Summary: Two-signal models have a rich history in immunology. In the classic two-signal model of T-cell activation, signal one consists of engagement of the T-cell receptor by antigen/major histocompatibility complex, whereas signal two arises from costimulatory ligands on antigen-presenting cells. A requirement for two independent signals helps to ensure that T-cell responses are initiated only in response to bona fide infectious threats. Our studies have led us to conclude that initiation of innate immune responses to pathogens also often requires two signals: signal one is initiated by a microbe-derived ligand, such as lipopolysaccharide (LPS) or flagellin, whereas signal two conveys additional contextual information that often accompanies infectious microbes. Although signal one alone is sufficient to initiate many innate responses, certain responses—particularly ones with the potential for self-damage—require two signals for activation. Many of our studies have employed the intracellular bacterial pathogen Legionella pneumophila, which has been established as a valuable model for understanding innate immune responses. In this review, we discuss how the innate immune system integrates multiple signals to generate an effective response to L. pneumophila and other bacterial pathogens.

Keywords: monocytes, bacterial, Toll-like receptors, inflammation, Legionella pneumophila

Introduction

Two-signal models, in a variety of forms, have helped shape our understanding of the adaptive immune response for over 40 years (1). In its simplest form, the current two-signal model of lymphocyte activation states that stimulation of B and T cells requires cooperation between two distinct signals: an antigen-specific signal that engages the B or T-cell receptor, and a second receptor-mediated ‘costimulatory’ signal that licenses the recipient lymphocyte to respond to antigen. In the case of T cells, this second signal is provided by an antigen-presenting cell that has been activated by contact with microbes. Activated T cells can then present a costimulatory signal to B cells. The two-signal model for B and T-cell activation provides a molecular mechanism for ensuring that the adaptive responses of lymphocytes are directed toward foreign microbial antigens. The requirement for two signals is thought to act as a safeguard that regulates the powerful
and potentially harmful immune reaction and prevents the accidental triggering of responses against the host’s own tissues.

Conceptual basis for two-signal models in innate immunity

The classical model of innate immune surveillance features germline-encoded pattern recognition receptors (PRRs) that recognize molecules, such as bacterial peptidoglycan (PGN) or viral single stranded RNA (ssRNA), that are conserved among broad classes of microbes but are absent in the host (2). While these molecular motifs are routinely referred to as pathogen-associated molecular patterns (PAMPs), they are actually common to both pathogenic and non-pathogenic microbes. Thus, the classical model does not clearly establish a mechanism for distinguishing pathogens from non-pathogens. A question that has interested us is whether the innate immune system can in fact discriminate between pathogens and non-pathogens, perhaps by integrating PRR signaling with additional contextual information. Recently, it has been proposed that in addition to microbial molecules, the innate immune system might also have evolved to detect ‘patterns of pathogenesis’ (3), which have been defined as the manipulations of host cell biology that pathogens use to infect, survive, and replicate in their hosts. In this review, we explore the possibility that these two modes of pathogen recognition—sensing of microbial molecules, and contextual recognition of pathogen-associated activities—might cooperate in ‘two-signal’ fashion to specify certain innate immune responses.

Legionella pneumophila as a probe of innate immune responses

While the study of model antigens and purified PAMPs has yielded a broad understanding of immune surveillance pathways, pathogens are defined in large part by their ability to evade and to manipulate these same pathways (4). Thus, to understand how the immune system detects and combats a virulent microbe, it is vital to study the interactions between virulent microbes and the immune system in the context of an infection. For this purpose, we employ the Gram-negative bacterial pathogen Legionella pneumophila. Common in water sources throughout the world, L. pneumophila evolved as a parasite of freshwater amoebae. However, this opportunistic pathogen can also infect macrophages in the mammalian lung. The ability to infect distinct cell types that have been separated by at least a billion years of evolution is surprising but may be due to the fact that L. pneumophila targets highly conserved host cell pathways to survive and replicate.

Upon phagocytosis by the host amoeba or macrophage, L. pneumophila employs a Type IV secretion system, called the Dot/Icm system, to secrete over two hundred effector proteins into the host cytosol (5–7). These effectors manipulate various host cell processes, resulting in delayed fusion with lysosomes, recruitment of ribosomes and ER-derived vesicles to the bacterial phagosome, and formation of a replicative vacuole. While the Dot/Icm system is absolutely required for bacterial replication and virulence, it also may inadvertently deliver bacterial ligands to immunosurveillance pathways in the host cytosol (discussed in detail throughout this review). Thus, the Dot/Icm system provides both a key determinant of pathogenicity and a potential way for the immune system to discriminate between virulent and avirulent L. pneumophila.

The evolutionary history of L. pneumophila makes it an excellent model pathogen for revealing innate immune surveillance strategies in mammals (8). Since L. pneumophila is believed not to be transmitted among mammals (9), it has not evolved significant mechanisms of immune evasion, allowing the characterization of innate immune pathways that better-adapted pathogens might inactivate or evade. Once such pathway is unmasked in L. pneumophila infection, we can study its role during infection with other, immune-evasive pathogens, where its involvement may have initially been less clear. This approach has led us to a broader understanding of immune recognition not only of L. pneumophila but also of intracellular bacterial pathogens in general.

