Published in final edited form as:
Curr Opin Microbiol. 2013 February ; 16(1): 17–22. doi:10.1016/j.mib.2012.11.004.

The Impact of Toll-like Receptors on Bacterial Virulence Strategies

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Abstract

The mammalian immune system has evolved in the presence of microbes, both pathogenic and commensal. The consequences of microbial recognition by the host has led to the development of compensatory mechanisms by both the host and microbe to either resist or tolerate the existence of the other. In this review we discuss examples of this co-evolutionary relationship. Due to space considerations and for conceptual clarity, we have focused on detection of bacteria by the Toll-like receptor (TLR) family and highlight examples of bacterial strategies to evade, subvert and in some cases even utilize these receptors.

Introduction

TLRs are a family of membrane-spanning innate immune receptors that recognize ligands derived from bacteria, fungi, viruses, and parasites. Recognition of conserved microbial features by TLRs leads to a variety of downstream signals in immune cells, including pro-inflammatory cytokine production, costimulatory molecule upregulation, anti-microbial peptide secretion, and phagosomal maturation (1, 2). While individual TLRs typically recognize a specific class of microbial ligands, collectively this family of receptors can detect a broad range of microbes. Of the thirteen TLRs present in mammals, ligands have been identified for twelve: lipopolysaccharide (LPS) for TLR4, lipopeptides for TLR2/1 and TLR2/6 heterodimers, flagellin for TLR5, unmethylated CpG motifs in DNA for TLR9, profilin and *Salmonella* flagellin for TLR11 (3), and various forms of RNA for TLRs 3, 7, 8 and 13, with no known ligand currently identified for TLR 12 (4). Thus, multiple TLRs can potentially recognize bacteria, although individual TLRs will play a more dominant role for certain bacterial species or in certain contexts (e.g., recognition of nucleic acids released from degraded bacteria).

Signaling

All TLRs share a common modular structure: a leucine rich repeat (LRR)-containing ectodomain responsible for ligand-binding, a membrane spanning region, and a cytosolic

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signaling domain called the Toll-interleukin 1 receptor homology domain (TIR). Ligand binding induces recruitment of TIR domain-containing signaling adaptors that associate with the TLR via homotypic TIR:TIR interactions. All TLRs, with the exception of TLR3, recruit MyD88 at this initial step. MyD88 can associate with IL-1R-associated kinase (IRAK) members. Upon dissociation from the TIR complex, IRAK proteins interact with tumor necrosis factor receptor-associated factor 6 (TRAF6)to propagate TLR activation signals that include the activation of transcription factors NF-κB, AP-1 (mediated by JNK, p38 and ERK), and IRF5 (5). TLR3 and TLR4 can recruit a different adaptor, TIR-domain-containing adapter-inducing interferon-β (TRIF), that leads to the dimerization and activation of inhibitor of NF-κB kinase (IKKi) and TANK (TRAF–family member associated NF-κB activator)-binding kinase 1 (TBK1). Activated TBK1/IKKi phosphorylates the transcription factor, IRF3, inducing its nuclear translocation and subsequent transcription of interferon-related genes (6).

Function

TLR activation leads to production of pro-inflammatory cytokines, including TNFα, IL-12, and IL-6. These cytokines induce local inflammation, support the survival and expansion of B and T cells and activate natural killer (NK) cells. A subset of TLRs can also induce type I interferon production (IFNα/β) (4, 6). This family of cytokines can inhibit translation and/or induce apoptosis in host cells, thereby exposing intracellular bacteria to the extracellular environment and killing by other infiltrating immune cells. TLR signaling also leads to the upregulation of costimulatory molecules and MHC molecules presenting bacterial antigens. Costimulatory molecules include CD80, CD86 and CD40 and have the overall outcome of generating protective adaptive immune responses by the activation of antigen-specific T cells (7).

