**Stimulus-specific autocrine and paracrine functions of macrophage-produced TNF**

**TNF’s autocrine and paracrine functions are determined by pathogen-specific signaling dynamics within the inflammatory network**

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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 auto- 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 production in response kinase activities controlled by MyD88- and TRIF-dependent pathways. By linking the model of the TNF production to models of the TLR-signaling modules, and TNFR-responsive NFκB signaling modules, we are able to explore computationally the possible functions of TNF during the immune response to diverse PAMPs. Contrary to expectation, we predict and then experimentally confirm that TNF’s autocine functions have little relevance in shaping the NFκB response to lipopolysaccaride although large amounts of TNF are produced and play important roles in paracrine responses. 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. 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. Proper control of the signaling pathways activated by TLRs is of particular importance, as aberrant signaling and chronic cytokine production can lead to disease states such as Crohn's disease, rheumatoid arthritis, and cancer. In macrophages, TLRs utilize two adaptors which mediate the signaling events leading to pro-inflammatory cytokine production: TRIF and MyD88. While all TLRs with the exception of TLR3 use the adaptor MyD88, TLR4 uses both MyD88 and TRIF, which signal from the cell membrane and endosome, respectively. These adaptors mediate the activation of transcription factors such as NFkB and IRF3, both of which have been implicated in the control of TNF production.

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. 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. 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. Previous reports have sought to determine whether the adaptors TRIF or MyD88 are responsible for these post-transcriptional production control mechanisms in macrophages, 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. Given the lack of consensus of which adaptor controls each of these post-transcriptional processes, an investigation that characterizes the TRIF and MyD88-mediated mechanisms of TNF production control is still needed.

The paracrine signaling functions of cytokines such as TNF, a cytokine which 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 responding to pathogens can respond to the cytokine that they secrete, has been documented as an essential aspect of TLR-induced inflammatory signaling. 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 signaling. In this paper, we investigated autocrine and paracrine TNF signaling functions in response to TLR agonists in the context of NFkB 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 NFkB 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 approach alone (Werner REF). By constructing simple mathematical models of regulatory modules identified by experimental studies, and linking these building blocks, we are able to build computational models 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 NFkB activation (Frank REF), as well as one for TNFR-mediated NFkB activation (Werner REF) 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 which utilizes both TRIF and MyD88: LPS. 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 NFkB 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. 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. Further, p38 and ERK have been shown to control TNF translation through the initiation factor eIF4E and TNF secretion through the enzyme TACE. 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 both. 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 NFkB 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 1E). 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 1F). 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 1G). 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.

These complex dynamical relationships can fit by a model, in which (biological mechanisms) and tested by mathematical modeling.

*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 REF). 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. 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 2B). 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 2C).

TNF nascent transcript analysis in LPS-stimulated macrophages revealed differential control between TRIF and MyD88. As NFkB was confirmed to be the transcription factor solely responsible for TNF transcription, we sought to characterize NFkB activation in *TRIF-/-* and *MyD88-/-* BMDMs in response to LPS. Electrophoretic Mobility Shift Assay (EMSA) using a G1G2 probe with NFkB binding sites revealed that NFkB activation is decreased in both *TRIF-/-* and *MyD88-/-* (Fig 2D). 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 NFkB activation is what drives the transcription of the TNF gene, this allows us to create a computational module for nascent TNF RNA using NFkB activity as an input (Fig 2E). This simple model consists of a TNF gene containing two kB sites, where NFkB is able to bind. Upon NFkB 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 NFkB activity and promoter binding sites, and a mass action equation for nascent processing (Eq 1.). Previous work as shown that PKR activity is important for processing nascent TNF RNA to mRNA (REF), and that this process is at least partially mediated by MyD88 (REF). 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 NFkB 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).

*Control of mRNA decay by TRIF-p38-MK2 potentiates mRNA expression stimulus-specifically.*

Previous reports have shown that the half-life of TNF mRNA can be modulated during TLR signaling, through a process whereby constitutive TNF mRNA degradation is inhibited. While it is clear that this process is important for the temporal dynamics of TNF production in TLR signaling, what is not clear is whether this mRNA stabilization process is controlled by MyD88, TRIF, or some combination of the two adaptors; while some reports have suggested that TRIF is needed for TNF mRNA stabilization, others have reported that TRIF is dispensable for stabilization, and further some have claimed that MyD88 is primarily responsible for control of TNF mRNA decay. The differing conclusions in these reports, combined with 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 first which stimuli activate the pathways leading to TNF mRNA stabilization, 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 3A). 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 (Fig 3A). 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 3B).

