Patterns of Pathogenesis: Discordn of Pathogenic and Nonpathogenic microbes by the Innate Immune System

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The dominant conceptual framework for understanding innate immunity has been that host cells respond to evolutionarily conserved molecular features of pathogens called pathogen-associated molecular patterns (PAMPs). Here, we propose that PAMPs should be understood in the context of how they are naturally presented by pathogens. This can be experimentally challenging, since pathogens, almost by definition, bypass host defense. Nevertheless, in this review, we explore the idea that the immune system responds to PAMPs in the context of additional signals that derive from common “patterns of pathogenesis” employed by pathogens to infect, multiply within, and spread among their hosts.

Introduction
The mammalian innate immune system provides an early and important response to microbial attack. Over the past decade, compelling evidence has accumulated that the response to invading microorganisms is stimulated by recognition of several distinct microbial structures by germline-encoded host receptors of the innate immune system (Beutler et al., 2006; Kawai and Akira, 2008; Medzhitov, 2007). In this review, we consider evidence that the response to pathogens is not limited to recognition of these structures. We suggest that, in addition, hosts may recognize distinct pathogen-induced processes that contribute to the progression of disease. The reason for thinking along these lines is that recognition of pathogen-induced events would provide the host with strategies for distinguishing a virulent organism from one that has lower disease-causing potential. The host could then escalate immune responses to a level commensurate with the attack being mounted.

As proposed by Janeway (Janeway, 1989), we will refer to the distinct microbial structures recognized by the host as pathogen-associated molecular patterns (PAMPs) and the host receptors that recognize them as pattern-recognition receptors (PRRs), despite some widely recognized problems with this nomenclature (see below). The details of the PRRs and their cognate PAMPs have been extensively reviewed elsewhere (Beutler et al., 2006; Kawai and Akira, 2008; Medzhitov, 2007).

Examples of PAMPs of particular relevance to bacterial pathogens include lipid A, an essential constituent of the lipopolysaccharide in the Gram-negative outer membrane, which is sensed by Toll-like receptor 4 (TLR4); bacterial flagellin, a protein subunit that polymerizes to form the flagellum, which is a ligand for TLR5; bacterial lipoproteins, from Gram-positive and Gram-negative bacteria, which are recognized by TLR2; bacterial DNA containing particular Cpg motifs that stimulate TLR9; and fragments of bacterial PGN that are sensed in the host-cell cytosol by the NOD1 and NOD2 receptors.

In addition to recognition of PAMPs, it has been suggested that the immune system responds to other signals commonly associated with infection. In particular, it has been proposed that cells dying of a “messy” necrotic death, as opposed to a programmed apoptotic death, may release molecules such as DNA, ATP, uric acid, and DNA binding proteins (HMGB1) into the extracellular milieu (Kono and Rock, 2008; Matzinger, 1994). These molecules have been variously termed DAMPs (damage-associated molecular patterns), alarmins, or endogenous adjuvants, and there is evidence that these host-derived molecules can stimulate immune responses, perhaps explaining the sterile inflammation in models such as ischemia-reperfusion injury (Kono and Rock, 2008). There is also evidence that DNA damage stimulates innate immune responses mediated by the NKG2D receptor (Gasser et al., 2005). What remains unclear, however, is whether damage-induced responses play a significant role in the host response to pathogens. One major issue is that much of the damage that occurs in an infection may be due to the host response rather than the pathogen. This self-inflicted damage may amplify immune responses, but cannot be used to explain the initiation of immune responses to pathogens, which is our primary concern here. In fact, many pathogens appear to go to considerable lengths to avoid host damage. Another unresolved question with damage-based models is whether or how hosts can distinguish sterile damage from pathogen-induced damage in order to initiate appropriate healing or inflammatory responses to each (Barton, 2008).

The plant immunity literature has long discussed a model conceptually related to the damage model, termed the “guard hypothesis” (Chisholm et al., 2006; Jones and Dangl, 2006), which proposes that specific kinds of cellular disruption—for example, of certain host signaling pathways—may trigger host
defense responses. In contrast to the damage-based models, in which generic cellular injury initiates immune responses, the Guard Hypothesis focuses immune responsiveness on the specific disruptions caused by pathogens. It remains unclear whether mammalian cells utilize a similar “guard” strategy. In this review, we attempt to extend these ideas and ask whether there are signals specifically associated with living, pathogenic microbes that could play a role in the initiation of innate immune responses. We focus exclusively on bacterial pathogens and consider two main questions: (1) Do living and dying bacteria produce distinct PAMPs, i.e., PAMPs-postmortem (PAMPs-PM) and PAMPs-per vita (PAMPs-PV), and if so, are they differentially recognized by the immune system? (2) Does the immune system initiate responses based not only on whether PAMPs are present, but on where and under what cellular context the PAMPs are presented? Our overall hypothesis is that PAMPs are delivered along with additional information that can be used by the host to distinguish pathogenic from nonpathogenic microbes and thereby guide the ensuing innate immune response.