Overview

In this review, we consider four distinct innate immune responses to L. pneumophila: (i) an inflammasome response, (ii) a Nod1/2-dependent response, (iii) a type I interferon response, and (iv) a unique ‘effector-triggered’ transcriptional response (Fig. 1). We discuss each of these four responses in the context of two-signal models. Application of this conceptual framework to innate immune recognition of virulent L. pneumophila yields insight into how the innate immune system may be able to integrate two crucial pieces of information about foreign material that it encounters: first, whether or not that material constitutes a microbe, and second, whether or not that microbe is a pathogen. These complementary signals may dictate both the quality and the strength of the innate immune response, resulting in both an innate and a subsequent adaptive response that is tailored to the threat at hand.
L. pneumophila-induced host cell death depends on a host protease called Caspase-1 (14–16) as well as on the putative pathogen-sensor proteins Naip5 (14–16) and Nlrc4 (also called Ipaf) (14, 17). Naip5 and Nlrc4 each contain a nucleotide-binding domain (NBD) and C-terminal leucine-rich repeat (LRR) domain and are thus classified as members of the NLR (NBD- and LRR-containing receptor) superfamily. Naip5 and Nlrc4 are thought to be involved in the formation of an ‘inflammasome’, a generic term used to describe a multiprotein platform for Caspase-1 activation (18). It must be emphasized that only limited biochemical data have been published demonstrating the existence of a multiprotein complex containing Nlrc4 and Naip5. However, Naip5 and Nlrc4 can be co-immunoprecipitated (14, 19), and our unpublished studies indicate that the association of Naip5 and Nlrc4 is inducible and results in the formation of a high molecular-weight complex (E. Kofoed and R. Vance, unpublished data).

After pioneering work that established other members of the NLR superfamily, namely Nod1 and Nod2, as microbe-sensor proteins (20–23), Naip5 and Nlrc4 were proposed to function as sensors of a L. pneumophila-derived ligand. The nature of the ligand remained a mystery, however, until two groups discovered that flagellin-deficient L. pneumophila did not trigger Naip5- or Caspase-1-dependent pyroptosis (15, 16). Over the last several years, a model has emerged in which the L. pneumophila Dot/Icm system (inadvertently) translocates flagellin into the host cell cytosol, where it is sensed by Naip5 and/or Nlrc4, leading to hetero-oligomerization of Naip5/Nlrc4, followed by recruitment and activation of Caspase-1. Similarly, activation of Caspase-1 by Salmonella typhimurium has also been found to be flagellin dependent (24, 25), with the notable difference that S. typhimurium-dependent activation of Caspase-1 requires only Nlrc4 and is largely independent of Naip5 (15, 26, 27). This peculiarity is discussed in more detail below.

In what sense can inflammasome activation by L. pneumophila be considered to depend on two signals? The nature of signal one is now clear: we have shown that retrovirally mediated expression of flagellin in the cytosol of macrophages is actually a programmed host cell suicide, called pyroptosis, that is mechanistically and morphologically distinct from apoptosis (12). Time-lapse imaging experiments have shown that dying pyroptotic cells literally explode, ejecting their cytosolic contents with little prior warning (13, J. von Molike and R. Vance, unpublished observations), whereas apoptotic cells display signals that promote their organized phagocytosis by macrophages prior to the loss of membrane integrity (13). A key finding was the observation that L. pneumophila-induced host cell death depends on a host protease called Caspase-1 (14–16) as well as on the putative pathogen-sensor proteins Naip5 (14–16) and Nlrc4 (also called Ipaf) (14, 17). Naip5 and Nlrc4 each contain a nucleotide-binding domain (NBD) and C-terminal leucine-rich repeat (LRR) domain and are thus classified as members of the NLR (NBD- and LRR-containing receptor) superfamily. Naip5 and Nlrc4 are thought to be involved in the formation of an ‘inflammasome’, a generic term used to describe a multiprotein platform for Caspase-1 activation (18). It must be emphasized that only limited biochemical data have been published demonstrating the existence of a multiprotein complex containing Nlrc4 and Naip5. However, Naip5 and Nlrc4 can be co-immunoprecipitated (14, 19), and our unpublished studies indicate that the association of Naip5 and Nlrc4 is inducible and results in the formation of a high molecular-weight complex (E. Kofoed and R. Vance, unpublished data).

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is that cytosolic access is essential for activation of the masome activation by signal model might be considered sufficient to explain inflammation (unpublished data). Thus, at first glance, a conventional one signal model might be considered sufficient to explain inflammasome activation by L. pneumophila. The critical point, though, is that cytosolic access is essential for activation of the Naip5/Nlrc4 inflammasome, and flagellin generally does not reach the host cytosol except in cases of pathogenic infection. Although extracellular flagellin is sensed in a one-signal fashion by the innate immune system (specifically by Toll-like receptor 5), the ensuing response involves NF-κB activation and cell survival—almost the polar opposite of the pyroptotic death response induced by the presence of flagellin in the cytosol. The existence of two distinct host responses to a single bacterial PAMP illustrates that host cells not only sense the presence of flagellin but also derive additional contextual information from where—extracellularly or cytosolically—the flagellin is sensed (3, 28). Thus, we propose that the subcellular location of flagellin is interpreted by the host as a second signal that dictates the functional outcome of signal one. While some may question whether or not cytosolic localization is truly a ‘signal’, the semantics are perhaps not worth disputing. At a minimum, it can probably be agreed that cytosolic localization provides contextual information (that we will call a signal) that is interpreted by host cells and used to direct distinct biological outcomes.