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 3C). This revealed that from 30 to 90 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 3D). 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 (Fig 3D).

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 3E). 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 without or with TRIF-mediated stabilization (Figure 3F). This model is able to explain the experimental observation that although *TRIF-/-* have increased nascent TNF RNA levels compared to wild-type, they have slightly decreased mRNA levels. TRIF-mediated mRNA stabilization plays a key role in LPS-induced TNF production.

*TRIF pathway accelerates TNF protein production and secretion.*

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. Therefore, we sought to characterize the mechanisms of TNF translation and secretion to determine whether TRIF or MyD88 controls these processes as well. TNF mRNA is translated into a membrane-bound trimer known as pro-TNF, which is cleaved by the enzyme TACE, allowing for its release from the membrane and subsequent secretion from the cell. In order to characterize adaptor-specific control TNF translation, western blots for pro-TNF expression were carried out from 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 4A). 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, unlike the p38-inhibitor condition (Fig 4B).

Given that eIF4E and TACE have been shown to be needed for TNF translation (REF) and secretion (REF), respectively, we next sought to characterize their activation. Previous reports have demonstrated that phosphorylation of eIF4E and TACE are necessary for the processive activity (REF, REF). Wild-type, *TRIF-/-*, and *MyD88-/-* BMDMs were stimulated with LPS and western blots for phospho-eIF4E and phospho-TACE performed (Fig 4D). 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. Taking the temporal kinetics of TNF mRNA into account, TRIF plays a larger role in promoting the translation of TNF. To determine whether this was the case for secretion as well, 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 4D). 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 model 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 4E). 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 4F). These simulations illustrate how although the *TRIF-/-* BMDMs have nearly the same levels of TNF mRNA as wild-type, they have significantly less TNF secretion, as the simulation for secreted TNF without the promotion of translation and secretions have similarly reduced levels of TNF secretion.

*A Mathematical model of PAMP-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 REF, 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 four modules for each step in TNF production with the model for TLR-induced NFkB activation coordinated through MyD88 and TRIF (Zhang TLR model) and the model for TNFR-induced NFkB signaling (Werner TNFR model), creating one model that computationally characterizes TLR-induced TNF production and signaling in the context of NFkB (Figure 5A). This mathematical model is informed by quantifying LPS-induced experimental results in wild-type, *TRIF-/-*, and *MyD88-/-* BMDMs, so is able to recapitulate experimental LPS induced TNF production, but is also able make predictions of how other TLR agonists that use TRIF and MyD88, such as PolyI:C (TLR3) and CpG (TLR9), respectively. Computationally simulating LPS-induced TNF production demonstrates that the model is able to recapitulate the experimental data at the level of nascent RNA, mRNA, and TNF production (Figure 5B). To test the model’s fitness, we simulated the model to predict PolyI:C and CpG-induced TNF production. Here, the model was able to successfully predict PolyI:C-induced TNF production at the level of mRNA, and protein secretion (Figure 5C). As PolyI:C induces less activation of NFkB through TRIF than LPS, the model predicted that PolyI:C-induced TNF production would be decreased compared to LPS. Further, the model predicted that PolyI:C would not lead to stabilization TNF mRNA message, even though that it uses TRIF which controls TNF mRNA half-life; experimental TNF mRNA half-life studies revealed that PolyI:C indeed does not stabilize TNF message (Supplement).

Next, we simulated the model to determine CpG-induced TNF production. The model predicted levels of nascent RNA and mRNA aligned with the experimentally derived levels; however, at the level of TNF protein secretion, the model is only able to predict the increased TNF secretion that occurs in response to CpG from 4-8 hours with the incorporation of the TNFR model (Figure 5D). As MyD88 does not control TNF mRNA stability nor promote the processing of TNF, CpG is predicted by the model to have delayed kinetics compared to LPS; however, as MyD88 leads to high induction of TNF mRNA, CpG stimulation does lead to significant TNF secretion. Here, the model predicts that the later peak of TNF secretion seen experimentally in response to CpG is due to autocrine TNF signaling. These results suggests that for certain stimuli, autocrine TNF signaling may be important for prolonging the inflammatory state.

