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Dynamical Characteristics of TLR-Responses Determined by Pathway-Specific Molecular Mechanisms

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Abstract

Toll-like receptors (TLRs) recognize specific pathogen-associated molecule patterns (PAMPs) and initiate innate immune responses via Myd88-dependent and/or TRIF-dependent sub-pathways. One of the members, TLR4, exploits both sub-pathways to respond to the bacterial outer membrane component lipopolysaccharide (LPS) to activate the transcription factor NFκB. Here, we constructed a macrophage TLR4 signaling mathematical model that recapitulates LPS-responsive NFκB dynamics. Our model reveals that specific topological features in each sub-pathway encode specific dynamical features of the LPS dose-response. In the Myd88-subpathway, the assembly of a macromolecule signal platform (MyDDosome) provides kinetic threshold and ultra-sensitivity for NF-κB activation, whereas the TRIF sub-pathway provides for a linear dose response. As TRIF signaling occurs in the endosome with a delay, the Mydd88 sub-pathway determines the response time of TLR4 signaling. We show here that ligand-induced receptor translocation is critical in determining a 15 min time delay between TRIF and MyD88 induced signals, but in providing for a multi-hour late phase of NF-κB activity. Accounting for stochasticity in receptor translocation generates two clusters of cellular dynamics that are observed experimentally. Taken together, we have demonstrated that the topological features of Myd88 and TRIF sub-pathways regulate specific dynamical features; their combination may provide for complex pathogen-specific signaling dynamics to control innate immune responses via TLR signaling spectrum.

INTRODUCTION

At a glimpse, Toll-like receptor (TLR) signaling is a complex network involving at least 12 different TLRs and interactions among a variety of proteins (1). Those TLRs perceive a large variety of PAMPs and determine the response to them through this seemingly vast signaling network. However, a closer look reveals that most signals merge into myeloid differentiation primary response gene 88 (MyD88)-dependent and/or TIR domain-containing adaptor protein-inducing interferon-β (TRIF)-dependent pathways, forming a more simplistic bow-tie structure (1). Thus it is intriguing how specificity can be achieved via these two sub-pathways. Among all the TLR family members, TLR4 is the first described in mammals (2) and the only one which employs both pathways to trigger inflammation and innate immune responses (3). Thus, it is a natural starting point to explore specificity in the TLR4 signaling pathway.

TLR4 functions as an indispensable receptor, which recognizes bacterial lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria and can activate at least two important transcription factors, NFκB and IRF3. In response to LPS, TLR4 recruits TIRAP at the plasma membrane and subsequently facilitates recruitment of MyD88 to trigger the initial activation of NFκB (3). TLR4 subsequently undergoes dynamin-dependent endocytosis and is trafficked to the early endosome (4), where it forms a signaling complex with TRAM and TRIF, to initiate the TRIF-dependent pathway that leads to IRF3 activation as well as the activation of the second wave of NFκB (3). It has been shown that NFκB dynamics play key roles in various

cellular responses (5) and thus it is important to understand how the converging pathways affect its dynamics.

It is known that NFκB dynamics display specificity among stimuli. TNFα stimulation triggers the transient NFκB activity, but in response to LPS nuclear NFκB activity is persistent (6). It has been proposed that the TNFα feedback in an auto-/paracrine manner is the key to the distinct NFκB dynamics (6-8). However, this seems to be limited to mouse embryonic fibroblasts (MEFs) (9). In dendritic cells (DCs), LPS induced TNFα production is independent of IRF3 (10) and cannot support the persistent NFκB in response to LPS. Furthermore, in the time window of the first 4 hours, the signaling processes are protein synthesis-independent and immediate early after stimulation, whereas the auto-/paracrine cytokine feedback requires cytokine production, usually late. Thus, the auto-/paracrine feedback contributing to the NFκB early dynamics in macrophage seems to be mild and will not to be considered here.

