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MyD88-Dependent and MyD88-Independent Pathways in Synergy, Priming, and Tolerance between TLR Agonists

Aranya Bagchi,* Elizabeth A. Herrup,† H. Shaw Warren,‡ James Trigilio,* Hae-Sook Shin,* Catherine Valentine,§ and Judith Hellman2§

TLRs sense components of microorganisms and are critical host mediators of inflammation during infection. Different TLR agonists can profoundly alter inflammatory effects of one another, and studies suggest that the sequence of exposure to TLR agonists may importantly impact on responses during infection. We tested the hypothesis that synergy, priming, and tolerance between TLR agonists follow a pattern that can be predicted based on differential engagement of the MyD88-dependent (D) and the MyD88-independent (I) intracellular signaling pathways. Inflammatory effects of combinations of D and I pathway agonists were quantified in vivo and in vitro. Experiments used several D-specific agonists, an I-specific agonist (poly(1:C)), and LPS, which acts through both the D and I pathways. D-specific agonists included: peptidoglycan-associated lipoprotein, Pam3Cys, flagellin, and CpG DNA, which act through TLR2 (peptidoglycan-associated lipoprotein and Pam3Cys), TLR5, and TLR9, respectively. D and I agonists were markedly synergistic in inducing cytokine production in vivo in mice. All of the D-specific agonists were synergistic with poly(I:C) in vitro in inducing TNF and IL-6 production by mouse bone marrow-derived macrophages. Pretreatment of bone marrow-derived macrophages with poly(I:C) led to a primed response to subsequent D-specific agonists and vice versa, as indicated by increased cytokine production, and increased NF-κB translocation. Pretreatment with a D-specific agonist augmented LPS-induced IFN-β production. All D-specific agonists induced tolerance to one another. Thus, under the conditions studied here, simultaneous and sequential activation of both the D and I pathways causes synergy and priming, respectively, and tolerance is induced by agonists that act through the same pathway. The Journal of Immunology, 2007, 178: 1164–1171.

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3 Abbreviations used in this paper: PAL, peptidoglycan-associated lipoprotein; BMDM, bone marrow-derived macrophage; D, MyD88 dependent; I, MyD88 independent; K0, knockout.

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The proximal D pathway is shown in black with white writing, and the I pathway is shown in white with black writing. Because TLR4 has effects on both the D and I pathways, the receptor is shown in both black and white. Putative connections from the I to the D pathways are shown using the curved arrow. Shared distal portions of the pathway are depicted in light gray with black writing to indicate that both the D and I pathways induce inflammatory cytokines through NF-kB.

production via the I pathway (31), additional pathways have also been identified that mediate induction of IFN-β by dsRNA, but do not use TLR3 (35). Fig. 1 depicts a simplified overview of TLR signaling via the D and I pathways, and includes the specific TLRs and their respective agonists that were used for the present studies. Details of specific intermediaries and pathway inhibitors are not included in Fig. 1. The D and I pathways share some distal intermediaries, such as TNFR-associated factor 6 (36) and NF-kB.

To date, no clear explanation has been advanced to explain how synergy, priming, and tolerance between TLR agonists occur. We tested the hypotheses that the split and the downstream interactions between the D and I intracellular TLR-signaling pathways are responsible for these distinct phenomena, and that patterns of inflammatory responses induced by combinations of TLR agonists can be predicted based on knowledge of the intracellular signaling pathways used by different TLR agonists. We characterized in vitro and in vivo inflammatory effects of a panel of naturally occurring and synthetic microbial TLR agonists on one another. In this study, we attempt to provide a simple framework to explain and to predict the patterns of inflammatory responses to combinations of TLR agonists based on differential engagement of the D and the I pathways.

