Immunology Taught by Bacteria

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Abstract
Introduction It has been proposed that the innate immune system might discriminate living and virulent pathogens from dead or harmless microbes, but the molecular mechanisms by which this discrimination could occur remain unclear. Although studies of model antigens and adjuvants have illuminated important principles underlying immune responses, the specific immune responses made to living, virulent pathogens can only be discovered by studies of the living, virulent pathogens themselves.
Methods and Findings Here, I review what one particular bacterium, *Legionella pneumophila*, has taught us about the innate immune response. Pathogens differ greatly in the mechanisms they use to invade, replicate within, and transmit among their hosts. However, a theme that emerges is that the pathogenic activities sensed by host cells are conserved among multiple pathogenic bacteria.
Conclusion Thus, immunology taught by *L. pneumophila* may lead to a more general understanding of the host response to infection.

Keywords *Legionella pneumophila* · inflammasome · innate immunity · flagellin · interferon

In 1996, Rolf Zinkernagel published a review in *Science* entitled "Immunology Taught by Viruses" [1]. Zinkernagel's message was targeted to a generation of immunologists that had, since Landsteiner [2], focused on dissecting immune responses to model antigens such as haptens or ovalbumin. While the use of model antigens has been essential for deciphering the basic mechanisms of adaptive immunity, Zinkernagel rightly argued that a deeper appreciation for viruses could provide new insights. Since the continued existence of viruses depends on their ability to evade the immune systems of their hosts, there may be no stronger evidence for the importance of a given immune function than a demonstration that a virus has evolved a mechanism to evade it. In urging immunologists to study viruses, Zinkernagel echoed Janeway's prior assertion that progress in immunology "will require a rediscovery of microbiology by immunologists" [3]. Certainly, the ensuing two decades of work in innate immunity, including the discovery of Toll-like and other innate receptors, has demonstrated how immunology can learn from microbiology.

Although viruses may be great teachers, they are also pedagogically problematic in many ways. Since viruses are highly co-evolved with their hosts, it is sometimes difficult to be certain whether an observed immune response to a virus is beneficial to the host or the virus. The evasion of many immune responses by viruses means that important immune responses are not always readily apparent or easy to study. And, although viral evasion of an immune response is strong evidence for the importance of that immune response, there is always the nagging worry that any immune response that can be easily evaded might not really be that important after all. Lastly, viruses are just one class of microbe that can cause disease, and it is unlikely that all the principles of immunity gleaned from viral infections will apply to other types of infection. Indeed, there is still much to learn about the fundamental biology of the immune response, and different infectious microbes can teach us different things. In this review, I illustrate how

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bacteria can teach us immunology by focusing on our recent progress in understanding the innate immune pathways that recognize *Legionella pneumophila*.

The Teacher: *L. pneumophila*

L. pneumophila is a gram-negative motile bacterium that causes a severe pneumonia called Legionnaires' disease. *Legionella* infection is typically acquired by humans through inhalation of a large dose of aerosolized bacteria, frequently arising from bacterial contamination of a cooling tower associated with a building's air-conditioning system. This mode of infection appears to be the explanation for the notorious outbreak of *Legionella* infections at the 1976 Legionnaires' Convention in Philadelphia, as well as several subsequent outbreaks. Once inhaled, *Legionella* is believed to replicate primarily in alveolar macrophages in the lung, though other cell types may also be targeted. Although *Legionella* infection can result in significant human morbidity and mortality, *Legionella*'s real "purpose" in life has little to do with us. Through most of its evolution, *Legionella*'s natural history appears to have occurred primarily in various species of freshwater amoebae. Humans are believed to be "accidental" and dead-end hosts, mere victims of "progress" that has come in the form of the recent invention of large-scale air-conditioning systems. *Legionella* does not appear to be transmitted from human to human [4], or even animal to animal [5], leading to the view that *Legionella* does not evolve in mammals and has thus probably not evolved mechanisms to evade mammalian immune responses.

