Published in final edited form as:

Microbiol Spectr. 2019 May ; 7(3): . doi:10.1128/microbiolspec.BAI-0011-2019.

Host-encoded sensors of bacteria—our windows into the microbial world

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Abstract

Bacterial pathogens can be very efficient at causing disease and are the cause of some of the worst epidemics that have affected humanity. However, most infections are prevented by the actions of our immune system. Immune activation depends on the rapid detection of bacteria by a diverse family of sensory proteins known as pattern recognition receptors. These receptors detect conserved features of bacteria that are not found in humans, but are often necessary for survival within the host or environment. In this review, we discuss the strategies used by pattern recognition receptors to detect bacteria and their products. We also discuss emerging evidence that some pattern recognition receptors can be activated by bacterial pathogens specifically, through the surveillance of host activities that are commonly targeted by virulence factors. This collection of surveillance mechanisms provides an interconnected network of defence, which is important to maintain the germ-free environment of the inner organs of humans and other multicellular organisms.

Overview

Outside of a mammalian host, bacteria face numerous challenges that can result in life-threatening risks. These challenges include variations in temperature, osmolarity, risk of predation, desiccation and nutrient shortage. For bacteria with the ability to survive within a mammalian host, several of these threats are less severe, as host cells exist at a fixed temperature, osmolarity, water and nutrient content. However, there is a cost associated with the benefits of an intracellular lifestyle. That cost is the threat of host-encoded immune defenses. Herein, we describe the molecular mechanisms used by mammalian hosts to detect bacterial infection. We discuss the receptors encoded by the host immune system that recognize infection and the bacterial molecules that these receptors detect. Finally, we illustrate how these detection strategies, which are diverse in mechanism of action, share a thematically similar goal. This goal is to induce inflammatory responses that are typified by the recruitment of the biggest threat to bacterial viability to the sites of infection—polymorphonuclear leukocytes, also known as neutrophils.

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Bacterial molecules that induce host defense

All multicellular organisms face the threat of microbial colonization, which poses a threat to the viability of the host. Studies over the last two decades, first by the late Charles Janeway Jr, and subsequently by many others, established a common strategy used by multicellular organisms to detect bacterial encounters. This common strategy of bacterial detection comes from the use of germline encoded host proteins that recognize molecules present within large classes of bacteria. The microbial molecules sensed by the host include structural components of the bacterial cell wall, such as lipopolysaccharides (LPS), lipoproteins, peptidoglycan fragments and flagellin subunits (Table 1). Additional microbial molecules include nucleic acids—DNA and RNA. As mammalian cells lack a cell wall, the presence of LPS and other cell wall components in the host provides a high-fidelity indicator of a bacterial encounter. Thus, the host encoded sensors of cell wall components, referred to as pattern recognition receptors (PRRs) most certainly evolved under the selective pressure to rapidly detect bacterial encounters. Nucleic acids, in contrast, are not exclusively bacterial and can be found in all living cells and viruses. Thus, while PRRs that detect nucleic acids can detect bacteria, the true selective pressure that drove the evolution of these receptors may have been based on the need to detect viral infection. There is one exception to this statement, as recent studies revealed that a specific sequence found in bacterial ribosomal RNA is a potent inducer of host defenses (1, 2). This ribosomal RNA sequence is highly conserved and is not found in eukaryotic ribosomal RNAs or viral genomes. As such, the receptors that detect bacterial ribosomal RNA likely evolved to detect these microorganisms.

The evolutionary focus of this discussion prompts consideration of the selective pressures placed on host and bacteria that directed the design of PRRs and their aforementioned microbial ligands. These microbial ligands are collectively referred to as pathogen associated molecular patterns (PAMPs) (Table 1). Within the human genome, genes exist that regulate numerous activities, including tissue development, body patterning, neurological, cardiac and hormonal activities. The molecular cues (ligands) that control these processes are encoded by the same genome that encodes the receptors and pathways that these cues stimulate. Thus, in developmental and metabolic systems, receptor and ligand are under positive selection to reinforce fidelity of the network. In contrast, PRRs and their microbial ligands are synthesized by different organisms. Moreover, robust detection of a PAMP by a PRR drives defensive responses that eliminate the bacterium that produced the very ligand that the receptor evolved to detect. Based on the separation of genomes that encode PAMP and PRR, and the antagonistic outcome of the receptor-ligand interaction, it is logical that host proteins involved in immune defense are encoded by some of the most rapidly evolving genes present in the human genome. Probably due to the rapid evolving nature of the receptors that inform our understanding of host-bacteria encounters, different multicellular organisms encode different repertoires of PRRs. These receptors, consequently, can induce defensive responses to infection that are tailored to suit the needs of the host. For example, PRRs that operate in humans link microbial detection to the initiation of T and B cell mediated adaptive immunity (3). As the fruit fly *Drosophila melanogaster* does not contain T and B cells, PRRs within this organism link bacterial detection to the production of antimicrobial peptides. Despite this diversity of receptor design and defensive strategy,