Materials and Methods

Reagents

*Escherichia coli* O111:B4 LPS was purchased (List Biological Laboratories). Two methods were used to confirm that the LPS was free of non-TLR4 agonists, as follows: 1) the LPS was determined to be free of protein using gold stains and immunoblots for PAL and murine lipoprotein; and 2) the LPS did not activate TNF production by bone marrow-derived macrophages (BMDM) from LPS-hyporesponsive (C3H/HeJ) mice. PAL was the LPS did not activate TNF production by bone marrow-derived macrophages and immunoblots for PAL and murein lipoprotein; and 2) TLR4 agonists, as follows: 1) the LPS was determined to be free of protein O111:B4 LPS was purchased (List Biological Laboratories) from *Escherichia coli* K12 CH202(pRC2), as described (37), and contained 0.5 pg of LPS/μg protein, based on the *Limulus* amebocyte lysate assay (Associates of Cape Cod) (38). The following TLR agonists and controls were purchased: Pam3Cys, a synthetic lipopeptide TLR2 agonist (EMC Microcollections); poly(I:C), a synthetic TLR3 agonist that resembles viral RNA (InvivoGen); mouse-specific stimulatory CpG DNA, a bacterial TLR9 agonist, and its control nonsense oligonucleotide (InvivoGen); and flagellin, a bacterial TLR5 agonist (Inotek Pharmaceuticals). Based on the *Limulus* amebocyte lysate assay, Pam3Cys and CpG DNA were LPS free, but flagellin and poly(I:C) contained appreciable amounts of LPS (1 and 2 ng of LPS/μg, respectively). Mouse rIFN-β was purchased (PBL Biomedical Laboratory).

**Mice**

The Institutional Animal Care and Use Committee at the Massachusetts General Hospital approved the animal studies. The mice used were C57BL/6 (Charles River Laboratories), C3H/HeJ (The Jackson Laboratory), and MyD88 knockout (KO) on a C57BL/6 background (a gift from W. Chao, Massachusetts General Hospital, Charlestown, MA). C3H/HeJ mice contain a mutation in the TLR4 gene that makes them hyporesponsive to LPS (2).

Preparation of mouse macrophages

BMDM were prepared from mice, as described (39). Briefly, stem cells were flushed from femurs and placed in Teflon bags (BioFOLIE; Sartorius). The cells were incubated in a differential medium for 7 days to allow for proliferation and differentiation, and were harvested. BMDM were seeded at a density of 4 × 10⁶ cells/cm² in 48- or 96-well plates for cytokine assays, and in T-25 flasks for EMSAs and immunoblots. BMDM were allowed to adhere for 24–48 h before use in assays. Cells were incubated at 37°C under humidified 5% CO₂.

Preparation of nuclear and cytoplasmic extracts for EMSAs and immunoblots

BMDM were washed twice with cold PBS, and the nuclear and cytoplasmic extracts were prepared using a commercially available extraction kit (PANomics), per the manufacturer’s instructions. The extracts were stored at −80°C until use.

**EMSA**

The protein content of the nuclear extracts was quantified by the Bradford method. A total of 5 μg of the extracts was incubated with a biotin-labeled NF-kB probe (PANomics) and was then subjected to electrophoresis on a 6% nondenaturing polyacrylamide gel. Control samples were also incubated with an excess of unlabeled NF-kB probe to confirm specificity of binding. The gel contents were then transferred to a nylon membrane (Bio-dyne B; Pall) and developed using a chemiluminescence system, as per the manufacturer’s instructions (PANomics EMSA Gel-Shift Kit), and exposed to film (Hyperfilm; Amersham Biosciences).

**Immunoblots for IkB-α**

The protein content of cytoplasmic extracts was quantified by the Bradford method, and the samples were run on 12% SDS-PAGE gels (25 μg of protein per lane). The proteins were transferred onto nitrocellulose membranes and were sequentially incubated with the following Abs (all from Santa Cruz Biotechnology): 1) mouse monoclonal anti-IκB-α (H-4; 1 μg/ml) or mouse monoclonal anti-actin (C-2; 1 μg/ml) as a loading control; and 2) HRP-conjugated goat anti-mouse IgG (1/10,000 dilution). The blots were developed using a chemiluminescence system (PerkinElmer).

**In vivo synergy experiments**

In separate experiments, 8-wk-old female mice (C57BL/6 and C3H/HeJ) were injected i.v. via tail veins with either of the following: 1) PAL, LPS, or a combination of PAL and LPS; 2) Pam3Cys, poly(I:C), or a combination of Pam3Cys and poly(I:C); or 3) Pam3Cys, CpG DNA, or a combination of Pam3Cys and CpG DNA. Control mice received an i.v. injection of the carrier for the TLR agonists (50 mM sodium phosphate (pH 7.4) or water). Injection volumes were 250 μl/mouse. Doses of TLR agonists are indicated in the figures. The mice were euthanized 2 h after injection, blood was collected by cardiac puncture, plasmas were prepared, and plasma TNF and IL-6 levels were quantified by ELISA (R&D Systems). Synergy was defined as a more than additive response to two simultaneously administered TLR agonists than would be predicted by adding the cytokine levels in the plasmas of mice injected with the individual components.

