Reprogramming of Murine Macrophages through TLR2 Confers Viral Resistance via TRAF3-Mediated, Enhanced Interferon Production

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Abstract

The cell surface/endosomal Toll-like Receptors (TLRs) are instrumental in initiating immune responses to both bacteria and viruses. With the exception of TLR2, all TLRs and cytosolic RIG-I-like receptors (RLRs) with known virus-derived ligands induce type I interferons (IFNs) in macrophages or dendritic cells. Herein, we report that prior ligation of TLR2, an event previously shown to induce “homo” or “hetero” tolerance, strongly “primes” macrophages for increased Type I IFN production in response to subsequent TLR/RLR signaling. This occurs by increasing activation of the transcription factor, IFN Regulatory Factor-3 (IRF-3) that, in turn, leads to enhanced induction of IFN-β, while expression of other pro-inflammatory genes are suppressed (tolerized). In vitro or in vivo “priming” of murine macrophages with TLR2 ligands increase virus-mediated IFN induction and resistance to infection. This priming effect of TLR2 is mediated by the selective upregulation of the K63 ubiquitin ligase, TRAF3. Thus, we provide a mechanistic explanation for the observed antiviral actions of MyD88-dependent TLR2 and further define the role of TRAF3 in viral innate immunity.

Introduction

The last few years have seen an explosion in the characterization of mechanisms for the recognition of microbial pathogens by the innate immune system. In particular, sensors that recognize molecular signatures of viral infection have been the subject of many exciting discoveries. Among the currently known innate immune antiviral sensors are the cytosolic RNA receptors, Retinoic acid-inducible gene 1 (RIG-I), and Melanoma differentiation-associated protein 5 (MDA5) [1–3], as well as, DDX21 and DHX36 (DDX/TRIF) [4]. A cytosolic DNA sensing multi-protein complex has been identified that responds to DNA virus infections, although the apical sensors for this pathway have not been fully elucidated [5,6]. In addition, the nucleic acid sensing endosomal Toll-like receptors (TLRs), i.e., TLR3, TLR7/8, and TLR9, as well as cell surface expressed TLR4, have known virus-derived ligands [7,8]. A common and critical feature of each of these innate viral surveillance systems is the ability to induce type I interferons (IFNs). IFNs are a family of pleotropic cytokines that are secreted and act in a paracrine or autocrine manner to induce an incredibly diverse array of genes with direct antiviral properties [9,10]. Additionally, type I IFNs serve as a point of connection between innate and adaptive responses, in that they educate and enhance the adaptive response and lead to viral clearance [11].

Among the TLRs, TLRs 2 and 3 are unique in that they utilize MyD88-dependent signaling exclusively and, thus, do not induce type I IFNs in macrophages and dendritic cells [12]. As a result, these receptors are generally described as being antibacterial. While there are no known virus-derived ligands for TLR5, there are a number of reports in the literature describing TLR2 activation by viral ligands, including a recent report that has shown that viral, but not bacterial, TLR2 ligands may induce IFN-α in a subset of monocytes by unknown mechanisms [13]. In addition, mouse infection models with several different viruses have shown deficiencies in viral clearance in mice with a targeted deletion in TLR2 [14–16]. Presently, the mechanism by which TLR2 contributes to an antiviral state in the absence of direct IFN induction is not clear.

Along with the well documented role that individual TLR and RLR sensors play in responding to infection, it is becoming increasingly clear that individual innate immune sensing systems do not operate in isolation, but that significant cross-talk between innate systems occurs [17]. Indeed, the biological relevance of such cross-talk has already been demonstrated in cases of polymicrobial infection [18,19].

Herein, we describe a TLR2-dependent mechanism for the governance of subsequent type I IFN production via both the TLR and RLR systems. This pathway shapes antiviral immunity in vitro in murine primary macrophages, and in vivo in mouse models of viral infection. In response to prior stimulation or “priming” with TLR2 ligands, subsequent type I IFN induction via all known IFN-β-inducing innate immune pathways is strongly potentiated. The underlying mechanism for this potentiation was identified as being largely due to the up-regulation of the E3 ubiquitin ligase,
Author Summary

In response to viral infection, cells of the innate immune system synthesize and release members of the type I interferon protein family. The interferons form an essential line of defense, both by slowing viral growth and by expanding the cellular immune response. The synthesis of interferon is initiated by recognition of viral constituents by one or more innate receptors. Among these receptors, Toll-like receptor 2 (TLR2) has been shown to be critical for the immune response to a number of viruses, yet TLR2 only directly initiates type I interferon production in a very small set of innate immune cells. We have discovered that TLR2 can contribute to the antiviral interferon response much more broadly by indirectly governing the production of interferon induced by other Toll-like receptors as well as downstream of the cytosolic RLRs.

These findings not only explain how bacterial or viral TLR2 ligands may selectively augment a subsequent TLR-TRAF3. We also demonstrate that this TLR2 dependent regulation of interferon may be important in biological scenarios involving co-infection of virus and Gram positive bacteria, but not Gram negative bacteria.