The ability of *L. pneumophila* to infect macrophages appears to derive from the fundamentally conserved cell biology of amoebae and macrophages [6]. Replication of *L. pneumophila* in amoebae and in macrophages occurs within a membrane-bound compartment called the *Legionella*-containing vacuole (LCV). The creation of the LCV by *L. pneumophila* requires a bacterial type IV secretion system called the Icm/Dot system, a macromolecular channel that inserts in the phagosomal membrane and delivers bacterial proteins, termed effectors, into the host cell cytosol. Well over 150 *L. pneumophila* effectors are believed to be translocated by the Icm/Dot system into the host cell cytosol, where they carry out diverse functions. There is evidence that the Icm/Dot system can also translocate nucleic acids [7] and probably fragments of peptidoglycan as well [8]. The primary function of the Icm/Dot-translocated protein effectors appears to be creation of the LCV. *Icm/Dot*-deficient mutants are unable to replicate in host cells and are instead rapidly targeted to a compartment harboring late-endosomal markers such as LAMP1. Consistent with these observations, several translocated effectors have been shown to regulate vesicular traffic in infected cells and recruit membrane to the *L. pneumophila*-containing phagosome [6]. However, other translocated effectors appear to have functions unrelated to vesicular traffic. For example, a small family of translocated effectors functions to inhibit host protein synthesis, whereas other translocated effectors appear to function to inhibit host cell death.

Amoebae and vertebrates are separated by at least a billion years of evolution. Given that many intracellular pathogens exhibit relatively restricted host ranges, it is surprising that there is sufficient conservation of biology to allow such a dramatic jump of host species. For this reason, it is possible that the adaptability of *L. pneumophila* is explained by infection of cryptic vertebrate hosts in the environment. Vertebrate hosts that can sustain transmission of *L. pneumophila* have not yet been found, however, and there is no strong evidence that *L. pneumophila* is at all immune evasive. Although amoebae certainly have some primitive immune defense mechanisms, immunity in amoebae obviously does not resemble the comparatively sophisticated multicellular innate and adaptive immune systems present in vertebrates. Thus, *L. pneumophila* can be thought of as a highly educated cell biologist, but also as an ignorant immunologist.

Why would an immunologist want to study such an immunologically unsophisticated pathogen? As is borne out by a few examples discussed briefly below, it appears that one of the advantages of studying *L. pneumophila* is that it provokes robust host responses—responses that would likely have to be suppressed by better-adapted pathogens. Moreover, since *L. pneumophila* is not closely related to any other bacterial species that have been the subject of extensive immunological analysis, we have reason to believe that *L. pneumophila* may be able to teach us new things. A third reason for learning from *L. pneumophila* is that just as *L. pneumophila* is not adapted to humans, nor is it likely that the human immune system has evolved specifically to recognize *L. pneumophila*. The selective pressure that has driven mammals to respond vigorously to *L. pneumophila* has likely come not from *L. pneumophila* itself, but from other pathogens that have made a more significant imprint on our evolutionary history. Thus, whatever we learn from *L. pneumophila* is not likely to be relevant only to one bacterial species, but should allow us to discern broader principles of immune recognition.

Patterns of Pathogenesis: Immunology Taught by Living Pathogens

Since Janeway's exhortation to rediscover microbiology [3], immunologists have done just that, dissecting innate responses to microbial ligands such as lipopolysaccharide

or flagellin. Janeway referred to these stimulatory molecules as pathogen-associated microbial patterns (PAMPs), and the discovery of Toll-like receptors (TLRs) and other innate receptors has resoundingly vindicated Janeway's prediction that the host would express germline-encoded receptors that recognize conserved microbial molecules. TLRs were discovered primarily by the use of purified PAMPs or killed microbes, but of course, pathogens are more than just a "bag of PAMPs", and so a recent goal has been to extend the vision of Janeway to the study of innate responses to living, virulent microbes. Our hypothesis is that, in addition to sensing of pathogen-encoded molecules (PAMPs), hosts might also sense particular pathogen-encoded activities that we have termed "patterns of pathogenesis" [9]. Despite the diversity of pathogens, we posit that there may be a few common virulence-related activities—for example, cytosolic invasion or disruption of actin—that the immune system could detect in order to trigger specific host responses. Our hypothesis resembles a longstanding hypothesis from the plant immunity literature called the Guard Hypothesis [10], but few examples of guard-type mechanisms have been shown to occur in mammals.

