Feature Review
Integration of Innate Immune Signaling

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The last decades of research in innate immunology have revealed a multitude of sensing receptors that evaluate the presence of microorganisms or cellular damage in tissues. In the context of a complex tissue, many such sensing events occur simultaneously. Thus, the downstream pathways need to be integrated to launch an appropriate cellular response, to tailor the magnitude of the reaction to the inciting event, and to terminate it in a manner that avoids immunopathology. Here, we provide a conceptual overview of the crosstalk between innate immune receptors in the initiation of a concerted immune reaction to microbial and endogenous triggers. We classify the known interactions into categories of communication and provide examples of their importance in pathogenic infection.

Introduction

Twenty-five years after the formulation of the theory of pattern recognition in innate immune sensing [1], research in the field of innate immunity has led to the discovery of several families of innate immune receptors, their intracellular signaling pathways, and the immune effector mechanisms that are triggered upon receptor engagement [2]. Defined microbial and non-microbial ligands as specific triggers of single pattern recognition receptors (PRRs) have been instrumental in delineating receptor specificities and downstream intracellular response circuitries. The field has now progressed to a state in which predictions can be made about the engagement of certain immune effector mechanisms, for example changes in transcription or phagocytic activity, in response to specific microbial ligands. However, innate immune sensing in a complex tissue in the *in vivo* setting involves a multitude of simultaneously triggered responses, interaction between signaling pathways, and concurrently-active effector mechanisms. As such, tissue-level innate immune sensing needs to integrate not only stimuli derived from different ligands of the triggering agent but at the same time must balance concomitantly-active signaling pathways to generate an appropriate, concerted, and self-limiting effector response. The mechanisms are only beginning to be understood, but recent studies have revealed multiple innate immune receptor interactions that provide insight into the principles of concerted tissue-level integration of innate immune signaling [3]. We discuss here several categories of PRR interaction in the coordination of the immune response, and focus on some of the most outstanding questions in the field, including the need to further delineate the cellular, tissue-level, and whole-organism facets orchestrating the compartmentalization of local and systemic innate immunity.

Integrated Components of Innate Immune Signaling

Recognition of microorganisms, including viruses, bacteria, and fungi, by the host immune system is based on the detection of conserved molecular structures that are shared by a large number of pathogens, termed pathogen-associated molecular patterns (PAMPs), as well as

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Coordination of innate immune receptor signaling in response to simultaneous stimuli in a complex tissue occurs at the levels of ligands, signaling transducers, and immune effector mechanisms.

Cross-activation between innate immune receptors involves all families of PRRs and serves as an important means of signal amplification in the initiation of an antimicrobial effector response.

Cross-inhibition between innate immune receptors, predominantly involving members of the NLR family, provides a means of signal prioritization, immune effector hierarchy, and regulated immune response termination.

PRR cooperation and cross-activation is an essential strategy of antipathogen defense.

Cell type- and tissue-specific expression patterns of innate immune sensors determine the biogeographical pattern of immune receptor crosstalk.

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damage-associated molecular patterns (DAMPs). According to protein structure and domain function, six families of PRRs can be distinguished:

(i) Toll-like receptors (TLRs) are a family of membrane-bound proteins that initiate innate immune responses through signaling pathways mediated by NF-κB and interferon (IFN)-regulatory factors (IRFs) [4]. With the exception of TLR3, all TLRs elicit signaling through MyD88 and the transcription factors NF-κB and IRF5 to induce the expression of proinflammatory cytokines such as IL-6, IL-12, and TNF [5]. In addition, some TLRs initiate signaling through MyD88 and IRF7 (TLR7, TLR8, and TLR9), or through TRIF/TRAM and IRF3 (TLR3 and TLR4) to induce type I IFNs [6].

(ii) C-type lectins (CTLs) are membrane-bound carbohydrate receptors that consist of two types: type I CTLs (such as DEC-205 and MMR) that contain multiple carbohydrate recognition domains (CRDs), and type II CTLs (including dectin 1, dectin 2, Mincle, DC-SIGN, DNGR-1) that contain a single CRD [7,8]. An additional member of the CTL family is the soluble carbohydrate receptor mannose-binding lectin (MBL). Intracellularly, CTLs trigger a pathway that is dependent on the kinase SYK and phospholipase C (PLC).

(iii) NOD-like receptors (NLRs) are cytoplasmic receptors of various microbial and non-microbial stimuli. While NOD1 and NOD2, upon stimulation, lead to the activation of NF-κB signaling and changes in gene expression, other members of this family do not directly alter transcription but initiate the formation of the inflammasome complex. The inflammasome recruits and activates caspase 1, which in turn activates proinflammatory cytokines by post-translational cleavage [9,10].

(iv) RIG-I-like receptors (RLRs), such as RIG-I, MDA5, and LGP2, are cytoplasmic sensors of RNA. Upon activation, they signal to the mitochondrial adapter MAVS to induce an IRF3- and IRF7-mediated type I IFN response [11].

(v) AIM2-like receptors (ALRs) are cytoplasmic DNA sensors. Some of these sensors, including IFI16 and DAI, signal through the endoplasmic reticulum-associated adapter STING to initiate downstream signal transduction. Others, such as AIM2, are capable of forming an inflammasome [12].

(vi) OAS-like receptors (OLRs) are a recently identified group of cytoplasmic innate sensors of nucleic acids. Members of this family are the oligoadenylate synthase (OAS) proteins and cyclic GMP-AMP synthase (cGAS). These PRRs are unique in their signal transduction mechanisms because they produce 2′–5′-linked second messenger molecules that initiate downstream antiviral immune responses [13].

Conceptually, PRRs can therefore be classified based on the type of their microbial or endogenous ligands (e.g., cell wall components, nucleic acids, metabolites), the signaling pathways they trigger (e.g., MAP kinase, NF-κB, IRFs, inflammasome), and the downstream immune effector modules they initiate (e.g., cytokine production, antimicrobial peptides, phagocytosis, antibodies) (Figure 1). As detailed below, some receptors can be activated by similar ligands, and initiate the same signaling and effector modules, thereby inducing a complex and multifaceted immune response.

