Uncovering evolutionary conserved activation processes and lysenin expression in earthworm immune cells

PhD thesis

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1. INTRODUCTION

1.1. Comparative aspects of innate and adaptive immunity

The immune system is of key importance for the integrity of organisms. It represents the basis of distinction between self and non-self structures. It plays a crucial role in the elimination of invading pathogens and modified self structures. Nowadays the comparative study of invertebrate and vertebrate immunity represents an importart part of basic science and a promising field of research.

Invertebrates have developed several innate immune mechanisms in order to keep self integrity, including leukocyte-like immune cells, that are capable to produce and secrete immunoprotective molecules. These immune cells can be divided into several subgroups, based on their morphological or functional heterogenity. Cellular and molecular components of innate immunity contribute in antimicrobial activity, coagulation, encapsulation, cytotoxicity and phagocytosis.

To understand the newly evolved adaptive immune system of vertebrates it is essential to study the phylogenetically ancient innate immunity of invertebrates, which is indicated by several evolutionarily conserved molecules and mechanisms. Consequently, the first line of defense in mammals is innate immunity til the slower adaptive immunity takes its turn.

1.2. The earthworm immune system: general characteristics, molecules and cell types

The class *Oligochaeta* (phylum *Annelida)* consists of a large number of different earthworms. The *Oligochaeta* earthworms are *protostomians,* their gut is tube-shaped, which is differentiated into three subparts. Uniquely among invertebrates, these species have a closed circulatory system and their blood harbor dissolved hemoglobin. Several various organs and tissue types are differentiated from the mesoderm. Mesoderm also forms the secondary body cavity, the so-called coelomic cavity. This chamber is filled with

coelomic fluid containing free-floating immune cells and humoral components. In case of danger the animals release the coelomic fluid through their dorsal pores to the environment.

Cellular components of earthworm immune system share functional similarities with vertebrate white blood cells. They participate in phagocytosis, cell-cell adhesion or in lytic processes. The typical lytic features of invertebrate immune cells can be investigated in experimental systems using microorganisms, red blood cells or tumor cells as targets. Humoral and cellular components of earthworm immunity can act separately or in concert.

Humoral factors with rapid activation provide more effective defense mechanisms than the slower cellular processes. Secretion of these soluble components guarantees the survival of earthworms in soil, which frequently consist microorganisms. However, this is only a single aspect of earthworm immunodefense, which is based on the subgroups of immune cells that produce and secrete soluble factors.

Earthworm immune cells, that are located in the coelomic cavity are called coelomocytes. They are considered to play a role in cytolytic and phagocytic events. Since the presence of clone specific receptor on invertebrate immune cells is still unknown, natural killer cell-like activity may be the mediator of cytotoxic reactions. This hypothetical NK cell-like lytic activity can explain the effective destruction of malignant cells.

Morphological observations revealed that one subgroup of coelomocytes is probably responsible for cytotoxic processes, while another mediates phagocytosis. According to other authors, granular and hyalin coelomocytes and free-floating chloragocyte (called as eleocyte) subpopulations can be distinguished. Chloragocytes are autofluorescent cells, caused by the riboflavin content of their intracellular granules (chloragosomes). Chloragocytes are considered to produce lytic molecules such as fetidins, lysenins.

According to the literature free chloragocytes (eleocytes) are terminally differentiated cells. These cells originate presumably from the chloragogen tissue surrounding the midgut. Chloragocytes start their life cycle as sessile cells, and then differentiate into free-floating cells.

Monoclonal antibodies were produced against coelomocyte subgroups to reveal their functional differencies and to identify distinct coelomocyte molecular markers (EFCC markers). Using this antibody pool, there were distinguished three subpopulations of *E.*

fetida. Two afore mentioned coelomocyte subgroups are effector immune cells participating in phagocytosis and encapsulation.