The purpose of pyroptosis and its strict control
What is the immunological purpose of pyroptosis, and why would the host only activate pyroptosis under stringent (two signal) circumstances? Several potentially beneficial roles of pyroptosis have been proposed, of which the most straightforward is the elimination of the intracellular replicative niche that bacterial pathogens might otherwise occupy to their benefit. While host cell death might prevent intracellular replication, it does not actually kill the expelled bacteria, which might then find another more hospitable (non-pyroptotic) host cell in which to reside. So pyroptotic cells must rely on other extracellular mechanisms to eliminate bacteria. These mechanisms may include phagocytes, complement, and antibody, but whether and how these classic immunological effectors cooperate with pyroptosis is still an area of investigation. In contrast to the relatively tidy and organized process of apoptotic cell death, pyroptosis is also associated with the uncontrolled release of cellular contents, some of which appear to promote inflammatory responses. The inflammatory mediators not only include cytokines processed directly by Caspase-1, such as IL-1β and IL-18, but may also include other cellular molecules that have been shown to promote inflammation, such as DNA, IL-1α, uric acid crystals, and HMGB proteins (29). Certain of these mediators are thought to recruit neutrophils to sites of infection, which may then specialize in killing any bacteria expelled from the extracellular niche by pyroptosis (30). It appears that the classic Caspase-1-dependent cytokines (IL-1β/IL-18) are not essential either for restriction of L. pneumophila replication in vitro (15–17) or for Caspase-1-dependent elimination of bacteria in vivo (30). Instead, the phagocyte oxidase appears to be important for pyroptosis-dependent elimination of bacteria in at least one in vivo model, implicating neutrophils in bacterial clearance following pyroptosis (30).

Weighing heavily against the above-mentioned benefits of pyroptosis are several significant potential costs to the host. First, pyroptosis results in elimination of phagocytes that might otherwise be able to play a key role in bacterial killing. Elimination of a few macrophages might be a viable immunological containment strategy, particularly if additional phagocytes such as neutrophils can be recruited that are ultimately able to eliminate the infection. As a general rule, though, elimination of immune cells seems potentially detrimental. A second potential cost of pyroptosis is immunopathology resulting from the release of cellular contents from dying cells and the resulting inflammatory responses. If activated inappropriately, necrotic-like cell death, such as is seen in pyroptosis, can lead to sterile inflammation (29). These considerations provide a rationale as to why pyroptosis is regulated by a stringent mechanism that requires the presence of two independent signals. The inverse application of this reasoning predicts that immune responses with a lower potential for self-damage should have lower thresholds of activation. Consistent with this hypothesis, ‘one signal’ recognition of flagellin by TLR5 results in transcriptional and pro-survival responses, rather than irreversible host cell death and release of inflammatory mediators.

A three signal model of inflammasome-dependent cytokine production?
Although inflammasome activation can result in rapid pyroptotic cell death, for many years the primary function
of Caspase-1 was believed to be the proteolytic processing of pro-interleukins-1β and -18 into their active, secreted forms (31, 32). Production of these cytokines also requires multiple independent signals. The first signals required are those leading to the production of the pro-interleukin proteins themselves. These signals are typically derived from Toll-like receptor signaling in response to classic microbial ligands such as LPS. As discussed above, additional signals then activate Caspase-1, which is required for cytokine processing and release. Since Caspase-1 activation itself seems to require two signals, it could be argued that release of IL-1β and IL-18 requires a total of three signals: an extracellular PAMP to stimulate production of pro-IL-1β/18, an additional PAMP to activate the inflammasome, and cytosolic invasion to deliver that PAMP to the cytosol. This raises the interesting question as to why cytokine processing would be more tightly regulated than pyroptosis. One possible reason may be that cytokine release can lead to systemic responses, whereas cell death may result in a more localized inflammation.

In collaboration with Petr Broz and Denise Monack, our laboratory demonstrated that cytokine processing and pyroptosis are indeed separable functions of inflammasomes (33). Cytokine processing appears to depend on a host adapter protein called Asc (34) that is recruited to active inflammasomes and forms a massive (>1 μm) supramolecular structure within cells called the Asc-speck or focus. One group calls the Asc focus the 'pyroptosome' (35), but this turns out to be a misnomer, since the structure appears to be specialized for cytokine processing rather than pyroptosis. Indeed, Asc-deficient cells appear to undergo normal pyroptosis in response to L. pneumophila and S. typhimurium (36, 37). Strangely, the pyroptosis that occurs in Asc-deficient cells is not associated with autoproteolytic processing of pro-Caspase-1. In fact, we showed that mutant pro-Caspase-1 alleles that are unable to undergo autoproteolytic processing are nevertheless able to execute pyroptosis almost normally (33). These results contradicted a widely held dogma that pro-Caspase-1 requires proteolytic cleavage to become active. Instead, for reasons that remain unknown but are perhaps related to substrate accessibility, it seems that proteolytic processing of Caspase-1 is required only for cytokine processing and not for pyroptosis. In addition, our results highlighted Asc as another potential control point that cells (or pathogens) could theoretically use to adjust the relative efficiency of cytokine processing versus pyroptosis. For example, inhibition of Asc would reduce cytokine processing while having no effect on pyroptosis. This is a tantalizing idea, but there are yet to be any data that specifically demonstrate that Asc expression or function is regulated by cells or pathogens.

Do Naip5 and Nlrc4 respond to distinct signals?

