

## REVIEW

# The molecular mechanisms of TLR-signaling cooperation in cytokine regulation

Qian Liu and Jeak Ling Ding

Innate immune cells recognize pathogens through pattern recognition receptors (PRRs), and activation of PRRs induces downstream signaling pathways to mount appropriate immune responses. Pathogens usually carry multiple ligands, which can simultaneously activate multiple PRRs. The cooperation of multiple PRRs and consequential crosstalk between their downstream pathways could enhance cytokine expression, which is required for effective immune responses. On the other hand, immune over-activation could also harm the host if immune homeostasis is not restored. Therefore, it is important to understand the mechanisms of PRR cooperation during an infection. As the best characterized PRRs, Toll-like receptors (TLRs) have an important role in pathogen recognition, and crosstalk among TLRs is common. In this review, we provide an update on the recent findings on the mechanisms of TLR cooperation. We summarize the known mechanisms and provide a future perspective on TLR crosstalk study, with a caution against the use of multiple TLR ligands as adjuvants in therapeutic strategies.

*Immunology and Cell Biology* advance online publication, 8 March 2016; doi:10.1038/icb.2016.18

## INTRODUCTION

The innate immune system senses invading pathogens through pattern recognition receptors (PRRs), which specifically recognize evolutionarily conserved molecules of pathogens known as pathogen-associated molecular patterns (PAMPs). Of all PRRs, the Toll-like receptors (TLRs) are the best characterized. So far, 13 TLRs have been identified: TLRs 1–9 in both mouse and human, TLR10 only in human, whereas TLRs 11–13 only in mouse.<sup>1</sup> On the basis of their subcellular localization, TLRs may be divided into two subgroups. One group, TLRs 1, 2, 4, 5, 6 and 11, reside on the cell plasma membrane and recognize PAMPs such as lipids, lipoproteins and proteins. The other group composed of TLRs 3, 7, 8 and 9, are found on the membrane of intracellular vesicles, for example, endosome, lysosome and endolysosome, which mainly recognize nucleic acids.<sup>2</sup>

After activation, the TLRs mainly signal through two adaptor proteins, TIR-domain-containing adapter-inducing interferon- $\beta$  (TRIF) and myeloid differentiation primary response gene 88 (MyD88). All TLRs, except TLR3, use MyD88 as their adaptor. TLR3 uses only TRIF as its adaptor. TLR4 recruits both MyD88 and TRIF as its adaptors. Although both MyD88 and TRIF pathways could activate NF $\kappa$ B, interferon regulatory factor (IRF) family members and mitogen-activated protein kinase, they have preferences and distinct specificities and characteristics. MyD88 activates mainly NF $\kappa$ B family members and mitogen-activated protein kinase, and it tends to induce a pro-inflammatory response by producing pro-inflammatory cytokines such as IL6 and IL12. On the other hand, TRIF mainly activates IRF family members and tends to stimulate an anti-viral response through the induction of interferon.<sup>2</sup>

Besides TLRs, three other PRR families, including C-type lectin receptors (CLRs), retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and NOD-like receptors (NLRs), also exert surveillance on the invading pathogens. Among these PRRs, the CLRs are found on the cell surface, whereas RLRs and NLRs reside in the cytosol. The different cellular localization of PRRs enables innate immune cells to detect pathogens in different cellular compartments.

Pathogens usually carry with them multiple PAMPs, for example, viruses contain nucleic acids—ssRNA, which activates TLR7/8 and dsRNA, which activates TLR3. Another example is *Mycobacterium tuberculosis*; its lipoarabinomannan, phosphatidylinositol mannosides and DNA are sensed by TLR2, TLR4 and TLR9, respectively.<sup>3</sup> Apart from the activation of multiple TLRs by one pathogen, in many cases, polymicrobial infection occurs. Several diseases are becoming increasingly recognized as being attributable to polymicrobial infections, such as the disease of the oral cavity, diabetic foot wound infections and chronic infection in the lungs of cystic fibrosis patients.<sup>4</sup> Thus invading microorganisms are likely to interact with several TLRs and non-TLR PRRs, simultaneously. However, the consequences of combinatorial activation of PRRs may vary a great deal depending on different combinations of activated PRRs. For example, the combined activation of PRRs can lead to complementary, synergistic and/or antagonistic cytokine production, indicating crosstalk among the PRRs.