1.3. The role of calcium in invertebrate and vertebrate immune responses

Intracellular calcium levels are maintained by calcium binding proteins, calcium transporters and ion channels, bound either to plasma membranes or to cell organelles. Activation of ion channels can occur after potential changes or ligand binding. Ca^{2+}-ATPases are located in the plasma membranes (PMCA), or in the sarcoplasmatic reticulum (SERCA).

Activation of vertebrate immune cells occurs using signaling pathways involving intracellular calcium as second messengers. The increase of intracellular calcium level in leukocytes leads to several immunological processes e.g. chemotaxis, degranulation, phagocytosis, citokine release or proliferation.

1.4. Humoral factors involved in earthworm immunity

In addition to cellular mechanisms coelomocytes have impact on humoral immunity by secreting haemolytic and antimicrobial factors.

Coelomic fluid as a key component of earthworm innate immune system harbors the fetidin/lysenin protein family. According to the litarature this compartment may also contain antigen binding proteins. Initially fetidins were described as haemolytic lipoproteins of *E. fetida*, later coagulation of body fluid were linked to this protein as well.

Lysenin, also isolated from *E. fetida*, shows strong molecular homologue to fetidin. Lysenin and its related peptides may play a role in the elimination of invading pathogens after physical injuries.

2. OBJECTIVES

Earlier it was not known if the coelomocytes are terminally differentiated cells or they are able to proliferate. It was unclarified as well if mitogens or ionophores may influence the proliferation and the calcium levels of earthworm coelomocytes.

Using earthworm coelomocytes from various species (*Eisenia fetida* and *Allolobophora caliginosa*) we aimed to:

1. Detect the basal intracellular calcium levels.
2. Study the calcium mobilizing activity of ionophore (ionomycin) and mitogens (plant lectins and lipopolysaccharide).
3. Compare the percentage of immune cells in proliferation phase. For this purpose we have used different *in vivo* mitogen stimuli.

Members of fetidin/lysenin protein family might play an important role in earthworm immune response. Location of lysenin production and its exact immune functions are not clear yet. In our further studies, we have used monoclonal anti-lysenin antibody to achieve the following goals:

4. Localize of cellular and tissue expression pattern of lysenin
5. Monitor the changes of lysenin expression in free-floating coelomocytes after bacterial challenge.

3. MATERIALS AND METHODS

3.1. Coelomocyte harvesting

We used *E. fetida* and *A. caliginosa* earthworm species in the experiments. Prior to coelomocyte isolation earthworms were kept on wet paper towels to remove their gut content. Then earthworm were placed into Petri dishes filled with isolating buffer. Their coelomic content is released to the buffer. We diluted it with *Lumbricus* balanced salt solution (LBSS) buffer and then collected it. Living cells were enumerated with trypan blue staining.

3.2. Loading coelomocytes with a Ca^{2+}-sensitive fluorescent indicator

Isolated coelomocytes (stained with Fluo3-AM) were collected in DMEM or in Ca^{2+}-free PBS at 10^7 cell/ml concentration.

After the detection of basal Ca^{2+}-levels by flow cytometry, we have performed several treatments (ionophore, mitogen or chemoattractant stimuli in different concentrations). FL-1 channel was used to measure the fluorescence intensity.

3.3. Stimulation of coelomocytes, investigation of their proliferative capacity

To reveal the effects of repeated coelomocyte isolations and mitogen stimuli on proliferation earthworms were kept on wet towels overnight. Each treatment groups contained three animals.

Studying the effects of repeated cell isolations, the first isolation was followed by three further isolations. During the resting periods, the earthworms were let to regenerate in soil. The second isolation has followed the first one after 1 day, the third the second after 3 days, and at last the fourh the third after 7 days.

Earthworms were kept in Petri dishes filled with 5-5 ml LBSS overnight. Different concentrations of mitogens - lipopolysaccharid (LPS), phytohaemagglutinin (PHA),

concanavalin A (ConA), or pokeweed mitogen (PWM) - were added to the buffer. We have used eartworms kept in LBSS for control samples. On the next day we have isolated coelomocytes from the animals to reveal the short-term effects of the treatments. To study the long-term effects of stimuli, we have kept the earthworms in soil for three days after coelomocyte isolation, then they were placed to wet towel overnight. At the end we have isolated coelomocytes again from the animals.

