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Potential and tolerance of toll-like receptor priming in human endothelial cells

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Abstract

Repeated challenge of lipopolysaccharide (LPS) alters the response to subsequent LPS exposures via modulation of toll-like receptor 4 (TLR4). Whether activation of other TLRs can modulate TLR4 responses, and vice versa, remains unclear. Specifically with regards to endothelial cells, a key component of innate immunity, the impact of TLR cross-modulation is unknown. We postulated that TLR2 priming (via Pam3Csk4) would inhibit TLR4-mediated responses while TLR3 priming (via Poly I:C) would enhance subsequent TLR4-inflammatory signaling. We studied human umbilical vein endothelial cells and neonatal dermal microvascular (HMVECs) endothelial cells. Cells were primed with a combination of Poly I:C (10 μ g/ml), Pam3Csk4 (10 μ g/ml), or LPS (100 ng/ml), then washed and allowed to rest. They were then rechallenged with either Poly I:C, Pam3Csk4 or LPS. Endothelial cells showed significant tolerance to repeated LPS challenge. Priming with Pam3Csk4 also reduced the response to secondary LPS challenge in both cell types, despite a reduced proinflammatory response to Pam3Csk4 in HMVECs compared to human umbilical vein endothelial cells. Poly I:C priming enhanced inflammatory and interferon producing signals upon Poly I:C or LPS rechallenge, respectively. Poly I:C priming also induced interferon regulatory factor 7, leading to enhancement of interferon production. Finally, both Poly I:C and LPS priming induced significant changes in receptor-interacting serine/threonine-protein kinase 1 activity. Pharmacological inhibition of receptor-interacting serine/threonine-protein kinase 1 or interferon regulatory factor 7 reduced the potentiated phenotype of TLR3 priming on TLR4 rechallenge. These results demonstrate that in human endothelial cells, prior activation of TLRs can have a significant impact on subsequent exposures and may contribute to the severity of the host response.

INTRODUCTION

The innate immune system is comprised of a diverse array of cell types. One critical piece of the defense network that comprises the innate immune system is the endothelium.¹ Disruption of normal endothelial function, as seen during severe infections, results in

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“barrier failure” leading to detrimental alterations in vascular tone, fluid leak, leukocyte trafficking, and coagulation.^{1,2} Driving these pathophysiologic changes in endothelial cells is the activation of toll-like receptors (TLRs). These pattern recognition receptors are an integral component of the innate immune response and their activation are the result of host interactions with various viral, fungal, and bacterial components, as well as endogenous TLR ligands.³⁻⁵ Though a single organism may drive the initial immune response, studies have suggested that those patients coinfecting with more than one organism are at risk for an increased severity of illness and need for hospitalization.⁶⁻⁸ While having multiple organisms lead to a worse outcome appears intuitive, the pathophysiology behind the influence of coinfection is less clear.⁹ In animal models, coinfection with viruses and bacteria impair bacterial clearance as a result of enhanced interferon production, suggesting the presence of one infection alters the virulence of another.¹⁰ Likewise, animal models of bacterial coinfection are congruent with evidence in humans, supporting the concept that concurrent bacterial infection negatively impact outcome.^{11,12} Though the mechanisms responsible for the worse outcome remain elusive, it has been suggested that infections can overstimulate the immune system and induce a state of immunoparalysis which enhances susceptibility to secondary infections.¹³

The means through which infection induce a state of immunoparalysis are likely multifactorial; however, the reduced cytokine response to the repeated challenges of lipopolysaccharide (LPS), termed endotoxin tolerance, have been suggested to mimic the immunoparalysis phenotype.^{14,15} Though endotoxin tolerance is a TLR4-mediated process, it has been postulated that other TLRs, such as TLR2 and TLR3, and their respective ligands, Pam3Csk4 (a synthetic triacylated lipopeptide that mimics bacterial lipopeptides) and Poly I:C (structurally similar to double-stranded viral RNA), can induce states of cross-tolerance.^{16,17} The mechanisms responsible for this cross tolerance remain unclear as some have suggested the TLR tolerance is driven by use of conserved signaling pathways, whereas others have suggested a multitude of pathways regulate tolerance.^{16,17} This lack of clarity is likely enhanced by use of mouse-derived macrophages, which can have a different phenotypic response compared to humans.¹⁸ Further confounding these results, we have demonstrated that the mechanisms of TLR4 signaling tolerance in endothelial cells are differentially regulated compared to those of leukocytes, suggesting that modulation of TLR signals in human endothelial cells may not be conserved to that reported in mouse macrophages.¹⁹ Understanding this difference in the human endothelium is emphasized by the role it plays in vascular homeostasis and innate immunity activation, making its modulation a key driver of the host response to infection.²⁰

Stimulation of TLRs leads to activation of 2 distinct pathways, the myeloid differentiation primary response protein 88 (MyD88)–dependent pathway and the TIR-domain-containing adapter-inducing interferon- β (TRIF)–dependent pathway. While TLR2 and TLR3 use predominately the MyD88 or TRIF pathway, respectively, TLR4 uses both pathways in a sequential fashion in leukocytes.²¹ Stimulation of conserved pathways is thought to induce tolerance (eg, MyD88-repeated stimulation), whereas divergent pathways lead to potentiation (eg, MyD88 priming before TRIF stimulation).¹⁶ However, we have demonstrated that in endothelial cells, TLR4 binding to LPS results primarily in MyD88 activation with little TRIF activation, making the role of MyD88 and TRIF in tolerance less

clear.¹⁹ Given the fundamental difference between endothelial cells and leukocytes, we sought to explore how the differential regulation of TLR-mediated MyD88 and TRIF pathway activation altered the response of endothelial cells to multiple TLR ligands compared to that published in leukocytes. In addition, based on clinical data that coinfections lead to worse outcomes and previous work on TLR signaling, we hypothesized that TLRs that used the same signaling pathway (eg, MyD88 only) would induce cross-tolerance while TLRs that signal via different pathways (TRIF vs MyD88) would lead to potentiation. By understanding the relationship of TLRs to one another on human endothelial cells, therapeutic avenues could be explored to augment the negative impact that coinfections have on the vasculature and innate immunity.