Initial studies on the collaborations between TLRs expressed by mouse macrophages showed that ligands of TLR3 and TLR9 could induce the production of more than additive levels of TNF, IL6 and IL12p40,<sup>5</sup> which is known as cytokine synergy. These findings confirmed that crosstalk between TLRs does exist. Subsequent studies

showed both gene expression and protein production of TNF, IL1 $\beta$ , IL10, IL6, IL12 and IL23, which are several fold higher in dendritic cells (DCs) stimulated with combinatorial TLR ligands compared with single stimulations.<sup>6,7</sup> Some *in vivo* experiments also support the notion that TLRs collaborate. For example, *Thr2*<sup>-/-</sup> and *Thr9*<sup>-/-</sup> mice are more susceptible than their wild-type counterparts to a high dose of *M. tuberculosis* challenge.<sup>8</sup> In addition, both *Thr3*<sup>-/-</sup> and *Thr9*<sup>-/-</sup> mice have reduced resistance to murine cytomegalovirus and this decreased resistance is associated with reduced synthesis of type I IFN and IL12,<sup>9</sup> which would have been produced by the presence of both TLRs 3 and 9.

Apart from collaboration within the TLR family members, cooperation of TLRs with non-TLR PRRs is more common as they may respond to different components of a single pathogen or to the same single component of a pathogen. For example, both TLRs and RLRs, which are positioned at different cellular locations, can recognize viral RNAs.<sup>3</sup> Both TLR2 and some CLR recognize fungal components such as glucan and mannose.<sup>10</sup> In addition, a large body of evidence has shown the collaboration of TLR2, NOD1 and NOD2 in synergizing the production of TNF and IL12p40, which is likely due to the breakdown products of bacterial peptidoglycans, recognized by TLR2. These resulting peptidoglycan products act as ligands, which activate NOD1 and NOD2.<sup>11–14</sup> Thus, it is common for TLRs to cooperate with other PRRs in pathogen recognition, resulting in collaborative immune defense.

Cytokine synergy induced by PRR cooperation has a critical role in immune response. On one hand, insufficient cytokine production may hamper appropriate levels of immune responses to pathogen infection. This is especially profound in age-dependent dysregulation of the immune system, where impaired TLR signaling fails to mount sufficient immune responses to fight infections.<sup>15–18</sup> On the other hand, uncontrolled cytokine synergy can be lethal and is associated with various diseases such as septic shock, chronic inflammatory and autoimmune diseases.<sup>19,20</sup> Thus an understanding of PRR collaboration mechanism is very important, as the amount of cytokines produced and the magnitude and quality of innate immune response, to a large extent, depends on the coordinated sum of multiple PRR signals.

The phenomenon of PRR crosstalk and the mechanisms of collaboration between TLR and other PRRs have been studied and summarized repeatedly.<sup>3,21</sup> However, the mechanism of crosstalk among TLRs has not yet been reviewed. As the most well characterized and most common family of PRRs, the mechanisms of TLR cooperation under pathogen infection condition deserve a thorough consolidation. In this review, we summarize and discuss the emerging findings of the mechanisms behind TLR collaboration to provide perspectives for the future study of TLR crosstalk.

## THE MECHANISMS OF TLR-SIGNALING COOPERATION

### The collaboration of MyD88 and TRIF pathways

It has been reported that the activation of both MyD88 and TRIF pathways is required for TLR activation. In both mouse and human DCs, cytokines such as IL12p70, IL6 and TNF are produced at synergistic levels when certain MyD88-associated TLRs are activated together with TRIF-associated TLRs.<sup>7</sup> When mouse bone marrow-derived DCs from TRIF- or MyD88-knockout mouse were mixed together and stimulated with CpG+Poly (I:C) or LPS+Gardiquimod, no synergy was observed, indicating the collaboration of MyD88 and TRIF pathways at a single cell-type level.<sup>22</sup> Similarly in macrophages and monocytes, both MyD88 and TRIF are required for cytokine synergy and combinatorial stimulation with only MyD88-associated TLRs will cause tolerance.<sup>23–25</sup>

