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SHORT COMMUNICATION

Distinct immune- and defense-related molecular fingerprints in sepatated coelomocyte subsets of *Eisenia andrei* earthworms

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Abstract

During phylogenesis different types of immunocytes such as amoebocytes and eleocytes have developed in earthworms to defend the host against microbial pathogens.

Previously we applied a cell sorting-based approach to untangle the morphological and functional properties of these aforementioned coelomocyte subsets. In order to compare their constitutive gene expression patterns, cell-sorting was performed and followed by semiquantitative RT-PCR in the distinct, separated coelomocyte subpopulations of unmanipulated *Eisenia andrei* earthworms. We targeted a variety of genes with diverse functions ranging from pattern recognition through intracellular signaling to oxidative stress.

Several immune-related genes (*CCF*, *TLR*, *lumbricin*, *LuRP*, *MyD88*) were only manifested in the amoebocytes. In contrast, other immune response genes (*lysozyme*, *lysenin*), lysosomal hydrolases (*cathepsin L* and *cathepsin C*) and *cystatin B* were expressed in both subpopulations. In addition, cell signaling molecules (*MyD88*, *PKC1*) and oxidative stress-related genes (*Cu/ZnSOD*, *MnSOD*) were mainly observed in amoebocytes, while other stress-related genes (*Cd-metallothionein*, *catalase*) were apparent in both subsets.

We conclude that these characteristic differences of the molecular signatures manifest in the functional heterogeneity of distinct coelomocyte subtypes.

Key Words: *Eisenia andrei*, coelomocytes, cell sorting, gene expression, immune response, oxidative stress

Introduction

Evolutionary conserved immune mechanisms are reported from diverse invertebrate organisms (Loker *et al*., 2004). A surprising complexity and close cooperation between cellular and humoral immune components can be observed in several invertebrate models including earthworms (Cooper *et al*., 2002; Cooper and Roch 2003; Bilej *et al*., 2010; Engelmann *et al*., 2016b). Earthworm coelomocytes are divided into amoebocyte and eleocyte subpopulations. Similarly to other invertebrate immunocytes, these cells are derived from the mesoderm (eleocytes are considered to be originated from the gut surface-located, liver equivalent chloragogenous tissue), and possess various functions during the immune response (Engelmann *et al*., 2005). In this regard, amoebocytes are mainly involved in the phagocytosis and encapsulation (Fuller-Espie, 2010), while eleocytes have no phagocytic properties, but they produce a handful of bioactive molecules (Stein *et al*., 1977; Valembois *et al*., 1985).

Recently we applied a cell-sorting-based approach to separate these distinct coelomocyte subsets upon their light-scatter properties (from the perspective of physical parameters; size and granularity). After separation we characterized their differences in morphological, cytochemical, functional and lectin-binding properties (Engelmann *et al.,* 2016a).

In the last two decades several immune proteins have been identified in earthworms; however, little is known about their differential gene expression in the coelomocyte subgroups (Bilej *et al.,* 2010; Engelmann *et al.,* 2016b).

Aware of the phenotypic and functional differences in the coelomocyte subsets, we aimed to analyze the distinct expression patterns of several immune and stress-related target genes in the separated amoebocytes and eleocytes.

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Table 1 List of primers were applied for semi-quantitative RT-PCR experiments

Target Gene	Gene Bank accession #	Sequence (5'-3')[a]	Amplicon size (bp)
TLR	JX898685	ATT GTG TCA AAC GCC TTC GC GTC GGC GAT CTC TTC CAA CA	123
CCF	AF030028	CAT TAA GCC GAC GTT GCT GG CGT CCT GTA GCA TCC GTT GT	145
LBP/BPI	JQ407018	GGT TCG ACC TCC GAC GAT AC GGT CAA CAG GGC GTC CAT TA	107
Lysozyme	DQ339138	GTC GCA TGG ATG TCG GAT CT GCG AGC AGT CCA TCT GAG TT	120
Lumbricin	KX816866	ACT CGG AAC GCA AGA ACC AA GGT TCT GCG TGA CCT CCT TC	139
LuRP	KX816867	GGT CGA GAG AAT CAA CCC AAC TA CTT GTG AGC GAT GTC GGC TA	133
Lysenin	D85846	TGA TCC ACA CTG GTG CTT CC CAG GTG CCA AGG AGA AGA AG	117
MyD88	EH670202	TGC GAG TAC AGG CTC GTT AAC CGT GCA GAT GTG GTT TAG GA	100
MEKK 1	EH672240	CAA GGA ACG ATC CCA TTC AT GTA TCA TGG TGC AAC CAA CG	147
PKC1	DQ286716	TTT TAT GCG GCC GAA GTC A GTC GGC GAT TTT GCA GTG A	120
Mt	AJ236886	CTT GTT GCT GCA CAA ACT GC TTT CCA CAT TTG CCC TTC TC	129
Catalase	DQ286713	TAC AAA CTG GTG AAC GCC GA AAA GGT CAC GGG TCG CAT AG	139
Cu/ZnSOD	KR106132	TGC CAA GTT TGA AGT GAC GG TTT GCC AAG ATC GTC CAC CA	103
MnSOD	KU057379	CCG AAG AAA AGC TGG CTG AA TGT CCT CCG CCG TTG AAT	91
Cystatin B	BP524680	TGG AGG GGA TGC TTT GCA TT ACG CAG ACA AGG TAC GAA GA	123
Cathepsin B	HO001247	TCC TGC CTT TCC AGA TTC ATT T GAA CCA CAG GAG CCC TGA TC	90
Cathepsin C	GR228740	CGG CTA CTT CCG CAT CGT T AGC GCC TGC TCA GAA GGA	120
Cathepsin L	EY892565	CAA CGG CTG TTT CCT ATC CAA GAA AAC ACA CGA TGC AAT GCA	110
COI	HQ534065	GGA TTT GGA AAC TGA CTT C TCG TTC TAG TCG AAG CCC AC	312
18S	AY365460	ATT AAG CCA TGC ATG TCT AAG CAC CTT TGT GGC ATG TAT TAG CTC CAG	135
RPL17	BB998250	GCA GAA TTC AAG GGA CTG GA CTC CTT CTC GGA CAG GAT GA	159
β-actin	JQ038870	ATG TGG ATC AGC AAG CAG GAG TA ATC GCC GAG ATC GGA ATC TT	90

