**Network dynamics determine the autocrine and paracrine signaling functions of TNF**

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**Abstract**

A hallmark of the inflammatory response to pathogen exposure is the production of TNF that coordinates innate and adaptive immune responses, by functioning in an autocrine or paracrine manner. Numerous molecular mechanisms contributing to TNF production have been identified, but how they function together and their relative contributions within the TNF production network remains unclear. We use an iterative Systems Biology approach of quantitative measurement and mathematical modeling to develop a quantitative understanding of each regulatory module that controls TNF mRNA synthesis, mRNA half-life, translation, protein processing, and secretion. Combing these modules yields a predictive model of TNF controlled by MyD88- and TRIF-dependent pathways. By linking the model of TNF production to models of the TLR-signaling module and the TNFR-responsive NFκB signaling module, we are able to explore computationally the possible functions of TNF during the inflammatory response to diverse TLR agonists. Contrary to expectation, we predict and then experimentally confirm that TNF’s autocrine functions have little relevance in shaping the NFκB response to lipopolysaccaride, despite the large amounts of TNF it induces. However, in response to CpG DNA, autocrine TNF is important in extending the duration of NFκB activity and shaping CpG-induced gene expression programs. These stimulus-specific autocrine and paracrine functions are determined by the stimulus-specific dynamics of TNF production interfacing with MyD88 and TRIF-pathway dynamics. Thus, the mathematical model delineates the signaling network design principles that underlie TNF’s distinct functions in the immune responses of intra-cellular and extra-cellular pathogens.

**Introduction**

Tumor necrosis factor (TNF) is a key inflammatory cytokine produced by macrophages exposed to pathogens. The Toll-Like Receptor (TLR) family of receptors recognize a variety of molecular substances derived from pathogens such as bacteria, viruses, and fungi, eliciting signaling events that coordinate inflammatory and innate immune responses (Akira 2006). TLRs are expressed in many cell types, but perhaps one of the most relevant types for the innate immune response are those of classically activated (M1) macrophages. A hallmark of M1 macrophages and a primary role that they carry out in the innate immune response is the production of pro-inflammatory cytokines, including the ubiquitously expressed TNF (Dosser 2008, Parameswaren 2010). Proper control of the signaling pathways activated by TLRs is of particular importance, as aberrant signaling and chronic TNF production can lead to disease states such as Crohn's disease, rheumatoid arthritis, and cancer (MacDonald 1990, Murch 1993, McInnes 2007, Waters 2013). In macrophages, TLRs utilize two adaptors which mediate the signaling events leading to pro-inflammatory cytokine production: TRIF and MyD88 (Hoebe 2003, Yamamoto 2003, Sato 2003, Kawai 1999, Hacker 2000). While all TLRs with the exception of TLR3 use the adaptor MyD88, TLR4 uniquely uses both MyD88 and TRIF, which signal from the cell membrane and endosome, respectively (Akira 2006). These adaptors mediate the activation of transcription factors such as NFκB and IRF3, both of which have been implicated in the control of TNF production (Wesche 1997, Yamamoto 2003, Drouet 1991, Covert 2005, Lee 2009).

Gene transcription is not the only level of control of TNF production, however; TNF production has been shown to be regulated post-transcriptionally through the control of its mRNA half-life, protein translation, and secretion (Han 1991, Andersson 2006, Black 1997). In unstimuated macrophages TNF mRNA has a relatively short half-life, but upon LPS stimulation, TNF mRNA half-life increases as much as six-fold through the dowregulation of mRNA degradation pathways (Carballo 1998, Kotylarov 1999, Lai 1999, Kontoyiannis 1999, Mackenzie 2002, Stoecklin 2004, Hitti 2006, Ronkina 2007, Hao 2009). At the level of protein translation, TNF production is modulated in response to LPS through the activation of the translation initiation factor eIF4E. TNF is expressed as a membrane-bound form known as pro-TNF, which is cleaved and secreted by the catalase TACE, the activity of which is unregulated following LPS stimulation (Black 1997, Soond 2005, Xu 2010). Previous reports have sought to determine whether the adaptors TRIF or MyD88 are responsible for these post-transcriptional production control mechanisms, but the conclusions have been mixed; while some reports have argued that TRIF is essential for TNF mRNA half-life control, others have suggested that TRIF is dispensable, or that MyD88 is indeed necessary (Data 2004, Hitti 2006, Ronkina 2007, Gais 2010, Wang 2011). Given the different conclusions that have been made in various cellular systems of which adaptor controls each of these post-transcriptional processes, we sought to characterize the TRIF and MyD88-mediated mechanisms of TNF production control in primary macrophages.

The paracrine signaling functions of cytokines such as TNF, a cytokine that is abundantly produced by macrophages to alert diverse cell types of pathogen infection, play a key role in the determination of inflammatory states. However, the autocrine signaling functions of cytokines, whereby cells activated by pathogens can respond to the cytokine that they secrete, has been documented as an essential aspect of TLR-induced inflammatory signaling (Wu 1993, Blasi 1994, Xaus 2000, Coward 2002, Kuno 2005, Lombardo 2007). While these autocrine functions have been explored for TNF, it is not clear whether there is a stimulus-specific encoding of the balance between autocrine and paracrine TNF signaling. In this paper, we investigated autocrine and paracrine TNF signaling functions in response to TLR agonists in the context of NFκB signaling, revealing that the kinetics of TRIF- and MyD88-mediated TNF modulation plays an essential role in the balance of autocrine and paracrine TNF signaling back to the NFκB system.

A fruitful trend in signaling biology has been the approach to combine experiment studies with computational models to achieve quantitative and qualitative insights that would not be possible with either alone, an approach that our lab has used extensively (Werner 2005, Kearns 2006, O’Dea 2007, Werner 2008). By constructing simple mathematical models describing regulatory modules identified by experimental studies, and linking these building blocks, we are able to build computational modules of signaling networks that may be used to develop novel predictions that can be tested experimentally. In order to characterize the production of TNF in macrophages in the context of TLR-induced signaling, we experimentally identified TRIF and MyD88-controlled signaling processes in the production of TNF, and built mathematical models to describe these mechanisms. We then integrated these TNF production modules with a model for TLR4-mediated NFκB activation (Frank REF), as well as one for TNFR-mediated NFκB activation (Werner 2008) to characterize TNF feedback. Using this iterative computational and experimental approach allows us to characterize TNF production and feedback in a TLR stimulus-specific manner.