Results

To characterize further the effects of TLR cross-talk on the induction of important inflammatory genes, primary mouse peritoneal macrophages were treated with media alone, or media supplemented with ligands for TLR 2 (P3C) or TLR4 (LPS). After overnight stimulation, the primary stimulus was removed and the cells washed extensively and allowed to rest for 60 minutes. The macrophage cultures were next re-stimulated with the TLR4 ligand, E. coli LPS, for 2 or 4 hrs and examined for gene induction by qRT-PCR. LPS induction of both the classical pro-inflammatory genes IL-6 and IL-12 p40 was strongly inhibited by prolonged TLR2 pre-stimulation (Figure 1, A and B). This is the predicted pattern described previously and known as “homotolerance” or “heterotolerance,” respectively [20]. Unexpectedly, when we examined the effect of TLR pre-stimulation on the LPS-mediated induction of type I interferon (IFN-β), we found the nature of the effect to be critically dependent on whether the initial stimulation had come through TLR2 or TLR4. Pretreatment with LPS (TLR4) sharply inhibited subsequent IFN-β induction in response to LPS (Figure 1C). However, pretreatment with P3C (TLR2) markedly potentiated IFN-β by nearly 10-fold at the mRNA level when compared to media-pretreated macrophages (Figure 1C). These TLR2-dependent changes in LPS-mediated gene induction were enhanced by TLR2 stimulation. To test this hypothesis, we initially profiled MAPK signaling in response to LPS in media-pretreated, LPS-pretreated, or P3C-pretreated macrophages. Stimulation of naive macrophages with LPS resulted in robust activation of the MAPKs p38, ERK p42/p44, and JNK as evidenced by increased phosphorylation (Figure 2). Pretreating macrophages with LPS overnight completely ablated LPS-driven MAPK activation (Figure 2), a result consistent with previous reports on endotoxin tolerance [20,22]. Pretreatment of macrophages with the TLR2 ligand P3C also reduced the activation of p38 and ERK, and almost completely inhibited activation of JNK by LPS (Figure 2). These data confirm prior reports that TLR2-mediated “heterotolerance” is less efficient than TLR4 “homotolerance” with respect to MAPK activation [20]. Based on these data, augmentation of MAPK signaling induced by TLR2-mediated “priming” cannot account for TLR2-mediated upregulation of IFN-β mRNA.

As a role for TLR2 in potentiating Type I interferon has not been described we sought to distinguish the molecular determinants of this effect. TLR2 functions as an obligate heterodimer with either TLR1 or TLR6, depending on the nature of the ligand [21]. We used ligands for TLR2/6 (P2C) or TLR2/1 (P3C) heterodimers in pretreatment experiments, and both potentiated subsequent IFN-β production in response to LPS via TLR4 (Figure 1D). The effect of TLR2 pre-stimulation on IFN-β was long-lived, lasting for at least 72 hrs following the removal of the pre-treatment TLR2 stimulus (Figure 1F). We also observed a requirement for a prolonged initial exposure (at least 8 h) to TLR2 ligands to elicit a measurable increase in IFN-β induction (Figure S3).

The unexpected result that “priming” peritoneal macrophages with TLR2 ligands enhanced TLR4-dependent IFN-β mRNA expression led us to speculate that components of the signal transduction apparatus downstream of TLR4 were being augmented by TLR2 stimulation. To test this hypothesis, we initially profiled MAPK signaling in response to LPS in media-pretreated, LPS-pretreated, or P3C-pretreated macrophages. Stimulation of naive macrophages with LPS resulted in robust activation of the MAPKs p38, ERK p42/p44, and JNK as evidenced by increased phosphorylation (Figure 2). Pretreating macrophages with LPS overnight completely ablated LPS-driven MAPK activation (Figure 2), a result consistent with previous reports on endotoxin tolerance [20,22]. Pretreatment of macrophages with the TLR2 ligand P3C also reduced the activation of p38 and ERK, and almost completely inhibited activation of JNK by LPS (Figure 2). These data confirm prior reports that TLR2-mediated “heterotolerance” is less efficient than TLR4 “homotolerance” with respect to MAPK activation [20]. Based on these data, augmentation of MAPK signaling induced by TLR2-mediated “priming” cannot account for TLR2-mediated upregulation of IFN-β mRNA.

Two discrete signaling arms are initiated upon TLR4 signaling and contribute to IFN-β activity [20,23,24]. Classical NF-κB activation through the canonical kinase, IKKβ, results in phosphorylation of the transcription factor, p65, that subsequently contributes to induction of not only most proinflammatory cytokines and chemokines, but also IFN-β. Activation of the non-canonical kinase, TBK-1, via the TRIF/TRAM arm of the TLR4 signaling pathway leads to activation of transcription factor, IRF-3, that, together with NF-κB and other transcription factors, activates transcription of IFN-β and other similarly regulated genes. We examined IKKβ activation by phospho-specific Western analysis of medium-pretreated and P3C-primed macrophages. LPS induced strong IKKβ activation in naive macrophages by 20 min that declined significantly by 60 min (Figure 3A). In marked contrast, LPS induced extremely weak IKKβ activation in the TLR2 (P3C)-pretreated cells that was detected transiently at 40 min (Figure 3A). LPS-pretreated TBK-1 phosphorylation in TLR2-primed cells activity was reduced when compared to that achieved in naive cells, but was not inhibited to the degree as the IKKβ (Figure 3B). As neither IKKβ nor TBK-1 activity could account for the increase in IFN-β induction in TLR2-primed macrophages, we sought to examine the effect of TLR2 priming directly on key transcription factors known to be involved in regulating the IFN-β promoter [25]. We observed that phosphorylation of the NF-κB constituent p65 on residue serine 536 was dramatically enhanced in the P3C-pretreated cells when compared to the naive macrophages (Figure 3C). Unexpectedly, we also observed a highly significant increase in the steady-state levels of total p65 in TLR2-primed cells that likely contributes to the increase in phosphorylated p65 (Figure 3C). Phosphorylation of

PLOS Pathogens | www.plospathogens.org 2 July 2013 | Volume 9 | Issue 7 | e1003479
The transcription factor IRF3 (on serine 396) was increased in P3C-primed macrophages; however, activation was not accompanied by increase in the levels of total IRF3 and, therefore, increased total IRF3 cannot explain the observed increase in phosphorylation (Figure 3D).