Wild-type and isogenic Icm/Dot-deficient *L. pneumophila* mutants provide a simple genetic model for comparing infections with virulent and avirulent microbes. A theme that has emerged from this model is that, although the Icm/Dot system is essential for *L. pneumophila* replication, Icm/Dot expression is also associated with induction of several robust innate immune responses, some of which act to restrict bacterial replication. Below, I briefly discuss several independent immune surveillance pathways stimulated specifically by virulent Icm/Dot⁺ bacteria.

Cytosolic Sensing of Flagellin by the Naip5/Ipaf Inflammasome

More than 20 years ago, Friedman and colleagues discovered that macrophages from the A/J strain of mouse were several logs more permissive to *L. pneumophila* replication than were macrophages from other strains, including C57BL/6 [11]. Years of genetic analysis traced the resistance of C57BL/6 to the function of a single genetic locus, *Lgn1*, and ultimately to a single gene, *Naip5*, within this locus [12, 13]. *Naip5* bears homology to other genes such as *Nod1* and *Nod2* that had previously been shown to function as cytosolic sensors of different fragments of peptidoglycan. Thus, the molecular cloning of *Naip5* led to the immediate hypothesis that Naip5 detects a *L. pneumophila*-derived ligand, leading to induction of an anti-bacterial immune response. However, the nature of the ligand and the ensuing response were far from clear.

Nod1 and Nod2 both contain caspase activation and recruitment domains (CARDs) that recruit the same downstream kinase, Rip2, leading to NF–κB activation and transcriptional responses. By contrast, Naip5 lacks CARDs; moreover, Naip5 does not seem to activate NF–κB, and, in our hands, at least, does not appear to regulate significant transcriptional responses (though others report modest transcriptional effects [14]). Several studies have demonstrated that a functional *Naip5* allele correlates with altered maturation of *L. pneumophila*-containing phagosome [15–17], but there is no direct evidence these observed maturation differences are causally responsible for the observed restriction of bacterial replication. Nor is there a clear molecular understanding of how Naip5 regulates the phagosomal maturation process. We tend to favor the idea that host cell suicide, rather than altered phagosome trafficking, is primarily responsible for restriction of bacterial replication. Since *L. pneumophila* requires a host cell niche in which to replicate, rapid host cell death would be expected to curtail bacterial replication. This model originated from studies showing that a functional *Naip5* allele (from the C57BL/6 strain) correlated with increased cell death [18] and caspase-1 activation [19] in response to *L. pneumophila* infection. Evidence against the host cell death model has come from studies showing that permissive macrophages from the A/J strain of macrophage appear to activate caspase-1 and die as efficiently as restrictive C57BL/6 macrophages [20]. However, other reports using quantitative single cell assays [21, 22] have observed differences in host cell death between A/J and C57BL/6 macrophages. Moreover, consistent with the cell death model, macrophages from *Naip5*-deficient mice are permissive to *L. pneumophila* and fail to activate caspase-1 or die in response to *L. pneumophila* infection [23]. Nevertheless, future studies are necessary to resolve the signaling events that occur downstream of Naip5.

By contrast, the signaling events upstream of Naip5 are better understood. A breakthrough was the finding that flagellin-deficient mutants of *L. pneumophila* evade *Naip5*-mediated restriction and fail to activate caspase-1-dependent cell death, leading to the hypothesis that the cytosolic presence of flagellin activates Naip5 [22, 24]. These studies were the first to suggest that host cells survey the cytosol for the presence of flagellin. It was also shown that a cytosolic surveillance pathway detected cytosolic flagellin delivered by *Salmonella* [25, 26]. In the case of *Salmonella*, a type III secretion system delivers flagellin to the cytosol, whereas for *L. pneumophila*, the Icm/Dot type IV secretion system is the relevant translocon. Subsequent studies demonstrated conclusively that the C-terminus of flagellin is a sufficient signal to activate Naip5 and caspase-1 [23]. Naip5 appears to function with its paralog, Ipaf, which is also essential for detection of

flagellin and restriction of *L. pneumophila* replication [19, 25, 26].