### Activation of multiple signaling pathways might be responsible for cytokine synergy induced by TLR cooperation

A global microarray analysis of mouse macrophage cell line, RAW 264.7, stimulated with CpG ODN and/or poly (I:C) revealed that cytokine synergy accelerates gene expression and is characterized by persistent activation of one or more regulatory pathways, resulting in a prolonged signaling cascade that increases, magnifies and diversifies gene expression.<sup>26</sup> Another global kinome analysis of chicken monocytes stimulated with CpG and/or poly (I:C) also showed that combinatorial stimulation could affect multiple pathways uniquely, for example, calcium signaling pathway, endocytosis and adipocytokine signaling pathways.<sup>27</sup> In this study, the researchers also revealed, by protein inhibitors, that the synergistic production of NO requires calcium signaling pathway protein, CaM and CaMK2.<sup>27</sup>

Other studies on signaling pathways that contribute to synergy mainly focused on several key signaling pathways downstream of TLRs, including mitogen-activated protein kinase, NF $\kappa$ B and PI3K. In both human and mouse DCs, inhibitors of NF $\kappa$ B, JNK, p38, cJun and ERK signaling<sup>7,28–30</sup> all caused impairment of cytokine synergy, indicating the association of these signaling pathways with cytokine synergy. Interestingly, the contribution of each pathway in synergy is gene-dependent. JNK and ERK pathways mainly affect the synergistic production of TNF, IL6 and IL12p40, whereas p38 mainly affects IL12p70.<sup>29–31</sup> This is not surprising, as cytokine genes are usually regulated by different sets of transcription factors induced by different signaling pathways. In mouse macrophages, sustained ERK phosphorylation has been reported to be concordant with synergy after TLR3 and TLR7 co-stimulation, and inhibition of JNK reduces the synergistic production of TNF upon TLR4 and TLR9 activations.<sup>32,33</sup> In addition, the peak of expression of I $\kappa$ B $\zeta$  mRNA, which is required for driving the expression of several cytokine genes, was prolonged when DCs were stimulated by synergistic TLR combinations such as TLR4+TLR7 or TLR3+TLR7, indicating the involvement of I $\kappa$ B $\zeta$  signaling in cytokine synergy.<sup>7</sup> Therefore, multiple signaling pathways are responsible for cytokine synergy induced by TLR cooperation.

Indeed, combinatorial TLR activation results in stronger and broader changes in gene expression. Activation of multiple pathways is required to induce such significant changes. More importantly, activation of multiple pathways should occur at the right timing, which is supported by the observation that poly (I:C) priming exerts more significant cytokine synergy.<sup>23</sup> This might be due to an assembly of a competent transcriptional regulatory complex, which requires the cooperation of transcription factors activated by different signaling pathways at the right point in time. This phenomenon will be further discussed in the next section.

### TLR signaling cooperate at the transcriptional level

Interaction of multiple signaling pathways transmits signals to the nucleus, resulting in the activation of numerous transcription factors. The activation of various transcription factors upon infection suggests a simple model in which a given gene is activated by a defined set of transcription factors that binds to the DNA motif of the gene promoter.<sup>34</sup> In this model, transcription of the given gene is enhanced synergistically, probably by binding of the transcription factors cooperatively on its promoter and forming a three-dimensional structure suitable for the recruitment of cofactors and chromatin remodeling complex. One example of such a model is the activation of human *IFNB*. It is possible that activation of multiple TLRs induces an appropriate set of transcription factors that augments cytokine transcription. Indeed, Liu *et al.*<sup>35</sup> recently demonstrated that TLR3 and TLR7 activate different sets of transcription factors, which are all

required for the maximum cytokine expression. They showed that the TLR7-MyD88 signaling axis mainly activates JunB and CEBP $\beta$ , whereas TLR3-TRIF pathway mainly activates IRF1. JunB, CEBP $\beta$  and IRF1 are all required for cytokine expression, and simultaneous activation of both TLR7-MyD88 and TLR3-TRIF pathways synchronize and sustain the activation of these three core transcription factors, leading to cytokine synergy.<sup>35</sup> Thus, TLRs could directly cooperate with each other by activating different sets of transcription factors, which are required for the expression of synergistic levels of cytokines. This phenomenon is illustrated in Figure 1. Tan *et al.*<sup>23</sup> showed that pretreatment with poly (I:C) followed by R848 treatment enhances cytokine synergy, and a time window of 8 h between the two PAMP treatments exerted strongest synergy effect compared with 0 and 24 h intervals. This might be explained by the assembly of an optimal set of transcription factors required for cytokine gene expression. An 8-h duration of pretreatment with poly (I:C) might prepare IRF1 to be on standby before R848-induced CEBP $\beta$  and JunB are recruited to cooperate in cytokine transcription. Thus the crosstalk of TLRs is subject to the time and order of PAMP treatments, which might contribute to innate immune memory against sequential pathogen infection.