**Pathogen-Associated Molecular Patterns**

**Dual Recognition of PAMPs**

A relatively select group of bacterial molecules are able to function as PAMPs, and importantly, several PAMPs are recognized by at least two different sensors, often in different contexts (Figure 1). For example, flagellin is a ligand for TLR5 at the cell surface but is also recognized cytosolically by an entirely distinct sensor, the Naip5/lpafl inflammasome. The two flagellin sensors appear to have evolved independently, as they recognize distinct domains of the flagellin molecule (Lightfield et al., 2008). Likewise, DNA is recognized in a specialized intracellular compartment by TLR9 (Ahmad-Nejad et al., 2002; Hacker et al., 1998; Honda et al., 2005) but is also sensed in the cytosol by several distinct sensors, some of which remain to be identified (Burckstummer et al., 2009; Fernandes-Alnemri et al., 2009; Hornung et al., 2009; Ishii et al., 2006; Muruve et al., 2008; Stetson and Medzhitov, 2006). RNA is recognized by TLR3 within a vacuole and by RIG-I and Mda5 in the cytosol. The cytosolic nucleic acid sensors recognize features of their ligands distinct from the features recognized by TLRs (Yoneyama and Fujita, 2008).

Importantly, the multiple receptors involved in recognizing a single PAMP do not appear to be redundant but instead often lead to unique signaling outcomes.

Two major categories of responses elicited by PAMPs are transcriptional and posttranslational. TLRs signal through several signaling adaptors, notably MyD88 and Trif, leading to a transcriptional response primarily dependent on the NF-κB and IRF-3/7 families of transcription factors (Kawai and Akira, 2008). Several cytosolic PRRs, on the other hand, appear to stimulate the inflammasome, a multiprotein complex that triggers a posttranslational response, the cleavage and activation of the cysteine protease caspase-1 (Franchi et al., 2009; Petrilli et al., 2007a). Caspase-1 in turn cleaves and activates the secretion of a variety of substrates, including pro-IL-1β and pro-IL-18. Caspase-1 is also required for a rapid, nonapoptotic death termed pyroptosis (Bergsbaken et al., 2009). Examples of PAMPs that activate the inflammasome include flagellin and DNA, but these PAMPs activate the inflammasome only upon delivery to the cytosol and not from the cell surface (Franchi et al., 2006; Lightfield et al., 2008; Miao et al., 2006; Molofsky et al., 2006; Muruve et al., 2008; Ren et al., 2006). Thus, the immune system can initiate responses based not only on whether PAMPs are present, but on where those PAMPs are presented.

The dual transcriptional and posttranscriptional responses downstream of PAMP recognition can be collaborative in important ways. For example, TLR signaling is required for the transcription of pro-IL-1β and pro-IL-18, and the posttranslational inflammasome response is then required for subsequent cleavage and secretion of the active cytokine (Franchi et al., 2009; Petrilli et al., 2007a). Such dual control may be an important regulatory mechanism to limit inappropriate or unnecessary production of cytokines that trigger potent responses that are potentially damaging to the host.

It is important to acknowledge that there is not always a clear-cut distinction between signals originating from extracellular, vacuolar, or cytosolically detected PAMPs. For example, nucleic acid ligands can induce expression of type I IFNs from a phagosome or the cytosol, and cell-surface TLRs and the cytosolic Nod1/2 sensors both activate NF-κB (Ishii et al., 2008). Nevertheless, there may be important differences in the kinetics,
intensity, or cell types involved in cytosolic versus TLR signaling, even if the fundamental signaling pathways are similar. For example, most cell types can produce type I IFNs, primarily via cytosolic sensing pathways, but the rapid and potent release of type I IFNs into the serum by plasmacytoid dendritic cells occurs uniquely downstream of TLR signaling. Both cytosolic and TLR-mediated production of type I IFNs appear to play essential roles in distinct contexts (Delale et al., 2005; Kato et al., 2005; Krug et al., 2004). Taken together, numerous observations support the idea that single PAMPs can trigger dual responses. Moreover, host cells appear to assess the context in which PAMPs are sensed, and this contextual information is used to generate distinct responses.

Issues that Complicate the PAMP Hypothesis

One oft-heard criticism of the PAMP hypothesis is that PAMPs are not restricted to pathogens but are instead produced by all microbes. Thus, one suggestion has been that PAMPs should really be renamed microbe-associated microbial pathogens (MAMPs) (Benko et al., 2008; He et al., 2007; Mackey and McFall, 2006; Sirard et al., 2006). Indeed, the PAMP model cannot by itself explain how pathogens and nonpathogens might be distinguished by the innate immune system and might even lead to the view that pathogens and nonpathogens are indistinguishable. Our view is that pathogenic and nonpathogenic microbes are distinguishable, but the challenge is to understand how they are distinguished.