In the case of multiple stimuli, the earthworms were kept in soil for 13 days after the first cell isolation and overnight stimulus. This was followed by one night on wet towel, then we have repeated the same stimuli used before. Then the animals have regenerated for two days in soil, and the last isolation has ended the experiment.

After washing steps and trypan blue staining, coelomocytes were collected in LBSS and divided into the same volume in FACS tubes. We have collected coelomocytes in RNase A and propidium iodide containing PI buffer. Samples were incubated and measured by flow cytometry.

3.4. Ca^{2+}-ATPase enzyme cytochemistry

Harvested coelomocytes were settled to glass slides and incubated in ATP and calcium-chloride containing sodium-barbital buffer. Samples were treated then with calcium-chloride, cobalt-chloride and ammonium-sulphide.

After incubation, the coelomocytes were stained with Mayer haematoxilin. The reaction appeared as a black precipitate in enzyme-positive cells.

3.5. Production of monoclonal anti-lysenin (a-EFCC5) antibody

Lysenin was dissolved in distilled water at 1 mg/ml concentration. Female BALB/c mice were injected using 10 µg lysenin in complete Freund adjuvant, followed by two other intraperitoneal injections in three week intervals using incomplete Freund adjuvant. Mice chosen by antibody titer and isotype were injected three days before the

splenocyte/myeloma fusion. Supernatants were tested with indirect ELISA method using lysenin, coelomocyte lysate and BSA control.

3.6. Earthworm coelomocyte immunocytochemistry

Coelomocytes ($5*10^5$ cells/ml in LBSS) were settled onto slides and fixed with ice cold acetone. We have blocked the endogenous peroxidase activity by phenil-hydrazine-hydrochloride. Slides were incubated in BSA solution, then stained with anti-lysenin supernatants (a-EFCC5) or purified IgG fractions. In the following step HRP- conjugated anti-mouse IgG was added. Immunreactions were visualized with 3-amino-9-ethyl carbazol containing acetate buffer.

We have performed double immunofluorescent stainings with a-EFCC5 IgG fractions from mice and NL557 conjugated donkey anti-mouse immunoglobulins as secondary antibody. After this step, we have applied FITC conjugated a-EFCC1, a-EFCC2, a-EFCC3 or a-EFCC4 mAbs. In the other set of experiments the anti-EFCC1-4 IgG fractions were detected by NL557 conjugated donkey anti-mouse immunoglobulin and then we have used a-EFCC5-FITC antibodies.

3.7. Immunohistochemistry and immunofluorescence of earthworm tissues

Earthworms were dissected and the tissues were fixed in Zamboni solution and embedded in succrose. Sectioning was performed by cryostate ans sections were placed to gelatine coated glass slides. Tissue samples were investigated by immunohistochemical or immunofluorescent methods. For washing steps 0.1% Triton-X 100 containing PBS was used.

3.8. *In vitro* bacterial treatments

Harvested earthworm coelomocytes were incubated with heat inactivated *Staphylococcus aureus* (OKI II2001) or *Escherichia coli* (ATCC 25922) bacteria. Coelomocytes (10^6) were mixed with bacteria (10^7) in 1 ml in Eppendorf tubes. After the

incubation samples were centrifuged. We have made coelomocyte lysate from the pellet, the supernatant was kept on -80 ^{o}C- for further experiments.

3.9. Preparation of coelomocyte lysate

Coelomocytes were lysed with RIPA buffer containing protease inhibitors on ice. Protein concentration of lysate and supernatants were measured using BCA kit, and used in further applications such as ELISA and Western blot.