[a]Upper and lower sequences represent forward and reverse primers

Materials and methods

Earthworm husbandry

Adult *Eisenia andrei* earthworms were maintained in breeding stocks at standard conditions (Molnár *et al*., 2012). Prior to coelomocyte harvesting, earthworms were placed onto moist tissue paper allowing defecation to avoid contamination during coelomocyte collections.

Coelomocyte harvesting

Coelomocytes were isolated as we described earlier (Engelmann *et al.,* 2004) and enumerated by 0.14% trypan-blue dye-based exclusion.

Cell sorting and flow cytometry

Collected coelomocytes were resuspended in *Lumbricus* balanced salt solution (LBSS) (Engelmann *et al.,* 2005) supplemented with 1% fetal bovine serum (FBS, Biowest, Nuaillé, France) and 5 mM EDTA (Sigma-Aldrich, Hungary) to prevent cell aggregation. Coelomocytes were sorted according to their basic forward and side scatter (FSC/SSC) characteristics reflecting their cell size and granularity, respectively. Sorting procedure was performed by a FACSAria III (BD Biosciences) cell sorter as we described earlier (Engelmann *et al.,* 2016a). The efficacy of sorting and the coelomocyte viability was controlled by 7-amino-actinomycin D (7-AAD) using a FACSCalibur flow cytometer.

Hematoxylin-eosin staining

Sorted coelomocyte subsets (80 µl of 5 x 10^5/ml) were spread onto glass sides using Cytospin 3 (SHANDON, ThermoScientific, Waltham, MA, USA) apparatus. Hematoxylin-eosin staining was employed following standard protocols.

RNA isolation, cDNA synthesis and semiquantitative RT-PCR

Total RNA was isolated from unseparated coelomocyte, sorted amoebocyte and eleocyte samples using NucleoSpin® RNA isolation kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. The quality and quantity of RNA samples were measured by NanoDrop 1000 spectrophotometer (ThermoScientific). Following DNAse I digestion (Sigma-Aldrich) the reverse transcription reaction was performed by Hi-Capacity Reverse Transcription Kit applying random hexamers (ThermoScientific). DNAse I-treated total RNA was reverse transcribed and subsequently used in the PCR reactions. Gene specific primers were designed based on the available sequences from NCBI GenBank Database and their major characteristics are detailed in Table 1.

The following PCR conditions were applied: an initial denaturation step at 95 °C for 10 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 54 °C for 30 s, and elongation at 72 °C for 30 s. Amplification cycles were terminated by a final extension at 72 °C at 10 min. Finally, PCR mixtures were analyzed on 1% (w/v) agarose gel, and PCR products were visualized by GelRed (Biotium, Inc., Fremont, CA, USA) dye. Gel pictures were photographed by GelDoc XR system (BioRad, Hercules, CA, USA).

Results and Discussion

Pattern recognition receptors (PRRs) are attributed to separated amoebocytes

To analyze the distinct gene expression patterns we separated the coelomocyte subpopulations based on their physical manifestation (size and granularity) (Fig. 1a). Post-sort cell viability measurements (7-AAD staining) indicated a high survival rate (82-85%) for sorted amoebocytes, while we were not able to evaluate the ratio of alive/dead eleocyte due to their high autofluorescence.

Hematoxylin and eosin staining was performed to check the efficacy of the sorting process. Majority of the sorted population was composed by hyaline amoebocytes (Fig. 1b), whereas a small percentage of granular amoebocytes appeared as well. Separated eleocytes (Fig. 1c) were easily perceptible by their small nucleus and the cytoplasm filled with chloragosomes.