TLR activation can also induce cell-intrinsic antimicrobial activity. For example, TLR2 and TLR4 activation can recruit NADPH oxidase assembly as well as mitochondria relocalization to the bacteria-containing phagosome, leading to a burst of reactive oxygen and nitrogen species within this compartment (8–10). Evidence also suggests that TLR signaling can lead to a rapid acidification of the phagosome in which TLR signaling has occurred, likely through recruitment of vacuolar-ATPase subunits to the phagosomal membrane (11–14). Both of these activities increase the antimicrobial capacity of the phagosome, although some bacteria have actually co-opted these signals to regulate their virulence programs (discussed in greater detail below). Detection of microbial ligands by TLRs can also induce the expression and secretion of antimicrobial peptides (AMPs), such as beta-defensins, and cathelicidin, further supporting the role of TLR-mediated detection in cell-intrinsic antimicrobial activity (15–17).

Evasion and Subversion of Immunity by Bacteria

Because the specificity of TLRs are fixed in the germline and activation initiates the earliest aspects of the immune response to infection, these receptors have applied tremendous selective pressure on the virulence mechanisms of potential pathogens. Not surprisingly, pathogens have evolved a variety of strategies to survive despite recognition by the innate immune system. Here we identify and focus on three general themes, each representing disruption or interference at distinct stages of the host response (Figure 1): 1) evasion of host detection by shielding ligands, 2) interference with TLR signaling pathways, and 3) inhibiting, escaping, or subverting phagocytosis (18–20) (21). Our discussion of these strategies cannot be exhaustive; instead, we highlight key examples.

Surface structure modification

The outer-membranes of gram-negative bacteria (including *Salmonella* and *Yersinia* species, for example) contain LPS, a potent activator of TLR4 (22–27). Activation of this TLR can lead to the production of pro-inflammatory cytokines, co-stimulatory molecule upregulation and secretion of type I IFN (1, 2, 5). These signals depend on the activation of NF-κB and MAP kinase pathways (1). Other surface proteins such as flagellin, a potent TLR5 ligand, can also lead to immune activation, albeit without the production of IFN, via similar pathways(28–30). It is therefore not surprising that bacteria have evolved mechanisms to make these surface structures less detectable by their corresponding TLRs.

LPS, crucial to the growth and survival of gram-negative bacteria, consists of a lipid A moiety conjugated to an O-linked polysaccharide. Lipid A is typically hexa-acylated, yet changes in the LPS structure (importantly the level of acylation) correlate with the ability for LPS-isolates from different bacteria to activate TLR4(31–34). For example, isolates of *Helicobacter pylori*, a human pathogen, have been shown to express penta-acylated LPS, making them less immunostimulatory (35). Further, some bacteria are capable of actively altering their LPS composition in order to make them less stimulatory during infection(31, 32, 36). For example, *Salmonella* is able to decrease the stimulatory capabilities of its LPS by expressing a lipid A deacetylase, PagL, that is specifically expressed during infection (via control of the PhoP/PhoQ two-component system) (37). A similar mechanism is used by the causative agent of bubonic plague, *Yersinia pestis*, which expresses a TLR4-stimulatory hexa-acylated lipid A component of its LPS during growth at 21–27 °C, but upon entry into humans or rodents (and an increase in temperature to 37 °C) expresses a non-stimulatory tetra-acylated form (36). Flagellin, much like the LPS of certain pathogenic bacteria (including isolates of *Bartonella, Helicobacter, and Campylobacter*), has also been shown to be less capable of stimulating TLR5. In these scenarios it was found that these pathogenic bacteria have acquired mutations within the N-terminal domain of their flagellin, specifically within regions responsible for activating TLR5 (28, 38). In turn, these mutations make bacteria less immunostimulatory and able to evade detection by the immune system during infection.