METHODS AND MATERIALS

Cells and culture conditions

Primary neonatal, male human dermal microvascular endothelial cells (HMVECs, Lonza, Basel, Switzerland), and primary mixed-gender human umbilical vein endothelial cells (HUVECs, Lonza) from multiple donors were plated on gelatin-coated plates at approximately 30,000 cells/cm² and grown to confluence in Endothelial Growth Media (EGM, Lonza) using 2% FBS (HUVECs) or 5% FBS (HMVECs). Cells were tested between passages 1 to 6.

HUVEC priming and treatment conditions

HUVECs were grown to confluence and treated with media (control), 100-ng/ml Ultra-Pure LPS (List Biological Labs, Campbell, Calif), 10- μ g/ml Poly I:C (Sigma-Aldrich, St. Louis, Mo), or 10- μ g/ml VacciGrade Pam3CSK4 (Invivogen, San Diego, Calif). After 16 hours, the cells were washed with 1 ml of media, and fresh media was added to the cells where remained undisturbed for another 24 hours. Afterward, cells were exposed to combinations of LPS, Poly I:C, Pam3CSK4, or media at the previously mentioned doses for an additional 6 hours for ELISAs.

HMVEC priming and treatment conditions

HMVECs grown to confluence were treated with media (control), 100-ng/ml Ultra-Pure LPS (List Biological Labs), 10- μ g/ml Poly I:C (Sigma-Aldrich), or 10- μ g/ml VacciGrade Pam3CSK4 (Invivogen). After 16 hours, the cells were washed with 1 ml of media, and fresh media was added to the cells where remained undisturbed for another 32 hours. The media was then replaced with either LPS, Poly I:C or Pam3CSK4 or media at the previously mentioned doses for an additional 1 hour for Western blot analysis or 16 hours for ELISAs. Prolonged exposure times for HMVECs compared to HUVECs were secondary to published differences in immune phenotype of HMVECs compared to HUVECs, as well as internal testing demonstrating prolonged development of a LPS tolerant phenotype in HMVECs at 16 hours after the second agonist exposure compared to a similar LPS tolerance phenotype seen in HUVECs after 6 hours of the second agonist (see Fig S1).²²

In some experiments, HMVECs underwent exposure to control media or 10- μ g/ml Poly I:C for 16 hours followed by a 32-hour exposure to fresh media. Afterward, cells were exposed

to either control (media with <0.1% DMSO), 1- μ M or 3- μ M 7-Cl-O-Nec1 (Nec-1s, EMD Millipore, Billerica, Mass) or 0.1- μ M 0.3- μ M Trichostatin A (TSA, Invivogen, San Diego, Calif) in the presence of 100 ng/ml of LPS for 16 hours after which supernatants were assayed for cytokine production. These doses were based on their effect on LPS-induced IP-10 production (see Fig S5) as well as previously reported doses of Nec-1s inhibition of receptor-interacting serine/threonine-protein kinase 1 (RIP1) phosphorylation and TSA inhibition of interferon regulatory factor 7 (IRF7) transcription.^{23,24}

In separate experiments to test TLR2 differences between HUVECs and HMVECs, both cell lines underwent incubation with LPS (100 ng/ml), Pam3Csk4 (10 mg/ml), Purified Lipotechoic Acid from *S. aureus* (LTA, 10 μ g/ml, Invivogen, San Diego, Calif), or media (control) for 16 hours. Afterward, supernatants were collected and assayed for cytokines.

Western blot analysis of proteins

HMVECs were exposed at time intervals previously mentioned. Cells were then washed with 1-ml cold PBS. The cells were scraped into 30- to 50- μ l cold phospho-protecting lysis buffer containing 20- μ l/ml Protease Inhibitor Cocktail (Sigma-Aldrich, St. Louis, Mo) and centrifuged at 21,100 $\times$ g for 5 minutes. Supernatant containing approximately 20 μ g of protein were separated by SDS-PAGE electrophoresis on a polyacrylamide gel (7.5%). The separated proteins were then transferred to nitrocellulose membranes using the Mini Trans-Blot (Bio-Rad, Hercules, Calif) following the recommended procedures. Membranes were blocked for 1 hour with Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, Neb) and incubated overnight with the primary antibody in TBST 0.1%. Primary antibodies included: I κ B kinase (IKK α), p-IKK β , p-p38, p38, p-ERK 1/2, extracellular signal-regulated kinase 1/2 (ERK 1/2), p-JNK, c-Jun amino-terminal kinase (JNK), p-RIP, RIP, p-IRF7, IRF7, TLR2, TLR3, α -tubulin (Cell Signaling Technology, Danvers, Mass), IRF3 (Santa Cruz Biotechnology, Inc, Dallas, Tex), p-IRF3 (EMD Millipore, Billerica, Mass), and TLR4 (Thermo-Fisher Scientific, Waltham, Mass). After washing, the membranes underwent incubation with the appropriate fluorescently conjugated secondary antibody (LI-COR Biosciences, Lincoln, Neb), and images were acquired and quantified using the Odyssey Imaging System (LI-COR Biosciences).

Enzyme-linked immunosorbent assay of cytokines

Supernatants were recovered at the time point mentioned above and stored at -80°C until used in the assays to examine cytokine production. IL-6 (eBioScience, San Diego, Calif) and IP-10 (R&D Systems, Minneapolis, Minn) concentrations were determined using a commercially available enzyme-linked immunosorbent assay (ELISA) kit and performed according to the manufacturer's specifications.

