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Evasion of phagotrophic predation by protist hosts and innate immunity of metazoan hosts by *Legionella pneumophilla*

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Summary

Legionella pneumophila is a ubiquitous environmental bacterium that has evolved to infect and proliferate within amoebae and other protists. It is thought that accidental inhalation of contaminated water particles by humans is what has enabled this pathogen to proliferate within alveolar macrophages and cause pneumonia. However, the highly evolved macrophages are equipped more sophisticated innate defense mechanisms than protists, such as the evolution of phagotrophic feeding into phagocytosis with more evolved innate defense processes. Not surprisingly, the majority of proteins involved in phagosome biogenesis (~80%) have origins in the phagotrophy stage of evolution. There are a plethora of highly evolved cellular and innate metazoan processes, not represented in Protist biology, that are modulated by *L. pneumophila*; including TLR2 signaling, NF-κB, apoptotic and inflammatory processes, histone modification, caspases, and the NLRC-Naip5 inflammasomes. Importantly, *L. pneumophila* infects hemocytes of the invertebrate *Galleria mellonella*, kill *G. mellonella* larvae, and proliferate in and kill *Drosophila* adult flies and *Caenorhabditis elegans*. Although co-evolution with protist hosts has provided a substantial blueprint for *L. pneumophila* to infect macrophages, we discuss the further evolutionary aspects of co-evolution of *L. pneumophila* and its adaptation to modulate various highly evolved innate metazoan processes prior to becoming a human pathogen.

Legionella pneumophila is an intriguing environmental organism for its co-evolution with various protist hosts in the aquatic environment and its further evolution and ability to cause disease in humans upon accidental aerosolization due to recent human history of anthropogenic manipulation of water (Boamah, Zhou, Ensminger, & O'Connor, 2017; Rowbotham, 1980). Amplification of *L. pneumophila* upon growth within amoebae in the aquatic environment allows for sufficient delivery to alveolar macrophages where *L. pneumophila* replicate intracellularly resulting in pneumonia (Barker & Brown, 1994). Protists have long been considered to be primitive macrophages and, therefore, may have been capable of providing all the "training" *L. pneumophila* required to infect human macrophages (Molmeret, Horn, Wagner, Santic, & Abu Kwaik, 2005). However, macrophages have undergone numerous evolutionary changes into the cells they are today,

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with vastly superior pathogen sensing and innate defense mechanisms for fighting off invading microbes. This indicates some immune evasion functions of *L. pneumophila,* which have allowed it to be successful pathogen in macrophages, have likely been acquired through further evolution and selection in metazoan hosts with more advanced innate defense mechanisms of protist hosts.

Growth within the environmental amoebae host primes *L. pneumophila* for infection of human alveolar macrophages (M. R. W. Brown & Barker, 1999; Hoppe et al., 2017; Molmeret et al., 2005). Intracellular replication of *L. pneumophila* within any host requires the establishment a replicative niche, known as the *Legionella*-containing vacuole (LCV) (Molofsky et al., 2006; Richards, Von Dwingelo, Price, & Abu Kwaik, 2013; Segal & Shuman, 1999). Within the LCV, bacterial metabolism and nutrition-based genetic regulation based in nutrient availability governs bacterial proliferation and differentiation (W. Eisenreich, Heesemann, Rudel, & Goebel, 2013; Wolfgang Eisenreich, Rudel, Heesemann, & Goebel, 2017; Fonseca & Swanson, 2014; Grubmuller, Schauer, Goebel, Fuchs, & Eisenreich, 2014; Häuslein et al., 2017; Lama, Drennan, Johnson, Rubenstein, & Cambronne, 2017; Manske & Hilbi, 2014; Oliva, Sahr, & Buchrieser, 2018; C. T. Price, Richards, & Abu Kwaik, 2014). Biogenesis of the LCV depends on the Dot/Icm type IVb translocation/secretion system (T4SS) that injects >320 effectors into the host cytosol (Burstein et al., 2009; J. Coers et al., 2000; de Felipe et al., 2008; Ghosh & O'Connor, 2017; Schroeder, 2018; W. Zhu et al., 2011). Effectors are responsible for preventing lysosome fusion, recruitment of ER-derived vesicles to the LCV, evasion of the innate immune response, and modulation of a plethora of other host processes to promote intracellular survival and replication of *L. pneumophila* (Allgood et al., 2017; Bärlocher, Welin, & Hilbi, 2017; Fontana et al., 2011; Horwitz, 1983; Horwitz & Maxfield, 1984; Isberg, O'Connor, & Heidtman, 2009; ubori, Bui, Hubber, & Nagai, 2017; Luo, 2011; C. Price et al., 2017; C. T. Price, Al-Quadan, Santic, Rosenshine, & Abu Kwaik, 2011; Qiu & Luo, 2017; Swanson & Isberg, 1995a, 1995b). A large number of the effectors have been acquired from eukaryotic hosts through inter-kingdom horizontal gene transfer (Burstein et al., 2016; Gimenez et al., 2011; Gomez-Valero et al., 2011; Lurie-Weinberger et al., 2010). Eukaryotic-like effectors allow the pathogen to manipulate host processes to establish a replicative niche (de Felipe et al., 2008; Lurie-Weinberger et al., 2010; C. T. Price, Al-Khodor, Al-Quadan, & Abu Kwaik, 2010; C. T. Price, Al-Quadan, Santic, Jones, & Abu Kwaik, 2010). The precise hosts from which these genes have been acquired are unknown. It is more likely that the large arsenal of *L. pneumophila* effectors evolved through the long term adaptation to various protist hosts (Best and Abu Kwaik, 2018).

Environmental hosts of *L. pneumophila*

Protists are unlikely be the only natural environmental hosts of *L. pneumophila* (Fields et al., 1989, Rowbotham, 1986). When examining natural aquatic biofilms, *L. pneumophila* has been found with diverse Protists and metazoans, possibly as a parasite or as food (Abu Khweek & Amer, 2018; Abu Khweek et al., 2013; Rasch et al., 2016). Due to low detection of *L. pneumophila in situ*, infection of metazoans could not be determined (Rasch et al., 2016). *Caenorhabditis elegans*, a temperate soil nematode, has been shown to be a possible environmental reservoir of *L. pneumophila* (Brassinga et al., 2010; Rasch et al., 2016).