**In vitro synergy experiments**

BMDM from C3H/HeJ mice were incubated for 20 h with individual TLR agonists or with combinations of two TLR agonists. TNF and IL-6 levels were quantified in the culture supernatants by ELISA (R&D Systems). Because of significant LPS contamination of the poly(I:C) and flagellin preparations, BMDM from LPS-hyporesponsive (C3H/HeJ) mice were used for experiments involving these TLR agonists. A nonsense oligonucleotide was used to control for nonspecific stimulatory effects of CpG DNA.

**FIGURE 1.** Overview of the TLRs and TLR agonists used for these studies, and corresponding I and D pathways. The proximal D pathway is shown in black with white writing, and the I pathway is shown in white with black writing. Because TLR4 has effects on both the D and I pathways, the receptor is shown in both black and white. Putative connections from the I to the D pathways are shown using the curved arrow. Shared distal portions of the pathway are depicted in light gray with black writing to indicate that both the D and I pathways induce inflammatory cytokines through NF-kB.
To explore the role of IFN-β in synergy, C3H/HeJ BMDM were incubated with IFN-β (150 and 1000 U/ml), poly(I:C), PAL, flagellin, or CpG DNA alone and in various combinations, and TNF levels were quantified in the culture supernatants.

**In vitro priming/tolerance experiments**

BMDM from C57BL/6 and C3H/HeJ mice were incubated for 20 h with medium or a TLR agonist (agonist 1). Supernatants were then removed, and cells were washed with medium. The cells were then incubated with medium or a TLR agonist, which was either the same or a different agonist (agonist 2). The duration of treatment with agonist 2 varied depending on the method of analysis: cytokine levels were quantified in culture supernatants 20 h, whereas nuclear translocation of NF-κB and activation of IkB were assessed by EMSAs and immunoblots, respectively, after 30 min. Concentrations of TLR agonists are indicated in the figure legends. In some experiments, two concentrations of LPS were tested (100 ng/ml and 1 μg/ml).

To assess priming of non-TLR-mediated NF-κB pathways, C3H/HeJ BMDM were treated with Pam3Cys or poly(I:C) as agonist 1, and then with mouse TNF (100 ng/ml; PeproTech) as agonist 2. IL-6 levels were quantified in the culture supernatants.

**Experiments using BMDM from MyD88 KO mice**

In vitro studies were also performed using BMDM from homozygous MyD88 KO mice. For studies of synergy, BMDM were treated for 20 h with Pam3Cys alone, poly(I:C) alone, or a combination of Pam3Cys and poly(I:C). In priming/tolerance experiments, BMDM were sequentially treated with medium, Pam3Cys, or poly(I:C), as indicated in the figure. TNF levels were quantified in the culture supernatants.

**Assessment of IFN-β production**

BMDM were sequentially treated with PAL and poly(I:C) (C3H/HeJ), or Pam3Cys and LPS (C57BL/6 and MyD88 KO), using the procedure outlined above for the priming and tolerance studies. IFN-β was quantified in the culture supernatants by ELISA (40) after treatment with agonist 2.

**Statistics**

Representative data from at least three experiments are presented in the figures. Data are expressed as means, and error bars represent SEM. The data were analyzed by one-way ANOVA with Bonferroni’s post hoc tests. Values of *p* < 0.05 were considered to be statistically significant. The following notations have been used to denote *p* values in the figures: *, *p* < 0.05; **, *p* ≤ 0.01; ***, *p* ≤ 0.001. For in vivo synergy studies, *p* values were calculated based on comparisons of plasma cytokine levels of mice treated with the combination of two agonists vs the levels that would be predicted if effects were additive, as defined by the sum of cytokine levels in plasmas of mice treated with the individual agonists. For in vitro synergy studies, *p* values were calculated based on comparisons of cytokine levels in supernatants of BMDM treated with the combination of two agonists vs the levels that would be predicted if effects were additive.

