Title: Ctenophore immune cells produce chromatin traps in response to pathogens and NADPH-independent stimulus

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Abstract

The formation of extracellular DNA traps (ETosis) is a mechanism of first response by specific immune cells following pathogen encounters. Historically a defining behavior of vertebrate neutrophils, cells capable of ETosis were recently discovered in several invertebrate taxa. Using pathogen and drug stimuli, we report that ctenophores – thought to represent the earliest-diverging animal lineage – possess cell types capable of ETosis, suggesting that this cellular immune response behavior likely evolved early in the metazoan stem lineage.

Introduction

Immune cells deploy diverse behaviors during pathogen elimination, including phagocytosis, secretion of inflammatory cytokines, and expulsion of nuclear material by casting extracellular DNA "traps" termed ETosis. Specific immune cell types have not been identified in early diverging non-bilaterian phyla and thus conservation of cellular immune behaviors across Metazoa remains unclear. Ctenophores are thought to have diverged very early from the animal stem lineage and may represent the most ancient extant animal phylum (1, 2; Figure 1A). Previous work has reported that ctenophores possess putative immune cells competent for phagocytosing bacteria (3), however it is unknown whether ctenophore immune cells display other antimicrobial behaviors. Here we present results demonstrating that the model ctenophore *Mnemiopsis leidyi* possesses cells capable of ETosis. These results suggest that cellular immune behaviors and signaling cascades that produce extracellular DNA traps are an evolutionarily ancient defense against pathogens and were likely present prior to the divergence of extant metazoan lineages.

Results

The release of extracellular DNA traps is an immune-based process during which nuclear material (chromatin) is cast into the surrounding extracellular space when ETosis-competent immune cells are exposed to cytokines or pathogen-associated molecular patterns (PAMPs) (4). Though initially believed to be exclusive to vertebrate neutrophils, recent studies have highlighted ETosis as an immune defense mechanism present in other vertebrate immune cell types and in some invertebrates (5; 6; 7; 8; 9; Fig. 1A). To address whether ctenophores possess cells capable of ETosis in response to pathogen challenge, we isolated total cells from *Mnemiopsis* and performed immune challenges *in vitro*. When *Mnemiopsis* cells were exposed to *E. coli*, we observed large areas of extracellular DNA that are positive for histones and *E. coli* (Fig. 1B, C), indicating that distinct populations of motile cells are undergoing nucleic material release (Fig. 1B; Supp. Video 1). Most *Mnemiopsis* cells showed intact nuclei with stereotypical complements of concentrated DNA and histone labeling (Fig. 1B, C). After exposure to *E. coli,*

the behavior of discrete motile, stellate *Mnemiopsis* cells changed dramatically by retracting their processes, undergoing nuclear rotation, and subsequently rapidly extruding nuclear material (Fig. 1D; Supp. Video 2). These cellular behaviors are reminiscent of the morphological changes that occur as vertebrate monocytes undergo cytoskeletal rearrangements preceding ETosis (9). Together these data suggest that specific ctenophore cells are competent for ETosis, releasing chromatin in response to exposure to microbes.

To assess whether *Mnemiopsis* cells were initiating intracellular signaling pathways in response to microbes, we harvested total protein from isolated cells after 30 minutes exposure to either LPS or zymosan. Western blot assays show MAP kinase pathway activation following PAMP exposure, with a significant increase in phosphorylation events in p42/44 MAPK (ERK) (Fig. 2A). Many immune receptor pathways, including Toll-like receptors, activate MAPK pathways, indicating that major components of immune-mediated intracellular signaling pathways are highly conserved to the earliest-diverging branches of Metazoa.