Furthermore, *in vitro* studies using *C. elegans* from environmental samples demonstrated the ability of *L. pneumophila* to colonize this metazoan (Rasch et al., 2016), which opens the possibility that if other lower metazoans were examined, new environmental hosts or reservoirs of *L. pneumophila* may be identified. Within the environmental sample, *L. pneumophila* was found to not be capable of *in vitro* infection of rotifera, copepod, or nauplius larvae (Rasch et al., 2016). However, the exact species were not determined and thus does not exclude other members of those phyla, class, or other naupli.

Additionally, *L. pneumophila* is capable of killing *Galleria mellonella* larvae, indicating the propensity to infect or persist with metazoans (Harding et al., 2012; Sousa, Silva, Moreira, Verissimo, & Costa, 2018). *L. pneumophila* replicates in the hemocytes, the phagocytes of invertebrates, of *G. mellonella* in a vacuole that resembles an LCV (Harding et al., 2012). *In vitro, L. pneumophila* can infect a wide range of metazoan host cells, including *Drosophila*derived cells (Table 1). In addition, the adult fruit fly is a model host for *L. pneumophila* proliferation and host lethality. Interestingly, *L. pneumophila* harbors a chitinase, which breaks down the cell wall component of fungi, arthropods, and some protists, further suggesting the ability to infect metazoans (DebRoy, Dao, Soderberg, Rossier, & Cianciotto, 2006). This relatively unexplored area of *L. pneumophila* ecology and its interaction with multicellular eukaryotes may provide better understanding of further evolution that enabled *L. pneumophila* to be an effective and successful pathogen that replicates within human macrophages, which degrade most other bacteria.

The draw-back to understanding how metazoan hosts have contributed to *L. pneumophila* evolution is that the evolutionary biology of the immune response has been studied in only two *L. pneumophila* natural hosts, *Dictyostelium discoideum* and *C. elegans* (Buracco, Peracino, Andreini, Bracco, & Bozzaro, 2017; Cardenal-Muñoz, Barisch, Lefrançois, López-Jiménez, & Soldati, 2018; Dunn et al., 2017; Mori, Mode, & Pieters, 2018; Swart, Harrison, Eichinger, Steinert, & Hilbi, 2018; L. Li & Faucher, 2016). Because the biology of many environmental organisms remains poorly understood, especially given their great diversity, it is reasonable that *L. pneumophila* would have encountered various innate pathogen recognition systems with various degrees of sophistication that would have aided in evolution of *L. pneumophila* to become an effective parasite of macrophages. For now, *D. discoideum* and *C. elegans* are the primary lower eukaryotic hosts for understanding how *L. pneumophila* has the ability to interact with the primitive hosts but it is not clear how this pathogen has evolved further to deal with advanced innate immune processes found in human macrophages (see below).

Protist resistant to *L. pneumophila* have also been identified but their relationship is poorly understood (Amaro, Wang, Gilbert, Roger Anderson, & Shuman, 2015). Failure to establish a replicative niche in an environmental non-permissive hosts could indicate host defense mechanism that *L. pneumophila* is unable to mitigate and provide more information on the pathogenicity of this organism. These protists may contain unique autonomous defense mechanism that *L. pneumophila* has not evolved to counteract. Resistance to *Legionella* is crucial to the protist hosts, as this ubiquitous environmental microbe and other environmental bacteria are part of the bacterial nutritional supply for unicellular protists.

Phagotrophy by protists versus phagocytosis

Phagotrophic feeding is the process by which unicellular eukaryotes acquire nutrients, and involves the extension of pseudopodia that envelop the food particle (Boulais et al., 2010). For ciliates, a specialized mouth-part, the cytostome, is the site where phagocytosis occurs. Bacteria serve as the most common food source for phagotrophic Protists and the nematode *C. elegans* (Arndt, 1988; Félix & Braendle, 2010). Multicellular *C. elegans* take up bacteria or unicellular eukaryotes through the mouth. If *L. pneumophila* survive through the pharyngeal grinders, it can colonize the intestinal lumen and invade into intestinal cells (Brassinga et al., 2010; Hellinga et al., 2015). Due to colonization in the gut, *C. elegans* can excrete *L. pneumophila* in mature intracellular forms (MIFs), which is an infectious cyst-like form of *L. pneumophila* that provides environmental resilience (R. A. Garduno, Garduno, Hiltz, & Hoffman, 2002; Hellinga et al., 2015; Robertson, Abdelhady, & Garduño, 2014).

In the laboratory, axenic strains of *D. discoideum* are used, circumventing the need to grow with a bacterial food source. These axenic strains have a null mutation in Ras-mediated neurofibromin, resulting in enlarged macropinosomes which allow for sufficient uptake of nutrients from the media (Bloomfield et al., 2015). Additionally, these strains are capable of phagocytosing larger particles (Bloomfield et al., 2015). This is also true for *Acanthamoeba*, *Hartmanella,* and other protist species used in research, but the exact mechanism for axenic growth is not always known. These axenic strains are used for studies with *L. pneumophila*. How the changes to phagocytic feeding have impacted uptake and intracellular replication of *L. pneumophila* is unknown.

Over a billion years of evolution, phagocytic feeding of unicellular eukaryotes has evolved into specialized pathogen-killing cells of multicellular eukaryotes (Boulais et al., 2010). In contrast, to phagotrophy by protists, phagocytosis was revealed by Elie Metchnikoff in 1882 and entails endocytosis and vesicular internalization of large materials such as bacteria, and is the mechanism to eradicate pathogens by phagocytic cells. Transitional organisms demonstrate this phenomenon; when encountering nutritional stress, *D. discoideum* will aggregate into a motile, multicellular slug (Kessin, 2001). Once nutrient conditions are favorable, it will differentiate into a fruiting body and release spores. Surveillance in the *D. discoideum* slug by Sentinel cells (S cells) retain their phagocytic ability to protect the multicellular slug and are selfsacrificing (G. Chen, Zhuchenko, & Kuspa, 2007). This ancient defense mechanism may have been adapted for defense functions in higher eukaryotes, which may have led to a more specialized cells like macrophages (Cosson & Soldati, 2008).