**Results**

Patterns of TLR agonist interactions are defined, as follows: 1) synergy: a supra-additive inflammatory response to simultaneous exposure to two TLR agonists; 2) tolerance: a reduction in inflammatory responses to a TLR agonist by cells that have been pretreated with a TLR agonist; 3) priming: an increase in inflammatory responses to a TLR agonist by cells that have been pretreated with a TLR agonist.

**D and I agonists are synergistic in vivo**

Mice were injected with the following: 1) PAL and LPS; 2) Pam3Cys and poly(I:C); and 3) Pam3Cys and CpG DNA at the doses indicated in Fig. 2. Plasmas from mice that were treated with a combination of the D-specific agonist, PAL, and the mixed D and I agonist, LPS, contained higher levels of IL-6, TNF, and MIP-1α vs mice that were treated with the individual components, or than would have been predicted for an additive effect, indicating that PAL and LPS are synergistic (Fig. 2a; *p* < 0.001). Similarly, a combination of D- and I-specific agonists (Pam3Cys and poly(I:C)) synergistically induced cytokine production (Fig. 2b; *p* < 0.001). However, the combination of two D-specific agonists, Pam3Cys and CpG DNA, was not synergistic (Fig. 2c; *p*: NS; shown for TNF).
IFN-α obtained with flagellin and CpG DNA, and using a higher dose of poly(I:C) on activation of BMDM by PAL. Similar results were shown with combinations of poly(I:C) (PIC, 25 μg/ml), or flagellin (FLG, 2.5 μg/ml), or CpG DNA (Cpg, 50 nM), or with the combination of PAL and CpG DNA (far right). n = 3 replicates per condition. PAL and flagellin were also not synergistic (data not shown). Similar results were obtained for IL-6 (data not shown). α: IFN-β does not contribute to the synergy between D- and I-specific agonists. TNF levels in supernatants of macrophages from C3H/HeJ mice that were simultaneously treated with PAL (1 μg/ml) and poly(I:C) (25 μg/ml), or PAL and IFN-β (150 U/ml). n = 3 replicates per condition. Similar results were obtained using flagellin and CpG DNA instead of PAL to treat the BMDM, and using IL-6 as the readout (data not shown).

D- and I-specific agonists are synergistic in vitro

Simultaneous exposure of C3H/HeJ macrophages to poly(I:C) (an I-specific agonist) and either PAL, flagellin, or CpG DNA (D-specific agonists) caused synergistic production of TNF (p < 0.001; Fig. 3a). The nonsense oligonucleotide (CpG DNA control) did not activate the macrophages or alter macrophage responses to poly(I:C) (data not shown).

The combination of two D-specific agonists is not synergistic

Unlike the combination of D and I pathway agonists, there was no synergy between the following: 1) PAL and CpG DNA; 2) CpG DNA and flagellin; or 3) PAL and flagellin (Fig. 3a, far right, shown for PAL and CpG DNA only).

IFN-β is not responsible for synergy between D and I agonists

To test the hypothesis that IFN-β, a major product of the I pathway, could be involved in synergy between poly(I:C) and the D agonists, we compared TNF production by BMDM incubated with the combination of the D specific agonists and either IFN-β or poly(I:C). There was not synergy between IFN-β and any of the D-specific agonists. Fig. 3b shows the effect of IFN-β (150 U/ml) vs poly(I:C) on activation of BMDM by PAL. Similar results were obtained with flagellin and CpG DNA, and using a higher dose of IFN-β (1000 U/ml; data not shown).

Sequential exposure of macrophages to PAL and LPS induces tolerance or priming to the second agonist depending on the order of exposure

C57BL/6 BMDM were incubated with PAL or LPS (agonist 1) for 20 h, and then with PAL or LPS (agonist 2) for 20 h, and TNF was measured in the culture supernatants after incubation with agonist 2. PAL and LPS each induced self-tolerance, as indicated by reduced TNF production when macrophages were treated with PAL followed by PAL, or LPS followed by LPS, as compared with macrophages that were treated with medium followed by PAL or LPS (Fig. 4a; p < 0.05). Pretreatment with LPS led to reduced TNF production with subsequent treatment with PAL vs treatment with medium followed by PAL (Fig. 4a; p < 0.001). This indicates that exposure to LPS tolerizes cells to PAL. Conversely, pretreatment with PAL caused an augmented response to a subsequent treatment with LPS (Fig. 4a; p < 0.001), indicating that PAL primes BMDM to LPS. A similar pattern of priming occurred when Pam3Cys or the TLR9 agonist, CpG DNA, was substituted for PAL (p < 0.01; data not shown). An identical pattern of tolerance/priming between LPS and D-specific agonists was observed using LPS concentrations of 100 ng/ml and 1 mcg/ml (shown for 100 ng/ml).

