Bacterial Secretion Systems and Regulation of Inflammasome Activation

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Abstract
Innate immunity is critical for host defenses against pathogens, but many bacteria display complex ways of interacting with innate immune signaling, as they may both activate and evade certain pathways. Gram-negative bacteria can exhibit specialized nano-machine secretion systems for delivery of effector proteins into mammalian cells. Bacterial type III, IV and VI secretion systems are known for their impact on caspase-1 activating inflammasomes, necessary for producing bioactive inflammatory cytokines IL-1β and IL-18, key participants of anti-bacterial responses. Here we discuss how these secretion systems can mediate triggering and inhibition of inflammasome signaling. We propose that a fine balance between secretion-system mediated activation and inhibition can determine net activation of inflammasome activity, and control inflammation, clearance or spread of the infection.

Innate Immunity

Innate immunity plays a critical role in controlling the spread of microbes which breach physical and integumentary barriers. Innate immune cells - such as macrophages, neutrophils, dendritic cells - sense conserved and recognizable pathogen- and danger-associated molecular patterns (PAMPs and DAMPs) through pattern recognition receptors (PRRs)(1,2). Activation of such receptors turns on intracellular pathways to relay the signal to the DNA level and activate transcription of cytokines, chemokines, interferons, and other factors important for priming an immune response. PRR families include NOD-like receptors (NLRs), Toll-like receptors (TLRs), AIM2-like receptors, and others.

TLRs represent an key class of PRRs, capable of recognizing a range of bacterial and viral molecules to initiate an innate immune response arrest a potential infection in its early stages. TLRs 2, 4, 5, and 6 are located at the cell surface and recognize PAMPs including Pam2Cys4 from Gram-positive bacteria (TLRs 2 and 6), LPS from Gram-negative bacteria (TLR4), and bacterial flagellin (TLR5) among others(1). TLRs 3, 7, 8, 9, 11, and 13 are found on endosomal membranes and primarily recognize nucleic acids.

Activation of transcription factor NF-kB downstream of certain TLRs results in production of chemotactic and pro-inflammatory cytokines including TNFa, IL-6, IL-8, IL-1β, and IL-18. The latter two cytokines, IL-1β, and IL-18, are particularly powerful immune orchestrators but require post-translational modification by enzymatic cleavage in order to be secreted in their mature forms. This enzymatic processing is classically accomplished via molecular complexes called inflammasomes. These complexes form upon activation of additional PRR sensors, ensuring the specificity of IL-1β/IL-18-dependent immune responses.

Inflammasomes and their Role in Disease

Inflammasomes are increasingly recognized as critical orchestrators of immunity. These multi-molecular protein complexes are at the center of a variety of pathways in innate immune cells, including cytokine production(3), cytoskeletal remodeling(4), and cell death(5). Inflammasome formation is initiated when a pathogen or danger-associated molecular patterns (PAMP or DAMP) are recognized and triggers signaling often via NOD-like receptor (NLR) protein such as NLRP3 or NLRC4. This results in nucleation and oligomerization of the adaptor protein Asc at the site of the NLR, and recruitment of pro-caspase-1 to the CARD domain of Asc(6). Dimers of pro-caspase-1 are then cleaved to active caspase-1 through

autoproteolysis, which then catalyzes the final processing of pro-IL-1β and pro-IL-18 into their mature secreted forms. Activation of caspase-1 is also accompanied by an inflammatory form of apoptosis, termed pyroptosis. Non-canonical caspase-11 inflammasomes, as well as pathways dependent on caspase-8 or neutrophil proteases have also been described(7-10).

Inflammasome-dependent secretion of IL-1β and IL-18 is critical for immune control of many microbes(11-16), and may play an important role in vaccine adjuvant-induced responses(17). However, dysregulation or inappropriate activation of inflammasomes can also produce severe autoinflammation(18-21) and contribute to autoimmune disorders(22-24), Alzheimer's disease(25), Parkinson's Disease(26), and many other pathologic processes. To some extent the roles of IL-1β and IL-18 overlap(27). Prominent effects of IL-1β include recruitment of neutrophils to sites of infection, promoting endothelial cell adhesion, and stimulating adaptive Th17 responses. An important role of IL-18 is to induce NK cells and T-cells to produce IFN-g, which activates macrophages. IL-1β in particular tends to cause host tissue damage, whereas IL-18 tends to have a less detrimental effect while still helping to control infection. This can be critical for the clearance of intracellular pathogens, and for efficient activation of adaptive immune responses. Consequently, inflammasome-activated caspase-1 and subsequent levels of IL-1β and IL-18 secretion are key events in many infectious and non-infectious diseases.

Heterogeneity of Inflammasome structure, activation, and regulation mechanisms

Consistent with the delicate balance needed between a sufficiently robust immune response and minimal tissue damage, sophisticated mechanisms exist to tightly regulate the specificity and sensitivity of inflammasome pathways. While the general model of NLR-Asc-Caspase-1 from early inflammasome studies is useful for a basic conceptualization of this system, the extent of its heterogeneity is being increasingly recognized and appreciated.

Recent findings have suggested that inflammasome complexes involve organized helical structures and the creation of fibril like structures(28), where the NLR or AIM2 nucleates Asc fibril polymer formation and finally caspase-1 polymers, culminating in formation of cleaved caspase-1. The structures can be viewed as supramolecular organizing centers (SMOCs).

NLR molecules such as NLRP1, NLRP3, NLRP6, and NLRP12 contain LRRs which are believed to be involved in activation, an ATPase NACHT domain (except NLRP1), and a pyrin domain through which they interact with Asc. However NLRC4 (sometimes referred to as IPAF) contains a CARD domain, which recruits Asc but can also directly recruit caspase-1. Although NLRC4 activation is more robust in the presence of Asc, it is not required. NLRP1b(29) and NOD1(30) can also activate caspase-1 independently of Asc.

Non-NLR sensors such as Pyrin, IFI16, and AIM2 also exist; these proteins contain Asc-interacting pyrin domains, but lack the LRR domains present on many other signaling molecules. Instead, AIM2 and IFI16 contain DNA-sensing HIN domains(31), and have been reported to respond to viral as well as bacterial DNA in the cytosol(32-34). In the case of Pyrin, a directly activating pathogen ligand has not been established but it has been proposed that this pathway responds to pathologic Rho-GTPase activity induced by multiple Gram-negative pathogens(18).

The case of Pyrin also demonstrates that inflammasome activation is not necessarily the result of a direct interaction of a sensor with a PAMP or DAMP ligand. The Pyrin inflammasome can be made hyper-active by mutations in the SPRY domain(35) or other domains(36); in humans such mutations are the cause of the most common autoinflammatory disease - Familial Mediterranean Fever (FMF)(37). The mechanisms of Pyrin activation and regulation are still being actively studied. Activity of the Pyrin inflammasome may be influenced by PSTPIP1(38), Siva(39), certain 14-3-3 isoforms(36,40), the leading edge of polymerizing actin(41), and a diverse variety of microbial molecules(42). Very recently, PKN1/2 kinases have also been implicated in controlling Pyrin activation by phosphorylating Pyrin to an inactive, 14-3-3 bound form(43), and the mevalonate pathway also regulates Pyrin inflammasomes(44). Many aspects of the activation mechanism remain unknown, but the emerging picture is one where Pyrin is triggered by perturbations in intracellular homeostasis, which are sensed by the endogenous signaling partners of Pyrin rather than by direct binding of a pathogenic ligand to Pyrin.

Other inflammasomes require cofactors for activation as well. The NLRC4 inflammasome is well known for recognizing flagellin, yet NLRC4 does not bind flagellin directly; instead, the presence of flagellin is relayed to NLRC4 by NAIP proteins which directly bind the ligand(45,46). NLRP3, often regarded as the quintessential classical inflammasome component, also has a complex mechanism of activation which senses DAMPs and PAMPs indirectly. NLRP3 can be activated by a variety of triggers including excessive influxes of calcium and/or efflux of potassium, oxidative damage, elevated ATP levels, and bacterial pore-forming toxins, crystallized molecules such as silica or uric acid, oxidized mitochondrial DNA, and many others(47). Activation by mitochondrial DNA appears attractive as a unifying mechanism, since the other activating events may trigger the upstream damage which causes the release and oxidation of mitochondrial DNA. How NLRP3 is involved in signaling in response to mitochondrial DNA is still incompletely understood. The mitochondrial DNA hypothesis may also explain observations that autophagy is associated with reduced inflammasome activation(48), as turnover of damaged mitochondria as well as ubiquitinated inflammasome components increases(49-51). If so, a recent study by Orlowski et al showing that multiple endogenous cathepsins potentiate NLRP3 activity may reveal another important mechanism of inflammasome regulation(52), considering that cathepsin activity is known to inhibit autophagy(53,54). However, other regulators may also contribute to signaling via this important pathway. Several studies have proposed NEK7 as a key participant in NLRP3-induced caspase-1 cleavage and cell death(55-57).

Another important NLRP3-activating mechanism occurs through upstream recognition of intracellular LPS by caspase-11. In this pathway, termed the non-canonical inflammasome, LPS-activated caspase-11 cleaves gasdermin D, which is involved in caspase-11 and caspase-1 dependent pyroptosis and NLRP3-dependent caspase-1 activation(58,59). Mechanistically, it has been proposed that gasdermin D forms pyroptotic pores in host cell membranes, and may even kill intracellular bacteria(60-63). Caspase-11 activity depends on interferon pathways, as TLR4, TRIF, and IFNAR1 deficient cells show heavily impaired caspase-11 processing(64). Recently it was shown that type-I interferons activate guanylate binding proteins (GBPs) which are involved in trafficking proteins to the plasma membrane or membranes of intracellular organelles, and are required for activation of the inflammasomes pathways as well as other antimicrobial actions in response to vacuolar Gram-negative bacteria(65-69). However, while the non-canonical caspase-11 inflammasome plays a critical role in host defense against intracellular Gram-negatives(64,70-72), mice lacking gasdermin D or caspase-11 are also protected from high dose LPS-mediated septic shock(58,73).