Previous studies have shown different roles of MyD88- and TRIF-dependent pathways in determining NFκB dynamics (9, 11-13). In Myd88-deficient cells, the NFκB peak is delayed (13), whereas in TRIF-deficient cells, the early activation of NFκB is normal but the response is more transient (11). Furthermore, in the double knockout cells, the NFκB activation in response to LPS is lost (11). Thus, the NFκB dynamics are completely determined by MyD88-dependent and the TRIF-dependent pathways and signaling via MyD88 is quicker and transient vs. late and persistent in TRIF-dependent pathway (9). But such a description currently lacks a quantitative and systematic characterization.

There are specific topological features in TLR4 sub-pathways. First, in the MyD88-dependent pathway, there are death domain (DD) containing adapters that form a macromolecular complex, called the MyDDosome, which is composed of 7 or 8 MyD88, 4 IRAK4 and 4 IRAK2/1 (14). However, in the TRIF-dependent pathway, such a DD complex is absent. How this difference affects signaling dynamics remains to be explored. Besides the MyDDosome, endocytosis of TLR4 receptor is found to separate the signaling branches both temporally and spatially (4). The ligand-bound TLR4 first induces MyD88 signaling from the plasma membrane and then is endocytosed and activates TRIF signaling from the early endosome (4). However, there is also homeostatic TLR4 trafficking from the plasma membrane to the endosome by endocytic recycling compartments, which could also initiate the signaling after binding to endocytosed LPS (15). It is not clear how these receptor trafficking modes affect the sub-pathway specificity.

We sought to study the above questions by combining biochemical experiments with mathematical modeling. Mathematical modeling has been demonstrated as a powerful tool to investigate complex cellular mechanisms (16, 17). Although several models have already been proposed to study the dynamics of TLR4 signaling (7, 18-21), few of them include the events upstream of IKK, which was found critical in defining NFκB dynamics in response to TNFα (22).

In this work, we studied the different dynamical features of NFκB (i.e. peak, response time and duration) in response to LPS in primary macrophages. We found these features

are differentially encoded in distinct sub-pathways. The MyD88-dependnent pathway determines the peak and timing of the response, while the TRIF-dependent pathway is responsible for the duration of the response. Underlying the differential sub-pathway specificity, two main molecular mechanisms determine the specificity. In the MyD88-subpathway, the assembly of a macromolecule signal platform (MyDDosome) provides a kinetic threshold and ultra-sensitivity for NF-κB activation, whereas the TRIF sub-pathway provides for a linear dose response. As TRIF signaling occurs in the endosome with a delay, the MyD88 sub-pathway determines the response time of TLR4 signaling. We show here that ligand-induced receptor translocation is critical in determining a 15 min time delay between TRIF and MyD88 induced signals, but in providing for a prolonged late phase of NF-κB activity. Accounting for stochasticity in receptor translocation generates two clusters of cellular dynamics that are observed experimentally. Taken together, we have demonstrated that the topological features of Myd88 and TRIF sub-pathways regulate specific dynamical features; their combination may provide for complex pathogen-specific signaling dynamics to control innate immune responses via the TLR signaling spectrum.

MODEL and METHODS

Model description. Four modules constitute the model: the receptor module, the IKK module, IRF3 activation module and the NFκB module (Fig. 1*A*). Figure S1 shows the details of the model, which is modeled as chemical reaction networks. In the TLR4 module, LPS first binds to the CD14 and then with the help of LBP, it binds the TLR4 receptor. Subsequently, the adaptor MyD88 is recruited and activated. On the other

branch, the LPS-TLR4 complex is transported to the early endosome, in a dynamin and CD14 dependent manner (4, 23). Then TRIF is recruited to the early endosome and activated. The activated MyD88 and TRIF are considered as output of the TLR4 module and the input of the IKK module, where they will activate TRAF6 (3). The active form of TRAF6 can recruit TAK- IKK complex (IKKK) and promote the activation of IKKK. Active IKKK phosphorylates the IKK complex, which is the output of the IKK module. Also the activated TRIF can activate TBK1, which phosphorylates IRF3 and promotes its dimerization and nuclear import. In the NFκB module, the active IKK phosphorylates the IκBα/β/ε, promoting their degradation and releasing the NFκB to translocate to the nucleus. Free nuclear NFκB can trans-activate the expression of *iκb* α/β/ε forming negative feedback loops.