Sequential exposure of cells to a D-specific and an I-specific agonist results in augmented inflammatory responses to the second agonist (cross-priming)

To further explore the role of the D and I pathways in priming and tolerance, we sequentially exposed C3H/HeJ BMDM to D- and I-specific agonists for 20 h each, and measured TNF levels in the supernatants after incubation with agonist 2 (Fig. 4b). Sequential exposure to poly(I:C) and any D-specific agonist (PAL, CpG DNA, or flagellin) resulted in augmented responses to the second agonist (p < 0.05). The same results were obtained using Pam3Cys and poly(I:C) (p < 0.01), and IL-6 levels followed the same pattern as TNF (data not shown).

D-specific agonists induce cross-tolerance

To test for cross-tolerance between different D-specific agonists, BMDM from C3H/HeJ mice were sequentially treated with two different D-specific agonists. TNF production was markedly reduced when cells were sequentially treated with any of the D-specific agonists (Fig. 4c; p < 0.01).

D and I agonists prime macrophages to TNF

To assess whether TLR agonists also modulated non-TLR NF-κB-mediated pathways, C3H/HeJ BMDM were sequentially treated with Pam3Cys or poly(I:C), followed by TNF. IL-6 levels were quantified after treatment with TNF. Both the Pam3Cys and poly(I:C) led to a primed subsequent response to TNF (Fig. 4d).

Effects of sequential treatment with TLR agonists on NF-κB translocation and IκB activation

To probe the pathways involved in priming and tolerance, we assessed the effects of sequential exposure to TLR agonists on cytoplasmic IκB-α levels by immunoblotting, and on nuclear translocation of NF-κB by EMSAs. BMDM were incubated with agonist 1 for 20 h, and then with agonist 2 for 30 min. Cells that were treated with Pam3Cys followed by poly(I:C), and vice versa, showed a combination of increased nuclear NF-κB translocation (Fig. 5a; data not shown for poly(I:C), followed by Pam3Cys) and reduced cytoplasmic IκB-α, indicating IκB activation (Fig. 5b). Conversely, cells that were treated sequentially with poly(I:C) followed by poly(I:C) or with Pam3Cys followed by Pam3Cys showed reduced nuclear NF-κB translocation (Fig. 5a; data not shown).
shown for poly(I:C) followed by Pam3Cys) and increased IκB-α (Fig. 5b), as compared with cells that were treated with medium followed by poly(I:C) or Pam3Cys. Control nuclear extracts that were incubated with the unlabeled probe did not show any shift, confirming the specificity of the binding (data not shown).

**FIGURE 4.** Priming and tolerance between D and I pathways. a–c, TNF levels in the supernatants of BMDM that were sequentially incubated for 20 h each with agonists 1 and 2. For all conditions of agonist 1, TNF was not detected when medium was used as agonist 2, indicating that there was no residual TNF production induced by agonist 1 that persisted during treatment with agonist 2. For all panels, n = 3 replicates per condition, and p values are compared with baseline levels for each TLR agonist, as established by incubating cells with medium as agonist 1, followed by the TLR agonist as agonist 2. The concentrations of the agonists were as follows: PAL, 1 μg/ml; Pam3Cys, 1 μg/ml; CpG DNA, 50 nM; flagellin, 2.5 μg/ml; LPS, 100 ng/ml and 1 μg/ml; and poly(I:C), 25 μg/ml. a, LPS tolerizes to PAL; PAL primes to LPS. Macrophages from C57BL/6 mice were incubated sequentially with PAL and LPS, and vice versa. Similar results were obtained for IL-6 (data not shown), and for both concentrations of LPS (shown for 100 ng/ml). b, Priming between D- and I-specific agonists. Macrophages from C3H/HeJ mice were incubated sequentially with poly(I:C) (PIC, 25 μg/ml) and Pam3Cys (P3C, 1 μg/ml). a, NF-κB translocation. Nuclear extracts were tested for NF-κB-binding activity by EMSA. b, IκB-α activation. Immunoblots were performed on cytoplasmic extracts to assess for IκB-α activation. Samples were probed with anti-IκB-α IgG. Samples were also probed with anti-actin IgG to verify equal loading between lanes.