Shared Ligands

Multiple PAMPs trigger more than one receptor (Figure 1A). The recognition of identical microbial ligands by more than one PRR likely provides an opportunity for the host to develop 'back-up' mechanisms for compensatory microbial sensing, and creates the possibility to sequentially add additional information to the recognition event, resulting in a continuum of signaling ranges instead of a 'digital' output denoting only the presence or absence of an inflammatory trigger. However, activation of several innate immune pathways by the same shared signals also poses the conceptual challenge of understanding the code determining which receptors are to be engaged in a given situation, and at which priority, signaling strength, and temporal and spatial
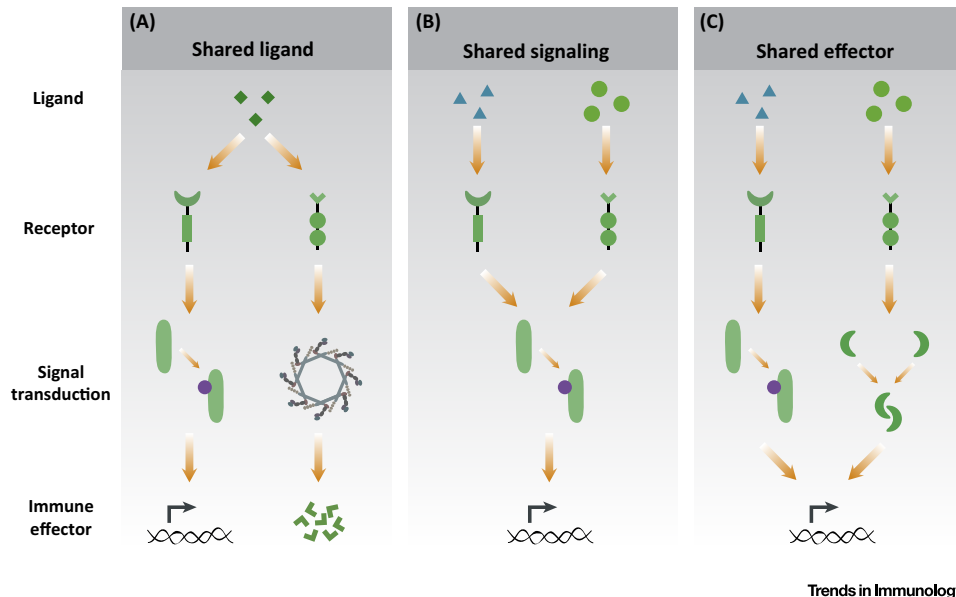
Figure 1. Levels of Innate Immune Signaling Integration. The interaction between two distinct PRR pathways can occur at the level of the microbial ligand (A), the signal transduction (B), or the downstream immune effector (C).

order. PRR signaling pathways triggered by the same ligand can be entirely independent from one another, as demonstrated by the example of bacterial flagellin, which is the ligand for both TLR5 and NLRC4 [14,15]. While TLR5 activation causes MyD88-dependent NF-κB translocation and the induction of gene expression, NLRC4 forms an inflammasome to activate caspase 1 and induce post-translational cleavage of pro-IL-1β and pro-IL-18. A major difference between the two receptors is their subcellular localization: TLR5 is expressed on the cell surface and detects extracellular flagellin, whereas NLRC4 is cytoplasmic. Similarly, dsRNA activates both TLR3 and RIG-I, but in distinct subcellular compartments. TLR3 is a surface/endosomal receptor for extracellular RNA, while RIG-I detects cytoplasmic RNA. Unlike TLR5 and NLRC4, TLR3 and RIG-I share common downstream elements, namely the transcriptional activator IRF3 and the induction of IFN-β. Thus, while several microbial ligands trigger more than one PRR, the subcellular localization of the recognition event may serve as a second crucial piece of information to evaluate the most appropriate cellular response [16,17].

Shared Signaling Components

As in the example of TLR3 and RIG-I, distinct PRRs feature common downstream signaling elements. In fact, the large number of innate immune receptors activate, upon ligand binding, a relatively small number of signal transduction pathways, such as the MAPK/p38/JNK pathway, TAK1/NF-κB activation, and IRF3 or IRF7 pathways, thereby creating another layer of integrated regulation [18]. In many cases, sharing of signaling components in the response to PAMP activation permits cost-effective signal amplification, enables the integration of various stimuli, and facilitates the modular use of pathway components for fine-tuning the host response. At the same time, such pathway cross-activation poses the risk of initiation of non-relevant and non-specific downstream host activities (Figure 1B). Studies in yeast have proposed pathway insulation or cross-inhibition as a potential solution to this problem [19,20]. Insulation of pathways can be accomplished by spatial separation through subcellular scaffolding. Cross-inhibition of the response to PAMPs and DAMPs is mediated by mutual suppression of pathways, for example by the use of binding site blockage. Indeed,

cross-inhibition is a strategy frequently found in PRR interactions [21], as we discuss in detail below.

Shared Effector Mechanisms

A third integration strategy occurs at the level of downstream effector mechanisms (Figure 1C). Several PRR signaling pathways result in the induction of identical innate immune responses. For instance, signaling through TLR3, RIG-I, MAVS, cGAS, STING, or IFI16 will all induce an IRF3 response that leads to the induction of type I IFNs and downstream IFN-stimulated genes. Thus, simultaneous triggering of more than one of these receptors will converge at the level of effector module induction. In principle, such interaction can be cooperative (synergistic), complementary (additive), or compensatory (redundant) [22]. Synergistic induction of an effector mechanism by two or more pathways will result in a higher activation than the sum of each pathway individually. Complementary signal integration works through parallel pathways that induce distinct effector mechanisms which can form a functional unit, such as antibody production and phagocytosis. Compensation occurs to ensure that, if one pathway is defective (or is blocked by a pathogen), the downstream effector mechanism remains active because another pathway triggers the same response instead. These three strategies can be found in a wide variety of effector mechanisms [22] and are pivotal for determining the type of immune response.

Mechanisms of Integration of Innate Immune Signaling

The levels of integration of innate immune signals described above may lead to an overall cross-activation or cross-inhibition of the host response to a pathogenic insult or tissue damage (Figure 2). Cross-activation occurs through positive interaction between innate immune receptors, triggering of signaling pathways, and initiation of effector modules, thereby resulting in potentiation of the immune response against the trigger compared to the response achieved by one sensor alone. The mechanisms underlying cross-activation of PRR pathways have been studied extensively, and we provide a few prototypical examples for such interactions below. By contrast, cross-inhibition occurs when one PRR pathway blocks the signal transduction mediated by another receptor. Inhibitory interactions could serve the resolution of a signaling event, and the temporal switch to a different immune effector response. Inhibitory interactions could also provide a means of prioritization between concomitant signaling events, thus establishing a hierarchy between PRRs, potentially to evaluate the urgency of intracellular signal transduction. We discuss prominent examples of PRR cross-inhibition below.