In *D. discoideum,* the phagosome matures into the post-lysosome, which is a process absent from macrophages, where the phago-lysosome is a dead-end for the internalized particle (Padh, Ha, Lavasa, & Steck, 1993). To finish the digestion process and expel undigested particles in *D. discoideum*, the WASH complex is recruited to the phagosomal membrane as the actin- nucleating promoting factor of Arp2/3 (Derivery et al., 2009). The V-ATPase is recycled from the post-lysosome and the compartment starts to reach a neutral pH. The actin coat fuses with the plasma membrane to release the undigested material (exocytosis). These post-lysosomal regulatory components are present in macrophages as well, but the post-

lysosomal-like stage is absent (Gomez & Billadeau, 2009). Interestingly, *L. pneumophila* Dot/Icm-translocated effector LegK2 contributes to preventing actin and vATPase localization to the LCV within *D. discoideum* (Fig. 1) (Clarke et al., 2002; Michard et al., 2015). Without V-ATPase at the LCV the vacuole fails to acidify, the lack of a proton gradient prevents lysosomal enzymes from functioning and the ability to transport ions for metal poisoning, all involved in bacterial killing. The LepA and LepB effectors of *L. pneumophila* are SNARE-like proteins that allow for nonlytic release of *L. pneumophila* in *Acanthamoeba castellanii* and *D. discoideum* (Fig. 1) (J. Chen et al., 2004). In *Tetrahymena* infected at high MOI, *L. pneumophila* has been shown to be released via pellets (Berk et al., 1998; Gardunow et al., 2008; Denoncourt et al., 2014; Faulkner et al., 2008). Post-lysosomal compartments of other protists are formed, but have not been well-studied (Stewart & Weisman, 1972).

Within both *D. discoideum* and human macrophages, Rab5 and Rab7 act as the masterminds of governing phagosome maturation (Dunn et al., 2017; Gutierrez, 2013). Rab5 drives phagosome maturation by transporting V-ATPases from the trans-Golgi network (Gorvel, Chavrier, Zerial, & Gruenberg, 1991; C. Zhang, Li, Zhang, & Xiao, 2011). Not surprisingly, the VipD, but not VipA effector of *L. pneumophila* interacts with activated Rab5 to prevent the downstream functions of Rab5, blocking vacuolar acidification (Fig. 1) (Gaspar & Machner, 2014; Ku et al., 2012; Prashar et al., 2018). Some of the LCVs will still acquire Rab7, a marker of the late endosome (Clemens, Lee, & Horwitz, 2000). While these interactions were tested in mammalian cells, Rab functions are well conserved in lower eukaryotes and would presumably function similarly in Protists (Boulais et al., 2010; Gotthardt et al., 2002). For an extensive review of the development of the LCV see Finsel *et al*. 2015 (Finsel & Hilbi, 2015).

The majority of phagosomal proteins (~80%) have origins in the phagotrophy stage of evolution (Boulais et al., 2010; Herweg et al., 2015). Overall, eukaryotic cellular processes involved in phagocytosis and trafficking have been well-conserved throughout eukaryotic evolution, aiding in the ease of *L. pneumophila* evolution to replicate in macrophages. However, far greater evolution from lower to higher eukaryotes represent in innate immune processes represent a larger challenge for evolution of *L. pneumophila* to adapt to macrophages of multicellular eukaryotes.

The primitive versus the sophisticated innate response of protists and macrophages

Protists and lower metazoans do not have immune responses, per se, but have aspects of defense that have evolved into sophisticated innate responses in higher metazoan through gene duplication and evolution (Pujol et al., 2001; Boulais et al., 2010). For *L. pneumophila*, experience with primitive defenses may have prepared the organism to infect higher metazoan but not when it comes to metazoan-specific defenses, as outlined below.

Each bacterial meal taken up by phagotrophy presents the opportunity for the protists to become infected. Therefore, protists have developed primitive immune responses to counteract parasitosis, which have served as the foundation for complex innate immune

functions in higher organisms (Desjardins et al., 1994; Desjardins, Houde, & Gagnon, 2005; Jutras & Desjardins, 2005). The foundation for the innate immune response came from cell-autonomous mechanisms in protists, which include lysozymes, ROS, metal poisoning, etc. (see (Dunn et al., 2017) for extensive review).

The *D. discoideum* transitional multicellular state is resistant to *L. pneumophila* (G. Chen et al., 2007). Within the motile slug of *D. discoideum*, S cells trap invading bacteria and are subsequently shed to protect the multicellular structure, functioning as a primitive innate immune system (X. Zhang & Soldati, 2016). When *D. discoideum* undergoing slug formation is exposed to *L. pneumophila*, the bacteria are swept into the slug by adhering to amoebae (G. Chen et al., 2007). After few hours, the majority of *L. pneumophila* could be found within the S cells (G. Chen et al., 2007). By 18h, the S cells containing *L. pneumophila* are shed from the slugs and found in the sheaths left behind (G. Chen et al., 2007). Even if *L. pneumophila* is directly injected into the slug, the bacteria still follow the same fate (G. Chen et al., 2007). Interestingly, *L. pneumophila* is not visible within the shed S cells but appears to be surrounded by cell debris (G. Chen et al., 2007). At the time of these studies, it was not known that S cells will release extracellular DNA traps (ETs) of mitochondrial DNA involved in NETosis, which could have been the observed “cell debris” (Fig. 1) (X. Zhang, Zhuchenko, Kuspa, & Soldati, 2016). ET release by *D. discoideum* S cells requires the NOX enzyme to generate reactive oxygen species (ROS) (Fig. 1) (X. Zhang et al., 2016). The emergence of NOX enzymes and multicellularity have been proposed to coincide with the origin of DNA-based defense strategies (X. Zhang & Soldati, 2016). NOX enzymes can also be found in *Naegleria gruberi*, a Protist host of *L. pneumophila*, which may have lost multicellular traits (Rowbotham, 1980; X. Zhang & Soldati, 2016). ETs appear to be an ancient defense mechanism that *L. pneumophila* would likely encounter in the environment. However, how *L. pneumophila* deals with ETs is unknown. There is evidence to suggest that ETs are an effective mechanism for controlling *L. pneumophila* with the motile slug of *D. discoideum* (G. Chen et al., 2007).

