Immunology

A new way to detect the danger: Lysosomal cell death induced by a bacterial ribosomal protein

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The death of immune cells in response to pathogens often dictates the outcome of an infection. In some contexts, pathogens specifically kill immune cells by producing highly potent toxins or by triggering host cell death pathways, thus ensuring successful infections. But for intracellular pathogens and viruses, the death of host cells normally is disastrous for their intracellular life cycle. Our recent experiments with the pathogen *Legionella pneumophila* revealed that the bacterial ribosomal protein RpsL is able to trigger lysosomal membrane permeabilization (LMP) and the subsequent macrophage cell death. Interestingly, a lysine to arginine mutation at the 88th residue, which also confers resistance to the antibiotic streptomycin, substantially impaired the cell death inducing activity of RpsL and allowed *L. pneumophila* to succeed in intracellular replication, suggesting the convergence of resistance mechanisms to innate immunity and antibiotics. The discovery of lysosomal cell death as an immune response to a bacterial ligand has expanded the spectrum of reactions that host cells can mount against bacterial infection; these observations provide a model to study the pathways that lead to the induction of LMP, a currently poorly understood cellular process involved in the development of many diseases. Journal of Nature and Science, 1(6):e107, 2015

Legionella | cathepsins | apoptosis | innate immunity | caspases

A fundamental aspect of immune function is the ability to distinguish between "self" from "non-self". An important component of this capacity is the innate immune system's arsenal of germ-line coded PRRs (pattern-recognition-receptors) which are distributed at different cellular locations to detect the presence of the MAMPs (microbe-associated-molecular-patterns) from micro-organisms (1). The PRRs can be categorized based on the subcellular location from which the receptor initiates signaling. Toll-like-receptors (TLRs) and C-type lectins are among the best-characterized membrane associated receptors that mainly function to recognize extracellular ligands (2). In parallel, a large number of cytoplasmic PRRs guard the intracellular space of mammalian cells from invading microbes, including the NOD-like receptors (NLRs), pyrin and HIN domain containing family (PYHIN), RIG-I-like receptors (RLRs) and cytosolic nucleic acid sensors (3). Cytosolic NLRs, engaged by their ligands, are capable of initiating effective defense through diverse downstream mechanisms, such as NF- B activation, IFN-β production and the assembly of large protein complexes termed inflammasomes. Inflammasome activation results in turn in the production of cytokines and the initiation of the pyroptotic cell death necessary for the clearance of infection (3). The assembly of certain inflammasomes and the subsequent activation of caspase-1 can be achieved through prion-like, self-propagating protein polymerization (4, 5), which appears to allow robust signal amplification even when the concentration of the ligand is extremely low. This makes these NLRs very sensitive immune sentinels (4, 5).

The roles of NLRs as guardians of the cytoplasm are further enhanced by their capacity to recognize many and diverse ligands. For example, NLRP1b responds to both the protease activity of the lethal toxin from *Bacillus anthracis* and infections by *Toxoplasma gondii* (6), whereas the AIM2 (absent in melanoma 2) inflammasome responds to cytosolic DNA molecules (7). NAIP (neuronal apoptosis inhibitory protein) proteins appear to expand the specificity of the NLRC4 inflammasome by recognizing diverse bacterial MAMPs including the rod and needle proteins of the Type III secretion systems as well as flagellin (8). The NLRP3 inflammasome has the broadest ability reported so far to respond to widely varied signals, ranging from infections by bacterial, fungal and viral pathogens to pore formation toxin, protein amyloid, extracellular ATP, and even inorganic substances such as silica and alum (9). It remains unknown whether such promiscuous activation is mediated by direct interactions between the signaling molecules and NLRP3 or by indirect mechanisms that employ other signal sensing proteins. Differing from other inflammasomes that require multiple proteins for their activation, recent studies revealed that caspase-11 is a non-canonical inflammasome that is activated by directly engaging intracellular LPS (10, 11).

Legionella pneumophila is an intracellular pathogen that causes a severe, atypical pneumonia termed Legionnaires' disease (12). Upon entering the host cell, *L. pneumophila* resides in a membrane-bound vacuole, in which the bacterium replicates. The establishment of the vacuole requires the Dot/Icm (defect in organelle trafficking/intracellular multiplication) transport system, which translocates a large number of protein substrates into host cells to re-orchestrate various cellular processes, including intracellular trafficking, lipid metabolism, protein synthesis and host cell death (13, 14). Effective targeting of such a large array of host processes renders *L. pneumophila* an excellent model to study cell biology in the context of bacterial infection (15, 16).