Mechanistically, crosstalk between PRR pathways typically utilizes one of the following modes of interaction: (i) modification of post-translational modifications (PTMs) and (ii) transcriptional coactivation (Box 1). We highlight the appearance of these mechanisms in our discussion of cross-activation and cross-inhibition in the following sections.

Cross-Activation

Cross-activation of two or more PRR pathways is achieved by positive regulation enhancing the involved signaling pathways (Table 1). Cross-activation between two or more PRR pathways may be conditional or non-conditional (Figure 2A). Conditional activation occurs when the activation of one PRR signaling pathway crucially depends on the activation of another pathway. A prototypical example for conditional PRR crosstalk is the so-called 'signal I' for inflammasome signaling. In the case of the NLRP3 inflammasome, two defined stimuli are necessary for caspase 1 activation [23]. Signal I induces expression of inflammasome components (e.g., NLRP3) and the pro-forms of IL-1 family cytokines, while signal II triggers inflammasome assembly and caspase 1 activation, leading to cleavage of pro-IL-1β and pro-IL-18 into their active forms [24]. Importantly, many TLRs and other PRR ligands that activate NF-κB signaling constitute 'signal I' for NLRP3 inflammasome component transcription [25,26]. Without such
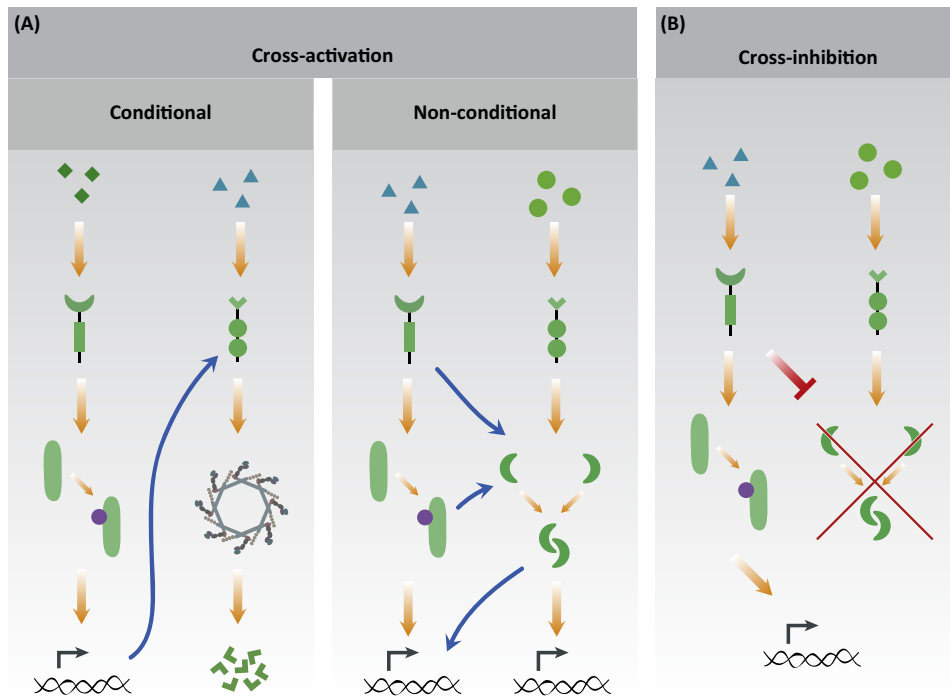
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Figure 2. Coactivation and Coinhibition Between Innate Immune Pathways. Coactivation of PRR pathways involves positive interaction between the signaling components downstream of different receptors. This coactivation can be mandatory (conditional), if one pathway crucially depends on the activity of another, or facultative (non-conditional), if both pathways can function independently of one another. Coinhibition involves blockade of one signaling pathway downstream of another PRR.

priming signal through a distinct set of innate sensors, inflammasome signaling does not occur, thus providing a classical example for conditional innate receptor cross-activation.

Another example of conditional cross-activation, in which two members of the same receptor family depend on one another, is the NAIP–NLRC4 interaction. The NLR family of apoptosis inhibitory proteins (NAIPs) were initially demonstrated to have a crucial role in the response against bacterial infection [27,28]. It is now clear that NAIPs function as innate immune receptors for specific bacterial-derived molecules in the cytoplasm [29,30]. Following detection of these ligands, NAIP proteins coassemble with the NLR protein NLRC4 to form an inflammasome complex [31]. Thus, while most other inflammasomes comprise only a single type of NLR, the NAIP–NLRC4 inflammasome is a unique example for the mandatory cooperation of two different NLR proteins. Recent studies in mice provided evidence that this cooperation is crucial for the protection against enteric infection in an intestinal epithelium-intrinsic manner [32,33]. NLR–NLR interaction in inflammasome activation has also been documented in the case of NLRC4 and NLRP3, which are recruited to the same inflammasome complex in response to *Salmonella* infection [34]. Cooperative inflammasome signaling has also been described between an NLR and a non-NLR receptor, such as in the case of NLRP3 and AIM2 during *Plasmodium* infection [35].

By contrast, non-conditional cross-activation is a scenario in which sensing events and downstream pathways can generally proceed independently of one another, but are potentiated by their concomitant activation. Non-conditional cross-activation has been found in the majority of PRR interactions and often works through transcriptional coactivation (Box 1). In a pioneering

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**Box 1. Mechanisms of PRR Pathway Crosstalk**

PRR crosstalk typically involves common mechanisms, most prominently modulation of post-translational modifications (PTMs) and transcriptional coactivation, as discussed in the following.

Modulation of PTMs

Alteration of protein activity by modulation of PTMs is frequently observed in the regulation of innate immune pathways [126]. Although a comprehensive picture of PTM-mediated regulation of PRR crosstalk is far from being reached, several examples of how PTMs influence innate immune signaling indicate that regulation at the PTM level might be the rule rather than the exception. For instance, NLRP3 is regulated by the ubiquitination state of the LRR domain through the enzyme BRCC3 [127,128]. The inflammasome adaptor ASC undergoes phosphorylation at Tyr144, mediated by the kinases JNK or SYK, to augment NLRP3 inflammasome activation [129]. Linear ubiquitination is also involved in ASC activation [130], and polyubiquitination of ASC upon inflammasome assembly targets the active complex for degradation by autophagy [131]. Tripartite motif (TRIM) proteins have been recognized as central regulators of innate immune signaling [132]. They function as E3-ubiquitin ligases, and several PRR signal transduction components are substrates of TRIM family members [133]. For instance, the E3-ubiquitin ligase TRIM25 is required for the activity of RIG-I, and the influenza virus component NS1 hijacks the TRIM25–RIG-I interaction to block the induction of an antiviral immune response [134,135]. RIG-I and MDA5 are also modulated by phosphorylation. The phosphatase PP1 dephosphorylates both viral sensors and thereby renders them active [136].