It is also important to note that some NLRs, such as NLRP6 and NLRP12, may have both pro- and anti-inflammatory functions(74-76). NLRP6 was shown to negatively regulate NF-kB driven innate immune responses and actually impede clearance of bacterial pathogens(77). Interestingly, in gut epithelial cells and neurons NLRP6 has a protective effect independent of inflammasome activity(78,79). NLRP6 was recently shown to recognize dsRNA together with Dhx15 (another potential cofactor), and play an important role in defense against norovirus in the gut independently of caspase-1(80). The unusual functions of NLRP6 complicate the evaluation of mechanisms of its involvement in inflammasome processes.

In some disease contexts NLRP12 may also forms an Asc-dependent inflammasome and promote caspase-1 activation. NLRP12 contributes to caspase-1 activity and IL-1β production in response to *Y. pestis*(76) and *K. pneumoniae*(81) and *Plasmodium(82)*. To date, however, no specific trigger of an NLRP12 inflammasome has been identified. Like NLRP6, NLRP12 has been suggested to negatively regulate NF-kB(83) and to limit inflammatory immune responses both in the intestines and in neurons(84,85), and NLRP12 can suppress immune responses to Salmonella infection.

A number of pathways are also capable of processing IL-1β and IL-18 independently of inflammasomes. Indeed, the IL-1β response to certain stimuli can be nearly unchanged in mice lacking caspase-1 or Asc(86-88). Several neutrophil proteases including serine proteinase-3, cathepsin G, and neutrophil elastase are known to directly process IL-1β and IL-18(89). In addition, caspase-8 can activate caspase-1 through an incompletely understood mechanism(90-92); however, caspase-8 has also been reported to process IL-1β independently of caspase-1(10,93,94).

Finally, it should be noted that inflammasome expression varies by cell type, stage of maturation, and type of activation. Expression of NLRP3, for example, is generally too low under resting conditions and

needs to be induced by priming (usually with lipopolysaccharide) before it may be activated. By contrast, expression of NLRC4 relative to NLRP3 may inverse within hours of stimulation in some cells(95), which may have important implications for studies involving long periods of priming or infection. Priming is also sometimes necessary to induce expression of Pyrin(96), which Gavrilin and colleagues showed to be lost in macrophages upon differentiation; however, monocytes and PBMCs differentiated in the presence of additional growth factors restores Pyrin expression(97). Similarly, NLRP12 may have low expression in fully differentiated macrophages but is present in neutrophils(98). For these reasons, inflammasome studies in any cell line should be carefully scrutinized for appropriate expression of relevant components. However, some inflammasome components may not even be properly expressed in certain mouse strains. A known example is NLRP1b, as several common strains including C57Bl/6 lack a functional protein, and this correlates with lack of sensitivity of cells towards anthrax lethal toxin (99). Expression of cofactors involved in regulation of various inflammasomes may also differ significantly. Therefore, caution is warranted before drawing conclusions following negative results in inflammasome studies, both *in vitro* and *in vivo*.

In summary, the remarkable variety in these pathways raises questions about what defines an inflammasome, and should caution against generalizations about their mechanisms. New developments have uncovered additional members of several pathways. Considerable effort (for example, by utilizing CRISPR/Cas9 technology) is being spent on expanding the numbers of players, and will undoubtedly help with further characterization of signaling events.

Secretion systems of bacterial pathogens

The ability to export molecules to manipulate the host environment is an essential ability of bacterial pathogens. Some of the most virulent Gram-negative bacteria have evolved type 3, 4 and 6 secretion systems capable of penetrating host cells and injecting effector proteins to alter normal cellular processes in ways that benefit the pathogen. Thus, these secretion systems are typically essential virulence factors. Examples of such bacteria - *Salmonella, Shigella, Francisella, Legionella, Burkholderia, Pseudomonas, Yersinia*, and others - infect millions of patients worldwide, with a large number of deaths. In addition to the health and economic burden owed to these pathogens, some are candidates for bioterror and biowarfare.

There has been significant progress in understanding the structural and mechanistic aspects of bacterial secretion systems(100). Yet the complex roles they play in the host-pathogen interaction, particularly as they pertain to immune responses, are only beginning to be recognized and appreciated. Considering the breadth of pathways involved in inflammasome regulation described earlier, it should be reasonably expected that molecules delivered by bacterial secretion systems with the design to manipulate host cell homeostasis would in one way or another influence inflammasome pathways. The functions of many of these molecules remain unknown or incompletely characterized, keeping this field rich with questions and opportunity for inquiry.

Of the six secretion systems known in bacteria, the type III secretion system, (T3SS), type IV secretion system (T4SS), and type VI secretion system (T6SS) are associated with the most virulent human pathogens. Examples of pathogens with a T4SS are *Legionella* and *Burkholderia* bacteria, as well as *Helicobacter pylori* (not discussed in this review). The T6SS was discovered relatively recently, and is present in *Vibrio*, *Pseudomonas*, *Burkholderia*, and *Francisella* species.

Of these secretion systems, the T3SS is the best studied and common to some of the most important and harmful bacterial pathogens (*Yersinia, Salmonella, Shigella, Burkholderia, Pseudomonas*, and others). The delivery apparatus of the T3SS has remained well conserved across species, and consists of the basal body, the needle, and a pore-forming complex at the tip. This structure is critically important for virulence; however, some key components cannot be easily altered without significantly compromising the ability to deliver effectors(101). Perhaps for this reason the secretion systems of several pathogenic species have become recognizable immune targets, or pathogen-associated molecular patterns (PAMPs) - molecules which are pathognomonic with bacterial infection for host immunity. Cytokine responses to the T3SS tend to be quite robust and may involve the activation of toll-like receptors and inflammasomes(45,102). Likewise, T3SS “injectisome” proteins, particularly those

involved in attachment and penetration of the host cell, have a disproportionate number of immune epitopes compared to other bacterial proteins (IEDB.org); several of these are established protective antigens that confer adaptive immunity against the pathogen(103). Consequently, there is constant evolutionary pressure on T3SS pathogens to limit or manipulate the host response to its T3SS, and likewise there is pressure on the host to develop sophisticated methods of immune recognition with minimal immunotoxic harm to self.

Interactions of specific bacterial secretion systems with inflammasomes

Salmonella

Species of the Gram-negative *Salmonella* genus are the leading source of acute gastroenteritis worldwide, resulting from foodborne poisoning through consumption of contaminated poultry, pork, eggs, and milk. In total, *Salmonella* causes estimated 1.3 billion cases of human disease each year and as many as 800,000 deaths (104-106). *Salmonella enterica* serovar Typhi, spread through contaminated water, causes up to 20 million cases and 220,000 deaths per year globally(107). The combined 15.2 million disability-adjusted life years (DALYs) lost per year due to typhoidal and non-typhoidal *Salmonella*(108) make it the second greatest bacterial contributor to global disease burden after tuberculosis.

S. enterica serovar Typhimurium, one of the most common serovars causing nontyphoidal salmonellosis, is a facultative intracellular bacterium able to survive and reproduce both inside and outside of host cells. This adaptability requires a large number of genes which are distributed throughout the *Salmonella* genome, distinguishing it from many other pathogens whose virulence genes are typically more compartmentalized(109). Horizontal transfer of pathogenicity islands (so called because of their absence in nonpathogenic serovars) gives some *S. enterica* serovars the ability to survive inside host cells and effectively evade the immune system.

The two major virulence determinants of *S. enterica,* such as serovars Typhimurium and Typhi, are the pathogenicity islands SPI-1 and SPI-2. These gene clusters encode two type III secretion systems (T3SS) capable of forming needle-like structures on the surface of the bacteria through which more than thirty specialized effector proteins can be injected directly into host cells(110,111).

SPI-1 is a 40-kb region which encodes two distinct regulatory proteins, InvF and HilA, in addition to a T3SS termed Inv/Spa and a cluster of effector proteins. This secretion system was shown to be necessary for bacterial contact with host cells, and effectors secreted through this system trigger host cell pathways to internalize the bacteria(112).

The second pathogenicity island, SPI-2, encodes a two-component regulatory system as well as another distinct T3SS (Spi/Ssa) which is a major virulence factor found in all subspecies of *S. enterica*(113,114). The Spi/Ssa T3SS of the SPI-2 pathogenicity island differs in structure and function from the Inv/Spa T3SS of the SPI-1, and while Inv/Spa mediates uptake of the bacterium, Spi/Ssa enables the survival and replication inside the host cell(113,114).