**Results**

*Dynamics of TNF production are dependent on kinetics of TRIF and MyD88 mediated signaling events*

To investigate the TRIF- and MyD88-specific control mechanisms and temporal dynamics of TNF production in TLR signaling, we used the TLR agonist that utilizes both TRIF and MyD88: lipopolysachharide (LPS) (Poltorak 1999, Kawai 1999, Alexopolou 2001, Yamomoto 2003). These two adaptors have been shown to activate multiple kinase signaling pathways, including IKK and TBK1 which lead to the activation of the transcription factors NFκB and IRF3, respectively, as well as the MAP kinases p38 and ERK. In the context of TNF production, p38 and ERK have been implicated in activating MK2, the kinase which phosphorylates TTP (Stoecklin 2004, Hitti 2006, Ronkina 2007, Deleault 2008). In unstimulated cells, TTP binds the ARE elements in the 3’end of TNF mRNA, designating it for degradation. Upon phosphorylation, TTP undergoes a conformational change, which prevents it from binding TNF mRNA, allowing for the prolonging of TNF mRNA half-life (Stoecklin 2004). Further, p38 and ERK have been shown to control TNF translation through the initiation factor eIF4E and TNF secretion through the enzyme TACE (Andersson 2006, Black 1997, Snood 2005, Xu 2010). This allows us to establish a signaling network that chronicles the four primary steps in TNF production: gene transcription, mRNA stabilization, translation, and secretion (Fig 1A). While the immediate effectors of stabilization, translation, and secretion are well-supported, it still remains unclear to what extent these processes are controlled by p38 or ERK, and more significantly, whether they are mediated by MyD88, TRIF, or some combination of the two. Therefore, this present study will experimentally characterize the complex mechanisms leading to TNF production. Further, the experimental quantification of these mechanisms will be incorporated into computational modules for TNF transcription, mRNA stabilization, translation, and secretion that can recapitulate the experimental results and make predictions about TLR-induced autocrine and paracrine TNF production in the context of NFκB signaling.

Bone Marrow Derived Macrophages (BMDMs) were derived from wild-type mice, and time course stimulations with LPS were performed. Numerous reports have investigated the dynamics of TNF production through the signaling adaptors TRIF and MyD88, but it is still unclear how TNF production control in response to TLR agonists occurs in macrophages. To investigate the processes of TNF production that TRIF and MyD88 each control, wild-type, *TRIF-/-*, and *MyD88-/-* BMDMs were stimulated with LPS and TNF secretion in the supernatant was measured by ELISA. While both *TRIF-/-* and *MyD88-/-* have significant defects in TNF secretion, *TRIF-/-* surprisingly exhibited lower TNF secretion than *MyD88-/-* (Fig 1B). To determine if the defects in TNF secretion seen in TRIF*-/-* and *MyD88-/-*  is due to decreased mRNA production, BMDMs were stimulated with LPS and mRNA levels were measured by RT-PCR (Fig 1C). Unsurprisingly, *MyD88-/-* had decreased TNF mRNA production compared to wild-type, showing no early mRNA production within 30 minutes, and reaching its peak around 1 hour. However, *TRIF-/-* BMDMs had only a small defect in TNF mRNA production, suggesting that TRIF may control translational or post-translational processing of TNF. To determine the TNF RNA synthesis rates, wild-type, *TRIF-/-*, and *MyD88-/-* BMDMs were stimulated with LPS and nascent transcripts collected. TNF RNA was measured by RT-PCR, revealing that while *MyD88-/-* showed significantly decreased TNF RNA for the first 25 minutes of LPS stimulation, nascent TNF RNA levels were slightly increased over wild-type after 60 minutes of LPS stimulation (Fig 1D). In contrast, *TRIF-/-* BMDMs exhibited slightly increased TNF RNA levels over wild-type for the first 30 minutes of LPS stimulation, decreasing compared to wild-type after 60 minutes. The fact that *TRIF-/-* have increased nascent TNF RNA for the first 30 minutes of LPS stimulation compared to wild-type but decreased total mRNA production throughout the time course suggests that TRIF may control post-transcriptional processing of TNF as well.

*TNF mRNA production is regulated by NFκB, but not IRF.*

In addition to leading to the activation of NFκB, the signaling adaptor TRIF also activates the transcription factor IRF3, leading to production of IFN-B and activation of the IFNAR signaling pathway. Previous reports had suggested that in addition to NFκB, IRF3 activation was an important factor in TNF production in response to TLR agonists such as LPS (Covert 2005). To investigate whether TNF gene transcription was controlled by solely NFκB or by IRF3 as well, Fetal Liver Derived Macrophages (FLDMs) were derived from wild-type and *RelA-/-RelB-/-cRel-/-* mice and stimulated with LPS. Total mRNA levels were measured by RT-PCR, revealing that the *RelA-/-RelB-/-cRel-/-* FLDMs have no appreciable TNF mRNA production, supporting the model that NFκB is essential for TNF gene transcription (Fig 1E). To investigate whether IRF3 was involved in TNF gene transcription, *IRF3-/-IRF7-/-* BMDMs were stimulated with LPS and TNF mRNA production determined by RT-PCR. This revealed that *IRF3-/-IRF7-/-* had no defects in TNF mRNA production (Fig 1F). To ensure that this was true for nascent TNF production as well, nascent transcript analysis in *IRF3-/-IRF7-/-* BMDMs stimulated with LPS demonstrated that there is no defect in TNF gene transcription and that IRF3 activation is not needed for TNF production (Fig 1G).

