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REVIEW

TLR Cross-talk Confers Specificity to Innate Immunity

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Cross-talk within the innate immune pathways is highly complex and contains many unknowns. Here, we discuss the different combinations of PAMPs, together with the sequence, order, and dosage of consecutive PAMP challenges, which determine the nature of the immune response by macrophages. The engagement of different Toll-like receptor (TLR) ligands leads to quantitatively and qualitatively unique cytokine production, showing that TLR pathway crosstalk enables the innate immune system to orchestrate immediate local and global responses. It is likely that multiple pathways are involved in the regulation of cytokine synergy, including many that have yet to be discovered.

Keywords cytokines, macrophage, pathogen recognition, signaling cross-talk, toll-like receptors

Abbreviations: CLRs: C-type lectin receptors; DUSP: dual specificity phosphatases; HMGB1: high-mobility group box protein; PPR: Pattern-recognition receptor; RLRs: RIG-like receptors

INTRODUCTION

In contrast to adaptive immunity, which partially limits damage to the host through active peripheral tolerance and the highly specific recognition of antigens, the innate immune system has been considered broadly promiscuous and relatively non-specific. Unlike the diverse array of T and B cell receptors generated through random rearrangement of the V, D, and J segments, which ensures high level of specificity attributable to *de novo* events within the lifetime of the mammalian immune system, the innate immune receptors are germline-encoded and are thus considered

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invariant. Hence, innate immunity is at best described as "pathogen-specific" through interaction with various pattern recognition receptors (PPRs), while adaptive immunity is considered "antigen-specific" [1]. Distinctions such as these imply that innate immunity has a limited repertoire of recognition, while adaptive immunity has almost unlimited receptor specificities. This assumption is somewhat misleading, as there is also degeneracy and cross-reactivity in adaptive immune receptors [2], with a recent study demonstrating a single T cell receptor (TCR) found to recognize more than a million different peptides [3]. Furthermore, the innate immune system is able to detect most, if not all microbes [4], by recognizing evolutionarily conserved molecules essential to pathogen survival. However, how specificity could be generated from receptors which recognize different pathogens nonspecifically remains to be explained.

Despite the lack of a detailed mechanistic understanding on the "specificity" of innate immunity, the invertebrates, which only possess innate immunity, have been shown to elicit strain-specific immunity in certain cases [5]. On the other hand, specificity in the vertebrate innate immunity has been relatively overshadowed by the adaptive immune system which is highly selective. However, several lines of evidence suggest that the innate immune system is also capable of a certain degree of specificity [6]. For example, in the Toll-like receptor (TLR) system, activation by microbial agonists (such as LPS, recognized by TLR4) leads to the activation of adaptive immunity, inflammation, and tissue repair. However, activation of the same TLR by endogenous ligands including heat-shock proteins and high-mobility group box protein (HMGB1) leads only to limited immune responses, without the activation of adaptive immunity and potential unwanted inflammatory insults or autoimmune reactions [1]. This shows that although the same TLR is employed, there are additional context-determinants that regulate the specificity of responses. In addition, these factors are also important in the discrimination between pathogenic or commensal microbes, which are achieved through the concomitant detection of "danger signals" or virulence factors [7]. This is analogous to the "two-signal" paradigm in T cell activation, whereby antigen-specific binding between the MHC-peptide/TCR complex, together with CD28-CD80/CD86 costimulatory interaction, is required for a full cellular activation [8]. This review highlights various examples of PRR cross-talk, which act as context-determining mechanisms in conferring specificity to innate immunity.

Specificity Through Receptor Diversity

Although T cells are known to have incredibly diverse receptor rearrangements with a theoretical 10^{12} possible VDJ recombinations, in reality, only 10^7 are observed in normal human TCR diversity [9]. Unlike the adaptive immune system described above, during infection the host inflammatory reaction is initiated by the recognition of pathogen-associated molecular patterns (PAMPs) by the PRRs. The four main PRR families are TLRs, RIG-like receptors (RLRs), NOD-like receptors (NLRs), and C-type lectin receptors (CLRs) which share overlapping ligand specificities and converge on common downstream signaling pathways such as NF-κB.