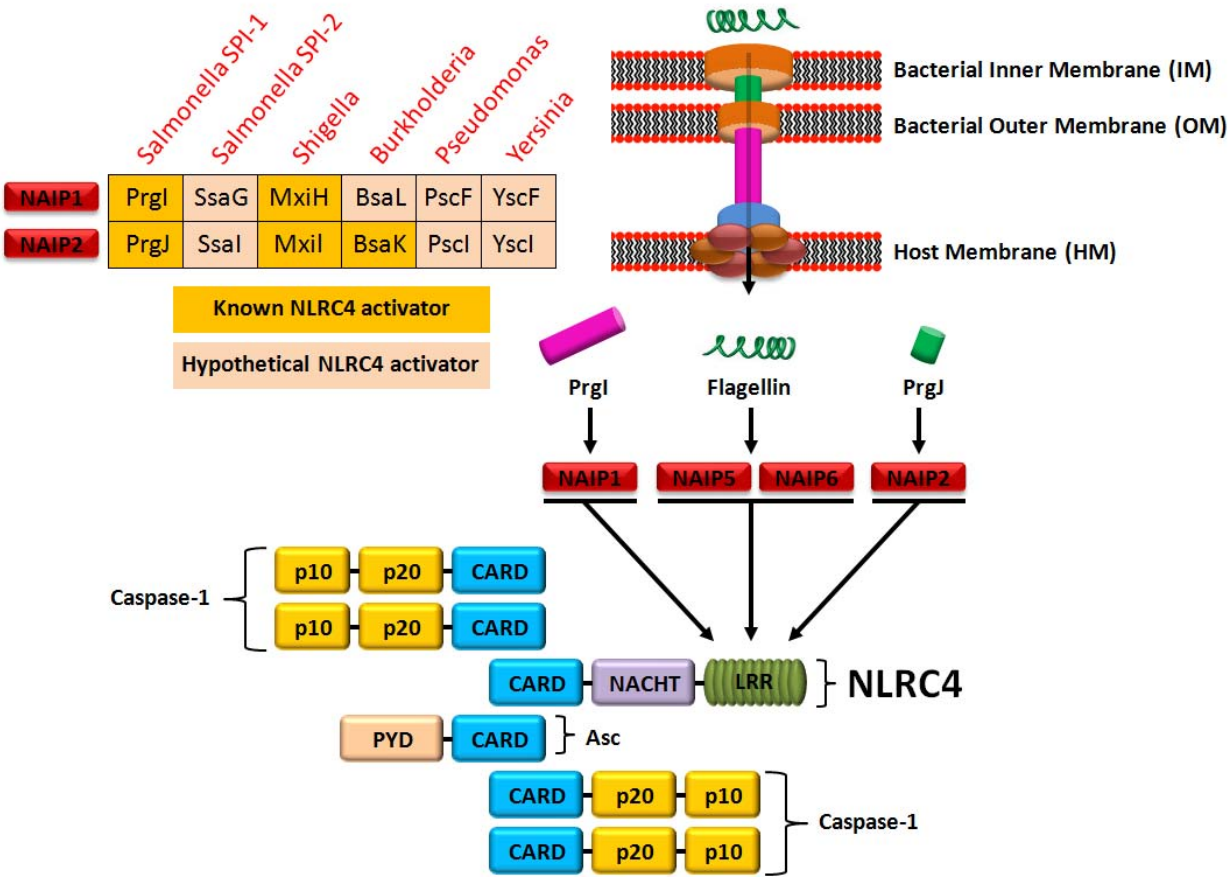

Figure 1. NLRC4 inflammasome structure and common recognition patterns of flagellin and T3SS molecules. Known activators include *Salmonella*, *Shigella*, *Burkholderia*, *Pseudomonas*, and *Yersinia*. In the upper left are shown homologs of PrgI and PrgJ which are known or predicted to activate NLRC4 via NAIP1 or NAIP2, respectively.

During enteric infection *S. enterica* invades the intestinal mucosa, followed by phagocytic uptake or entry into non-phagocytic enterocytes(105). The bacteria restricts the expression of SPI-1 and SPI-2 until it encounters the appropriate host environment, at which point it expresses the T3SS genes required for further survival and propagation(115,116). Culturing *S. Typhimurium* at different conditions can mimic different host environments. For example, bacteria grown to log-phase will increase expression of SPI-1(117), but at stationary phase expression of SPI-1 decreases while expression of SPI-2 will increase(118). Thus, the bacterium seems to sense whether it is in an extracellular or intracellular environment, and alter the expression of its virulence factors for optimal adaptation.

The host immune system is able to sense and react to these bacterial factors. *S. Typhimurium* expressing SPI-1 and the Inv/Spa T3SS induces rapid macrophage cell death and IL-1β production, which is dependent on NLRC4 as well as the NLR apoptosis inhibitory proteins (NAIPs)(45,119). As mentioned previously, NAIPs interact with NLRC4 upon sensing PAMPs and DAMPs, and trigger activation of the NLRC4 inflammasome. Mice express four NAIP paralogs (NAIP 1, 2, 5 and 6) of which NAIP5 and NAIP6 detect bacterial flagellin, NAIP2 detects the Inv/Spa T3SS inner rod protein PrgJ(45,119), and NAIP1 and its human homolog NAIP detect the Inv/Spa T3SS needle protein PrgI(102,120-122). Interestingly, there is only one known human NAIP protein, and this molecule may be responsible for detecting both PrgI and flagellin(123).

As mentioned above, *S. Typhimurium* grown to stationary phase (mimicking an intracellular niche) will upregulate SPI-2 while downregulating SPI-1. Macrophages infected with these bacteria will

undergo a much slower cell death (12-17 hours compared to 1-2 hours for log-phase bacteria), which is not dependent on the Inv/Spa T3SS. Instead, this cell death is triggered by the Spi/Ssa T3SS and occurs predominantly through NLRP3 and the noncanonical caspase-11 inflammasome, and to a lesser extent through NLRC4(124). The Spi/Ssa T3SS is used by the bacteria to inject effector proteins into the cell cytoplasm, but it also allows translocation of flagellin protein which triggers the NLRC4 inflammasome. Using a Δfla mutant which does not produce flagellin, Broz et al showed that *S. typhimurium* initiates two host inflammasome pathways, with the presence of flagellin and SPI-2 respectively triggering NLRC4 and caspase-11, likely with the SPI-2 needle mediating transfer of flagellin and LPS (124).

Caspase-11 is capable of detecting intracellular LPS through an TRIF/interferon assisted pathway(64) and activate what has been termed a non-canonical inflammasome, leading to release of IL-1β and IL18 and initiation of pyroptosis(7,73,125). It was proposed that caspase-11 binds directly to LPS(126) leading to cleavage of gasdermin D and initiation of cell death(58,127). The Spi/Ssa T3SS encoded by SPI-2 may introduce bacterial LPS into the host cytoplasm, activating caspase-11. The fact that some caspase-11 dependent IL-1β production still occurs in the absence of SPI-2 suggests that LPS may be delivered into the cytosol through an alternative mechanism, perhaps through the function of guanylate binding proteins (GBPs).

S. Typhimurium has several mechanisms to avoid immune detection and maintain an intracellular growth niche. The bacteria shifts from SPI-1 to SPI-2 expression, and also down-regulates flagellin expression in order to minimize activation of NLRC4. SPI-2 drives the expression of the Spi/Ssa T3SS, which the bacterium uses to secrete effector proteins that help it persist in its vacuolar niche. One secreted protein, SifA, induces stabilization of the vacuole by microtubules, and is critically important for virulence(71,128). Yet the Spi/Ssa T3SS, which is required by the bacteria for virulence, also activates inflammasome pathways by the mechanisms described above and results in the eventual clearance of the pathogen.

Yersinia

Yersinia pestis is the etiologic agent of some of the deadliest pandemics in human history, with total deaths in the hundreds of millions. *Y. pestis* continues to cause disease worldwide, particularly impacting the African sub-continent(129). However, *Y. pestis* is endemic in rodents in the Western North America, and sporadic cases of infection and death in the United States.

Human-pathogenic *Yersiniae* share a pCD1/pYV plasmid-encoded conserved T3SS with largely similar needle structure and injected effector proteins (Yops). These Yops play an important role in suppressing host immune functions and promoting bacterial survival. Avoiding immune surveillance is particularly important to the biological strategy of the *Y. pestis*, the etiologic agent of plague; immune evasion enables this pathogen to cause systemic disease with high mortality. Lack of a functional T3SS renders *Y. pestis* and the related enteropathogens *Y. pseudotuberculosis* and *Y. enterocolitica* essentially avirulent(130-135), although some aspects of disease may be observed with *Y. pseudotuberculosis* lacking T3SS(136).

In the case of *Yersinia*, a robust early immune response orchestrated by Interleukin-1β (IL-1β) and IL-18 favors host survival(76,137). The expression of these cytokines is effectively suppressed by injected Yops, despite evidence that *Yersinia* can activate the NLRP3, NLRC4, and NLRP12 inflammasomes(76,138), as well as a non-canonical caspase-8 pathway(91,92). How specific *Yersinia* molecules activate and inhibit these pathways is not fully understood.

In the case of NLRP3 and NLRC4, activation depends on the presence of the functional T3SS apparatus(90,138,139), and may be triggered by parts of the injectisome structure itself, such as YscI(140) or YscF(141). Other possible mechanisms for NLRP3 activation include hyper-translocation of the YopB/D translocon into the cell cytoplasm(139), and the destabilizing effects of a large pore in cell membrane or endosomes(142). It is also possible that in the presence of a functional needle, other molecules (e.g. LPS) pass from the bacterium into the host cytoplasm and activate inflammasome pathways. While the details of the mechanism(s) are not yet clear, inflammasome activation by the

injectisome is effectively blocked by YopK(138). Brodsky, Marketon and colleagues propose that this effector operates at the host side site of the translocon and regulates the delivery of other Yops(139,143). It is not clear if YopK may prevent the unintended entry of bacterial components other than Yops into the host cell. Potentially, YopK could also conceal inflammasome-activating components of a hyper-translocated injectisome, or stabilize the pore to prevent membrane-damage associated inflammasome activation.