Transcriptional Coactivation

Transcription factor activation is a common endpoint of intracellular signaling cascades elicited by PRR activation, including AP-1, NF-κB, IRF3, and IRF7. These transcription factors have both unique and shared sets of target genes, enabling combinatorial activation of gene transcription downstream. Furthermore, binding between transcription factors can modulate their target spectrum, a mechanism that further expands the versatility of transcriptional responses to stimuli and that can be found, for instance, in the regulation of cellular metabolism [137]. Similarly, in the case of PRR triggering, global reprogramming of the transcriptome is specific to the microbial ligand [138]. Concomitant detection of distinct ligands leads to transcription factor interactions and target gene activation not observed with the individual ligands, as has been described for CpG/poly(I:C) co-stimulated monocytes [139]. Importantly, such transcriptional coactivation not only broadens the repertoire of induced target genes but also enhances the magnitude of gene expression [36].

Modification of PTMs	Transcriptional coactivation

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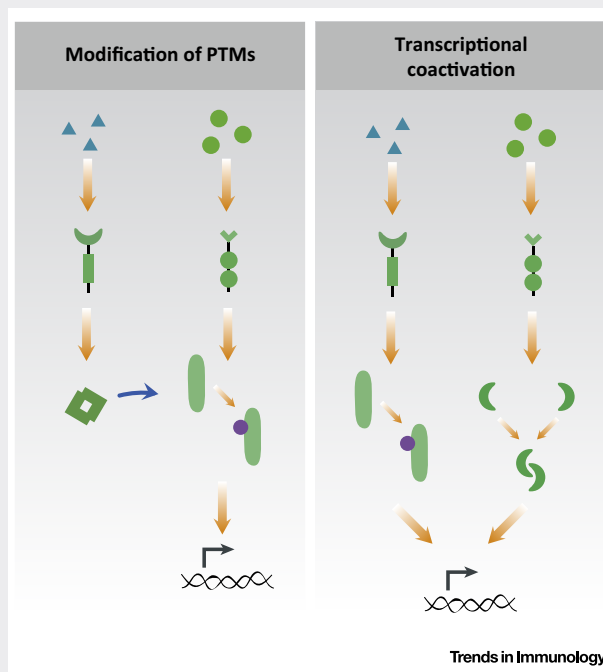
study, Napolitani *et al.* stimulated dendritic cells with multiple TLR ligands and found potent synergy in their signaling pathways and in the induction of around 1% of all genes induced by the separate ligands [36]. Gautier *et al.* suggested that this synergistic effect might be explained through an IFN feedback loop that amplifies the responses through TLR3/4 and TLR7/8 [37].

Table 1. Examples of PRR Cross-Activation[a]

Type of Interaction	PRRs Involved	Consequence of Interaction	System Investigated	Refs
Conditional cross-activation	TLRs 2, 3, 4, 7 NOD2, NLRP3	Transcriptional priming of inflammasome signaling	Human PBMCs, mouse macrophages	[25,140–142]
	TLR4, NLRP3	Priming of inflammasome signaling by PTM	Mouse macrophages	[127]
	TLR4, NLRP3	Priming of inflammasome signaling through IRAK1	Mouse macrophages	[143,144]
	TLR3, NLRP3	Priming of inflammasome signaling through TRIF/RIPK1/FADD	Mouse macrophages	[145,146]
	TLR2, TLR4	Transcriptional induction	Endothelial cells	[147]
	TLR3, TLR7	Transcriptional induction	Mouse and human DCs	[148]
	NAIPs, NLRC4	NLRC4 inflammasome formation	Human macrophages, mice	[29,31]
Non-conditional cross-activation	TLR2, TLR4	Synergy and cross-tolerance	Mouse macrophages, PBMCs from sarcoidosis patients and controls	[50,149,150]
	TLR9, NOD2	Synergistic cytokine induction	PBMCs	[94]
	TLRs 3, 4, 7, 8, 9	Synergistic gene transcription	Human and mouse DCs	[36,151]
	NOD1, TLRs 1, 2, 4–8	Synergistic cytokine induction	Primary human cells, mice	[93,152]
	TLRs 3, 4, 7, 8	Synergistic cytokine induction	Human DCs from arthritis patients and controls	[153]
	TLRs 2, 3, 5, 9	Synergistic cytokine induction through IRF5	Mouse macrophages and *in vivo*	[38,154]
	TLRs 3, 9	NO induction, kinome activation	Chicken monocytes	[139,155]
	TLRs 2, 3	Synergistic cytokine induction	Mouse DCs, mouse macrophage cell line, mouse model of preterm birth, human nasal epithelial cells	[156–158]
	Dectin 1, TLRs 2, 4, 5, 7, 9	Synergistic signaling through SYK	Mouse macrophages, human macrophages	[101,159,160]
	TLRs 3,4,8	Synergistic cytokine induction	Human macrophages	[161,162]
	TLR2, mannose receptor	Synergistic cytokine induction	*Pseudomonas* infection of human monocytes	[163]
	TLRs 2, 4, 9	Synergistic antibody response	Human cells	[164]
	TLRs 3, 7	Synergy and cross-inhibition depend on the time window through IRF1, JunB, and C/EBP	Human macrophages	[165,166]
	NOD1/2, TLR4	Synergistic NKT cell activation	Mouse DCs	[167]
	TLR4, TLR9	Tuberculosis vaccine	Mice	[168]
	TLR4, TLR7	Synergistic gene transcription	Chicken PBMCs	[169]

[a]Abbreviations: DCs, dendritic cells; PBMCs, peripheral blood mononuclear cells.

Together, these studies indicate that specific TLRs and their signal transduction pathways cooperate in inducing gene expression in a fraction of the transcriptome to optimize the response to a given stimulus, a concept that has been applied to a multitude of TLR–TLR and other TLR–PRR interactions (Table 1).