The PAMP model is also complicated by the extent to which pathogens can modify their PAMPs to avoid host recognition. Although PAMPs are usually portrayed as invariant or highly constrained structures that are extremely difficult for microbes to alter, the extent of PAMP plasticity is appreciable and is often neglected in discussions of the PAMP model. Almost by definition, a pathogen is a microorganism that causes disease by avoidance or manipulation of host innate immunity, and it is presumed that pathogens must be able to avoid innate immunity to some extent (Hedrick, 2004; Hornet et al., 2002). LPS provides several interesting examples of variants that appear to confound the host. For example, the oral pathogen Porphyromonas gingivalis can produce an astonishing variety of at least 12 different lipid A molecules (Reife et al., 2006), which can be stimulatory, invisible (R. Darveau, personal communication), or antagonistic to TLR4 (Coats et al., 2007). As an additional example, Yersinia pestis modifies its LPS via altered acylation, rendering it a poor ligand for TLR4 (Kawahara et al., 2002; Montminy et al., 2006; Rebeil et al., 2006). Avoidance of PAMP recognition is not limited to LPS. The flagellin of Helicobacter pylori, for example, is not efficiently recognized by TLR5 (Andersen-Nissen et al., 2005; Gewirtz et al., 2004; Lee et al., 2003); moreover, several bacterial pathogens have efficient mechanisms for downregulating flagellin expression within hosts (Akerley et al., 1995; Shen and Higgins, 2006; Wolfgang et al., 2004). Pathogens are also able to manipulate downstream signaling pathways to prevent responses downstream of PAMP recognition (Bhavsar et al., 2007). The selective pressure for certain pathogens to circumvent responses to PAMPs is evidence in favor of the model that PAMP recognition plays an important role in stimulating host innate immunity. Further supporting this idea is the evidence that mutations in PRRs often (but do not always) lead to increased host susceptibility (Ishii et al., 2008; Puel et al., 2005). Although it is unlikely that any pathogen is able to render its PAMPs entirely invisible to the immune system, we suspect that, to the extent required for their replication and transmission, pathogens can avoid recognition of their PAMPs. This is an important consideration that leads us to consider whether additional mechanisms of pathogen sensing may complement PAMP sensing. These additional mechanisms might, for example, permit specific recognition of microorganisms that have high pathogenic potential.

The barcode hypothesis is one model that has been proposed to explain how responses could be tailor-made to certain microbes (Adern, 2003). This model suggests that the different combinations of PAMPs found on different microbial species could be interpreted in a way that would allow for unique responses to distinct classes of pathogens. For example, a Gram-negative (but not a Gram-positive) pathogen would stimulate TLR4, leading to the unique transcriptional response, dominated by production of IFNβ, that is downstream of TLR4. However, many pathogens defy strict categorization in this way, indicating that more factors may come into play. For example, many Gram-negative pathogens lack LPS that is stimulatory for TLR4 (Munford and Varley, 2006), and while Gram-positive bacteria lack LPS, many still potently induce IFNβ via a cytosolic immunosurveillance pathway that remains to be fully defined (Perry et al., 2005). Thus, while a PAMP barcode provides some useful information to the immune system, it seems likely that PAMP signals are interpreted in the context of other cues that occur in the course of infection.

Patterns of Pathogenesis

Concept Defined

The PAMP framework has been useful because it provides a relatively straightforward system for categorizing responses to microbes based on the PAMPs that are expressed. However, the idea that the innate immune system views pathogens as mere “bags of PAMPs” ignores important contextual information that accompanies PAMPs when they are delivered by virulent microorganisms. Here, we propose a complementary framework for understanding innate responses to pathogens that we call “patterns of pathogenesis” (Figure 2). This proposal is based on decades of research that has led to the recognition that pathogens use a few common strategies to cause disease (Finlay and Falkow, 1997). As each strategy is found in a broad swath of pathogens, each can be considered conceptually similar to a common molecular pattern recognized by PRRs. Below is a broad and nonexhaustive description of some of these patterns. Later, we will explore whether immune recognition of the events that result from these patterns potentiates innate immune signaling.

Pattern I: Growth

With the exception of a very small subset of toxin-producing organisms, the most common pattern associated with pathogens is their ability to grow in their hosts upon invasion. It should be beneficial for the immune system to distinguish growing and dying bacteria, especially in the context of an acute infection, in order to organize the appropriate response. Here, we ask if there are bacterial molecules that signify growing versus dead and dying bacteria. We propose that a PAMP signifying life be named PAMP-PV (per vita) and one that signifies death as PAMP-PM (postmortem). Molecules potentially associated with bacterial
death and lysis would include any large macromolecule that is only released during bacteriolysis, such as DNA or RNA. Small molecules that could be associated with growth include those that could be secreted by living bacteria, such as PGN fragments, quorum-regulating autoinducers (Zimmermann et al., 2006), bacterial pyrophosphates such as HMB-PP (Hintz et al., 2001), or perhaps bacterial nucleotide-based second messengers such as c-di-GMP (Karaolis et al., 2007; McWhirter et al., 2009).

Below, in the section on innate immune recognition, we will attempt to illustrate the concept of molecules generated during growth and death by examining the factors that control the production of PGN fragments recognized by PRRs.

**Pattern II: Cytosolic Access**

A primary characteristic of many pathogens is the ability to deliver microbial molecules into the host cell cytosol. One of the best-characterized versions of this strategy is the deployment of AB toxins, in which a B subunit mediates binding and translocation of the enzymatic A subunit into the host cytosol (Finlay and Falkow, 1997). For example, the B subunit of anthrax toxin, called protective antigen (PA), mediates the delivery of the A subunits (lethal factor and edema factor) from an acidified phagosome into the host cell cytosol (Young and Collier, 2007). This is not the only means by which pathogens access the host cell cytosol. In fact, other mechanisms, such as dedicated systems for secretion of bacterial proteins into the host cell cytosol, are even more closely linked with the presence of viable bacteria.

