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SYMPOSIUM

Still Enigmatic: Innate Immunity in the Ctenophore *Mnemiopsis leidyi*

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Synopsis Innate immunity is an ancient physiological response critical for protecting metazoans from invading pathogens. It is the primary pathogen defense mechanism among invertebrates. While innate immunity has been studied extensively in diverse invertebrate taxa, including mollusks, crustaceans, and cnidarians, this system has not been well characterized in ctenophores. The ctenophores comprise an exclusively marine, non-bilaterian lineage that diverged early during metazoan diversification. The phylogenetic position of ctenophore lineage suggests that characterization of the ctenophore innate immune system will reveal important features associated with the early evolution of the metazoan innate immune system. Here, we review current understanding of the ctenophore immune repertoire and identify innate immunity genes recovered from three ctenophore species. We also isolate and characterize *Mnemiopsis leidyi* cells that display macrophage-like behavior when challenged with bacteria. Our results indicate that ctenophores possess cells capable of phagocytosing microbes and that two distantly related ctenophores, *M. leidyi* and *Hormiphora californiensis*, possess many candidate innate immunity proteins.

Introduction

Phylogenetic placement of ctenophores and the role of immunity in non-bilaterian phyla

Ctenophora is a globally distributed phylum of gelatinous marine predators (Chun 1880; Harbison et al. 1978; Mills and Haddock 2007). A resurgent interest in ctenophore biology primarily stems from phylogenomic analyses highlighting their early divergence from other animals (Fig. 1A; Dunn et al. 2008; Shen et al. 2017; Whelan et al. 2017). The biradially symmetric body plan of ctenophores is composed of an outer ectodermal layer with eight ciliary ctene rows and an inner endodermal layer, separated by a thick collagenous mesogleal "middle" layer rich in extracellular matrix (Fig. 1B). Ctenophores have unique modifications of many traits observed in other animals. Due to their suite of distinctive traits, ctenophores have become an important group for providing phylogenetic insights to infer both the origins of, and changes associated with, early metazoan character trait evolution (Jager et al. 2011; Dunn et al. 2014; Presnell et al. 2016; Norekian and Moroz 2019).

Innate immunity in non-bilaterians

The innate immune system is an ancient defensive mechanism for distinguishing self/non-self and detection/elimination of pathogens (Fujita 2002). It is comprised of physical epithelial barriers, cellular defenses, and humoral (circulating) proteins. When pathogens breach the primary physical barrier, specific cell types with immune function are activated. These cells express membrane proteins called pathogen recognition receptors (PRRs) that initiate a signaling cascade upon pathogen detection, leading to a broad immune response that includes engulfment of foreign pathogens by phagocytic cells (phagocytes) (Fig. 3; Dzik 2010; Gourbal et al. 2018). The humoral immune system is comprised of immune proteins capable of detecting and destroying pathogens

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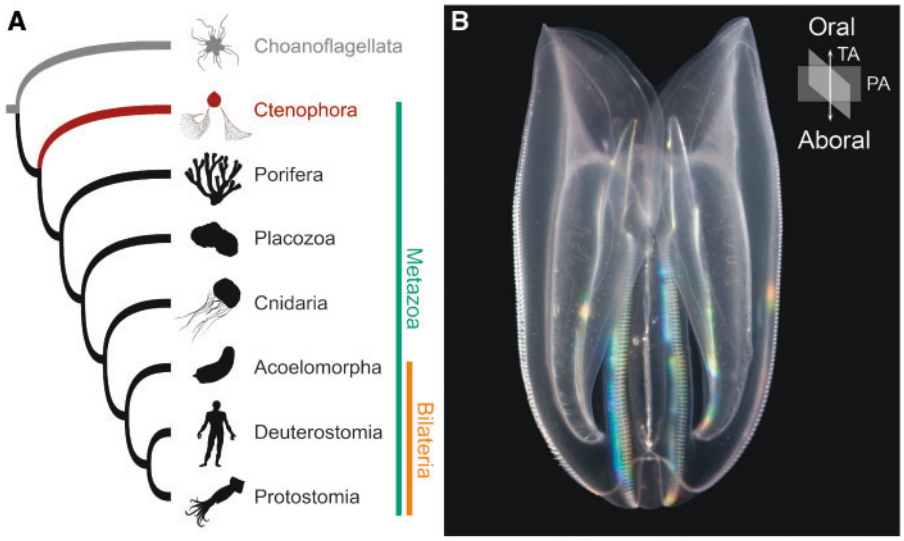