As NFκB was confirmed to be the transcription factor solely responsible for TNF transcription, we sought to characterize NFκB activation in *TRIF-/-* and *MyD88-/-* BMDMs in response to LPS. Electrophoretic Mobility Shift Assay (EMSA) using a HIV G1G2 probe with κB binding sites revealed that NFκB activation is decreased in both *TRIF-/-* and *MyD88-/-* (Fig 1H). The *TRIF-/-* have normal early activation (0-30 minutes) but significantly decreased activation following; conversely, *MyD88-/-* have decreased early activation, but late activation (45 minutes to 4 hours) is unchanged compared to wild-type. Given that TRIF and MyD88-mediated NFκB activation is what drives the transcription of the TNF gene, this allows us to create a computational module for nascent TNF RNA using NFκB activity as an input (Fig 1I). This simple model consists of a TNF gene containing two κB sites, where NFκB is able to bind. Upon NFκB binding, the activity of which is determined through quantification of EMSA experiments, nascent TNF RNA is produced, which can be processed into mRNA. In this mathematical model, the rate of nascent TNF transcription can be determined by a Hill equation based on NFκB activity and promoter binding sites, and a mass action equation for nascent processing. Previous work as shown that PKR activity is important for processing nascent TNF RNA to mRNA (Osman 1999), and that PKR activation is at least partially mediated by MyD88 (Horng 2001). Given that MyD88 and TRIF both contribute to p38 and ERK activation, which are upstream of PKR, in the computational module MyD88 and TRIF equally contribute to nascent RNA processing. Using experimentally determined NFκB activity in LPS-induced signaling as an input, this model is able to recapitulate the experimentally determined nascent TNF RNA transcription seen in WT, *TRIF-/-*, and *MyD88-/-* BMDMs (Fig 2F).

*TRIF controls TNF mRNA stabilization, translation, and protein secretion stimulus-specifically*

Previous reports have shown that the half-life, translation, and secretion of TNF mRNA can be modulated during TLR signaling. While it is clear that these processes are important for the temporal dynamics of TNF production in TLR signaling, what is not clear is whether MyD88, TRIF, or some combination of the two adaptors control these regulation steps. The discrepancy between nascent TNF RNA production levels and whole-cell TNF mRNA levels in *TRIF-/-* BMDMs in response to LPS, prompted us to investigate which stimuli activate the pathways leading to TNF mRNA stabilization, translation, and protein secretion, as well as which TLR adaptor, or combination of both, control these processes.

To investigate the stimulus-specific half-life control of TNF mRNA, wild-type BMDMs were stimulated with TNF alone, which induces TNF mRNA expression but not TNF mRNA stabilization, or TNF and LPS. Wild-type BMDMs were treated with stimulus for 30 minutes, and then treated with actinomycin-D, a drug which intercolates into DNA and arrests transcription. TNF mRNA levels are measured by RT-PCR in 15 minute increments following actinomycin-D treatment, and trend lines constructed to determine mRNA half-lives. Stimulation with TNF set a baseline of constitutive TNF mRNA half-life of around 10 minutes (Fig 2A). When stimulated in conjunction with LPS, the half-life of TNF mRNA increased 3.5 fold to 35 minutes. To determine whether this LPS-induced stabilization of TNF mRNA was TRIF or MyD88-specific, we stimulated *TRIF-/-* and *MyD88-/-* BMDMs with LPS followed by treatment with actinomycin-d. This revealed that while the *MyD88-/-* showed no decrease in LPS-induced TNF mRNA half-life compared to wild-type, the *TRIF-/-* showed a complete loss of the LPS-induced mRNA stabilization, with a half-life of 10 minutes. This data demonstrates that TRIF, and not MyD88, is necessary for TNF mRNA stabilization in macrophages. To determine whether this stabilization was p38-dependent, 30 minutes prior to LPS stimulation, wild-type BMDMs were treated with p38 inhibitor. After actinomycin-D treatment, p38-inhibitor treated TNF mRNA half-life was determined by RT-PCR to be around 13 minutes, showing that the TRIF mediates TNF mRNA stabilization through p38 (Figure 2B).

As p38 and ERK pathways have been implicated in the control of post-transcriptional processing of TNF mRNA, and both TRIF and p38 are essential for stabilization of TNF mRNA, we sought to characterize the activation of the p38 and ERK pathways. Since 30-90 minutes after LPS stimulation is the timeframe within the mRNA half-lives are measured, western blots for phopsho-p38 and phospho-ERK in LPS stimulated wild-type, *TRIF-/-*, and *MyD88-/-* BMDMs were performed (Fig 2C). This revealed that from 30 to 75 minutes, *TRIF-/-* BMDMs have decreased p38 activation. Similarly, while ERK activation dynamics are more transient than p38 activation dynamics, *TRIF-/-* BMDMs showed decreased ERK activation from 30-60 minutes. Given that previous reports had showed that the MAPK target MK2 is important for TNF mRNA stabilization and translation, we performed western blots for phospho-MK2 as well. This revealed that while the *MyD88-/-* may have slightly decreased activity at 30 minutes only, the *TRIF-/-* have decreased MK2 activity from 30-60 minutes. To determine whether this MK2 phosphorylation is p38 or ERK-dependent, WT BMDMs were pre-treated with p38 or ERK inhibitor for 1 hour prior to LPS stimulation, and western blots for phospho-MK2 performed (Fig 2D). This revealed that p38, and not ERK, is essential for MK2 activation, as p38 inhibition completely abolished phosphorylation of MK2, while ERK inhibition had no effect. Previous reports have shown that the MK2 target TTP is a primary regulator of TNF mRNA degradation. We found that phosphorylation of TTP, which leads to its inactivation and prevents TNF mRNA degradation, is decreased in p38-inhibitor treated wild-type BMDMs stimulated with LPS, but not significantly in ERK-inhibitor treated cells.