PRR Collaboration and Non-additive Responses

Underhill [10] has proposed several theories to explain why PRR cross-talk has evolved; these include (i) robustness against microbial evasion, (ii) compensation against genetic diversity in host population, (iii) multiple receptors which allow for a scaled response, and finally (iv) multiple receptors which facilitate a tailored, hence more specific response [10]. In agreement with these proposals, the optimal host response to many pathogens indeed requires the collaboration of multiple receptors and possibly, their cognate adaptors. For example, the double-stranded DNA virus modified vaccinia Ankara (MVA) is cooperatively recognized by three different PRRs

(TLR2–6, RIG-I, and NALP3) [11] whereas response to rhinovirus is controlled by both the TLR and RLR pathways (TLR3, RIG-I, and MDA5) [12]; *Mycobacterium paratuberculosis* is recognized by TLR2, TLR4 and NOD2 [13]. However, PRRs may also negatively regulate each other, especially when different types of pathogens are involved. One such observation is that prior infection by viruses increases subsequent susceptibility to bacterial infection due to RLR interference with TLR signaling [14]. Accordingly, the phrase, receptor "collaboration," is favored over costimulation or cooperation [10], as it captures the essence of PRR interactions, which can either be positive (synergistic) or negative (antagonistic) in regulating the eventual immune responses.

We next elaborate and provide examples of collaborations between PRRs that result in the following three types of nonadditive responses: (i) strict requirement for two or more PRRs, (ii) interactions for robustness or redundancy, and (iii) negative regulation between PRRs.

Strict requirement for two or more PRRs to achieve non-additive responses

Several immune processes require the concomitant activation of at least two different PRRs in order to proceed. In signaling systems theory, this mechanism of regulation is known as "coincidence detection," which reduces the chance of false detection. For example, when a sensor detects a signal amidst random noise, there is a probability (p), that the detected signal is actually noise. However, if two sensors are required to simultaneously detect a signal, the probability of falsely sensing noise is reduced to (p^2). In the neural system, such "coincidence detection" mechanisms are known to underlie many important processes such as long-term potentiation and long-term depression in synaptic plasticity [15]. With the immune system such "coincidence detection" is required to obviate random activation, thereby avoiding nonspecific responses and minimizing damage to the host or the potential onset of autoimmunity.

One well-documented example of cooperation between innate immune receptors is the production and release of mature IL-1β in macrophages. IL-1β mRNA is upregulated in the presence of TLR ligands, but the release of bioactive IL-1β protein depends on the activation of caspase-1, which is regulated by an NLR-containing complex, known as the inflammasome [16]. Thus, macrophages require two signals, one from the TLR system and one from the NLR system, in order to release bioactive IL-1β. Interestingly, this strict requirement for "coincidence detection" is not necessary in monocytes, where caspase-1 is constitutively active and TLR activation alone is sufficient to trigger IL-1β release. This reflects a functional adaptation to the different environments encountered by monocytes and macrophages in host defense and inflammation, respectively. Monocytes are present in the circulatory system which is normally a microbe-free site and should respond quickly upon detection of pathogens. In contrast, macrophages are found in peripheral and mucosal tissues and are in constant contact with commensals or nonpathogenic microbes, where uncontrolled proinflammatory activation could lead to substantial immunopathology to the host [16].