Fig. 1 The lobate ctenophore, *Mnemiopsis leidyi*. **A**) Metazoan relationships with choanoflagellates as outgroup, based on recent phylogenomic inference. **B**) Adult *M. leidyi* with schematic located at upper right depicting orientation of the tentacular axis (TA) and pharyngeal axis (PA). Light refraction causes the ctene row prism effect.

that circulate at low levels throughout the animal (Dzik 2010; Lubbers et al. 2017). Classical receptor-initiated signaling pathways and proteins critical to innate immune system function include: members of the NF-κB/Toll-like receptor (TLR) pathway, NOD-like receptors (NLR), lectins, and perforin-2 (P2)/MPEG-1 (Fig. 2 and Supplementary Table S1). Many proteins associated with these pathways have been identified in non-bilaterians, including corals and sponges (Wiens et al. 2005; Gauthier et al. 2010; Detournay et al. 2012; Richter et al. 2018; Kamm et al. 2019) and a restricted complement appear to have pre-metazoan origins (Richter et al. 2018).

In contrast, our understanding of the immune gene repertoire in ctenophores is sparse. A limited number of candidate immune genes have been identified in the genome of the cydippid ctenophore *Pleurobrachia bachei*: 56 scavenger receptors, 2 peptidoglycan recognition proteins, interferon regulatory factor (IRF), and proteins containing a membrane attack complex component/perforin (MACPF) domain (Moroz et al. 2014). The lobate ctenophore *Mnemiopsis leidyi*, when challenged with Gram-negative or Gram-positive bacteria, up-regulates *lectins* and *complement factor b* genes, indicating an innate immune response (Bolte et al. 2013). It has been shown that ctenophores interact with unique bacterial and viral communities, but how these relationships are maintained is not known (Daniels and Breitbart 2012; Breitbart et al. 2015). The presence of these microbial associations suggests that ctenophores have a cellular innate immune system capable of both regulating pathogens as well as maintaining symbiotic microbial partners (Harvill 2013).

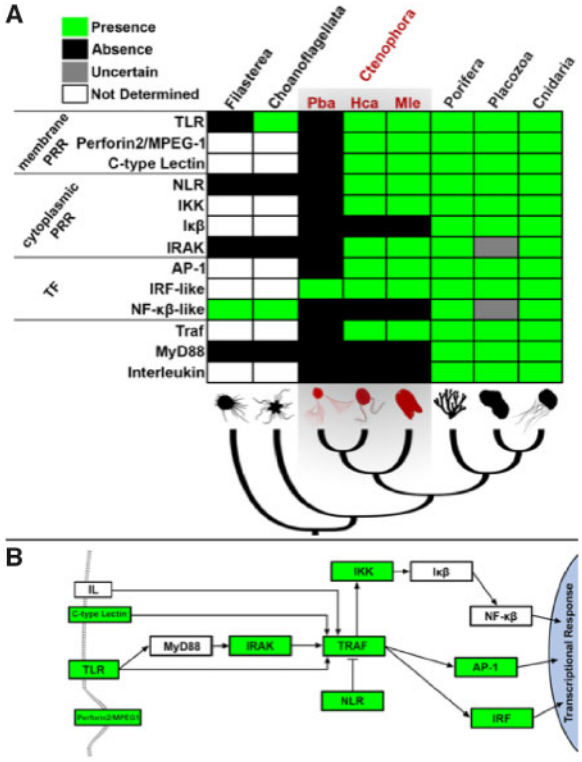

Fig. 2 Components of major innate immune signaling pathways. **A**) Components of classical immune signaling pathways found in non-bilaterian, choanoflagellate and filasterea genomes. Some immune genes appear to be absent in *Pleurobrachia* (*Pba*) (black) but present in *Mnemiopsis* (*Mle*) and *Hormiphora* (*Hca*) (green). **B**) Simplified signaling cascade following TLR stimulation. Pathway components identified in ctenophores are highlighted in green.