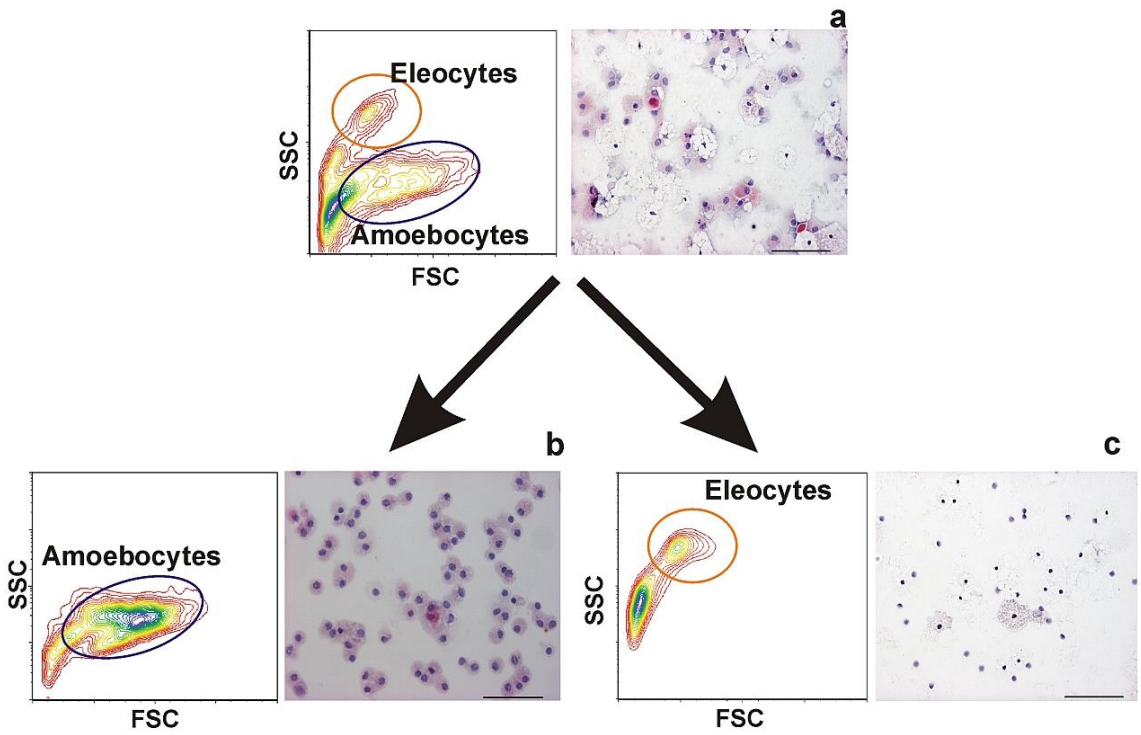

Fig. 1 Pre- and post-sorting analyses of coelomocyte subsets and cytochemical properties of sorted populations. Total coelomocyte population (a) was separated to amoebocyte (b) and eleocyte (c) subpopulations upon their physical manifestations. Post-sort analyses demonstrated that amoebocytes were mainly undamaged; however, eleocytes showed a certain fragility evidenced by the increased amount of debris. Hematoxilin-eosin staining revealed mixed total coelomocytes prior to sorting while homogenous amoebocytes (b) and eleocyte subpopulations (c) can be observed following the separation. Scale bars: 100 µm. Representative dot-plots are presented from three independent experiments.

Innate immunity operates with a panel of PRRs to discriminate between non-self and self structures. Recently, some unique and evolutionary conserved PRR molecules have been identified in *E. andrei* earthworms (Bilej *et al*., 2010; Engelmann *et al.,* 2016b). First, we investigated the expression of pattern recognition receptor (PRR) genes including *coelomic cytolytic factor* (*CCF*), *toll-like receptor* (*TLR*), and *LPS-binding protein/bacterial permeability-increasing protein* (*LBP/BPI*). CCF is an unique LPS, peptidoglycan and β-1,3-glucan/N,N'-diacetylchitobiose-binding protein that is expressed at higher level in the chloragogenous tissue and lower level in large coelomocytes (Beschin *et al*., 1998). In the case of coelomocyte subsets, we found that *CCF* was only expressed in separated amoebocyte subpopulation, but it was not present in sorted eleocytes (Fig. 2a).

First evidence of annelid TLRs was emerged from the analyses of polychaete and hirudean species (Davidson *et al.,* 2008; Cuviller-Hot *et al*., 2011). Shortly, the coding sequence of TLR was identified in *E. andrei (EaTLR). EaTLR* expression level was relatively low in coelomocytes (Škanta *et al*., 2013) and -according to our observation- only occurred in amoebocytes (Fig. 2a), however its presence is not restricted exclusively to immunocompetent tissues (Škanta *et al., 2*013; Engelmann *et al*., 2016b). Further genomic investigations have shown the high diversity of *TLRs* in annelid earthworms (Fjøsne *et al*., 2015).

Recently, a homologue of evolutionarily conserved LBP/BPI molecule was isolated from *E. andrei*. The highest expression level of *EaLBP/BPI* was observed in coelomocytes, seminal vesicles, while the lowest level appeared in the intestine (Škanta *et al*., 2016). We observed that *LBP/BPI* expression only occurred in amoebocytes, but not in eleocytes (Fig. 2a).