These results are tested by a simple module for TNF mRNA half-life control, whereby TRIF leads to the activation of p38, p38 phosphorylates and activates MK2, and MK2 phosphorylates TTP which prevents TTP from binding the 3’ ARE elements in TNF mRNA, leading to the stabilization of the TNF message and an increase in half-life (Fig 2E). This module uses the experimentally determined nascent TNF RNA levels as input, with total TNF mRNA as the output. The effect of TNF mRNA stabilization can be illustrated by contrasting the output of total TNF mRNA simulations for four potential half-life control mechanisms (Figure 2F). Here, (name?) scoring reveals that the TRIF-controlled scenario best recapitulates the experimental data, confirming that although *TRIF-/-* have increased nascent TNF RNA levels compared to wild-type, they have slightly decreased mRNA levels.

While post-transcriptional control of TNF mRNA stabilization by TRIF accounts for the discrepancy between nascent TNF RNA and whole cell TNF mRNA levels in *TRIF-/-* BMDMs, these cells still show a significant lack of TNF secretion compared to wild-type cells that is not apparent at the level of mRNA production. In order to characterize adaptor-specific control TNF translation, western blots for pro-TNF expression were carried out in wild-type, *TRIF-/-*, and *MyD88-/-* BMDMs pre-treated with TACE inhibitor TAPI-1 to block secretion and stimulated with LPS. Western blots for TNF were performed, revealing that while wild-type cells produce significant amounts of pro-TNF peaking at 60 minutes, *TRIF-/-* and *MyD88-/-* have serious defects in pro-TNF expression (Fig 2G). While *MyD88-/-* show little to no TNF mRNA induction at 30 minutes so the lack of pro-TNF protein expression at 60 minutes is unsurprising, *TRIF-/-* have severely decreased pro-TNF expression, demonstrating that TRIF regulates the translation of TNF as well. Further, we sought to determine whether this regulation of translation by TRIF was mediated through p38 or ERK. Wild-type BMDMs were pre-treated with TACE inhibitor and either DMSO, p38 inhibitor, or ERK inhibitor for 1 hour followed by LPS stimulation. The western blot for pro-TNF shows that TRIF control of translation is exerted through p38 and not ERK, as the ERK-inhibitor condition showed no decrease in pro-TNF expression (Fig 2H).

Given that eIF4E and TACE have been shown to be necessary for TNF translation (Andersson 2006) and secretion (Black 1997), respectively, we next sought to characterize their activation. Previous reports have demonstrated that phosphorylation of eIF4E and TACE are necessary for the processive activity (Wang 1998, Topisirovic 2004, Fan 1999, Diaz-Rodriguez 2002, Xu 2010). Wild-type, *TRIF-/-*, and *MyD88-/-* BMDMs were stimulated with LPS and western blots for phospho-eIF4E and phospho-TACE performed (Fig 2I). Here, *MyD88-/-* show decreased eIF4E phosphorylation around 30-45 minutes, while *TRIF-/-* show significantly decreased eIF4E phosphorylation from 60-75 minutes. While both adaptors contribute to TNF translation, the levels of TNF mRNA able to be translated are significantly lower at 30 minutes, where MyD88 primarily contributes to eIF4E activity, than at 60 minutes, where TRIF primarily contributes to eIF4E activity. Further, western blots for TACE phosphorylation were performed. While wild-type and *MyD88-/-* cells exhibited significant TACE phosphorylation, peaking at 75 minutes, the *TRIF-/-* cells showed decreased TACE phosphorylation in comparison. To determine whether TRIF-controlled phosphorylation of TACE was mediated through p38 or ERK MAPK, wild-type BMDMs were pre-treated with p38 or ERK inhibitor for one hour, followed by stimulation with LPS (Fig 2J). This revealed that both p38 and ERK have an effect on TACE phosphorylation, as inhibition of p38 or ERK decreased TACE phosphorylation. However, it is ERK that is essential for TACE phosphorylation, demonstrated by the lack of induction of TACE phosphorylation seen in the ERK inhibitor pre-treated condition.

These experimental results led us to construct a computational module for the control of TNF translation and secretion. In this module, TRIF leads to the activation of p38 and ERK pathways; p38 controls the activity of eIF4E through phosphorylation which promotes TNF translation, whereas ERK and, to a lesser extent, p38, controls the activity of TACE through phosphorylation which promotes the cleavage and secretion of TNF (Figure 2K). This module uses experimentally determined LPS-induced mRNA levels as an input, allowing us to make computational simulations of the role that TRIF plays in promoting TNF translation and TNF secretion (Fig 2I). These simulations illustrate that TRIF-mediated control of translation and secretion is necessary to recapitulate the experimentally determined levels of pro-TNF expression and TNF secretion, confirming that TRIF controls TNF translation and secretion.

*A Mathematical model of TLR agonist-responsive TNF production.*

Computationally modeling inflammatory processes and immune signaling networks presents the challenge of creating models that can be predictive and recapitulate experimental data without becoming prohibitively large in scale. Further, modeling a signaling process, such as the production of a cytokine in response to TLR agonists, needs to be placed in the wider context of the inflammatory signaling network of which it is involved. In this paper, we have quantified experimental results to inform computational modules for key steps in the production of TNF. These individual modules not only allow for the determination of which signaling events are sufficient for each respective step in TNF production, but as the output of each module is also the input for each subsequent one, also allow for the construction of a model that predicts TNF production that aligns with experimental results. Our lab has demonstrated the benefits to using this modular approach to signaling networks previously, publishing modules for TNFR and TLR-induced signaling (Werner 2008, Zhang REF). Given the modular nature of these computational models, this allows us to place our computational characterization of TNF production within the larger context of TLR and TNFR signaling.