The best characterized of these auxiliary secretion systems are the type III secretion systems (T3SSs) encoded by numerous Gram-negative bacterial pathogens (Galán and Wolf-Watz, 2006). These secretion systems are often described as molecular syringes that deliver bacterial proteins, usually enzymes, into the host cell cytosol. The secreted proteins are referred to as “effectors” and are analogous to the A subunit of AB toxins. Indeed, one can consider auxiliary secretion systems as elaborate B subunits that deliver toxins directly into target cells. Once in host cells, the effectors perform a variety of functions that contribute to pathogenesis. Extracellular pathogens such as Yersinia can utilize T3SSs to deliver effectors that block uptake of bacteria into cells; conversely, intracellular pathogens such as Salmonella can utilize T3SSs to enter cells and establish intracellular compartments that support replication (Galán and Wolf-Watz, 2006). It is worth noting that while the apparatus of the T3SS itself is conserved across species, the effectors and their activities are distinct and sometimes encode unusual biochemical functions (Bhavsar et al., 2007). A T3SS is thus a flexible scaffold that is compatible with a highly diverse set of pathogenic lifestyles. Bacterial pathogens lacking T3SSs often encode evolutionarily unrelated systems that nevertheless fulfill the same basic function. Examples include type IV secretion systems (T4SSs) (e.g., of Legionella, Coxiella, and Brucella) and the more recently discovered type VI secretion system (T6SS) (e.g., in Pseudomonas and Vibrio) (Pukatzki et al., 2006). What all these systems have in common is that they promote cytosolic access of microbial molecules.

The use of dedicated secretion systems is not limited to Gram-negative bacteria. In Gram-positive microorganisms, including Mycobacteria, functionally similar mechanisms appear to exist. The ESX-1 secretion system of Mycobacterium tuberculosis (Simeone et al., 2009) is evolutionarily distinct from those described above, but it fulfills the same basic function of delivering bacterial products to the cytosol of host cells. In the case of Gram-positive organisms, some pore-forming toxins such as streptolysin O may serve as portals for the injection of bacterial molecules into the host cell cytosol (Madden et al., 2001). Again, in its natural setting, the pore-forming toxin appears to function as an elaborate B subunit. Some pore-forming toxins deliver not just a few effectors to the cytosol but are also involved in phagosome disruption, allowing an entire pathogen to access the cytosol. This is the case for lysteriolysin O, a pore-forming toxin required for Listeria monocytogenes to escape the phagosome, replicate in the cytosol, and cause disease in hosts (Schnupf and Portnoy, 2007). A key point is that pathogens that access the cytosol require cytosolic access as a critical component of their virulence strategy, and thus, mutants lacking auxiliary or pore-forming systems are typically avirulent.

**Pattern III: Hijacking and Disrupting Normal Host Cytoskeleton Function**

Several highly divergent species of bacteria, including Listeria, Shigella, Mycobacterium marinum, and Rickettsial species, not only access but also replicate in the host cytosol (Gouin et al., 2005). The events associated with cytosolic replication emphasize that a third major pattern may mark a pathogen for recognition by the host: many microorganisms can either hijack or disrupt host cytoskeletal function. These four bacterial species, as well as poxvirus, exploit the host cell system of actin-based motility, allowing their movement within cells and from cell to cell, thereby spreading the infection without exiting the cells (Gouin et al., 2005). In each case, proteins associated with the surface of the pathogen recruit key regulators of the cytoskeleton to initiate novel rounds of actin polymerization within the host cell.

A large number of additional pathogens disrupt the host cytoskeleton for completely distinct purposes. Some pathogens, such as Salmonella, manipulate host actin in order to invade cells (Galán and Wolf-Watz, 2006), whereas other pathogens disrupt...
host actin in order to block phagocytosis (Viboud and Bliska, 2005). The molecular mechanisms by which pathogens modulate host actin are also diverse. A common way to target the cytoskeleton is to directly manipulate the function of Rho family small GTPases that control cellular cytoskeletal processes (Heasman and Ridley, 2008). Many bacterial pathogens disrupt function of these proteins either by mimicking key regulators of the cycle or chemically modifying Rho family members. For instance, multiple bacterial pathogens translocate proteins that cause GTP hydrolysis and inactivation of the Rho family member (Black and Bliska, 2000; Fu and Galán, 1999; Goehring et al., 1999; Von Pawel-Rammingen et al., 2000). There also exist AB toxins or translocated substrates of specialized secretion systems that change the activation state of Rho family members by chemical alteration, usually disrupting protein function (Aktories et al., 1989; Chardin et al., 1989; Just et al., 1995; Schmidt et al., 1997; Yarbrough et al., 2009). Finally, some bacterial proteins act more directly after they access the host cytoplasm and target either actin itself or an actin-associated protein, resulting in alterations of cytoskeletal dynamics (Fullner and Mekalanos, 2000; Hayward and Koronakis, 1999; Zhou et al., 1999). Despite the remarkably diverse ways that pathogens affect the host cytoskeleton, the key point of emphasis here is that disruption of the host cytoskeleton is a common “pattern” employed by many pathogens (but not nonpathogenic microbes) and could therefore be an important cue to the host immune system.