The transcription factor c-Jun is a constituent of the heterodimeric transcription factor, AP-1, that has also been shown to be essential for activation of IFN-β transcription [26]. In the case of TLR4, c-jun phosphorylation is also known to be downstream of MAPK signaling. In contrast to p65 and IRF3, LPS-driven

Figure 1. TLR2 ligands can selectively potentiate IFN-β production induced via TLR4. (A–C) Primary peritoneal macrophages were treated overnight with media or the indicated TLR ligands, and then re-stimulated with E. coli LPS (100 ng/ml) for 4 hrs (A and B) or 2 hrs (C) and gene expression for IL-6 (A), IL-12 p40 (B), or IFN-β (C) was analyzed by qRT-PCR. (D) Primary peritoneal macrophages were treated overnight with media or Pam3Cys or Pam2Cys (250 ng/ml) and re-stimulated after overnight incubation with E. coli LPS (100 ng/ml) for 2 hrs before extraction of RNA and analysis of gene expression. (E) Cell supernatants from macrophages treated as in (A–C) were analyzed for IL-12 p40 and IFN-β protein by ELISA. (F) Peritoneal macrophages were treated overnight with media or Pam3Cys (250 ng/ml) and re-stimulated either immediately with LPS for 2 hrs or following an additional 24, 48, or 72 hr resting period. Experiments were performed at least three times. Results from a single representative experiment are shown.

doi:10.1371/journal.ppat.1003479.g001
phosphorylation of c-jun was significantly reduced in P3C-primed peritoneal macrophages (Figure 3E). This result is in agreement with the previous MAPK results showing a loss of JNK activation in P3C-pretreated macrophages (Figure 2). Both p65 and IRF3 are phosphorylated by their proximal kinases, IKKβ and TBK-1, respectively, while in the cytosol. Following phosphorylation, both p65 and IRF3 enter the nucleus where they are competent to bind to the IFN-β promoter. To examine whether the observed increased phosphorylation of p65 and IRF3 resulted in increased nuclear import, we performed nuclear fractionation of both medium-pretreated and P3C-primed macrophages after stimulation with medium only or with LPS for 60 min, followed by Western analysis for p65 and IRF3 (Figure 3F). We observed significantly augmented nuclear accumulation of both p65 and IRF3 in the P3C pre-treated cells when compared to the medium-pretreated, naïve macrophages (Figure 3F).

Since nuclear accumulation of p65 may not correlate directly with DNA binding, we performed an EMSA using our nuclear fractions from naïve, LPS-pretreated, or P3C-primed macrophages that were re-stimulated with media or LPS for 60 min (Figure 3G). Nuclear proteins from naïve (media-pretreated) macrophages exhibited strong binding to the NF-kB probe when stimulated with medium only or with LPS for 60 min, followed by Western analysis for p65 and IRF3 (Figure 3F). We observed significantly augmented nuclear accumulation of both p65 and IRF3 in the P3C pre-treated cells when compared to the medium-pretreated, naïve macrophages (Figure 3F).

A key question that arises from these observations is the following: why, as in the case of the P3C-primed macrophages, where NF-kB activity is not strongly diminished, do we not see an increase in the activity of all known NF-kB-responsive genes, e.g., IL-6 or IL-12 p40 (Figures 1A and 1B)? One answer may lie in the works of Medzhitov et al. and McCall et al. who showed that in LPS homotolerance, some normally LPS-responsive genes are transcriptionally silenced through chromatin remodeling such that these promoters are no longer able to interact with transcription factors, regardless of the levels of activated transcription factors [27,28]. Therefore, we hypothesized that if such a state also occurs in TLR2-induced heterotolerance, it may be possible to abrogate the inactive/tolerant phenotype by preventing remodeling during the initial (“tolerizing”) stimulus. To test this hypothesis, primary macrophages were treated with media alone, Pam3Cys, or Pam3Cys in the presence of Trichostatin A (TSA), a known inhibitor of histone deacetylase activity. Each group of cells was then re-stimulated with LPS as before and assayed for the induction of IL-12 p40 mRNA by qRT-PCR. As expected (Figure 1B), TLR2 priming of macrophages eliminated induction of IL-12 p40 mRNA by LPS (Figure 4A). Concurrent treatment of macrophages with TSA and TLR2 ligand significantly preserved the LPS inducibility of IL-12 p40 (Figure 4A). P3C-primed macrophages exhibited a significance increase in IFN-β mRNA induced by LPS that was reversed by the presence of TSA. Thus, manipulation of histone deacetylase activity reverses “heterotolerance.”

While the treatment with TSA strongly argues for a role for chromatin remodeling in TLR2 heterotolerance leading to diminished IL-12 p40 mRNA expression, treatment with chemical inhibitors does not directly assay for transcription factor binding.
We therefore also performed chromatin immunoprecipitation (ChIP) analysis on medium- vs. P3C-pretreated macrophages (Figure 4, C and D). Following 60 min of LPS stimulation in naïve or P3C-primed cells, cross-linked chromatin was immunoprecipitated with antibody against either IRF3 or p65/RelA. PCR was used to amplify bound chromatin fragments corresponding to the IFN-β enhancer region. Consistent with our results with the EMSA, p65 binding activity was maintained in P3C-primed cells at levels seen in medium-pretreated macrophages. However, IRF3 binding to the IFN-β promoter was significantly enhanced in the P3C-primed cells (Figure 4C). As an additional control, we assayed for the recruitment of RNA pol II to the IL-12p40 promoter following 60 minutes of LPS stimulation in naïve and TLR2 primed macrophages (Figure S4). TLR2 priming strongly inhibited RNA pol II recruitment to IL-12 p40, arguing for negative chromatin remodeling in the regulation of this promoter.

