a shift from viewing the tolerant state as immune paralysis, to being potentially protecting against maladaptive overstimulation of toll-like receptor 4 (TLR4) signaling. Thus, it is now frequently referred to as “immune reprogramming” [8]. While these effects have been well described in leukocytes, the presence of endotoxin tolerance in non-myeloid cells of innate immunity, namely endothelial cells, is less clear [9, 10].

Induction of immune reprogramming has been shown to impact several signaling pathways including IL-1 receptor associated kinase-M (IRAK-M) induced inhibition of TLR4 [11] and enhanced IL-10 production [12]. While the exact mechanisms contributing to tolerance induction remain to be fully elucidated, the common theme among the postulated mechanisms is suppression myeloid differentiation primary response gene 88 (MyD88)-dependent signaling and enhancement of the TIR-domain-containing adapter-inducing interferon- β (TRIF) pathway [13]. In leukocytes, following TLR4 activation, MyD88 activation occurs at the cell surface. Subsequently, TLR4 is endocytosed resulting in TRIF activation and late pro-inflammatory cytokine and interferon production [14, 15]. It has been postulated that the sequential activation of MyD88 and TRIF pathways is preserved across multiple cells; however, endothelial TLR4 primarily stimulates the MyD88 pathway with very little TRIF activation [16, 17]. Given the predominance of MyD88 signaling in endothelial cells, it remains unclear if the absence of significant TRIF signaling is responsible for the reduced tolerance phenotype seen in endothelial cells [9].

Though LPS has been well described to induce tolerance and reprogramming, the toxicity of LPS has limited its clinical use. Monophosphoryl lipid A (MPLA) is a TLR4 agonist that is

currently employed clinically as an FDA-approved vaccine adjuvant due to its immunomodulating properties and minimal immunotoxicity [18]. Recent studies have demonstrated the ability of MPLA to augment the innate host response to infection. Priming with MPLA prior to infectious challenge non-specifically improves bacterial clearance and survival, an effect that is largely dependent on enhanced neutrophil recruitment [19]. It had been postulated that in leukocytes, the immunomodulatory functions of MPLA are preferentially mediated through activation of the TRIF-dependent signaling pathway, the interferon producing arm of TLR4 signaling [20]. However, we have shown that in endothelial cells, MPLA acts primarily through the cytokine producing MyD88 pathway [17]. Given the differential effects both TLR4 signaling and tolerance have in endothelial cells compared to leukocytes, we sought to answer several fundamental questions. First we wanted to determine whether endothelial cells were capable of endotoxin tolerance and additionally, whether MPLA priming could confer the same tolerant effects as LPS. Next, we wanted to determine if the tolerant effects induced by MPLA priming to LPS challenge were the result of activation of the MyD88 pathway. Lastly, we sought to determine if established mechanisms postulated to control endotoxin tolerance in leukocytes, such as IRAK-M induction, were conserved in endothelial cells. By answering these questions, we could gain new insight into the potential for priming of endothelial cells with the immunomodulator, MPLA, to reduce the negative consequences of subsequent TLR4 activation.

Materials and Methods

Cells and culture

Primary human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (Basel, Switzerland) and grown in Endothelial Growth Media-2 (Lonza) supplemented with 2% FBS. HUVECs were plated at a density of approximately 30,000 cells/cm² and grown to confluence. Experiments were conducted between the 2nd and 6th passages. Media was exchanged every 3 days.

Tolerance conditions

To determine the effect of MPLA and LPS on inducing tolerance in HUVECs, cells were exposed combinations of 10 µg/ml of VacciGrade-MPLA (*S. enterica* serotype minnesota Re 595, Invivogen, San Diego, CA), 100 ng/ml of Ultra-Pure LPS (*E. coli* 0111:B4, List Biological Laboratories, Inc. Campbell, CA), or control (2% FBS media) for 16 hours in media containing 2% FBS (1st treatment). Cells were then washed with media twice and allowed to remain undisturbed in fresh media containing 2% FBS for 24 hours. Afterwards, cells were challenged with of LPS (100 ng/ml) in 2% FBS media (2nd treatment). LPS derived from *E. coli* was chosen based on previous experiments examining the inhibitory kinetics of MPLA [17], in addition to the more common use of *E. coli* LPS in models of endotoxic shock. The dose of MPLA used was based on previously published dose-response curves in endothelial cells [17], as well as similar doses used in animal models of tolerance [19].

siRNA transfection

HUVECs were treated with siRNA (scrambled siControl, siTICAM1(TRIF), siMyD88) according to the manufacturer's protocol. In brief, siRNA were procured from Dharmacon (Lafayette, CO). siRNA (25 nM) was incubated with Dharmafect (Dharmacon) in serum-free medium for 20 min. The resultant complex of siRNA-Dharmafect was added to the cells in 2% FBS media without antibiotics for 24 hours either prior to the initial priming exposure or during the wash period prior to secondary LPS exposure. At the end of experiments, cells were collected and MyD88 and TRIF transcription and protein levels were determined by Real-time PCR and western blot analysis as described below.