In his landmark paper, Janeway proposed that the targets of innate receptors must be highly conserved and difficult for pathogens to alter; otherwise, pathogens will quickly evolve to avoid detection (39). Based on the examples discussed above (as well as many others), pathogens are clearly capable of modifying TLR ligands to avoid detection. What prevents all pathogens from rapidly evolving away from the specificity of innate receptors? Certainly there are multiple answers to this intriguing question, but one explanation is that modification of the features targeted by TLRs reduces the overall fitness of the pathogen. For example, in the case of *Yersinia*, tetra-acylated lipid A may affect the integrity of the outer membrane or reduce the fitness of bacteria in non-human hosts (otherwise bacteria would only express the tetra-acylated form). Perhaps in rodents and humans this loss of fitness is countered by the avoidance of TLR4 recognition. A similar case is true for flagellin mutations. These mutations lead to reduced bacterial motility, unless coupled with secondary compensatory mutations that can rescue the motility defect in certain bacteria. Hence, the cost of reduced motility is overcome by the benefit afforded by survival via TLR5 evasion (28, 38).

Modulation of Intracellular Signaling Pathways

Instead of avoiding detection altogether, some bacteria have evolved to inhibit the signaling pathways downstream of TLR signaling. These mechanisms lead to the same overall outcome; inhibiting production of inflammatory cytokines as well as other anti-microbial activities that are initiated downstream of TLR activation (40–49). To do so, many different

pathogens encode secretion systems that puncture host cell membranes and inject bacterially-encoded effector proteins that mimic or degrade members of TLR signaling pathways or directly interfere with normal signaling by covalently modifying signaling intermediates. For example, the *Yersinia* effector protein YopJ is an acetyltransferase that modifies key residues within MAP kinases and IKKβ, preventing their phosphorylation and inhibiting activation of the MAPK cascade and NF-κB(40–42). In another set of examples, enteropathogenic *E. coli* (EPEC) type 3 secretion system (T3SS) effector NleC cleaves RelA (p65), a subunit of NF-κB, and another EPEC effector, NleD, cleaves JNK to inhibit activation of AP-1(43–46). A similar bacterial strategy for targeting TLR signaling molecules can be found in an E3 ubiquitin ligase T3SS effector protein, IpaH9.8, encoded by the gastrointestinal pathogen *Shigella flexenri*. Upon injection into the cytosol, IpaH9.8 is able to bind to the IKK regulator, NEMO, and the ubiquitin adaptor protein ABIN-1, leading to ubiquitination and destruction of NEMO and inhibition of the NF-κB signaling pathway after TLR activation(47). Other forms of TLR signaling inhibition take place more proximally to the receptor. For example, *Salmonella, Brucella, E. coli* and certain *Yersinia* species encode different TIR-domain containing proteins that interfere with the homotypic TIR:TIR interactions between TLRs and their signaling adaptors (50, 51).

Inhibiting, Escaping or Subverting Phagocytosis

The nucleic acid sensing TLRs (TLR3, TLR7/8, and TLR9) are localized intracellulary and are recruited to phagosomes (4, 52), and surface localized TLRs, such as TLR4 and TLR2, can be internalized and sense bacterial products within the phagosome (53). Certain pathogens try to avoid phagocytosis by immune cells as a means to avoid this detection and the induction of antimicrobial mechanisms. Numerous virulence mechanisms resulting in inhibition of phagocytosis have been described, including inhibition of complement deposition on the bacterial cell surface, as is the case for *Streptococcus pyogenes* M protein, and shielding of the bacterium in fibrin clots via coagulase expression by *Staphylococcus aureus* (54, 55). Other classes of pathogens escape phagosomes and replicate within the cytosol. This strategy avoids detection by TLRs, but renders bacteria susceptible to cytosolic innate immune sensors. Both *Shigella* and *Listeria monocytogenes* utilize this virulence strategy (21, 56)—mutants that are unable to escape have a heightened production of pro-inflammatory cytokines, presumably due to increased activation of phagosomal TLRs.