3.10. Indirect ELISA with anti-EFCC5 antibody

Microtiter plates were coated either with coelomocyte lysate or with lysenin. Non-specific binding was blocked with gelatine. The plates were incubated with a-EFCC5 supernatant. After washing steps HRP conjugated goat anti-mouse IgG antibody was added. The isotype of a-EFCC5 mAb were detected with indirect ELISA method using mouse antibody isotype detecting kit. The reaction was visualized using o-phenilenediamine, and stopped with sulfuric acid. Washing steps were performed with 0.05 % Tween 20 containing PBS. The intensity of the reaction was measured at 490 nm.

3.11. Sandwich ELISA with a-EFCC5 antibody

Microtiter plates were coated with polyclonal anti-lysenin antibody. Non-specific binding was blocked with gelatine. Calibration curve was prepared using different lysenin concentrations between 2 µg/ml and 50 µg/ml. Wells were treated either with control or with supernatants of coelomocyte lysates. Then we have followed the indirect ELISA protocol with anti-EFCC5 antibody.

3.12. SDS-polyacrylamide gel electrophoresis and Western blot

We have used 10% polyacrylamide gel for protein separation. Protein samples were then blotted to nitrocellulose membrane. The membranes were blocked with 1% BSA/TBS-

Tween. Samples were incubated with monoclonal a-EFCC5 antibody or with rabbit polyclonal anti-lysenin antibody for 60 minutes. In the next step HRP conjugated anti-mouse or anti-rabbit IgG antibody were used at room temperature. Washing steps were performed using TBS/ 0.05% Tween buffer, reactions were visualized with ECL reagent.

3.13. Flow cytometry with FITC conjugated a-EFCC5 antibody

Isolated coelomocytes were washed in PBS and fixed in 4% paraformaldehyde (10^5 cells/sample). We have washed the samples with PBS/0.1 % sodium azide, then added permeabilizing solution (PBS/0.1% sodium azide/0.1% saponine). Samples were incubated with FITC conjugated a-EFCC5 antibody containing permeabilizing solution for 30 minutes at room temperature.

The probes were washed twice using saponine containig permeabilizing solution and once with BSA and sodium azide containing PBS. Samples were fixed in 0.5 % paraformaldehyde containing PBS. Fluorescence intensities were measured with FACSCalibur flow cytometer in FL1 channel.

4. RESULTS

4.1. Basal calcium levels of *E. fetida* and *A. caliginosa* coelomocyte subpopulations

Basal intracellular calcium levels were detected using flow cytometry. Signal intensity of Fluo3-AM fluorescent staining correlates with the free calcium level of cells. We have described significant differences in the basal calcium levels between coelomocyte subgroups. *E. fetida* chloragocytes showed higher basal calcium levels than granular or hyalin coelomocytes. In the case of *A. caliginosa,* we have observed similar trends.

4.2. Effects of ionophore treatment on calcium levels of coelomocyte subpopulations

Coelomocytes were loaded with Fluo3-AM in calcium containing DMEM. We have measured the basal calcium levels, then added 7 µM ionomycin to the samples. *E. fetida*

coelomocytes have showed significant, approx. three-four fold calcium level increase in a few seconds after the treatment. In contrast, the chloragocyte/eleocyte subpopulation have not reacted with calcium level increase upon ionomycin treatment.

We have speculated, if there is any difference in calcium signal in the coelomocytes of different earthworm species. Ionomycin have triggered also a significant Ca^{2+}-level increase in the case of *A. caliginosa* coelomocytes ($p<0.05$). However, this response was smaller than the signal of *E. fetida* coelomocytes. We have observed no calcium signal in *A. caliginosa* chloragocytes/eleocytes upon ionomycin treatment similarly to *E. fetida* chloragocytes/eleocytes.