To characterize TLR-induced TNF production, we connected our three modules for each step in TNF production with the model for TLR-induced NFκB activation coordinated through MyD88 and TRIF, creating one model that computationally characterizes TLR agonist-induced TNF (Figure 3A). Computationally simulating LPS-induced TNF production in the wild-type demonstrates that the model is able to recapitulate the experimental data at the level of nascent RNA, mRNA, pro-TNF production, and TNF secretion (Figure 3B). Expanding the computational simulations to the *TRIF-/-* and *MyD88-/-* conditions, however, reveals that while the model is able to capture TRIF dynamics (in the *MyD88-/-*), it is not able to accurately capture MyD88 dynamics, particularly at the level of nascent RNA and mRNA production (Figure 3C). To investigate these adaptor-specific differences further, we then used the model to predict the TNF production dynamics of two other TLR agonists, PolyI:C (TLR3/TRIF agonist) and CpG DNA (TLR9/MyD88 agonist). Here, the model was able to successfully predict PolyI:C-induced TNF production at the level of mRNA and protein secretion (Figure 3D). However, the current form of the model was not able to recapitulate CpG-induced dynamics of TNF mRNA production or secretion . Given the previous reports on the ability of TNF to signal in an autocrine manner, we posited that perhaps autocrine TNF in response to CpG led to the persistence of TNF mRNA and protein secretion, so we sought to investigate this further.

*Autocrine function of TNF augments NFκB activation in response to CpG.*

Incorporating these three modules into one signaling network in a straightforward, stepwise fashion allows us to characterize the temporal dynamics of TNF production. Reports suggest that TNF may signal in an autocrine fashion and play a role in augmenting NFκB activation in response to certain stimuli conditions. The nature of the TNF model, with various TLR-agonist inputs that induce different adaptor-mediated kinetics, gives us the ability to investigate the potential autocrine function of TNF and make predictions that can be tested experimentally. Therefore, we expanded the computational model to include autocrine TNF signaling by incorporating the module for TNFR-mediated NFκB activation (Werner 2008) (Figure 4A). We then simulated the model for LPS, CpG, and PolyI:C stimulation conditions to determine whether autocrine feedback in the model would allow for a better prediction of experimental data. Here, we found that autocrine TNF signaling had little effect on levels of the LPS and PolyI:C conditions, but drastically improved the ability of the model to predict CpG-induced TNF mRNA production and protein secretion (Figure 4B). To determine whether this prolonging of CpG-induced dynamics was due to persistent NFκB activity induced by autocrine TNF, we simulated the model for NFκB activation with and without TNF autocrine feedback. In this scenario, the model predicts that while LPS-induced NFκB will not be affected by the loss of autocrine TNF, the model predicts that CpG-induced autocrine TNF is needed for persistent NFκB activity (Figure 4C). To test this experimentally, NFκB activity was measured in the absence of TNF autocrine signaling using *tnf*-/- mice, which are deficient in TNF production. As in previous experiments, BMDMs from wild-type and *tnf*-/- mice were generated, and stimulated with either LPS or CpG. EMSAs for NFκB activation confirmed the computational prediction, demonstrating that while *tnf*-/- BMDMs did not have reduced NFκB activation in response to LPS, they did have reduced NFκB activation in response to CpG from 4-8 hours (Fig 4D). This aligns with the result from the model, which predicted that stimuli that signal transiently, such as a CpG mediated through MyD88, would be more dependent on TNF autocrine feedback for late NFκB activation. This result suggests that in response to CpG, secreted TNF serves a primarily autocrine role.

*TLR-agonist induced kinetics of TNF production encodes paracrine and autocrine functions stimulus-specifically*

Computational simulations of the TNF production model led to the prediction that sustained NFκB activity in response to CpG would be dependent on CpG-induced TNF autocrine signaling, which was confirmed by experiments in *tnf-/-* BMDMs. As NFκB is a transcription factor that controls many inflammatory genes, we next sought to determine the role that CpG-induced autocrine TNF signaling plays at the level of gene transcription. To do this, wild-type and *tnf-/-* BMDMs were stimulated with CpG up to 24 hours, RNA extracts collected, and RNA-seq performed. Analysis of RNA-seq data revealed that 267 genes were significantly upregulated by CpG stimulation (Figure 5A). K-means clustering produced 6 clusters of genes, clusters characterized by either a strong early peak induction (cluster 5), a peak at 8 hours but persistent induction (cluster 2 and 6), a peak at 8 hours followed by a decrease by 24 hours (clusters 1 and 4), or a slow induction resulting in a peak at 24 hours of stimulation (cluster 3). Of these CpG-induced genes, xxx were found to be significantly decreased in the *tnf-/-* condition. Not surprisingly, numerous genes found to be decreased in the *tnf-/-* are involved in controlling inflammation and cell-cell signaling events during inflammatory processes (Figure 6B). Further, a number of the genes are known-NFκB controlled genes, demonstrating that TNF autocrine signaling not only has a general effect on prolonging the inflammatory state, but that the observed decrease in NFκB activity seen in *tnf-/-* BMDMs stimulated with CpG leads to a phenotype of decreased NFκB.

Next, we sought to investigate the paracrine role that secreted TNF serves in response to LPS and CpG. Tissue-resident macrophages exist in an environment where they secret cytokines and signal to other cell types in the tissue, such as fibroblasts, which respond and are activated by the macrophages signals. To construct an experimental system that mimics this tissue-resident macrophage environment, BMDMs generated from *tnfr-/-* mice were co-cultured with *myd88-/- trif-/-* 3T3s, the latter of which cannot activated NFκB. In this setup, the initial stimulus (LPS or CpG) activates the *tnfr-/-* BMDMs, but not the *myd88-/- trif-/-* 3T3s. However, the TNF secreted by the *tnfr-/-* BMDMs is able to activate the *myd88-/- trif-/-* 3T3s, which is measured by immunofluorescent staining for NFκB subunit p65 (Fig 5C). This microscopy experiment reveals that LPS-induced TNF secretion plays a strong paracrine role, as more *myd88-/- trif-/-* 3T3s near TNF-secreting BMDMs show significant p65 nuclear translocation (Fig 5D). However, in the CpG-stimulated condition, *myd88-/- trif-/-* 3T3s showed less p65 translocation the LPS-stimulated condition. These results lead us to conclude that LPS-induced TNF secretion plays a primarily paracrine role, while CpG-induced TNF secretion plays an autocrine role in NFκB activation.