multicellular organisms from every kingdom of life appear to have evolved to use a similar set of PAMPs as indicators of infection. Below we will focus on the best-characterized experimental systems to study PRR activities in the context of bacterial encounters—the human and mouse. We refer the reader to several excellent reviews that describe host defense strategies that operate in insects and plants (4–6).

Host-encoded sensors of bacterial molecules.

Several families of PRRs exist in humans and mice. Within each family are proteins that exhibit significant structural similarity, but there is limited similarity across PRR families. Thus, the unifying feature that links these diverse families of proteins is function—the ability to detect and respond to bacterial encounters. The first described and best-characterized family of PRRs consists of the Toll-like Receptors (TLRs) (7–11). TLRs are type I transmembrane proteins that are structurally characterized as containing a large extracellular leucine rich repeat domain that recognizes PAMPs and an intracellular signaling domain that shares homology with the intracellular domain of the interleukin-1 (IL-1) receptor and various plant resistance proteins. Consequently, cytosolic tail of TLRs is classified as a Toll-IL1 Receptor-Resistance (TIR) domain (7). Several TLRs are considered to be specific sensors of bacteria, in that they recognize specific components of the cell wall. These cell wall components include lipopolysaccharides (LPS), which are detected by TLR4 (8–11), lipoproteins (detected by TLR2/1 or TLR2/6 heterodimers) (12, 13) or flagellin (TLR5) (14). TLR9, the receptor for unmethylated CpG containing DNA, is also implicated in the detection of bacteria (15). However, as described above, TLR9, along with the other nucleic acid sensing TLRs (TLR3, 7 and 8) probably evolved to detect viruses. TLR13 is the most recently defined member of this family and perhaps the most intriguing, in the context of bacterial detection. TLR13 recognizes an RNA sequence that corresponds to that found in bacterial ribosomal RNA (1, 2). In contrast, all other RNA sensing TLRs do not detect their cognate PAMP in a sequence specific manner. Interestingly, the sequence detected by TLR13 corresponds to the sequence targeted by several naturally occurring fungal metabolites that display potent antibacterial activity. This sequence of RNA is highly conserved across bacteria, which may explain why it is targeted by mammalian TLRs and fungal metabolites. Further evidence in support of the unusual nature of the target of TLR13 is based on the surprising finding that TLR13 is only found in mice. Despite the lack of TLR13, humans induce potent inflammatory responses to the RNA ligands that activate this receptor. Based on these findings, one must conclude that there is substantial selective pressure to detect bacterial encounters, and multiple PRRs likely evolved independently in diverse species to serve this purpose.

Whereas most TLRs detect bacterial PAMPs directly, one notable exception derives from studies of LPS detection by TLR4. TLR4 interacts weakly with LPS, yet picomolar concentration of this PAMP can induce TLR4-dependent inflammatory responses (16). This high degree of sensitivity to LPS has been explained by the finding that TLR4 is not the sole sensor of extracellular LPS. Indeed, three LPS-binding proteins act upstream of TLR4 to promote high efficiency detection. The extracellular LPS-binding protein (LBP) is the first of these receptors to act, as it binds directly to the gram-negative bacterial outer membrane, bacterial outer membrane vesicles (OMVs) or micelles. These interactions somehow disrupt

the tight packaging of LPS and facilitate the extraction of a monomer of LPS by the GPI-anchored protein CD14. CD14 then transfers LPS to the small molecule MD-2, which is constitutively associated with the extracellular domain of TLR4. Only upon binding MD-2 can LPS form contacts with TLR4, which results in its dimerization. The dimerized ectodomain is the first step in the signaling process, whereby the intracellular TIR domains also dimerize and promote NF- κ B dependent inflammatory responses. The directionality of LPS transfer to TLR4 is ensured by the increasing affinity of LBP, CD14 and MD-2 for LPS (17–22).