Redundancy and robustness in nonadditive responses

Apart from restricting host damage by being strictly dependent on the activation of two or more PRR pathways, detection of a pathogen by multiple PRRs confers robustness to the innate immune system. Substantial redundancy of TLR and NLR pathways have been reported in the development of T cell immunity to replication-defective adenovirus serotype 5, a potent inducer of $CD8^+$ T cell responses [17]. In this scenario, TLR- and NLR-driven pathways are able to compensate for each other, ensuring a robust immune response in the event of microbial evasion of one pathway or the other. There is redundancy even within each individual PRR family, as seen in the TLR

system where the lack of a single TLR member does not result in primary immunodeficiency although patients lacking the shared components such as MyD88 or IRAK4 exhibit impaired production of proinflammatory cytokines together with increased susceptibility to pyrogenic bacteria. Such impairments are typically associated with primary immunodeficiency [18]. Other than robustness against pathogen evasion, the detection of pathogens via multiple PRRs allows for the generation of an efficient response with minimal immunopathology [19], since the engagement of multiple effector mechanisms enables the immune system to effectively clear the pathogen without exerting maximal activation of any one pathway. This may explain why autoimmune diseases paradoxically develop in immunocompromised patients [20], as the lack in specific immune components causes the remaining pathways to be overactivated.

Alternatively, activation via a single PRR may induce a mild response whose magnitude, speed or duration is enhanced in the presence of an additional PRR activation. This kind of collaboration allows the innate immune system to rapidly scale up the response in the face of severe threats where multiple PAMPs are present. An example of this would be the case of fungal infection whereby both CLRs and TLR2 are activated. Stimulation with β-glucan (CLR dectin-1 ligand) alone does not result in TNF production, even at high doses. A low dose of Pam_3CSK_4 (TLR2 ligand) alone similarly results in only a low level of TNF secretion. However, when macrophages encounter both ligands simultaneously, TNF production is synergized via the prolonged degradation of IκB, which in turn enhances NF-κB nuclear translocation [21]. Furthermore, the crosstalk between dectin-1 and TLR2 does not only quantitatively enhance the magnitude of the response, but also qualitatively modifies it, synergizing the levels of IL-6 and IL-23 while downregulating IL-12, conditions which promote the development of T_H17 immunity, which is the hallmark of antifungal response [22].

Negative PRR interactions result in nonadditive responses

In spite of the many examples of synergistic PRR collaborations, not all PRR interactions are positive. To prevent excessive immune activation, it is beneficial for the host to possess mechanisms to attenuate proinflammatory signals. Indeed, the trigger of PRRs induces the upregulation of inhibitory pathways, such as the suppressor of cytokine signaling [23] and Tyro3/Axl/Mer [24] systems. Other mechanisms of indirect inhibition include the induction of anti-inflammatory IL-10 and the internalization and degradation of receptors. However, different PRRs may also directly antagonize each other at the signaling level, as in the case of RLR mediated cross-interference of TLR signaling [14], and dendritic cell immunoreceptor inhibition of TLR signaling via SH2-domain-containing protein tyrosine phosphatase recruitment [25]. Within TLR signaling, MyD88 has been shown to be an inhibitor of TRIF signaling, with one study showing enhanced TRIF-mediated RANTES production in the absence of MyD88 in corneal epithelial cells but not macrophages [26], and another demonstrating that MyD88 inhibits TRIF-mediated IFN-β and RANTES by suppressing IKKε-dependent IRF3 phosphorylation in macrophages [27]. These two studies also highlight that PRR-PRR interactions are highly cell- and context-dependent, as seen previously with IL-1β processing in monocytes compared to macrophages. In general, negative cross-talk between different PRR families is one mechanism of preventing immunopathologies [28].

TLR-TLR Cross-talk

Generally, RLRs are specialized in the recognition of viruses, whereas CLRs are predominantly responsible for detecting and clearing fungal infections. On the other hand, TLRs have receptors for all classes of pathogens, ranging from viruses, and bacteria to fungi and parasites [29]. In addition, as previously described, RLR or CLR

signaling alone is insufficient to mount an effective immune response in certain cases, whereas TLR-mediated signaling is essential and adequate. The TLR system is also the most well-understood, which facilitates the study of the network since the major nodes have been identified. Furthermore, the expression of TLRs is generally restricted to immune cells, in contrast to the RLRs which can be found in almost all cell types. This further simplifies the problem by allowing us to consider TLR-TLR interactions within a single cell type, rather than the more complex interactions between immune and non-immune cells. Therefore, the rest of this review focuses on specificity as an outcome of TLR-TLR cross-talk.