The transcription factor, IRF5, has also been shown to be involved in cytokine synergy in mouse macrophages under the stimulation of TRIF- and MyD88-activating ligands.<sup>13</sup> Peritoneal macrophages from IRF5 knockout mice exhibited a severe defect in the production of IL-12p40 in response to stimulation with a combination of poly (I:C) and CpG-DNA or LPS and CpG-DNA, whereas IRF3 knockout macrophages exhibited an almost normal response. This is also true *in vivo*. When poly (I:C) and CpG were administered intraperitoneally, the high levels of production of IL12p40 and IL6 were impaired in IRF5 knockout mouse compared with their wild-type counterparts. Apparently, IRF5 is needed for cytokine synergy under combinatorial stimulation. However, the authors did not describe how MyD88 and TRIF might contribute to IRF5 induction, and neither was there any clarification on whether IRF5 regulates cytokine expression in a direct or indirect manner. In future, it would be interesting to test the IRF5

expression under either MyD88- and/or TRIF-activated conditions to analyze the individual and combined contributions of these two pathways on IRF5 activation. It would also be interesting to perform ChIP with IRF5 antibody to check whether IRF5 binds directly to the cytokine promoter. Such studies will enlighten us on how IRF5 has a role in MyD88-TRIF crosstalk during infection.

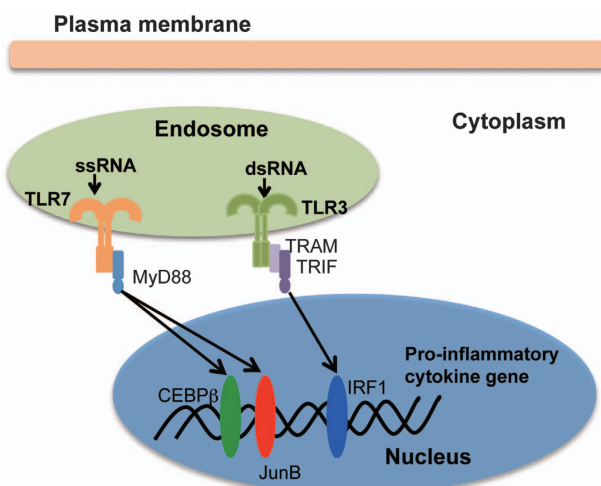
### The role of autocrine interferons in TLR cooperation

On the basis of the notion that a TRIF-dependent TLR is essential for cytokine synergy, one postulate was raised that IFN produced by the TRIF pathway might selectively enhance the cytokine production, as the TRIF pathway is more specialized in type I interferon production than the MyD88 pathway. Thus, some research groups have studied the involvement of IFN in cytokine synergy in DCs. However, controversial views have emerged from different research groups. Synergistic production of IL12 induced by CpG+poly (I:C) or R848 +poly (I:C) or R848+LPS is abolished in DCs from IFNAR knockout mouse compared with that of the wild type.<sup>6,22</sup> However, in both human and mouse DCs, blocking type I IFN with either IFN-neutralizing antibody or IFNAR2-blocking antibody, or adding type I IFN barely changed the level of IL12 production under synergistic activation of TLRs.<sup>6,7,22,28,29</sup> This controversy might be a result of different cell culture conditions and experimental methods. IFNAR knockout might completely block the type I IFN autocrine pathways, whereas other methods of study showed only partially blocked pathway without exerting a significant effect. It could also imply that endogenous IFN is the 'synergy factor,' or, that IFNAR might have the additional synergy role besides sensing IFN in DCs. Interestingly, the autocrine effect of IFN is unlikely to be the basis of the synergistic production of other proinflammatory cytokines such as IL6 and IL12p40, because neither IFNAR knockout nor neutralization, or adding type I IFN have any significant effect on their expression level under combinatorial TLR activation.<sup>6,7,22-24,28,29</sup> This is probably due to the lack of interferon-sensitive response element on the promoter region of these cytokine genes. However, the TRIF pathway may still contribute through other type I IFN-independent manner to cooperate with MyD88 to synergize cytokine expression. This possibility needs to be examined in future.