Although relatively little is known about the biology of ctenophore immunity, these findings suggest that ctenophores likely possess an immune system with components similar to other metazoans. However, beyond the limited identification of putative candidate immune genes, cellular mechanisms associated with the immune response in ctenophores remain undescribed. In this report, we identify additional putative innate immunity candidate homologs in *M. leidyi* and *Hormiphora californiensis*, two distantly related ctenophores. We also directly challenge cells isolated from the lobate ctenophore *M. leidyi* with a microbial pathogen and identify a motile cell population capable of microbial phagocytosis. We compare these new observations with what is known in other non-bilaterians and highlight remaining gaps in our current knowledge of ctenophore immune biology.

Materials and methods

Identification of putative immune gene homologs

To identify putative homologous protein sequences, *M. leidyi* genome protein models 2.2 (Ryan et al. 2013; Moreland et al. 2014) and *H. californiensis* transcriptome (Francis et al. 2015) were scanned for candidate immune proteins using BLASTp (Supplementary Table S1). Sequences from human and *Nematostella vectensis* were used as initial queries. All recovered *M. leidyi* sequences, regardless of *E*-value, were reciprocally blasted against the BLASTp non-redundant protein sequences database. After reciprocal blast, sequences were kept if a similar protein was identified with a cut off value $\leq 3e^{-05}$. Additionally, previously published genomes of *P. bachei*, *Amphimedon queenslandica*, *Monosiga brevicollis*, *Trichoplax adhaerens*, and *N. vectensis* were queried via the NCBI Genome database (Putnam et al. 2007; King et al. 2008; Srivastava et al. 2008, 2010; Moroz et al. 2014).

Isolation of ctenophore cells

Founder animals for the laboratory culture of *M. leidyi* were collected in Miami, FL, USA. Animals were maintained as previously described (Presnell et al. 2016; Vandepas et al. 2017). Single cell isolations were achieved using both explants and maceration of adult animal tissue following Vandepas et al. (2017).

Phagocytosis assays and live cell imaging

Isolated cells were incubated either in media containing 10 ng/µL Hoechst 33342 and 500 nM Lysosensor Green (Thermo Fisher Scientific, USA) or 100 nM LysoTracker (Red DND-99, Molecular Probes). Cells were then incubated for 20 min with either 0.3 mg/mL pHrodo Red *Escherichia coli* particles (Thermo Fisher Scientific) or 0.1 $\mu g/\mu L$ Alexa488 conjugated BioParticles and washed. Cells were imaged with a Nikon Ti (Eclipse) inverted microscope with Ultraview Spinning Disc (CSU-X1) confocal scanner (Perkin Elmer) and Axio Imager.Z2 (Zeiss). Images were captured with an Orca-ER Camera using Volocity software (Perkin Elmer) and AxioCam MRm Rev3 using Zen Blue software (Zeiss), and post-assembled in Adobe Photoshop (Adobe).

To track cells in wounding experiments, animals were incubated in 3 mg/L neutral red (Sigma–Aldrich) for 24 h prior to wounding the ectoderm epithelium by scalpel. Tissues were then exposed to Alexa488 conjugated BioParticles (Thermo Fisher Scientific) and imaged 15 min post-wounding. Images were acquired using a SteREO Discovery.V8 with AxioCam MRc Rev3, using Zen Blue software (Zeiss), and post-assembled in Adobe Photoshop (Adobe).