RNA isolation and real-time PCR of mRNA

RNA was isolated from HUVEC and HMVEC cultures using the MasterPure RNA Purification Kit (Epicentre Technologies, Madison, Wis) following the instructions provided in the kit. 3 μ g of total RNA was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Thermo-Fisher Scientific, Waltham, Mass) according to the manufacturer's protocol. Efficiency of the PCR was tested by amplification of the target

from serially diluted cDNA generated from reverse transcription to achieve an efficiency of 90%. Real-time PCR was performed using the TaqMan Fast Advanced Master Mix (Thermo-Fisher Scientific, Waltham, Mass) on a StepOnePlus Real-Time PCR System (Applied Biosystems, Thermo-Fisher Scientific, Waltham, Mass). PrimeTime qPCR assay (Integrated DNA Technologies Inc, Coralville, Iowa) used to amplify the target TLR2 mRNA contained the following sequences: 5'-CCATTGCTCTTTCACTGCTTTC and 5'-ATGACCCCCAAGACCCA. GAPDH primers were as follows: 5'-ACATCGCTCAGACACCATG and 5'-TGTAGTTGAGGTCAATGAAGGG. Data were normalized as a ratio of threshold cycle of target mRNA to GAPDH and corrected for efficiency using the StepOne software.

Statistical analysis

Data are expressed as means $\pm$ standard error of individual, replicated experiments. Comparisons of treatment groups and conditions were done via unpaired *t* test for single comparisons and 1-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, Calif). A *P* value of <0.05 was considered statistically significant.

RESULTS

Poly I:C-mediated TLR3 priming enhanced subsequent Poly I:C IL-6 production

Repeated exposure LPS induces a state of tolerance to subsequent exposure, known as endotoxin tolerance.²⁵ The tolerance phenomenon however is not thought to be unique to LPS and TLR4, but conserved across other TLRs.¹⁶ To examine if variations in TLR priming existed among endothelial cells and how these compared to known effects on macrophages, we first exposed HMVECs or HUVECs to Poly I:C, a TLR3 agonist, or LPS, then allowed the cells to remain undisturbed in fresh media. Afterward, cells were re-exposed to Poly I:C and cell supernatants were collected and examined for IL-6, indicative of MyD88/TNF receptor associated factor activation, and IP-10, as a surrogate of TRIF/interferon-regulatory factor (IRF) signaling.²⁶ As shown in Fig 1A (and Fig S1A), Poly I:C increased IL-6 production in both cells types and further, priming with Poly I:C potentiated IL-6 production when the cells were re-exposed to Poly I:C. LPS priming had little effect on Poly I:C-induced IL-6, though there were subtle differences where LPS priming induced a small inhibition of IL-6 secretion on Poly I:C rechallenge in HMVECs, whereas it enhanced IL-6 production in HUVECs. In comparison (Fig 1B), while Poly I:C induced significant secretion of IP-10 in both cells types, there was only evidence of tolerance when cells were primed with Poly I:C and rechallenged in HUVECs, and not HMVECs. Likewise, LPS priming was able to induce a slight tolerance to subsequent Poly I:C exposure in HMVECs but less so in HUVECs. Despite the minor differences between HMVECs and HUVECs, these data show that in general, Poly I:C priming strongly potentiates its own proinflammatory cytokine induction after rechallenge, whereas having little overall effect on modulation of interferon stimulation.

TLR2 priming and activation was differentially regulated among endothelial cell types

Though we noted subtle differences in Poly I:C priming and subsequent responses between HMVECs and HUVECs, the general responses were similar between the endothelial cell types. To examine if a conserved phenotype was observed via priming and subsequent re-exposure with TLR2 stimulation, HMVECs and HUVECs were primed with the TLR1/2 agonist, Pam3Csk4, or LPS, washed, and allowed to rest in fresh media and then rechallenged with Pam3Csk4 at the time intervals previously mentioned. As shown in Fig 2A, there were significant differences in the responses to priming and overall stimulation of TLR2 by Pam3Csk4 between HMVECs and HUVECs. HMVECs had a poor response to Pam3Csk4 during either the initial stimulation or rechallenge. In contrast, HUVECs had a comparatively robust response to Pam3Csk4. Similar to HUVECs undergoing Poly I:C challenge in the previous experiment, HUVECs primed with LPS and re-exposed to Pam3Csk4 had more IL-6 production than unprimed cells. In addition, cells primed with Pam3Csk4 produced more IL-6 when rechallenged with Pam3Csk4 compared to LPS or unprimed cells, suggesting a potentiation of Pam3Csk4 priming. We then turned our attention to IP-10 production. As seen with IL-6 secretion, HMVECs and HUVECs displayed different phenotypic responses to Pam3Csk4 (Fig 2B). Again, HMVECs lacked a discernable response to Pam3Csk4, either alone or after priming. However in HUVECs, Pam3Csk4 was able to induce a significant, albeit small, induction of IP-10. In addition, similar to IL-6, priming with either LPS or Pam3Csk4 induced progressive elevations in IP-10 secretion, suggesting a synergistic connection between MyD88 activation by either TLR4 or TLR2 and enhanced interferon production. Given that Pam3Csk4 acts as a TLR1/2 agonist, we tested the IL-6 signaling differences between HMVECs and HUVECs using a more pure TLR2 agonist, lipoteichoic acid (LTA). LTA continued to induce IL-6 production in HUVECs, whereas HMVECs remained hyporesponsive (Fig S2A). Interestingly, we detected higher TLR2 mRNA production in HMVECs compared to HUVECs, without noticeable differences between TLR2 total protein, suggesting either post-translational or TLR1 heterodimerization issues with TLR2 accounting for the differences between TLR2 responses in HMVECs and HUVECs (Fig S2B).