Simulate the knockouts. To study the different receptor shuttling modes, we perturbs the model by setting the shuttling rate to zero (both directions)

Model construction. The NFκB module is adapted from our previous publication (22). The remaining modules are formalized by Ordinary Differential Equations (ODEs) based on mass action except the MyD88 activation, where the Hill equation is used to represent MyDDosome formation. Since the NFκB module has been well established (22), we only parameterized the other modules. The model is numerically implemented in MATLAB 7.11.0 (MathWorks) and fitted by using lsqnonlin, a constrained minimization algorithm from Matlab Optimization Toolbox (MathWorks). The final parameter values along with the chemical reactions are shown in Table SI.

RESULTS

Decomposition of TLR4 signaling by knockouts reveals distinct dynamics in MyD88-dependent and TRIF-dependent pathways. In order to study the dynamics of Myd88- and TRIF-dependent pathways individually, we first checked IKK dynamics using a quantitative kinase assay in wt, myd88 knockout and *trif* knockout bone marrow derived macrophages (BMDMs). As shown in Fig. 1*B top*, the IKK dynamics are in different phases in each knockout for both low and high concentrations of LPS. In *trif* knockout, IKK activity is induced early and transiently, reaching a peak 15 mins earlier than the *myd88* knockout. In contrast, *myd88* knockouts show late and more persistent IKK signaling. Consistent with previous results in MEFs (6), the wt IKK activity is sustained after LPS stimulation, lasting for at least 2 hours (Fig. 1*B top*). However, the results here show robust IKK activity within 15 minutes even at the low dose, which is faster than in MEFs.

Next, we moved to the downstream transcriptional factors NFκB and IRF3. We measured the nuclear NFκB activity by EMSA (Fig. 1*B middle*) and nuclear IRF3 phosphorylation by western blot (Fig. 1*B bottom*). The activities of NFκB and IRF3 displayed distinct dynamical features. In general, NFκB activity is more persistent while IRF3 activity is transient. NFκB activity reaches a peak at 30 mins in the 1ng/ml LPS case and then sustains at least to 4 hours. In addition, the response is faster and stronger as the concentration of LPS is increased from 1ng/ml to 100 ng/ml (Fig. 1*B middle*). In contrast, the IRF3 phosphorylation profiles are bell-shaped, with higher peak levels in response to the higher dose of LPS (Fig. 1*B bottom*). Both NFκB and IRF3 showed induced activities

to a high and low dose of LPS, which suggests that BMDMs are quite sensitive to LPS stimulation.

We quantified those blots and used the data to calibrate our model. It is worth mentioning that the IKK activation cycle and downstream NFκB module used here are adapted from our previously published model (22). Receptor activation, subsequent activation of MyD88 and TRIF, and the IRF3 module are newly constructed here. Our model was able to recapitulate the data from Figure 1B for both high and low concentrations of LPS (Fig 1*C* and *D*) with a synergistic activation of TRAF6 in the model corresponding to the wt condition (8).

Next, we extended our simulation to sample a range of LPS concentrations, from 0.1 ng/ml to 100 ng/ml (Fig. S2). We observed quick but transient IKK activity in *trif* knockouts (Fig. S2*A left*); delayed but long lasting signaling in *myd88* knockouts (Fig. S2*A middle*) and a combination of both in *wt* (Fig. S2*A right*). The NFκB DNA-binding dynamics displayed a similar pattern (Fig. S2*B*). There is no IRF3 activity in *trif* knockout BMDMs because nuclear IRF3 activity is solely controlled by TRIF-dependent signaling (Fig. S2*C left*).

Consistent with previous studies, both experiments and simulation suggested a biphasic response of NFκB signaling for a wide range of LPS doses. The wt NFκB dynamics are composed of the MyD88-dependent quick and transient response and the TRIF-dependent persistent response.