Cytokine production

Culture supernatants were collected at the completion of the 1st ligand exposure (16 hours) and at the completion of the 2nd exposure (46 hours total duration). Collected supernatants were stored at -80 °C. Supernatant IL-6 (eBioScience, San Diego, CA), IP-10, G-CSF, e-Selectin (R&D Systems, Minneapolis, MN), and VCAM-1 (Cell Sciences, Canton, MA) concentrations were assessed using a commercially available enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's specifications. To serve as a positive control for overall IP-10 production, in separate experiments, HUVECs were exposed to 10 µg/ml of poly I:C (Sigma-Aldrich, St. Louis, MO) for 16 hours and supernatants were afterwards tested for IP-10 via ELISA.

RNA isolation and Real-time Polymerase Chain Reaction

RNA was isolated from cultured cells via GenElute Mammalian Total RNA Miniprep Kit (Sigma, St. Louis, MO) following the manufacturer's instructions at 6 hours after agonist exposure during the 2nd treatment for tolerance experiments and after 24 hours for siRNA experiments. 1.5 µg of total RNA was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Life Technologies, Grand Island, NY) according to the manufacturer's protocol. Efficiency of the PCR reactions was tested by amplification of the target from serially diluted cDNA generated from reverse transcription to achieve an efficiency of 95% ± 5%. Real-time PCR was performed using the TaqMan Fast Advanced Master Mix on a StepOnePlus Real Time PCR System (Applied Biosystems, Life Technologies, Grand Island, NY). PrimeTime® qPCR 5' Nuclease Assays (Integrated DNA Technologies, Inc., Coralville, IA) were used to amplify the target mRNAs for the follow transcription products; MyD88, TRIF, GAPDH. Data were normalized as a ratio of threshold cycle of target mRNA to GAPDH and corrected for efficiency using the StepOne software.

Western Blot and Electrophoresis

Cell lysates were collected at 1 hour after agonist exposure during the 2nd treatment for tolerance experiments and after 24 hours for siRNA experiments. Protein extracts (50 µg/sample) were denatured by boiling the samples in x2 Laemmli buffer (Bio-Rad, Hercules, CA) for 5 minutes. Samples were then separated by SDS electrophoresis on a polyacrylamide gel (10%) and transferred to nitrocellulose membranes. Membranes were blocked with Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE) for 1 hour at

room-temperature. Membranes were incubated with primary antibodies overnight at 4°C on a rocker. Antibodies were as follows: IKK α β , phospho-IKK β , IRAK-M, p-p38, p38, p-ERK, ERK, p-JNK, JNK, MyD88, TRIF (Cell Signaling Technology, Danvers, MA), IRF3 (Santa Cruz Biotechnology, Inc., Dallas, TX), p-IRF3 (EMD Milipore, Billerica, MA), Tubulin (Vanderbilt Antibody Core, Nashville, TN). Afterwards, membranes were incubated with fluorescent secondary antibodies and analyzed using the Odyssey Imaging System (LI-COR Biosciences, Lincoln, NE). Protein quantification was performed via densitometry and normalized as a ratio of expressed protein to Tubulin or phosphorylated protein to respective total protein.

Statistical Analysis

Data are expressed as means $\pm$ SE of a multiple, individual experiments. Comparisons of treatment groups and conditions were done via unpaired t-test for single comparisons and one-way ANOVA, with Bonferroni correction, for multiple-group comparisons. All analysis was done using GraphPad Prism 5.03 statistical software (GraphPad Software Inc., La Jolla, CA). A p value of <0.05 was considered statistically significant.

Results

MPLA is a weak inducer of MyD88-dependent cytokine and adhesion molecules compared to LPS