One of the unusual aspects of the Naip5/Nlrc4 inflammasome, as compared to other inflammasomes, is the involvement of two NLR proteins, Naip5 and Nlrc4. By contrast, all other inflammasomes described to date appear to contain only one NLR protein, e.g. Nlrp1b, Nalp3, or Aim2. Interestingly, Naip5 and Nlrc4 do not seem to be redundant with each other, since deletion of either one produces a strong phenotype, and deletion of both does not result in a stronger phenotype than deletion of Nlrc4 alone (J. Persson, N. Trinidad, and R. Vance, unpublished observations). Thus, it appears that Naip5 and Nlrc4 cooperate in some manner that is yet to be fully understood. One possibility is that Naip5 and Nlrc4 each respond to distinct signals (38). However, our data do not seem consistent with this notion. Expression of a C-terminal domain of flagellin in the host cytosol from a mammalian promoter appears to be sufficient to activate Caspase-1-dependent cell death, and this single relatively specific signal appears to require both Nlrc4 and Naip5. Instead, the existing data support the idea that Naip5 and Nlrc4 are both in the same genetic pathway, but it is not yet clear whether Naip5 is upstream, parallel, or downstream of Nlrc4. One interesting observation is that although Naip5 is strictly required for the response to the C-terminal domain of flagellin, there are additional stimuli that can activate Nlrc4 completely independently of Naip5 (26, 27). S. typhimurium and Pseudomonas aeruginosa, for example, appear to activate Nlrc4 and Caspase-1 in a partially Naip5-independent manner. The most striking of these Naip5-independent stimuli is the bacterial protein PrgJ, which, like flagellin, activates Caspase-1 in a manner dependent on Nlrc4 when expressed in the macrophage cytosol from a retroviral promoter (39). We showed that Naip5 is not required for detection of PrgJ (27), which led us to speculate that perhaps Naip proteins act upstream of Nlrc4 and confer the ability to recognize and distinguish specific bacterial ligands. Indeed, our preliminary studies suggest that another Naip paralog could be specialized in detection of PrgJ (E. Kofoed and R. Vance, unpublished data). Although Nlrc4 is widely assumed to be the direct sensor of flagellin, this has never been formally demonstrated, and the existing data are equally consistent with a model in which Nlrc4 is not a sensor at all but acts downstream of Naip5 as an adapter to recruit and activate Caspase-1. Resolving these possibilities will
require a more tractable system for biochemical analysis of the activation of Naip5/Nlrc4 by flagellin.

Relevance of two-signal models to other inflammasomes

The Naip5/Nlrc4 inflammasome is now recognized as just one of several inflammasomes capable of activating Caspase-1 in response to infectious or noxious stimuli. Can the principles underlying activation of Naip5/Nlrc4 be applied to other inflammasomes? One intriguing case is the Nlrp1b inflammasome, which has been shown to activate Caspase-1 in response to lethal factor (LF) from Bacillus anthracis (40). The related Nlrp3 inflammasome responds to a diverse set of stimuli, including extracellular ATP (signaling via cell-surface P2X7 receptors) as well as phagocytosed crystalline or crystal-like material, such as uric acid, asbestos, or alum (reviewed in 41). The direct ligands that bind and activate Nlrp1b and Nlrp3, should they even exist, remain uncertain. By contrast, the ligand for the Aim2 inflammasome appears to be cytosolic double-stranded DNA of almost any sequence (42–45). Nlrp1b and Nlrp3 are both NLR family members, whereas Aim2 is a member of a small family of proteins that contain PYRIN and HIN200 domains. Despite considerable sequence divergence and remaining uncertainty surrounding their regulation, it does nevertheless appear that all these inflammasomes follow a two-signal model for their activation. Nlrp1b, for example, responds to anthrax LF, but only in the presence of a pore-forming protein called PA that delivers lethal factor to the host cell cytosol. It is not clear whether the PA pore itself provides the second signal or whether the function of PA is merely to deliver LF to the cytosol. Likewise, signal one for Aim2 is DNA, but Aim2 does not respond to extracellular DNA and instead is only activated upon cytosolic delivery of DNA (typically upon viral invasion of the cytosol). It is therefore only in the presence of alarming second signals—cytosolic invasion by a DNA virus or a pore-forming toxin—that Aim2 and Nlrp1b activate Caspase-1.

By contrast, the Nlrp3 inflammasome is a bit of an enigma. Some stimuli that activate Nlrp3—for example, ATP or phagocytosed crystalline particles—can be of extracellular origin. It has been proposed that phagocytosed crystals activate Nlrp3 by induction of reactive oxygen intermediates (46), or by induction of phagosomal rupture and subsequent release of lysosomal enzymes into the cytosol (47). So, these crystals may in fact be associated with second (cytosolic) signals in a manner reminiscent of other inflammasomes. But what about extracellular ATP? This stimulus functions by engaging cell-surface P2X7 receptors, and thus cytosolic invasion is not required for it to induce inflammasome activation. However, two considerations suggest that Nlrp3 might be the exception that proves the two-signal rule. First, while Nlrp3 is a robust inducer of IL-1β/IL-18 release, it is not always clear whether or not Nlrp3 is as potent an inducer of pyroptosis as other inflammasomes. Since production of pro-IL-1β/IL-18 already requires separate (TLR-dependent) signals, perhaps two-signal regulation of Nlrp3 activation itself is not entirely critical. Second, unlike the Naip5/Nlrc4 and Nlrp1b inflammasomes, the Nlrp3 inflammasome appears not to be constitutively active in resting cells. For example, extracellular ATP does not induce potent Nlrp3-dependent release of IL-1β unless the Nlrp3 inflammasome is first primed (by LPS or other signals) (48). The molecular basis of the requirement for Nlrp3 priming is still not completely clear but appears likely to include transcriptional induction of Nlrp3 itself (48).