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

Incorporating these three modules into one signaling network in a straightforward, stepwise fashion allows us to characterize the role that TLR-induced TNF production plays in NFkB signaling. TNF secreted by tissue-resident macrophages in response to pathogen challenge has a well-know paracrine signaling role in recruiting other cells and cell types to sites of infection. However, TNF may also signal in an autocrine fashion and play a role in augmenting NFkB activation in response to certain stimuli conditions. The nature of the TNF model, with various TLR-agonist inputs that induce different temporal dynamics, gives us the ability to investigate the potential autocrine function of TNF and make predictions that can be tested experimentally. Previous work on the TLR signaling module gave insights into the dynamics of signaling events mediated by MyD88 and TRIF. While MyD88-mediated IKK and NFkB activation are earlier and transient, TRIF-mediated IKK and NFkB activation are later and persistent. Using the three TLR agonists as inputs for the TNF model, simulations revealed that the model is able to recapitulate TNF production dynamics for LPS, PolyI:C, and CpG, and that CpG-induced TNF production may be dependent on TNF autocrine feedback. Given the difference of temporal NFkB dynamics that are induced by TRIF and MyD88, we next sought to determine whether TNF autocrine signaling may be necessary for sustained NFkB activation in response to certain stimuli.

These computational results result led us to investigate further whether TNF autocrine feedback is important for sustained NFkB activation. The mathematical model was simulated for LPS and CpG response for NFkB activity either with or without autocrine TNF signaling. Here, the model predicts that while the loss of autocrine TNF signaling has no effect on LPS-induced NFkB activation, it does have an effect on CpG-induced NFkB activation (Figure 6A). To test this experimentally, NFkB activity was measured in the absence of TNF auctrine 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 NFkB activation confirmed the computational prediction, demonstrating that while *tnf*-/- BMDMs did not have reduced NFkB activation in response to LPS, they did have reduced NFkB activation in response to CpG from 4-8 hours (Fig 6B). This aligns with the result from the TNF 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 NFkB activation. This result suggests that in response to CpG, secreted TNF serves a primarily autocrine role.

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 which mimics this tissue-resident macrophage environment, BMDMs generated from *tnfr-/-* mice were co-cultured with *myd88-/-/trif-/-* 3T3s, the latter of which cannot activated NFkB. 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 NFkB subunit p65. 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 6C). However, in the CpG-stimulated condition, *myd88-/-/trif-/-* 3T3s showed less p65 translocation the LPS-stimulated condition (Fig 6D). 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 NFkB activation.

*CpG-induced autocrine TNF signaling contributes to prolonging the inflammatory state*

Computational simulations of the TNF production model led to the prediction that sustained NFkB activity in response to CpG would be dependent on CpG-induced TNF autocrine signaling, which was confirmed by experiments in *tnf-/-* BMDMs. As NFkB 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 7A). 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-NFkB controlled genes, demonstrating that TNF autocrine signaling not only has a general effect on prolonging the inflammatory state, but that the observed decrease in NFkB activity seen in *tnf-/-* BMDMs stimulated with CpG leads to a phenotype of decreased NFkB (Figure 6C).

**Discussion**

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. This iterative approach led us to incorporate this TNF production module to the previously published modules for TNFR signaling, creating a single model for NFkB activation, TNF production, and TNF feedback in TLR-induced signaling in order to account for the differential dynamics of stimulus-specific NFkB activation and TNF production. Furthermore, a benefit of this iterative approach also led us to suspect that sustained TLR-induced NFkB 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 NFkB activity is indeed dependent on autocrine TNF, demonstrating the robustness and predictive ability of the model.