Pam3Csk4 induced tolerance while Poly I:C both tolerized and potentiated the response of TLR4 to LPS secondary challenge

Knowing that instances of viral or bacterial coinfections lead to poorer outcomes and that based on our previous data, both TLR3 and TLR2 have the ability to potentiate their subsequent responses, we wanted to explore what role cross tolerance or potentiation had on endothelial cells in the setting of endotoxin exposure. For these experiments, cells were primed with Pam3Csk4 or Poly I:C, as well as LPS, to study TLR cross-tolerance. As shown in Fig 3A (and Fig S1B), both HMVECs and HUVECs displayed endotoxin tolerance, as endothelial cells primed with LPS showed reduced IL-6 response to subsequent LPS challenge. Likewise, in both HMVECs and HUVECs, IL-6 production was reduced in Poly I:C-primed cells compared to unprimed cells. Surprisingly, in both HMVECs and HUVECs, the priming of Pam3Csk4 induced tolerance to subsequent LPS exposure, despite the lack of an observable inflammatory response to Pam3Csk4 to HMVEC in previous experiments. Next, we examined if a similar pattern of potentiation and tolerance was observed with IP-10 production. Based on previous studies, LPS causes limited production of IP-10, owing to

poor induction of the TRIF pathway after TLR4 stimulation in endothelial cells.¹⁹ However, as shown in Fig 3B (and Fig S1C), when both types of endothelial cells were primed with Poly I:C, there was a significant induction of IP-10 with a greater than 17-fold increased level compared to unprimed cells in HMVECs and greater than 7-fold in HUVECs. Moreover, priming by Pam3Csk4 reduced IP-10 production by LPS rechallenge compared to unprimed cells in HMVECs, though this effect was not as significant as LPS priming. This tolerance induced by Pam3Csk4 was not observed in HUVECs, again suggesting a variance in TLR2 signaling between HMVECs and HUVECs. To ensure that the enhanced IP-10 production by LPS after Poly I:C priming was not just the result of residual IP-10 production from the initial priming event, we examined IP-10 levels in cells that had been primed by Poly I:C but had no secondary stimulus, to those primed by Poly I:C and rechallenged with LPS. As shown in Fig 3C, Poly I:C priming continued to induce elevated IP-10 despite removal of the agonist for more than 24 hours. However, the re-exposure of the primed cells to LPS further enhanced IP-10 production, suggesting synergy between the priming and the secondary challenge.

Poly I:C priming strongly induced IRF7 and decreased RIP1 phosphorylation

Priming endothelial cells with Poly I:C and rechallenging with Poly I:C induced significant elevations in IL-6 production without significant alterations in IP-10. To further explore the mechanisms that could explain these findings, we examined protein expression and phosphorylation of several key elements of inflammatory signaling, including MAPK, nuclear factor kappa beta (NF- κ B), and IRF activity. For these experiments, we limited our examinations to only HMVECs, since they displayed a similar phenotype to HUVECs with regard to Poly I:C priming, as well as being a cell type more characteristic of inflammation-mediated endothelial dysfunction seen in patients.¹ HMVECs primed with Poly I:C had a notable upregulation of total IRF7 compared to LPS primed or unprimed cells (Fig 4). This upregulation of IRF7 was associated with increases in TLR3 expression. We detected no difference in total IRF3 protein in cells primed with Poly I:C or LPS. In addition, we were unable to detect any changes in phosphorylation of IRF3 or IRF7 compared to control in any of the primed or unprimed cells stimulated with Poly I:C (Fig S3A), suggesting that greater abundance of IRF7 contributed to the potentiation of Poly I:C priming. Next, we examined whether the enhanced IL-6 production in Poly I:C primed cells could be the result of enhanced MAPK or NF- κ B activation. We used phosphorylation of IKK, the upstream activator of NF- κ B, as our surrogate of NF- κ B activation. In addition, we examined the 3 arms of downstream MAPK activation: p38, JNK, and ERK 1/2. TLR3 has been postulated to activate these downstream pathways via activation of RIP1, which interacts with TNF receptor associated factor 6, providing a link between the MyD88 and TRIF arms of TLR signaling.²⁷ First, we examined the effect Poly I:C had in both the setting of priming and single challenge. Compared to unstimulated, Poly I:C induced significant phosphorylation of RIP1 under nonprimed conditions. However, when cells were primed with LPS or Poly I:C and then rechallenged with Poly I:C, the amount of relative phosphorylated RIP1 to total RIP1 was reduced significantly. Despite evidence suggesting that RIP1 leads to NF- κ B activation, we were unable to demonstrate any phosphorylation of IKK by 1 hour (Fig S4B). Similarly, in both primed and unprimed cells, Poly I:C did not have a measurable effect on the MAPKs p38 or JNK. Instead, we found that while Poly I:C did not enhance significant

phosphorylation of ERK 1/2 by itself, Poly I:C primed cells had enhanced p-ERK 1/2 in response to Poly I:C rechallenge, suggesting that Poly I:C priming did impact MAPK activation, albeit minimally at the time interval examined.

LPS-mediated interferon was associated with enhanced IRF7 upregulation by Poly I:C

Based on our previous data, demonstrating that priming by Poly I:C greatly enhanced LPS-mediated IP-10 production and that Poly I:C priming induced significant expression of IRF7, we sought to determine the mechanism of interferon production by LPS. In addition, we were interested in examining the proinflammatory and anti-inflammatory effects of Poly I:C priming on LPS signaling and the reduced inflammatory effect of Pam3Csk4 priming. We found as with previous experiments, the priming of Poly I:C induced significant enhancement of total IRF7, while neither LPS nor Pam3Csk4 priming had any notable effect on IRF7 production (Fig 5). In addition, none of the agonists induced any detectable changes in IRF3, neither TLR2 nor TLR4 (Fig S4). Next, we tested the effect of priming on RIP1 activity. Similar to previous experiments, LPS alone enhanced RIP1 phosphorylation compared to controls. Likewise, cells primed with either LPS or Poly I:C had less RIP1 phosphorylation compared to unstimulated cells or those cells stimulated with LPS alone. Interestingly, despite the reduced IL-6 production seen with Pam3Csk4 priming, LPS exposure after Pam3Csk4 priming induced the same amount of RIP1 phosphorylation as LPS alone, demonstrating that RIP1 modulation was unlikely to be involved in TLR2-mediated tolerance. Knowing that endotoxin tolerance is also associated with reductions in NF- κ B signaling, we examined phosphorylated and total IKK. LPS priming was associated with a significant reduction in total compared to unprimed. Interestingly, Poly I:C priming was similarly associated with a significant reduction of total IKK; however, it was also associated with a significant increase in phosphorylated IKK. As with RIP1, priming with Pam3Csk4 showed no significant alterations in IKK expression compared to unprimed cells challenged with LPS. Since our previous data suggested that Poly I:C priming enhanced ERK 1/2 signaling, we turned to the effect of priming MAPK activity after LPS rechallenge.