The differential requirement for priming of distinct inflammasomes may also reflect the fact that Nlrp3, but not Nlrc4, is capable of responding to endogenous (self) ligands. Thus, non-constitutive expression of Nlrp3 may serve to limit the potential self-reactivity of this inflammasome. In general, although the nature of the two signals can differ, it does appear that a requirement for two or more signals is a general feature for inflammasome activation that presumably acts to constrain Caspase-1 activation to scenarios in which the host is gravely threatened.

Two-signal activation of cytosolic transcriptional responses

Several cytosolic pattern recognition receptors other than Naip5/Nlrc4 also contribute to the host response to L. pneumophila: the canonical Nod-like receptors Nod1 and Nod2 sense PGN fragments, while an Irf3-dependent pathway responds to an unidentified ligand, probably a nucleic acid. In contrast to inflammasome recognition of L. pneumophila, these other PRRs activate transcriptional responses rather than rapid inflammatory cytokine release and cell death.

Induction of pro-inflammatory cytokines by Nod1 and Nod2

The cytosolic PRRs Nod1 and Nod2 recognize cytosolic fragments of bacterial PGN (di-amino-pimelic acid and muramyl dipeptide, respectively) and activate host transcription via their common adapter, Rip2 (20–23, 49). Lipoproteins associated with bacterial cell walls can stimulate the extracellular sensor TLR2 but do not activate the intracellular Nods. Thus, as for Naip5/Nlrc4, the cytosolic localization of Nod1 and
Nod2 provides contextual evidence that a pathogen is present, since non-pathogenic bacteria generally cannot access the cytosol. Viala et al. (50) illustrated this point by demonstrating that cells can sense the PGN of the extracellular Gram-negative pathogen Helicobacter pylori in a Nod1-dependent manner. It was proposed that extracellular H. pylori could deliver PGN to the host cytosol via its bacterial secretion system, resulting in Nod1 signaling and restriction of bacterial growth. While the transcriptional targets of Nod1 and Nod2 largely overlap with TLR-induced genes, there are some recent data suggesting that Nods may additionally activate unique downstream events, such as autophagy of invasive bacteria, in a Rip2-independent manner (51–53).

Nod1 and Nod2 perform somewhat redundant roles with TLRs during L. pneumophila infection both in macrophages and in vivo (54, 55). Yet while TLRs can recognize both wildtype L. pneumophila and the avirulent Dot/Icm− mutant, Nod1 and Nod2 specifically respond to virulent L. pneumophila expressing a functional Dot/Icm system (55–57). Several studies have noted a role for Nod1 and/or Nod2 in L. pneumophila infection in the mouse lung, although myeloid differentiation factor 88 (MyD88)-dependent pathways (likely to include both TLR and IL-1 signaling) are also crucial (54, 58–62). Frutuoso et al. (63) observed that neutrophil recruitment to L. pneumophila-infected lungs was almost entirely dependent on Nod signaling, while several other groups have noted a more modest requirement for the Nods in both neutrophil recruitment and clearance of bacteria (56, 58, M. Fontana and R. Vance, unpublished data). The effects of Nod1 and Nod2 deficiency become much more pronounced in the absence of compensatory Myd88-dependent signaling; for example, Myd88/−/− mice, which lack both TLR and Nod signaling, are strikingly susceptible to low doses of L. pneumophila, while Myd88+/− mice are moderately resistant (58).

The activation of multiple redundant innate signaling pathways by L. pneumophila is perhaps to be expected, given the pathogen’s lack of significant immune evasion mechanisms. At the same time, study of host responses to other, better adapted, pathogens suggests that cooperation between the Nod proteins and other immune surveillance pathways is common, serving to enhance or even specify the innate response. For example, while Nod2 signaling alone does not typically result in induction of type I interferons, it does significantly increase the Irf3-dependent induction of IFN-β in macrophages infected with the bacterial pathogen Listeria monocytogenes (64). In addition, a unique Nod2 ligand, N-glycocolyl MDP, produced by Mycobacterium tuberculosis, has been reported to have the capacity to induce IFN-β (65). Similarly, co-treatment of macrophages with the Nod2 agonist MDP greatly enhances cytokine responses (66, 67) and toxic shock induced by LPS (68). Thus, not only do the Nods require two signals (a bacterial ligand plus cytosolic delivery) for their own activation, but they also provide a second signal that greatly enhances immune responses—including those that cause morbidity and mortality—downstream of other receptors.

In addition to altering the magnitude of immune responses, the Nods have also been shown to act in concert with TLRs to generate qualitatively distinct responses to complex stimuli. In dendritic cells, the pro-inflammatory cytokine IL-23 is poorly induced by TLR2 or Nod2 ligands alone but robustly induced when the two are added together (69, 70). IL-23 plays an important role in mucosal immunity, polarizing the adaptive immune response toward a Th17 phenotype. Accordingly, dendritic cells activated with both TLR2 and Nod2 ligands (but not either one alone) are capable of potently activating IL-17 production in T cells (69). Inappropriate Th17 responses can drive harmful inflammation and autoimmunity in disease models such as collagen-induced arthritis and experimental autoimmune encephalomyelitis (71). Thus, the requirement for ligation of two innate immune receptors (one of which is sequestered from normal contact with commensal bacteria by cytosolic localization) may be a way to limit this powerful, potentially harmful host response to situations where a pathogen is present.