It is well know that TNF production by macrophages plays a critical role during the pathogen response, alerting more specific cell types to sites of infection and regulating their response. Numerous studies have shown the critical role that TNF plays in forming granulomas, inducing bacteria-killing iNOS production, and providing overall protection against intracellular bacteria such as *Mycobacterium bovis*, *Mycobacterium tuberculosis*, *Bucrella abortus*, and *Listeria monocytogenes*. (Kindler 1989, Flynn 1995, Zhan 1996, Serbina 2003) The various TLRs expressed by macrophages give them the ability to recognize and respond to a wide variety of bacteria, viruses, and fungi by producing cytokines tailored to each particular infection at hand. In this present study, we observed that in macrophages, TNF signals in a stimulus-specific matter. While in the case of the TLR4 agonist LPS, TNF secreted by macrophages plays a role as a paracrine signaler, for the TLR9 agonist CpG, secreted TNF primarily serves as an autocrine signaler. Although unlike TLR4, TLR9 recognizes CpG motifs in viral DNA, TLR4 and TLR9 recognize components of bacteria, giving rise to questions regarding TNF signaling: why does TNF signal differently when the response is mediated by receptors that both recognize bacteria, and what physiological role do stimulus-specific temporal kinetics of TNF production play? One major difference between TLR4 and TLR9 is that each plays a more primary role in recognize different types of bacteria; while TLR4 is often essential for the recognition of extracellular bacteria (REF), TLR9 plays a critical role in the recognition of intracellular bacteria including *M. tuberculosis*, *B. abortus*, and *Legionella pneumophila* (Bafica 2005, Surendran 2012, Bhan 2008). This difference can be summarized by a simple characterization of types of pathogens the two TLRs recognize: TLR4 primarily recognizes extracellular pathogens, namely bacteria, while TLR9 primarily recognizes intracellular pathogens, including virus, as well as types of intracellular bacteria and fungus. The endosomal expression of TLR9 gives it an ideal location for recognizing intracellular pathogens, whereas a potential consequence of TLR4’s plasma membrane location is that its response to intracellular bacteria may be limited. The nature of these two different types of infection, extracellular and intracellular, requires a tailored inflammatory and immune response. Whereas a macrophage infected with an intracellular bacteria will need to illicit a balanced response that successfully clears the pathogen from within itself without causing over-activation of neighboring cells that may not be infected, an appropriate response by a macrophage that senses LPS would be to swiftly secrete inflammatory signals into the bacteria infected microenvironment.

In this study we show that the dynamics of LPS-induced TNF production are fast and robust, as TLR4 signaling is mediated by both the adaptors MyD88 and TRIF, which provide the activation of early TNF gene transcription and promote TNF processing, respectively. In contrast, while CpG-induced TNF production does reach a high level, the kinetics are significantly slower than in the LPS condition because TLR9 does not connect to the TRIF signaling arm which accelerates TNF processing. However, the temporal kinetics of TLR-induced NFkB activation and TNF production is key to understanding this stimulus-specific regulation. LPS stimulation of TRIF KO and MyD88 KO BMDMs demonstrated that MyD88-mediated NFkB activation occurs early with a sharp peak (15-45 minutes), whereas TRIF-mediated NFkB 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 NFkB activity is still high due to the persistence of TRIF-mediated NFkB activation. In contrast, CpG mediates only through MyD88, which induces an NFkB temporal profile which 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 NFkB activity had decreased down to basal. Therefore, autocrine TNF serves to augment and prolong NFkB activity in response to CpG, which may be necessary for intracellular bacteria clearance.

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. Further, another study found that constitutively-expressed transmembrane-bound TNF could signal in an autocrine manner, leading to prolonged NFkB 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, Silfman 2003, Lee 2002). 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). Furthermore, recent computational studies on the dynamics of TNF production and TNFR internalization in *M. tuberculosis* granuloma formation suggests that receptor internalization in TLR-induced signaling may provide balance between paracrine and autocrine signaling (Fallahi-Sichani 2011). In this study, we show that stimulus-specific control of TNF production in macrophages leads to different temporal dynamics of TNF release and subsequent NFkB activation. The strong and early secretion of TNF in response to the TLR4 agonist LPS, which uses both adaptors MyD88 and TRIF to promote TNF transcription and processing respectively, ensures that TLR4-induced TNF signals in a strong paracrine manner, but does not augment NFkB activation through autocrine signaling due to the overlapping dynamics of TNF secretion and TRIF-mediated NFkB activation. In contrast, the temporally later secretion of TNF in response to the TLR9 agonist CpG, which lacks the strong promotion of TNF processing from TRIF, favors autocrine TNF signaling due to the temporal separation of early MyD88-mediated NFkB activation and later TNF secretion, which is essential for TLR9-induced persistent NFkB activation controlled by secreted TNF feeding back into NFkB activity through TNFR. This stimulus-specific control of TNF paracrine and autocrine signaling is important for pathogen-specific macrophage immune response.