Despite the abundance of findings in signaling pathways and transcription regulation, definitive evidence on TLR cooperation mechanisms is still lacking, and yet TLR pathway crosstalk has vital implications on the immune response, immune over-activation and the fine balance between these two forces for consequential restoration of homeostasis. It is important to note that TLR cooperation mechanisms are highly cell-type-specific and gene-dependent. For example, DCs and macrophages produce different repertoire of cytokines. Activated DCs produce a large amount of IL12, which is composed of IL12p35 and IL12p40, to mount effective adaptive immune response, whereas macrophages induce mainly IL12p40 but not IL12p35. Thus, the mechanism of TLR-induced cytokine synergy could be different in different cell types. In addition even in the same DC cell, the synergistic production of IL12p35, which possesses interferon-sensitive response element in its promoter, is regulated differently from IL12p40. Thus the mechanism of TLR cooperation in regulating cytokine expression may need to be investigated on a case-by-case basis and the findings carefully considered under different cell context.

### CONCLUSIONS AND FUTURE PERSPECTIVES

It is becoming clearer that TLRs do not work alone in the recognition of invading pathogens. They network with each other to mount



**Figure 1** TLRs cooperate directly at the transcription level. Upon activation of TLR7 and TLR3, the MyD88 pathway activates CEBP $\beta$  and JunB, and the TRIF pathway induces IRF1. CEBP $\beta$ , JunB and IRF1 are all required for maximum pro-inflammatory cytokine gene expression. Combinatorial TLR activation synchronizes the activation of necessary transcription factors and provides an optimal condition for cytokine expression.



appropriate wave(s) of immune responses. TLRs crosstalk mainly through MyD88- and TRIF-induced signaling pathways and their downstream transcription regulators. In addition, signaling pathways induced by autocrine cytokines also have indispensable roles, and multiple signaling pathways such as those regulated by mitogen-activated protein kinase and NF $\kappa$ B are involved.

TLR crosstalk has an important role in maintaining immune homeostasis. In many cases, TLR crosstalk is required for mounting an appropriate level of immune response against pathogen invasion. This is of particular importance in the aged population. Aged mice express lower levels of TLRs on their macrophages, and cytokine production is also impaired.<sup>15</sup> In humans, TLR1/2 signaling is diminished in peripheral blood monocytes from the elderly.<sup>36</sup> These TLR-associated immune impairment may contribute to the enhanced susceptibility to bacterial, yeast and viral infections and poor adaptive immune responses in the aging population. On the other hand, uncontrolled TLR crosstalk, which results in immune-over-activation, may hamper immune homeostasis. Numerous studies have revealed the involvement of multiple TLRs, such as TLR 3, 7, 8, 9 in autoimmune diseases such as systemic lupus erythematosus (SLE).<sup>37</sup> Dysregulation of cytokines IL12 and IL6 was reported to contribute to SLE pathogenesis, plausibly related to the abnormality in TLR signaling.<sup>20,38</sup> Thus TLRs and their crosstalk are crucial in maintaining homeostasis and mounting efficient immune responses. As TLRs and other non-TLR PRRs recognize different components of a single pathogen or even the same component of a pathogen at different cellular localizations, TLRs could collaborate with other non-TLR PRRs (for example, RLRs, NLRs and CLR). An example of TLR and RLR crosstalk may be illustrated with the West Nile virus infection. RLRs, which recognize virus nucleic acid, is essential for sensing West Nile virus infection and for the initial triggering of intracellular innate immune response. TLRs 3 and 7, which are localized at different subcellular compartments also sense nucleic acids, and serve a secondary role to drive specific cytokine production leading to the regulation of the adaptive immune response and programming of cell-mediated immunity.<sup>39,40</sup> Another example may be taken from how both TLR2 and some CLR recognize fungal components such as glucan and mannose, thus collaborating in fungal infection. A large body of evidence has also shown the collaboration of TLR2 and NLRs (NOD1 and NOD2) in synergizing the production of TNF and IL12p40 upon bacterial invasion. This is likely due to the bacterial peptidoglycans, which are recognized by TLR2. Furthermore, the breakdown product of bacterial peptidoglycans act as ligands for NOD1 and NOD2.<sup>11–14</sup> Conceivably, multiple PRRs form many layers of surveillance against the invading pathogens to ensure secure and complete recognition of microbial PAMPs, resulting in the induction of appropriate levels of defense signaling for efficient removal of the pathogens. Notably, researchers need to be very cautious when evaluating the effect of TLR cooperation in cytokine expression. Importantly, the crosstalk between TLRs and other PRRs cannot be ignored, as this phenomenon leads to cytokine synergy and/or antagonism, which have pathophysiological implications on immune response and homeostasis. This is especially pertinent to the use of multiple TLR ligands as adjuvants in therapeutic strategies. The potential amplification of the effects of these ligands on other PRRs should be taken into account.