Recognition of Patterns of Pathogenesis by the Innate Immune System

The above discussion presents the idea that although there are numerous mechanisms of pathogenesis, there appears to be a relatively select group of cellular targets, or hubs, that many pathogens exploit as points of vulnerability. Just as relatively few bacterial structures are targeted as PAMPs, perhaps it is also the case that relatively few host pathways are suitable points of vulnerability that can be exploited by pathogens. If so, it would make sense for the host to monitor these host structures for signs of distress that could indicate the presence of a pathogen. A similar model has been termed the guard hypothesis in the plant innate immunity literature (Chisholm et al., 2006; Jones and Dangl, 2006). Here, we extend this idea to consider how PAMP sensing and other forms of immunosurveillance might be coordinated.

Sensing Pathogen Replication and Death

It remains an open question whether there are molecules whose structure signifies either growing or dying microbes. Clearly, it might be helpful to the immune system if such discrimination were possible. For example, the presence of dead bacteria might indicate that the immune response has been successful and therefore be a signal to begin contracting and resolving the response, perhaps in conjunction with additional signals (Harty and Badovinac, 2008). Pathogen growth or death could be sensed by the host in a variety of ways. For example, pathogen replication could alter local levels of host amino acids, other nutrients, or oxygen (Rius et al., 2008). Here, we focus on just one possible pathway in some detail by examining host responses to the most ubiquitous of bacterial PAMPs, PGN, an essential conserved and abundant structure that is shed during bacterial growth. PGN is a well-characterized PAMP, but when produced in the context of a natural infection, it may provide more specific signals: for example, signifying the presence of live pathogenic bacteria. In Gram-negative bacteria, approximately 60% of shed PGN is recycled (Park and Uehara, 2008), whereas in Gram-positive bacteria, it is released (Cloud-Hansen et al., 2006). Bacteria also remodel their PGN during assembly of macromolecular structures such as flagella and auxiliary secretion systems (Vollmer et al., 2008). To accommodate growth and PGN remodeling, bacteria utilize a collection of PGN-specific degrading enzymes called autolysins (Vollmer et al., 2008). The term autolysin is misleading, because these enzymes are essential for PGN remodeling, and their regulated activity prevents autolysis. It is their deregulation, such as during treatment with β-lactam antibiotics, which causes autolysis (bacteriolysis). Autolysins can be divided into families based on their site of cleavage and precise enzymatic mechanisms. Bacteria often express more than a dozen autolysins with many distinct activities. Animals also express enzymes that degrade PGN, although with limited substrate specificity, including lysozyme (N-acetyl muramidase) and PGRPs (amidase activity). Lysozyme and PGRPs can be bacteriocidal and act to limit the inflammatory properties of PGN (Ganz et al., 2003; Royet and Dziarski, 2007). Not surprisingly, PGN from many pathogens is resistant to lysozyme (Boneca et al., 2007).

It is reasonable to suspect that PGN released by growing bacteria may be structurally distinct from nongrowing bacteria or those undergoing bacteriolyis. One example may be tracheal cytotoxin (TCT) (Luker et al., 1995). TCT consists of a single monomeric unit of PGN (GlcNAc, MurNAc, glutamic acid, diaminopimelic acid, and two alanines). The receptors for TCT include murine Nod1 (Magalhaes et al., 2005) and some Drosophila PGRPs (Aggarwal and Silverman, 2008; Chang et al., 2006). Although TCT generation requires cleavage at a glycosidic bond that is also targeted by lysozyme, active TCT is not generated by lysozyme but rather requires the activity of a bacterial-specific lytic transglycosylase that leaves a 1,6-anhydro-bond upon cleavage (Cloud-Hansen et al., 2008). Autolysins of this class are used by bacteria to remodel PGN during biosynthesis and assembly of macromolecular structures such as auxiliary secretion systems (Koraimann, 2003) and are hence necessary for pathogenesis. TCT is normally recycled by Gram-negative bacteria, but its specific release is associated with the induction of inflammation and pathogenesis of Bordetella pertussis and Neisseria gonorrhoeae. Thus, TCT production signifies bacterial growth and stimulates inflammation, fulfilling the requirements of a PAMP-PV.

In contrast, another bioactive PGN fragment may fulfill the criteria of a PAMP-PM. Muramyl dipeptide (MDP), originally isolated from killed M. tuberculosis and an active component of Freund’s complete adjuvant (Ellouz et al., 1974), is recognized by Nod2 (Benko et al., 2008). Generation of MDP requires the activity of a bacterial-specific endopeptidase (Humann and Lenz, 2008). Unlike TCT, its presence is not specific for growing bacteria and, in fact, the PGN cleavage that generates MDP also destroys TCT. Further emphasizing the connection between MDP and lack of viability, generation of ligands for Nod2 (presumably MDP) occurs in the phagolysosomes of activated macrophages, but only upon bacteriolyis (Herskovits et al.,
The immunological consequences of Nod2 stimulation are still controversial and range from NF-κB stimulation (Ferwerda et al., 2008; Hsu et al., 2008; Maeda et al., 2005; Marina-Garcia et al., 2008; Pan et al., 2007) to suppression of TLR signaling (Watanabe et al., 2004, 2006) to promotion of Th2-like polarization in the gut (Magalhaes et al., 2008) and downregulation of PGN-induced colitis (Yang et al., 2007). Hence, it is conceivable that the presence of MDP represents either harmless flora or killed and degraded bacteria: exactly as expected for a PAMP-PM.