The dynamical features of the NFκB response are specific to distinct sub-pathways. Now, it is known that temporal dynamics encode information that is important to signal transduction (24). We used the dose sampling data in Fig. S2*B* and plotted all the $NF\kappa B_n$ time courses in Fig. 2*A*. The color gradient represents the increase in dose. Then we studied different features of NFκB dynamics including duration, response time (half-peak time), and time of peak activity.

We first studied the duration of the NFκB dynamics. We defined the duration by determining the time that nuclear NFκB levels stay above a threshold set at 50 nM (see Fig. 2*A* black dashed line) (22, 25). The threshold is usually required by transcription factors to generate burst gene expression. Here, our result demonstrates that in distinct knockouts the duration is affected differently in response to increasing concentrations of LPS (Fig. 2*B*). We can see in the MyD88-dependent pathway the duration is constant for doses of LPS above 0.2 ng/ml. But in wt and the trif-dependent pathway the duration is dose-dependent. In other words, the trif-dependent pathway encodes the duration of the response in a dose-dependent manner. However, when we look at the response speed (Fig. 2*C*) and the timing of the maximum response (Fig. 2*D*), the cases are different. The MyD88-dependent pathway has the same response speed as wt, but in the TRIF-dependent pathway the response is delayed (Fig. 2*C*), indicating the speed is determined by MyD88-dependent pathway. The peak time displayed similar results as response speed (Fig. 2*D*). Thus distinct sub-pathways encode different aspects of NFκB dynamics.
In summary, the MyD88-dependent pathway determines the peak and response speed while the duration of the response is controlled by the TRIF-dependent pathway.

Signalosome formation is responsible for the MyD88 signaling specificity.

Signalosome is ubiquitous in different signaling pathways, for example, PIDDosome in the apoptosis pathway and prion-like aggregation of MAVS protein in the antiviral innate immune pathway (26, 27). MyDDosome provides another instance (14, 28). It is interesting to study why form those macro signaling molecules. In order to study this, we modeled the MyDDosome formation following the sequential binding proposal from Lin et al (14) (Fig. 3*A*). The results demonstrate a threshold in activation with hill coefficient between 2 and 3.2 can be generated during the process (Fig. 3*B* and Supplemental materials Fig. S3).

Then, we use the TLR4 model to study effects with or without such a threshold in MyD88 activation by tuning the value of Hill coefficient. It turns out with the Hill kinetics the model can generate different sensitivity of the peak-value respect the LPS dosage in *trif*$^{-/-}$ and *myd88*$^{-/-}$. As shown in Fig. 3*C*, the sensitivity of IKK peak is higher at low dose but lower at high dose in *trif*$^{-/-}$ than *myd88*$^{-/-}$, when using Hill coefficient in the range where k_f/k_b are from 0.1 to 1 in Fig. 3*B*. Thus, the threshold generated in MyDDosome formation is critical in generating those distinct peak dose responses.

To test this prediction, we have measured the dose-response of IKK to LPS in 4 different concentrations: 0.1 ng/ml, 1 ng/ml, 10 ng/ml and 100 ng/ml (Fig. 3*D* and Fig. S4). Since the response is quick, we only measured the IKK activity in first 45 mins where peak locates. By quantifying these blots, we get the transient peak dose response, which is

quite similar to the case in Fig. 3. Taken together, our model and data suggested a capability of fine-tune the response by the signalosome.

We also did sensitivity analysis. We studied the response time sensitivity by perturbing each parameter 5% to see which parameter most easily affect the response time (Fig. S5). It turns out the parameters related to the receptor level, i.e. the degradation and generation rate constant of TLR4, stand out, which should not be a surprise. One can easily imagine that the more receptor the more capable the cell to receive and response to the ligand signals. Most interestingly, k_{mMyD88}, i.e. the threshold in the MyD88 activation, ranked at the top showing that the response speed is most sensitive to the threshold in the MyD88 dependent pathway formed by the signalosome.