Several other mechanisms have been suggested to drive PRR coactivation. In a systematic study of interactions between TLRs that either share the same pathway (MyD88-dependent: TLR2, TLR5, TLR9), act through a different pathway (MyD88-independent: TLR3), or combine both pathways (TLR4) it was found that simultaneous activation of distinct pathways resulted in synergy, while repeated activation of the same pathway caused mutual cross-inhibition [38]. Synergy between pathways has also been observed across members of distinct PRR families, as exemplified by the coactivation of TLR9 and STING [39], as well as AIM2 and TLR7/9 [40]. These studies suggest a 'signal transduction model' of PRR cooperation.

In addition, a spatiotemporal aspect of PRR regulation has been suggested as a crucial player in innate immune receptor interaction [41]. According to this model, stimulation of a particular receptor opens a 'temporal window' for additional stimulation [36]. The kinetics of such a window, the gene regulatory and signal transduction mechanisms that may explain it, and its cell type-specificity remain to be explored. It is important to note that the majority of studies focusing on PRR cross-activation have reached their conclusions based on *in vitro* studies of cultured cells stimulated with combinatorial microbial ligands. These might not fully reflect the spatial and temporal characteristics of the *in vivo* situation, as detailed below. Nonetheless, these examples collectively highlight the variety of cross-activation mechanisms between different PRRs, illustrating the close interconnection between their signaling pathways.

Cross-Inhibition

Cross-inhibition between two PRRs occurs when the activation of one receptor negatively influences signaling events initiated by another receptor (Table 2). Negative regulation between components of the immune system is crucial to avoid immunopathology in the form of autoimmunity [42,43], cancer [44–46], and chronic inflammation [47,48]. Such negative cross-talk can be found within families of receptors, between different families, and at different levels of signal transduction (Figure 2B).

Early evidence came from *in vitro* studies of simultaneous administration of two or more TLR ligands. For instance, concomitant TLR2 stimulation blocks the induction of IL-12 by TLR3 and TLR4 in human DCs, while the induction of IL-15 and IFN-β was not affected [49]. The mechanism mediating the inhibitory activity of TLR2 stimulation involved autocrine signaling of IL-10. TLR2 was also reported to induce partial tolerance to TLR4 stimulation through the abrogation of JNK and NF-κB signaling, suggesting cross-inhibitory interaction between shared signal transduction pathways downstream of TLRs 2 and 4 [50].

Other families of innate immune receptors can also negatively interact with the TLR receptor family (Figure 3). Mice lacking the NLR family member NLRP6 are highly resistant to infection with the bacterial pathogens *Listeria monocytogenes* and *Salmonella* Typhimurium [51]. *Nlrp6*-deficient myeloid cells feature enhanced activation of canonical NF-κB and MAPK signaling in response to the TLR2 ligand Pam3CSK4 and the TLR4 ligand lipopolysaccharide (LPS), suggesting that NLRP6 interferes with the signaling pathways downstream of TLRs [51].

Another member of the NLR family that can function as a negative regulator of TLR signaling is NLRX1. NLRX1 was originally described as a negative modulator of MAVS and type I IFN signaling [52]. While the participation of NLRX1 in the negative regulation of MAVS-dependent antiviral responses remains controversial [53,54], another report showed that NLRX1 can

Table 2. Examples of PRR Cross-Inhibition.

Type of Interaction	PRRs Involved	Consequence of Interaction	System Investigated	Refs
Cross-inhibition	TLR2, TLR3, TLR4	TLR2 stimulation blocks the induction of IL-12 by TLR3 and TLR4	Human DCs and PBMCs	[49]
	TLR2, TLR4	TLR2 induces partial tolerance to TLR4 stimulation through abrogation of signal transduction	Mouse macrophages	[50]
	NLRP6, TLR2, TLR4	NLRP6 interferes with the signaling pathways downstream to TLRs	Mouse macrophages	[51]
	NLRX1, TLR	NLRX1 negatively regulates TLR-induced NF-κB activation	HEK293T cells, RAW264.7 cells, bone marrow-derived macrophages	[21]
	NLRX1, RIG-I, TRAF6	NLRX1 interacts with TRAF6 and inhibits NF-κB activation	Mouse macrophages	[55]
	NLRC3, STING	NLRC3 inhibits STING–TBK1 association	Mouse macrophages, primary mouse embryonic fibroblasts	[56]
	NLRC3, TLRs	NLRC3 modifies the signaling adaptor TRAF6 and NF-κB	Mouse macrophages	[57]
	NLRP12 and an undetermined PRR	NLRP12 attenuates NF-κB signaling	*In vitro* study of human monocytes, bone marrow-derived dendritic cells, mouse model	[58,59]
	NLRP12	NLRP12 as a negative regulator of NF-κB in T cells	Human T cells	[61,62]
	C-type lectin DCIR and TLR8	DCIR inhibits TLR8-mediated IL-12 and TNF-α production	Human cultured DCs	[63]
	Dectin 1, TLR9	Dectin 1 engagement can modulate TLR9 signaling via induction of SOCS1	Mouse bone marrow-derived dendritic cells and macrophages	[64,65]
	MBL, TLR3, TLR4	Binding of MBL to TLR ligands or to TLR directly	Human monocytes, THP-1 cells or DCs	[65–67]

negatively regulate TLR-induced NF-κB signaling [21]. Mechanistically, NLRX1 was found to interact with TRAF6 in the steady-state, but to dissociate from TRAF6 upon TLR stimulation. In TLR-activated cells, NLRX1 binds to the IKK complex, inhibiting phosphorylation of IKK and propagation of the signaling pathway. Further evidence came from NLRX1 knockdown mice, which exhibit elevated IKK phosphorylation in macrophages upon LPS treatment, and enhanced susceptibility to septic shock [21]. In addition to TLRs, NLRX1 was also reported to inhibit RIG-I signaling [55]. RIG-I interacts with the signaling adapter MAVS only upon viral infection. In the absence of NLRX1, however, RIG-I constitutively binds to MAVS, causing persistent type I IFN stimulation. As a consequence, *Nlrx1*-deficient mice feature an enhanced antiviral response upon exposure to influenza, resulting in decreased viral titers and exacerbated immunopathology in the lung [55].