Ionomycin caused calcium influx in the cytoplasm by opening Ca^{2+} channels in the plasma membrane or/and intracellular Ca^{2+} stores. However, the calcium source in the coelomocytes was unclear in our experiments. To solve this question, we have loaded coelomocytes with Fluo3-AM in Ca^{2+} -free PBS. Nextly, we added ionomycin to reveal its effects on coelomocytes. *E. fetida* coelomocytes showed a characteristic Ca^{2+}-signal increase upon the treatment, but the increase was lower compared to the response evoked in DMEM. Although, the increase of cytoplasmatic free Ca^{2+} level was significantly higher compared to the basal Ca^{2+} level ($p<0.001$), suggesting that ionomycin opens intracellular calcium stores. We have not observed significant Ca^{2+} -influx changes using *A. caliginosa* coelomocytes loaded in Ca^{2+} -free PBS.

4.3. PMA treatment induced no calcium signal in coelomocyte subgroups

Phorbol 12-myristate 13-acetate (PMA) is a phorbol esther with specific protein kinase C activating characteristics. According to our observations neither earthworm species reacted with basal Ca^{2+} level changes after PMA addition. However, after 1 minute treatment of coelomocytes with ionomycin we have added 1 nM or 10 nM PMA that caused an immediate attenuation of intracellular Ca^{2+} signal. Although, the detected attenuation of calcium level was not significantly different from the ionomycin treated samples, but it was a concentration dependent and transient process. At 200 seconds of the observation period the ionomycin and PMA treated samples showed similar intracellular calcium levels as the ionomycin treated coelomocytes.

4.4. Thapsigargin sensitive calcium pumps in earthworms

Thapsigargin is a specific inhibitor of the intracellular endoplasmatic reticulum Ca-ATPases (SERCA pumps). We have tested whether SERCA pump-like channels are involved in refilling the intracellular calcium stores of coelomocytes. Thapsigargin inhibited the provisory SERCA pumps of coelomocytes revealed by the increase of intracellular free calcium content.

Applying 2 µM of thapsigargin, first we observed a weak ($p<0.05$), then a delayed and strongly significant ($p<0.001$) Ca^{2+} efflux in *E. fetida* coelomocytes.

In addition *E. fetida* coelomocytes were preincubated with 2 µM thapsigargin for 30 minutes and then ionomycin were added we have detected significantly attenuated ($p<0.01$) calcium influx compared to the signal in coelomocytes without thapsigargin pretreatment.

Thapsigargin had a similar Ca^{2+}-mobilization effect in the case of *A. caliginosa* coelomocytes. Using same thapsigargin concentration a similar delayed effect was measured. However, this significant ($p<0.001$) calcium increase was lower compared to the *E. fetida* coelomocytes.

Moreover, enzyme cytochemical reaction was also performed to localize and detect the intracellular Ca^{2+}-ATPases in earthworm coelomocytes. Ca^{2+}-ATPase enzyme positive coelomocytes were found in both earthworm species. The most prominent enzyme reaction was observed near to the nucleus. However, in some occasions strong reactivity was observed in the whole cytoplasm. Coelomocytes with less prominent cytoplasm and large nucleus were stained positively, while chloragocytes and large coelomocytes appeared to be negative.

4.5. Ca^{2+}-mobilizing effect of phytohaemagglutinin in *E. fetida* coelomocytes

We tested commonly used mitogens of vertebrate experiments (LPS, PHA, ConA, or PWM) to study their effect on earthworm coelomocyte calcium levels. We only observed increased calcium levels when PHA was added to *E. fetida* coelomocytes. We applied several PHA concentrations and detected elevated calcium levels ($p<0.05$) at 30 µg/ml and 60 µg/ml concentrations. Both concentrations caused transient calcium influxes.

However, this effect was not strictly concentration dependent. *E. fetida* chloragocytes showed no reaction to the used stimuli. Various concentrations of PHA were tested on *A. caliginosa* coelomocytes but there was no significant response.