Activation of TLR4 by MPLA has been suggested to preferentially activate TRIF-dependent responses in leukocytes compared to LPS [20]. However, we have previously demonstrated that in endothelial cells, MPLA preferentially stimulates MyD88-dependent responses over TRIF-dependent responses [17]. First, we confirmed that vaccine grade MPLA preferentially activated MyD88-dependent pathway. LPS induced increases in both IL-6 (MyD88-dependent) and IP-10 (TRIF-dependent) production after 16 hours of exposure in HUVECs. MPLA was also able to induce IL-6 and IP-10 production, albeit weakly (IL-6 control 87.7 pg/ml $\pm$ 5.3 vs MPLA 219.9 pg/ml $\pm$ 5.7, $p < 0.001$; IP-10 control 0.7 pg/ml $\pm$ 0.5 vs MPLA 11.0 pg/ml $\pm$ 2.3, $p < 0.01$) (Figure 1A), though the induction of IP-10 was below the standard detection level of the assay. Knowing that the protective effects of MPLA in *in vivo* models of infection are thought to be related to enhanced neutrophil recruitment to sites of infection [19], we next turned our attention to secretion of MyD88-dependent cytokine and adhesion molecules that are related to leukocyte recruitment and attachment to the endothelium. As shown in Figure 1B, while LPS challenge enhanced granulocyte colony stimulating factor (G-CSF) release from endothelial cells, MPLA produced little G-CSF by comparison. Conversely, MPLA induction of endothelial soluble adhesion molecules including vascular cell adhesion molecule (VCAM) and e-selectin was approximately 45% and 57% of the total induced by LPS stimulation over controls, respectively. These results confirm a preferential, albeit weak, activation of MyD88-dependent processes by MPLA in endothelial cells compared to LPS.

MPLA generates tolerance to LPS challenge for MyD88-dependent responses

MPLA has been used as an immunomodulatory agent in models of cecal ligation and perforation or wound infection and has been demonstrated to be an effective treatment when

administered prior to infectious challenge [19]. In order to examine whether MPLA priming could induce tolerance to endothelial cells subsequently challenged with LPS, we exposed HUVECs to MPLA or control conditions for 16 hours. To compare to the well-described phenomenon of endotoxin tolerance, cells were alternatively exposed to LPS for 16 hours. Afterwards, cells were washed and allowed to rest in media for 24 hours. Cells were then challenged with LPS for 6 hours. As shown in Figure 2A, with regards to IL-6 secretion, MPLA induced tolerance to LPS with a similar efficacy to LPS priming. LPS priming was also able to minimally induce tolerance to IP-10 production after LPS rechallenge, as was MPLA priming, though this effect was not statically significant given the overall low IP-10 induction levels by LPS alone. Next we examined whether MPLA priming was able to tolerize or potentiate MPLA rechallenge. For these experiments, HUVECs were primed with MPLA or LPS for 16 hours, allowed to rest for 24 hours and then rechallenged with MPLA for 6 hours. Though LPS was able to induce tolerance to subsequent MPLA challenge, MPLA priming did not have any effect on MPLA rechallenge for IL-6 (Figure 2B). The results were slightly less clear for IP-10, as MPLA priming induced tolerance to MPLA rechallenge compared to LPS priming which did not. However, though there was statistical difference, the overall true difference was likely negligible considering the weak induction of IP-10 by TLR4. By comparison to TLR4 stimulation of IP-10, poly I:C a TRIF-biased TLR3 agonist, induces IP-10 levels that are approximately 500-fold greater than LPS (see Supplemental Figure 1). Thus overall, it appears that MPLA does not tolerize or potentiate itself on rechallenge.

When examining other MyD88-mediated processes, such as G-CSF, MPLA induced tolerance with equal efficacy to LPS priming. Interestingly, endothelial cells demonstrated less tolerance overall when examining markers of endothelial-cell adhesion. As demonstrated in Figure 3, soluble VCAM production was reduced in cells primed with MPLA or LPS compared to LPS alone by 34% and 37%, respectively. With regards to e-Selectin, LPS induced no tolerance compared to non-primed controls. Though MPLA priming induced a statistically significant reduction in e-Selectin production after LPS rechallenge compared to unprimed cells, the overall impact of MPLA priming on e-Selectin expression was not statistically different than LPS primed cells. We were unable to detect any TNF α or IL-10 production in response to either LPS or MPLA (data not shown). These data demonstrate that with regards to MyD88-mediated cytokine and soluble adhesion molecule production, MPLA induced tolerance to a very similar degree as LPS, however an effect of MPLA on TRIF-mediated cytokine production was less clear given the poor induction of TRIF-mediated responses to TLR4 activation.

MyD88, more than TRIF, contribute to tolerance in endothelial cells

In myeloid cells, it has been postulated that the driving forces related to endotoxin tolerance are upregulation of TRIF-pathway mediated processes and downregulation of MyD88 responses [13]. To examine if this phenotype was conserved in endothelial cells, HUVECs were exposed to scrambled, MyD88, TICAM (also known as TRIF) siRNA or a combination of MyD88 and TRIF siRNA either prior to the priming event or prior to the rechallenge. Cells were exposed to siRNA 24 hours prior to agonist exposure and achieved greater than 80% reduction of mRNA for both MyD88 and TRIF, though MyD88 and TRIF