TCT or MDP lead to RIP2 and NF-κB activation downstream of Nod1 or Nod2, so it is not clear that host signaling distinguishes one as a PAMP-PV and the other as a PAMP-PM (Benko et al., 2008). Although TCT and MDP target Nod1 and Nod2 respectively, either may have additional receptors and/or target other responses, leading to differential readouts for these two molecules. For example, MDP may activate the inflammasome (Hsu et al., 2008; Martinon et al., 2004; Pan et al., 2007). Additional contextual clues are likely to be critical for the immune system to interpret Nod1/2 signals. As we have suggested here, it is possible that, in different scenarios, specific PGN fragments can differentially signify growing or dying bacteria, depending on the infectious context in which the fragments are generated. Another complication is that designating any PGN fragment a PAMP-PM is difficult, as there is no easy way for material from a dead organism to access the cytosol. However, a role for peptide transporters has been suggested (Swaan et al., 2008; Vavrivcka et al., 2004), and PGN fragments may enter intestinal epithelial cells by normal cellular damage (Miyake et al., 2006).

**Sensing Cytosolic Access**

Although secretion systems and AB/pore-forming toxins provide pathogens with the ability to access the cytosol and thereby control the inner workings of host cells, much recent evidence supports the idea that “violation of the sanctity of the cytosol” also triggers significant host responses (Lamkanfi and Dixit, 2009). There are two mutually nonexclusive models that describe how host cells sense cytosolic access by secretion systems. The first is that secretion systems are detected via cytosolic recognition of specific translocated bacterial molecules (PAMPs) or their activities. A central premise of this hypothesis is that the bacterial molecules that are sensed cannot diffuse across the plasma or phagosomal membrane and therefore only reach the cytosol by active or inadvertent translocation by the secretion system. The presence of such molecules in the cytosol is therefore a strong indication that a secretion system (or B subunit) is present. A second hypothesis is that secretion or pore-forming systems are sensed by host cells via detection of the physical damage associated with bacterial structures penetrating the plasma or phagosomal membranes. For example, there has been the suggestion that pore-forming toxins or the needles of type III and type IV secretion systems cause damage or lead to ion efflux when they insert into host membranes (Kirby and Isberg, 1998; Shin and Cornelis, 2007; Viboud and Bliska, 2005). Both hypotheses have some merit and are discussed in turn below.

**Sensing of Translocated PAMPs as a Signal for Cytosolic Access**

One of the first pathogens shown to translocate a PAMP into the cytosol was the extracellular pathogen *Helicobacter pylori*, which delivers PGN-derived ligands for Nod1 via its T4SS (Viala et al., 2004). The pore-forming toxin pneumolysin from *Streptococcus pneumoniae* (Ratner et al., 2007) has also been shown to allow fragments of bacterial PGN to access the host cell cytosol and trigger Nod1.

Another well-characterized example of a translocated PAMP that is sensed in the cytosol is flagellin. In order to assemble a flagellum, flagellin monomers are normally secreted by the flagellar secretion system, an apparatus that shares considerable homology with T3SSs (Chevance and Hughes, 2008). Interestingly, and perhaps because of this homology, recent data have demonstrated that flagellin can be translocated into the host cell cytosol via T3SSs (Sun et al., 2007). Flagellin also appears to reach the host cell cytosol via the T4SS of *L. pneumophila*, though the mechanism of translocation remains unclear (Molofsky et al., 2006; Ren et al., 2006). Once in the cytosol, flagellin triggers the Nalp5/Ipaf inflammasome, leading to IL-1β/IL-18 release and pyroptotic cell death (Franchi et al., 2006; Lightfield et al., 2008; Miao et al., 2006; Molofsky et al., 2006; Ren et al., 2006). In this case, it is clear that the cytosolic presence of flagellin is sufficient to activate the Nalp5/Ipaf inflammasome and that secretion-system-induced pores or membrane damage do not appear to play an essential role (Lightfield et al., 2008). Since delivery of flagellin to the host cell cytosol is strictly dependent on type III or IV secretion systems, the cytosolic presence of flagellin is a strong signal to the immune system that a pathogen (as opposed to a commensal) is present, since presumably only pathogens will encode such secretion systems. Interestingly, recent work has established that the Ipaf inflammasome is also capable of responding to the conserved inner rod component of the T3SS itself (E. Miao and A. Aderem, personal communication). This conserved component is apparently translocated into host cells, where it functions as a PAMP that directly indicates the presence of a pathogen with a secretion system.

Anthrax lethal factor (LF) is another example of a translocated molecule that activates a host response in the cytosol. The host sensor protein that responds to LF was recently identified as Nalp1b (Boydien and Dietrich, 2006), but it remains unclear how LF is sensed. The protease activity of LF appears to be essential in order to trigger a host response, so it is likely that the activity of LF is sensed as opposed to the molecule itself or the pore formed by the lethal toxin B subunit (PA).