Despite being an outstanding "cell biologist", *L. pneumophila* is considered a poor "immunologist", as the infection of mammalian immune cells such as macrophages with this bacterium generates robust and successful immune responses, which often are less pronounced or even undetectable in cells infected by better-adapted pathogens (17). *L. pneumophila* is readily sensed by extracellular PRRs such as TLR4 and TLR5 (18) and its presence can activate multiple intracellular NLR and TLR sensors can be activated by *L. pneumophila* in a manner that requires a functional Dot/Icm transporter (15, 19). For instance, *L. pneumophila* is detected by the NOD receptors *in vivo*, since mice lacking NOD1 are impaired in neutrophils infiltration and in their ability to clear the bacterium (20) (Fig. 1). Further, a *L. pneumophila* mutant that aberrantly enters the cytosol triggers the activation of the noncanonical caspase-11 inflammasome, which senses intracellular LPS (10, 11) (Fig. 1). *L. pneumophila* can also be recognized by the AIM2 inflammasome (Fig. 1), probably by bacterial DNA "leaked" into the host cytosol by the Dot/Icm system (20). Since *L. pneumophila* also triggers Type I Interferon production in a STING-(stimulator of interferon genes) dependent manner, it is tempting to postulate that the "leaked" bacterial DNA also engages the cGAS (Cyclic GMP-AMP synthase)→c-di-AMP-GMP→STING pathway (21, 22) (Fig. 1). Moreover, infection by Dot/Icm-competent *L. pneumophila* significantly induces Type I IFN production probably by bacterial RNA "accidentally" delivered into the host cytosol by the Dot/Icm system (19, 23) (Fig. 1). These observations suggest that the Dot/Icm transporter delivers a wide variety of immune ligands into host cells or that some of the effectors are able to activate the immune responses when they biochemically attack host cellular processes. Indeed, such effector-triggered immunity (ETI) has been documented for effectors involved in inhibiting host protein synthesis (24). The potential ability of the Dot/Icm transporter to

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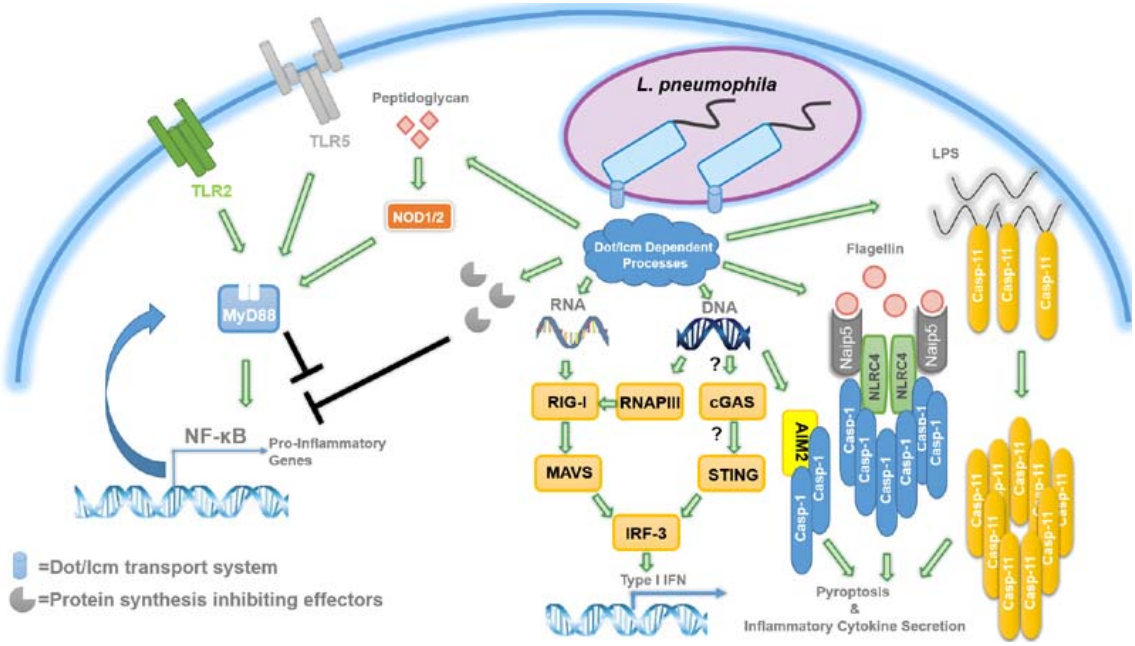