protein knockdown were only approximately 33% and 46% at this time point, respectively ($p < 0.05$ for siControl versus siMyD88 or siTRIF for mRNA and protein, see Supplemental Figure 2). Figure 4A demonstrates the effect of MyD88, TRIF and combined siRNA, with scrambled siRNA as controls, on LPS- vs. MPLA-induced IL-6 secretion. In the presence of scrambled siRNA (siControl), LPS again induced much greater IL-6 production compared to MPLA. However, in the presence of siRNA to MyD88 (siMyD88), the induction of IL-6 was completely abolished after MPLA exposure and nearly abolished after exposure to LPS. This is in contrast to incubation with TRIF siRNA (siTRIF) which also inhibited LPS-induced IL-6 expression, though to a lesser degree than the siMyD88. MPLA continued to have a minimal IL-6 production in the presence of siTRIF, though the expression was not statistically different that control. The dual presence of siRNA to both MyD88 and TRIF (siMyD88/TRIF) continued to render the cells unresponsive to MPLA, though LPS still continued to have some, albeit minimal, effect on IL-6 production ($209.1 \text{ pg/ml} \pm 13.89$ for Control vs $496.3 \text{ pg/ml} \pm 106.1$ for LPS, $p < 0.05$). To examine the effects of the siRNA groups on TRIF-mediate cytokines in response to MPLA and LPS, supernatants were also tested for IP-10 expression. Unexpectedly, while siControl had no major effects on the relative expression of IP-10 by MPLA or LPS, the presence of siMyD88 or siTRIF significantly enhanced IP-10 production irrespective of the presence of MPLA or LPS (Supplemental Figure 3). Next, we assessed the impact of siMyD88, siTRIF or siMyD88/TRIF selectively after priming and prior to the secondary LPS challenge. For these experiments, HUVECs were exposed to control, MPLA or LPS for 16 hours, washed for 24 hours in the presence of siRNA, then exposed to control or LPS for 6 hours. In cells exposed to siControl, tolerance to LPS was maintained with both MPLA and LPS priming, though with MPLA to a slightly lesser degree (Figure 4B). In the presence of siMyD88 or siMyD88/TRIF, the tolerant effect of priming with either MPLA or LPS was lost. In contrast, exposure to siTRIF after priming had no effect on tolerance, and IL-6 levels remained similar to the siControl group.

To further examine the effects of priming on MyD88 and TRIF, cells exposed to siControl during the wash period underwent PCR to determine production of mRNA of both MyD88 and TRIF at 6 hours after LPS rechallenge. As shown in Figure 5A, in all HUVECs exposed to LPS, there was a significant reduction in MyD88 mRNA, with a slightly larger reduction in cells primed with MPLA. In comparison, TRIF mRNA production was unchanged in any of the cells exposed to MPLA or LPS relative to controls. To determine if the changes in MyD88 mRNA after LPS exposure resulted in change in MyD88 protein expression as a mechanism of tolerance, HUVECs underwent priming with MPLA or LPS and were rechallenged with LPS. At 1 hour after re-exposure, cell lysates were collected and examined for MyD88 protein levels (Figure 5B). Despite changes in MyD88 mRNA in the LPS exposed groups, there was no evidence of diminished total MyD88 protein levels compared to controls. Taken together with the impact of siMyD88 on endothelial responses to LPS, these results suggest that the MyD88 pathway more so than the TRIF pathway, contributes to endothelial tolerance.

LPS, but not MPLA, priming induce IRAK-M production

Several mechanisms have been suggested to contribute to the phenotype of endotoxin tolerance. Particularly, enhanced production of IRAK-M leading to reduced I κ B kinase (IKK) production and subsequently reduced NF- κ B has been well described [11]. Additionally, it has been hypothesized that tolerance induces upregulation of TRIF-mediated interferon regulatory factor 3 (IRF3) activities, leading to protection through the production of interferon- β [21]. To examine the role of these intracellular signaling mechanisms in tolerant endothelial cells, we analyzed protein expression 1 hour after rechallenge with LPS in HUVECs primed with MPLA or LPS. As shown in Figure 6, priming with LPS induced a 42% increase in IRAK-M production compared to controls. Furthermore, this increase in IRAK-M was complimented by a 47% reduction in phosphorylated IKK (p-IKK). In contrast, MPLA priming was unable to induce any IRAK-M production compared to controls. Additionally, though MPLA was able to reduce p-IKK during rechallenge by approximately 14% compared to unprimed cells, this result was not statistically significant (relative protein density 1.55 ± 0.16 for MPLA $\rightarrow$ LPS versus 1.81 ± 0.22 for Control $\rightarrow$ LPS, $n = 4$ per group, N.S.). To examine alterations in TRIF-mediated intracellular signaling, we examined the effect of MPLA or LPS priming on the most downstream portion of the TRIF-induced interferon production, IRF3. Consistent with our previous data showing a limited role of TRIF, LPS did not induce significant changes in p-IRF3 compared to control cells. Likewise, tolerance had no effects on inducing elevated levels of p-IRF in cells primed with MPLA or LPS. These results demonstrate that while LPS-mediated tolerance does involve negative regulation of NF- κ B via upregulation of IRAK-M, an IRF3-dependent mechanism does not appear to be involved in LPS or MPLA-mediated tolerance.