Another cytotoxic pathway triggered in response to pathogen access of the cytosol is a TLR-independent pathway leading to the induction of IFNβ and other coregulated genes. This pathway is apparently activated by an extremely diverse group of Gram-positive and Gram-negative pathogens (Charrel-Dennis et al., 2008; Henry et al., 2007; O’Riordan et al., 2002; Roux et al., 2007; Stanley et al., 2007; Stetson and Medzhitov, 2006). In each case, it has been demonstrated that induction of IFNβ requires pathogen expression of an auxiliary secretion system, but the variety of secretion systems is impressive, ranging from multidrug-resistance transporters (Crimmins et al., 2008) to type III (V. Auerbuch and R.R.I., unpublished data), IV (Roux et al., 2007; Stetson and Medzhitov, 2006), VI (Henry et al., 2007), and other secretion systems (Stanley et al., 2007). It remains unclear whether the induction of type I IFN in all these cases proceeds via the same basic mechanism. Although
transfected DNA can recapitulate the response (Ishii et al., 2006; Leber et al., 2008; Stetson and Medzhitov, 2006), and the addition of exogenous poly I:C can stimulate type I IFN induction dependent on the type III system (V. Auerbuch and R.R.I., unpublished data), the identity of the actual ligand(s) sensed and its receptor(s) is still not known. T4SSs are able to translocate DNA (Hamilton and Dillard, 2006; Segal et al., 1998; Vogel et al., 1998), but this has not been established for the other secretion systems. What is clear is that, in all cases, the responses are associated with auxiliary secretion systems of live, growing bacteria that access the host cell cytosol and may thus represent examples of host recognition of a PAMP-PV. **Sensing of Pores or Membrane Damage as a Signal for Cytosolic Access**

The idea that pores formed by bacterial secretion systems or toxins can be sensed by the innate immune system is attractive, because it could provide a unified mechanism by which diverse bacterial pathogens could be sensed (Freche et al., 2007). As mentioned above, the broader idea that the immune system can respond to cellular damage (Matzinger, 1994) is supported by some data (Kono and Rock, 2008), but there is surprisingly little evidence that secretion systems themselves cause damage when expressed by wild-type bacteria at physiologically relevant multiplicities of infection. For example, the macrophage cell death provoked by secretion-competent pathogens such as *Legionella* and *Salmonella*, previously suspected to be due to the damage induced by the pore-forming activity of secretion systems, has turned out to be due primarily to a host response triggered by a molecule (flagellin) translocated by the secretion system (Franchi et al., 2006; Miao et al., 2006; Molofsky et al., 2006; Ren et al., 2006). In cases where pore-forming toxins or secretion systems apparently trigger host cell damage, it is often difficult to know whether the apparent damage is directly inflicted by the toxin or secretion system or whether it results from a host response, such as pyroptosis (Bergsbaken et al., 2009; Fink and Cookson, 2005), that is triggered in response to an unknown translocated PAMP.

Strains of *Yersinia* lacking expression of all known translocated effectors still trigger host responses dependent on the T3SS translocon (Bergsbaken and Cookson, 2007; Shin and Cornelis, 2007; Viboud and Bliska, 2001; V. Auerbuch and R.R.I., unpublished data), consistent with responses made to the translocon itself or pores. However, it is very difficult to rule out the existence of an unknown translocated effector or PAMP. The apparent response to the *Yersinia* translocon does not require very high multiplicities of infection. However, in other cases, very high and possibly nonphysiological multiplicities of infection seem to be required for bacterial secretion systems to trigger pore formation. For example, although excessive extracellular application of the pore-forming toxin listeriolysin O can lead to activation of host caspase-1 and IL-1β secretion, *Listeria* itself delivers listeriolysin O in a highly regulated manner and appears to go to considerable lengths to avoid damaging the host cell in which it must replicate (Schnupf and Portnoy, 2007).

Nevertheless, under certain scenarios, membrane pore formation does seem to trigger specific host immune responses (Aroian and van der Goot, 2007). For example, treatment of cells with pore-forming toxins such as aerolysin, nigericin, or maitotoxin led to activation of caspase-1 via an inflammasome that contains the Nlrp3 protein (Freche et al., 2007; Gurcel et al., 2006; Mariathasan et al., 2006). Nigericin comes from the nonpathogenic soil organism *Streptomyces* and has therefore presumably not evolved to target mammalian cells, so its physiological significance and the mechanism by which it activates Nlrp3 remain uncertain. Nigericin and other activators of the Nlrp3 inflammasome, such as stimulation of cation-selective P2X7 channels by millimolar concentrations of extracellular ATP, induce K+ ion efflux from cells. Thus, one suggestion is that K+ efflux is a common mechanism by which cells can sense membrane pores and activate the Nlrp3 inflammasome (Mariathasan et al., 2004; Petrilli et al., 2007b). However, other studies have demonstrated that potassium efflux is not sufficient to activate Nlrp3 (Pelegrin and Surprenant, 2006) and have identified a hemichannel pore protein, pannexin-1, as an essential and more proximal activator of Nlrp3. Again, the large pores formed by pannexin-1 do not seem to correlate with Nlrp3 activation (Pelegrin and Surprenant, 2007). Thus, there is little evidence that most naturally delivered toxins activate the inflammasome via pore formation, and the physiological mechanism by which the Nlrp3 inflammasome is activated in the context of infection remains very poorly understood and may involve lysosomal disruption (Hornung et al., 2008) and/or the generation of reactive oxygen species (Cassel et al., 2008; Dostert et al., 2008). Whatever the mechanism of Nlrp3 activation, there is nevertheless considerable evidence that Nlrp3 can play an important role in regulating a variety of immune responses in vivo (Eisenbarth et al., 2008; Kool et al., 2008; Li et al., 2008; Sutterwala et al., 2006).