Results and discussion

Innate immune protein searches

Our searches for putative immune protein homologs were based on reciprocal BLASTp searches directed against the genomes of *M. brevicollis*, *M. leidyi*, *P. bachei*, *N. vectensis*, and *A. queenslandica* as well as a transcriptome from *H. californiensis* (Supplementary Table S1). The absence of putative homologous genes cannot be verified based solely on sequence similarity, thus our conclusions regarding apparent absences of putative homologous immune protein sequences from reciprocal BLASTp searches alone are limited. Additionally, while it is possible that apparent absences of specific immune genes in *Pleurobrachia* are biologically meaningful, it is also possible that those absences are due to assembly ascertainment bias. Thus, we focus our results and discussion on putative immunity protein homologs that were recovered from the *M. leidyi* genome and *H. californiensis* transcriptome for comparison to other non-bilaterians (Fig. 2).

Toll-like receptors

TLRs are a class of membrane bound PRRs that sense microbial pathogen-associated molecular patterns (PAMPs) through the utilization of an extracellular domain composed of leucine-rich repeats (LRRs) that selectively bind to an array of pathogens. A nearly complete suite of genes involved in the

canonical TLR signaling cascade have been identified in cnidarians (Miller et al. 2007; Putnam et al. 2007; Poole and Weis 2014). Components of the TLR signaling pathway have also been identified in sponges (Wiens et al. 2006; Gauthier et al. 2010); however, these putative TLR homologs appear to lack the LRR domain required for canonical pathogen cell wall binding (Gauthier et al. 2010; Nie et al. 2018). Putative TLR homologs have been identified in choanoflagellates, suggesting cell surface recognition-mediated TLR interactions appear to predate the origin of the Metazoa (Richter et al. 2018). While domains associated with TLRs have not been recovered from initial screens of the *P. bachei* reference genome (Moroz et al. 2014), we identified two putative TLR homologs in both *M. leidyi* and *H. californiensis* that contain predicted LRR domains typically required for mediating microbial pathogen interactions (Ng et al. 2011; Fig. 2 and Supplementary Table S1).

Perforin-2/MPEG-1

P2/MPEG-1 is a glycoprotein that forms pores in the cell membrane of Gram-negative bacteria, causing them to lyse (Osińska et al. 2014). P2/MPEG-1 consists of both a MACPF domain and a P2 domain (McCormack and Podack 2015). In the sponge *Suberites domuncula*, P2/MPEG-1 is activated in response to lipopolysaccharides (Wiens et al. 2005). Within the *P. bachei* genome, seven genes were reported to contain MACPF domains, but none possess a P2 domain suggesting they lack a true functional P2/MPEG-1 homolog. In contrast, in *M. leidyi* and *H. californiensis* we identified a putative P2/MPEG-1 homolog that contains both the MACPF domain and P2 domain (Fig. 2 and Supplementary Table S1). The presence of P2/MPEG-1 in both ctenophores suggests that this protein may have a more ancient role in attacking Gram-negative bacteria than previously hypothesized (McCormack and Podack 2015).

NOD-like receptors

NLRs are cytosolic PRRs that surveil extracellular and endosomal spaces. In humans, NLRC3 is an important negative regulator of the inflammatory response through the inhibition of the TLR-activated NF-κB pathway (Schneider et al. 2012). In cnidarians, the NLR system is complex, with a diverse set of paralogs (Shinzato et al. 2011). While no NLR homolog has been recovered from the *P. bachei* genome (Moroz et al. 2014), we identified a single homolog for a NLR showing sequence similarity to human NLRC3 from both *M. leidyi* and *H. californiensis* (Fig. 2 and Supplementary Table S1). The apparent paucity of identifiable NLRs in ctenophores is suggestive of a simplified cytosolic defense system relative to other metazoan lineages (Lange et al. 2011; Shinzato et al. 2011; Yuen et al. 2014).