Considering the complexity of PRR network, it would be rather myopic to just focus on TLRs (and other non-TLR PRRs), and search for specific genes, proteins and signaling pathways involved for understanding the mechanism of TLR crosstalk. It is also necessary to understand the whole PRR signaling crosstalk from a systems

perspective, taking into account all the participating PRRs as well as other cellular components. Thus systems biology approach may be employed to computationally model the signaling networks in future. Efforts have been made on quantitative modeling of TLRs, mainly TLR3 and TLR4 pathways,<sup>41,42</sup> to elucidate various aspects of TLR signaling. However, none of these models took into account the dynamic crosstalk between the different TLR pathways. A model reflecting the interaction of multiple PRR crosstalk is lacking, and information from such a model would be excellent to provide future directions in the study of TLR cooperation as well as other PRR crosstalk without overlooking any component of the PRR-mediated pathways. Knowledge on the networking principles of PRRs, and the predicted and testable pathways could help us understand the sentinels of frontline immune defense and pathogenesis, and provide us with clues to control infection-inflammation-mediated diseases. Such an effort is ongoing collaboratively among empirical and computational experts.<sup>43</sup>

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## ACKNOWLEDGEMENTS

We thank the National Medical Research Council (NMRC/CBRG/0055/2013) for financial support.

- De Nardo D. Toll-like receptors: activation, signalling and transcriptional modulation. *Cytokine* 2015; **74**: 181–189.
- Takeuchi O, Akira S. Pattern recognition receptors and inflammation. *Cell* 2010; **140**: 805–820.
- Trinchieri G, Sher A. Cooperation of Toll-like receptor signals in innate immune defence. *Nat Rev Immunol* 2007; **7**: 179–190.
- Peters BM, Jabra-Rizk MA, O'May GA, Costerton JW, Shirliff ME. Polymicrobial interactions: impact on pathogenesis and human disease. *Clin Microbiol Rev* 2012; **25**: 193–213.
- Whitmore MM, DeVeer MJ, Edling A, Oates RK, Simons B, Lindner D *et al.* Synergistic activation of innate immunity by double-stranded RNA and CpG DNA promotes enhanced antitumor activity. *Cancer Res* 2004; **64**: 5850–5860.
- Gautier G, Humbert M, Deauvieu F, Scullier M, Hiscott J, Bates EE *et al.* A type I interferon autocrine-paracrine loop is involved in Toll-like receptor-induced interleukin-12p70 secretion by dendritic cells. *J Exp Med* 2005; **201**: 1435–1446.
- Napolitani G, Rinaldi A, Berton F, Sallusto F, Lanzavecchia A. Selected Toll-like receptor agonist combinations synergistically trigger a T helper type 1-polarizing program in dendritic cells. *Nat Immunol* 2005; **6**: 769–776.
- Bafica A, Scanga CA, Feng CG, Leifer C, Cheever A, Sher A. TLR9 regulates Th1 responses and cooperates with TLR2 in mediating optimal resistance to *Mycobacterium tuberculosis*. *J Exp Med* 2005; **202**: 1715–1724.
- Tabeta K, Georgel P, Janssen E, Du X, Hoebe K, Crozat K *et al.* Toll-like receptors 9 and 3 as essential components of innate immune defense against mouse cytomegalovirus infection. *Proc Natl Acad Sci USA* 2004; **101**: 3516–3521.
- Ferwerda G, Meyer-Wentrup F, Kullberg BJ, Netea MG, Adema GJ. Dectin-1 synergizes with TLR2 and TLR4 for cytokine production in human primary monocytes and macrophages. *Cell Microbiol* 2008; **10**: 2058–2066.
- van Heel DA, Ghosh S, Butler M, Hunt K, Foxwell BM, Mengin-Lecreux D *et al.* Synergistic enhancement of Toll-like receptor responses by NOD1 activation. *Eur J Immunol* 2005; **35**: 2471–2476.
- Fritz JH, Girardin SE, Fitting C, Werts C, Mengin-Lecreux D, Caroff M *et al.* Synergistic stimulation of human monocytes and dendritic cells by Toll-like receptor 4 and NOD1- and NOD2-activating agonists. *Eur J Immunol* 2005; **35**: 2459–2470.
- Uehara A, Yang S, Fujimoto Y, Fukase K, Kusumoto S, Shibata K *et al.* Muramyl dipeptide and diaminopimelic acid-containing desmaramylpeptides in combination with chemically synthesized Toll-like receptor agonists synergistically induced production of interleukin-8 in a NOD2- and NOD1-dependent manner, respectively, in human monocytes in culture. *Cell Microbiol* 2005; **7**: 53–61.
- Tada H, Aiba S, Shibata K, Ohteki T, Takada H. Synergistic effect of Nod1 and Nod2 agonists with toll-like receptor agonists on human dendritic cells to generate interleukin-12 and T helper type 1 cells. *Infect Immun* 2005; **73**: 7967–7976.
- Renshaw M, Rockwell J, Engleman C, Gewirtz A, Katz J, Sambhara S. Cutting edge: impaired Toll-like receptor expression and function in aging. *J Immunol* 2002; **169**: 4697–4701.
- Nyugen J, Agrawal S, Gollapudi S, Gupta S. Impaired functions of peripheral blood monocyte subpopulations in aged humans. *J Clin Immunol* 2010; **30**: 806–813.