4.6. Bacterial formyl peptide activates coelomocytes and triggers intracellular calcium level increase

Short bacterial fMLP peptide is a chemotactic molecule and a potent activator for vertebrate phagocytes exerting intracellular calcium mobilization. We tested if fMLP exerts calcium influx in earthworm immune cells. We could not observe strong intracellular calcium increase. However, analyzing the kinetic curves there was a significant ($p<0.05$) intracellular calcium increase after adding fMLP compared to the basal Ca^{2+} levels. The fMLP treatment caused a transient rise of intracellular calcium. Using Ca^{2+} -free PBS we observed a similar rise of calcium level after fMLP treatment, but there was no significant difference between the samples loaded in Ca^{2+} -free PBS or in DMEM. *A. caliginosa* coelomocytes showed no response after fMLP treatment.

4.7. Cell cycle of coelomocytes upon various stimuli

We studied the proliferation activity of freshly harvested coelomocytes using PI staining and flow cytometry. We performed repeated coelomocyte isolations from the same earthworms and applied regeneration periods. We could detect the increase of cell numbers in G0-G1 and M phase. We found the highest amount of cells in mitotic phase after the second and third coelomocyte isolation. Samples after the last, fourth immune cell harvesting contained a lower number of M phase cells.

We also measured the rise of coelomocyte proliferation activity after cell isolation and various stimuli. We treated the earthworms with 12 μg/ml LPS, 25 μg/ml PHA, 25 μg/ml ConA or 25 μg/ml PWM stimuli. Our results revealed effects of PWM and ConA lectins, and a small increase of proliferating cell number upon LPS treatment, compared to LBSS treated controls.

4.8. Lysenin expression of harvested coelomocytes

Previous papers revealed the role of earthworm coelomocytes not only in cellular immunological responses but also in humoral mechanisms. They participate in immunodefense by producing haemolytical and antibacterial factors. We raised monoclonal antibody against lysenin (a-EFCC5) to investigate the lysenin expression of *Eisenia* coelomocytes.

Our experiments identified three subgroups of free floating immune cells of coelomic cavity. All three cell groups showed a-EFCC5 positivity, but with different intensity. We found the highest number of a-EFCC5 positive cells in the eleocyte subpopulation.

Antibody positive cells have large granular cytoplasm and relatively small nucleus. Immunopositivity were mostly found in the granular cytoplasm. The morphology of a-EFCC5 positive cells marks the free chloragocyte subgroup of coelomocytes. Another cell type characterized with large nucleus and smaller cytoplasm was negative for a-EFCC5 immunostaining. This cell group could be the hyaline amoebocytes. We have performed double immunostainings using previouly raised monoclonal antibodies against the coelomocyte subgroups. The a-EFCC3 positive coelomocytes (marking hyaline amoebocytes) are tending to attach together and form aggregates, while a-EFCC5 positive cells are mainly solitary cells.

4.9. Lysenin expresssion of free-floating coelomocytes

According to immunohistochemical and *in situ* hybridization findings, lysenin is produced by the chloragocytes of typhlosolis. Our immunohistochemical and immunocytochemical stainings identified free-floating coelomocytes as a-EFCC5 positive (lysenin expressing) cells. Lysenin expressing cells appeared in small groups of coelomocytes in coelomic cavity or as solitary cells bound to muscle tissue or nerve cord.

4.10. Effects of Gram positive bacteria on lysenin expression

It was necessary to reveal whether our mAb recognises specifically lysenin or not. Western blot analysis were performed and the results proved that a-EFCC5 recognises one discrete band in the coelomocyte lysate and its molecular size was identical with the purified lysenin protein. The control anti-lysenin polyclonal antibody recognized a protein band in the coelomocyte lysate similarly to a-EFCC5 antibody, but using the polyclonal antibody we observed a much stronger background compared to the reaction with mAb. The polyclonal antibody recognises the lysenin protein both in purified form and in coelomocyte lysate. The negative control Jurkat cell lysate showed no staining with our anti-lysenin antibodies.