There is also some evidence indicating that YopE, an effector with GTPase activating protein (GAP) activity which inhibits RhoA/G, Rac1, and Cdc42, can also inhibit inflammasome activation by stabilizing the injectisome pore (142,144). An early report by Schotte and colleagues suggested that the Rho-GTPase inhibitor YopE may modulate caspase-1 activation in a manner dependent on Rac1(4). Although this finding has not received much followup in the inflammasome field, it points to an understudied role of cytoskeletal guanine nucleotide exchange factors (GEFs) and GAPs in inflammasome regulation. Many pathogens target host GTPases to inhibit motility and phagocytosis, and there is compelling recent evidence that these pathways can play important roles in regulating inflammasomes(7,42,145). Examples of other bacterial effectors with GAP functions include *Salmonella* SptP and *Pseudomonas* ExoS/ExoT(146). *Yersinia* YopT has protease activity towards Rho GTPases and can thus also inactivate this pathway(147). In the case of YopE, it is also possible that its Rho-GTPase inhibiting function induces activation of one inflammasome pathway, such as Pyrin (42,148), but inhibits another (perhaps, NLRP3) (4,142,144,149); however, this perceived inhibition could also be influenced by YopE regulation of T3SS effector secretion. Our own studies (Ratner et al, submitted) strongly suggest that YopE activates a Pyrin inflammasome pathway.

The *Yersinia* effector YopM also limits caspase-1 mediated IL-1β/IL-18 production through another incompletely understood mechanism. YopM was originally proposed to directly bind and inhibit caspase-1(150), yet subsequent results could suggest an alternative indirect ability of YopM to inhibit caspase-1, dependent on the presence of the cytoskeletal scaffolding protein Iqgap1(151). Furthermore, bindings partners of YopM include kinases Prk1/2 (also called PKN1/2) and Rsk1/2(152,153); interaction with the latter (also known as S6 ribosomal kinase) with the C-terminus of YopM has also been suggested to be important for caspase-1 inhibition and promotion of virulence by this effector(151,153). Recently, Pkn1/2 have been implicated in Pyrin inflammasome regulation(43). YopM is a homolog of E3-ubiquitin ligases IpaH (*Shigella*) and SspH1 (*Salmonella*), which have no known roles in caspase-1 regulation. Yet YopM does control IL-1β and IL-18 production in vivo, and contributes to virulence in a manner dependent on IL-1β, IL-18, and caspase-1(90). Our recent results (Ratner et al, submitted) suggest that YopM binds a complex of RSK1, PKN1 and Pyrin, and blocks YopE-induced Pyrin inflammasome activation and not needle/rod induced NLRP3/NLRC4 activation.

Another effector, YopJ, robustly suppresses IL-1β and IL-18 precursors as well as other NF-kB dependent cytokines(90,154,155), but also triggers caspase-8 dependent activation of caspase-1, IL-1β and IL-18 at low levels(91,92,156). Caspase-8 is important in host defense against *Y. pestis*(92,137), but it is not clear whether this is due to its role in processing IL-1β/IL-18, its pro-apoptotic activity, or its role in regulating other NF-kB dependent cytokines. *In vitro* studies indicate that YopJ is an acetyltransferase targeting IKKβ(157), MAP Kinase Kinases(158,159), and the MAP3K, TAK1(160,161). YopJ has also been reported to behave as a deubiquitinase(162,163). Interestingly, the catalytic activity of YopJ positively correlates with its ability to induce caspase-8 dependent cytotoxicity and IL-1β secretion, and may affect virulence(164,165). Indeed, some studies in non-microbial systems indicate that inhibition or lack of IKK β /MAPK leads to a paradoxical increase in IL-1β secretion and caspase-8 activation despite an expected anti-inflammatory effect(166-168). One possibility is that the non-canonical caspase-8 pathway could be part of a host trapdoor mechanism for IL-1β/IL-18 production, designed to be triggered when effectors participating in disease processes such as YopJ attempt to suppress the critical NF-kB/MAPK pathways after surface receptor activation.

Burkholderia

Burkholderia species are closely related to *Pseudomonas*, and include several opportunistic pathogens which can cause serious disease in humans. *B. pseudomallei* causes the highly lethal disease

melioidosis, and has even been considered as a candidate for biowarfare. Many species of *Burkholderia* are considered harmless; however, cystic fibrosis (CF) patients are uniquely susceptible to chronic lung infection with *Burkholderia* species, including ones which normally do not cause disease in humans.

Burkholderia pathogens are able to survive inside macrophages, and infection is typically eventually resolved by adaptive immunity. Nevertheless, in the early stages of infection, the *Burkholderia* secretion systems interact with several inflammasomes with important consequences for the course of disease. Some polymorphisms of NLRC4, for example, significantly impact survival in melioidosis in humans(169).

In general, it is difficult to distinguish whether effectors or secretion systems themselves are responsible for activating an inflammasome, and it is even more challenging when multiple interacting secretion systems are present. *B. cenocepacia* has a T2SS, T3SS, T4SS, and T6SS, each of which may contribute to activation NLRP3 and possibly to a lesser extent NLRC4(170). The T6SS and T2SS cooperate in the delivery of metalloproteinases zmpA and zmpB, which are essential for intracellular survival and also partially contribute to NLRP3 activation. Yet there seem to be other NLRP3 activators which have yet to be identified, and may include structural components of the secretion systems or other translocated proteins.

By contrast, *B. pseudomallei* does not seem to trigger NLRP3 activation in macrophages, but instead the early inflammasome response appears entirely dependent on NLRC4(171). This inflammasome activity arrests replication of intracellular bacteria. The NLRC4 activation appears to be primarily driven by the flagellin protein FliC and the basal body protein BsaK - a homolog of the NLRC4-activating PrgJ protein in *Salmonella*. Later in infection, IL-1β secretion is driven by an NLRC4-independent pathway, and is curiously accompanied by caspase-1 independent cell death. Both NLRC4 and TLR5 are required for host survival and resolution of *B. pseudomallei* lung infection in vivo, but indeed there appears to be another unidentified inflammasome activated later during infection(169). Although some inflammasome activity is essential for the host response, production of IL-1β specifically leads to excessive neutrophil recruitment and elastase-mediated lung damage(172). Rather than resolution of infection, this results in increased host mortality and systemic invasion by the pathogen. Instead, it is IL-18 production by inflammasome activity which appears to assist survival and bacterial clearance.

B. cenocepacia, a particularly antibiotic-resistant bacterium that is often problematic when appearing in CF patients, has also been shown to activate the Pyrin inflammasome in human monocytic cells by Gavrilin, Wewers and colleagues(173), and this type of activation was recently also demonstrated in mice(42). The T6SS, but not the T3SS activates Pyrin and induces its recruitment to phagosomes. The *B. cenocepacia* T6SS is known to disrupt Rho-GTPases and cytoskeletal regulation(174-176), which are events that have been hypothesized to activate Pyrin(18,41,42,177,178). This ability may be associated with the effector TecA, which induces RhoA covalent modification (deamidation) in the GTPase switch-I region(179). Loss of Pyrin is associated with increased intracellular bacterial survival, but also reduced inflammation in the lungs of infected mice(42). This may be an important insight for CF lung infection with *Burkholderia* species, as evidence suggests higher mortality and ineffective clearance of the related *P. aeruginosa* pathogen associated with increased inflammasome responses.

Pseudomonas

Certain *Pseudomonas* species, particularly *Pseudomonas aeruginosa*, are important opportunistic and nosocomial pathogens. They are particularly dangerous for immunocompromised and severely ill patients, as well as individuals with cystic fibrosis (CF). *P. aeruginosa* is able to establish chronic lung infection in CF patients due to the uniquely permissive environment of the CF lung; the ensuing inflammation results in progressive lung damage and is currently the leading cause of death among CF patients. Given the high hazard that *P. aeruginosa* poses to the sizable population of individuals with CF worldwide, inflammasome responses to this pathogen in the context of the CF lung deserve special attention.

The relationship between *Pseudomonas* infection and inflammasomes is complex and controversial. Some studies indicate reduced bacterial clearance when inflammasome activation is

defective(180). Yet a majority of studies suggest that inflammasome activation is counterproductive to bacterial clearance, particularly in the lungs, and may even exacerbate tissue damage and mortality(95,181-185). In the study by Faure et al, it appears that inflammasome-driven IL-18 dampens IL-17 activity, which is critical for clearing *Pseudomonas* lung infection. It is possible that these discrepancies highlight a difference between corneal infection, where an IL-1β/IL-18 response is beneficial to the host, and lung infection, where the same type of response is inappropriate. A curious observation is that *P. aeruginosa* appears to exploit the regulation of host autophagy by IL-1β and caspase-1, with the net result that inflammasome activation promotes the survival of the pathogen(95,186,187).

Pseudomonas is primarily known to activate NLRC4 and NLRP3, although a caspase-1 independent pathway will also be discussed. The AIM2 inflammasome does not appear to be activated(188). The *Pseudomonas* RhsT protein has been suggested to be an inflammasome activator, however the authors do not elaborate on possible mechanisms(184). RhsT contains conserved sequence homology with the *Clostridium difficile* toxin B (TcdB), a RhoA modifier and inhibitor, and a known activator of the Pyrin inflammasome. Whether this protein indeed activates Pyrin or another inflammasome warrants investigation, as the RhsT family proteins are widespread and conserved among many pathogens, including several that have been shown to activate Pyrin.(42)

The NLRC4 inflammasome is activated by multiple *Pseudomonas* molecules, likely including the T3SS injectisome itself, both in vivo and in vitro(181,189). Potentially recognized injectisome components include PscI (a homologue of the NLRC4-activating *Salmonella* basal body protein PrgJ), and PscF (a homologue of *Yersinia* needle protein YscF). NLRC4 is also known to be activated by flagellin proteins of many bacterial species, and *Pseudomonas* flagellin follows this pattern as well(185). It is worth noting that NLRC4 activation correlates with bacterial motility, and some investigators have suggested that it may be flagellar motility, rather than the flagellin protein per se, which leads to phagocytosis and inflammasome activation(190,191). Yet this view is challenged by experiments showing that surfactant protein A directly binds recombinant flagellin as well as live *Pseudomonas in vitro* and *in vivo*, enhancing the phagocytosis and capacity of both to activate NLRC4(192). Findings by Anantharajah and colleagues also suggest that IL-1β release and pyroptosis are not correlated to flagellar motility(193). Thus, it is possible that decreased inflammasome activation by non-motile *Pseudomonas* is due to reduced contact with host cells; this is especially worth considering given that clinical isolates of *Pseudomonas* from chronically infected lungs are typically mucoid strains, which are resistant to contact with immune cells and phagocytosis.