Differential and collaborative relay of signals from TLRs to the nucleus

A number of important questions remain to be addressed within TLR signaling. If all signaling converges on either MyD88 and TRIF, how are discriminatory signals relayed from the TLR to the cell nucleus [30]? Downstream of MyD88 and TRIF, the signaling pathways activated also appear similar and seem largely redundant [31]. And yet the activation of different TLRs can result in different responses. For example, LPS-induced activation of TLR4 leads to high production of IL-12p70, a Th1 promoting cytokine, while activation of TLR2 results in only low production of IL-12p70, high production of IL-10 and skewing towards Th2 responses [32]. Nevertheless, the specific context-determining mechanisms apart from the activation of common pathways that result in such divergent responses are still not fully understood.

In addition, while we might now be aware of the differential *in vitro* and *in vivo* effects of single TLR ligands studied in isolation, hitherto researchers are still unable to predict the outcome in a simplified but more realistic scenario whereby two TLR ligands are encountered over the course of an infection. This scenario is plausible, as even a single pathogen often contains multiple PAMPs, which may be released simultaneously or at different stages of the infection. For example, ssRNA molecules from ssRNA viruses processed in the endosome is detected by TLR7 while dsRNA produced during viral replication is detected by TLR3. Furthermore, in the case of a polymicrobial infection, PAMPs from different classes of pathogens may be present concurrently. In such complex scenarios, it is likely that there is a significant degree of crosstalk between the TLR-mediated pathways. Thus, the net response is determined not only by whether a single TLR ligand is activated or not as is the case in many studies solely relying on LPS as an activation factor, but also by which combination of TLRs are responses simultaneously activated [10].

Synergy in TLR cross-talk

Numerous studies have documented the importance of TLR-TLR cross-talk. For example, it was reported that simultaneous stimulation with MALP2 and LPS (TLR2 and TLR4 ligands, respectively) results in the production of TNF at levels much greater than that observed for each of the ligands alone [33], a phenomenon termed synergy. More recently, TLR4 and TLR9 were shown to synergize in the production of TNF in mouse macrophages [34] in a manner associated with enhanced MAPK signaling. However, another study [35] using human monocyte-derived dendritic cells demonstrated that TLR4 and TLR9 were the only combination of receptors that led to the synergistic expression of IL-12p70, and this phenomenon was not observed for other cytokines such as TNF. These differences highlight the divergent responses of macrophages and dendritic cells (DCs); while both types of cells are antigen presenting cells (APCs) expressing TLRs, they have specialized functions in innate (TNF) and adaptive (IL-12p70) immunity, respectively. Different groups [36, 37] working with DCs have found other pairwise combinations of TLR ligands which are able to cause the greater-than-additive production of IL-12p70 as well as other cytokines,

TABLE 1. A summary of TLR-TLR crosstalk resulting in cytokine synergy.