Having narrowed the likely mechanism for TLR2 priming of Type I interferon to increased transcriptional activity, we interrogated the larger biological significance of this phenomena. Given that the primary function of IFN-β is in antiviral defense, we next examined the effect of TLR2 priming on the course of a model virus infection. To this end, WT primary peritoneal macrophages were infected in vitro with Vesicular Stomatitis Virus (VSV Indiana Strain) at increasing MOI, without or with prior P3C priming. Additionally, we primed IFN-β−/− macrophages with TLR2 ligand and these cells were similarly infected with VSV. Significant inhibition of VSV-induced cytopathic effect (CPE) was observed in cells pretreated with P3C (Figure 5A). The protective capacity of TLR2 priming was dependent on having an intact IFN-β gene (Figure 5A) as evidenced by the failure of P3C to protect IFN-β−/− macrophages. TLR2 priming inhibited viral growth, rather than simply inducing cell death, as plaque titration of VSV showed a >2 log inhibition (Figure 5B, left panel). Quantity of CPE by measuring the crystal violet stain eluted from cells remaining bound to the plate strongly paralleled quantification of viral replication obtained by plaque assay (Figure 5B, right panel). We next sought to determine whether VSV infection of P3C-primed cells elicited greater production of IFN-β mRNA than seen in naïve cells. To this end, naïve or P3C-primed macrophages were infected with VSV, and IFN-β mRNA was measured by qRT-PCR 6 hours post-infection. As predicted, VSV infection induced greater levels of IFN-β mRNA than seen in naïve cells. To this end, naïve or P3C-primed macrophages were infected with VSV, and IFN-β mRNA was measured by qRT-PCR 6 hours post-infection. As predicted, VSV infection induced greater levels of IFN-β mRNA than seen in naïve cells. To this end, naïve or P3C-primed macrophages were infected with VSV, and IFN-β mRNA was measured by qRT-PCR 6 hours post-infection. As predicted, VSV infection induced greater levels of IFN-β mRNA than seen in naïve cells. To this end, naïve or P3C-primed macrophages were infected with VSV, and IFN-β mRNA was measured by qRT-PCR 6 hours post-infection. As predicted, VSV infection induced greater levels of IFN-β mRNA than seen in naïve cells.
influenza strain, PR8, also elicited greater production of IFN-β in P3C-primed cells as well as diminished production of IL-6 mRNA (Figure 5D). As both VSV and influenza are relatively small RNA viruses, we also utilized Vaccinia Virus (WR strain) as a model DNA virus. We observed enhanced IFN-β induction by Vaccinia Virus at 8 hours post-infection, although we were not able to detect IL-6 at this time point (Figure 5E).

The significant protection afforded by P3C priming in vitro encouraged us to extend our virus studies in vivo. Female BALB/c mice were administered 500 μl of PBS or PBS containing 100 μg Pam3Cys i.p. Twenty-four hrs later, mice were infected intranasally (i.n.) with 1×10⁹ pfu VSV. Mice were monitored for mortality and morbidity for up to 10 days post-infection. We observed complete protection from a VSV LD50 in mice that received a single priming dose of P3C (Figure 5F; p<0.05). Similar experiments were carried out in which medium- or P3C-pretreated mice were challenged with 5000 TCID 50 influenza PR8 (LD100). P3C-primed mice exhibited a delayed mean time to death upon PR8 challenge compared to unprimed, infected mice (data not shown). To examine this issue, peritoneal macrophages were incubated with heat-killed Gram positive bacteria Staphylococcus aureus overnight at an MOI of 1. Macrophages pre-incubated with S. aureus were subsequently stimulated with E. coli LPS or transfected with poly I:C to simulate a viral infection. Macrophages exposed to S. aureus displayed strongly ablated induction of IL-12 p40 and enhanced induction of IFN-β in response to LPS (Figure 6A). Similarly, transfected poly I:C induced far greater IFN-β in bacterially primed cells (Figure 6B). We confirmed these observations with a second clinically relevant Gram positive bacteria, S. pneumoniae (Figure 6B). Our initial experiments demonstrating divergent effects for TLR2 and TLR4 on subsequent type I IFN induction (Figure 1C) led us to speculate that a priming effect for IFN-β might be a common effect of Gram positive, but not Gram negative bacteria. To test this hypothesis, peritoneal macrophages were pre-incubated with media alone or heat-killed E. coli and then stimulated either with LPS or by transfected poly I:C. In marked contrast to Gram positive bacteria, pre-treatment with E. coli strongly suppressed IFN-β mRNA.

While it is clear that TLR2 can contribute to the regulation of IFN-β and innate antiviral immunity, our previous experiments utilized a pure, synthetic ligand, Pam3Cys, to establish TLR2 priming. Whether such priming could be accomplished by biological organisms in the context of infection has not been shown. To address this issue, peritoneal macrophages were incubated with P3C to establish TLR2 priming. Whether such priming could be accomplished by biological organisms in the context of infection has not been shown. To address this issue, peritoneal macrophages were incubated with heat-killed Gram positive bacteria Staphylococcus aureus overnight at an MOI of 1. Macrophages pre-incubated with S. aureus were subsequently stimulated with E. coli LPS or transfected with poly I:C to simulate a viral infection. Macrophages exposed to S. aureus displayed strongly ablated induction of IL-12 p40 and enhanced induction of IFN-β in response to LPS (Figure 6A). Similarly, transfected poly I:C induced far greater IFN-β in bacterially primed cells (Figure 6B). We confirmed these observations with a second clinically relevant Gram positive bacteria, S. pneumoniae (Figure 6B). Our initial experiments demonstrating divergent effects for TLR2 and TLR4 on subsequent type I IFN induction (Figure 1C) led us to speculate that a priming effect for IFN-β might be a common effect of Gram positive, but not Gram negative bacteria. To test this hypothesis, peritoneal macrophages were pre-incubated with media alone or heat-killed E. coli and then stimulated either with LPS or by transfected poly I:C. In marked contrast to Gram positive bacteria, pre-treatment with E. coli strongly suppressed IFN-β mRNA.