ETs of neutrophils (NETs) are a recent discovery and have been popularized in the field of pathogenesis, but they are not the only immune cells capable of extruding DNA. Less studied, is the ability of macrophages to produce ETs (METs) (Boe Devin, Curtis Brenda, Chen Michael, Ippolito Jill, & Kovacs Elizabeth, 2015; Chow et al., 2010). METs are comprised of lysozyme, myeloperoxidase, and nuclear and mitochondrial DNA that are released in response to microbes, microbial products, or cytokines (Fig. 1) (Boe Devin et al., 2015). *Mannheimia hemolytica* elicit MET formation in bovine alveolar macrophages but not in bovine monocyte-derived macrophages, indicating this event may be a pathogen- or site-specific event (Aulik, Hellenbrand, & Czuprynski, 2012). Within the population of macrophages infected by a pathogen, only a small population (<25%) undergoes METosis (Aulik et al., 2012; Chow et al., 2010; Liu et al., 2014). High levels of inflammatory cytokines, like TNF-α, induce generation of METs *in vitro* (Mohanan, Horibata, McElwee, Dannenberg, & Coonrod, 2013). *L. pneumophila* is known to induce TNF-α expression during infection, which may contribute to the production of METs (Blanchard, Djeu, Klein, Friedman, & Stewart, 1987; Chang, Amemura-Maekawa, Kura, Kawamura, & Watanabe, 2004). It is currently unknown whether METs are produced in response to *L. pneumophila*. This could be one way to control large numbers of *L. pneumophila* being released

extracellularly during infection. In humans, this may contribute to the alveolar damage seen in the lungs during infection (Jäger et al., 2013).

Control of most antimicrobial functions of *Drosophila melanogaster,* which is a model host for *Legionella*, is under the control of the Immune deficiency (Imd) pathway, responsible for activating NF-κB (Dorer, Kirton, Bader, & Isberg, 2006; Myllymäki, Valanne, & Rämet, 2014). Many proteins within this pathway contain a death domain, which can be found in combination with ankyrin repeats, leucine-rich repeats (LRR), TIR domains, and others (L. Aravind, Dixit, & Koonin, 1999; Finn et al., 2016; Myllymäki et al., 2014). *D. discoideum* has one death domain containing protein, *C. elegans* contains 24, while *Homo sapiens* have 124 (Cosson & Soldati, 2008; Finn et al., 2016). Interestingly, four *Legionella* species contain death domains: *Legionella norrlandica, Legionella sainthelensi, Legionella tusonenis,* and *Legionella longbeachae*, but their function is unknown (Finn et al., 2016). To note, death domains are not exclusive to eukaryotes, they can be found in many prokaryotes and archaea, but have only been studied in eukaryotes.

Modulation of Toll-like receptors signaling, a late evolutionary process, by *L. pneumophila*

It is thought that nematodes diverged before TLRs were co-opted for immune signaling (Irazoqui, Urbach, & Ausubel, 2010). The TLRs of higher organism recognize bacterial patterns through LRRs and signal through adaptor proteins with Toll/interleukin-1 receptor (TIR) domains (Akira, Uematsu, & Takeuchi, 2006; Burch-Smith et al., 2007; Xu et al., 2000). In mice, TLR4 is unresponsive to *L. pneumophila*; instead TLR2 is activated in response to the atypical LPS of the pathogen with long branched fatty acids in the lipid A moiety (Akamine et al., 2005; Girard et al., 2003). In human macrophages but not murine macrophages, the type-II secretion system (T2SS) dampens signaling through the MyD88-TLR2 pathway (Fig. 1) (Mallama, McCoy-Simandle, & Cianciotto, 2017). What environmental factors selected for or conserved the ability for *L. pneumophila* to dampen TLR2 signaling is unknown. Additionally, human TLR4 polymorphisms are associated with disease resistance (Fig. 1) (Thomas R Hawn et al., 2005). *L. pneumophila* also interacts with TLR5 through recognition of flagellin (T. R. Hawn, Smith, Aderem, & Skerrett, 2006). Two TIR domain-containing protein are present in *D. discoideum*, TirA and TirB (Fig. 1) (Table 2) (G. Chen et al., 2007). The S cells of *tirA*-deficient *D. discoideum* are killed by *L. pneumophila* (G. Chen et al., 2007). However, the exact function of these proteins is unknown.

C. elegans does express one TLR, TOL-1, which is most similar to *Drosophila* Toll-8, that is required for protection from *Salmonella enterica* but not all bacterial species tested (Table 2) (Pujol et al., 2001; Tenor & Aballay, 2008). Interestingly, TOL-1 plays a more important role in development for *C. elegans* and it is thought that nematodes diverged before TLRs were coopted for immune signaling (Irazoqui et al., 2010). If TOL-1 in *C. elegans* contributes to dampening the ability of *L. pneumophila* to replicate within the nematode is unknown.

Along with coding for TOL-1, *C. elegans* contains homologs of the mammalian downstream signal transduction components TRF-1, PIK-1, and IKB-1 (homologs of human TRAF1, IRAK, and IκB, respectively) but they do not seem to play a role in pathogen resistance, supporting the idea that some genes were coopted for immune functions likely following major gene duplication events in Euteleostomi and Bilatera (Table 2) (Boulais et al., 2010; Pujol et al., 2001). In contrast to higher metazoans, *C. elegans* does not contain the downstream signaling protein, of TRF-1, PIK-1, and IKB-1 - NF-κB (Pujol et al., 2001).

Modulation of metazoan Caspases, apoptotic/anti-apoptotic pathways, and the NLRC4 inflammasome by *L. pneumophila*

Caspases or caspase-like proteins consist of a conversed group of enzymes involved in programmed cell death or cell cycle regulation proteins in eukaryotes and even some prokaryotes (Bell & Megeney, 2017). Paracaspases can be found in animals and *D. discoideum*, while metacaspases are present in fungi, plants, and some Protists (L Aravind & Koonin, 2002; Trzyna, Legras, & Cordingley, 2008; Anthony G. Uren et al., 2000). Metacaspase of *Acanthamoeba castellanii* stimulates encystation and is over expressed at <20°C, contributing to elimination of *L. pneumophila* (Ohno, Kato, Sakamoto, Kimura, & Yamaguchi, 2008).

Within *C. elegans* CED-3, the only caspase of the nematode, acts as both the initiator and executioner but also regulates stem cell-like seam cells (Ellis & Horvitz, 1986; Weaver et al., 2014). The cell death pathway in *C. elegans* can be simplified to a four-protein pathway: EGL-1 (human equivalent BID, BIM) blocks CED-9 (Bcl2), which in turn blocks CED-4 (Apaf-1), allowing for CED-3 (caspase 9) to induce apoptosis (Table 2) (for extensive review of caspase regulation with human and nematode comparisons see review (Riedl & Shi, 2004)).