The *P. aeruginosa* T3SS has been shown to activate both NLRP3 and, surprisingly, NLRC4 by inducing mitochondrial damage and DNA release(95,186). In the latter study, Jabir et al demonstrated mitochondrial DNA binding to NLRC4 downstream of *Pseudomonas* infection, uncovering aspects of the NLRC4 mechanism which may have been previously overlooked(187,194). *Pseudomonas*-triggered inflammasome activation induces autophagy, which seems to be associated with defective killing of the bacteria. Moreover, in an acidic microenvironment, as is typically the case in bacterial infection foci, *Pseudomonas* T3SS triggers enhances inflammasome activation immune cells(195). This may be significant because acidic conditions are known to favor autophagy(196-198), further assisting bacterial survival. Cumulatively this adds to a growing body of evidence that in most cases, inflammasome activation infection with *P. aeruginosa* is ineffective and histotoxic - particularly in the lung where excessive inflammatory damage is associated with worse clinical outcome.

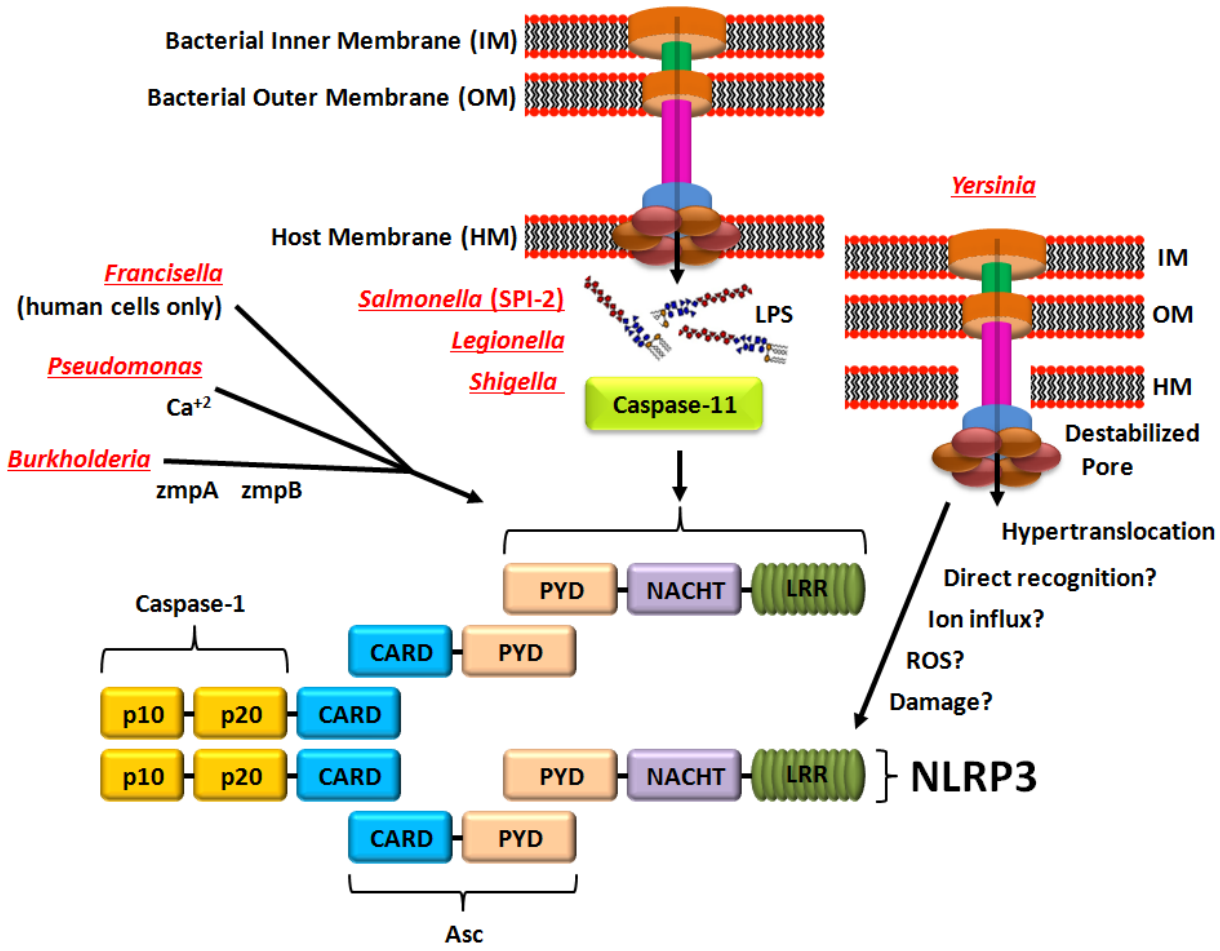

Figure 2. NLRP3 inflammasome structure and function, including the non-canonical caspase-11 pathway. Known activators are in red, and triggering molecules and processes are detailed.

These findings may help explain why CF patients are more vulnerable to *P. aeruginosa* lung infection. First, the pH of the CF lung is more acidic than in healthy individuals(199,200), which was shown to negatively impact bacterial killing by Pezzulo and colleagues(201). According to the studies cited earlier, this lower pH would be associated with even greater inflammasome activation and decreased bacterial clearance. Second, a recent elegant study by Rimessi and colleagues demonstrated that *Pseudomonas* activates NLRP3 and NLRC4 more strongly in CF cells due to intrinsically impaired calcium homeostasis(202). CFTR is a chloride ion channel, and its deficiency leads to abnormally high intracellular and mitochondrial calcium levels. *P. aeruginosa* infection triggers additional calcium entry via the mitochondrial calcium uniporter, resulting in greater mitochondrial damage, oxidative stress and subsequent NLRP3 activation in CF cells. Thus, the intrinsically aberrant calcium homeostasis and increased acidity exacerbate the inappropriate inflammasome activation in response to *P. aeruginosa*, and lead to exaggerated neutrophil influx with subsequent lung damage by neutrophil elastase despite perpetual failure to clear the bacteria. Indeed, inhibiting the Pannexin-1 (P2X7) channel with probenecid prior to *Pseudomonas* infection successfully prevents calcium influx-driven caspase-1 activation, and reduces the severity of infection *in vivo*(203,204).

Yet if caspase-1 inhibition in the lung is beneficial for the host and detrimental for *Pseudomonas*, then what is the significance of ExoU - a *Pseudomonas* T3SS toxin which is reported to be a potent caspase-1 inhibitor? According to Anantharajah and colleagues, IL-1β secretion is abrogated in the presence of ExoU, and pyroptosis is replaced with rapid cell death(193), which is not caspase-8

dependent(92) and is likely necrotic(205). Neither the mechanism of cell death nor caspase-1 inhibition are fully understood. ExoU is a phospholipase, which is unique among T3SS effectors(205), and it is apparently able to effectively suppress both NLRC4-dependent and independent caspase-1 processing. However, *Pseudomonas* strains which lack ExoU appear to have a competitive advantage over ExoU(+) strains(206). Over time, the clinical isolates recovered from chronic *Pseudomonas* lung infections tend to becomes ExoU(-), non-motile, and often completely lacking a T3SS.

In addition to NLRP3 and NLRC4, *Pseudomonas* also appears to trigger a non-canonical inflammasome pathway. This pathway appears to be influenced by pilin and requires a functional T3SS, but is not dependent on NLRP3, NLRC4, or Asc(207). Karmakar et al also identified a pathway of IL-1β production by neutrophils in response to *Pseudomonas* corneal infection which is independent of Asc and caspase-1, but dependent on the activity of neutrophil elastase and serine proteases(180). Here, IL-1β was found to be necessary for bacterial clearance from the cornea, in contrast to the detrimental effects of IL-1β in *Pseudomonas* lung infection. Others have also reported a neutrophil-driven pathway in response to *Pseudomonas* with similar non-canonical characteristics(182,208), with potential regulation by Pstpip2(208). If these studies indeed describe a single pathway, then the fact that it is independent of caspase-1 narrows the possibilities of enzymes known to directly cleave IL-1β to caspase-8, neutrophil elastase, proteinase 3, and cathepsin G. Although evidence to confidently exclude a role for caspases-8 and 11 is incomplete, currently it appears *Pseudomonas* does not strongly activate these pathways(7,64,92). Synthesizing all of these results may suggest a scenario where pilin is secreted by the *Pseudomonas* T3SS, and perhaps activates direct processing of IL-1β and IL-18 by neutrophil serine proteases independently of inflammasomes or caspase-1, although there may be other interpretations as well.

Francisella

The facultative intracellular bacterium *Francisella tularensis* is the causative agent of tularemia, an acute systemic disease typically presenting as pneumonia, with high mortality. It is a highly virulent pathogen which, like *Y. pestis*, is classified as a category A select agent with the potential to be used for bioterror and biowarfare. For non-select agent research, the model of choice is often *F. tularensis* subspecies *holarctica* (Live Vaccine Strain, LVS) or *F. novicida,* as it is virulent in mice but attenuated in humans(209-211).