Figure 4. Enhanced transcription factor occupancy on the IFN-β promoter. (A) and (B). Primary peritoneal macrophages were treated for 8 hrs with media alone, or with 250 ng/ml Pam3Cys, or Pam3Cys plus 50 nM Trichostatin A (TSA). Following stimulation, cells were washed and allowed to rest overnight, at which time the cells were re-stimulated with 100 ng/ml E. coli LPS for 4 (A) or 2 (B) hrs. Gene expression was analyzed by qRT-PCR. (C) and (D) Macrophages treated overnight with media alone or 250 ng/ml Pam3Cys were re-stimulated with E. coli LPS (250 ng/ml) for 60 min and used in Chromatin Immuno-precipitation (ChIP) assays with antibodies against the indicated transcription factors. Precipitated DNA was amplified using primers directed against a region of the IFN-β promoter. Experiments were performed at least three times and the results were obtained from a single representative experiment.

doi:10.1371/journal.ppat.1003479.g004
induced by both LPS and transfected poly I:C (Figure 6C). In addition to bacteria, some viruses are known to signal through TLR2, including Vaccinia Virus [13]. We assayed the ability of UV-inactivated Vaccinia Virus to regulate IFN-β production by LPS and transfected poly I:C. Vaccinia Virus was capable of effective priming for both TLR4 and the RLRs (Figure 6D). To

Figure 5. TLR2 ligation enhances in vitro and in vivo viral resistance and IFN-β production. (A) WT C57BL/6 or IFN-β−/− primary peritoneal macrophages were treated overnight with media alone or with 250 ng/ml Pam3Cys and subsequently infected with the indicated MOI of Vesicular Stomatitis Virus. After 24 hrs of infection, adherent monolayers were stained with crystal violet. (B) (left panel) Supernatant from triplicate wells of macrophages infected with VSV (MOI = 10) for 24 hours were harvested and viral titer was determined by plaque titration on BHK cells. (right panel) Trypan Blue was used to stain macrophages infected with VSV (MOI = 50) for 24 hrs. A minimum of 10 fields per well were counted. (C) Macrophages were pretreated with media alone or Pam3Cys for 24 hrs and subsequently infected with indicated MOI of VSV and harvested after six hrs for gene expression analysis. (D) Macrophages were pretreated with media alone or Pam3Cys for 24 hrs and subsequently infected with influenza strain PR8 at an MOI = 1 for 6 hrs and harvested for gene expression analysis. (E) Macrophages were pretreated with media alone or with Pam3Cys for 24 hrs and subsequently infected with Vaccinia Virus (WR strain) at an MOI of 5 for 8 hours prior to harvesting for gene expression analysis (F) WT 6–8 week female BALB/cJ mice were injected i.p. with 0.5 ml saline or 100 mg Pam3Cys in saline 24 hrs prior to i.n. infection with 1 × 10⁶ pfu VSV. N = 12 mice per treatment group (G) WT or TLR2−/− mice were infected i.n with 1 × 10⁷ pfu of VSV in saline. N = 12 mice per group. All experiments were performed at least three times and data from a representative experiment is shown for each.

doi:10.1371/journal.ppat.1003479.g005

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demonstrate that in vivo priming by one organism might influence an infection by a second, we injected BALB/c mice i.p. with PBS or heat-killed S. aureus 24 hours prior to i.n. infection with VSV. We observed a significant protective effect induced by S. aureus against VSV infection (Figure 6E).

While it is clear that both VSV and influenza infections can involve TLR4 [29,30], the cytosolic nucleic acid innate immune receptors play a large role in detecting RNA viruses and initiating an IFN response via the TBK-1/IRF3 axis. It was therefore important to determine if the antiviral effects of TLR2 priming are limited to the TLRs, and specifically TLR4, or whether IFN-β induction is potentiated by TLR2 priming through other families of cytosolic pattern recognition receptors. To this end, we examined the expression levels of the known cytosolic RNA sensors MDA5, RIG-I, and MAVS in medium- and P3C-treated macrophages. We observed a striking up-regulation in the expression of MDA5 protein following 24 hrs of P3C stimulation (Figure 7A). We also observed a much more modest, but reproducible, increase in RIG-I protein levels. As seen previously [31], MAVS appears as several bands of differing electrophoretic mobilities, of which only one appears to be increased in P3C-treated macrophages. The increase in abundance of these cytosolic RNA sensors led us to speculate that there may be a functional increase in sensitivity of this system in P3C-treated macrophages. Therefore, naive and P3C-treated primary macrophages were transfected with High Molecular Weight (HMW) and Low Molecular Weight (LMW) poly I:C to stimulate MDA5 and RIG-I, respectively. As a control, we additionally treated control and P3C-pretreated macrophages with “free” poly I:C to stimulate TLR3. We examined a time course of IFR3 activation by Western analysis in each case (Figure 7, B–D) and IFN-β mRNA induction by qRT-PCR (Figures 7 E–G). HMW and LMW poly I:C, as well as soluble poly I:C, elicited dramatically greater activation of IRF3 in P3C-primed cells,!correlating in each case with a strong increase in induction of IFN-β at the mRNA level. Interestingly, in the cases of transfected poly I:C, we did not observe an inhibition of IL-6 induction in P3C-primed cells.

In seeking a common signaling element that might account for the potentiation in IFN-β induction by both TLRs and RLRs, we postulated that the ubiquitin ligase TRAF3 would be a strong candidate to mediate this effect. TRAF3 is immediately upstream of IRF3 activation in both the TLR and RLR pathways and TRAF3-null immune cells are defective in IFN induction via TLR3 [32–34]. Initially, we profiled TRAF3 mRNA over a time course of Pam3CSk treatment and found a late increase in TRAF3 consistent with the time of IFN-β potentiation (Figure 8A). We examined the levels of TRAF3 and TRAF6 in the naive and P3C-pretreated macrophages by Western analysis. Dramatically, we found that protein levels of TRAF3, but not TRAF6, were significantly higher in P3C-primed cells (Figure 8B). As a control, we tested additional innate immune ligands, and found that while TLR3 and TLR4 were capable of inducing TRAF3 mRNA to limited extents, the effect was significantly weaker than for TLR2 ligands (Figure S5). To ascertain whether elevated levels of TRAF3 might lead to enhanced signaling complex formation, we performed co-immunoprecipitations from macrophages following 45 min of LPS treatment utilizing an antibody against IRF3 and blotting for co-precipitating TRAF3. We observed significantly greater amounts of TRAF3 in complex with IRF3 in TLR2-primed cells (Figure 8C). While TRAF3 is an essential component of TRIF signaling to IFN-β, it is not known whether an increase in TRAF3 by itself can potentiate TLR4-dependent IFN-β induction and IRF-3 activation. To test this hypothesis, we over-expressed TRAF3 in MAT4 cells, HeLa cells that constitutively express TRAF3, stimulated them with LPS, and examined TBK-1 and IRF3 activation. TRAF3 over-expression significantly increased both signaling leading to P-IRF3 (Figure 8D, left panel) and IFN-β gene induction (Figure 8E, left panel). Over-expression of TRAF3 also increased IRF3 activation and IFN-β gene induction induced by transfection of cells with HMW poly I:C (Figures 8D and 8E, left panels respectively). Overexpression of TRAF3 did not significantly affect LPS-driven p65 phosphorylation (data not shown). To complement our over-expression studies, we performed TLR2 priming and LPS restimulation studies using littermate control and Lys-Cre TRAF3+/lox (TRAF3−/−) peritoneal macrophages. Loss of TRAF3 severely compromised LPS-driven IFN-β induction irrespective of prior TLR2 priming (Figure 8F, left panel). The overall enhancement of LPS-dependent IFN-β induction due to priming in TRAF3−/− macrophages was 4.2-fold compared to unprimed cells. In contrast, TLR2 priming only enhanced IFN-β induction by an average of 1.7-fold in TRAF3−/− macrophages. The loss of TRAF3 had little effect on the induction of IL-12 p40 (Figure 8F, right panel), thus illustrating the specificity of TRAF3 for the interferon inducing pathway.