Some bacteria survive within the phagosome despite its antimicrobial nature. This virulence strategy has several implications for the coevolutionary relationship with TLRs—those that survive within the phagosome must have a means of inhibiting the antimicrobial mechanisms induced by engaging these innate receptors. *Salmonella, M. tuberculosis, Legionella,* and *Chlamydia* are among those pathogens capable of surviving within phagosomes by preventing fusion with lysosomes (57–59). The process by which this occurs is different for each bacterial species, and in some cases greatly depends on recognizing features of the phagosome in order to induce virulence genes required for inhibiting the phagosomal maturation process or protecting the bacterium from antimicrobial onslaught. For example, in order to neutralize radicals that are produced via TLR-induced recruitment of NADPH oxidase to the phagosomal membrane, *Brucella abortus* and *Staphylococcus aureus* express superoxide dismutase and catalase (60, 61). *Salmonella* inhibits recruitment of NADPH oxidase via injection of effectors into the host cell (62). Several bacteria also alter their cell wall structure in order to make them less susceptible to antimicrobial peptides and other intra-phagosomal antimicrobial mechanisms induced by TLR activation (17, 32, 63–66). These critical virulence mechanisms rely on timely induction upon entry into host cells. The process by which this regulated expression occurs is a common theme for many intracellular bacteria and relies on the use of cues provided, in many cases, by the innate immune system. In the next section we focus on examples of bacteria coopting innate

signals to coordinate expression of virulence genes for survival in diverse host environments.

Coopting Innate Immune Signals

Various phagosomal parameters induced upon TLR activation can be used by bacteria to identify their presence within host cells. A number of bacteria utilize phagosomal acidification for this purpose. For example, *Salmonella* requires TLR-dependent phagosomal acidification to coordinate expression of the SPI-2 T3SS, which is required for intracellular replication (12). *Brucella suis* also relies on acidification to induce virulence genes (67). Several cytosolic pathogens, including *Listeria* and *Shigella,* require phagosomal acidification to activate lysins and escape into the cytosol (56). *M. tuberculosis* utilizes signals associated with phagosomal maturation to regulate expression of efflux pumps that increase resistance to certain antibiotics (68). Also, the *Salmonella* two-component sensor PhoP/PhoQ, responsible for mediating anti-microbial-resistant LPS modifications, is induced by cationic antimicrobial peptides present in the phagosome upon activation (64).

These examples suggest that innate immune signals may represent common phagosomal features used by bacteria to coordinate virulence gene expression and raise an interesting point of discussion regarding *why* bacteria would become dependent on immunity-driven phagosomal signals to induce virulence mechanisms. While we can only speculate about "*why*" questions relating to host-pathogen interactions, an emerging theme of these relationships is that many of the cues leading to virulence gene induction are linked to innate immune signaling. Certainly all pathogens must regulate expression of their virulence genes, and a defining feature of a given pathogen may be which cues it uses to induce the genes required to transition between distinct niches. For example, to cause systemic infection after oral ingestion *Salmonella* must traverse the intestinal epithelium, encounter phagocytic immune cells (mainly macrophages and dendritic cells), survive and replicate within these cells, and eventually disseminate to systemic sites. This process relies on the expression of adhesion molecules, two different T3SSs, and a multitude of additional evasion mechanisms. Moreover, inappropriate induction of virulence genes can negatively impact each of these steps, and detection of the virulence factors themselves or the consequences of their action can activate additional innate immune pathways (69, 70). For these reasons, pathogens must quickly identify signals associated with these transitions between niches. Thus, while the consequences of innate immune activation may select the emergence of virulence strategies, these same features of the host response may best define the context in which these strategies are required.

Conclusion

Throughout this review we have focused on the interaction between pathogenic bacteria and TLRs, highlighting examples in which bacteria have attempted to evade TLR signaling by altering their surface structures, interfering with TLR signaling pathways, or escaping, inhibiting, or subverting phagocytosis. We have focused on examples of bacteria using innate immune signaling to regulate induction of virulence strategies. The examples and discussion presented herein speak to the evolution of pathogens with and in response to the innate immune system and highlight the ability of pathogens to evade or exploit host responses to enhance their virulence

Acknowledgments

We apologize to any colleagues whose work was not cited due to space constraints. Research in the Barton laboratory related to the topic of this review is supported by the NIH (AI095587, AI06332) and a Burroughs Wellcome Fund Investigator in the Pathogenesis of Infectious Disease Award (to GMB).