NLR family members can also regulate the DNA-sensing pathway that signals through STING. Zhang *et al.* identified NLRC3 as a direct negative regulator of STING by inhibiting the STING–TBK1 association that is crucial for IFN activation [56]. NLRC3 also blocks trafficking of STING to perinuclear signaling 'hotspots'. Consistent with increased IFN secretion in the absence of
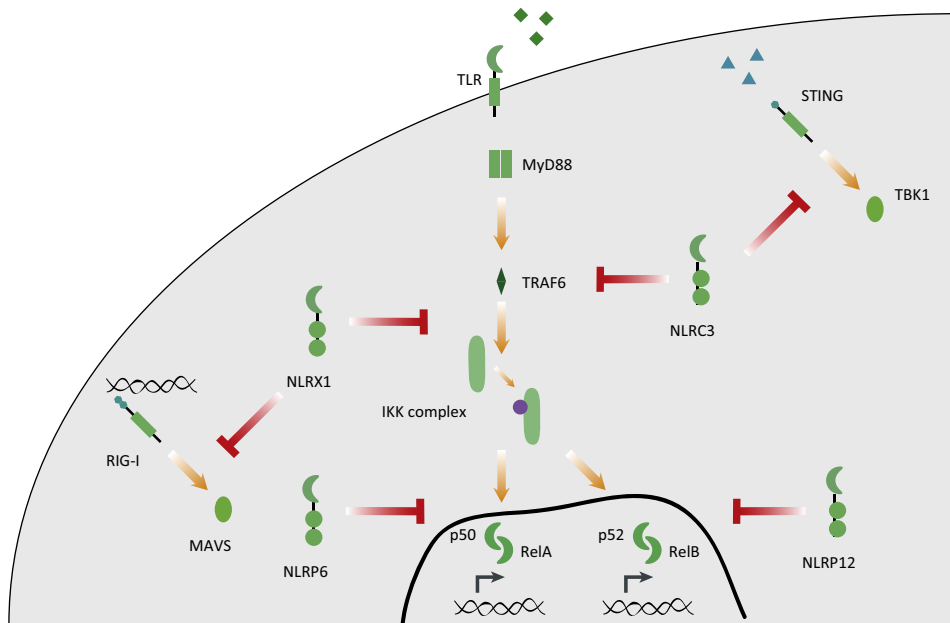
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Figure 3. Inhibition of PRR Pathways by NLR Family Members. The signal transduction pathway downstream of TLR signaling to canonical and non-canonical NF-κB activation can be blocked by NLRC3, NLRX1, NLRP6, and NLRP12. In addition, NLRC3 inhibits the STING–TBX1 interaction downstream of STING ligands. NLRX1 has been reported to inhibit MAVS activation in response to viral RIG-I stimulation.

NLRC3, $Nlrc3^{-/-}$ mice feature enhanced resistance to herpes simplex virus type 1 (HSV-1) infection [56]. The crosstalk of NLRC3 with other innate immune pathways also includes TLR signaling. Schneider *et al.* demonstrated that NLRC3 attenuates TLR singling via modification on the ubiquitination state of signaling adaptor TRAF6 [57]. NLRC3 binding to TRAF6 leads to diminished K63-linked ubiquitination and resultant abrogation of NF-κB activation. $Nlrc3^{-/-}$ mice injected with LPS exhibit higher serum levels of IL-6 and TNF, and are more susceptible to endotoxic shock.

A further NLR family member, NLRP12, also attenuates NF-κB signaling, although the precise upstream PRR inducing NF-κB signaling has not been determined to date. First evidence for the role of NLRP12 came from an *in vitro* study of human monocytes, in which NLRP12 was suggested to regulate the non-canonical pathway of NF-κB activation through NIK [58]. *Nlrp12*-deficient mice feature elevated NF-κB and ERK activation, increased proinflammatory cytokine expression, and enhanced susceptibility to colonic inflammation and tumorigenesis [46,59]. NLRP12 was found to interact with both NIK and TRAF3, thereby limiting non-canonical NF-κB signaling [59]. At the same time, elevated inflammatory signaling in $Nlrp12^{-/-}$ mice is associated with resistance to infection with *Salmonella* Typhimurium [60]. While the above studies have focused on the role of NLRP12 in myeloid cells, this NLR was also found to function as a negative regulator of NF-κB in T cells [61,62].

In addition to NLRs, C-type lectin receptors also negatively modulate other PRRs. Stimulation of the dendritic cell immunoreceptor (DCIR) in cultured DCs blocks cytokine secretion induced by TLR8, but not by other TLRs [63]. Furthermore, dectin 1 engagement can modulate TLR9 signaling via induction of suppressor of cytokine signaling (SOCS1). Coadministration of zymosan, a dectin 1 ligand which lacks any possible TLR-binding moiety, and CpG DNA, a

prototypical ligand for TLR9, results in an abrogation of IL-12p40 production, which is not found in the absence of SOCS1 [64]. Of note, the soluble receptor MBL has been reported to directly interact with the TLR9 ligand CpG, and this interaction is associated with reduced CpG DNA-induced inflammatory cytokine production in human monocytes [65]. Likewise, MBL has been suggested to bind to the TLR4 ligand LPS [66] and the TLR3 ligand poly(I:C) [67].

Many common principles of PRR signaling interaction can be found in the case of the negative feedback regulator A20. A20 is induced by NF-κB, thus making it a transcriptional output of many PRR signaling pathways [68]. In turn, A20 exerts its anti-inflammatory effect by inhibiting NF-κB signaling [69]. Mechanistically, A20 functions by modulating the ubiquitination state of its target proteins [70], and is itself recruited to ubiquitin residues [71], thus exemplifying the prominent role of PTMs in PRR signaling regulation (Box 1), although non-catalytic activities of A20 are also involved in blocking NF-κB signaling [72]. A20 abrogates TLR-induced TRAF6 ubiquitination [73] and NOD2-induced RIP2 ubiquitination [74]. Consequently, mice lacking A20 specifically in myeloid cells develop autoimmunity [75], rheumatoid arthritis [76], and colitis [77]. Because A20 suppresses NF-κB signaling, it also inhibits the 'signal 1' for NLRP3 inflammasome signaling [78,79].

Taken together, these examples reveal multifaceted crosstalk between the signaling pathways of various PRRs, in particular NLRs with other families, resulting in context-specific coactivation or coinhibition of the respective downstream immune response (Figure 3).