The ligand induced-shuttling is responsible for the duration specificity. Next, we would like to ask which molecular mechanisms are responsible for those features. In TLR4 signaling, there are at least two main shuttling processes related to the receptor: the ligand induced endocytosis and the homeostatic shuttling (Fig. 4*A*). The ligand-bound TLR4 first induce MyD88 signaling from the plasma membrane and then is endocytosed and activates TRIF signaling from the early endosome (4). Homeostatic TLR4 traffics from the plasma membrane to the endosome by utilizing endocytic recycling compartment (15). It is not clear how these endocytosis modes affect the sub-pathway specificity of NFκB signaling.

By setting the corresponding reaction constants to zero, we are able to simulate the model with constitutive shuttling only or ligand-induced shuttling only. Then we examined what are the effects of these different shuttling on the duration dose responses (Fig. 4*B* and *C*). It is clear that the nuclear NFκB profiles were not affected in different shuttling modes in MyD88 dependent pathway but changed dramatically in wt and models containing TRIF-dependent pathway only (Fig. 4*B* and *C left*). This indicates the shuttling only affects the signaling from early endosome, i.e. from TRIF-dependent pathway. Dose-response capability for the duration remained in the ligand-induced, but abrogated in the constitutive shuttling only case (Fig. 4*B* and *C middle*). However, the peak-time dose responses didn't change because it is controlled by the MyD88 pathway (Fig. 4*B* and *C left*). Thus, we conclude here that the ligand induced receptor shuttling is key to generate the duration sub-pathway specificity.

From cell population to single cell. With the progress of microscopy and florescent proteins, more and more interesting observations are found by measurements at single cell level. When come to single cell, the heterogeneity is one of the prominent phenomena. In terms of LPS induced NFκB dynamics, single cell data revealed two clusters of distinct responsive cell population (8) – cluster 1 is transient dynamics and cluster 2 is persistent dynamics. Here we wonder can it due to the variability in activation of sub-pathway, since previous results in this paper demonstrated specific dynamical features are directly related to those two sub-pathways.

In order to test this hypothesis, we introduced two uncorrelated uniform random parameters between 0 and 1 as fraction of activation for each pathway in the reaction MyD88 activation (f_m) and TRIF activation (f_t) (Fig. 5*A*). Quantities f_m and f_t can be considered as activation efficiency with value between 0 and 1 – 0 means defected activation and 1 means normal activation. Then we simulated the model 200 times to mimic 200 single cells. We then clustered those $NF\kappa B_n$ trajectories, which are shown in Fig. 5*B*. It is clear that there are two clusters (cluster 1 and cluster 2), which are similar to the experimental observation (8). The cluster 1, where most cells demonstrated transient NFκB responses, are most likely due to low efficiency of TRIF-dependent pathway activation (Fig. 5*C*). But for the persistent responses in another cluster (cluster 2), TRIF-dependent pathway is more efficiently activated (Fig. 5*D*). In the condition that TRIF-dependent pathway are always normal activated and only MyD88-dependent pathway has stochasticity, the cluster 1 disappears (Fig. S6*A*). In the case that only stochasticity happens in TRIF-dependent pathway, the two clusters remain (Fig. S6*B*). However, the heterogeneity in the peak time disappears (Fig. S6*B* and *C*). Thus, we revealed that the specific roles of noise in the sub-pathways: noise in MyD88-dependent pathway renders the variability in the quickness of the response; whereas the noise in the TRIF-dependent pathway determines persistent second phase activity. In this section, we demonstrated the possibility that heterogeneity in the NFκB response in single cell level could be due to the differential levels of sub-pathway activation at the upstream reactions (29).

DISCUSSION

In this work, we have studied the different dynamical features of NFκB in responses to LPS in macrophage. We found these features are differentially encoded via distinct sub-pathways. Specifically, MyD88-dependent pathway encoding the peak and timing of the response and TRIF-dependent pathway encodes the duration of the response. Underlying the different sub-pathway encoding, two main molecular mechanisms determined the specificity. The ligand-induced shuttling is responsible for the duration encoding and the signalosome formation render the peak and quick responses.