Interestingly, despite the symmetrical operation of many stages of microbial detection and inflammation induction across different PRR families, this complex process of PAMP binding is unique to TLR4. All other well-characterized PRRs detect their ligand directly, with high affinity. One possible explanation for why PAMP detection by TLR4 is so complex is based on the nature of the ligand itself. Like other PAMPs derived from the bacterial cell wall, LPS is surface exposed. However, it is not the exposed substructure of LPS that is bound by MD-2 and TLR4, but rather the hydrophobic acyl chains that are buried in the membrane. Thus, the non-exposed structures within LPS must be extracted by CD14 and presented to downstream PRRs. But why extract LPS in the first place? Why not simply couple initial detection of the exposed LPS substructures with inflammation inducing activities? While the answer to this question is unknown, it is worth noting that the very nature of LPS detection by TLR4 involves a single molecule of LPS being extracted by a bacterium that contains thousands of such molecules. Importantly, it has been estimated that 1000 molecules of LPS can be extracted from an individual *E.coli* cell, each of which could (in principle) be used to activate TLR4-induced inflammatory responses on 1000 different macrophages (16). The use of the term overwhelming force, which is often associated with military dominance, is appropriate for this discussion, as we can consider the unusual strategy of LPS detection a means to amplify the number of cells that respond to a single bacterial encounter.

In recent years, new LPS receptors have been identified, as well as new functions for known LPS receptors. For example, caspase-11 in mice, and caspases-4 and -5 in humans are now recognized to bind directly to LPS (23, 24). Recent biophysical analysis illustrated that, unlike the aforementioned receptors that bind monomers of LPS, caspase-4 (and likely the functional orthologues) do not bind monomers. Rather, these caspases appear to bind large aggregates of LPS (25). Additionally, the G-protein coupled receptor BAI-1 was identified as a protein that binds the surface-exposed core oligosaccharides of LPS. Finally, CD14, in addition to transferring LPS to TLR4, acts to promote endocytosis—thereby promoting the transfer of TLR4 dimers into endosomes, which is an important site of inflammatory signal transduction (26).

Whereas the TLRs are the primary sensors of extracellular bacteria, several PRRs detect cytosolic bacteria. In terms of number of receptors that participate in cytosolic bacteria detection, the nucleotide binding leucine rich repeat (NLRs) proteins are most notable. Among the NLRs, NOD1 detects γ -d-glutamyl-meso-diaminopimelic acid (iE-DAP) (27, 28), NOD2 detects muramyl dipeptide (MDP) and NLRP3 detects the activities of N-acetylglucosamine (NAG) fragment of peptidoglycan (29, 30). The NLRs NAIP2, 5, 6 also

detect bacterial products directly, with NAIP2 detecting a bacterial secretion system structure and NAIP5 and NAIP6 detecting a distinct substructure within flagellin than what is detected by TLR5 (31). Members of the RIG-I like Receptor (RLR) family also detect bacteria, as do the DNA sensors cGAS and AIM2. Each of these receptors survey the cytosol for nucleic acid ligands. The first described RLR, RIG-I, detects short double stranded RNA sequences with a di- or tri-phosphorylated 5' terminus and the RLR MDA5 detects long branched chains of double stranded RNA (32–36). Genetic analysis has implicated RLRs in the detection of bacterial infections, most notably the cytosolic pathogen *Listeria monocytogenes* (37, 38). Rather than sensing RNA sequences in the cytosol, cGAS and AIM2 detect DNA in this subcellular compartment (39, 40). The presence of DNA in the cytosol is an unusual event as all cellular sources of DNA should be confined to the nucleus or mitochondria. cGAS and AIM2 each contain high affinity DNA binding domains, which can detect any DNA that leaks into the cytosol, such as during genotoxic stress, mitochondrial dysfunction or bacterial lysis. While one would consider bacteriolysis in the cytosol to be a rare event, the number of infections that activate cGAS is ever-increasing. For example, cGAS appears to detect *Mycobacterium tuberculosis*, *Chlamydia* species, and *Legionella pneumophila* (41) (42). Several of the aforementioned studies have proposed that the DNA detected during these infections is derived from living (not lysed) intracellular bacteria. At present, it is unclear how DNA would be released from bacteria without lysis, although bacteria Type III, IV and VII secretion systems (T3SS, T4SS, T7SS) may provide conduits to the cytosol.