F. novicida avoids degradation by phagocytes by escaping from the phagosome into the cytosol, where it is then free to replicate. However, upon escape from the phagolysosome, *F. novicida* triggers caspase-1 cleavage and IL-1β secretion(212). *Francisella* activates AIM2 in mice but, unlike the other pathogens discussed in this review, does not seem to activate either NLRC4 nor NLRP3(213,214). In human cells both NLRP3 and AIM2 are triggered(214). Activation of NLRP3 was recently corroborated by another study where human monocytes produced IL-1β in response to *Francisella* bacteria and ATP(215); this IL-1β secretion also required K+ influx, suggesting an NLRP3-dependent mechanism. Perhaps a *Francisella* effector is able to inhibit NLRP3 activation in mice but not in humans(216), or mice and humans have other potential differences in NLRP3 regulation. Another mouse-human difference concerns *Francisella* activation of the Pyrin inflammasome. Gavrilin and colleagues showed that in human monocyte-derived macrophages and THP-1 cells, *Francisella* triggers the Pyrin inflammasome(97). This contrasts with findings by Fernandes-Alnemri et al, who showed that mice lacking Pyrin still produce IL-1β in response to *Francisella*, while mice lacking AIM2 produce little to none. Significant differences in the protein sequences of mouse Pyrin versus human Pyrin may partly explain this discrepancy(217).

The mechanism by which *F. novicida* activates AIM2 is also unusual, occuring through an IRF-1 dependent pathway(218). Activation of the cytosolic DNA sensor cGAS and STING in response to cytosolic *F. novicida* leads to IRF-1 mediated transcription of GBPs; specifically, GBP 2 and 5 were found to lead to activation of AIM2, but not NLRP3, in a dsDNA dependent manner in mouse macrophages. These GBPs have been proposed to be involved in the lysis of the bacteria or the bacteria containing vacuole, thereby releasing bacterial DNA into the cytoplasm(69,218,219). As mentioned

earlier, the AIM2 inflammasome assembles upon directly binding dsDNA via the HIN domain(31,220-222). Yet the study by Man and colleagues shows that although AIM2 and cGAS can both bind dsDNA, cGAS activation is upstream of AIM2 and is necessary for inflammasome formation in response to *F. novicida*. Both AIM2 and IRF1 were required for restriction of *F. novicida* replication in vitro, and survival in vivo(218). Potentially, interferon signaling could be necessary to increase AIM2 expression, however other studies show that even small amounts of transfected dsDNA are enough to rapidly trigger activation of the AIM2 inflammasome(223). A specific trigger of this pathway is not known, although one possibility is that the *F. novicida* activator of STING is a secreted cyclic nucleotide, similar to *L. monocytogenes*(224).

Cytosolic LPS from the intracellular *F. novicida* would be expected to also trigger caspase-11 activation. However, *Francisella* produces tetra-acylated rather than hexa-acylated LPS, which loses its ability to bind and activate caspase-11(73). This is a similar strategy to that of *Y. pestis*, limiting activation of TLR4(137), and downstream expression of inflammasome factors such as NLRP3, pro-caspase-1, pro-IL-1β, and pro-IL-18.

The *Francisella* pathogenicity island (FPI) encodes 16-19 genes which express a Type VI secretion system (T6SS) which is required for virulence. The IglC T6SS protein induces phagosome rupture and allows *Francisella* to escape into the cytosol(210). This is in contrast to *S. typhimurium*, which secretes factors in order to stabilize the phagosome and avoid cytosolic entry. *Francisella* lacking functional IglC fail to escape the phagosome, and also fail to trigger the AIM2 inflammasome(218). This suggests that activation of AIM2 requires the presence of the bacteria in the cytosol, and bacterial secretion of effectors and other factors into the cytosol from inside the phagosome may not be sufficient to trigger inflammasome activity.

The function of IglC is still under investigation, and it may potentially be part of the T6SS apparatus itself(225); indeed, it appears to be a homolog of Hcp, which is thought to form the tube-like structure of the T6SS for delivery of effectors(226,227). In this case, an IglC mutant may fail to activate AIM2 simply because the activating molecule (presumably, DNA) is not translocated into the cytosol.

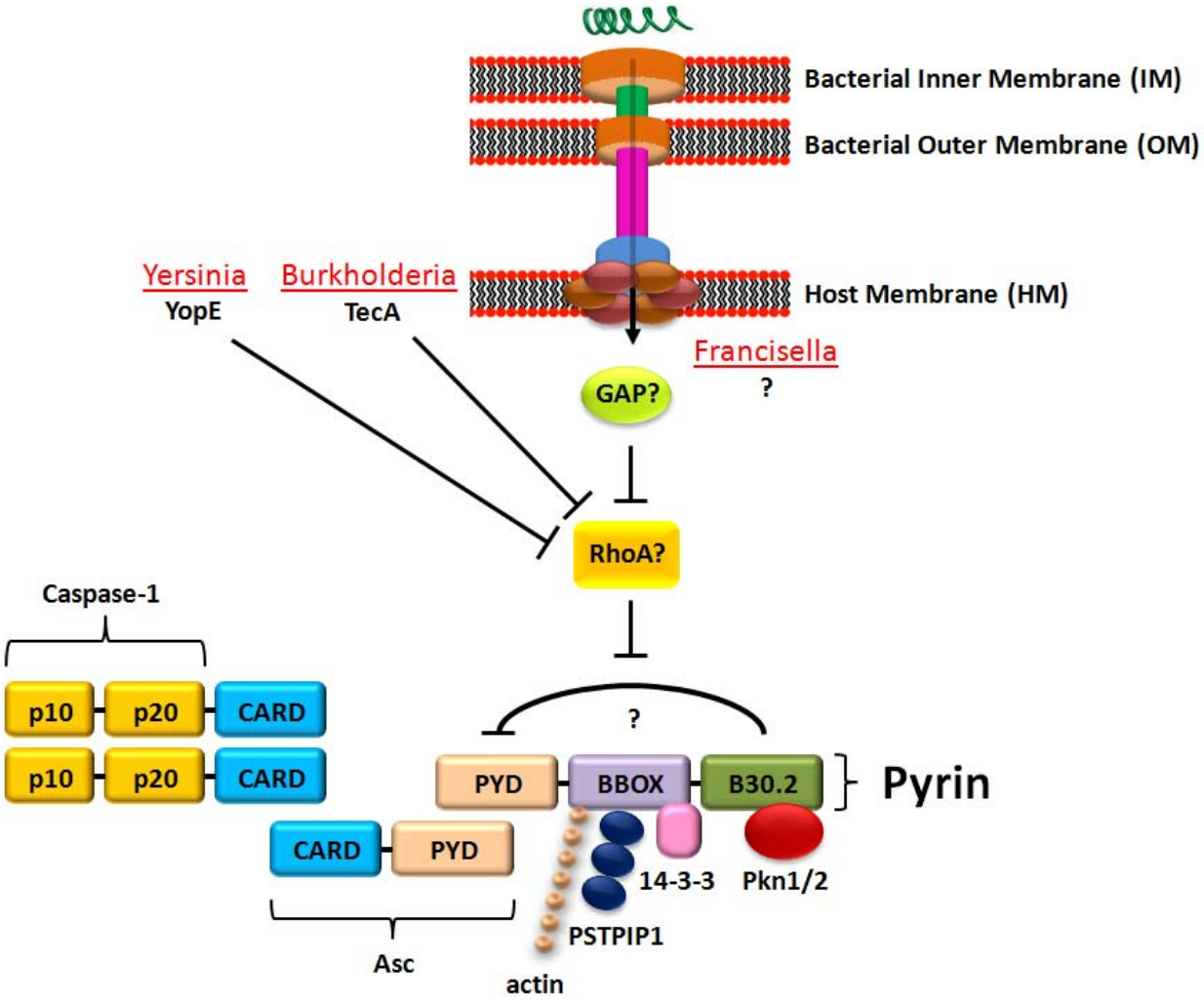

Figure 3. Pyrin inflammasome structure and function. *Francisella* and *Burkholderia* are known activators of Pyrin, the *Burkholderia* T6SS effector TecA inhibits RhoA and triggers Pyrin. *Yersinia* YopE is a proposed Pyrin activator based on its ability to inhibit RhoA via the YopE GAP activity.

Legionella

Legionella pneumophila is a Gram-negative intracellular pathogen responsible for the respiratory infection known as Legionnaire's disease. The ability of *Legionella* to survive inside macrophages and the way it interacts with inflammasomes is in many ways comparable to *Salmonella*. After phagocytosis, survival and replication by *L. pneumophila* requires inhibition of phagosome-lysosome fusion, so that the bacteria may persist in a protected vacuole. The requirement to stabilize this intracellular niche is evident from the fact that bacteria that are incapable of growing inside host cells are also incapable of causing disease in animals(228). However, upon sufficient replication the bacteria induces rupture of the vacuole followed by lysis of the infected cell(229). This releases the bacteria into the host environment, allowing it to infect more cells and for the infection to continue.

Legionella has a type IV secretion system (T4SS) encoded by a region of the genome called *icm* (intracellular multiplication). The T4SS translocates hundreds of effector proteins into the cytoplasm in order to stabilize the bacterial vacuole and establish a replicative niche(228-231); this high number of effectors distinguishes *Legionella* among pathogens with secretion systems. Most of the effectors are involved in manipulating host pathways to prevent fusion of the bacterial vacuole with lysosomes(232).

Some effectors such as SidF and SdhA prevent the host cells from undergoing apoptosis in order to limit inflammatory responses and immune detection(233,234).