It is known that the temporal profiles of the activity of key signaling players contain information of downstream signaling programs (24). For example, compared to the transient NFκB dynamics in response to TNFα stimulation, the LPS induced a quick and prolonged nuclear NFκB profiles, demonstrate the stimulus specificity (6, 7). Usually, dynamical responses have different features, like amplitude and duration (24). Here our results indicate that those features can be separately regulated by different sub-pathways of the complex signaling pathway, providing a new insight of the topic.

Traditionally, endocytosis was considered only as an event to down-regulate the signaling players on the plasma membrane (30, 31). Recently, people begin to reveal novel role of endocytosis by signaling from early endosome (30, 31). Our result clearly showed that the biphasic signal could be originate from this process. Also our model has compared two passages for the endosomal signaling: one is via ligand binding in the endosome and the other is the endocytosis of the ligand-receptor complex and demonstrated that indeed

ligand-induced shuttling is the key one to generate the duration dose responses in both wt and myd88 knockouts.

Cellular signaling pathways usually involve the formation of large signaling molecular complexes (Signalosome) (28). The oligomerization of the receptor can provide multiple ligand binding sites, and based on the allosteric mechanism, it could bring in cooperatively into the system (32). Such cooperativity would be helpful to generate bistability in the apoptosis decision process (33). But, it is not known whether similar roles could play at the adaptor level (34). Recently, It is shown that the signalosome can provide specificity by selectively recruitment substrates (35). But how these assembling processes affect the dynamics of signal transduction is not known (28). Our study clearly demonstrated that according to the possible formation path, the inherent kinetic cooperativity can be generated and can be approximated by classic Hill equation. Interestingly, threshold was suggested in the TNFα signaling (36). Such cooperativity will have effects at the signaling dynamics, rendering different sensitivities as compared with signaling in solution. Thus, we linked the molecular level details with signaling dynamics here. In future, spatial modeling together with single molecule imaging of fluorescence would be helpful to validate our results (37).

Moving to single cell data, the heterogeneity is the first thing we met. There are two sources of heterogeneity in general 1) the genetic ones come from the mutation and etc 2) the non-genetic ones come from the thermodynamical fluctuation in chemical reaction and protein abundance variations (38). In TNF signaling, the paracrine secretion can

render the population robustness despite of noisy single cell responses (36, 39). But in LPS, here we showed that the heterogeneity could due to the intrinsic pathway activation variations directly. Here after establish distinct roles for the MyD88- and TRIF-dependent pathways, the two cluster data from single cell measurement can be easily interpreted as randomness in activation both pathways. The simulation results here validated that randomness happens at the adapter activation level is sufficient to explain the experimental results. The next step should be testing this hypothesis experimentally.

[Insert Acknowledgements here]

1. Oda K & Kitano H (2006) A comprehensive map of the toll-like receptor signaling network. *Mol. Syst. Biol.*:2006.0015.

2. Medzhitov R, Preston-Hurlburt P, & Janeway CA, Jr. (1997) A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature* 388(6640):394-397.

3. Kawai T & Akira S (2010) The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat. Immunol.* 11(5):373-384.

4. Kagan JC*, et al.* (2008) TRAM couples endocytosis of Toll-like receptor 4 to the induction of interferon-beta. *Nat. Immunol.* 9(4):361-368.

5. Hoffmann A & Baltimore D (2006) Circuitry of nuclear factor kappaB signaling. *Immunol. Rev.* 210:171-186.

6. Werner SL, Barken D, & Hoffmann A (2005) Stimulus specificity of gene expression programs determined by temporal control of IKK activity. *Science* 309(5742):1857-1861.

7. Covert MW, Leung TH, Gaston JE, & Baltimore D (2005) Achieving stability of lipopolysaccharide-induced NF-kappaB activation. *Science* 309(5742):1854-1857.

8. Lee TK*, et al.* (2009) A noisy paracrine signal determines the cellular NF-kappaB response to lipopolysaccharide. *Sci. Signal.* 2(93):ra65.