In vertebrate immune cells, ETosis can also be triggered in the absence of microbes by defined stimuli that induce release of reactive oxygen species (ROS) from cell membrane-bound NADPH or from mitochondria (5, 9). To quantify whether microbial and drug stimuli induce significant immune responses, we developed a novel method for quantifying ETosis using a combination of cell imaging and large-scale analyses (Fig. 2B). Imaged cells were automatically scored based on fluorescent signal intensity of Hoechst (membrane permeable DNA staining) and Sytox Green (membrane impermeable DNA staining) using Cell Profiler (10) and then grouped by population in FlowJo. During ETosis, chromatin unravels, and open DNA is expelled from the cell in filamentous nets. ETotic cells are therefore characterized by diffuse DNA content and loss of membrane integrity, indicated by low fluorescence intensities for both DNA stains (Fig. 2B). In pooled image fields between replicates, we observed a significant increase in ETosis among populations of *Mnemiopsis* cells exposed to PAMPs including heat-killed *Staphylococcus aureus*, lipopolysaccharide (LPS, a bacterial cell wall component), and the fungal cell wall derivative zymosan (Fig. 2C). Notably, total cell death did not significantly increase under any treatment condition (Fig. 2D), indicating that the observed cell death responses were specific to ETotic immune cells.

We distinguished between forms of caspase-driven cell death and the caspase-independent process of ETosis by applying caspase inhibitor ZVAD to *Mnemiopsis* cells and subsequently treating with PAMPs. No significant difference in numbers of cells that underwent ETosis were detected and total cell death decreased (data not shown), indicating that caspase inhibitors successfully blocked caspase-mediated apoptosis in *Mnemiopsis* cells but did not affect pathogen-mediated “NET” production.

Using the Pacific oyster *Crassostrea gigas*, we show that both *Crassostrea* hemocytes and *Mnemiopsis* stellate cells ETose after exposure to nigericin, a K+ ionophore known to stimulate an ETosis pathway through mitochondrial ROS (9; Fig. 2E, F; Supp. Video 3). These are the first reports of activation of the NADPH-independent ETosis pathway outside Bilateria. Cellular mechanisms underlying ET formation in vertebrates are not well understood (9) and data on these signaling pathways in invertebrate taxa is sparse. Effective stimulation of ETosis in bivalve mollusc hemocytes using PMA (phorbol 12-myristate 13-acetate) to activate protein kinase C (PKC) and stimulate NADPH-dependent ROS production, has been debated (6, 7, 11). We found significant induction of ETosis in *Mnemiopsis* cells and *C. gigas* hemocytes following PMA and nigericin exposure, indicating that ETosis in both lineages is effectively stimulated by multiple signaling pathways.

Discussion

The survival and success of multicellular organisms is driven by the ability to recognize self from non-self. Identifying the evolutionary origins of immune cells is crucial not only for understanding the function of metazoan immunity but also the evolution of multicellularity. The phylogenetic position of Ctenophora suggests that characterization of the innate immune

system in this enigmatic clade will reveal important features associated with the early evolution of the innate immune system in metazoans. We demonstrate that the ctenophore *Mnemiopsis* has cells capable of ETosis as a response to pathogen challenges and stimulation of NADPH-dependent and -independent ROS production pathways. This suggests that ETosis is a more ancient and fundamental immune defense mechanism across animals than previously realized. Intriguingly, some *Mnemiopsis* cells and *Crassostrea* hemocytes were observed phagocytosing large numbers of bacteria without undergoing ETosis. Future studies should address whether the ETosis-competent and highly phagocytic cells observed in both taxa represent discrete immune cell types.

Methods

Adult *Mnemiopsis* were maintained in the laboratory as described previously and cells were isolated following established protocols (12). *Crassostrea gigas* were maintained under flowing seawater at approximately 13°C and hemolymph was extracted from the adductor muscle with a syringe. Cells from both taxa were maintained under sterile conditions in filtered seawater (FSW) + 1% penicillin/streptomycin. For stimulation of ETosis, cells were exposed to pHrodo-*E. coli*, *Staphylococcus aureus*, nigericin (Thermo Fisher Scientific), LPS (Sigma Aldrich), PMA (Sigma Aldrich), or ZVAD (Calbiochem). Live cell staining was performed following (2) and live imaging was performed using a JuLI Stage (NanoEntek). For immunofluorescence, ctenophore cells were prepared following (12, 13) and labeled with mouse anti-histone H11-4 (EMD Millipore) and anti-mouse Alexa Fluor 488 (Thermo Fisher Scientific). Imaging of ETotic cells was performed using a Cytation Cell Imaging Multi-Mode Reader (BioTek), followed by quantification using CellProfiler 3.0 and FlowJo v10. Western blots and confocal imaging were performed following (13).