**FIGURE 5.** Effect of sequential treatment with different TLR agonists on NF-κB translocation, IκB activation, and IFN-β production. a and b, BMDM from C3H/HeJ mice were treated sequentially with agonist 1 for 20 h and agonist 2 for 30 min, as indicated in the figures. Nuclear and cytoplasmic extracts were prepared after treatment with agonist 2. Agonists included poly(I:C) (PIC, 25 μg/ml) and Pam3Cys (P3C, 1 μg/ml). a, NF-κB translocation. Nuclear extracts were tested for NF-κB-binding activity by EMSA. b, IκB-α activation. Immunoblots were performed on cytoplasmic extracts to assess for IκB-α activation. Samples were probed with anti-IκB-α IgG. Samples were also probed with anti-actin IgG to verify equal loading between lanes.

**FIGURE 6.** Lack of synergy and priming between D- and I-specific agonists in macrophages from MyD88 KO mice. TNF levels were quantified in supernatants of BMDM from MyD88 KO mice after simultaneous (a) and sequential (b) treatment with poly(I:C) (25 μg/ml) and Pam3Cys (1 μg/ml). n = 3 replicates per condition.
as the IFN-γ pathways also mediate poly(I:C)-induced IFN production (35), we (poly(I:C)) (Fig. 7a).

Augmented LPS-induced IFN-γ from wild-type, but not from MyD88 KO mice, resulted in a following sequential treatment with agonist 1 and then agonist 2 for 20 h. Whereas simultaneous or sequential activation of the D and I pathways engagement of TLR-mediated intracellular pathways. Whereas si-


data show that at the doses and time frames used for these studies the patterns of inflammatory responses elicited by combina-

tions of TLR agonists can be predicted based on differential engagement of TLR-mediated intracellular pathways. Whereas simultaneous or sequential activation of the D and the I pathways results in augmented production of inflammatory cytokines, cross-tolerance results from sequential exposure to two different D-spe-

cific agonists and there is not synergy between different D-specific agonists. The data support the hypothesis that the underlying mechanisms lie within the D and I TLR signaling pathways. To our knowledge, these are the first studies that provide a predictive framework to explain the seemingly disparate phenomena of syn-

ergy, priming, and tolerance between TLR agonists.

Further studies will be required to pinpoint the specific factors involved in the modulatory effects of the D and I pathways on one another. The complexity of responses suggests that multiple inter-

mediares may be involved. We speculate that tolerance and cross-tolerance result from inhibition of the proximal pathway, that priming results from up-regulation of activity of downstream shared pathway components, and that synergy is caused by simul-

taneous activation of both pathways. Proximal pathway inhibition could be caused by up-regulation of proximal pathway inhibitors and/or by down-regulation of activity or production of proximal pathway intermediaries. A number of inhibitors of the D pathway have been identified that potentially could be involved (41). For instance, LPS treatment has been reported to increase expression of IL-1R-associated kinase-M, an inhibitor of TLR signaling (42). There is evidence that there are shared intermediaries be-

 tween the D and I pathways, such as TNFR-associated factor 6 and NF-κB (43).

Perhaps activation through either the D or I pathway causes up-regulation or activation of distal intermediaries, leading to primed distal pathways. When the second agonist activates the same limb, the signal encounters a down-regulated proximal path-

way that results in tolerance. However, when the second agonist activates through the complementary pathway, the signal is able to reach the primed distal pathway, leading to augmented cytokine production. Our data that TNF and IFN-γ production are both primed by pretreatment with D-specific agonists support the concept that D pathway engagement up-regulates activity of distal portions of both the inflammatory cytokine and IFN-γ/β limbs of the I pathway. The notion that activation via either the D or the I pathways results in priming of distal NF-κB-mediated pathways is supported by the finding that both D and I agonist pretreatment causes an augmented IL-6 response to treatment with TNF.