9. Kawai T & Akira S (2007) Signaling to NF-kappaB by Toll-like receptors. *Trends Mol. Med.* 13(11):460-469.

10. Sakaguchi S*, et al.* (2003) Essential role of IRF-3 in lipopolysaccharide-induced interferon-beta gene expression and endotoxin shock. *Biochem. Biophys. Res. Commun.* 306(4):860-866.

11. Yamamoto M*, et al.* (2003) Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. *Science* 301(5633):640-643.

12. Kawai T*, et al.* (2001) Lipopolysaccharide stimulates the MyD88-independent pathway and results in activation of IFN-regulatory factor 3 and the expression of a subset of lipopolysaccharide-inducible genes. *J. Immunol.* 167(10):5887-5894.

13. Kawai T, Adachi O, Ogawa T, Takeda K, & Akira S (1999) Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity* 11(1):115-122.

14. Lin S-C, Lo Y-C, & Wu H (2010) Helical assembly in the MyD88-IRAK4-IRAK2 complex in TLR/IL-1R signaling. *Nature* 465(7300):885-890.

15. Husebye H*, et al.* (2010) The Rab11a GTPase controls Toll-like receptor 4-induced activation of interferon regulatory factor-3 on phagosomes. *Immunity* 33(4):583-596.

16. Basak S, Behar M, & Hoffmann A (2012) Lessons from mathematically modeling the NF-kappaB pathway. *Immunol. Rev.* 246(1):221-238.

17. Kholodenko BN (2006) Cell-signalling dynamics in time and space. *Nat. Rev. Mol. Cell Biol.* 7(3):165-176.

18. Selvarajoo K (2006) Discovering differential activation machinery of the Toll-like receptor 4 signaling pathways in MyD88 knockouts. *FEBS Lett.* 580(5):1457-1464.

19. An G (2009) A model of TLR4 signaling and tolerance using a qualitative, particle-event-based method: Introduction of spatially configured stochastic reaction chambers (SCSRC). *Math. Biosci.* 217(1):43-52.

20. An GC & Faeder JR (2009) Detailed qualitative dynamic knowledge representation using a BioNetGen model of TLR-4 signaling and preconditioning. *Math. Biosci.* 217(1):53-63.

21. Selvarajoo K*, et al.* (2008) Signaling flux redistribution at Toll-like receptor pathway junctions. *PLoS ONE* 3(10):e3430.

22. Werner SL*, et al.* (2008) Encoding NFκB temporal control in response to TNF: distinct roles for the negative regulators IκBα and A20. *Genes Dev.* 22(15):2093-2101.

23. Zanoni I*, et al.* (2011) CD14 Controls the LPS-Induced Endocytosis of Toll-like Receptor 4. *Cell* 147(4):868-880.

24. Behar M & Hoffmann A (2010) Understanding the temporal codes of intra-cellular signals. *Curr. Opin. Genet. Dev.* 20(6):684-693.

25. Longo DM*, et al.* (2013) Dual delayed feedback provides sensitivity and robustness to the NF-kappaB signaling module. *PLoS Comput. Biol.* 9(6):e1003112.

26. Park HH*, et al.* (2007) Death domain assembly mechanism revealed by crystal structure of the oligomeric PIDDosome core complex. *Cell* 128(3):533-546.

27. Hou F*, et al.* (2011) MAVS forms functional prion-like aggregates to activate and propagate antiviral innate immune response. *Cell* 146(3):448-461.

28. Ferrao R, Li JX, Bergamin E, & Wu H (2012) Structural Insights into the Assembly of Large Oligomeric Signalosomes in the Toll-Like Receptor-Interleukin-1 Receptor Superfamily. *Sci. Signal.* 5(226).

29. Shokhirev MN & Hoffmann A (2013) FlowMax: A Computational Tool for Maximum Likelihood Deconvolution of CFSE Time Courses. *PLoS One* 8(6):e67620.

30. Platta HW & Stenmark H (2011) Endocytosis and signaling. *Curr. Opin. Cell Biol.* 23(4):393-403.

31. Miaczynska M, Pelkmans L, & Zerial M (2004) Not just a sink: endosomes in control of signal transduction. *Curr. Opin. Cell Biol.* 16(4):400-406.