Figure 1. Innate immune recognition of *L. pneumophila.* A schematic depiction of the immune surveillance engaged by *L. pneumophila*. Note that the inhibition of protein synthesis conferred by the *L. pneumophila* is sensed by the host via a MyD88 dependent mechanism. Host perception of these activities promotes selective translation of pro-inflammatory genes such as IL-1α. RIG-I, retinoic acid-inducible gene 1; IRF-3, Interferon regulatory factor 3; STING, stimulator of interferon genes; MAVS, mitochondrial antiviral-signaling protein; RNAPIII, RNA polymerase III; cGAS, cyclic GMP-AMP synthase; NOD1/2, Nucleotide-binding oligomerization domain-containing protein 1/2; TLR2, Toll like receptor 2; TLR5, Toll like receptor 5; MyD88, Myeloid differentiation primary response gene 88; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; Naip5, NLR family, apoptosis inhibitory protein 5; NLRC4, NLR family CARD domain-containing protein 4; AIM2, Absent in melanoma 2; Casp-1, capase-1; Casp-11, caspase-11; IFN. Interferon; LPS, lipopolysaccharide.

deliver non-cognate substrates, including immune ligands flagellin and RpsL may arise from the necessity to recognize numerous cognate effectors with diverse secretion signals (14, 25).

The discovery of flagellin as a cytosolic PAMP is particularly intriguing; it led to the uncovering of the mystery behind the long-known fact that bone marrow derived macrophages (BMDMs) from most mice inbred strains are refractory to *L. pneumophila* infection (26). For example, challenging BMDMs from C57BL/6 mice with *L. pneumophila* results in bacterial clearance accompanied by pyroptosis (27). However, macrophages from the A/J mouse strain, allow robust intracellular replication of *L. pneumophila* (26). Taking advantage of the sharply different responses of these mouse lines to *L. pneumophila*, Dietrich *et al*. mapped the genetic element responsible for the permissiveness of A/J mice to the Lgn1 locus, and Diez *et al*. further pinpointed it to the *Naip5* gene (28, 29). The very same phenotype also was utilized to identify flagellin as the bacterial factor that dictates the outcome of the infection by screening for *L. pneumophila* mutants capable of successful intracellular replication in BMDMs from C57BL/6 mice (30, 31). Flagellin later was shown to accomplish this function by directly engaging Naip5 (8, 32). These exciting successes clearly demonstrate the great potential to elucidate unknown or underappreciated host responses using less adapted pathogens such as *L. pneumophila*.

The host cell death pathways are integrated components of the innate immune system (33) and the different types of host cell death distinctly influence the outcome of infection: pyroptosis and necrosis are pro-inflammatory, because the execution of these types of cell death is accompanied by the release of cellular contents into the extracellular milieu; whereas, apoptosis is considered to be immunologically silent, because the cellular contents are sealed in apoptotic bodies as the cell dies (34-36). In addition to these conventional pathways, accumulating evidence suggests the existence of lysosomal cell death, which appears to bear features of both pyroptosis and apoptosis (37). Lysosomal cell death has been shown to play pivotal roles in numerous pathological conditions, such as stroke, acute pancreatitis, parasitic and viral infections (37). The lysosome is the central signaling organelle for lysosomal cell death; permeabilization of the lysosomal membranes in response to distinct signals leads to the release into the cytosol of acidic contents as wells as various hydrolases such as the cathepsins (38). Because of their potentially broad substrate repertoire, hydrolases are capable of inducing and/or accelerating apoptosis, pyroptosis, necrosis, or cell death with mixed features. Certain cathepsins appear to be selectively released into the cytosol, and some of them have been shown to play a more dominant role than others under specific conditions (39). For example, cathepsin B plays a predominant role in involution and in tumor necropsies induced by Granulysin, while cathepsin D is more important in triggering apoptosis during the early commitment phase in T lymphocytes (40, 41). After the induction of LMP, many components of the classical apoptotic pathways are involved in lysosomal cell death (37). Thus, it is not unexpected that the latter form of cell death bears some features of the canonical apoptosis and, under certain conditions can be partially blocked by apoptosis inhibitors (42, 43).