Cell type	Derivation	Combinations	Cytokine	Mechanism	Refs
Dendritic cells					
Human moDC	CD14 selection, GM-CSF, IL-4	TLR3/4 and TLR8	IL-12p70	Sustained c-Jun	[36]
Human moDC	CD14 selection, GM-SCF, IL-4	TLR4 and TLR8	IL-12p70, IL-6, IL-10, TNF	p38	[55]
Human moDC	CD14 selection, GM-SCF, IL-4	TLR3 and TLR2/6/5	IL-12p70	Not studied	[55]
Human moDC	Negative selection, GM-CSF, IL-4	TLR3/4 and TLR8	IL-12p70, IL-6, IL-10, TNF	p38, NF-κB, PI3K	[56]
Mouse BM-DC	GM-CSF	TLR3 and TLR9	IL-12p70	Autocrine type I IFN	[48]
Mouse BM-DC	Negative selection, GM-CSF, TNF	TLR3/4 and TLR8	IL-12p70	Type I IFN loop	[46]
Mouse BM-DC	Negative selection, GM-CSF	TLR2 and TLR4	IL-10	p38, JNK	[57]
Mouse BM-DC	GM-CSF	TLR2, TLR3 and TLR9	IL-12p70 and IL-15	Not studied	[38]
Macrophages					
Human moMϕ	Adherence, GM-CSF	TLR3/4 and TLR8	No synergy	Not studied	[56]
Mouse BM-DM	Teflon adherence	TLR3/4 and TLR2/5/9	TNF, IL-6	NF-κB, not IFN-β	[43]
Mouse BM-DM	M-CSF	TLR4 and TLR9	TNF, IL-6	JNK	[34]
PEC	Thioglycollate-induced	TLR3/4 and TLR7/9	IL-12p40	IRF5	[44]

This table compares the various studies investigating cytokine synergy in TLR-TLR stimulation, based on the cell type studied, derivation method, combination of TLR ligands, cytokines shown to be synergistically regulated, proposed mechanisms, and the respective references. moDC, monocyte-derived dendritic cells. BM-DM, bone marrow-derived macrophages. moMϕ, monocyte-derived macrophages. PEC, peritoneal exudate cells. GM-CSF, granulocyte-macrophage colony stimulating factor. M-CSF, macrophage colony stimulating factor.

leading to a Th1 polarizing phenotype. This synergistic effect of combinatorial TLR stimulation has been exploited to design an adjuvant that enhanced T cell responses to vaccination [38]. For example, while dual TLR stimulation quantitatively enhanced the number of responding T cells through IL-12p70-driven clonal expansion, triple TLR stimulation by MALP-2, poly(I:C), and CpG was able to qualitatively modulate the immune response, enhancing the levels of IL-15 and thereby driving the development of T regulatory (Treg) cells. These and other studies are collated in Table 1.

Antagonism in TLR cross-talk

Apart from the above examples of TLR-TLR cross-talk leading to cytokine synergy, TLR cross-talk can also result in less-than-additive responses, or antagonism. One well-known example would be the phenomenon of LPS tolerance, whereby cells stimulated chronically with LPS become refractive to subsequent TLR stimulation. Several mechanisms have been proposed, such as the downregulation of TLR4/MD2 surface expression, and the upregulation of negative regulators such as IL-10, IRAK-M, and SARM (sterile α– and HEAT/armadillo-motif-containing protein) [39]. However, consensus

has not yet been reached, with conflicting reports that remain to be resolved. It has been shown that LPS-tolerized cells have reduced rather than enhanced expression of certain negative regulators such as suppressor of IkappaB kinase-epsilon [40]. In addition, LPS induced cross-tolerance to MALP2 stimulation could not be explained by enhanced IL-10 production [33]. Conversely, MALP2 induced cross-tolerance to LPS was not due to the downregulation of TLR4 surface expression. Rather, it has been suggested that TLR2-induced tolerance is specific to TLR4 and TLR7 ligands but does not affect TLR3 and TLR5 signaling, and this is achieved via inhibition of paracrine type I interferon amplification which in turn abrogates IL-12p70 production [41]. The complexities demonstrated by these studies show that the outcome of the encounter with several TLR ligands is potentially diverse and merits further investigations.