32. Changeux JP (2012) Allostery and the Monod-Wyman-Changeux model after 50 years. *Annual review of biophysics* 41:103-133.

33. Ho KL & Harrington HA (2010) Bistability in apoptosis by receptor clustering. *PLoS Comput. Biol.* 6(10):e1000956.

34. Scott JD & Pawson T (2009) Cell Signaling in Space and Time: Where Proteins Come Together and When They're Apart. *Science* 326(5957):1220-1224.

35. Schrofelbauer B, Polley S, Behar M, Ghosh G, & Hoffmann A (2012) NEMO Ensures Signaling Specificity of the Pleiotropic IKKbeta by Directing Its Kinase Activity toward IkappaBalpha. *Mol. Cell* 47(1):111-121.

36. Turner DA*, et al.* (2010) Physiological levels of TNFalpha stimulation induce stochastic dynamics of NF-kappaB responses in single living cells. *J. Cell Sci.* 123(Pt 16):2834-2843.

37. Gay NJ, Gangloff M, & O'Neill LAJ (2011) What the Myddosome structure tells us about the initiation of innate immunity. *Trends Immunol.* 32(3):104-109.

38. Niepel M, Spencer SL, & Sorger PK (2009) Non-genetic cell-to-cell variability and the consequences for pharmacology. *Curr. Opin. Chem. Biol.* 13(5-6):556-561.

39. Paszek P*, et al.* (2010) Population robustness arising from cellular heterogeneity. *Proc. Natl. Acad. Sci. U. S. A.* 107(25):11644-11649.

[Insert Tables here]

Supplemental material

Kinetic threshold generated by signalosome formation. We examine the impact of MyDDosome formation on the signaling dynamics. Based on its structure and the mutagenesis results, Lin et al. has suggested a sequential assembly model for MyDDosome (14). In essential, LPS binding induces both dimerization and higher-order oligomerization of TLR4 (28). MyD88 is then recruited to the receptors through homotypic TIR-TIR interactions and facilitates MyDDosome assembly with IRAK4 and IRAK1 or IRAK2 through homotypic DD-DD interactions (28). According to this hypothesis, we proposed a six-step model for MyDDosome formation shown in Fig. 3*A*. Step 1, the TLR4-LPS dimmer (C) recruits two MyD88 (M) molecules forming a complex (CM_2). Step 2, two CM_2 complex bind and form C_2 M_4. Step 3, C_2M_4 binds to CM_2 forming C_3M_6. Step 4, in C_3M_6 complex, six myd88 molecules can reach closely to form heximer (M_6) and release TLR4 dimmers. M_6 then can either disassociate to monomers (step 5) or continue to form MyDDosome (step 6). The k_f is the rate constant of the forward reactions, including binding and M_6 formation. Whereas the k_b is the rate constants of the backward reactions, i.e. disassociation.

Here, we supposed that the steps 1 to 5 are fast relative to reaction 6. So based on the quasi-steady-state hypothesis, these reactions can reach equilibrium fast given an initial concentration of the TLR4-LPS dimmer (C_0), which is considered as the input of the MyDDosome model here. The MyDDosome formation rate then is in proportion to the corresponding steady-state level of M_6 (M^{ss}_6).

We draw the relative concentrations of M^{ss}_6/M_0 versus the relative input concentration C_0/M_0 in up left panel of Fig. S3*A* (circles). Parameters are k_f =1, k_b = 0.1 and M_0 = 1. These dots can be fitted by a Hill equation with Hill constant n = 3 (line). In Fig. S3*B*, we varied the fraction k_f/k_b by changing k_f only and presented the dose-response between M^{ss}_6 and C_0 in a heat map. The right panel of Fig. 3*B* shows that those dose-response curves can be fitted by Hill equation and the resultant Hill constants are in proportion to k_f/k_b in a range between 2 and 3. Thus, we can use a Hill equation to describe the MyD88 activation in the whole model as we did in previous section.