Previously, we were able to show that priming by Poly I:C enhanced ERK 1/2 activity on rechallenge, despite the absence of detectable ERK 1/2 phosphorylation by Poly I:C itself. As shown in Fig 6, Poly I:C priming enhanced subsequent ERK 1/2 phosphorylation to LPS exposure, similar to previous experiments. In addition, LPS priming also enhanced ERK 1/2 phosphorylation to LPS rechallenge, though to a lesser degree. This was again despite little phosphorylation of ERK 1/2 by LPS alone. When examining JNK activity, Poly I:C priming was able to enhance phosphorylation of JNK after LPS exposure; however, LPS priming was unable to significantly enhance phosphorylated JNK to LPS rechallenge compared to unprimed. Finally, phosphorylation of p38 was most strongly enhanced by LPS, though priming by Poly I:C or LPS enhanced the phosphorylation of p38 after LPS rechallenge only to a mild degree. Interestingly, we were unable to detect any significant changes in MAPK activity after Pam3Csk4 priming, suggesting the effects of Pam3Csk4 priming are independent of MAPK activity.

RIP1 and IRF7 inhibition negatively regulated TLR4 interferon induction after TLR3 priming

In both set of experiments where Poly I:C priming occurred, we observed 2 phenomena; a relative decrease in RIP1 phosphorylation and an enhancement in IRF7 production. To test if inhibition of either of these processes would alter the potentiating effect of TLR3 priming on TLR4 output, specifically with regards to interferon production, we primed cells with Poly I:C or control, washed the cells and then exposed them to LPS in the presence or absence of the RIP1 specific inhibitor, 7-Cl-O-Nec-1 (Nec-1s), or a nonspecific histone deacetylase inhibitor known to inhibit IRF7 activity, Trichostatin A (TSA). First, we tested Nec-1s and TSA in LPS-induced IP-10 production and detected a reduction in IP-10 with both inhibitors, with TSA reducing IP-10 more than Nec-1s (Fig S5A). Next, we tested these inhibitors in the presence of LPS after the cells had been primed by Poly I:C. As shown in Fig 7, we noted a subtle dose dependent reduction in IP-10 by both Nec-1s (dose range 1–3 μ M) and TSA (dose range 0.1–0.3 μ M), with an overall reduction in IP-10 at the higher dose tested by 48.6% and 61.6%, respectively. Testing higher doses of Nec-1s and TSA did not lead to any further significant reduction in IP-10 levels and in fact, higher doses of TSA offered no reduction in IP-10 compared to vehicle control, possibly due to off target effects of histone deacetylase inhibition (Fig S5B). These results confirm the contribution of RIP1 and IRF7 to the potentiated phenotype of TLR3 priming on TLR4 interferon induction and suggest a potential pharmacological approach to mitigate this potentiated response.

DISCUSSION

It has been postulated that cells of innate immunity develop tolerance in the setting of repeated TLR stimulation.^{16,17} While these observations have been demonstrated in murine macrophages, humans have been shown to have both similar and divergent mechanisms of TLR modulation, suggesting that the mechanisms involved in the tolerant phenotype may not be conserved across all the models.^{18,28} In these experiments, we show that in human endothelial cells, which contribute directly to innate immunity, priming by various TLR ligands can induce both tolerance and potentiation in the setting of secondary TLR challenges. These studies provide new insight into the complexity of TLR signaling and suggest that cross-modulation may lead to an impaired or enhanced inflammatory response depending on the stimuli.

Activation of TLRs leads to the activation of 2 main intracellular pathways: MyD88 and TRIF.²⁹ TLR3 has been shown to primarily induce inflammation via TRIF-mediated processes, whereas TLR2 induces inflammation in a MyD88-dependent manner.³ TLR4 is unique among the TLRs in that it has been shown to activate both MyD88 and TRIF-dependent inflammation.³ Owing to the conservation of two primary pathways in TLR-mediated inflammation, it has been demonstrated in mouse myeloid cells that priming with TRIF-biased ligands, such as Poly I:C, could prevent induction of interferon-inducible products, such as IP-10, when cells were restimulated with LPS.¹⁷ Likewise, priming with MyD88-biased ligands, such as Pam3Csk4, could impair production of proinflammatory cytokines after rechallenged with LPS.³⁰ These processes were postulated to be independent as MyD88 priming suppressed subsequent MyD88-mediated processes and TRIF priming suppressed TRIF responses.¹⁷ However, using human endothelial cells, we found greater

complexity in the TLR cross-modulation that was cell type specific. With regards to TLR3 signaling, priming with Poly I:C significantly enhanced IL-6 production upon Poly I:C rechallenge, without inducing any significant effects on IP-10 (Fig 1). Furthermore, we found that LPS priming had little effect on subsequent Poly I:C challenge. Turning our attention next to TLR2 signaling, we found vast differences in the ability of HUVECs and HMVECs to respond to the TLR2 ligand, Pam3Csk4 (Fig 2). HUVECs displayed a robust response to Pam3Csk4 that was not observed in HMVECs with either with priming or rechallenge. There are several potential reasons for the discrepancies in TLR output observed between HUVECs and HMVECs in our study, including differences in cell passage, donor gender, and timing of inflammatory activation.³¹⁻³³ However, the more likely explanation is the heterogeneity of endothelial subpopulations. In this regard, our results are consistent with previous data showing the heterogeneity of TLR2 responses across different endothelial subtypes and suggest regional differences in cellular responses to different TLR ligands.^{34,35}