**Discussion**

Macrophages play an early, key role in the innate immune response to a variety of pathogens through the activation of TLRs. In this paper, we investigated the mechanisms of production of one ubiquitous cytokine secreted by macrophages, TNF, and the autocrine and paracrine roles that it plays in innate immunity. While previous reports were unclear to what extent the adaptors TRIF and MyD88 control TNF mRNA induction, mRNA stabilization, translation, and secretion, here we describe in mechanistic detail how these two adaptors contribute to TNF production in response to TLR agonists. Furthermore, we use biochemical rates and mechanistic insights to build computational modules for each step in TNF production, culminating in a mathematical model for TNF production in the context of TLR-induced NFκB activation and TNF autocrine feedback. Simulating this mathematical model allowed us to make computational predictions of how autocrine TNF signals in a stimulus-specific manner, a prediction validated through experimentation in *tnf-/-* and macrophages. Finally, as CpG-induced autocrine TNF signaling leads to sustained NFκB activity, we investigated gene induction in the absence of TNF by RNA-Seq, demonstrating that autocrine TNF produced in response to CpG is important for multiple stages of the macrophage inflammatory and innate immune response.

While there have been many reports concerning the mechanisms by which TNF production is modulated in response TLR agonists, the cell systems used were diverse; as a result, it was unclear whether these mechanisms are stimulus-specifc or whether they are controlled by TRIF or MyD88 in macrophages. In this paper, we demonstrate that while MyD88 is primarily responsible for early nascent TNF RNA induction as well as TNF mRNA production, TRIF is essential for the activation of post-transcriptional mechanism that promote the processing of TNF: mRNA stabilization, pro-TNF translation, and TNF secretion. Stabilization of TNF mRNA, leading to the increase in TNF mRNA half-life, is controlled through a TRIF-p38-pMK2 axis whereby TRIF leads to the activation of MAP kinases which downregulate mRNA degradation mechanisms. Further, the translation of TNF mRNA to pro-TNF is shown to be controlled by TRIF, as TRIF deficient macrophages have significantly decreased pro-TNF expression than would be predicted by computational simulations given the relatively high levels of TNF mRNA seen in TRIF deficient cells. At the level of TNF secretion, the enzyme TACE has been previously shown to cleave membrane-bound pro-TNF, and that it is controlled by p38 and ERK. Here, we confirm that both p38 and ERK activity are needed for proper TACE activation, and that TACE activation is primarily controlled by TRIF. These three TRIF-controlled post-transcritional mechanisms are essential for proper temporal kinetics of TNF production in response to LPS, as a deficiency in TRIF leads cells to secrete significantly less TNF protein despite their near-wild-type levels of TNF mRNA.

A hallmark of the systems biology approach is the fruitful collaboration of experimental and computational approaches to characterize signaling networks in a quantitative manner. However, the vast and interconnected nature of immune signaling networks does not lend them well to comprehensive and predictive studies; computational networks that sufficiently account for signaling processes are difficult to develop. One approach that was presented early on in the move towards systems biology is ‘modular’ biology (Hartwell 1999). In this framework, signaling networks can be broken down into discrete modules that describe separate molecular events, which can be mechanistically characterized and quantified through experimentation. The essence of the systems biological approach is that rather than studying individual systems in isolation, systems are studied through quantitative experimentation in order to characterize their context and the system as a whole. In this study, we break down TNF production into three discrete modules: transcription, mRNA stabilization, and translation/secretion. The architecture for these individual modules have been developed by investigating and quantifying stimulus-specific (LPS) and adaptor-specific (MyD88, TRIF) mechanisms. However, the fitness of signaling modules are limited unless they can be used as building blocks for larger signaling networks. To this end, we have combined the three modules together to make one stimulus-specific predictive module for TNF production. By performing this dual experimental and computational approach to characterizing TNF production with an iterative approach from the bottom-up, we are able to test the sufficiency of the network architecture by simulating the module for different stimuli.

The experimental approach revealed LPS stimulation of macrophages confers an strong, early, and persistent TNF secretion through the combination of fast MyD88-mediated NFκB activation leading to RNA transcription and later TRIF-mediated promotion of TNF processing. In contrast, while CpG induces significant NFκB activation and TNF RNA transcription rapidly, TNF secretion takes longer to reach LPS-stimulated levels due to the lack of TRIF-induced promotion of TNF processing, as TLR9 does not use TRIF as an adaptor. While the computational TNF module was able recapitulate LPS-stimulated TNF secretion dynamics, it was not able to recapitulate CpG-stimulated dynamics. This led us to incorporate this TNF production module to the previously published modules for TNFR signaling, creating a single model for NFκB activation, TNF production, and TNF feedback in TLR-induced signaling in order to account for the differential dynamics of stimulus-specific NFκB activation and TNF production. By including autocrine TNF signaling into the mathematical model, we were able to capture the dynamics of TNF secretion seen experimentally for CpG stimulation. Furthermore, a benefit of this iterative approach also led us to suspect that sustained TLR-induced NFκB activation may be dependent on autocrine TNF, a phenomenon that the model indeed predicted through simulation for CpG, but not for LPS. Testing this experimentally revealed that CpG-induced sustained NFκB activity is indeed dependent on autocrine TNF, demonstrating the robustness and predictive ability of the model.

The temporal kinetics of TLR-induced NFκB activation and TNF production is key to understanding this stimulus-specific regulation. LPS stimulation of *trif-/-* and *myd88-/-* macrophages demonstrated that MyD88-mediated NFκB activation occurs early with a sharp peak (15-45 minutes), whereas TRIF-mediated NFκB activation occurs later and is sustained for considerably longer (45 minutes to 4 hours). The combination of MyD88 and TRIF adaptors working in conjunction leads to high TNF secretion that signals in a paracrine fashion, but could potentially signal in an autocrine manner as well. However, the peak of LPS-induced TNF production occurs within a timeframe (1-2 hours) where NFκB activity is still high due to the persistence of TRIF-mediated NFκB activation. In contrast, CpG mediates only through MyD88, which induces an NFκB temporal profile that is early and strong, but decreases faster than what LPS induces. As CpG-induced TNF production reaches a peak later (4 hours), this TNF is able to signal in an autocrine manner because the MyD88-mediated NFκB activity has decreased down to a near basal. Therefore, CpG-induced autocrine TNF serves an analogous role to LPS-induced TRIF signaling: to augment and prolong NFκB activity.