Sequential TLR Stimulation

Combinatorial or sequential TLR stimulation could potentially modulate or fine tune the immune response, allowing a greater degree of control in terms of the qualitative and quantitative responses. Indeed, the recognition of several pathogens has been shown to depend on sequential activation. For example, the recognition of a particular strain of herpes simplex virus depends on an initial interaction via cell surface TLR2, followed by intracellular TLR9 recognition of internalized viral genomic DNA. Furthermore, this sequential recognition is required to occur within the same cell through direct recognition of viral particles, rather than activation via a bystander effect [42]. It has been observed that murine DC pretreated with CpG DNA (a TLR9 ligand) followed by LPS (a TLR4 ligand) showed an enhanced IL-12 production, but when the order of TLR ligand exposure was reversed with the addition of LPS followed by CpG DNA stimulation, such enhancement was abrogated [35].

In contrast, rather than inducing tolerance, De Nardo et al. [34] have shown that LPS pretreatment promotes pro-inflammatory response, including IL-6 and TNF production of mouse BMMs to the TLR9 ligand, CpG DNA. The priming effects of LPS, which correlated with enhanced Erk1/2, JNK, and p38 MAP activation, appeared to be mediated via both c-Fms-dependent and -independent mechanisms. Similarly, macrophages pretreated with poly(I:C) for 20 h followed by stimulation with TLR2 or TLR9 ligands showed synergy for TNF production, and this synergy was preserved when the order of stimulation was reversed [43]. Macrophages simultaneously stimulated with TLR2 and TLR5 ligands showed a synergistic increase in TNF production; however, when pretreated with either LPS or MALP2 for 24 h and then challenged again with either LPS or MALP2, the synergy observed under simultaneous stimulation was abrogated [33]. All these examples illustrate that TLR-TLR collaboration is highly sensitive to the timing or order of stimulation as well as the length of time between the first and second stimulations, and potential differences may prevail in various APC populations. What remains to be discovered are whether such positive and negative sequential effects are seen for other TLR combinations, and the mechanism driving such outcomes.

Mechanisms Regulating Cytokine Synergy

Arising from the discoveries of diverse and complex responses to TLR-TLR cross-talk, several different mechanisms of synergy have been proposed. Firstly, multiple lines of evidence suggest that **MyD88-TRIF adaptor cross-talk** mediates synergy. Most of the combinations resulting in synergy involve either TLR3 or TLR4, which provides the TRIF signal, and another MyD88-activating ligand (e.g., Pam_3CSK_4 or MALP2 TLR2 ligands, R848 TLR7 ligand, or CpG TLR9 ligand). Consistent with the hypothesis that synergy arises from MyD88-TRIF collaboration, pairwise combinations of TLR ligands which activate only MyD88 signaling do not induce synergism [43]. Ouyang et al. [44]

have also suggested that MyD88-TRIF synergy in the production of IL-12p40 mRNA is due at least in part to IRF5 activity, although it remains unclear whether IRF5 is specifically required for synergy or is simply necessary for gene transcription. However, there are exceptions such as synergy between TLR2 and TLR5 [41] or TLR9 [43] where TRIF signaling is absent, which indicate that other mechanisms play a role. A recent study showed that multiple TLRs (TLR2, TLR4, and TLR9) in macrophages regulate and coordinate multiple virulence mechanisms of *Salmonella typhimurium*, to support the intracellular survival and virulence of this pathogen [45]. This is consistent with the crosstalk of multiple signaling events driven by multiple TLRs. Moreover, the bone-marrow derived macrophages from mice deficient in TLR2, TLR4, and TLR9 were found to be more resistant to *S. typhimurium* infection compared to macrophages from TLR2- and/or TLR4-deficient animals [45], suggesting that the presence of other residual TLR-mediated host-defense responses could alter the ultimate pathogen survival.