Coelomocytes were incubated *in vitro* for 6 hours with heat-inactivated *E. coli* or *S. aureus* bacteria strains. We investigated the lysenin expression in coelomocytes with Western blot analysis, while the secreted lysenin in the supernatant was assessed by sandwich ELISA. Lysenin expression of coelomocytes incubated with *S. aureus* was significantly elevated ($p=0.0043$) compared to *E. coli* treated coelomocytes. It revealed non-significant elevation compared to untreated control samples ($p=0.058$).

Moreover *E. coli* treated coelomocyte samples had a significantly decreased lysenin expression ($p=0.042$) compared to control samples.

Lysenin content of supernatant (secreted by coelomocytes) showed a similar pattern to the coelomocyte lysates. In the coelomocyte supernatant exposed to *S. aureus* we measured elevated lysenin concentration compared to untreated control samples ($p=0.018$) or to *E. coli* treated coelomocytes ($p=0.012$). There was no significant lysenin level change in *E. coli* exposed samples compared to controls ($p=0.55$).

5. DISCUSSION

5.1. Basal calcium levels of coelomocytes, induction of Ca^{2+}-influx with ionomycin or inhibition using PMA

Efficiency of innate immunity depends on the recognition of pathogen components (pathogen associated molecular patterns- PAMPs) that mediated by the non-clonally distributed receptors such as pattern recognition receptors (PRRs).

PAMP recognition followed by downstream events: activation of signalling pathways, production of inflammatory mediators antimicrobial peptides, or influence the activity of genes controlling phagocytosis (Figure 1). The activation of vertebrate immune cells of innate and adaptive immunity depends on calcium-related signaling pathways. Oscillation of intracellular calcium levels participates in several immunological processes, such as chemotaxis, degranulation, phagocytosis, proliferation or cytokine release.

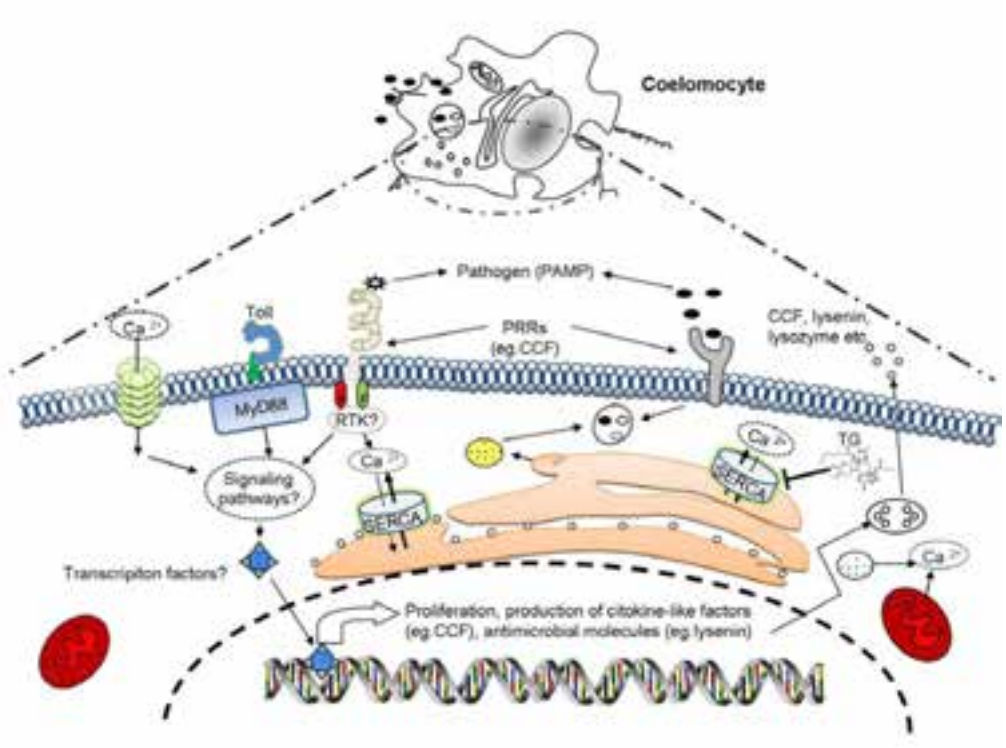

Figure 1.: *PAMP recognition by PRRs activates coelomocytes using several- hypothetical- signaling pathways. These patways can lead to phagocytosis, cell division or secretion of antimicrobial factors (Figure is based on Inv Surv J, 2011, 8: 78-84).*

Invertebrates use similar, evolutionarily conserved biochemical signaling pathways.