NF-κB pathway

The NF-κB pathway plays a central role in innate immunity. Characterized by the transcription factor NF-κB, this pathway is activated by dissociation of NF-κB from its inhibitor Iκ-B (Gilmore and Wolenski 2012). NF-κB and many constituent pathway components are present in several non-bilaterian taxa, including the sponge *A. queenslandica* and the cnidarians *N. vectensis*, *A. digitifera*, and *Hydra* (Wolenski et al. 2011; Gilmore and Wolenski 2012). Searches of the *P. bachei* genome yielded no putative homologs for any NF-κB pathway components Supplementary Table 1 and Fig. 2B; Moroz et al. 2014). In contrast, we recovered several putative homologs for NF-κB pathway components from *M. leidyi* and *H. californiensis* including TRAF, IRAK, and IKK. Interestingly, no studies to date in ctenophores have recovered MyD88, NF-κB, and Iκ-B homologs (Fig. 2 and Supplementary Table S1). Previously, *NF-κB* was identified in both *Capsaspora* and *A. queenslandica* (Sebé-Pedrós et al. 2010; Gilmore and Wolenski 2012; Richter et al. 2018), suggesting that ctenophores have lost this transcription factor and other proteins containing Rel homology domains.

Transcription factors IRF and AP-1

IRF and activator protein-1 (AP-1) are proinflammatory transcription factors involved in activating downstream innate immune factors (Rauscher et al. 1988; Yanai et al. 2012). Four IRF sequences were recovered from initial screens of the *P. bachei* reference genome as well as two partial sequences from *Pleurobrachia pileus* (Nehyba et al 2009; Moroz et al. 2014). Our searches of *M. leidyi* and *H. californiensis* recovered a single putative IRF homolog (Fig. 2 and Supplementary Table S1). To form a functional AP-1, heterodimerization of Fos and Jun proteins must occur (Rauscher et al. 1988). A single putative Fos protein and a single putative Jun protein were identified from *M. leidyi* and *H. californiensis* suggesting a similar heterodimerization interaction may result in a functional AP-1 homolog in ctenophores.

Motile phagocytic cells

Immune cells are typically motile, with pseudopodia-like processes that engulf foreign material, as well as apoptotic or necrotic cells (Horsthemke et al. 2017).