Toll-like receptor 5 (TLR5) is a cell surface receptor that also recognizes flagellin. Notably, Naip5/Ipaf-dependent recognition of flagellin does not involve TLR5 [22, 24–26], and in fact, the two sensor systems respond to distinct parts of the flagellin molecule [23]. Why would host cells have evolved separate cytosolic and cell-surface detection systems for flagellin? We suspect that each flagellin-sensing system has its purpose and that the presence of flagellin in the cytosol is interpreted by the host as signifying a different and more serious threat than the presence of extracellular flagellin, which could result from transient bacteremia with harmless commensal bacteria. Flagellin can only reach the cytosol if delivered there by a microbe with a secretion system, and such microbes are almost certain to be pathogens. In fact, although it is a PAMP (flagellin) that is sensed by Naip5/Ipaf, it seems likely that flagellin serves only as a proxy—its detection is simply a way to detect a pathogen-associated activity, namely secretion of bacterial molecules into the cytosol.

Transcriptional Responses to Virulent *L. pneumophila*

Genetic elimination of the flagellin-sensing pathway by use of flagellin-deficient *L. pneumophila* mutants, or Naip5/Ipaf knockout mice, has little effect on the transcriptional responses made specifically to virulent *L. pneumophila*. For example, one prominent transcriptional response to *L. pneumophila* involves the induction of the cytokine interferon-β. This response requires bacterial expression of the Icm/Dot system, but is independent of Toll-like receptors, flagellin, Naip5, or Ipaf [27]. Instead, induction of type I IFN appears to involve, at least in part, the cytosolic RNA sensors RIG-I and/or MDA5 [28–30]. A role for RIG-I/MDA5 was unexpected, since cytosolic RNA sensors were previously thought only to detect viruses. The mechanism by which RIG-I and/or MDA5 are activated by *L. pneumophila* remains uncertain [29]. Regardless, the observed RIG-I-dependent induction of type I IFNs only occurs upon infection with live, virulent *L. pneumophila* and would not have been discovered using purified PAMPs or killed bacteria.

Cytosolic RNA sensors do not appear to be the only transcriptional pathways induced by virulent *L. pneumophila*. In addition, Icm/Dot-dependent activation of NF–κB downstream of Nod1 and Nod2 has been observed [8], presumably in response to translocated fragments of peptidoglycan. There also appears to be an Icm/Dot-dependent TLR/Nod1/Nod2/flagellin-independent pathway leading to induction of MAP kinases and several important inflammatory cytokines [8]. The molecular basis for this novel pathway is still uncertain, but it seems to be a specific host response to virulent *L. pneumophila*.

Conclusion

Studies with *L. pneumophila* illustrate the important point that pathogenic and non-pathogenic microbes can be discriminated by the innate immune system. In the cases discussed above, the sensors involved in pathogen-specific responses are not TLRs, but are instead a variety of cytosolic sensors that are selectively activated in response to virulent Icm/Dot+ *L. pneumophila* that accesses the cytosol as part of its virulence strategy. In other infection scenarios, TLRs may also be activated specifically in response to pathogenic microbes. For example, selective basolateral expression of TLRs might permit responses to occur only upon infection with microbes capable of disrupting epithelial barriers. It is clear that pathogens employ diverse mechanisms of pathogenesis and each pathogen is likely to provoke a distinct set of host responses. Regardless of what might also be learned from studies of other living virulent pathogens, *L. pneumophila* has proven to be a powerful tool for discovery of several cytosolic immunosurveillance pathways and serves as an example of how immunology can be taught by bacteria.

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