Finally, several papers have reported physical and functional interactions between the Nods, the actin cytoskeleton, and the plasma membrane, that may have consequences for pathogen recognition (reviewed in 72). Both Nod1 and Nod2 appear to associate with the plasma membrane in resting epithelial cells. This association requires an intact actin cytoskeleton, since pharmacological perturbation of actin polymerization abrogates membrane association (72, 73). Legrand-Poels et al. (73) went on to show a physical interaction between Nod2 and the small GTPase Rac1, which regulates multiple cell processes including cytoskeletal remodeling. Interestingly, pharmacological inhibitors of actin polymerization actually enhance the responses of both Nod1 and Nod2 to purified ligands (72, 73), while point mutants of Nod1 that cannot localize to the plasma membrane also fail to activate NF-κB (72) or autophagy (51–53). In addition, in epithelial cells infected with invasive Shigella flexneri, Nod1 relocalizes to membrane sites of bacterial internalization, where it associates with NEMO (a kinase that acts in the NF-κB pathway) (72), and ATG16L (a protein involved in autophagy) (51–53). Taken together, these observations suggest that dynamic associations with both the plasma membrane and the cytoskeleton are
important for Nod signaling, although the precise roles of each interaction remain to be dissected (74).

It is not yet clear whether or not interactions between Nods and the cytoskeleton are important in pathogen recognition. However, multiple bacterial pathogens (e.g. *L. monocytogenes, Rickettsia species, S. flexneri*) manipulate the host actin cytoskeleton to move within cells or spread from cell to cell. Thus, perturbations of the cytoskeleton may in fact be a ‘pattern of pathogenesis’ that acts in concert with Nod recognition of PGN to trigger a strong immune response.

**Cytosolic induction of Type I interferons**

Type I interferons (herein referred to simply as IFN) consist of a single IFN-β subtype, and numerous other subtypes (α, δ, ε, etc.), that all signal via the same type I IFN receptor (called Ifnar). Type II IFN (also called IFN-γ) signals via a distinct receptor and is not discussed herein. While IFN induction has classically been studied in the context of viral rather than bacterial infections, multiple bacterial pathogens potently induce IFN through engagement of either TLRs or cytosolic receptors (reviewed in 75). Bacteria induce IFN through one of two distinct mechanisms. The first involves cytosolic recognition of nucleic acids. In fact, early evidence for the existence of a cytosolic DNA sensor came from the observation that macrophages deficient in TLR signaling produced IFN in response to *L. pneumophila* infection in a manner dependent on a functional Dot/Icm system (75). Thus, induction of IFN by *L. pneumophila* follows a two-signal model: it requires both a bacterial ligand (discussed below) and cytosolic delivery of that ligand by a bacterial secretion system. Since the Dot/Icm system is evolutionarily related to bacterial conjugation systems, it was initially suggested that the Dot/Icm system may translocate bacterial DNA into the host cytosol (75). Subsequently, we and others have demonstrated a partial role for the cytosolic RNA sensors (e.g. MDA5 and RIG-I) in IFN induction by *L. pneumophila* (76–78). The precise nature of the bacterial ligand in this case is still uncertain; although MDA5 and RIG-I sense RNA, two reports have described an unusual mechanism in which RNA polymerase III can transcribe double-stranded DNA into a ligand for RIG-I (76, 79). It appears likely that *L. pneumophila* induces type I IFN via multiple mechanisms.

Other bacteria also induce IFN in a manner dependent on both PAMPs and cytosolic access. One example is IFN induction by cyclic di-nucleotides, such as c-di-GMP and c-di-AMP, which are unique molecules that function as second messengers in bacteria and archaea but that are not produced by mammals (80, 81). Cyclic-di-AMP and cyclic-di-GMP induce IFN through a currently unidentified cytosolic sensor (82–85). Pathogenic *L. monocytogenes* triggers this pathway through release of cyclic-di-AMP, while an avirulent mutant that fails to escape from the phagosome into the cytosol does not induce IFN (64, 84, 86). Similarly, virulent *Francisella tularensis* induces IFN upon escape into the host cytosol, while mutant *F. tularensis* lacking a functional secretion system does not induce IFN (87). While the ligand and receptor are both unknown in this instance, *F. tularensis* is known to release DNA into the cytosol of infected macrophages through bacterial lysis (88). Thus, a cytosolic DNA sensor may be involved. *Yersinia pseudotuberculosis* also induces a cytosolic IFN response dependent on its pore-forming bacterial proteins YopB or YopD (89). Again, although both the ligand and receptor are unknown in this particular case, overall it seems clear that cytosolic invasion or access is a requirement for induction of IFN by many species of pathogenic bacteria (90).

The second major mechanism by which bacteria induce IFN is through TLR signaling. In theory, the endosomal TLR7 and TLR9, which sense single-stranded RNA and unmethylated CpG DNA, respectively, are capable of recognizing bacterial ligands and inducing IFN. However, few examples of such recognition occur in the literature (91), perhaps because these TLRs induce IFN most robustly in plasmacytoid dendritic cells, which are not highly phagocytic (92) and are not believed to play a large role in immune responses to bacteria [but interestingly, Ang et al. (93) report a role for pDCs, but not type I IFN, in *L. pneumophila* infection]. Instead, the major TLR-dependent way in which bacteria induce IFN is through TLR4-dependent recognition of LPS on Gram-negative bacteria. Membrane-bound TLR4 is expressed on the surface of immune cells, bypassing a requirement for a second signal such as cytosolic access. This innate pathway is therefore an exception to the two-signal models of IFN induction.