- 17 Panda A, Qian F, Mohanty S, van Duin D, Newman FK, Zhang L *et al*. Age-associated decrease in TLR function in primary human dendritic cells predicts influenza vaccine response. *J Immunol* 2010; **184**: 2518–2527.
- 18 Qian F, Wang X, Zhang L, Lin A, Zhao H, Fikrig E *et al*. Impaired interferon signaling in dendritic cells from older donors infected in vitro with West Nile virus. *J Infect Dis* 2011; **203**: 1415–1424.
- 19 Nimah M, Brill R. Coagulation dysfunction in sepsis and multiple organ system failure. *Crit Care Clin* 2003; **19**: 441–458.
- 20 Huang X, Hua J, Shen N, Chen S. Dysregulated expression of interleukin-23 and interleukin-12 subunits in systemic lupus erythematosus patients. *Mod Rheumatol* 2007; **17**: 220–223.
- 21 Kawai T, Akira S. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity* 2011; **34**: 637–650.
- 22 Krummen M, Balkow S, Shen L, Heinz S, Loquai C, Probst HC *et al*. Release of IL-12 by dendritic cells activated by TLR ligation is dependent on MyD88 signaling, whereas TRIF signaling is indispensable for TLR synergy. *J Leukoc Biol* 2010; **88**: 189–199.
- 23 Suet Ting Tan R, Lin B, Liu Q, Tucker-Kellogg L, Ho B, Leung BP *et al*. The synergy in cytokine production through MyD88-TRIF pathways is co-ordinated with ERK phosphorylation in macrophages. *Immunol Cell Biol* 2013; **91**: 377–387.
- 24 Bagchi A, Herrup EA, Warren HS, Trigilio J, Shin HS, Valentine C *et al*. MyD88-dependent and MyD88-independent pathways in synergy, priming, and tolerance between TLR agonists. *J Immunol* 2007; **178**: 1164–1171.
- 25 Bekerjian-Ding I, Roth SI, Gilles S, Giese T, Ablasser A, Hornung V *et al*. T cell-independent, TLR-induced IL-12p70 production in primary human monocytes. *J Immunol* 2006; **176**: 7438–7446.
- 26 Tross D, Petrenko L, Klaschik S, Zhu Q, Klinman DM. Global changes in gene expression and synergistic interactions induced by TLR9 and TLR3. *Mol Immunol* 2009; **46**: 2557–2564.
- 27 Arsenault RJ, Kogut MH, He H. Combined CpG and poly I:C stimulation of monocytes results in unique signaling activation not observed with the individual ligands. *Cell Signal* 2013; **25**: 2246–2254.
- 28 Zhu Q, Egelston C, Vivekanandhan A, Uematsu S, Akira S, Klinman DM *et al*. Toll-like receptor ligands synergize through distinct dendritic cell pathways to induce T cell responses: implications for vaccines. *Proc Natl Acad Sci USA* 2008; **105**: 16260–16265.
- 29 Bohnenkamp HR, Papazisis KT, Burchell JM, Taylor-Papadimitriou J. Synergism of Toll-like receptor-induced interleukin-12p70 secretion by monocyte-derived dendritic cells is mediated through p38 MAPK and lowers the threshold of T-helper cell type 1 responses. *Cell Immunol* 2007; **247**: 72–84.