Alternatively, **type I interferons**, rather than direct TRIF-induced signaling, might explain cytokine synergy, since the activation of TLR9 is capable of inducing the production of type I interferons, at least in plasmacytoid DCs. For example, the synergistic production of IL-12p70 in DCs might be explained by the IFN amplification loop, whereby type I interferons induced by encounter with the first TLR ligand increases the levels of the transcription factor IRF7. This might enhance the usage by the second TLR ligand and synergistically promote the level of IL-12p70 produced [46, 47]. In support of this hypothesis, it has been observed that the synergy of IL-12p70 in DCs is reduced in Type I common chain IFN receptor (IFNAR) gene knockout mice. However, the IFN amplification hypothesis is inadequate. Blocking of the IFNAR with antibodies was found to have no effect on IL-12p70 production [48]. Using a different approach, exogenously added IFN-β induced only a modest and variable effect on IL-12p70 synergy that was independent of the nature of the stimulus [36], indicating that other mechanisms are possibly involved. Furthermore, while type I IFNs are known to influence the production of IL-12p70, the production of other proinflammatory cytokines is not known to depend on the IFN amplification loops. Therefore, other mechanisms must be involved although they may act in concert with type I interferons, especially for cytokines other than IL-12p70. One such mechanism may be the novel TRIF-dependent pathway which licenses NLRP3 inflammasome activation by Gram-negative bacteria such as *E. coli* and *C. rodentium* [49]. The phagocytosed bacterial mRNA was shown to trigger NLRP3 assembly leading to caspase-11 activation via the TLR4-TRIF-IFN-β pathway, which then synergizes with the NLRP3 to mediate caspase-1-dependent IL-1β and IL-18 production. Thus, TRIF was identified to be a regulator of caspase-11, which highlights the importance of TLRs as master regulators of inflammasomes during Gram-negative bacterial infection [49]. The cross-talk between TLR-TLR and TLR-NLR pathways supports the integration of signaling events with synergistic outcome against the infection.

Using several chemical inhibitors of the **MAPK, NF-κB, and PI3K pathways**, various researchers have also claimed that these pathways are important for the synergistic production of cytokines (see Table 1). Aside from the conflicting claims regarding the relative importance of each pathway, many studies showed that chemical inhibition of the various signaling pathways not only reduced the amount of cytokines produced when two ligands were simultaneously present, but also reduced the amount of cytokines produced when single TLR ligands were applied instead. Therefore, such reduction in the amount of cytokines in the presence of the chemical inhibitor cannot be solely attributed to the abolishment of cross-talk; another equally valid interpretation would be that the pathways inhibited are necessary, but not sufficient for cytokine synergy.

Another possible mechanism of synergistic cytokine production would be the **inhibition of suppressors** of TLR signaling. Indeed, it has been demonstrated that TLR7 costimulation with TLR4 inhibits negative regulators IRAK-M and BCL3, which normally attenuate the cytokine response, leading to increased levels of TNF, IL-10, and IL-12 [50]. Given the importance of the MAPK pathway in cytokine production, the spatiotemporal regulation of MAPK phosphorylation represents an attractive target for immunomodulation. In particular, a family of MAPK phosphatases known as the dual specificity phosphatases (DUSP), have been shown to play important roles in the negative regulation of immunity via the inhibition of MAPK phosphorylation and localization [51, 52]. Two members (DUSP1 and DUSP2) have been shown to be positively associated with combinatorial TLR stimulation [36, 53]. Interestingly, Tan et al. recently identified that synergistic TLR stimulation is associated with downregulation of DUSP6, which in turn results in sustained ERK phosphorylation and cytokine synergy [54]. However, it is not yet known whether members of the DUSP family directly participate in cytokine synergy or are merely associated with combinatorial TLR stimulation.

In conclusion, the engagement of different TLR ligands leads to quantitatively and qualitatively unique cytokine responses, showing that TLR-TLR crosstalk enables the innate immune system to orchestrate immediate local and global responses. However, there is a lack of consensus on the mechanism of synergy. It is likely that multiple pathways are involved in the regulation of cytokine synergy, including many that have yet to be discovered.

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Declaration of Interest

The authors declare no conflict of interest. The authors are responsible for the content and writing of the article.

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Notice of Correction

Changes have been made to this article since its original online publication date of 9 June 2014.