It is not yet clear why TLR4 should be specially licensed to induce IFN independent of other signals. However, we note that the ligands for cytosolic induction of IFN (i.e. RNA and DNA) are widely distributed among viral and bacterial pathogens, and are also produced by the host. Thus, in the context of nucleic acid sensing, signal two may be important to provide additional information about the nature of the pathogen and to restrict the potentially harmful recognition of self-nucleic acid. In contrast, TLR4 recognizes a molecule found exclusively on Gram-negative bacteria that produce hexa-acylated LPS, which are especially prevalent in the intestinal flora. Therefore, TLR4 may be unlikely to induce an inappropriate self-directed response. In addition, TLR4’s capacity to induce
IFN in the absence of a pathogen-associated signal two may indicate a role for this cytokine in regulating interactions between the host and commensal bacteria in the intestine.

Rationale for two-signal induction of IFN in response to bacteria

The roles of IFN in host defense against bacteria are complex and poorly understood. IFN actually appears to be harmful to the host during infection with L. monocytogenes (94–96), F. tularensis novicida (97), and various strains of Mycobacterium tuberculosis (98, 99), perhaps because it polarizes the adaptive response away from a protective Th17 outcome (97), affects responses to IFN-γ (100), or sensitizes lymphocytes to apoptosis (95).

On the other hand, type I IFN restricts bacterial growth in macrophages infected with L. pneumophila (77, 101) and plays a protective role in mice infected with E. coli and multiple Streptococcus species (102). The pleiotropic effects of IFN may make it extremely important for the immune system to regulate production of this cytokine. The requirement for multiple signals may therefore serve to tailor the specificity and magnitude of the IFN response to the threat at hand.

Two-signal activation of bacterial effector-triggered responses

In plants, detection of harmful pathogenic activities has long been considered an important mode of innate immune recognition, termed ‘effector-triggered immunity’ (reviewed in 103). By expressing ‘guard’ proteins that monitor the integrity of vital host cell processes, plants are able to detect and respond to pathogen-mediated disruptions in their own cellular processes (103). Several groups have postulated the existence of similar surveillance mechanisms in metazoans (3, 104), but until recently, there was little evidence to support these speculations. However, in the last few years, several papers have clearly shown specific innate immune responses that depend on the activity of pathogen-derived toxins or effector proteins, which themselves target host processes such as cytoskeletal remodeling and protein synthesis. In some cases, these ‘effector-triggered’ responses act in concert with classical PRR signaling to induce robust, qualitatively distinct responses to virulent bacteria and viruses (55, 105).

Effector-triggered responses to inhibition of host protein synthesis

Macrophages infected with L. pneumophila make distinct transcriptional responses to wildtype (Dot/Icm+) and avirulent (Dot/Icm−) bacteria (55, 57, 106), including the robust Dot/Icm-dependent induction of inflammatory cytokines and chemokines (Csf1, Csf2, Il23a, Cd20), stress response genes (Gadd45a, Egr1, Hsp70), modulators of the mitogen-associated protein kinase (MAPK) and NF-κB pathways (Dusp1, Dusp2, Nfkbia, Iruk2, Tnfip3), and cell-surface markers (Selr, Cd83, Cd44) (55, 57, 106, 107). While the cytosolic PRRs discussed above do play a role in inducing a subset of Dot/Icm-dependent transcriptional targets, the molecular basis of the full Dot/Icm-dependent transcriptional response has only recently begun to be deciphered.

First, Shin et al. (57) reported that wildtype L. pneumophila, but not an avirulent Dot/Icm− mutant, induced MAPK activation and transcriptional responses in macrophages lacking both TLR and Nod1/Nod2 signaling. The requirement for a functional Dot/Icm secretion system suggested that induction of this host response involved cytosolic access and/or secretion of bacterial effectors (57). In collaboration with Simran Banga and Zhao-Qing Luo, we have since demonstrated that the specific host transcriptional response to pathogenic Dot/Icm+ L. pneumophila involves the activity of five bacterial effectors that are secreted into infected macrophages (55). Once delivered, these effectors inhibit host protein synthesis via enzymatic modification of a host elongation factor (107–109). When translation is inhibited, several repressors of inflammation, including the inhibitor of NF-κB (IkB), fail to be resynthesized as they turn over. The loss of these repressors results in sustained activation of NF-κB (55). An analogous mechanism appears likely to account for the Dot/Icm-dependent induction of MAPKs (S. Shin and M. Fontana, unpublished data). It appears that the sustained activation of signaling pathways that occurs in the presence of translation inhibition underlies the subsequent enhanced transcription of inflammatory cytokines, including IL-23 and granulocyte/macrophage colony-stimulating factor (GM-CSF) (55). Thus, short-lived inhibitors like IκB appear to serve as ‘guards’ of host translation, leading to a strong host response when this vital cellular process is disrupted.

Inhibition of translation alone cannot recapitulate the entire macrophage transcriptional response to L. pneumophila (55). Rather, translation inhibition acts in concert with TLR or Nod signaling to generate the full transcriptional response. The combination of these two signals, as in macrophages infected with L. pneumophila or treated with a TLR ligand and a translation inhibitor (such as cycloheximide), results in robust transcription and translation of pro-inflammatory cytokines (55). Thus, TLRs and Nods provide signal one, indicating the presence of a microbe, while inhibition of host protein synthesis serves as the second signal that alerts the host that the microbe is likely to be pathogenic. As discussed above, cytokines such
as IL-23 can be harmful to the host when expressed inappropriately; thus, the two-signal requirement here may again serve to limit a potentially damaging host response to situations where a pathogen is present.