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Highlights

- Pathogens have evolved multiple mechanisms to evade TLR recognition and signaling
- Alterations in bacterial surface structures make pathogens less immunostimulatory
- Pathogens encode virulence factors that directly inhibit TLR signaling pathways
- Inhibition, escape, or subversion of phagocytosis is a method to avoid TLR detection
- Some pathogens coopt innate immune signals to initiate virulence

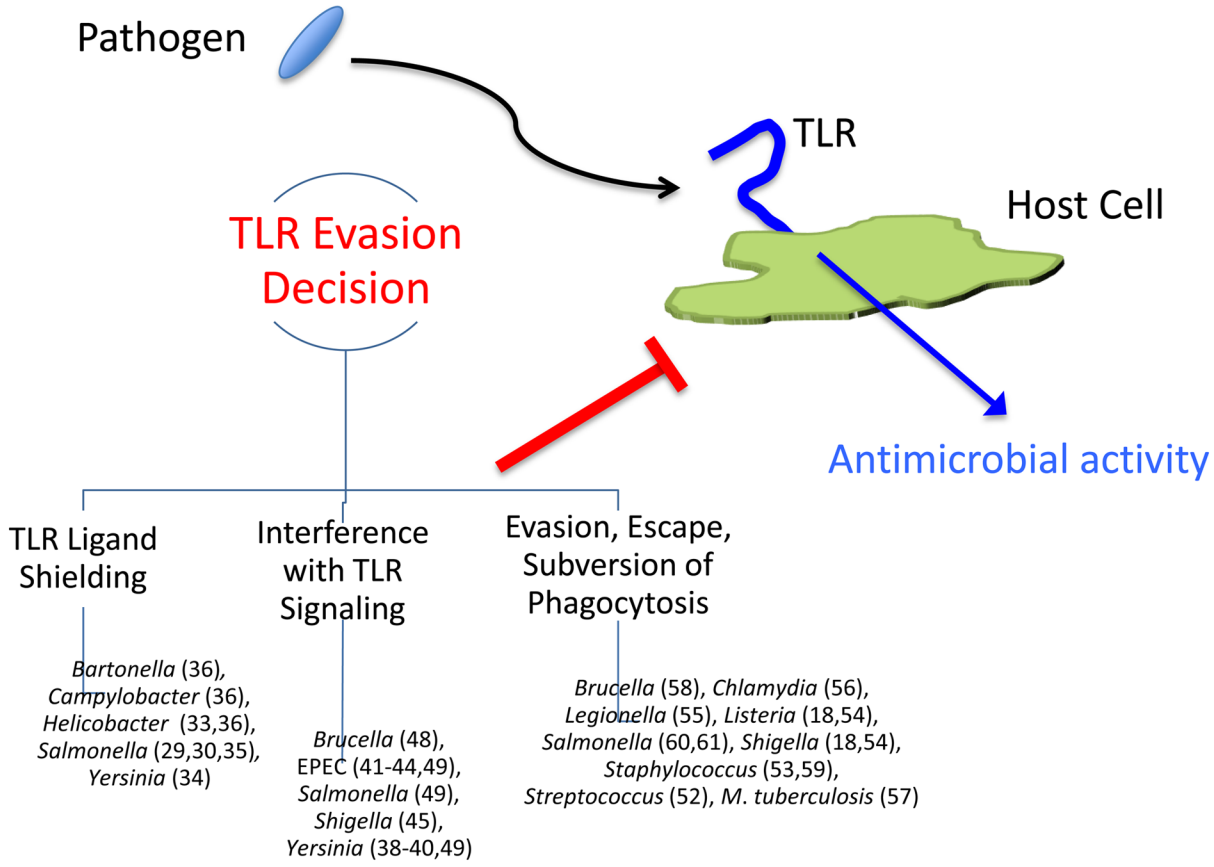

Figure 1. Strategies used by pathogens to evade TLR signaling

Three common evasion strategies utilized by different pathogens are illustrated. Bacteria that employ these mechanisms (and relevant citations) are indicated beneath each strategy. See text for discussion.