In conclusion, we have identified a novel mechanism that accounts for the ability of TLR2 to increase antiviral immunity despite an inability to increase IFN levels directly. TLR2 priming of macrophages results in increased TRAF3 levels that lead to the selective priming of IFN-β production by multiple innate immune surveillance pathways and increased viral resistance.

Discussion

The distinct subcellular localizations and the overlapping ligand chemistries among the receptors in the TLR and RLR families underscores the complementarity between these systems in providing a comprehensive surveillance network against viral infection. While often regarded as a receptor for bacterial lipoproteins, a critical role for TLR2 in antiviral defense has been established in several systems. The human viral pathogens Cytomegalovirus (CMV), Respiratory Syncytial Virus (RSV) and Varicella Zoster all have been shown to either directly encode TLR2 ligands among their structural proteins, or to require functional TLR2 for immune clearance in vivo [14,16,35]. Of particular interest for our present study, splenic levels of IFN-β are diminished in TLR2−/− mice infected with mouse CMV [15]. A recent report has extended the connection between TLR2 and the innate response to virus [13]. In this work, using Vaccinia Virus as a model, an as yet unknown ligand on UV-inactivated Vaccinia was shown to be a potent TLR2 agonist. Unexpectedly, while Vaccinia could trigger TLR2-dependent cytokines from all TLR2-expressing cell types tested, it also selectively induced type I IFN in a TLR2-dependent manner, from Ly6Chigh inflammatory monocytes.” However, the molecular mechanism underlying this phenomenon has yet to be fully elucidated. Our current work also provides evidence that UV-inactivated Vaccinia is a potent TLR2 agonist, and we propose that TLR2-based “priming” may be an additional mechanism by which TLR2 contributes to type I IFN production in tissues where innate immune cells other than inflammatory monocytes predominate. Such priming might work systemically during virus infection as free viral proteins capable of acting as TLR2 ligands are shed into the circulation from an infected tissue and travel to uninfected sites and encounter TLR2-expressing innate immune cells. However, it is important to note that a TLR2 priming-based mechanism need not only be of significance during infection with a single viral pathogen. We have demonstrated that TLR- and RLR-mediated IFN-β production is
Figure 6. Gram positive bacteria and select viruses, but not Gram negative bacteria, can prime for TLR- and RLR-driven IFN-β production. (A–C) Primary peritoneal macrophages were incubated overnight with media alone or with the indicated heat killed bacteria (moi = 1). Following treatment, macrophages were washed 3X with PBS and re-stimulated either with \textit{E. coli} LPS (100 ng/ml) for 2 hours, or transfected with poly I:C (1 μg/ml) for 6 hours. (D) Macrophages were incubated with media or UV inactivated Vaccinia Virus (moi = 0.5) overnight and re-stimulated with \textit{E. coli} LPS (100 ng/ml) for 2 hours or transfected with poly I:C for 6 hours. (E) BALB/cJ mice were injected i.p. with saline or heat-killed \textit{S. aureus}.
potently enhanced by TLR2 ligands derived from both Gram positive bacteria as well as virus. This could play a role in the pathogenesis of polymicrobial co-infections involving both a bacterial and a viral component. Indeed, synergistic IFN production has already been demonstrated in vivo in a model involving co-infection of Streptococcus pneumoniae and influenza A in the upper airways of mice [36]. In this model, Gram positive bacterial infection enhances viral induced IFN-β, that in turn, enhances bacterial growth [36].

Recent literature also suggests that TLR-RLR cross-talk with respect to IFN production may not only occur under dynamic conditions of infection and inflammation. Steady-state priming of innate IFN production in macrophages by unknown components of the intestinal microbiota has been definitively demonstrated [37]. Our data also support the notion that prior ligation of TLR2 leads to a reduction in the subsequent induction of classical pro-inflammatory cytokines such as IL-12 and IL-6, while simultaneously increasing IFN-β responsiveness. In fact, a dynamic balance between IL-12 and IFN-β was recently suggested and shown to depend mechanistically on the levels of IRF transcription factor activation [19]. Whether such a mechanism is operative in our experimental systems remains to be determined.