Host encoded sensors of pathogen activities

While most PRRs detect microbes by directly sensing the presence of PAMPs, others detect infection through sensing of activities carried out by bacteria as part of their virulence strategies. The consequences of these activities can be cellular stress, damage or biochemical modification of host factors. Inflammasomes are sensors that detect pathogen activities and will be discussed in greater detail in other chapters of this book. Of note, the NLRP3 inflammasome detects disturbances in cellular homeostasis including damage to membranes caused by bacterial toxins and secretion systems including T3SS and T4SSs. Secretion systems are a common virulence mechanism of many bacterial pathogens of plants and animals. Bacteria use these organelles to inject factors (known as effector proteins) into target cells. T3SSs and T4SSs are molecular syringes that span bacterial membranes and insert themselves into the host cell membrane (and cell wall in the case of plant cells). The pore formed by the insertion of translocon components into host membranes causes activation of the NLRP3 inflammasome, leading to pyroptosis via caspase 1 activation (43).

In addition to the physical disruption caused by the presence of bacteria and their secretion systems, the biochemical activities of secreted effectors themselves are now emerging as signals detected by host PRRs. Translocated effectors carry out a number of biochemical functions to manipulate host cell functions, including affecting membrane trafficking, cytoskeletal dynamics, or inhibiting the host immune response. The injection of this arsenal of toxins does not go unnoticed and we now know that host cells are able to detect certain modification of host targets. This phenomenon of 'indirect interaction' was long thought to be unique to plant innate immunity and is often-discussed in the context under the name of

‘guard hypothesis’. This theory stipulates that important host proteins that are targeted by bacterial effectors are ‘guarded’ by sensors. Sensor activation leads to the activation of an innate response (4, 44). For example, *Arabidopsis thaliana* RIN4 is an important negative regulator of plant immunity. It is phosphorylated and inhibited by a number of bacterial factors including the *Pseudomonas syringae* effectors AvrB and AvrRpm1 (45). This phosphorylation event is detected by two plant proteins: the guards RPM1 and RPS2, activating an immune response (46–48).

Such a relationship has now been described in metazoans, including insects and vertebrates. In both cases, the ‘guarded targets’ identified are small GTPases of the Rho family (RhoA, Rac1/2, Cdc42). Rho GTPases sit at the apex of a number of signaling pathways that regulate critical cellular functions including cytoskeletal dynamics and activation of inflammation. As such, they are targeted by a range of bacterial factors. For example, the *E.coli* cytotoxic necrotizing factor 1 (CNF1) activates the Rho GTPase Rac2. This modification is detected by the immune adaptor IMD in flies and Rip1-Rip2 in mammalian cells, leading to the expression of pro-inflammatory cytokines (49). Similarly, the intracellular pathogen *Salmonella* activates Rac1 and Cdc42 in order to induce its internalization into non-phagocytic cells. This process of Rac1 manipulation is detected by Nod1, in a complex with Hsp90 and Rip2 (50). Finally, RhoA inhibition by a number of pathogens is sensed by the cytosolic protein Pyrin, leading to subsequent activation of proptosis mediated by the inflammasome (51).

Neutrophil recruitment to the sites of infection—a common outcome of bacterial detection.

The varying sensors of bacterial molecules or virulence factor activities lead to the activation of defense responses that can be general classified as inflammatory (Figure 1). These responses involve the upregulation of genes that promote numerous aspects of host defense. Many downstream host defense responses are context dependent, but a common response associated with all innate immune responses is the induction of inflammation at the site of bacteria detection. Upon bacterial detection, all of the PRR pathways described herein induce the production of chemokines that promote the recruitment of large numbers of neutrophils to infected tissues. Many neutrophil chemokines are expressed by the action of the transcription factors NF- κ B and AP-1, which are commonly activated by transcription inducing PRR pathways and inflammasome pathways via the release of IL-1 (3, 52). In most cases of bacterial encounters, neutrophils are the first cells recruited from the bloodstream to the infected tissue. Neutrophils are the most potent antibacterial phagocytes known to operate in the human body. Unlike dendritic cells, monocytes and macrophages, which are also professional phagocytes, neutrophils rarely live longer than 24 hours and are not considered to play a role in antigen presentation to T lymphocytes (53, 54). Rather, the primary function of neutrophils is to kill bacteria and other microorganisms. These cells display several activities supporting such an anti-bacterial function, including potent phagocytic activity and the ability to produce copious amounts of reactive oxygen species. Neutrophils also contain secretory granules and lysosome-like organelles that are rich in anti-microbial peptides and enzymes that operate to degrade virtually every component of

the bacterial cell wall. Finally, neutrophils are adept at undergoing an unusual form of cell death known as netosis, where the cells release genomic DNA into the extracellular space to ensnare bacteria. These DNA-rich nets not only capture and prevent bacterial spread, but the associated histones display potent anti-bacterial activity (55, 56).