Despite having a T4SS rather than a T3SS, *Legionella* activates inflammasomes by mechanisms similar to those of *Salmonella*. The *Legionella* T4SS transolcates LPS into the cytosol where it triggers activation of the non-canonical caspase-11 dependent NLRP3 inflammasome (235,236). Pyroptosis requires caspase-11, but not NLRP3 in cells infected with *Legionella*, which is consistent with the gasdermin D-dependent mechanism proposed by Kayagaki and colleagues(58). Interestingly, it has also been reported that activated caspase-11 induces fusion of the *L. pneumophila*-containing phagosome to the lysosome through actin remodeling(72).

Legionella flagellin translocated into the cytoplasm through its secretion system is detected by the NLRC4 inflammasome through the adaptor molecule Naip5(16,236-239). In other bacteria, the T3SS needle protein PrgJ and its homologs may also trigger NLRC4, however *Legionella* lacks a T3SS and flagellin may be the only NLRC4 activator in this pathogen. This NLRC4 pathway seems to be sufficient for controlling bacterial replication, based on evidence that deficiency of caspase-1 but not caspase-11 impairs bacterial clearance both in vitro and in vivo(240). However, lack of caspase-1 is functionally similar to a lack of caspase-11 and NLRC4, so it is not possible to evaluate the relative importance of the caspase-11 pathway using a capase-1 deficient model. Given the robust caspase-11 dependent activation of caspase-1 by *L. pneumophila* lacking flagellin(236), as well as its role in fusing the bacterial vacuole with lysosomes(72), it is conceivable that caspase-11 may be redundant with NLRC4 and sufficient for bacterial control on an NLRC4 $^{-/-}$ background. *Legionella* lacking the T4SS (dotA $^{-/-}$) does not show any inflammasome activation or cell death(236), most likely because these mutant bacteria fail to secrete LPS and flagellin along with essential effector proteins to stabilize the vacuole. This results in normal trafficking of the bacterium to the lysosome where it is efficiently neutralized(237).

Shigella

The Gram-negative *Shigella* is the causative agent of shigellosis, a foodborne illness prevalent in developing countries. *Shigella* results in severe gastrointestinal disease in humans, but does not seem to cause significant disease in other animals. It invades the colonic and rectal mucosa leading to leukocyte recruitment, severe inflammation, and often bloody diarrhea (dysentery) which leads to further spread of infection in poorly sanitized regions. Like several other pathogens discussed in this review, the ability of *Shigella* to survive intracellularly is a central part of its infection strategy.

Shigella is closely related to *Salmonella*, but one major distinction is that *Shigella* may lackflagella. Like *S. typhimurium*, pathogenic *Shigella* species, like *S. flexneri*, *S. sonnei*, *S. dysenteriae* and *S. boydii*, are capable of entering gut epithelial cells as well as macrophages, and trigger rapid cell death. *Shigella* also uses a T3SS to secrete effectors which induce vacuole rupture and release the bacteria into the cytosol. Although *Shigella* lacks flagellin it still readily triggers the NLRC4 inflammasome through the same T3SS it uses to escape this vacuole. NLRC4 is activated by Naip2, which detects the inner rod protein MxiI(241), and Naip1, which recognizes the needle component MxiH(102,122,242).

Release of *Shigella* into the cytoplasm of the host cell also triggers IFN dependent caspase-11 activation(64), with downstream activation of caspase-1 through the non-canonical inflammasome, secretion of IL-1β and IL-18, and pyroptosis via gasdermin D(58). It was also reported that caspase-4, the human homolog of caspase-11, is involved in host resistance to *Shigella*(243); however, *Shigella* secretes the effector protein OspC3 which inhibits caspase-4 activation. It is interesting that OspC3 is highly specific to caspase-4 and does not inhibit caspase-11, suggesting the preference *Shigella* has for infecting humans

The *Shigella* T3SS also appears to induce autophagy(244), which is known to suppress inflammasome activation; inhibition of autophagy promoted cell death in infected macrophages, which is again suggestive of pyroptosis. In the absence of caspase-1 or NLRC4 autophagy was dramatically enhanced, which is consistent with reports that caspase-1 negatively regulates autophagy(187).

Similar to *Yersinia*, *Shigella* modifies its LPS in order to evade immune detection(245). Paciello and colleagues show that during intracellular replication, *Shigella* predominantly expresses tri- and tetra-

acylated LPS with fewer acyl chains in lipid A than when it is cultured in growth media. This hypoacylated LPS is much less potent in activating TRL4, resulting in limited expression of pro-caspase-1, pro-IL-1β, and pro-IL-18. However, the authors also suggest that in late infection, when *Shigella* is obligated to proliferate extracellularly due to decreasing access to live local cells to infect, the bacteria reverts to production of immunopotent hexa-acylated LPS. This allows leukocytes to respond to the pathogen more effectively and eventually clear it from the body.

Escherichia

Several *Escherichia coli* types affecting human health, such as Entero-Pathogenic *E. coli* (EPEC) and Entero-Hemorrhagic *E. coli* (EHEC) harbor a T3SS and trigger IL-1β release, however, it is possible that the mechanisms behind the IL-1β production in some settings may differ from other bacteria. One study indicated that NLRP3-dependent EHEC-induced IL-1β was independent of the T3SS but dependent upon formation of RNA:DNA hybrids(246), and another report suggested that inflammasome activation via NLRP3 was triggered by viable *E. coli* and their mRNA(247). However, *E. coli* also is able to trigger T3SS-dependent effects, such as activating NAIPs/NLRC4 via T3SS needle/rod components EprJ, EscI and EprI(45,248), and the effector NleA may inhibit NLRP3 stimulation(249). Other human pathogens, like *Vibrio* and *Chlamydia* also harbor secretion systems with potential of modulating inflammasome activities.

Conclusion

Bacterial secretion systems interact with inflammasome pathways in many different fashions, both with activating and inhibitory functions. Responses can be initiated by translocon/pore formation, directed by secreted effector proteins or by components such as flagellin or LPS channeled through the needle. Each pathogen has its distinct way of interacting with the host innate immune system, and can harbor inhibitory proteins suppressing inflammasome activation, but the host may have evolved mechanisms to sense these key virulence factors. However, it is often a battle between blocking and activating forces with regard to net effect on innate immunity, and there is likely a delicate balance that will decide if and how the pathogen may cause disease. This is a fascinating field, more progress is likely to increase the perceived complexity of these mechanisms, but may also to help in the design of new therapeutics for inflammatory diseases.