Our data strongly supports the hypothesis that this potentiation likely occurs, in part, via a TLR-dependent amplification of the levels of the K63 ubiquitin ligase TRAF3. TRAF3 is uniquely positioned at a common node in the IFN-inducing pathways downstream of both TLRs and RLRs, and our data clearly show that TLR2 pre-stimulation greatly increases the amount of IRF3 activation for a given dose of challenge ligand. Precisely how, on an inter-molecular level, this priming effect occurs remains to be elucidated. This question is made all the more interesting, given that the positive effects of TRAF3 in our system take place in a context in which we see diminished TBK-1 activation downstream of TLR4 (Fig. 3B). The elegant studies by Ogunseyan et al. [33] and Hacker et al. [32] support a model in which in response to TLR4 ligation, TRAF3 is part of a multi-protein complex that contains both TBK-1 and IRF3 and in which TRAF3 is clearly required for IRF3 activation. Since over-expression of TRAF3 alone is not sufficient to initiate signaling to the IFN-β promoter ([33] and Figure 8D), we propose that the principal effect of elevated TRAF3 levels is to dramatically enhance the kinetics by which TBK-1 can interact with and phosphorylate IRF3. Such a schema would be logical given the enzymatic activity of TRAF3 as a K63 ubiquitin ligase capable of assisting in the assembly of multi-protein complexes. This scenario is the first of which we are aware, in which TRAF3 levels are used as a rheostatic mechanism by which the innate immune response is tuned. The potential cell- and tissue-type specificity of the effects of TLR2 priming, and by extension, TRAF3, in reshaping the transcriptional responses to innate immune receptor ligation remains an area for future investigation. This is particularly relevant given that in contrast to macrophages and dendritic cells, TRAF3−/− B cells exhibit increased responses to TLR ligands [38,39]. Additionally, TRAF3 has a well-established role as a negative regulator of the non-cannonical NF-kB pathway in B cells [40] that may provide greater complexity to the crosstalk between TLR2 and other pathways such as those downstream of CD40. It should be noted that the presence of residual priming effects of TLR2 ligands in the TRAF3−/− macrophages (Figure 8F) indicates that additional TLR2-responsive factors beyond TRAF3 contribute to this phenomena and need to be further investigated. For example, one possible additional factor that might contribute to enhanced IFN-β induction could be the cytosolic receptor MDA5, as steady-state levels of MDA5 protein are dramatically up-regulated following TLR2 priming (Figure 7A).

In addition to TRAF3, we found that levels of the NF-κB family member, p65, were greatly enhanced following TLR2 priming. Such TLR-dependent regulation of p65 has not been previously reported, but is analogous to the accumulation of RelB reported in endotoxin tolerance [41]. The molecular mechanism of this p65 regulation remains to be elucidated, but may represent an exciting new area of governance for NF-κB responses. The enhanced accumulation of p65 protein levels may be important in maintaining NF-κB activation downstream of TLR4 in situations of reduced IKKβ kinase complex activity such as is found in TLR2-primed cells (Figure 3A).

It should also be noted that the present work has several important implications with respect to our understanding of macrophage TLR tolerance. Tolerance in macrophages as a result of prolonged exposure to ligands for TLRs has long been seen as a means to limit excessive acute inflammation in cases of disseminated infection. At the same time, not all TLR-responsive genes are “tolerizable” [42], although the mechanism for the discrimination between tolerized and non-tolereized genes is far from clear. Our work suggests that a macrophage made “tolerant” by exposure to TLR2 ligands may, in fact, selectively prime some classes of promoters, such as those responsive to IRF3, by up-regulating certain downstream signaling nodes (e.g., TRAF3) and in this way render the host hyper-vigilant to viral infection.

Materials and Methods

Ethics statement

Animal work performed for this study complied with all applicable provisions of the Animal Welfare Act, the U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training, the Public Health Services (PHS) Policy on the Humane Care and Use of Laboratory Animals and the Guide for the Care and Use of Laboratory Animals (8th Ed.). The IACUC protocol governing this work was reviewed by the IACUC committee of the University of Maryland Baltimore School of Medicine. IACUC protocol A2000-01. This committee specifically approved the protocol for this work.

Cell lines and mice

Primary murine peritoneal macrophages were prepared as described previously [20]. Briefly, 3 ml of 3% sterile fluid thioglycollate (Remel) was injected i.p. into 6–8 wk old, wild-type (WT) C57BL/6J mice or BALB/cJ (Jackson Laboratories, Bar Harbor, ME). Four days later, macrophages were harvested by peritoneal lavage with sterile saline. IFN-β-null mice (IFN-β−/−) and MyD88-null mice (MyD88−/−), backcrossed onto a C57BL/6J background (N=8), were bred in-house as described previously [43]. IFNAR−/− mice and control littermates were a kind gift of Dr. Matthew Frieman (University of Maryland Baltimore). TLR2 null mice (TLR2−/−) were a kind gift of Dr. Rose Viscardi (University of Maryland Baltimore). TRAF3−/− and littermate control MEFs were a kind gift of Dr. Genhong Cheng (UCLA Medical School) and were maintained in DMEM (BioWhittaker).
Figure 7. TLR2 enhances RLR expression and signaling. (A) Primary peritoneal macrophages were treated overnight with media alone or 250 ng/ml Pam3Cys and were analyzed by Western blot for expression of RLR family members. (B–D) Macrophages treated with media or Pam3Cys overnight were transfected with HMW poly I:C, (B) LMW poly I:C, (C) or non-transfected extracellular poly I:C (D) and analyzed for IRF3 activation by Western blot. (E) Cells treated as in (B) were used to examine gene expression by qRT-PCR. (F) Cells treated as in (C) were used to examine gene expression by qRT-PCR. (G) Cells treated as in (D) were used to examine gene expression by qRT-PCR. Experiments were performed three times with a representative experiment shown for each.