These findings confirmed the notion that molecular recognition of pathogens is dedicated mainly to the amoebocyte subpopulation. In comparison to the earlier functional studies (Valembois *et al*., 1985; Valembois and Lasségues, 1995), our results support the concept that amoebocyte subpopulation is priorly involved in the pathogen-triggered phagocytic response.

Distinct antimicrobial molecular fingerprints of coelomocyte subsets

Nowadays, antimicrobial proteins (AMPs) are recognized as the first line of defense against microbial pathogens (Boman, 1991; Zasloff, 2002).

Lysozyme is a highly conserved AMP present in many different organisms ranging from plants to human. Previous investigations have revealed that *E. andrei* lysozyme represents strong sequence similarity with other invertebrate lysozymes (Josková *et al.,* 2009). Furthermore its expression is increased in coelomocytes upon Gram-positive and Gram-negative bacteria exposure. We found that both separated coelomocyte subpopulation expressed this AMP, however we observed a more augmented *lysozyme* expression in separated amoebocytes compared to eleocytes (Fig. 2b).

Lumbricin is a distinctive earthworm AMP that was initially isolated from *Lumbricus rubellus*. So far several additional lumbricin homologues have been described from other annelid species (Cho *et al.,* 1998; Wang *et al.,* 2003; Schikorski *et al*., 2008; Li *et al*., 2011). Its expression was observed in several tissues, but not in coelomocytes (Li *et al*., 2011). Recently, we have identified the coding sequence of lumbricin and its novel-related peptide (LuRP) in *E. andrei.* Lumbricin and LuRP show close relationship with other lumbricin homologues (Bodó *et al*., 2019). Additionally, we observed that *lumbricin* and *LuRP* exert ubiquitous expression in several earthworm tissues (including coelomocytes), but their highest expression was evidenced in the foregut. Among other tissues *Lumbricin* and *LuRP* expression was the lowest in coelomocytes. As for their distribution in the subpopulations we observed that only amoebocyte subpopulation turned out to be weakly positive, but eleocytes were negative for these genes (Fig. 2b).

A species-specific bioactive molecule lysenin has been described from *Eisenia* earthworms. Lysenin protein family consists of sphingomyelin-(and phosphocholine)-binding molecules, and possess diverse biological activities (cytotoxicity, antimicrobial activity, and opsonization) (Engelmann *et al.,* 2016a). Our immunohistochemical analysis showed that mostly eleocytes were the lysenin expressing cells (Opper *et al.,* 2013), in contrast to previous observations where chloragocytes were suggested as the major players in lysenin production (Ohta *et al.,* 2000). *Lysenin* expression was manifested in both subpopulations (Fig. 2b) that is concordant with our previous flow cytometry-based observations (Opper *et al*., 2013).

Conserved signaling molecules in coelomocyte subsets

Our knowledge is very limited concerning on the intracellular signaling in earthworm immunity (Engelmann *et al.,* 2011). We gained recent knowledge of different signaling pathways and we tested their expression pattern in the separated coelomocyte subsets. Certain signaling pathways such as MAPK cascade are fundamental and evolutionarily conserved; since several publications are available from various organisms (Sakaguchi *et al.,* 2004; Ragab *et al.,* 2011). Hayashi *et al.,* 2012 observed down-regulation of *MEKK1* level in silver nanoparticle (AgNP)-exposed *Eisenia* coelomocytes. We measured that *MEKK1* expression is present in the isolated amoebocytes, besides we found a very weak signal in the eleocyte population (Fig. 2c).

Innate immunity is largely dependent on the engagement of TLRs. Following PAMP recognition intracellular molecular events are initiated by the cytosolic components of TLR signaling pathway, one such is the MyD88. Hayashi *et al.,* 2012 have observed a delayed induction of *MyD88* gene in coelomocytes upon AgNP exposure. *MyD88* was only expressed in the amoebocyte subpopulation that is relevant with the amoebocyte-restricted *TLR* expression (Fig. 2c).

Protein kinase C (PKC) has fundamental functions in cellular homeostasis, and its role is well implicated in the immune response (Larsen *et al*., 2002). Earthworm *PKC1* and *PKC2* were recently