L. pneumophila infection of human macrophages triggers robust caspase-3 activation, the executioner of cell death, but *L. pneumophila* prevents apoptosis (Fig. 1) (Abu-Zant et al., 2007; Gao & Kwaik, 1999; Molmeret et al., 2004; Wenhan Zhu, Hammad, Hsu, Mao, & Luo, 2013). This tug-of-war is conducted by Dot/Icm T4SS effectors (Krause & Amer, 2016). The effectors VipD, Ceg18, Lem12, LegS2, and Lpg0716 induce caspase-3 activation in mammalian cells (Wenhan Zhu et al., 2013). VipD destabilizes the mitochondrial membrane, releasing cytochrome *c* (Fig. 1) (Wenhan Zhu et al., 2013). The mechanism of action for the other four effectors is unknown. On the other end of the spectrum, *L. pneumophila* upregulates antiapoptotic genes in macrophages notably ones involved in NF-κB activation (TRAF5, TNF, Bcl10, etc) or genes whose expression is regulated by NF-κB (bcl2 and xiap) (Abu-Zant et al., 2007).

L. pneumophila T4SS effector, SidF, interacts with two pro-apoptotic members of the Bcl2 family to inhibit their pro-death functions in macrophages (Fig. 1) (Banga et al., 2007). This may be possible through conserved domain homology with lower metazoans that this ability was developed in a host like the nematode and remains functional within macrophages. *D. discoideum* does undergo programmed cell death but its only paracaspase is not required (RoisinBouffay et al., 2004).

Inhibitor of apoptosis proteins (IAPs) all contain baculoviral IAP repeats (BIR) and are also conserved among metazoans (Anthony G. Uren, Coulson, & Vaux, 1998; Verhagen, Coulson, & Vaux, 2001). These proteins inhibit apoptosis by acting as direct inhibitors of caspases. However, in *C. elegans* the two IAPs, BIR-1 and BIR2, are unlikely to act as general cell death inhibitors, a function that likely evolved later in the metazoan lineage, but instead are more similar to human Birc5 (Survivin) which is also involved in the regulation of cell division alongside its ability to inhibit caspase activation (Table 2) (Fraser, James, Evan, & Hengartner, 1999; Verhagen et al., 2001). Interestingly, through BLAST identification, one protein in *Legionella steeli* contains a BIR domain with conserved binding sites. BIR proteins have been characterized in vertebrates, yeast, viruses, and nematodes (Anthony G. Uren et al., 1998). BLAST analysis of amoebozoa and ciliphora indicate few BIR-containing proteins exist among sequenced species within either of these taxonomic groups. Of note, one strain of *Tetrahymena thermophila*, a natural host of *L. pneumophila* contains a single BIR-containing protein, as determined by BLAST (Kikuhara, Ogawa, Miyamoto, Nikaido, & Yoshida, 1994). Whether *L. steeli* and *T. thermophila* BIR proteins function like metazoan IAPs or *C. elegans* BIR proteins is unknown nor is their contribution to intracellular replication known.

In human macrophages, Xiap and Birc3/Ciap2 are upregulated during infection of *L. pneumophila,* which likely aid in preventing apoptosis (Abu-Zant et al., 2005; Losick & Isberg, 2006). The most notable IAP is mouse baculoviral IAP repeating-containing 1 protein (Birc1 or Naip5) which restricts *L. pneumophila* replication in mice (Diez et al., 2003). NLRC4 (also known as Ipaf) interacts with Naip5 and recognizes *L. pneumophila* flagellin, leading to the NLRC4-Naip5 inflammasome, activation of caspase-1, −7 and −11, and delivery of *L. pneumophila* to the lysosome (A. Amer et al., 2006; Appelt & Heuner, 2017; Casson & Shin, 2013; Jörn Coers, Vance, Fontana, & Dietrich, 2007; Halff et al., 2012; He & Amer, 2014; M. Lamkanfi et al., 2007; Lamkanfi, Kanneganti, Franchi, & Núñez, 2007; Lightfield et al., 2008; Molofsky et al., 2006; Speir et al., 2017). However, in human macrophages and the permissive A/J mouse strain, the Naip5 allele is defective in detecting *L. pneumophila* flagellin, thus caspase-1, −7, and −11 are not activated, allowing for robust intracellular replication (Fig. 1) (Akhter et al., 2012; Akhter et al., 2009; A. Amer et al., 2006; Molmeret et al., 2004; Wright et al., 2003; Yamamoto, Klein, Newton, Widen, & Friedman, 1988).

CED-4 in *C. elegans* is homologue to NLRC4- and Apaf-1-like protein that is involved in the cytochrome *c*-dependent activation of caspase-3 (humans) or CED-3 (*C. elegans*) (Table 2) (Geddes et al., 2001; Poyet et al., 2001; Zou, Henzel, Liu, Lutschg, & Wang, 1997). VipD membrane destabilization and subsequent release of cytochrome-*c* would likely function similar in the nematode host due to these conserved mechanisms (Wenhan Zhu et al., 2013). Ced4/Apaf1 family acts as critical regulators of apoptosis in humans and *C. elegans* and NF-κB signaling pathways in humans (Geddes et al., 2001).

Like the tug-of-war with apoptosis, *L. pneumophila* also interferes with host autophagy pathways (Joshi & Swanson, 2011; Khweek et al., 2013). In permissive mice, when Atg5, involved in extending the membrane of autophagic vesicles and also acts as a pro-apoptotic molecule targeted to the mitochondria, is silenced by RNAi, replication of *L. pneumophila* is

enhanced (Matsuda, Fujii, & Yoshida, 2009). However, when autophagy is induced by 2-dexy-d-glucose, pathogen replication in permissive mice is inhibited (Matsuda et al., 2009). Similar to the progression of autophagosomes, during the early stages of LCV development, Atg7 and Atg8/LC3 are acquired on the LCV then lost (A. O. Amer & Swanson, 2005; Choy et al., 2012). While these studies were done in mice, Atg proteins are evolutionarily conserved and are also described in *D. discoideum* (Otto, Wu, Kazgan, Anderson, & Kessin, 2003). Atg9 mutants in *D. discoideum* are more permissive to intracellular replication by *L. pneumophila* but showed a strong defect in phagocytosis(Tung et al., 2010). Several T4SS effectors have are involved in modulation of host autophagy by *L. pneumophila*. The RavZ effector irreversibly deconjugates Atg8 in mammalian cells (Choy et al., 2012); the *Lp*SpI effector disrupts sphingolipid metabolism (Monica Rolando et al., 2016); while the Lpg1137 effector degrades syntaxin 17, blocking starvation-induced autophagy (Arasaki & Tagaya, 2017). These are highly evolved metazoan processes that are modulated by specific effectors of *L. pneumophila*, indicating selection and adaptation of the pathogen to multi-cellular eukaryotic hosts.