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Figure Legends

Fig. 1 (A) Simplified phylogeny of Metazoa and summary of ETotic cells and stimuli across Metazoa. (B) Untreated, intact *Mnemiopsis leidyi* nuclei stained with Hoechst. (C) *Mnemiopsis* cell challenged with TxRed-*E. coli* has undergone ETosis; chromatin has been exuded from the cell in a large web-like pattern. *E. coli* are entrapped by the chromatin filaments (white arrowheads). (D) Untreated *Mnemiopsis* nuclei stained with Histone 11-4 antibody (green) and Hoechst (white). (E) *Mnemiopsis* cells treated with ROS-stimulating drug nigericin to elicit ETosis. Arrows mark DNA+histone "nets". Intact nuclei are also visible. (B-E scale bar: 10 μm). (F) Stills from timelapse video showing a motile, stellate *Mnemiopsis* cell retracting its processes and exuding chromatin (blue) after being exposed to Tx-Red *E. coli*.

Fig. 2 Quantification of ETosis and cell death following exposure to diverse stimuli. (A) MAP Kinase pathway stimulation in total *Mnemiopsis* cells. ERK phosphorylation significantly increases 30 minutes after exposure to bacterial or fungal signatures compared to unstimulated controls. (B) Schematic synopsis of fluorescent quantification from novel quantitative imaging pipeline. Individual cells are scored based on fluorescent signal intensity. (C) Quantification of ETosis in *Mnemiopsis* cells following exposure to diverse stimuli. (D) Quantification of total *Mnemiopsis* cell death following pathogen exposure. No significant differences in total cell death were observed. (E, F) Analysis of effects of NADPH-dependent or NADPH-independent ROS production pathways in (E) total *Mnemiopsis* cells (F) *C. gigas* hemocytes. Exposure to NADPH-independent pathway-stimulating drug nigericin shows significant increase in ETosis while PMA exposure shows no significant difference in numbers of ETotic cells. (A, C, D, E, F) Two-tailed t-test *$p<0.05$, **$p<0.005$, ***$p<0.001$, ****$p<0.0001$.

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Author contributions
LEV and CS conceived this study with input from FWG and ALH. WEB and FWG supplied animals. LEV performed cell isolations, immune challenges, and protein assays. CS and LEV performed imaging. CS performed data analysis and statistical analyses. LEV and CS assembled the manuscript with input from FWG, ALH, NTK, WEB. All authors approved the final manuscript.

Competing interests
The authors declare no competing financial interests.