Disease Implications of Innate Immune Signaling Integration
Given the central importance of PRRs as inducers of innate and adaptive immune responses, the positive and negative interactions outlined above are of particular relevance in multiple diseases. In the following section we summarize the importance of PRR crosstalk in the host defense against pathogenic infection and the consequences of aberrations in coordinated signaling between PRR families.

Viral infection
Given the wide variety of viral PRR ligands [genomic DNA, single-stranded RNA (ssRNA), or double-stranded RNA (dsRNA)], many important principles of PRR interaction and their relevance in the antipathogen response were found in studies of viral infections. Innate recognition of viruses is mediated by several innate receptors, among them a subfamily of TLRs (TLRs 3, 7, 8, and 9) detecting viral components in endosomes [80–83], as well as the RLR, ALR, and OLR families of receptors that detect cytosolic nucleic acids [12].

Initial studies focused on the interactions between virus-sensing TLRs. Cooperation between TLR3 and TLR9, by stimulation of mouse macrophages with both the TLR3 ligand poly(I:C) and the TLR9 ligand CpG DNA, enhanced the innate immune response over and above the additive response [84], which can be explained by transcriptional changes and the reprogramming of global gene expression [85]. TLRs can also function as priming signals for the detection of viruses by other PRRs, such as NLRP3 in the case of respiratory syncytial virus [86].

Dengue virus infection results in one of the most common mosquito-borne viral diseases. Virus infection triggers numerous intracellular innate immune responses. Dengue virus RNA is recognized by TLR3, MDA5, and RIG-I, resulting in a signaling cascade that induces IFN-α/β production [87]. All three receptors synergize in viral restriction [88].

While the above examples illustrate PRR interactions to inhibit viral replication, a study of influenza virus infection proposed that PRR cooperation can in some cases promote virus

spread. RIG-I cooperates with TLR7 in the sensing of influenza A. Following sublethal viral challenge in the lung, the infection induced inflammatory pathways downstream of TLR7 and RIG-I, leading to enhanced recruitment of myeloid cells to the site of infection. These cells, in turn, are themselves target cells for influenza infection and thus provide an additional reservoir for viral replication [89]. Thereby, in some cases, concerted PRR triggering might be subverted by the virus to perpetuate infection.

Bacterial Infection

Several reports have highlighted the cooperation of TLRs with other PRRs in launching an immune response against bacterial infection (Table 1). As discussed below, such interactions can occur simultaneously or sequentially, illustrating the complexity of temporal signal integration that occurs upon bacterial infection. A prominent example is the recognition of peptidoglycan by NOD1 and NOD2, which can synergize with bacterial TLR ligands to induce cytokine production [90–93]. Interestingly, the synergistic induction of cytokines downstream of TLR9 and NOD2 is lost in Crohn's disease patients harboring a disease-associated NOD2 mutation, indicating that TLR–NOD syergy might be involved in maintaining mutualism between host and microbiota [94]. Recently it was found that *Staphylococcus aureus* activates both NOD2 and TLR2, and drives skin inflammation, in a mouse model of atopic dermatitis through the expansion of basophils and eosinophils [95].

Cooperation of TLR signaling with other PRRs in bacterial recognition and signal integration is indicative of the life cycle of the respective pathogen. The example of *Salmonella* Typhimurium infection best illustrates this concept. As mentioned above, both TLR5 and NLRC4 recognize bacterial flagellin, but at different subcellular localizations [14,15]. Both PRRs initiate distinct signaling pathways in response to *Salmonella* infection (NF-κB signaling and inflammasome, respectively), which proceed independently of one another [14]. This strategy accounts for the hierarchy of effector mechanisms and the fitness cost that is associated with it [96]: Extracellular *Salmonella* is detected by surface-bound TLR5, initiating a MyD88-dependent NF-κB response and the induction of cytokines. Only once *Salmonella*, an intracellularly replicating pathogen, has invaded the cell can cytoplasmic flagellin trigger the inflammasome, a pathway that causes pyroptotic cell death and the release of IL-1β, one of the most potent proinflammatory mediators and modulators of systemic organism physiology.

Fungal Infection

The innate immune response to fungal infection highlights some of the functional principles in the cooperation between two classes of PRRs: CLRs and TLRs.

The CTR dectin 1 recognizes fungal β-glucans [97]. Several studies demonstrated that dectin 1, which mediates phagocytosis of yeast, cooperates with TLR2 and MyD88 to augment proinflammatory cytokine production, allowing maximal responses to infection [98–101]. The cooperation between dectin 1 and TLR2 is a classical example for non-conditional synergistic coactivation on the level of immune effectors [100]. This cooperation results, for instance, in enhanced production of reactive oxygen species [99].

Another cooperative signaling pathway in antifungal defense involves dectin 1 and NLRP3. The NLRP3 inflammasome is activated by infection with *Candida albicans* [102], and requires a priming step provided by β-glucan recognition through dectin 1, leading to the production of pro-IL-1β. In contrast to the above cooperation between dectin 1 and TLR2, the dectin 1/NLRP3 axis is a conditional interaction because the inflammasome components are not produced in the absence of the dectin 1-dependent 'signal I', and thus NLRP3 activation cannot occur independently.

Parasites

Toxoplasma gondii is a parasite that can cause severe clinical syndromes including encephalitis, congenital infection, and even mortality. *T. gondii* infection is a major risk for immune-compromised individuals [103]. Several molecules derived from the parasite, including glycosylphosphatidyinositol (GPI) anchors, were shown to activate TLR2, TLR4, and TLR11 [104–106]. However, none of the single-knockout mice recapitulate the profound phenotype observed in MyD88 knockout mice infected with *T. gondii* [107]. It was found that TLR7 and TLR9 recognize the parasite RNA and DNA. In addition, both TLR11 and TLR12 were shown to be required for the recognition of *T. gondii* profilin protein. The triple knockout of TLR7/TLR9/TLR11 results in high susceptibility for infection [108], demonstrating cooperative TLR signaling in the response against parasite infection.

The Biogeography of Innate Immune Signaling Integration

An important aspect of integrated innate immune responses that is neglected in most studies is the anatomical distribution of the response monitored. Most PRRs feature a tissue- and cell type-specific expression pattern, as do the elements of their downstream signaling components [109]. Therefore, in many cases, crosstalk between innate immune receptors might actually occur in an intercellular rather than intracellular fashion. For instance, upon microbial infection, distinct cellular compartments will recognize different microbial components and therefore respond with different signaling pathways. Such differences can arise, for example, between those cells that are infected with the pathogen versus those that only undergo surface contact. They could also be due to differential receptor expression between hematopoietic and non-hematopoietic cell types, or even between the cell types of a given hematopoietic lineage. As a result, both cell types cooperatively regulate the immune effector modules instigated by the microbial trigger. For instance, the total tissue level of a cytokine or an antimicrobial peptide is the consequence of multiple different cell types producing these mediators.