There is discrepancy on whether the LCV is diverted to the macroautophagy pathway or the LCV is transformed to resemble the rough ER, without the need for macroautophagy (Otto et al., 2004). In *D. discoideum* macroautophagy has been shown to be dispensable for intracellular replication of *L. pneumophila* (Otto et al., 2004; Monica Rolando et al., 2016). Differences in trafficking and interaction with host pathways may be related to the evolution of the host. Controlling macroautophagy in higher eukaryotes, which acts a defense mechanism against intracellular pathogens, may be more important than in lower eukaryotes (Deretic, 2006, 2011).

Modulation of metazoan NF-κB by *L. pneumophila*

Although the evolutionary origin of NF-κB has yet to be determined (Friedman & Hughes, 2002; Irazoqui et al., 2010), its role in programmed cell death and innate immunity is likely only present in vertebrates (L. Aravind et al., 1999). NF-κB is activated and translocated to the nucleus of the human and mouse macrophages during infection of *L. pneumophila;* and a functional T4SS is required (Fig. 1) (Abu-Zant et al., 2007; Losick & Isberg, 2006). Early NF-κB activation in *L. pneumophila*-infected macrophages occurs by TLR5 recognition of *L. pneumophila* flagellin (Fig. 1) (Bartfeld et al., 2009; Thomas R. Hawn et al., 2003). Prolonged nuclear translocation of NF-κB is necessary for host cell survival after *L. pneumophila* infection but how that is sustained is unknown (Bartfeld et al., 2009; Losick & Isberg, 2006). The LnaB T4SS effector of *L. pneumophila* activates NF-κB and to a degree so do the LidA, SidM, SidA, SidE, SidH, VpdA, LegA12 and LegA5 effectors (Cambronne & Roy, 2007; Losick, Haenssler, Moy, & Isberg, 2010; Luo & Isberg, 2004; Machner & Isberg, 2006; Murata et al., 2006; VanRheenen, Luo, O'Connor, & Isberg, 2006). But none are shown to bind NF-κB. So, is NF-κB activation "accidental", a side-effect of some other function of these proteins, or a direct function that was honed in a yet-to-be-identified metazoan host of *L. pneumophila*? LegK1 of *L. pneumophila* interacts with IκB, which sequesters NF-κB, within mammalian host cells, an ability that could have been selected for through intracellular replication in *C. elegans* due to an IκB homolog (Ge et al., 2009; M. D. Jacobs & Harrison, 1998; Losick et al., 2010; Pujol et al., 2001). However, the role of NF-

κB in programmed cell death is likely only present in vertebrates (L. Aravind et al., 1999). Control over NF-κB is crucial to intracellular survival of *L. pneumophila*, which supports the notion that other higher eukaryotes in the environment have likely played an important part in additional "training" and further evolution of *L. pneumophila* to modulate highly evolved metazoan processes and infect human macrophages.

Modulation of phagocyte chemotaxis and cell migration by *L. pneumophila*

Leukocytes and *D. discoideum* move in a manner known as amoeboid migration (Artemenko, Lampert, & Devreotes, 2014). To move, cells rapidly protrude and retract pseudopods which are driven by actomyosin contractility, weak cell-substrate interactions, and lack of matrix degradation (Artemenko et al., 2014; Lämmermann et al., 2008). In *D. discoideum*, a small family of cAMP receptors drive chemotaxis, whereas in leukocytes, a much larger family of chemokine receptors govern chemotaxis (Dormann, Vasiev, & Weijer, 2000; Vasiev & Weijer, 2003). The core components are remarkably conserved either by sequence or functional homology between these leukocytes and *D. discoideum* (Artemenko et al., 2014). For a comprehensive review of the common chemotactic mechanisms between *D. discoideum* and leukocytes see Artemenko *et al.* (Artemenko et al., 2014).

Chemotaxis is an important but understudied function in the context of *L. pneumophila* infection. How intracellular infection alters host cell migration is unknown and if there is an altered phenotype, what bacterial factors are contributory? *L. pneumophila* inhibits aggregation and migration of *D. discoideum*, and migration of murine macrophages and human PMNs, in a T4SS-dependent manner (S. Simon, Wagner, Rothmeier, Muller-Taubenberger, & Hilbi, 2014). T4SS effector, LegG1, a Ran activator, aides in migration of *D. discoideum* and murine macrophages and in intracellular LCV motility (Rothmeier et al., 2013; S. Simon et al., 2014). Interestingly, a Δ*legG1* mutant of *L. pneumophila* inhibits migration even more so than WT *L. pneumophila* (S. Simon et al., 2014). LegG1 also alters the directionality of these host cells, preventing forward migration (S. Simon et al., 2014).

Autoinducer LAI-1 of *L. pneumophila* inhibits chemotaxis and cell migration of *D. discoideum* and murine macrophages (Sylvia Simon et al., 2015). In mammalian cells this is IQGAP-1- and Cdc42-dependent but not RhoA or Rac1 (Sylvia Simon et al., 2015). Full length homologs for IQGAP-1 have been found in *C. elegans* and partial protein homologs have been identified in *D. discoideum* (containing only the GAP-related domain that mediates binding of Cdc42 and Rac1 but not RhoA or Ras, and the RasGAP carboxyl terminus) (Table 2) (Briggs & Sacks, 2003; Faix et al., 2001). Additionally, homologs for Rho family, which includes Cdc42 and Rac1, have been identified in *D. discoideum*, indicating that the ability to manipulate mammalian cell migration has likely evolved in *L. pneumophila* within the Protist hosts (Bush, Franek, & Cardelli, 1993; Rivero et al., 1999; Vlahou & Rivero, 2006). Remarkably, IQGAP-1 is involved in many other signaling pathways and coordinates multiple cellular activities such as chemokine and growth factor-dependent cell proliferation, adhesion, and phagocytosis. However, its depletion does not affect the ability of *L. pneumophila* to replicate in A549 epithelial cells (M. D. Brown & Sacks, 2006; Sylvia Simon et al., 2015; White, Erdemir, & Sacks, 2012). Control of a large number of cellular processes through IQGAP-1could be very beneficial to *L. pneumophila*.