- 30 Makela SM, Strengell M, Pietila TE, Osterlund P, Julkunen I. Multiple signaling pathways contribute to synergistic TLR ligand-dependent cytokine gene expression in human monocyte-derived macrophages and dendritic cells. *J Leukoc Biol* 2009; **85**: 664–672.
- 31 Mitchell D, Yong M, Schroder W, Black M, Tirrell M, Olive C. Dual stimulation of MyD88-dependent Toll-like receptors induces synergistically enhanced production of inflammatory cytokines in murine bone marrow-derived dendritic cells. *J Infect Dis* 2010; **202**: 318–329.
- 32 Zhou L, Nazarian AA, Xu J, Tantin D, Corcoran LM, Smale ST. An inducible enhancer required for IL12b promoter activity in an insulated chromatin environment. *Mol Cell Biol* 2007; **27**: 2698–2712.
- 33 De Nardo D, De Nardo CM, Nguyen T, Hamilton JA, Scholz GM. Signaling crosstalk during sequential TLR4 and TLR9 activation amplifies the inflammatory response of mouse macrophages. *J Immunol* 2009; **183**: 8110–8118.
- 34 Smale ST. Selective transcription in response to an inflammatory stimulus. *Cell* 2010; **140**: 833–844.
- 35 Liu Q, Zhu Y, Yong WK, Sze NS, Tan NS, Ding JL. Cutting edge: synchronization of IRF1, JunB, and C/EBPbeta activities during TLR3-TLR7 cross-talk orchestrates timely cytokine synergy in the proinflammatory response. *J Immunol* 2015; **195**: 801–805.
- 36 van Duin D, Mohanty S, Thomas V, Ginter S, Montgomery RR, Fikrig E *et al*. Age-associated defect in human TLR-1/2 function. *J Immunol* 2007; **178**: 970–975.
- 37 Wu YW, Tang W, Zuo JP. Toll-like receptors: potential targets for lupus treatment. *Acta Pharmacol Sin* 2015; **36**: 1395–1407.
- 38 Linker-Israeli M, Deans RJ, Wallace DJ, Prehn J, Ozeri-Chen T, Klinenberg JR. Elevated levels of endogenous IL-6 in systemic lupus erythematosus. A putative role in pathogenesis. *J Immunol* 1991; **147**: 117–123.
- 39 Daffis S, Suthar MS, Szretter KJ, Gale M Jr, Diamond MS. Induction of IFN-beta and the innate antiviral response in myeloid cells occurs through an IPS-1-dependent signal that does not require IRF-3 and IRF-7. *PLoS Pathog* 2009; **5**: e1000607.
- 40 Suthar MS, Ma DY, Thomas S, Lund JM, Zhang N, Daffis S *et al*. IPS-1 is essential for the control of West Nile virus infection and immunity. *PLoS Pathog* 2010; **6**: e1000757.
- 41 Helmy M, Gohda J, Inoue J, Tomita M, Tsuchiya M, Selvarajoo K. Predicting novel features of Toll-like receptor 3 signaling in macrophages. *PLoS ONE* 2009; **4**: e4661.
- 42 Gutierrez J, St Laurent G 3rd, Urcuqui-Inchima S. Propagation of kinetic uncertainties through a canonical topology of the TLR4 signaling network in different regions of biochemical reaction space. *Theor Biol Med Model* 2010; **7**: 7.
- 43 Liu B, Liu Q, Yang L, Palaniappan SK, Bahar I, Thiagarajan PS *et al*. Innate immune memory and homeostasis may be conferred through TLR3-TLR7 pathway crosstalk. *Sci Signal*. (In revision) 2016.