In this study, we show that stimulus-specific control of TNF production in macrophages leads to differential temporal dynamics of TNF release and subsequent NFκB activation. Furthermore, these stimulus-specific TNF dynamics encode and determine the role that autocrine and paracrine TNF signaling will play during the states of macrophage inflammatory processes. This balance between autocrine and paracrine signaling has long been suggested to be important for determining macrophage function. Autocrine TNF signaling was proposed early on in the study of intracellular bacteria infections as an important feature of pathogen response (Kindler 1989), as was the need for a balance between paracrine and autocrine TNF signaling in mounting a response that addresses infection without developing autoimmune disorders (Zhan 1996). Previous studies have reported that mice with a bioactive transmembrane-bound TNF which is unable to be secreted are still able survive physiological doses of the intracellular bacteria *L. monocytogenes* (Alexopoulou 2006), even with an abrogation of paracrine TNF signaling. Another study found that constitutively-expressed transmembrane-bound TNF could signal in an autocrine manner, leading to prolonged NFκB activity (Haas 1999). However, secretion of TNF, even to signal in an autocrine manner, is likely still needed, as mice lacking TNF cleavage have reduced protection against intracellular bacteria (McIlwain 2012). This autocrine function of TNF in CpG-induced signaling has clinical relevance as well: multiple studies have reported that the drug Infliximab, an antibody that binds to soluble TNF to treat diseases like rheumatoid arthritis, makes individuals more prone to infection by intracellular pathogens like *M. tuberculosis*, *L. monotycogenes*, and *Histoplasma capsulatum* (Keane 2001, Slifman 2003, Lee 2002). Here, we present that CpG-induced autocrine TNF signals to sustain NFκB activity, leading to proper upregulation of genes controlling multiple levels of the macrophage pathogen response. The mechanistic insights of TNF production gleaned here, that TNF signaling encodes inflammatory information not only in the amount of TNF produced but in the temporal kinetics of how and when TNF is produced, should inform future studies at both the experimental and clinical level.

***Figure 1 TNF production is regulated by both MyD88 and TRIF pathways through NFκB but not IRF.* A** Diagram illustrating mechanisms potentially regulating the production of TNF; solid lines indicate known mechanisms, dashed lines indicate mechanism that have been reported in the literature in differing cell systems. **B** Secretion of TNF in cell media measured by ELISA in wild-type, *trif*-/-, or *myd88*-/- Bone Marrow Derived Macrophages (BMDMs). Cells stimulated with 10ng/mL LPS, n=3. **C** Levels of TNF mRNA (log2 fold) produced by wild-type, *trif*-/-, or *myd88*-/- BMDMs stimulated with 10ng/mL LPS, measured by RT-PCR. Wild-type, n=5. *trif*-/-, n=3. *myd88*-/-, n=3. **D** Levels of nascent TNF RNA (log2 fold) produced by wild-type, *trif*-/-, or *myd88*-/- BMDMs stimulated with 10ng/mL LPS, measured by RT-PCR. Wild-type, n=3. Nascent transcripts measured by RT-PCR with intron-exon spanning primers. Error bars indicated 1 standard deviation. **E** TNF mRNA levels (fold) measured RT-PCR in wild-type or *rela-/-relb-/-crel*-/- Fetal Liver Derived Macrophages (FLDMs) stimulated with 100ng/mL LPS (n=1). **F** Levels of TNF mRNA (log2 fold) produced by wild-type or *irf3-/-irf7-/-* BMDMs stimulated with 10ng/mL LPS, measured by RT-PCR (wild-type, n=5; *irf3-/-irf7-/*-, n=3). **G** Levels of nascent TNF RNA (log2 fold) produced by wild-type or *irf3-/-irf7-/-* BMDMs stimulated with 10ng/mL LPS, measured by RT-PCR as in Figure 1 (n=3). **H** Activation of NFκB measured by EMSA (G1G2 κB-containing HIV probe) in wild-type, *trif* -/-, or *myd88*-/- BMDMs stimulated with 10ng/mL LPS. Arrow indicates p65-p50 dimer. Right, quantification of NFκB EMSA bands normalized to peak activity (n=3). **I** Schematic module of the computational model for transcription of nascent TNF RNA; input is quantified NFκB activation data from 2D, output is nascent TNF RNA. **J** Lines: computational simulations of the model in 2E for nascent TNF in wild-type, trif-/-, or *myd88*-/- genotypes stimulated by 10ng/mL LPS. Data points: experimental data for nascent TNF RNA in wild-type, *trif* -/-, or *myd88*-/- BMDMs stimulated with 10ng/mL LPS as reported in 1D. For all graphs, error bars indicated 1 standard deviation.