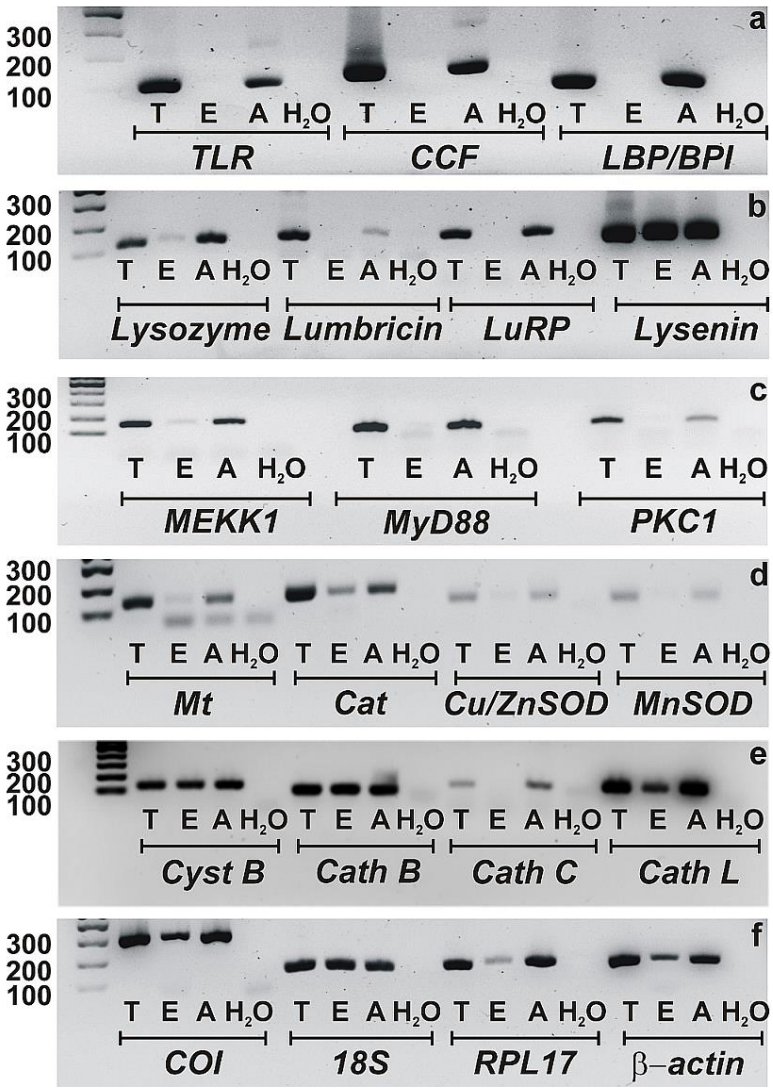

Fig. 2 Expression patterns of (a) pattern recognition receptors; PRRs, (b) antimicrobial peptides AMP, (c) signaling pathway genes, (d) metal-, oxidative stress-induced molecules, (e) hydrolytic proteases, and (f) housekeeping genes in coelomocytes of *Eisenia andrei*. (a) *TLR, CCF,* and *LBP/BPI*; (b*) Lysozyme, Lumbricin, LuRP* and *Lysenin; (*c) *MEKK 1, MyD88,* and *PKC1;* (d) *Mt, Cat, Cu/ZnSOD* and *MnSOD;* (e) *Cyst B, Cath B, Cath C,* and *Cath L;* (f) *COI, 18S,* 7 *RPL17* and *β-actin.* Total coelomocytes (T), and separated eleocytes (E) and amoebocytes (A), with H_2O as PCR negative control. Representative images are presented from three independent experiments.

partially cloned (Brulle *et al*., 2006). Homa *et al*., (2013) found that a phorbol ester (PMA), a potent activator of PKC caused the proliferation of earthworm coelomocytes. Interestingly, in the course of *in vitro* PMA administration we observed that PKC is not involved in the Ca^{2+}-dependent activation of coelomocytes (Opper *et al*., 2010; Engelmann *et al*., 2011). Among coelomocyte subgroups only the separated amoebocytes evidenced the expression of *PKC* (Fig. 2c). In fact, the amoebocyte subpopulation has a crucial role in the pathogen recognition, and in the downstream inflammatory response evidenced by the selective expression of signal transduction molecules and antimicrobial factors.

Expression of oxidative, metal stress genes in separated coelomocyte subsets

In addition to immune response-related genes, we examined the expression patterns of other defense-related genes involved in metal- and oxidative stress. Metallothioneins (Mt) are intensively studied metal-sequestrating proteins and ubiquitously expressed in a wide variety of organisms including earthworms (Calisi *et al.,* 2014; Kowald *et al.,* 2016). In recent years, earthworms are frequently applied as a sentinel organism to evaluate metal contaminations in soil (Calisi *et al.,* 2014). Homa *et al.,* (2005) previously described that *E. fetida* coelomocytes are able to accumulate various metal ions. Earlier results demonstrated that

Mt expression is mostly attributed to chloragocytes (Morgan *et al*., 2004); however we found that *Mt* manifestation occurred in both separated amoebocyte and eleocyte (free-floating chloragocyte) subpopulations. In contrast to the previous data we observed weak signal in eleocytes, while amoebocytes had a stronger *Mt* expression (Fig. 2d).

All living organisms possess a diversity of antioxidant defense mechanisms (Wang *et al.,* 2015). Catalase (Cat) is one conserved key enzyme of oxidative stress, and it exists in many different cell types including earthworm coelomocytes (Brulle *et al*., 2006). *Cat* expression was observed in both sorted coelomocyte subpopulations (Fig. 2d). Amoebocytes are involved in the early immune response against pathogens, but probably both subpopulations participate in maintaining the normal cellular homeostasis.