Acknowledgments

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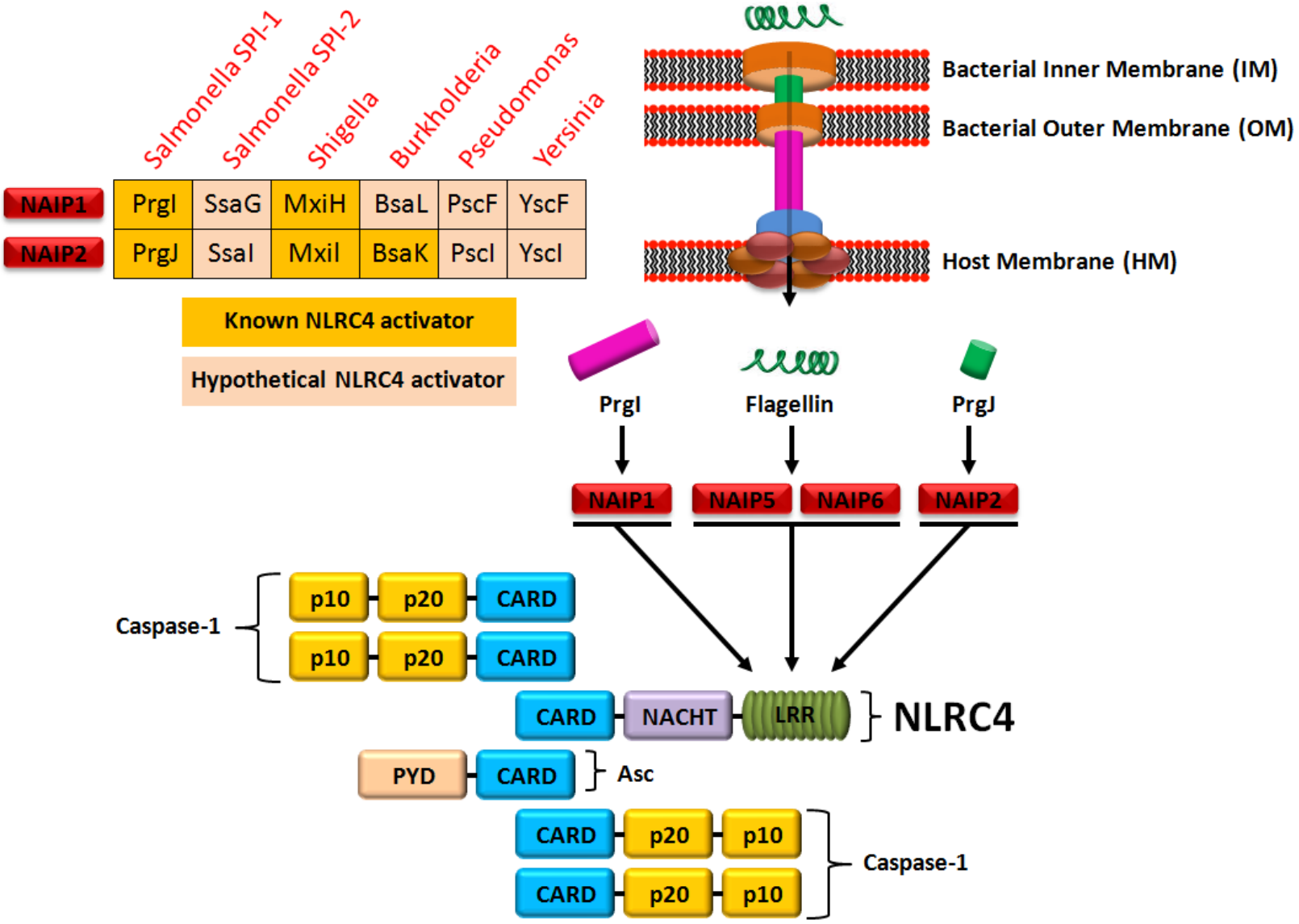
Salmonella SPI-1
Salmonella SPI-2
Shigella
Burkholderia
Pseudomonas
Yersinia
NAIP1
PrgI
SsaG
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PscF
YscF
NAIP2
PrgJ
SsaI
MxiI
BsaK
PscI
YscI
Known NLRC4 activator
Hypothetical NLRC4 activator
Bacterial Inner Membrane (IM)
Bacterial Outer Membrane (OM)
Host Membrane (HM)
PrgI
Flagellin
PrgJ
NAIP1
NAIP5
NAIP6
NAIP2
Caspase-1
p10
p20
CARD
p10
p20
CARD
CARD
NACHT
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NLRC4
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CARD
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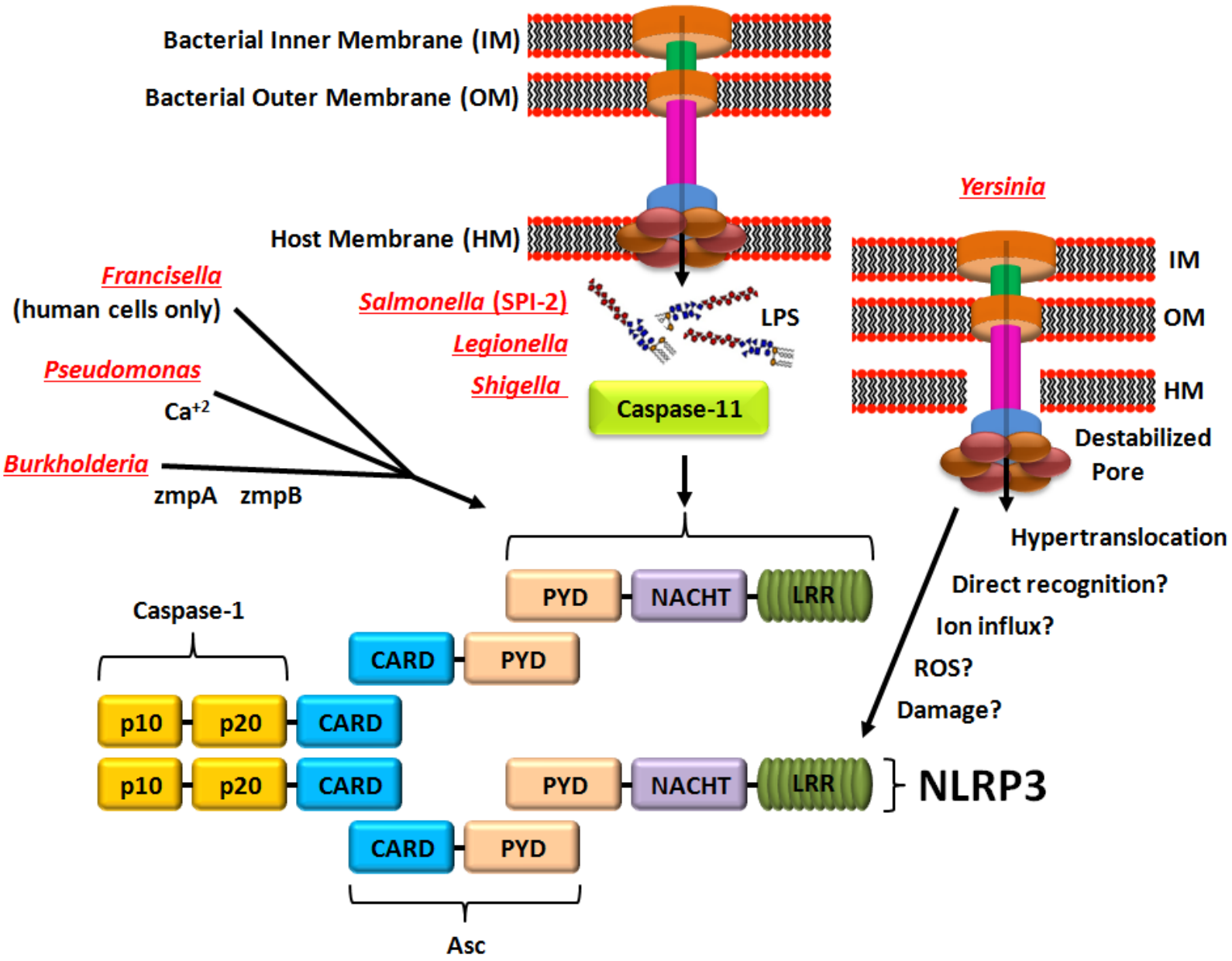
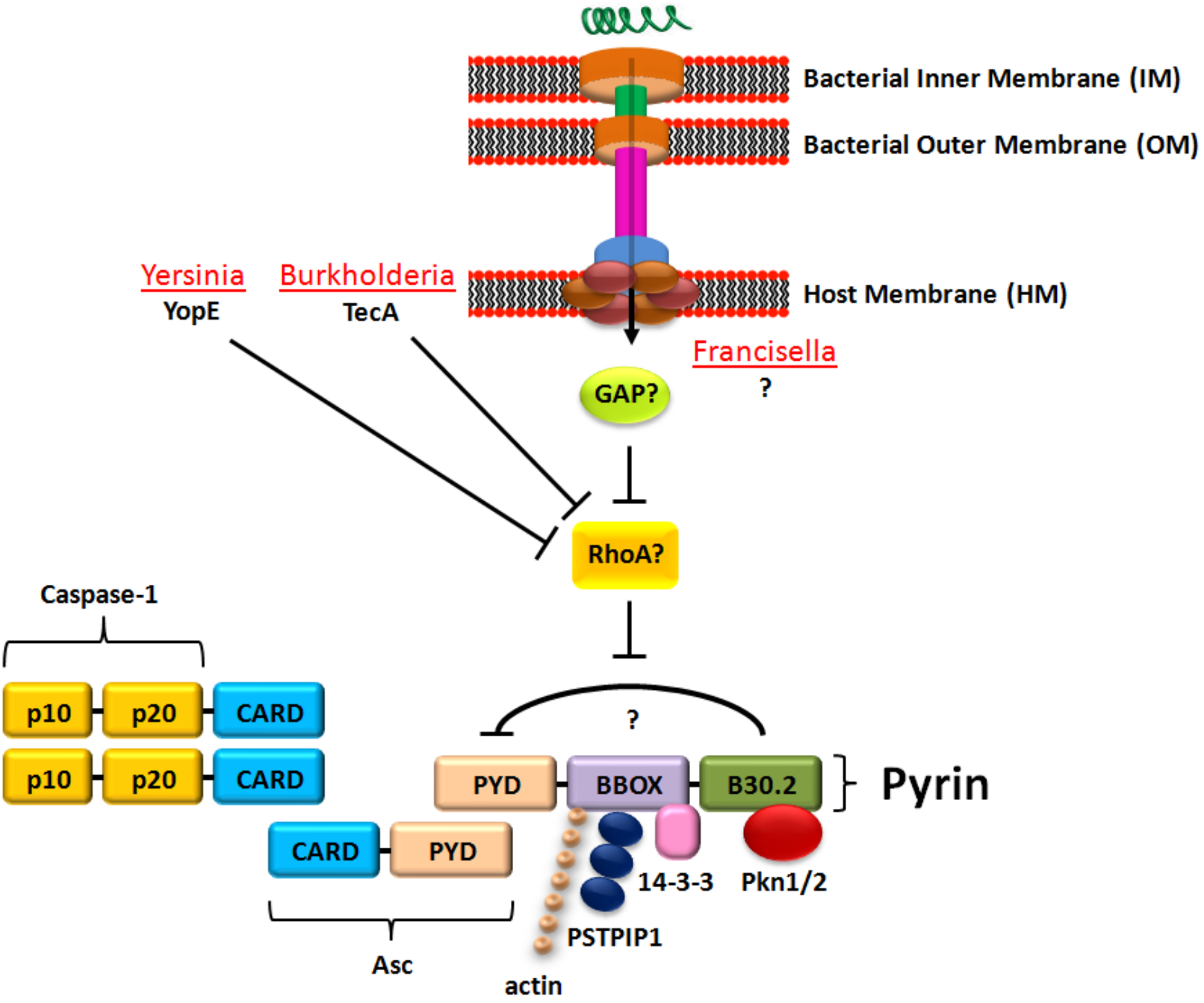
Caspase-1


Bacterial Inner Membrane (IM)
Bacterial Outer Membrane (OM)
Host Membrane (HM)
Francisella
(human cells only)
Pseudomonas
Ca+2
Burkholderia
zmpA zmpB
Salmonella (SPI-2)
Legionella
Shigella
LPS
Caspase-11
Yersinia
IM
OM
HM
Destabilized
Pore
Hypertranslocation
Direct recognition?
Ion influx?
ROS?
Damage?
Caspase-1
p10
p20
CARD
PYD
NACHT
LRR
NLRP3
Asc


Bacterial Inner Membrane (IM)
Bacterial Outer Membrane (OM)
Host Membrane (HM)
Yersinia
YopE
Burkholderia
TecA
Francisella
?
GAP?
RhoA?
Caspase-1
p10
p20
CARD
p10
p20
CARD
?
PYD
BBOX
B30.2
Pyrin
CARD
PYD
14-3-3
Pkn1/2
PSTPIP1
Asc
actin