Next, we primed cells with combinations of Poly I:C, Pam3Csk4, or LPS and rechallenged the cells with LPS (Fig 3). Priming with a TLR2 agonist induced a similar reduction in IL-6 production to those cells primed with a TLR4 agonist. On the other hand, TLR3 priming induced some time-dependent suppression, of IL-6 compared to LPS alone. The most interesting result however occurred when examining TLR3 priming of TLR4-mediated IP-10 production, where Poly I:C priming greatly enhanced the IP-10 production by LPS. These results run counter to previously published data looking at the interactions of repeated stimulation with TLR agonist.^{16,17} In those studies, it was demonstrated that Poly I:C priming inhibited production of IP-10 by LPS rechallenge.¹⁷ Though the reasons for the observed differences are likely multifactorial, the most obvious reasons for the differences are the species and the cell type studied. With regard to cell type, we have previously shown that in endothelial TLR4 signaling, the impact of MyD88 and TRIF pathways in the overall proinflammatory response appear different than those demonstrated in leukocytes.³⁶ Indeed, it is known that within both leukocytes and endothelial cell sub-populations, there is differential expression of TLRs, making it unlikely that responses are conserved across all cell types.^{20,37} Concerning the issue of species differences, the similarities and differences in TLRs, specifically TLR3 and TLR4, among human and other species has been well documented.³⁸⁻⁴⁰ Given the differences in cell type and species, it is unknown which of these is more important in determining the outcome of priming, but it is obvious from our data that priming in one cell type does not necessarily lead to the same outcome in another cell type. Examining intracellular mechanism responsible for these tolerant and potentiation effects was our next focus.

To determine how TLR3 priming via Poly I:C induced potentiation of subsequent TLR challenge, we examined several intracellular components of MyD88 and TRIF signaling. Poly I:C priming induced significant increases in IRF7 without any notable effects on IRF3 at the time point examined (Fig 4). In addition, Poly I:C induced RIP1 phosphorylation, which was reduced when cells were primed with Poly I:C. Despite a reduction in RIP1 phosphorylation, Poly I:C primed cells exhibited enhanced ERK 1/2 activity when rechallenged with Poly I:C. Similar observations were observed in Poly I:C-primed cells undergoing LPS rechallenge where Poly I:C priming enhanced IRF7 and ERK 1/2 levels as

well as both LPS and Poly I:C priming altering RIP1 activity during LPS rechallenge (Figs 5 and 6). These observations are notable for several reasons. One is the suggestion of dynamic and differential changes in proinflammatory signaling that exist between TLR3 and TLR4 where Poly I:C priming greatly enhanced IL-6 production upon Poly I:C rechallenge with a small, though significant increase in ERK 1/2, whereas challenge with LPS after Poly I:C induced large increases in ERK 1/2 phosphorylation with only an impaired increase in IL-6 output over time. The specific reasons for these observations are unclear, but it has been demonstrated in human macrophages that TLR4 activation induces quick proinflammatory output, whereas TLR3-mediated inflammation displayed delayed kinetics.⁴¹ With regard to ERK 1/2 specifically, it has been reported that both proinflammatory and anti-inflammatory cytokines can induce a similar impact on ERK 1/2 depending on the cell type.⁴² Similarly, ERK 1/2 has been thought to be an important mediator of cytokine signaling between MyD88 and TRIF responses in a time-dependent manner.⁴³ Thus, it is certainly possible that different TLR ligands induce distinctive kinetics on intracellular signals that in turn, lead to unique inflammatory outputs. This would certainly agree with our observation that while both LPS and Poly I:C priming reduced RIP1 phosphorylation to near equal degrees, the inflammatory phenotypes of Poly I:C and LPS are nearly opposite, suggesting that RIP1 has differential effects on TLR3 and TLR4-mediated inflammation.

The second important observation is the strong and persistent upregulation of IRF7 after Poly I:C priming. Production of interferon-dependent chemokines, such as IP-10, is known to be regulated by several different interferon regulatory factors. However, among the IRFs, IRF7 is thought to be least important as far as IP10 production.⁴⁴ With regards to endothelial cells, interferon-related chemokine production after LPS is weak compared to leukocytes. However, our data suggest that induction of IRFs, particularly IRF7, after Poly I:C priming allows for subsequent challenges via LPS to induce significant amounts of interferon-related chemokines. We should note however that observations are based only on total protein production as we were unable to detect any changes in IRF3 or 7 phosphorylation. This is possibly due to the delayed kinetics of IRF phosphorylation, though some studies have also suggested quick phosphorylation and dephosphorylation of IRF7.^{45,46} Thus, we are unable to definitively determine the role of IRF3, or other IRFs, have in Poly I:C priming amplification of LPS-mediated IP-10 production, but there does appear to be a link that is opposite of that observed in murine macrophages.¹⁷ The implication of TLR3-mediated amplification of TLR4-mediated interferon production is important in that one of the postulated mechanisms of bacterial superinfection after virus is via enhanced interferon production that impairs macrophage function and bacterial clearance.¹⁰ Human dendritic cells have been shown to display a similar phenotype with enhanced cytokine production after bacterial infection when primed with a virus and thus the enhanced interferon production in bacterial superinfection is not likely due solely to endothelial cells, but they clearly are an important source of interferon in viral-primed hosts.⁴⁷ To examine if we could disassociate TLR3- and TLR4-mediated processes, we focused on 2 proteins that appeared to be implicated in TLR3 and TLR4 interferon and inflammatory signaling, IRF7 and RIP1.