TLR4 is unique because it acts through both the D and I path-

ways, and it has been suggested that synergy between the pathways may be responsible for the potent inflammatory effects of LPS (29). We observed that pretreatment with D-specific agonists primed inflammatory cytokine and IFN-γ responses to subsequent treatment with LPS. Conversely, pretreatment with LPS tolerance.

d cells to D-specific agonists. This pattern of response supports the hypothesis that inhibition of proximal pathways and augmentation of distal pathway activity may contribute to tolerance and priming. Thus, LPS and other D agonists may inhibit the proximal D path-

way and increase activity of distal components of both the I and D pathways. The reduced response to a D-specific agonist following LPS would result from the signal not getting through the proximal D pathway. However, when cells are treated first with a D-specific agonist, despite proximal inhibition of the D pathway, LPS can still access the up-regulated distal pathways via the proximal, non-inhibited I pathway. Poly(I:C), a synthetic analog of viral dsRNA, has been reported recently to induce IFN-γ predominantly via a non-TLR3-mediated pathway that uses melanoma differentia-

on-associated gene-5 (35). TLR-independent pathways to poly(I:C)-

induced IFN production may, at least in part, explain our finding that pretreatment of BMDM with a TLR2 agonist does not cause a primed IFN-γ response to poly(I:C), but does cause a primed IFN-γ response to LPS.

D- and I-specific agonists do not induce synergy or priming in BMDM from MyD88 KO mice

We assessed responses of BMDM from homozygous MyD88 KO mice to simultaneous and sequential treatment with D (Pam3Cys)- and I (poly(I:C))-specific agonists. Synergy and priming between Pam3Cys and poly(I:C) were obliterated in MyD88 KO macro-

phages (Fig. 6). As expected, poly(I:C) did tolerate cells to itself (Fig. 6b).

Sequential engagement of the D then the I pathway causes augmented IFN-γ production

In initial experiments, we quantified IFN-γ levels in the superna-

tants of C3H/HeJ BMDM after sequential treatment with PAL and poly(I:C). BMDM from C3H/HeJ mice were treated with PAL (1 μg/ml) and poly(I:C) (25 μg/ml). b, Pam3Cys and LPS. BMDM from C57BL/6 and MyD88 KO mice were treated with Pam3Cys (1 μg/ml) and LPS (100 ng/ml). n = 3 replicates per condition. Values of p are compared with baseline levels for each TLR agonist, as established by incubating cells with medium as agonist 1, followed by the TLR agonist as agonist 2.

FIGURE 7. Priming and tolerance of IFN-β production between D and I agonists. a and b, IFN-β levels were quantified in BMDM supernatants following sequential treatment with agonist 1 and then agonist 2 for 20 h each. a, PAL and poly(I:C). BMDM from C3H/HeJ mice were treated with PAL (1 μg/ml) and poly(I:C) (25 μg/ml). b, Pam3Cys and LPS. BMDM from C57BL/6 and MyD88 KO mice were treated with Pam3Cys (1 μg/ml) and LPS (100 ng/ml). n = 3 replicates per condition. Values of p are compared with baseline levels for each TLR agonist, as established by incubating cells with medium as agonist 1, followed by the TLR agonist as agonist 2.

Discussion

We have shown that at the doses and time frames used for these studies the patterns of inflammatory responses elicited by combi-

nations of TLR agonists can be predicted based on differential engagement of TLR-mediated intracellular pathways. Whereas simultaneous or sequential activation of the D and the I pathways results in augmented production of inflammatory cytokines, cross-tolerance results from sequential exposure to two different D-spe-
cific agonists and there is not synergy between different D-specific agonists. The data support the hypothesis that the underlying mechanisms lie within the D and I TLR signaling pathways. To our knowledge, these are the first studies that provide a predictive framework to explain the seemingly disparate phenomena of syn-

ergy, priming, and tolerance between TLR agonists.