MPLA priming preferentially enhances ERK phosphorylation

Activation of TLR4 is known to regulate mitogen-activated protein kinases (MAPKs), which are intracellular pathways leading to cellular proliferation and survival or apoptosis and cell death [22]. With regards to tolerance, macrophages repeatedly exposed to endotoxin have been shown to have downregulated MAPK activity [23]. To determine the role of MAPKs in endothelial tolerance, MAPK protein phosphorylation was examined a 1 hour after LPS rechallenge in primed HUVECs. As shown in Figure 7, LPS induced significant phosphorylation of all MAPKs, with the largest effects on c-Jun N-terminal kinase (JNK) (45% increase in p-extracellular-signal-regulated kinase (ERK), 75% increase in p-p38 and 88% increase in p-JNK for LPS relative to controls, $n = 4$ per group). HUVECs primed with LPS and rechallenged with LPS had near absence of enhanced phosphorylation for p38 and JNK relative to unprimed HUVECs. HUVECs primed with MPLA also had reduced phosphorylation of p38 and JNK after LPS rechallenge compared to unprimed cells by 25% and 19%, respectively, though this difference was only significant for p38. Contrary to these findings, priming with LPS enhanced ERK phosphorylation after rechallenge; however this increase in phosphorylation was not statistically significant. ERK phosphorylation was further enhanced in HUVECs primed with MPLA and rechallenged with LPS (21% increase in p-ERK for MPLA $\rightarrow$ LPS vs Control $\rightarrow$ LPS, $p < 0.01$). These data show that despite activation of the same TLR4 receptor and similar effects on tolerance, MPLA and LPS priming have differential effects on intracellular signaling pathways, specifically in relation to MAPK activation.

Discussion

The phenomenon of endotoxin tolerance has often been thought to be the result of immune reprogramming of myeloid cells, particularly monocytes and macrophages [6–8]. Whether the tolerant phenotype could be extrapolated to other cells of innate immunity, particularly endothelial cells, has been controversial [9, 10]. Additionally, while the postulated mechanisms contributing to tolerance have varied, there has been consensus that tolerance results from enhancement of TRIF-mediating processes acting as negative regulators of MyD88-dependent signaling [13, 24]. In the current study, we demonstrate that endothelial cells are indeed capable of developing a tolerant phenotype, and this tolerance was more dependent on MyD88 signaling than on TRIF. Furthermore, we found that MPLA, a weak TLR4 agonist with diminished pro-inflammatory capabilities, was able to induce tolerance in endothelial cells to a similar degree as LPS. These studies provide new knowledge regarding the complexity of TLR4 signaling and provide support for the use of immunomodulators, such as MPLA, protecting against endotoxin induced endothelial dysfunction.

MPLA has long been known to possess immunomodulatory properties, leading to its use as a vaccine adjuvant [20]. It has been shown to have beneficial effects in animal models of infection [19] and ischemia-reperfusion injury [25]. The underlying reasons for the protective effect of MPLA in these models remain unknown, but it has been suggested to be the result of improved bacterial clearance in models of infection [19] and enhanced calcitonin production in ischemia-reperfusion [26]. How these beneficial effects of MPLA translate to endothelial-mediated inflammation is unknown. In the current study, the exposure of HUVECs to MPLA induced little pro-inflammatory cytokine production compared to LPS (Figure 1), confirming its role as a weak inducer of inflammation. We next turned our attention to the ability of MPLA to induce tolerance. Debate exists regarding the meaning and significance of endotoxin tolerance. Initially the phenotype of tolerance was believed to represent a type of immune paralysis. This understanding has slowly shifted to characterize tolerance as a reprogrammed, and potentially adaptive, immune status [8, 27]. To examine if the phenotype of endotoxin tolerance has similar characteristics in endothelial cells and whether MPLA could induce a tolerance, we primed HUVECs with MPLA or LPS and then rechallenged the cells with an additional dose of LPS. MPLA was able to reduce the production of MyD88-dependent cytokines, with a similar efficacy as LPS (Figure 2); however it overall had minimal effect on the TRIF-dependent chemokine, IP-10. Interestingly, in HUVECs exposed to LPS or MPLA, the tolerant phenotype was less dramatic for molecules related to adhesion, such as VCAM and e-Selectin (Figure 3). Though we did not test cell-cell adhesions directly, the observation of continued VCAM and e-Selectin expression despite priming with MPLA or LPS may contribute to the improved bacterial clearance seen in animal models of sepsis after TLR4 priming [19, 28]. Our next objective was to determine if the tolerant phenotype in endothelial cells was more dependent on MyD88 than TRIF.