It is worth noting that several of the activities described here are also observed in other phagocytic cells, such as the ability to produce reactive oxygen species and ingest bacteria. However, these antibacterial activities appear most active in neutrophils, and in the case of netosis, appear to be unique to this cell type. When considered in the context of the rapid accumulation of large numbers of these cells in infected tissues, it is reasonable to suggest that the biggest threat to bacterial survival in the host is the anti-microbial activities of neutrophils. Consistent with this suggestion are numerous findings that bacteria that have the ability to replicate within other phagocytes are readily killed by neutrophils. Thus, the mechanisms that link bacterial detection by PRRs to anti-bacterial activity of neutrophils are of prime importance for host defense. Other aspects of immunity, such as the activation of antigen-specific T lymphocytes and production of antigen specific antibodies are also important, but these activities are induced after neutrophil-mediated activities are mobilized to contain the infection.

Perspectives

In this essay, we discussed several means by which mammalian cells detect and respond to bacterial encounters. These means include the use of structurally diverse receptors and mechanistically distinct signaling pathways. Despite this diversity, the evolutionary pressures to develop these detection systems are likely constant—the need to ensure host survival by preventing bacterial replication. It is worth noting that much of our knowledge in this area derive from studies that have focused on the interactions between bacteria and phagocytes or fibroblasts, neither of which are the first cells to encounter bacteria in the natural course of events. Epithelial cells, either in the skin, airways or digestive tract, represent the barrier to the environment. As such, these cells are poised for rapid detection of bacterial infection, and the innate immune pathways that operate in epithelial cells likely influence many downstream innate and adaptive responses to infection. Indeed, there is ample evidence that epithelia are the first cells that experience the activities of bacterial type III secretion systems, in particular those involved in invasion. Studies over the next several years will likely expand on our knowledge of the innate immune system in phagocytes and fibroblasts to include similar inquiries into the biology of barrier tissues. This broadening of the scope of host-microbe analysis will allow the classification of innate immune responses as being common to all cell types or tissue-specific. The emergence of human and mouse organoid models that represent an increasingly diverse set of tissues should facilitate such inquiries and may allow for a more detailed view to emerge of the earliest stages of any host-bacterial interaction.

Acknowledgments

We thank the members of the Kagan and Odendall labs for helpful discussions. This work was supported by the NIH grants AI133524, AI093589, AI116550 and P30DK34854 to JCK. JCK holds an Investigators in the Pathogenesis of Infectious Disease Award from the Burroughs Wellcome Fund. C.O. is supported by a King's

College London Prize Fellowship and a Sir Henry Dale Fellowship from the Royal Society and the Wellcome Trust (Grant number 206200/Z/17/Z).

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Figure 1:

Evolutionary arms race between the host immune systems and bacterial virulence mechanisms

1. Pattern recognition receptors recognise PAMPs and activate conserved signaling pathways that induce transcriptional activation of inflammation with expression of cytokines and interferons.

2. Pathogenic bacteria use secretion systems (SS) to inject effector proteins into host cells. The diverse functions of these effectors include the ability to block different components of PRR-induced pathways.

3. The activities of pathogenic bacteria are detected by 'guard' proteins. In vertebrates these are mostly NLRs and other inflammasome stimulators. They detect:

3a. The presence of type III and IV SSs, usually through the formation of a pores in host membranes

3b. Post-translational modification of host proteins.

Consequently, NLRs and inflammasomes induce inflammation at the transcriptional and posttranslational levels through caspase-dependent activation of pyroptosis.

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Table 1:
Mammalian PRRs and their targets

PRRs and their associated PAMPs and localization are shown.

Sensor	Family	PAMP	Localisation
TLR2/1, TLR2/6	TLR	Lipoproteins	Plasma membrane
TLR4		LPS	Plasma membrane
TLR3		dsRNA	Endosomes
TLR5		Flagellin	Plasma membrane
TLR7		ssRNA	Endosomes
TLR8		ssRNA	Endosomes
TLR9		CpG DNA	Endosomes
TLR13		rRNA with specific sequence	Endosomes
Nod1	NLR	iE-DAP	Cytosol
Nod2		Muramyl dipeptide (MDP)	Cytosol
NLRP3		Peptidoglycan	Cytosol
NAIP2		T3SS rod	Cytosol
NAIP5		Flagellin	Cytosol
NAIP6		Flagellin	Cytosol
CGAS		DNA	Cytosol
AIM2	ALR	DNA	Cytosol
RIG-I	RNA	Short ds RNA with 5' terminus	Cytosol
MDA5		Long branched chains of ds RNA	Cytosol