While cycloheximide (or another translation inhibitor) alone does not recapitulate the full transcriptional response to L. pneumophila, it does robustly induce a subset of Dot/Icm-dependent genes, including the small GTPase Gem and the cytokine Ifi4 (55). Since these genes require only one signal for their induction, we would hypothesize that they are less likely to induce host damage when improperly expressed. While the roles of both IL-1α and Gem in L. pneumophila infection remain uncharacterized, we note that in contrast to secreted cytokines and surface proteins, the cytosolic factor Gem is unlikely to act in a cell-extrinsic fashion. Thus, in vivo, its effects are likely limited to infected macrophages, which represent a small fraction of host cells at the site of infection. The cytokine IL-1α is an interesting case. Although IL-1α is pro-inflammatory and signals through the same receptor as IL-1β, its mechanism of secretion is unclear, and it may only be released from dying cells (110). Thus, the transcript of this cytokine may be somewhat less stringently regulated because it still requires a second signal—host cell death—for its release.

Although many of the details remain to be worked out, our studies of L. pneumophila have suggested potentially general mechanisms by which the immune system re-aims in potentially destructive responses while allowing for effective defense. Indeed, we have found parallels between the immune response to L. pneumophila and the response to other bacterial toxins and effectors that inhibit host translation. We have shown that two of these toxins, diphtheria toxin and Pseudomonas aeruginosa exotoxin A, can cooperate with TLR signaling to elicit effector-triggered responses both in macrophages and in the mouse lung (55). This surveillance pathway may also be active in other host cell types, since an epithelial cell line treated with Shiga toxin, another bacterial inhibitor of translation, also superinduces characteristic cytokines (111).

Effector-triggered immune responses to other pathogens

Other examples of metazoan immune responses to pathogen-encoded effectors have recently begun to emerge. While few, if any, of these have been worked out in mechanistic detail, they do provide evidence for effector-triggered immunity as a general mode of innate immune surveillance in animals. Herein, we cite several examples of pathogen-encoded activities that disrupt two distinct cellular targets: the cytoskeleton, and host membrane integrity. Importantly, the ‘patterns of pathogenesis’ (3) we discuss below can aid in the detection of multiple classes of pathogens, including intracellular and extracellular bacteria and viruses.

Multiple bacterial and viral pathogens manipulate the host actin cytoskeleton to avoid phagocytosis by immune cells or, alternatively, to invade and move within host cells. Thus, the cytoskeleton is a logical candidate for monitoring by the innate immune system. In particular, a common host target for pathogens is the Rho family of GTPases, whose members direct cytoskeletal dynamics. Several groups have now demonstrated innate immune responses to bacterial effectors or toxins that constitutively activate members of the Rho GTPase family, including the S. typhimurium effectors SopE, SopE2, and SopB (112), and the uropathogenic E. coli toxin cytotoxic necrotizing factor-1 (CNF-1) (113). These innate immune responses, which include activation of NF-kB and MAPKs and secretion of cytokines, are independent of TLR and Nod signaling and, in the case of S. typhimurium, require a functional bacterial secretion system (112). It is perhaps significant that these studies were done in epithelial cells, which are not specialized immune cells and do not express a wide complement of PRRs. While many epithelial cells do possess some TLRs, their expression is often spatially restricted to the basal membrane, which may minimize inappropriate recognition of gut commensals (114). Thus, ‘effector-triggered’ responses might be particularly important (and non-redundant) in epithelial cells, since other modes of pathogen recognition are somewhat restricted. Interestingly, it has been proposed that S. typhimurium may actually induce intestinal inflammation as a survival strategy, resulting in decreased competition from commensals (115). Thus, as for classical PRR-based immune recognition, effector-triggered immune responses may be exploited by pathogens in order to gain a survival advantage (112, 115).

Viruses are also experts in manipulating host cell biology, raising the possibility that they too are sensed indirectly by host pathways that monitor the integrity of the cell. Multiple groups have observed that influenza virus activates the Nlrp3 inflammasome in dendritic cells and macrophages, leading to release of the cytokine IL-1β (116–119). Ichinohe et al. (105) recently reported that influenza activation of the Nlrp3 inflammasome appears to require the activity of the viral protein M2, a proton channel that plays multiple roles in the influenza replication cycle. As discussed above, activation of the Nlrp3 inflammasome requires a signal one to upregulate both Nlrp3 and pro-IL-1β. Ichinohe et al. (105) showed that during influenza infection, TLR7 provides the transcriptional signal one, presumably through recognition of viral RNA. The activity of
the influenza M2 protein therefore appears to serve as the vital signal two, though it is not yet understood how M2 activity results in Nlrp3 activation.

**Concluding perspective**

In the two-signal model of adaptive immunity, the innate immune system plays a crucial role in regulating the provision of signal two, thereby controlling the ability of B and T cells to respond to antigen. Thus, this two-signal model links innate with adaptive immunity, resulting in adaptive antigen-specific responses to microbes that trigger innate immune receptors. Herein, we have discussed the ways in which the innate immune system is itself regulated by two-signal frameworks. While certainly not all innate immune responses fall neatly into a two-signal model, many do—particularly when considering the immune response to virulent microbes, which both trigger and manipulate multiple innate pathways.

Within two-signal models of innate immune sensing, we have become particularly interested in cooperation between PRRs and pathways that detect pathogen-associated activities. Conceptually, the latter are intriguing because they offer a way to selectively respond to pathogenic bacteria, whereas both pathogens and non-pathogens have the potential to trigger pathways that sense conserved microbial ligands. As more examples of effector-triggered immunity are characterized in metazoans, we hope to better understand how the innate immune system integrates multiple signals to contain infection and elicit an appropriate adaptive response.

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