Activation of TLR4 leads to sequential activation of two distinct pathways, MyD88 and TRIF. To directly examine the roles of MyD88 and TRIF in tolerance, HUVECs were exposed to siRNA targeting MyD88, TRIF or both. The application of siMyD88 or

siMyD88/TRIF significantly impaired the impact of MPLA and LPS during priming as well as overall tolerance (Figure 4). These findings were in stark contrast to siTRIF, which did not have any effect on tolerance. This result was surprising in that several studies have suggested the TRIF-pathway to be a primary mediator of tolerance [13, 24]. One explanation for this is the insignificance of the TRIF-pathway in TLR4 activation of endothelial cells. While poly I:C, a TLR3 agonist, is a potent stimulator of the TRIF-pathway (Supplemental Figure 1), LPS does not generate significant levels of TRIF-mediated chemokines in endothelial cells [17, 30], suggesting that the pathways of TLR4 activation are not conserved across all cell types. Additionally, though MPLA has been postulated to preferentially activate TRIF in leukocytes [20], it does not appear to significantly stimulate TRIF responses in endothelial cells.

The bias of LPS and MPLA toward MyD88-dependent responses in endothelial cells was further demonstrated when examining transcription of MyD88 and TRIF (Figure 5A). Incubation with LPS significantly inhibited MyD88 transcription, a result that was slightly enhanced by priming with MPLA. In comparison, TRIF transcription was unaltered by LPS exposure, reaffirming a diminished role of TLR4-mediated TRIF activation in endothelial cells. Interestingly, the diminished MyD88 mRNA did not translate into reduced MyD88 protein levels (Figure 5B). The lifecycle of MyD88 after initiation of TRIF signals has not been well characterized, though it is thought to disengage from TLR4 shortly after endocytosis to allow for TRIF mediated responses, where it may undergo degradation [31]. Alternatively, MyD88 has been shown to sequester in phagosomes after TLR4 activation, suggesting a role for protein recycling [32]. Irrespective of what happens to MyD88 after TLR4 activation, it is clear that any disruption in new MyD88 synthesis has profound effects on the endothelial TLR4 response. Additionally, our data highlights the relative insignificance of TLR4-mediated TRIF activation previously suggested in endothelial cells [30]. It is likely that endothelial TLR4 activation leads to a differential pathway activation than that described in leukocytes and these variances account for the contrasts in inflammation measured between cell types challenged with the same TLR agonist [30]. Specifically related to tolerance, the observation that monocytes readily develop tolerance [24] while endothelial cells do not always do so [9] further suggests divergence of pathway stimulation and modulation despite activation of the same receptor. Our results highlight these discrepancies by demonstrating that while endothelial cells can undergo tolerance, the mechanisms responsible for the tolerant-phenotype are more MyD88-dependent.

While TRIF-mediated responses have been linked to a tolerant phenotype, one conserved observation is down regulation of MyD88 activation. Several mechanisms have been postulated to modulate MyD88 in tolerance, including IRAK-M [11], among others [33]. To examine the role of intracellular mechanisms contributing to tolerance, we examined phosphorylated and total protein expression for a number of inflammatory signaling pathways in HUVECs after tolerance. Consistent with published reports in leukocytes, LPS-induced tolerance was associated with reduced IKK phosphorylation and enhanced IRAK-M expression (Figure 6). Surprisingly, MPLA did not enhance IRAK-M production or significantly reduce IKK phosphorylation, showing IRAK-M was not responsible for the observed changes in cytokine production seen with MPLA priming. Instead, we found that MPLA priming significantly enhanced the amount of p-ERK in HUVECs rechallenged with

LPS, even more so than LPS priming (Figure 7). While the impact of enhanced ERK activation on tolerance is not known, in endothelial cells, ERK activation has been shown to have anti-inflammatory properties [36]. Indeed, support for the role of ERK in tolerance was demonstrated in dendritic cells where enhanced ERK activation rendered cells tolerant to LPS [38]. While the specific contribution of ERK in tolerance remains unknown, it is clear that TLR4 activation can induce tolerance via a variety of mechanisms [33]. Further studies will be necessary to delineate differences in the signals required to induce tolerance in endothelial cells compared to leukocytes and how these differences translate into different clinical phenotypes.