***Figure 2 TRIF regulates TNF mRNA half-life, translation, and protein secretion.* A** TNF mRNA half-life measure by RT-PCR. wild-type, *trif* -/-, or *myd88*-/-  BMDMs pre-stimulated for 30 min with 10ng/mL TNF alone (-) or 10ng/mL TNF and 10ng/mL LPS (LPS) and then treated with actinomycin-d to arrest transcription (wild-type, n=5; *trif* -/-, n=4; *myd88*-/- , n=3). **B** TNF mRNA half-life measure by RT-PCR in wild-type BMDMs. Cells pre-stimulated with 10ng/mL TNF alone (-), 10ng/mL TNF and 10ng/mL LPS (LPS), or 10ng/mL TNF, 10ng/mL LPS, and 10µM p38-inhibitor for 30 min, followed by actinomycin-d treatment (TNF, n=5; LPS, n=5; p38, n=2). **C** Western blots for phospho-p38, phospho-ERK, phospho-MK2, and actin in wild-type, *trif* -/-, or *myd88*-/- BMDMs stimulated with 10ng/mL LPS. Blots shown representative of >3 experiments. Below, quantification of western blots normalized to wild-type peak phosphorylation. Error bars indicate 1 standard deviation; \* indicates a p value < 0.05, \*\* indicates a p value < 0.02 for difference between wild-type and *trif* -/- timepoints. **D** Western blots of phospho-MK2, phospho-TTP, and actin in wild-type BMDMs pre-treated with DMSO, 10µM p38 inhibitor, or 10µM ERK inhibitor for 1 hour followed by stimulation with 10ng/mL LPS. **E** Schematic of the module 2 for TRIF-mediated stabilization of TNF mRNA; input: nascent TNF RNA from simulations of module 1; output: TNF mRNA. **F** Lines: simulations of module 2 for TNF mRNA production in the wild-type, *trif* -/-, or *myd88*-/- genotype in response to 10ng/mL LPS with either no stabilization control (top left), stabilization by TRIF and MyD88 (top right), stabilization by MyD88 alone (bottom left), or stabilization by TRIF alone (bottom right). Data points: experimental data for TNF mRNA in wild-type, *trif* -/-, or *myd88*-/- BMDMs stimulated with 10ng/mL LPS as reported in 1C. **G** Left: Westernblot for proTNF and actin in wild-type, *trif* -/-, and *myd88*-/- BMDMs stimulated with 10ng/mL. Data representative of 3 experiments. Right: Quantification of proTNF bands normalized to peak wild-type protein levels. Error bars indicate 1 standard deviation; \* indicates a p value < 0.05, \*\* indicates a p value < 0.02 for difference between wild-type and trif-/- timepoints. **H** Westernblot for proTNF and actin in wild-type BMDMs pre-treated with DMSO, 10µM p38 inhibitor, or 10µM ERK inhibitor for 1 hour followed by stimulation with 10ng/mL LPS. Blot is representative of 2 experiments. **I** Westernblot for phospho-eIF4E, phopsho-TACE, and actin in wild-type, *trif* -/-, and *myd88*-/- BMDMs stimulated with 10ng/mL. Phospho-eIF4E, n=2; p-TACE, n=3. **J** Westernblot for phospho-TACE and actin in wild-type BMDMs pre-treated with DMSO, 10µM p38 inhibitor, or 10µM ERK inhibitor for 1 hour followed by stimulation with 10ng/mL LPS. Blot is representative of 2 experiments. **K** Schematic of module 3 describing the promotion of TNF translation and secretion controlled by TRIF. Input: TNF mRNA levels from Module 2 simulations; Output: secreted TNF. **L** Lines: simulations of module 3 measure proTNF expression (top) and secreted TNF (bottom) with and without the promotion of TNF procession through TRIF-mediated translation and secretion regulation. Data points: experimental data for pro-TNF expression (top 2 graphs) or TNF secretion (bottom 2 graphs) in wild-type, *trif* -/-, or *myd88*-/- BMDMs stimulated with 10ng/mL LPS as reported in 2G and 1B, respectively.

***Figure 3 The multi-modular model accounts for TNF production in response to some TLR agonists but not others.* A** Schematic of the computational model combining modules for TLR receptor activation to adaptors TRIF and MyD88, activation of IKK and NFκB, and the 3 modules for TNF production. **B** Model simulations and experimental data for wild-type cells in response to 10ng/mL LPS; solid lines indicate values of model simulations, points represent experimental data represented in previous figures. **C** Model simulations and experimental data, represented as in 3A, for *trif-/-* and *myd88-/-* cells in response to 10ng/mL LPS. **D** Model simulations and experimental data for TNF mRNA and secreted TNF, represented as in 3A, for wild-type cells in response to 500nM CpG and 50µg/mL PolyI:C. For experimental data points, n=3.

***Figure 4 TLR-responsive TNF production functions in an autocrine manner in response to some TLR ligands but not others.* A** Expandedschematic of the computational model in Figure 3A, incorporating TNF autocrine feedback into NFκB. **B** Model simulations and experimental data for TNF mRNA and secreted TNF in wild-type cells stimulated with 10ng/mL LPS, 500nM CpG, or 50µg/mL PolyI:C; solid lines indicate values of model simulations, points represent experimental data represented in previous figures. **C** Model simulations for NFκB activity in wild-type or *tnf-/-* stimulated by 10ng/mL LPS or 100nM CpG. Solid lines indicate wild-type simulation, dashed lines indicate *tnf-/-*. **D** Experimental validation of model simulations in 4C. Activation of NFκB measured by EMSA (G1G2 κB-containing HIV probe) in wild-type and *tnf-/-* BMDMs stimulated with 10ng/mL LPS or 100nM CpG. Graphs are quantification of experimental data shown below, normalized to peak wild-type NFκB activation. Gel and quantification is representative of 4 experiments.

***Figure 5 TLR agonist-induced paracrine and autocrine-specific functions of TNF.* A** RNA-seq data from wild-type and *tnf-/-* BMDMs stimulated with 100nM CpG. K-means clustering led to 6 clusters. Cluster median for each cluster represents the data where the peak RNA induction has been normalized to 1, and the median for each genotype graphed. **B** Select genes that have significantly decreased RNA induction in the *tnf-/-* condition in response to CpG, grouped by the function and the reported role each plays in inflammation and macrophage function. **C** Co-culturing of *tnfr-/-* BMDMs with *trif-/-myd88-/-* 3T3s. First panel, p65-staining of *trif-/-myd88-/-* 3T3s stimulated with 1µg/mL LPS for 25 min. Second panel, co-culture of untreated *tnfr-/-* BMDMs with *trif-/-myd88-/-* 3T3s stained for p65. Third panel, co-culture of *tnfr-/-* BMDMs with *trif-/-myd88-/-* 3T3s stimulated with 1µM CpG for 75 min and stained for p65. Fourth panel, co-culture of *tnfr-/-* BMDMs with *trif-/-myd88-/-* 3T3s stimulated with 1µg/mL LPS for 75 min and stained for p65. Images representative of 4 separate experiments. **D** Bar graphs showing the average number of cells with nuclear p65 in a given field of view, 20-30 images for each experiment. error bars indicate 1 stardard deviation from the mean, n=4

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