TLR signaling primarily occurs through either the MyD88 or TRIF pathway.²⁹ Though these pathways are distinct, there are links between the two. In these experiments, we observed alterations in 2 proteins, RIP1 and IRF7, which both have been implicated in crosslinking of

the proinflammatory and interferon-producing arm of TLR signaling.^{48,49} We employed 2 pharmacological inhibitors undergoing clinical evaluation, 7-Cl-O-Nec-1 (Nec-1s) and TSA. Nec-1s has been shown to downregulate RIP1 activity and inhibits its inflammatory effects.²³ TSA, a histone deacetylase inhibitor, is known to inhibit IRF7 activity.⁵⁰ Though histone deacetylase inhibitors are typically nonspecific regulators of gene transcription, TSA has been shown to preferentially alter IRF7 transcription compared to IRF3.²⁴ Both Nec-1s and TSA caused a dose-dependent decrease in IP-10 production in cells primed with Poly I:C and subsequently challenged with LPS (Fig 7). These results confirm a role of both RIP1 and IRF7 in the enhanced interferon production to TLR4 signaling through TLR3 priming. Interestingly, both Nec-1s and TSA appeared to have similar effects on IP-10 after priming, suggesting a possible synergy between the proteins. Though we did not test this hypothesis directly, it has been suggested that RIP1 and IRF7 do directly bind to each other and that RIP1 is required for IRF7 activity.^{51,52} Given this link, it is possible that TLR3-mediated RIP1 activation drives IRF7 production and this upregulation in turn, drives TLR4-mediated interferon production. In addition, our data suggest that this process continues long after the initial TLR3 stimulation, as Nec-1s and TSA reduced IP-10 production by TLR4 at greater than 32 hours after the Poly I:C removal. The prolonged upregulation of IRF7 may provide opportunities to pharmacologically prevent late interferon activation that could be detrimental to clearance of bacteria coinfections.

In summary, our data demonstrate a complex web of TLR cross tolerance and potentiation in human endothelial cells. Interestingly, we found results that differ dramatically from those previously published in macrophages, specifically in relation to TLR3 priming by Poly I:C and subsequent TLR responses. Though the reasons for the differences are not clear, effects of cell type (endothelial vs macrophage) and species (human vs mouse) may account for the observations. In addition, we demonstrated that by priming TLR3 through viral mimicry using Poly I:C, interferon production by TLR4 stimulation through LPS is enhanced in a RIP1- and IRF7-dependent manner. The importance of this lies in the clinical observation that viruses can enhance bacterial coinfection. These data may suggest novel strategies to reduce the consequences of bacterial coinfection after viral challenge.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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All authors have read the journal's authorship agreement and have made the following contributions: S. R. Koch and R. J. Stark designed and performed the experiments; S. R. Koch, F. S. Lamb, J. Hellman, E. R. Sherwood, and R. J. Stark analyzed the data, wrote, and revised the manuscript.

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AT A GLANCE COMMENTARY

Koch SR, et al.

Background

Human endothelial cells are a key component of the innate immune system that control vascular tone and leukocyte trafficking. Their activation by toll-like receptors (TLRs) during infectious challenge can alter their response to rechallenge, a clinical scenario observed during viral or bacterial coinfections.

Translational Significance

Stimulation of human endothelial cell TLR3 potentiated TLR4-inflammatory processes, a finding that is opposite of that reported in murine macrophages. In addition, using pharmacological inhibitors undergoing clinical evaluation, we attenuated TLR3-induced potentiation of TLR4 interferon production. This increased interferon production after TLR3 stimulation is one of the postulated mechanisms behind enhanced susceptibility to bacterial superinfection after viral exposure.

Fig 1.

Poly I:C priming potentiated IL-6, but not IP-10, upon Poly I:C rechallenge. HMVECs (left) or HUVECs (right) were primed with the TLR3 agonist, Poly I:C (10 μ g/ml), the TLR4 agonist, LPS (100 ng/ml), or medium (control) for 16 hours and then exposed to fresh media for 24 hours (HUVECs) or 32 hours (HMVECs). Afterward, cells were exposed to Poly I:C (10 μ g/ml) for 6 hours (HUVECs) or 16 hours (HMVECs). Culture supernatants were collected at the completion of the experiments and examined for IL-6 (**A**) or IP-10 (**B**) by ELISA. Data are expressed as means $\pm$ standard error of replicated, individual experiments ($n = 4$). * indicates $P < 0.05$, ** indicates $P < 0.01$, *** indicates $P < 0.001$ compared to unprimed exposed to Poly I:C; † indicates $P < 0.05$, NS indicates nonsignificant between designated groups, as determined by ANOVA. Dashed line represents baseline cytokine levels in the absence of stimulus. LPS, lipopolysaccharide; TLR, toll-like receptor; HUVECs, human umbilical vein endothelial cells.

Fig 2.

The effect of Pam3Csk4 is differentially regulated between HMVECs and HUVECs. HMVECs (left) or HUVECs (right) were primed with the TLR2 agonist, Pam3Csk4 (10 μ g/ml), LPS (100 ng/ml), or medium (control) for 16 hours and then exposed to fresh media for 24 hours (HUVECs) or 32 hours (HMVECs). Afterward, cells were exposed to Pam3Csk4 (10 μ g/ml) for 6 hours (HUVECs) or 16 hours (HMVECs). Culture supernatants were collected at the completion of the experiments and examined for IL-6 (**A**) or IP-10 (**B**) by ELISA. Data are expressed as means $\pm$ standard error of replicated, individual experiments ($n = 4$). * indicates $P < 0.05$, ** indicates $P < 0.01$, *** indicates $P < 0.001$ compared to unprimed exposed to Pam3Csk4; †† indicates $P < 0.01$, NS indicates nonsignificant between designated groups, between designated groups as determined by ANOVA. Dashed line represents baseline cytokine levels in the absence of stimulus. LPS, lipopolysaccharide; HUVECs, human umbilical vein endothelial cells.

Fig 3.