Figure 1

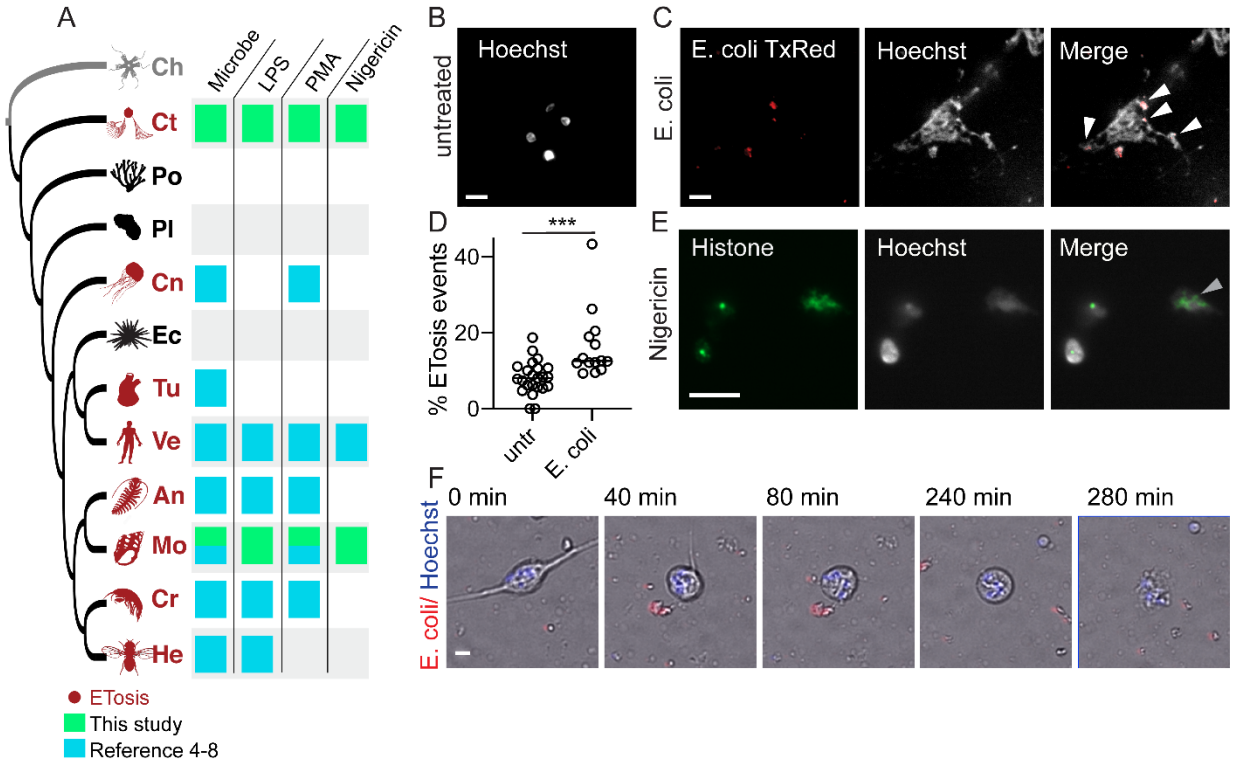
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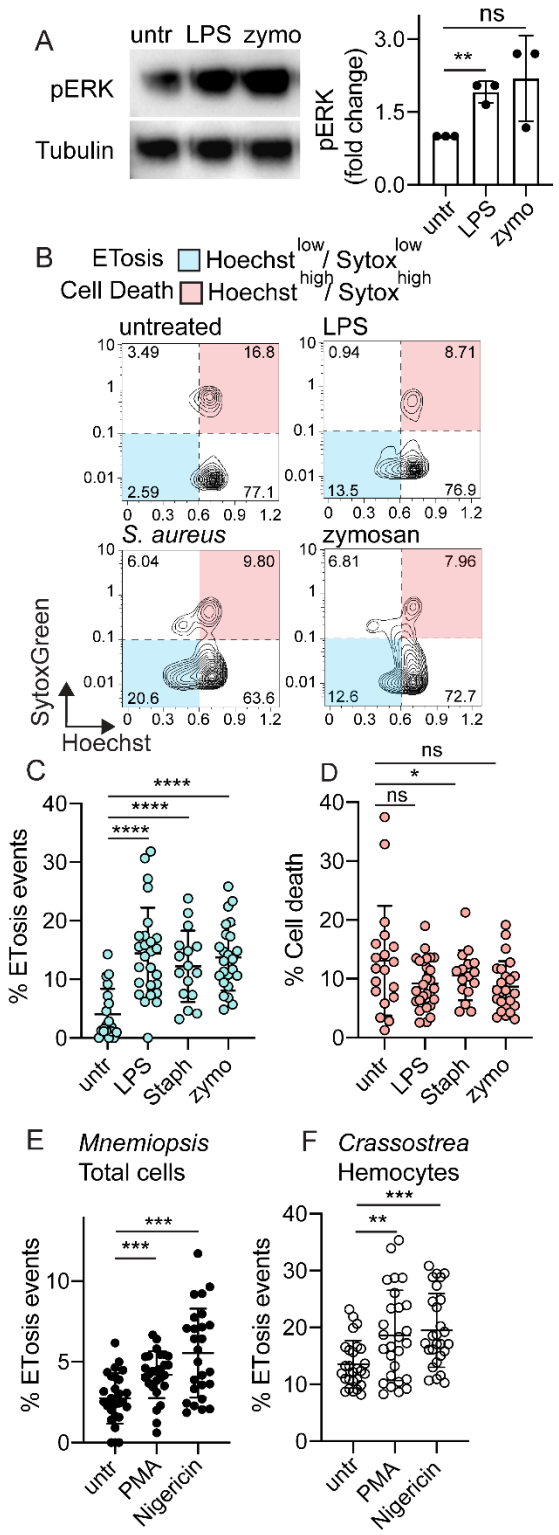
Figure 2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