Another oxidative stress-related enzyme, *Cu/Zn-SOD* has been recently cloned and characterized in *E. fetida* (Xiong *et al*., 2012). The predicted amino acid sequence was excavated that genetic distance of Cu/Zn-SOD in *E. fetida* was far from other invertebrate SOD molecules. Indeed, it showed strong sequence similarity with homologue sequences from *Tubifex tubifex* and *L. rubellus* (Xiong *et al.,* 2012). In addition, the sequence of *MnSOD* has been recently assessed in *E. andrei* (Roubalová *et al*., 2018). Interestingly, its role in innate immune responses now has been elucidated (Wang *et al.,* 2015). In contrast to *Mt* and *Cat* expression *Cu/Zn-SOD* and *MnSOD* were only manifested in separated amoebocyte subpopulation (Fig. 2d). Indeed, following the phagocytosis the intracellular "killing" in amoebocytes is mediated by the free reactive oxygen (and nitrogen) species that needs to be terminated by certain antioxidative enzymes (e.g. Cat, SOD).

Hydrolytic endopeptidases and their inhibitor are present in coelomocyte subsets

Cathepsins (Cath) are ubiquitous lysosomal proteases involved in many aspects of the cell life cycle. Cath B, Cath L and Cath C have been identified in several invertebrate organisms. Undoubtedly, Cath B is one of the most typical member of this molecular family that takes essential part in the immune response against bacterial infections (Balaji *et al*., 2002). Cath B is involved in the regulation of apoptosis, and this lysosomal protease is implicated to be involved in the immune mechanisms of the echinoderm, *Apostichopus japonicus* (Chen *et al.,* 2016). Cath L is also identified in several invertebrates including the leech *Theromyzon tessulatum.* Cath L was involved in the phagocytic responses of leeches (Lefebvre *et al*., 2008).

Cystatin B (Cyst B) is an endogenous cathepsin inhibitor, which localization was observed in the cytosol, mitochondria and nucleus (Kopitar-Jerala, 2015). Previously, cystatin B gene was described in the leech *T. tessulatum,* and upregulated after bacterial challenge (Lefebvre *et al*., 2004). Interestingly, leech coelomocytes possess a differential expression of cathepsin L (in chloragocytes and amoebocytes) and cystatin B (only in chloragocytes) (Lefebvre *et al*., 2008). According to our results all of the observed cathepsins (*Cath B, C* and *L*) were present in the sorted amoebocyte subpopulation, while only *Cath C* is absent from the sorted eleocytes (Fig. 2e). Interestingly, *Cath C* expression evidenced a low expression in total coelomocytes, while it appeared relatively higher in isolated amoebocytes. The inhibitor of these lysosomal proteases, *Cyst B* was expressed in both coelomocyte subpopulations (Fig. 2e). Their immune function in *E. andrei* is still unknown; however we cannot rule out the option that these proteases might be the possible regulators of lysenin-mediated cell lysis and also involved in the phagocytic machinery (Engelmann *et al*., 2016a).

We have chosen four „housekeeping" genes to prove the intact RNA quality of the sorted coelomocyte subsets. All of tested genes including *COI*, *18S*, *RPL17* and *β-actin* expressed in both populations, however *RPL17* and *β-actin* genes showed a lower level in eleocytes (Fig. 2f).

Conclusions

Taken together, hereby we report initially the differential expression patterns of immune and defense-related genes in sorted coelomocyte subsets of *E. andrei*. Our results verify the previously observed cytochemical, immunological and functional differences of coelomocyte subsets at the molecular level. Accordingly, amoebocytes are the main effector cells participating in pathogen recognition, and elimination. Eleocyte subpopulation is mainly involved in the stress response and production of bioactive molecules. These results provide fine details about the substantial molecular functions of separated coelomocyte subsets.

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References

Balaji KN, Schaschke N, Machleidt W, Catalfamo M, Henkart PA. Surface cathepsin B protects cytotoxic lymphocytes from self-destruction after degranulation. J. Exp. Med. 196: 493-503, 2002.

Beschin A, Bilej M, Hanssens F, Raymakers J, Van Dyck E, Revets H, *et al*. Identification and cloning of a glucan-and lipopolysaccharide-binding protein from *Eisenia foetida* earthworm involved in the activation of prophenoloxidase cascade. J. Biol. Chem. 273: 24948-24954, 1998.

Bilej M, Procházková P, Silerová M, Josková R. Earthworm immunity. Adv. Exp. Med. Biol. 708: 66-79, 2010.

Bodó K, Boros Á, Rumpler É, Molnár L, Böröcz K, Németh P, *et al*. Identification of novel lumbricin homologues in *Eisenia andrei* earthworms. Dev. Comp. Immunol. 90: 41-46, 2019.

Boman HG. Antibacterial peptides: key components needed in immunity. Cell. 65: 205-207, 1991.

Brulle F, Mitta G, Cocquerelle C, Vieau D, Lemière S, Leprêtre A, *et al*. Cloning and real-time PCR testing of 14 potential biomarkers in *Eisenia fetida* following cadmium exposure. Environ. Sci. Technol. 40: 2844-2850, 2006.

Calisi A, Lionetto MG, De Lorenzis E, Leomanni A, Schettino T. Metallothionein induction in the coelomic fluid of the earthworm *Lumbricus terrestris* following heavy metal exposure: a short report. Biomed. Res. Int. 2014: 109386, 2014.