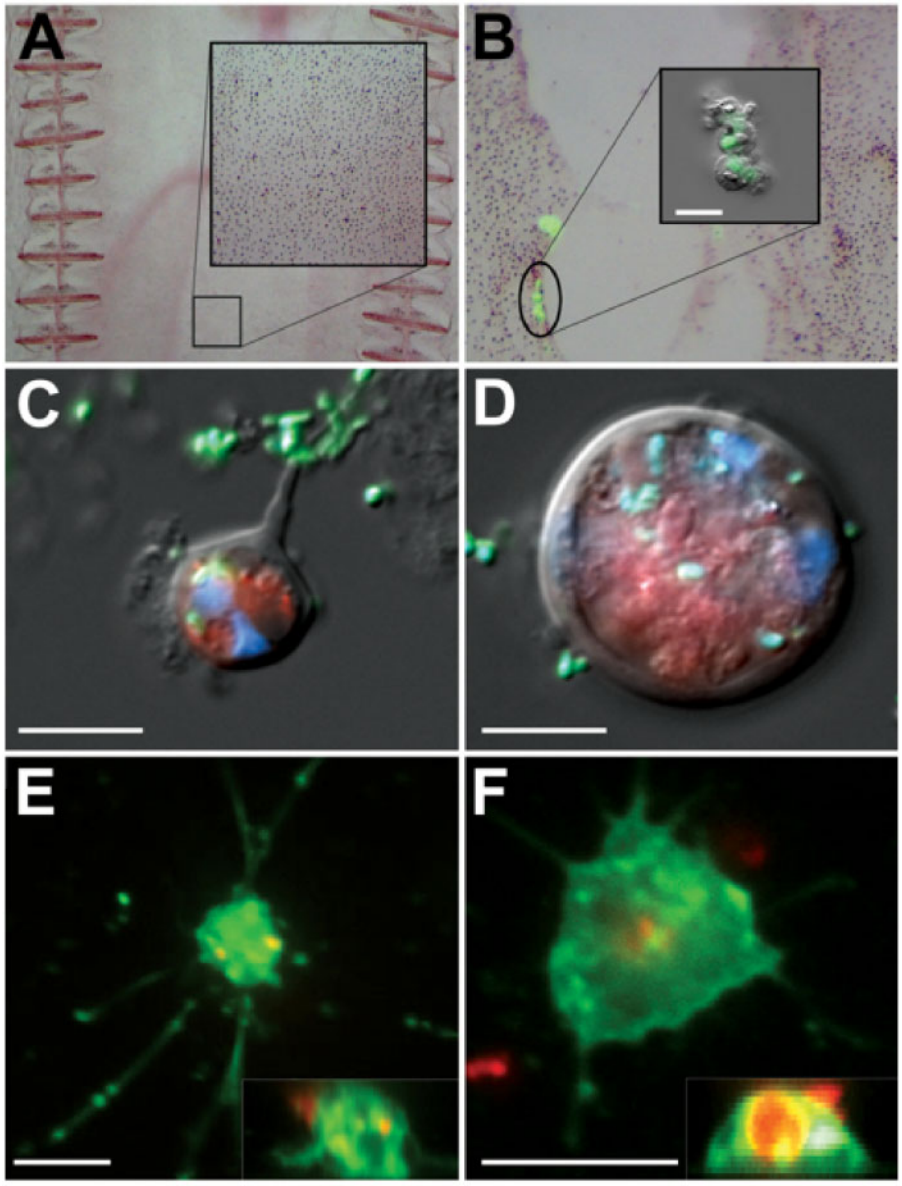

Fig. 3 Phagocytic cells isolated from M. leidyi. Scale bar is 10 μm in all images. A) Adult M. leidyi stained with neutral red vital dye which preferentially accumulates in lysosomes. Inset is magnified view of ectodermal epithelial sheet prior to wounding. B) Same ectodermal field inset from (A) 15min post-wound and seeded with Alexa488 conjugated E. coli. Oval and inset show isolated phagocyte cells with engulfed/internalized fluorescent bacteria. C, D) Combined brightfield (DIC) and fluorescent images of ctenophore phagocytic cell types. Nuclei are labelled with Hoescht (blue), bacteria are labelled with Alexa488 (green), and lysosomes are labelled with Lysotracker (red). C) A motile phagocytic cell displaying extended pseudopodia interacting with and trapping bacteria. The cell has also ingested bacteria. D) Multinucleated round, endodermally-derived digestive cell. E) An isolated phagocytic cell displaying long pseudopodia. The cell has ingested E. coli (red). Inset z-plane cross-section shows bacteria located within the cell body. F) An isolated stellate cell that has engulfed fluorescent bacteria. Inset z-plane cross-section shows bacteria are concentrated within the cell.

With the recent establishment of methods for maintaining and experimentally manipulating ctenophore cells in primary culture (Presnell et al. 2016; Vandepas et al. 2017), we have identified a class of highly motile cells displaying amoebocyte-like morphology (Fig. 3C–F). These cells move throughout the mesoglea and converge in areas under the dermis that have been experimentally wounded (Fig. 3A, B). When challenged with live or heat-killed bacteria, *in vivo* and *in vitro*, these motile amoebocyte-like cells display both pinocytic and phagocytic behavior in which bacteria are quickly engulfed over the course of minutes (Fig. 3). The engulfed bacteria are subsequently shuttled into acidic compartments within cells (Fig. 3E, F, insets).

Previously, Franc (1985) described several varieties of mesogleal cells in the ctenophores *Beroe* and *Pleurobrachia*. Some of these cells contain electron-dense granules, as well as similar amoebocyte-like morphologies and phagocytic behavior to the motile phagocytic cells observed in *M. leidyi* in this study (Franc 1985; Hernandez-Nicaise 1991; Fig. 3). While the functional significance of these mesogleal cells was not described in this earlier work, our results strongly support a role in pathogen response for motile phagocytic cells in *M. leidyi*.