**Sensing Actin Cytoskeleton Disruption**

As discussed above, a common host target of many pathogens is the actin cytoskeleton. Interestingly, agents that block actin polymerization promote transcriptional activation mediated by NF-κB (Kustermans et al., 2008). Cytoskeletal structures may control NLR responses, as both Nod1 and Nod2 localize to actin-rich regions near the plasma membrane (Kufer et al., 2008; Legrand-Poels et al., 2007). Furthermore, stimulation of the NOD1 pathway that leads to transcriptional responses is enhanced by cytochalasin D (Magalhaes et al., 2005). There are at least two models consistent with these observations. Perhaps NODs are located at the plasma membrane, as this is the site of bacterial attachment, invasion, and PGN release (Kufer et al., 2008). Indeed, it was suggested that NOD1 is activated by PGN released through the *Helicobacter pylori* T4SS (Viala et al., 2004). Alternatively, NODs may act as “guards” of the actin cytoskeleton and may be released and activated upon perturbations, such as those associated with bacterial toxins and effectors (Legrand-Poels et al., 2007).

There may also be a link between the actin cytoskeleton and inflammasome activation. Two proteins associated with inflammation, pyrin and ASC, localize to the plasma membrane at sites of actin polymerization (Wai et al., 2009). Even more intriguingly, ASC and pyrin localized to the actin tails of *L. monocytogenes* during intracellular actin-based motility (Wai et al., 2009). It is not yet possible to make any conclusions from this single observation, but it does suggest that components of the inflammasome associate with regions of active actin polymerization. Though not the focus of this review, there are...
suggestions that cytosolic sensors of viruses, such as RIG-I, are also associated with the actin cytoskeleton (Mukherjee et al., 2009), though the significance of this observation remains to be fully elucidated.

**Conclusions**

Here, we have considered the hypothesis that, in addition to sensing of PAMPs, the host innate immune system is able to respond to patterns of pathogenesis—signals that derive from the strategies that live pathogens use to invade, manipulate, replicate within, or spread among their hosts. Our discussion has clearly not been exhaustive, and we suspect that there may be additional patterns that we have not discussed in detail that might also be sensed by host cells. For example, adherence of pathogens to host cells is a common pattern of pathogenesis that may well trigger specific host responses. The production of extracellular enzymes (e.g., proteases, phospholipases) is another pattern of pathogenesis that may liberate host ligands that are sensed by the innate immune system or produce cellular damage that triggers host responses. Extracellular enzymes may be especially central to the sensing of certain eukaryotic pathogens that lack PAMP-like molecules (Sokol et al., 2008).

Although sensing of PAMPs is central to the generation of effective immune responses, it seems clear that PAMPs are not sensed in a vacuum and that infection by live pathogens provides additional contextual cues that shape the immune response. We suggest that these cues may potentially allow the immune system to distinguish living pathogens from dead or otherwise harmless microbes. Because of the complexity of many host-pathogen interactions, it can be experimentally challenging to use living pathogens to dissect immune responses, but it is probably crucial to do so, and the availability of a variety of host and pathogen genetic systems has made an analysis of innate immunity to pathogens increasingly feasible (Persson and Vance, 2007). One of the most striking observations that underscores the notion that living pathogens induce unique immune responses comes from the model of experimental listeriosis. In this model, immunization with live *L. monocytogenes* provides potent protection against a subsequent challenge, whereas immunization with killed *L. monocytogenes* confers no protective immunity (von Koenig et al., 1982). Moreover, *L. monocytogenes* strains that fail to access the host cell cytosol due to mutations in listeriolysin O not only fail to immunize against subsequent challenge with virulent *L. monocytogenes*, they also appear to actively suppress host immunity via a pathway dependent on IL-10 (K.S. Bahjat, N. Meyer-Morse, A.L. Philpott, and S.E. Girardin, unpublished data). Thus, host immune responses are shaped not only in response to PAMPs but also in response to contextual cues—e.g., those provided by growing bacteria or bacteria that access the host cell cytosol as part of their virulence strategy.

Live-attenuated pathogens have shown considerable utility as vaccines, but there is no theoretical consensus as to why live pathogens are especially immunostimulatory and not generalizable procedure, other than trial and error, for how best to attenuate pathogens for their use as vaccines. Based on the considerations we present above, it might be expected that a vaccine strain that has been attenuated through the deletion of an immunostimulatory secretion system may fail to elicit protective immunity. An example of such a vaccine may be the relatively ineffective BCG vaccine for *M. tuberculosis*, in which the ESX secretion system (RD1 locus) was deleted during its generation. A prediction following from our conceptual framework would therefore be that strains lacking secreted effectors (A subunits) but retaining the immunostimulatory translocation system (B subunits) would function as more effective live-attenuated vaccines. There is, at present, little direct evidence to support this specific idea, but the general notion that PAMPs are interpreted by the immune system in the context of other infection-derived signals is likely an important concept for future consideration.

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