Pam3Csk4 and LPS priming induced tolerance, while Poly I:C potentiated. HMVECs (left) or HUVECs (right) were primed with the Poly I:C (10 μ g/ml), Pam3Csk4 (10 μ g/ml), LPS (100 ng/ml), or medium (control) for 16 hours and then exposed to fresh media for 24 hours (HUVECs) or 32 hours (HMVECs). Afterward, cells were exposed to LPS (100 ng/ml) for 6 hours (HUVECs) or 16 hours (HMVECs). Culture supernatants were collected at the completion of the experiments and examined for IL-6 (**A**) or IP-10 (**B**) by ELISA. (**C**) HMVECs (left) and HUVECs (right) were exposed to Poly I:C (10 μ g/ml) or medium (control) for 16 hours, washed in fresh media for 24 hours and then exposed to LPS (100 ng/ml) or medium (control). Supernatants were collected at the end of the 16 hours and examined for IP-10 via ELISA. Data are expressed as means $\pm$ standard error of replicated, individual experiments ($n = 4$). * indicates $P < 0.05$, ** indicates $P < 0.01$, *** indicates $P < 0.001$ compared to unprimed exposed to LPS, ††† indicates $P < 0.001$ between designated groups, as determined by ANOVA. Dashed line represents baseline cytokine levels in the absence of stimulus. LPS, lipopolysaccharide; HUVECs, human umbilical vein endothelial cells.

Fig 4.

Poly I:C priming enhanced IRF7 and ERK 1/2, while attenuating RIP1. HMVECs were primed with Poly I:C (10 $\mu\text{g/ml}$), LPS (100 ng/ml), or medium (control) for 16 hours and then exposed to fresh media for 32 hours. Afterward, cells were exposed to Poly I:C (10 $\mu\text{g/ml}$) for 1 hour. Cell lysates were collected and run for Western blot analysis. Blots were probed for total and phosphorylated proteins. (A) Representative images showing phosphorylated (p-RIP1, p-ERK 1/2) and total proteins (IRF3, IRF7, TLR3, RIP1, ERK 1/2, and tubulin). (B) Bar graphs showing the relative abundance of phosphorylated or total proteins after normalization to respective total protein or tubulin expression. Data are expressed as means $\pm$ standard error of individual experiments ($n = 4$) after normalization to control quantities. * indicates $P < 0.05$, *** indicates $P < 0.001$ compared to control $\rightarrow$ Poly I:C group, NS indicates nonsignificant between designated groups, as determined by ANOVA. IRF7, interferon regulatory factor 7; ERK1/2, extracellular signal-regulated kinase 1/2; LPS, lipopolysaccharide; RIP1, receptor-interacting serine/threonine-protein kinase 1.

Fig 5.

IRF7 increased while RIP1 decreased in LPS-treated cells primed with Poly I:C. HMVEC cells were primed with Poly I:C (10 μ g/ml), Pam3Csk4 (10 μ g/ml), LPS (100 ng/ml), or medium (control) for 16 hours and then exposed to fresh media for 32 hours. Afterward, cells were exposed to LPS (100 ng/ml) for 1 hour. Cell lysates were collected and run for Western blot analysis. Blots were probed for total and phosphorylated proteins. (A) Representative images showing phosphorylated (p-RIP1, p-IKK) and total proteins (IRF7, RIP1, IKK, and tubulin). (B) Bar graphs showing the relative abundance of phosphorylated or total proteins after normalization to respective total protein or tubulin expression. Data are expressed as means $\pm$ standard error of individual experiments ($n = 4$) after normalization to control quantities. * indicates $P < 0.05$, ** indicates $P < 0.01$, *** indicates $P < 0.001$ compared to control $\rightarrow$ LPS. † indicates $P < 0.05$, †† indicates $P < 0.01$, ††† indicates $P < 0.001$ between designated groups, as determined by ANOVA. IRF7, interferon regulatory factor 7; LPS, lipopolysaccharide; RIP1, receptor-interacting serine/threonine-protein kinase 1; IKK, I κ B kinase.

Fig 6.

MAPKs are increased in Poly I:C primed cells challenged with LPS. HMVEC cells were primed with Poly I:C (10 μ g/ml), Pam3Csk4 (10 μ g/ml), LPS (100 ng/ml), or medium (control) for 16 hours and then exposed to fresh media for 32 hours. Afterward, cells were exposed to LPS (100 ng/ml) for 1 hour. Cell lysates were collected and run for Western blot analysis. Blots were probed for total and phosphorylated proteins. (A) Representative images showing phosphorylated (p-p38, p-JNK, p-ERK 1/2) and total proteins (p38, JNK, ERK 1/2). (B) Bar graphs showing the relative abundance of phosphorylated after normalization to respective total protein. Data are expressed as means $\pm$ standard error of individual experiments (n = 4) after normalization to control quantities. * indicates $P < 0.05$, ** indicates $P < 0.01$, *** indicates $P < 0.001$ compared to control $\rightarrow$ LPS. † indicates $P < 0.05$, †† indicates $P < 0.01$, ††† indicates $P < 0.001$ between designated groups group as determined by ANOVA. ERK1/2, extracellular signal-regulated kinase 1/2; LPS, lipopolysaccharide; JNK, c-Jun amino-terminal kinase.

Fig 7.

RIP1 and IRF7 inhibition decreased IP-10 after Poly I:C priming. HMVEC were primed with the Poly I:C (10 $\mu\text{g/ml}$) for 16 hours and then exposed to fresh media for 32 hours. Afterward, cells were exposed to LPS (100 ng/ml) for 16 hours in the presence of medium with <0.1% DMSO (control), 7-Cl-O-Nec1 (RIP1-specific inhibitor, Nec-1s, dose range 1 to 3 μM) or trichostatin A (IRF7 nonspecific inhibitor, TSA, dose range 0.1 to 0.3 μM). Culture supernatants were collected at the completion of the experiments and examined for IP-10 by ELISA. Data are expressed as means $\pm$ standard error of replicated, individual experiments ($n = 4$). * indicates $P < 0.05$, ** indicates $P < 0.01$, compared to Poly I:C $\rightarrow$ LPS vehicle controls, NS indicates nonsignificant between designated groups, as determined by ANOVA. IRF7, interferon regulatory factor 7; LPS, lipopolysaccharide; RIP1, receptor-interacting serine/threonine-protein kinase 1.