Chen H, Lv M, Lv Z, Li C, Xu W, Zhang W, *et al.* Molecular cloning and functional characterization of cathepsin B from the sea cucumber *Apostichopus japonicus.* Fish Shellfish Immunol. 60: 447-457, 2016.

Cho JH, Park CB, Yoon YG, Kim SC. Lumbricin I, a novel proline-rich antimicrobial peptide from the earthworm: purification, cDNA cloning and molecular characterization. Biochim. Biophys. Acta. 1408: 67-76, 1998.

Cooper EL, Kauschke E, Cossarizza A. Digging for innate immunity since Darwin and Metchnikoff. Bioessays. 24: 319-333, 2002.

Cooper EL, Roch P. Earthworm immunity: a model of immune competence Pedobiologia. 47: 676-688, 2003.

Cuvillier-Hot V, Boidin-Wichlacz C, Slomianny C, Salzet M, Tasiemski A. Characterization and immune function of two intracellular sensors, HmTLR1 and HmNLR, in the injured CNS of an invertebrate. Dev. Comp. Immunol. 35: 214-226, 2011.

Davidson CR, Best NM, Francis JW, Cooper EL, Wood TC. Toll-like receptor genes (TLRs) from *Capitella capitata* and *Helobdella robusta* (Annelida). Dev. Comp. Immunol. 32: 608-612, 2008.

Engelmann P, Hayashi Y, Bodó K, Ernszt D, Somogyi I, Steib A *et al.* Phenotypic and functional characterization of earthworm coelomocyte subsets: Linking light scatter-based cell typing and imaging of the sorted populations. Dev. Comp. Immunol. 65: 41-52, 2016a.

Engelmann P, Hayashi Y, Bodó K, Molnár L. New aspects of earthworm innate immunity: novel molecules and old proteins with unexpected functions. In Ballarin L, Cammarata M (eds): Lessons in immunity: from single cell organisms to mammals. Academic Press, Elsevier 53-66, 2016b.

Engelmann P, Molnár L, Pálinkás L, Cooper EL, Németh P. Earthworm leukocyte populations specifically harbor lysosomal enzymes that may respond to bacterial challenge. Cell Tissue Res. 316: 391-401, 2004.

Engelmann P, Opper B, Németh P. Interactions of intracellular calcium and immune response in earthworms. Inv. Survival J. 8: 78-84, 2011.

Engelmann P, Pálinkás L, Cooper EL, Németh P. Monoclonal antibodies identify four distinct annelid leukocyte markers. Dev. Comp. Immunol. 29: 599-614, 2005.

Fjøsne TF, Stenseth EB, Myromslien F, Rudi K. Gene expression of TLR homologues identified by genome-wide screening of the earthworm *Dendrobaena veneta*. Innate Immun. 21:161-166, 2015.

Fuller-Espie SL. Using flow cytometry to measure phagocytic uptake in earthworms. J. Microbiol. Biol. Educ. 11: 144-151, 2010.

Hayashi Y, Engelmann P, Foldbjerg R, Szabó M, Somogyi I, Pollák E. *et al*. Earthworms and humans *in vitro*: characterizing evolutionarily conserved stress and immune responses to silver nanoparticles. Environ. Sci. Technol. 46: 4166-4173, 2012.

Homa J, Olchawa E, Stürzenbaum SR, Morgan AJ, Plytycz B. Early-phase immunodetection of metallothionein and heat shock proteins in extruded earthworm coelomocytes after dermal exposure to metal ions. Environ. Pollut. 135: 275-280, 2005.

Homa J, Zorska A, Wesolovski D, Chadzinska M. Dermal exposure to immunostimulants induces changes in activity and proliferation of coelomocytes of *Eisenia andrei*. J. Comp. Physiol. 183: 313-322, 2013.

Josková R, Silerová M, Procházková P, Bilej M. Identification and cloning of an invertebrate-type lysozyme from *Eisenia andrei*. Dev. Comp. Immunol. 33: 932-938, 2009.

Kowald GR, Stürzenbaum SR, Blindauer CA. Earthworm *Lumbricus rubellus* MT-2: metal binding and protein folding of a true cadmium-MT. Int. J. Mol. Sci. 17: 65, 2016.

Kopitar-Jerala N. Innate Immune Response in Brain, NF-Kappa B Signaling and cystatins. Front. Mol. Neurosci. 8: 73, 2015.

Larsen EC, Ueyama T, Brannock PM, Shirai Y, Saito N, Larsson C, Loegerring D *et al.* A role of PKC-ε in FcγR-mediated phagocytosis by RAW 264.7 cells. J. Cell Biol. 159: 939-944, 2002.

Lefebvre C, Cocquerrelle C, Vandenbulcke F, Hot D, Huot L, Lemoine Y *et al.* Transcriptomic analysis in the leech *Theromyzon tessulatum*: involvement of cystatin B in innate immunity. Biochem. J. 380: 617-625, 2004.