Alveolar macrophages, lung fibroblasts and epithelial cells secrete IL-8, a potent chemotactic and activator of neutrophils. *L. pneumophila* infection of alveolar macrophages and epithelial cells induces IL-8, in an NF-κB-dependent manner (Chang et al., 2004; Kunkel, Standiford, Kasahara, & Strieter, 1991; Teruya et al., 2007). Although little is known about the role of IL-8 and cell migration during *L. pneumophila* infection, modulation of such highly evolved metazoan processes by *L. pneumophila* is a clear adaptation of the pathogen to more evolved metazoan hosts.

Blocking host cell motility could be beneficial to *L. pneumophila* to dampen the host immune response or to prevent energy expenditure. Alternatively, alterations in host cell migration could be an untargeted consequence of interference in other host cell processes that interact with host cytoskeletal and/or microtubular components, like formation of the LCV and recruitment of vesicles.

Host histone modification by *L. pneumophila*

Histones are conserved throughout the eukaryotic lineage (Nuñez-Corcuera, Birch, & Williams, 2011; Waterborg, 2012). However, the post-translational modification profile of H3 varies greatly among species and is more complex in mammals than lower eukaryotes, unlike H4 which is less modified and more consistent across species (Garcia et al., 2007). One T4SS effector, RomA, contains a eukaryotic SET-domain, which catalyzes lysine methylation of histones resulting in the downregulation of host gene expression (M. Rolando et al., 2013). RomA specifically targets histone H3 for trimethylation at a residue, K14, not previously known to be otherwise methylated in mammals (M. Rolando et al., 2013). It could be that posttranslational modification of this residue is more common in Protists or lower metazoans than mammals, and provided a greater replication benefit for *L. pneumophila* in the environment. RomA is required for intracellular replication in human macrophages and to a greater extent in *Acanthamoeba castellanii* (M. Rolando et al., 2013). RomA is also responsible for methylating non-histone proteins (Schuhmacher et al., 2018). Additionally, the T4SS effector, LegAS4, a homolog of RomA, has been shown to methylate K14 of H3, as well as K4 and K9 (T. Li et al., 2013; M. Rolando & Buchrieser, 2014; Son et al., 2015). Methylation of K9 by LegAS4 increases rDNA transcription (Son et al., 2015). It is currently unknown whether these two effectors are working synergistically or antagonistically in the host and if that is speciesdependent.

The foundations for epigenetics in histone modification may be conserved but the nuances of post-translational modifications vary. However, *L. pneumophila* modifies histones of may have evolved in lower eukaryotes but may not have the same effect in human macrophages, or the effect is accidental rather than being shaped by evolution and adaptation. It will be interesting to see how differences between these evolutionarily distant hosts change *L. pneumophila* epigenetic modifications.

Conclusion

Many aspects of the ability *L. pneumophila* to infect human macrophages can be defined by its relationship with protists. Although it is more likely that the large arsenal of *L.*

pneumophila effectors evolved through the long term adaptation to various protist hosts (Best and Abu Kwaik, 2018), it is unlikely that these lower eukaryotes could have provided all the training necessary for successful evolution of *L. pneumophila* to evade the more evolved innate defense processes and proliferate in metazoan macrophages. Many findings point towards a putative role for metazoans in the evolution of *L. pneumophila* and its ability to replicate in macrophages Effector modulation and control of NF-κB (Abu-Zant 2007, Losick 2006, bartfeld 2009, Ge 2009), including the ability to dampen TLR2 signaling (Mallama 2017), and the upregulation of anti-apoptotic genes regulated by NF-κB (Banga et al., 2007; Abu-Zant et al., 2007).

Difficulty in probing intracellular *L. pneumophila* within eukaryotes in environmental samples and difficulty identifying the putative eukaryotic hosts has been the major limitation in confirming a larger natural reservoir for *L. pneumophila*. Higher eukaryotes present greater challenge for *L. pneumophila* due to their more sophisticated innate immune processes. It could be that environmental metazoans serve only as reservoirs for *L. pneumophila*, where intracellular replication is limited but persistence or transient infection occurs. This could still allow for selective pressure and evolution of defense mechanism in *L. pneumophila* against process that are more evolved to what is found in macrophages.

Metazoans in the aquatic environment are unavoidable by *L. pneumophila*, which has the propensity to infect evolutionarily distant hosts. *L. pneumophila* manipulate many cellular processes that are highly conserved through evolution of eukaryotes, such as the endosomal-lysosomal degradation pathway, histone methylation, cell migration, prenylation, and the ubiquitin-proteasome system. However, it is highly unlikely that evolution of *L. pneumophila* from a Protists parasite into infection of human macrophages was simply due the accidental aerosols transmission after human industrialization and manipulation of the aquatic environment within the past ~100 years. However, *L. pneumophila* modulates various highly evolved metazoan processes absent from protist hosts. These include TLRs, mTOR, NF-κB, inflammasomes, caspases, and apoptotic and anti-apoptotic pathways. Therefore, we hypothesize that some lower metazoan species are likely to be natural hosts for *L. pneumophila* and these have played a key role in further evolution to enable *L. pneumophila* to manipulate numerous highly evolved metazoan-specific innate defense processes in order to proliferate within higher metazoan cells human macrophages and cause pneumonia.