doi:10.1371/journal.ppat.1003479.g007
Figure 8. TRAF3 expression is elevated by TLR2 priming and over-expression is sufficient to potentiate TLR- and RLR-mediated IRF3 activation. (A) Primary peritoneal macrophages treated overnight with media alone or 250 ng/ml Pam3Cys were washed 3X with PBS and were harvested at the indicated times and analyzed by qRT-PCR for TRAF3 expression. (B) Macrophages were treated overnight with Pam3Cys and harvested at the indicated times. TRAF3 and TRAF6 expression was determined by Western blot. (C) Macrophages were treated with Pam3Cys or LPS for 60 min and harvested. Total and phospho-IRF3, total and phospho-TBK-1, and TRAF3 expression was determined by Western blot. (D) Macrophages were transfected with TRAF3 expression vector or empty vector and treated with LPS or HMW pI:C for the indicated times. Total and phospho-IRF3, total and phospho-TBK-1, and TRAF3 expression was determined by Western blot. (E) Macrophages were treated with LPS or HMW pI:C for 6 hours and TRAF3 expression was determined by qRT-PCR. (F) Macrophages were treated with LPS or HMW pI:C for 6 hours and TRAF3 expression was determined by qRT-PCR.
supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. Lys-Cre TRAF3<sup>fl<sup>ox<sub>lox</sub></sup></sup> macrophages were generated by Dr. Ping Xie (Rutgers University). HEK293T (ATCC, Manassas, VA) cells were cultured in DMEM (BioWhittaker) supplemented with 10% FBS, (Serine 396), total IRF-3, phospho-ERK1(Thr202)/ERK2(Thr202) kinase (Invitrogen Life Technologies), as recommended by the manufacturer. Viral infections: Vesicular Stomatitis Virus (Indiana Strain; ATCC) was grown and tiered as previously described [45]. For infection of primary macrophages, macrophages were washed 1X with PBS and infected at the indicated multiplicity of infection (MOI) in serum-free RPMI for 1 hr at 37°C with occasional rocking. Infection media was removed and cells were cultured for an additional 24–48 hrs in RPMI containing 2% FBS. For in vivo infection, BALB/c mice were first anesthetized with isofluorane and subsequently infected intranasally (i.n.) with virus resuspended in PBS as described [43]. Influenza strain A/PR/8/34 ("PR8") was obtained from the ATCC and propagated as described previously [46] and was the kind gift of Dr. Donna Farber (Columbia University, NY). In vitro infections were conducted as described above for VSV. Vaccinia Virus (Western Reserve) was obtained from the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: Vaccinia WR from Dr Bernard Moss. In vitro Vaccinia infections were performed as described for VSV. UV inactivation of vaccinia was carried out using a StrataLinker (Stratagene corp.) at a dose of 5 J/cm².

Preparation of nuclear extracts and EMSA: Nuclear extracts were prepared using a nuclear extraction kit (Active Motif, Carlsbad, CA) according to the manufacturer’s instructions. The NF-kB consensus oligonucleotide, 5'-agggagaggtccttggcgc-3', from the murine IgkB light chain gene enhancer was synthesized by the Biopolymer and Genomics Core Laboratory (University of Maryland, Baltimore, MD). DNA probes were 32P end-labeled with T4 polynucleotide kinase (Invitrogen Life Technologies), as recommended by the manufacturer. EMSA was conducted as described previously [47].

Quantitative Real Time PCR (qRT-PCR): Total mRNA was isolated from peritoneal macrophages using TRIZOL (Invitrogen Carlsbad, CA) reagent according to manufacturer’s instructions. A total of 1 µg of RNA was utilized in oligo(dT) cDNA synthesis (Promega RT system A5500). qRT-PCR was carried out using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems) utilizing SYBR Green Reagent (Applied Biosystems) and transcript-specific primers. mRNA expression profiles were normalized to levels of the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT) in each sample and the fold change in expression was calculated by the 2<sup>-ΔΔCt</sup> method [48].
Figure S1 Priming of TLR4 induced IFN-β by ligands of the innate immune system. Primary peritoneal macrophages were treated overnight either with media alone or with Pam3Cys (250 ng/ml), soluble poly I:C (50 mg/ml), E. coli LPS (25 ng/ml), recombinant Salmonella flagellin (100 ng/ml), transfected poly I:C (500 ng/ml), or transfected poly dAdT (500 ng/ml). Cells were washed extensively and re-stimulated with E. coli LPS (25 ng/ml) for two hours. Total RNA was harvested and used to analyze IFN-β expression by qRT-PCR. (TIF)

Figure S2 TLR2 priming of TLR4 induced IFN-β is type I interferon-receptor-independent. Primary peritoneal macrophages harvested from C57BL/6J WT and IFNAR−/− mice were treated overnight with media alone or with Pam3Cys (250 ng/ml) and subsequently re-stimulated with E. coli LPS (25 ng/ml) for two hours. Total RNA was harvested and used to analyze IFN-β expression by qRT-PCR. (TIF)

Figure S3 TLR2 priming of TLR4 induced IFN-β requires prolonged exposure to TLR2 ligands. Primary peritoneal macrophages were treated for the indicated times with media alone or with Pam3Cys (250 ng/ml) and subsequently re-stimulated with E. coli LPS (25 ng/ml) for two hours. Total RNA was harvested and used to analyze IFN-β expression by qRT-PCR. (TIF)

Figure S4 Loss of RNA pol II recruitment to the IL-12 p40 promoter in TLR2 primed macrophages. Primary peritoneal macrophages were treated overnight either with media alone, or with Pam3Cys (250 ng/ml), soluble poly I:C (50 mg/ml), E. coli LPS (20 ng/ml), recombinant Salmonella flagellin (100 ng/ml), transfected poly I:C (500 ng/ml), or transfected poly dAdT (500 ng/ml). Total RNA was harvested and used to analyze TRAF3 expression by qRT-PCR. (TIF)

Figure S5 Differential TRAF3 induction by ligands of the innate immune system. Primary peritoneal macrophages were treated for 8 hours either with media alone or with Pam3Cys (250 ng/ml), soluble poly I:C (50 mg/ml), E. coli LPS (25 ng/ml), recombinant Salmonella flagellin (100 ng/ml), transfected poly I:C (500 ng/ml), or transfected poly dAdT (500 ng/ml). Total RNA was harvested and used to analyze TRAF3 expression by qRT-PCR. (TIF)

Supporting Information

Chromatin immunoprecipitation assays
ChIP assays were carried out using the Active Motif (Carlsbad CA) ChIP-IT Express Enzymatic kit according to manufacturer’s instructions.

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