Further studies will be required to pinpoint the specific factors involved in the modulatory effects of the D and I pathways on one another. The complexity of responses suggests that multiple inter-

mediares may be involved. We speculate that tolerance and cross-tolerance result from inhibition of the proximal pathway, that priming results from up-regulation of activity of downstream shared pathway components, and that synergy is caused by simul-
taneous activation of both pathways. Proximal pathway inhibition could be caused by up-regulation of proximal pathway inhibitors and/or by down-regulation of activity or production of proximal pathway intermediaries. A number of inhibitors of the D pathway have been identified that potentially could be involved (41). For instance, LPS treatment has been reported to increase expression of IL-1R-associated kinase-M, an inhibitor of TLR signaling (42). There is evidence that there are shared intermediaries be-

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Perhaps activation through either the D or I pathway causes up-regulation or activation of distal intermediaries, leading to primed distal pathways. When the second agonist activates the same limb, the signal encounters a down-regulated proximal path-

way that results in tolerance. However, when the second agonist activates through the complementary pathway, the signal is able to reach the primed distal pathway, leading to augmented cytokine production. Our data that TNF and IFN-γ production are both primed by pretreatment with D-specific agonists support the concept that D pathway engagement up-regulates activity of distal portions of both the inflammatory cytokine and IFN-γ/β limbs of the I pathway. The notion that activation via either the D or the I pathways results in priming of distal NF-κB-mediated pathways is supported by the finding that both D and I agonist pretreatment causes an augmented IL-6 response to treatment with TNF.

TLR4 is unique because it acts through both the D and I path-

ways, and it has been suggested that synergy between the pathways may be responsible for the potent inflammatory effects of LPS (29). We observed that pretreatment with D-specific agonists primed inflammatory cytokine and IFN-γ responses to subsequent treatment with LPS. Conversely, pretreatment with LPS tolerance.

d cells to D-specific agonists. This pattern of response supports the hypothesis that inhibition of proximal pathways and augmentation of distal pathway activity may contribute to tolerance and priming. Thus, LPS and other D agonists may inhibit the proximal D path-

way and increase activity of distal components of both the I and D pathways. The reduced response to a D-specific agonist following LPS would result from the signal not getting through the proximal D pathway. However, when cells are treated first with a D-specific agonist, despite proximal inhibition of the D pathway, LPS can still access the up-regulated distal pathways via the proximal, non-inhibited I pathway. Poly(I:C), a synthetic analog of viral dsRNA, has been reported recently to induce IFN-γ predominantly via a non-TLR3-mediated pathway that uses melanoma differentia-

on-associated gene-5 (35). TLR-independent pathways to poly(I:C)-

induced IFN production may, at least in part, explain our finding that pretreatment of BMDM with a TLR2 agonist does not cause a primed IFN-γ response to poly(I:C), but does cause a primed IFN-γ response to LPS.
Numerous studies have been published on TLR interactions, often with conflicting results (16–23). Other cell populations, such as dendritic cells and T cells, have been shown to manifest priming to combinations of TLR agonists (45, 46). MyD88-dependent TLR agonists have been reported to induce tolerance to LPS by inhibiting IL-1R-associated kinase-1 activation (47). Similar to our results, poly(I:C) has been found to impair activation of the I, but not of the D pathway (47). In contrast to our results, TLR2 and TLR9 agonists have been reported to cause tolerance to LPS (47, 48), and CpG DNA was reported to not prime RAW264.7 cells to LPS (49). There are some potential explanations for the discrepancies in results. The cell types, time courses, and readouts vary between studies. Different cell types may not behave identically, as is suggested by a study that showed that macrophages from various compartments of a mouse varied with respect to the induction of tolerance to LPS (50). Finally, it is possible that for some earlier studies, the purity of the TLR agonists used in the present studies were highlypure.

The interplay between and the modulation of inflammatory effects of different TLRs appear to be highly complex. Understanding these crucial processes is important to gain insights into how the host responds to different pathogens, and how responses to infection may be affected by factors such as the stage of infection, antecedent infections, or recurrent episodes of bacteremia. Our studies indicate that patterns of inflammatory responses to combinations of TLR agonists can be predicted based on engagement of the D and I pathways. These studies were performed using pairs of highly purified TLR agonists, and therefore cannot be directly extrapolated to predict responses in the more complex milieu that exists in infection. Nevertheless, the data suggest that engagement of the D and I pathways with different TLR agonists and at different times may profoundly influence host inflammatory responses during infections.

Disclosures

The authors have no financial conflict of interest.

References