This concept is illustrated by instances of PRR signal transfer between cells. Detection of viral DNA by cGAS in the cytosol results in the production of cyclic 2′–5′-linked GMP-AMP [110]. This second messenger is transferred between neighboring cells through gap junctions, leading to the activation of STING in cells that were not in direct contact with the virus [111]. Another example of PRR signaling cooperation between different cell types was found in the immune response against RNA virus infection. Newcastle disease virus (NDV) seems to induce a temporally and spatially separated PRR response in the lung [112]. Infected alveolar macrophages detect intracellular NDV through RIG-I, and subsequently produce type I IFNs. Plasmacytoid dendritic cells (pDCs) recognize NDV in endosomes via TLR7 and signal through MyD88 to induce an IFN response. Interestingly, when RIG-I signaling is suppressed by NDV, the alternative recognition pathways by pDCs becomes dominant, thus compensating for the loss of one detection pathway through another in a cell type-autonomous fashion.

Even more intriguing is the cooperation between hematopoietic and non-hematopoietic cells during hepatitis C virus (HCV) infection. Endosomal HCV is recognized by TLR3 (signaling through TRIF) and cytoplasmic HCV is detected by RIG-I (signaling through MAVS) in hepatocytes. However, HCV expresses a protease called NS3/NS4A that is able to cleave both TRIF and MAVS, thereby shutting down the IFN response. Intriguingly, in this situation, direct cell-to-cell contact between infected and hepatocytes and pDCs in the liver initiates transfer of viral RNA to the pDC, where it triggers a TLR7-dependent immune response [113–115]. While the exact transfer mechanism is unknown and could involve exosomes or autophagosomes, this example illustrates the crosstalk between immune receptors of distinct families in distinct cellular compartments in the efficient response to viral immune evasion.

The cooperation of innate immune receptor signaling spans even across different organs. Efficient innate immune responses against influenza infection in the lung require distal TLR signaling in the intestine [116]. This leads to priming of inflammasome signaling, migration of myeloid cells to the lung, and anti-influenza immunity, demonstrating that inter-organ cooperation might be at play in the crosstalk of different PRRs during viral infection. Another example of such long-range PRR interactions is in driving the progression of non-alcoholic fatty liver disease to steatohepatitis, both some of the most common complications of the metabolic syndrome [117]. NLRP3 and NLRP6 inflammasome dysfunction in the intestine leads to enhanced influx of microbial molecules to the portal circulation and the liver, where they trigger TLR4 and TLR9 signaling in hepatocytes, resulting in an elevation of local levels of proinflammatory cytokines, including TNF. This, in turn, is required for the exacerbation of disease and steatohepatitis development [117]. Together, a better understanding of how distinct PRR signaling events interact between different tissues may provide crucial insights into the mechanisms of a concerted antimicrobial immune response and the development of common inflammatory diseases.

Concluding Remarks

The first quarter century since the proposition of the innate immune recognition theory has been productive in the identification of individual PRRs, their ligands, and signal transduction pathways [2]. Our challenge ahead lies in understanding of the principles underlying the integration of innate immune sensing into its physiological context in a complex tissue. Information about cell type-specific expression of innate immune components, local concentrations and distributions of PRR ligands, and the kinetics and hierarchies of innate immune signaling pathways will be pivotal in advancing our understanding of the conceptual principles governing *in vivo* PRR interactions. Because innate immune receptors are promising drug targets, understanding the crosstalk between different PRRs in the physiological context might be crucial for targeted therapies for inflammatory diseases.

Looking ahead, it will be interesting to determine the interactions between innate immune receptors and other cellular sensors including environmental and nutritional sensors. Given the close connection between innate immunity and cellular metabolism [118,119], and the role of NLR family members as sensors of the cellular state [10], one can anticipate a scenario in which PRR signaling is not only regulated by other PRRs of the same cell but also by receptors determining the metabolic state of a cell, such as G protein-coupled receptors (GPCRs) or members of the solute carrier (SLC) family [120–122]. First examples of interactions between nutritional sensors and innate immune signaling have been suggested, for instance, in the case of the aryl hydrocarbon receptor that influences the cellular response to LPS [123,124]. As such, the interaction with non-microbial receptors might place innate immune sensing in the context of tissue homeostasis [125] to modulate appropriate cellular responses to the drastic deviation from the steady-state induced by PRR signaling.

Understanding the complexity of innate immune signaling may open new avenues for the rational design of novel therapies. Inflammatory processes triggered by PRR interactions contribute to the etiology of a wide variety of diseases, ranging from auto-inflammation to the 'metabolic syndrome' (obesity, type II diabetes mellitus, non-alcoholic fatty liver disease, as well as their associated diseases and complications). Thus, understanding the nature of involvement of integrated PRR signaling in the pathogenesis of multifactorial disorders offers wide potential for 'immune therapy' intervention. Nevertheless, such clinical translation first requires a detailed understanding of the *in vivo* characteristics, kinetics, and biogeography of PRR interactions. A parallel can be drawn to the study of signaling pathways regulating the strength of the adaptive immune responses – pathways that are now clinically targeted as treatments against cancer. Reaching the level of understanding that is necessary for therapeutic interventions may highlight the exciting next era in the field of innate immunology.

Outstanding Questions

What is the cell type specificity of PRR and signaling component expression? Can we classify hematopoietic and non-hematopoietic cells based on their ability to respond to different microbial stimuli, and can we integrate the localization of a cell type within a given tissue into this classification?

How is the interaction between PRRs arranged in spatial dimensions *in vivo*? What are the local concentrations of PRR ligands in a tissue? How common is PRR ligand transfer between cells?

How is PRR activity regulated in the case where an innate immune receptor has multiple distinct functions? For instance, what determines if NLRP6 acts as an inhibitor of NF-κB or triggers inflammasome assembly? When does NLRC3 block TRAF6, and when does it interact with STING signaling?

Do innate immune sensors interact with non-pattern recognition receptors, such as environmental and nutritional sensors? Is there regulation at the level of intracellular signaling between these sensors to adjust PRR signaling to the metabolic state of a cell?

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