Round digestive cells

Previously, ciliated digestive cells were shown to line defined territories within the gastrovascular canals of *M. leidyi* and other ctenophore taxa (Hernandez-Nicaise 1991; Presnell et al. 2016; Vandepas et al. 2017). In the current study, ciliated digestive cells were exposed to bacteria *in vitro* and displayed phagocytic behavior (Fig. 3D). Whether this behavior is indicative of digestion, immunity, or both is currently unclear. In diverse metazoan taxa, digestive cells are involved in mounting immune responses and have been proposed to share a common origin with immune cells (Mason et al. 2008; Ahluwalia et al. 2018). It is plausible that the observed phagocytosis of bacteria by ctenophore digestive cells is a generalized behavior in which any potential food items are engulfed. Given the potential for multiple physiological roles of digestive cells, a robust characterization of ctenophore digestive cell behavior would further our understanding of whether cells in the gut epithelium are sensing and engulfing pathogens, simply digesting potential food particles, or both. Transcriptomic analysis of these cell types will be useful in identifying whether ctenophore digestive cells express immunity and/or inflammatory response genes.

Conclusion

In this report, we present new evidence that the ctenophores *M. leidyi* (family Bolinopsidae) and *H. californiensis* (family Pleurobrachiidae) possess many putative homologs to immune proteins not previously identified in *P. bachei* (family Pleurobrachiidae; Moroz et al. 2014). Rigorous phylogenetic analyses will be crucial for elucidating the orthology/paralogy relationships among putative members of the major immune gene families found in non-bilaterian taxa. For example, our identification of candidate homologs to TRAF, IRAK, and IKK proteins in ctenophores suggests that an in-depth analysis of the evolutionary relationships between suites of proteins involved in intracellular immune responses and animal taxa will be critical for identifying potential lineage-specific losses and duplication events among TLR pathway components. Similarly, the identification of putative homologous protein sequences to IRF and AP-1 transcription factors highlights a need for further investigation of potential immune regulatory effector genes. Assessment of putative immune cell types and cellular behavior in ctenophores has been limited (Franc 1985; Hernandez-Nicaise 1991). Here we present new evidence for microbial phagocytosis by two distinct *M. leidyi* cell types, one of which displays an amoebocyte-like morphology typical of macrophage-type immune cells (Fig. 3). These phagocytic stellate cells are highly motile and capable of extensive migration throughout the organism (Fig. 3A, B). Further genetic and cell biological investigations are necessary to determine whether ctenophores possess multiple immune cell types capable of initiating distinct immune responses or behaviors in response to specific pathogens and different modes of infection.

Our results represent an initial step in understanding the immune protein repertoire and cellular behaviors associated with microbial phagocytosis in *M. leidyi*. Future studies focused on analyses of gene expression in the motile phagocytic cell types identified by this study will play a role in illuminating immune cell behavior and anti-microbial defense mechanisms deployed by ctenophores. Similarly an in-depth analysis of putative intracellular immune pathway signaling components in ctenophore immune cells would elucidate whether conserved signaling mechanisms, such as MAPK pathway activity are engaged in response to pathogens. To improve our broad understanding of cellular defenses in the metazoan ancestor and innate immunity evolution during subsequent metazoan lineage diversification, several additional critical aspects of cellular immune defenses that remain unexplored in ctenophores require further examination, including encapsulation, opsonization, and free radical function. Filling the gaps between gene sequence and cellular behaviors associated with immunity will inform our understanding of pathogen defense mechanism repertoires and the evolution of innate immunity. The early divergence of the ctenophore lineage from other animals, coupled with recent advances for performing cellular-level assays, provide a unique opportunity to explore both conserved and novel aspects of the animal immune system through deep evolutionary time in this enigmatic clade.

Author contributions

N.T.-K., W.E.B.: conceptualization. L.E.V. and W.E.B.: methodology. All authors contributed to data acquisition and drafting of this manuscript.

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Supplementary data

Supplementary data are available at *ICB* online.

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