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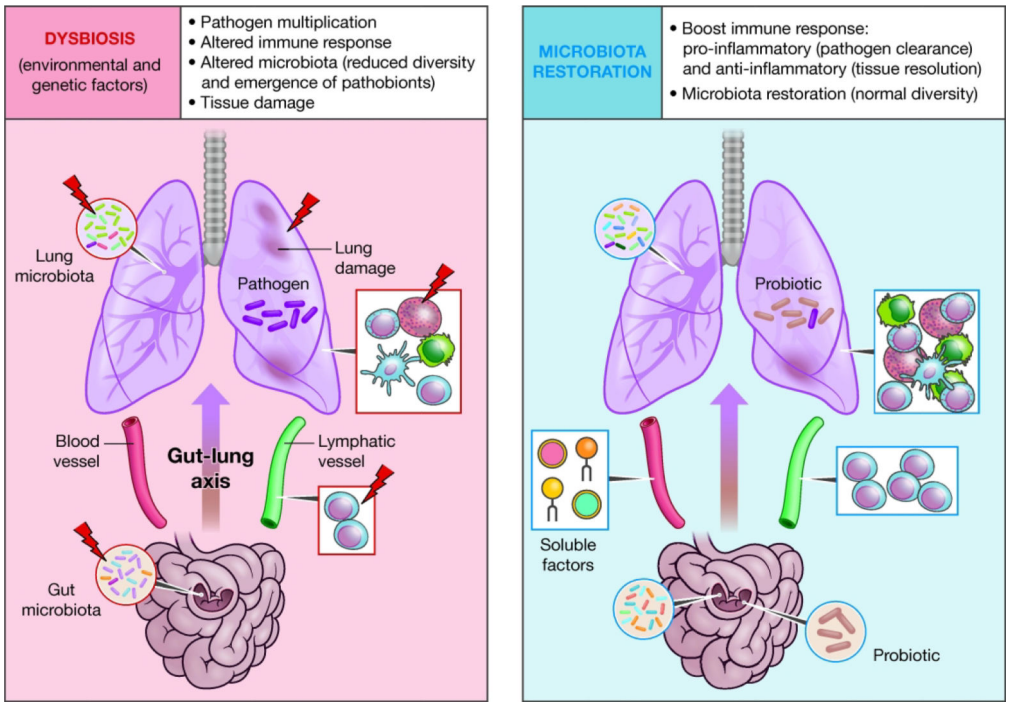

Figure 1.

Manipulation of evolutionarily conserved and metazoan-specific innate defense processes by *L. pneumophila*. The endosomal-lysosomal degradation pathway, which is effectively evaded by *L. pneumophila* in macrophages and Protists, is highly conserved through evolution. The VipD effector of *L. pneumophila* binds the host Rab5 to prevent vacuolar maturation and acidification. Extracellular traps (ETs) of mitochondrial DNA in *D. discoideum* requires NOX and ROS; whereas METosis in human macrophages consists of mitochondrial and nuclear DNA and is not ROS-dependent. A) In human macrophages but not Protist hosts, TLR5 engagement with bacterial flagellin triggers early activation of NF-κB. The LegK1 effector binds IκB allowing for NF-κB activation. TLR2 signaling is mediated by the bacterial LPS but is dampened by the T2SS. TLR4 polymorphisms contribute to disease phenotypes in human, but the role of TLR4 in humans is unclear. Autophagy pathways are activated in human macrophages through multiple mechanisms, such as the VipD effector releasing cytochrome *c* from the mitochondria. However, the SidF effector interacts with pro-apopotic members of the Bcl2 family to block cell death. B) In *D. discoideum* LegK2 prevents vATPase from localizing to the LCV, which blocks acidification, but whether this also occurs in human macrophages is unknown. The T4SS effectors LepA and LepB contribute to non-lytic release of *L. pneumophila* in amoebae but not macrophages. *D. discoideum* contains two TIR-domain proteins that are involved in host defense against *L. pneumophila,* but their function is unknown.

Table 1.

Legionella pneumophila-permissive metazoan hosts.

Human alveolar macrophages	(Nash, Libby, & Horwitz, 1984)
Human monocyte derived macrophages	(Horwitz & Silverstein, 1980)
Mouse macrophages permissive (A/J)	(Yamamoto et al., 1988)
Rat alveolar epithelial and macrophages	(Mody et al., 1993)
Human macrophage cell lines	(Kunishima, Takemura, Yamamoto, Kanemitsu, & Shimada, 2000; Neumeister, Faigle, Lauber, Northoff, & Wesselborg, 2002; Pearlman, Jiwa, Engleberg, & Eisenstein, 1988; Weissgerber, Faigle, Northoff, & Neumeister, 2003)
Mouse macrophage cell lines	(Hoffmann et al., 2013)
A549 (human lung epithelial)	(Kunishima et al., 2000; Maruta et al., 1998)
MRC-5 (human lung fibroblast)	(Daisy, Benson, McKitrick, & Friedman, 1981; Oldham & Rodgers, 1985)
HEK293 (human embryonic kidney)	(Habyarimana, Price, Santic, Al-Khodor, & Kwaik, 2010)
HeLa (human cervical epithelial)	(Daisy et al., 1981; Dreyfus, 1987; R. A. Garduno, Quinn, & Hoffman, 1998)
Hep-2 (CCL-23) (human epithelial, HeLa-derived)	(Daisy et al., 1981; Oldham & Rodgers, 1985)
WI-26 (CCL-95.1) (human epithelial-like)	(Stone & Abu Kwaik, 1998)
HepG2 (human liver epithelial)	Our unpublished data
CHO (Chinese hamster ovary, epithelial)	(Robinson & Roy, 2006)
L929 (mouse adipose fibroblast)	(Fernandez, Lee, Haldane, Sumarah, & Rozee, 1989)
McCoy (mouse fibroblast)	(Daisy et al., 1981)
Vero (*Cercopithecus aethiops* kidney epithelial)	(Oldham & Rodgers, 1985)
Macaca nemestrina alveolar macrophages	(R. F. Jacobs, Locksley, Wilson, Haas, & Klebanoff, 1984)
Jurkat (human T lymphocyte)	(Neumeister et al., 2002; Weissgerber et al., 2003)
Guinea Pig	(Berendt, Young, Allen, & Knutsen, 1980)
Drosophila melanogaster	(Dorer et al., 2006)
Leiostomus xantrurus macrophages	(Weeks, Sommer, & Dalton, 1988)
Caenorhabditis elegans	(Brassinga et al., 2010; Komura, Yasui, Miyamoto, & Nishikawa, 2010)

Table 2.

Human proteins involved in the immune response that are modulated by *L. pneumophila* and their homologs in *Caenorhabditis elegans* and *Dictyostelium discoideum*.

Humans	***C. elegans***	***D. discoideum***
NF-κB		
IκB	IκB-1	
TRAF1	TRF-1	
IRAK	PIK-1	
Caspase-9	CED-3	
Apaf-1	CED-4	
NLRC4	CED-4	
Bcl2	CED-9	
BID, BIM	EGL-1	
IAP (Birc5)	BIR-1, BIR-2	
TLRs	TOL-1	TirA/TirB