Since its discovery in 1976, *L. pneumophila* has been intensively studied to analyze the mechanisms of its interactions with hosts. However, most of our understanding of *L. pneumophila* pathogenesis has come from the use of several laboratory strains such as Lp02, JR32 and AA100, which are all derived from clinical isolates (44, 45). Very little is known about how environmental strains of *L. pneumophila*, which are the primary source of infection outbreaks, interact with mammalian hosts. In our experiments to test several *L. pneumophila* strains recently isolated from hospital water systems (46), we found that none of these strains was able to replicate in BMDMs from A/J mice, which are permissive to most, if not all laboratory strains. By focusing on one such strain, LPE509, we established that infection of A/J BMDMs by wild type environmental *L. pneumophila* causes extensive cell death (46).

We pursued the hypothesis that LPE509 codes for a unique factor(s) that is absent or altered in the laboratory strains, and identified RpsL (30S ribosomal subunit protein S12) as a bacterial ligand that is recognized by primary mouse macrophages and is responsible for the growth restriction phenotype (47). Further analysis revealed that a point mutation in this protein, Lys88→Arg,

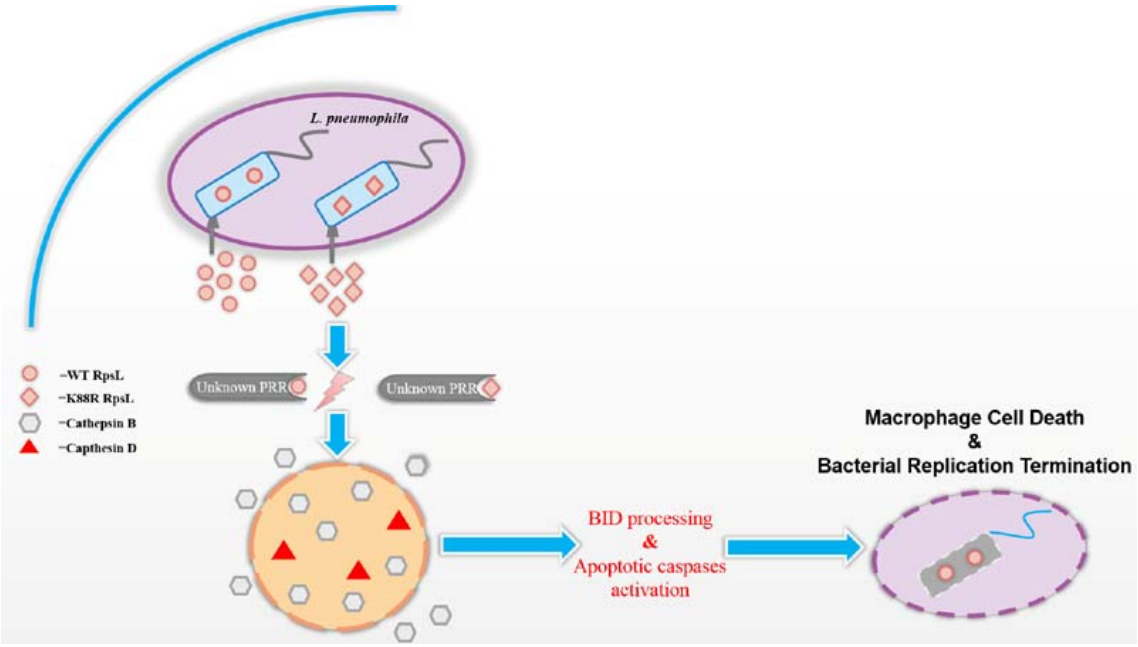

Figure 2. A schematic presentation of the model of the sensing of RpsL by macrophage. In this model, the bacterial ribosomal protein RpsL is leaked into the cytosol presumably by the promiscuous nature of the Dot/Icm secretion system. In the cytosol of the host cell, RpsL engages a yet unknown receptor to trigger the activation of pathways that lead to permeabilization of the lysosomal membrane, allowing the release of various hydrolases including cathepsin B into the cytosol. Cathepsin B then processes BID into tBID, which inserts into the outer membrane of the mitochondria to induce the release of cytochrome c and subsequent activation of the classic apoptotic cascade. These events eventually lead to host cell death and termination of bacterial replication.