Lefebvre C, Vandenbulcke F, Bocquet B, Tasiemski A, Desmons A, Verstraete M, Salzet M *et al.* Cathepsin L and cystatin B gene expression discriminates immune coelomic cells in the leech *Theromyzon tessulatum*. Dev. Comp. Immunol. 32: 795-807, 2008.

Li W, Li S, Zhong J, Zhu Z, Liu J, Wang W. A novel antimicrobial peptide from skin secretions of the earthworm, *Pheretima guillelmi* (Michaelsen). Peptides. 32: 1146-1150, 2011.

Loker ES, Adema CM, Zhang SM, Kepler T. Invertebrate immune systems – not homogeneous, not simple, not well understood. Immunol. Rev. 198: 10-24, 2004.

Molnár L., Engelmann P, Somogyi I, Mácsik LL, Pollák E. Cold-stress induced formation of calcium and phosphorous rich chloragocytes

granules (chloragosomes) in the earthworm *Eisenia fetida*. Comp. Biochem. Physiol. 163A: 199-209, 2012.

Morgan AJ, Stürzenbaum SR, Winters C, Grime GW, Aziz NA, Kille P. Differential metallothionein expression in earthworms (*Lumbricus rubellus*) tissues. Ecotox. Env. Saf. 57: 11-19, 2004.

Ohta N, Shioda S, Sekizawa Y, Nakai Y, Kobayashi H. Sites of expression of mRNA for lysenin, a protein isolated from the coelomic fluid of the earthworm *Eisenia foetida.* Cell Tissue Res. 302: 263-270, 2000.

Opper B, Németh P, Engelmann P. Calcium is required for coelomocyte activation in earthworms. Mol. Immunol. 47: 2047-2056, 2010.

Opper B, Bognár A, Heidt D, Németh P, Engelmann P. Revising lysenin expression of earthworm coelomocytes. Dev. Comp. Immunol. 39: 214-218, 2013.

Ragab A, Buechling T, Gesellchen V, Spirohn K, Boettcher AL, Boutros M. *Drosophila* Ras/MAPK signalling regulates innate immune responses in immune and intestinal stem cells. EMBO J. 30: 1123-1136, 2011.

Roubalová R, Dvořák J, Procházková P, Škanta F, Navarro Pacheco NI, Semerád J, *et al*. The role of CuZn- and Mn-superoxide dismutases in earthworm *Eisenia andrei* kept in two distinct field-contaminated soils. Ecotoxicol. Environ. Saf. 159: 363-371, 2018.

Sakaguchi A, Matsumoto K, Hisamoto N. Roles of MAP kinase cascades in *Caenorhabditis elegans*. J. Biochem. 136: 7-11, 2004.

Schikorski D, Cuvillier-Hot V, Leippe M, Boidin-Wichlacz C, Slomianny C, Macagno E *et al.* Microbial challenge promotes the regenerative process of the injured central nervous system of the medicinal leech by inducing the synthesis of antimicrobial peptides in neurons and microglia. J. Immunol. 181: 1083-1095, 2008.

Škanta F, Roubalová R, Dvořák J, Procházková P, Bilej M. Molecular cloning and expression of TLR in the *Eisenia andrei* earthworm. Dev. Comp. Immunol. 41: 694-702, 2013.

Škanta F, Procházková P, Roubalová R, Dvořák J, Bilej M. LBP/BPI homologue in *Eisenia andrei* earthworms. Dev. Comp. Immunol. 54: 1-6, 2016.

Stein E, Avtalion RR, Cooper EL. The coelomocytes of the earthworm *Lumbricus terrestris*: morphology and phagocytic properties. J. Morphol. 153: 467-477, 1977.

Valembois P, Lasségues M, Roch P, Vaillier J. Scanning electron-microscopic study of the involvement of coelomic cells in earthworm antibacterial defense. Cell Tissue Res. 240: 479-484, 1985.

Valembois P, Lasségues M. *In vitro* generation of reactive oxygen species by free coelomic cells of the annelid *Eisenia fetida andrei*: an analysis by chemiluminescence and nitro blue tetrazolium reduction. Dev. Comp. Immunol. 19: 195-204, 1995.

Wang X, Wang X, Zhang Y, Qu X, Yang S. An antimicrobial peptide of the earthworm *Pheretima tschiliensis*: cDNA cloning, expression and immunolocalization. Biotechnol. Lett. 25: 1317-1323, 2003.

Wang M, Wang L, Yi Q, Gai Y, Song L. Molecular cloning and characterization of a cytoplasmic manganese superoxide dismutase and a mitochondrial manganese superoxide dismutase from Chinese mitten crab *Eriocheir sinensis*. Fish Shellfish Immunol. 47: 407-417, 2015.

Xiong W, Bai L, Muhammad RU, Zou M, Sun Y. Molecular cloning, characterization of copper/zinc superoxide dismutase and expression analysis of stress-responsive genes from *Eisenia fetida* against dietary zinc oxide. Comp. Biochem. Physiol. 155C: 416-422, 2012.

Zasloff M. Antimicrobial peptides of multicellular organisms. Nature. 415: 389-395, 2002.