There are limitations in our current study. Application of siRNA was unable to completely inhibit all mRNA synthesis, and thus protein production (Supplemental Figure 2). In addition, siRNAs can stimulate innate immunity via direct activation of other toll-like receptors, potentially altering MyD88 and TRIF signaling [29]. The stimulatory properties of siRNA may explain the enhanced IP-10 production seen with siRNA exposure prior to LPS challenge (Supplemental Figure 3). These “off-target” effects of siRNA may have contributed to tolerance that was independent of protein knockdown. Further limitations include the constraints on exposure time and the absence of endothelial cell interactions with other innate immune cells *in vitro*. Certainly the tolerance of cells can be effected by the duration of agonist exposure [39] as well as innate immune system interaction within the host environment. Whether the time intervals employed for these experiments can be extrapolated to human physiology and whether the endothelial tolerant phenotype is altered in the presence of other primed or activated immune cells remains unknown.

In summary, we show for the first time that the immunomodulator and vaccine adjuvant, MPLA, can induce an LPS tolerance phenotype to endothelial cells. This tolerance appears to be induced in a MyD88-dependent manner, as TLR4-dependent TRIF-mediated processes are severely diminished in endothelial cells. Additionally, we provide evidence that TLR4-mediated tolerance in general is related to alterations in MyD88 but not TRIF signaling as TRIF-deficient HUVECs showed a tolerance pattern similar to control cells. In determining intracellular mechanisms contributing to tolerance, LPS priming enhanced IRAK-M expression, with associated decreases in p-IKK. Phosphorylation of p38 and JNK may also contribute to the LPS-induced, but less so for MPLA-induced tolerant phenotype. Instead, MPLA priming appeared to induce tolerance by enhancing p-ERK. These studies provide new evidence that MPLA can induce endothelial tolerance and explore the complex mechanisms regulating tolerance in endothelial cells. More importantly, they show that MPLA offers unique properties that may modulate endothelial injury to an infectious stimulus which may enhance its impact as a clinical immunomodulator.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Summary Statement

Monophosphoryl lipid A, a vaccine adjuvant, can render endothelial cells tolerant to subsequent challenge by lipopolysaccharide. Tolerance results in impaired MyD88-dependent signaling, leading to reduced cytokine production. This study highlights the potential use of immunomodulators to prevent infection-mediated endothelial dysfunction.

Clinical Perspectives

- Severe bacterial infections, known as sepsis, induce a pro-inflammatory phenotype in endothelial cells that can promote vasomotor dysfunction, capillary leak and thrombosis. Therapies specifically targeting endothelial inflammation are lacking.
- Monophosphoryl lipid A (MPLA) is employed as a human vaccine adjuvant. In previous studies, MPLA pre-treatment reduced mortality from subsequent sepsis. We now report that it can induce endothelial cell tolerance to gram-negative derived lipopolysaccharide (LPS) without inciting a significant pro-inflammatory response itself. The tolerant phenotype is dependent on TLR4 signaling via MyD88- as opposed to TRIF-dependent pathways and requires subsequent ERK phosphorylation to a much greater extent than tolerance induced by LPS itself.
- These results provide novel insight into the potential for a prophylactically applied immunomodulator, such as MPLA, to dampen endothelial inflammation during a subsequent infectious challenge.

Figure 1. MPLA is a weak agonist of MyD88-dependent cytokine production

Primary HUVECs (passages 2 to 6) were exposed to MPLA (10 μ g/ml), LPS (100 ng/ml) or control conditions (2% FBS media) for 16 hours. **A:** Culture supernatants were collected at 16 hours and examined for IL-6 or IP-10 production via ELISA. **B:** Culture supernatants were collected at 16 hours and examined for G-CSF production as well as soluble VCAM and e-Selectin expression via ELISA. Data are expressed as means $\pm$ SE of individual experiments ($n = 5$). ** indicates $p < 0.01$, *** indicates $p < 0.001$ by ANOVA.

Figure 2. MPLA induces tolerance to subsequent LPS challenge

HUVECs (passages 2 to 6) were exposed to MPLA (10 µg/ml), LPS (100 ng/ml) or control conditions (2% FBS media) for 16 hours. Afterwards, cells were washed twice with media and then allowed to sit undisturbed in 2% FBS media for 24 hours. **A:** Cells were then rechallenged with LPS (100 ng/ml) or control conditions for 6 hours. Culture supernatants were collected and examined for IL-6 or IP-10 production via ELISA. **B:** Cells were rechallenged with MPLA (10 µg/ml) or control conditions for 6 hours. Culture supernatants were collected and examined for IL-6 or IP-10 production via ELISA. Data are expressed as

means $\pm$ SE of individual experiments (n = 4 to 8). * indicates $p < 0.05$, *** indicates $p < 0.001$ by ANOVA.