camouflages the bacterium from host immune recognition. Consistent with this finding, all commonly used laboratory strains, such as Lp02, JR32 and AA100 turn out to harbor the K88R mutation in *rpsL* (45, 48). In contrast, original clinical isolates such as strain Philadelphia-1 that carries a wild type *rpsL* allele are unable to replicate in A/J BMDMs (47). We further demonstrated that replacing the wild type *rpsL* with the K88R allele allows strain Philadelphia-1 to replicate robustly in BMDMs from A/J mice. Conversely, replacing the K88R mutation in strain Lp02 with the wild type allele abolished its ability to replicate in A/J BMDMs. This growth restriction appears specific to primary murine macrophages, as this strain still replicates proficiently in its protozoan hosts, in the human macrophage-like cell line U937 and in murine embryonic fibroblasts (47). Consistently, these cell types do not detectably respond to intracellular RpsL, regardless of the delivery method, suggesting that RpsL itself cannot cause lysosomal membrane permeabilization (LMP) (47). These observations also suggest that RpsL functions by engaging a putative receptor whose expression is restricted to a narrow spectrum of immune cells or only in certain species, a phenomenon that has been described previously for PRRs such as cGAS and TLR13 (49-51). Furthermore, the murine macrophage-like cell line Raw 264.7, murine peritoneal resident macrophages and thioglycollate (TGC) induced peritoneal macrophages all respond differently to strains expressing wild type versus the K88R mutant of RpsL (Zhu and Luo, unpublished results). Such differences point toward potential variations in the expression of the putative RpsL receptor and/or other components of signaling pathway in these cell types. Further experiments aimed at examining how primary human macrophages such as monocytes respond to RpsL will be essential to establish whether the observed reactions occur in macrophages from species other than mouse.

The cell death associated the infections by *L. pneumophila* strains expressing wild type *rpsL* is unique, as it is independent of all known cell death pathways that are triggered by laboratory *L. pneumophila* strains (15, 47, 52). By pharmaceutical and genetic approaches, we established that the lysosomal cell death is involved in RpsL-sensing. Further analysis suggested that cathepsin B is important for the recognition of RpsL, as ablating this gene significantly delays macrophage death upon RpsL challenge. Despite this apparently critical role, macrophages lacking cathepsin B did not support productive intracellular growth of *L. pneumophila* harboring wild type *rpsL*, suggesting the involvement of other factors in the response to RpsL. This hypothesis is consistent with the fact that RpsL also induces the release of cathepsin D into the cytosol in macrophages (47). Cathepsin D is able to initiate cell death under certain conditions (53), and it may contribute to macrophage death at later time points after sensing RpsL. Based on these lines of evidence, we postulate a model in which a putative cytosolic receptor senses RpsL to initiate the destabilization of lysosomal membranes. The hydrolases that are subsequently released process various cytosolic proteins involved in apoptosis (e.g. Bid) to promote cell death, finally leading to the elimination of infected macrophages and the termination of bacterial replication (Fig. 2).

In summary, the identification of RpsL as a potential PAMP molecule sensed by mouse macrophages to trigger lysosomal cell death has once again demonstrated the effectiveness and complexity of the mammalian immune surveillance system. Apparently, the identification of the receptor(s) that senses RpsL is highly beneficial. Future studies of the pathways associated with the receptor are likely to advance our understanding of how the lysosomal pathway serves as part of the cytosolic immune defense against invading pathogens. On a broader sense, it will be interesting to test whether other pathogens are capable of inducing novel immune responses when placed in "unnatural" niches. For example, *Salmonella enterica* are divided into typhoidal and non-typhoidal serovars depending on their ability to establish systemic or local gastroenteritis infections (54). It will therefore be interesting to test whether non-typoidal *Salmonella* serovars, when artificially introduced to "unnatural" niches such as spleen and liver, would trigger robust host immune responses that are suppressed by typhoidal serovars. And if so, the identification of the microbial/host factor(s) that are responsible for such responses will surely advance our understanding of host-microbe interactions.

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