Figure 3. Adhesion molecules are less tolerant to TLR4 priming

HUVECs (passages 2 to 6) were exposed to MPLA (10 µg/ml), LPS (100 ng/ml) or control conditions (2% FBS media) for 16 hours. Afterwards, cells were washed twice with media and then allowed to sit undisturbed in 2% FBS media for 24 hours. Cells were then rechallenged with LPS (100 ng/ml) or control conditions for 6 hours. Culture supernatants were collected and examined for G-CSF production as well as soluble VCAM and e-Selectin expression via ELISA. Data are expressed as means ± SE of individual experiments (n = 5).

* indicates p < 0.05, *** indicates p < 0.001 by ANOVA.

Figure 4. MyD88 plays an important role in tolerance

A: HUVECs were exposed to 25 nM scrambled siRNA (siControl), siRNA to MyD88 (siMyD88), siRNA to TICAM1 (siTRIF) or a combination (siMyD88/TRIF) for 24 hours. Afterwards, media containing siRNA was removed and HUVECs were exposed to LPS (100 ng/ml) or MPLA (10 µg/ml) or control for 16 hours. Supernatants were collected afterwards and examined for IL-6 production by ELISA (n = 3). **B:** HUVECs were challenged with LPS (100 ng/ml) or MPLA (10 µg/ml) or control for 16 hours, then exposed to 25 nM scrambled siRNA (siControl), or siRNA to MyD88 (siMyD88), siRNA to TICAM1

(siTRIF) or a combination (siMyD88/TRIF) for 24 hours in media. Afterwards, media containing siRNA was removed and HUVECs were exposed to LPS (100 ng/ml) or control for 6 hours. Supernatants were collected afterwards and examined for IL-6 production by ELISA. Data are expressed as means $\pm$ SE of individual experiments (n = 3 to 7). * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$, [†] indicates $p < 0.05$ between siRNA group compared to siRNA control by ANOVA.

Figure 5. Endotoxin treatment leads to MyD88 downregulation

HUVECs were challenged with LPS (100 ng/ml) or MPLA (10 µg/ml) or control for 16 hours, then washed and left in media for 24 hours. Afterwards, cell were exposed to LPS (100 ng/ml) or control. **A:** Cellular RNA was transcribed into cDNA copies from the siControl samples and amplified using real-time PCR with primers for MyD88 and TICAM 6 hours after LPS rechallenge. Data are expressed as means ± SE of a multiple, individual experiments after normalization to GAPDH expression (n = 3). * indicates p < 0.05 between groups as shown with lines, *** indicates p < 0.001 compared to normalized control by ANOVA. **B:** Cell lysates were harvested and analyzed by western blots 1 hour after LPS

rechallenge. Blots were probed for total MyD88 protein and Tubulin. *Left*: Representative image showing MyD88 and Tubulin. *Right*: Bar graphs showing the relative abundance of MyD88 proteins after normalization to Tubulin expression. Data are expressed as means $\pm$ SE of individual experiments ($n = 4$) after normalization to control quantities.

Figure 6. LPS priming, but not MPLA priming, induces IRAK-M expression

HUVECs were exposed to LPS (100 ng/ml) or MPLA (10 µg/ml) or control for 16 hours. Afterwards HUVECs were allowed to rest for 24 hours in media. Then HUVECs were rechallenged with LPS (100 ng/ml) or control for 1 hour. Cell lysates were harvested and analyzed by western blots. Blots were probed for total IRAKM and Tubulin as well as total and phosphorylated IKK and IRF3. *Left:* Representative images showing phosphorylated or total protein (p-IKKαβ, p-IRF3, IRAK-M) and total proteins (IKKβ, IRF3) or Tubulin. *Right:* Bar graphs showing the relative abundance of phosphorylated proteins after

normalization to total protein expression. Data are expressed as means $\pm$ SE of individual experiments (n = 4) after normalization to control quantities. * indicates $p < 0.05$ between groups as shown with lines by ANOVA.

Figure 7. MPLA and LPS differentially regulate MAPKs in tolerance

HUVECs were exposed to LPS (100 ng/ml) or MPLA (10 µg/ml) or control for 16 hours. Afterwards HUVECs were allowed to rest for 24 hours in media. Then HUVECs were rechallenged with LPS (100 ng/ml) or control for 1 hour. Cell lysates were harvested and analyzed by western blots. Blots were probed for total and phosphorylated MAPK proteins. *Left:* Representative images showing phosphorylated (p-p38, p-ERK, p-JNK) and total proteins (p38, ERK, JNK). *Right:* Bar graphs showing the relative abundance of phosphorylated proteins after normalization to total protein expression. Data are expressed

as means $\pm$ SE of individual experiments ($n = 4$) after normalization to control quantities. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$ between groups as shown with lines by ANOVA.