We demonstrated distinct Ca^{2+} levels of earthworm coelomocyte subgroups and their changes after various treatments (ionophore, lectins, chemoattractant) by flow cytometry. First, we measured the basal intracellular Ca^{2+} levels of coelomocyte subgroups of two earthworm species. The chloagocyte/eleocyte subgroup showed higher basal calcium level in both species than the granular or hyaline groups.

Then we tested the effects of intracellular calcium level modulating reagents in coelomocytes. We detected a 3-fold increase of free intracellular Ca^{2+} level upon ionomycin treatment. After ionomycin administration *A. caliginosa* coelomocytes showed a significant Ca^{2+} influx compared to their basal Ca^{2+} level. However, this increase was lower than in *E. fetida* coelomocytes. Interestingly, the chloragocyte/eleocyte population showed no response to ionomycin treatment in both species. Possibly chloragocytes/eleocytes loose their Ca^{2+} mobilizing activity during differentiation/maturation. However, the absence of ionomycin effect can also be the consequence of the low Ca^{2+} permeability of Ca^{2+} channels located in plasma membrane. Ionomycin cause first the release of calcium content from intracellular calcium stores, then activates the Ca^{2+} channels of plasma membranes. To prove this hypothesis, we have tested the ionomycin effect on coelomocytes treated in Ca^{2+}-free PBS. According to our findings, coelomocytes reacted with lower calcium signal upon ionomycin treatment compared to the samples kept in 1.8mM Ca^{2+} containing medium. Consequently, ionomycin triggers significant intracellular Ca^{2+} level increase due to Ca^{2+} influx through plasma membranes and Ca^{2+} efflux from intracellular Ca^{2+} stores.

Our results suggest that the PKC activator PMA neither induce Ca^{2+} influx through the plasma membrane, nor mobilizes Ca^{2+} from intracellular stores. Our findings fits in the line with earlier results found in vertebrate organisms. This proves the existence of universally conserved signaling mechanisms. According to our hypothesis, different stressors can lead to distinct activation/Ca^{2+} mobilization in coelomocyte subgroups.

5.2 Ca^{2+}-ATPase activity in coelomocytes

We have used thapsigargin to demonstrate the presence of conserved Ca^{2+}-ATPases in earthworm coelomocytes. Through thapsigargin treatment we have demonstrated Ca^{2+}-

flux in both earthworm species, which provided functional evidence of the existence of earthworm Ca^{2+}-ATPases. Moreover, thapsigargin pretreatment decreased the ionomycin evoked Ca^{2+-}-influx, proving that one of the major calcium stores of coelomocytes is located in the ER.

In order to localize the Ca^{2+}-ATPase activity in coelomocytes we stained coelomocytes for Ca^{2+}-ATPase enzyme cytochemistry.

Previous studies showed Ca^{2+}-ATPase activity in eartworm and leech tissues with mesodermal origin. We found enzyme positive coelomocytes from both earthworm species. The most characteristic staining pattern was observed around the nucleus, which is consistent with the localization of ER Ca^{2+}-ATPases.

5.3. Mitogen and chemoattractant induced Ca^{2+-}-influx in coelomocytes

Plant lectins such as PHA, PWM and LPS endotoxin are commonly used mitogens in both *in vitro* and *in vivo* experiments. It is known that some of these molecules evoke calcium signal oscillations during the activation and proliferation of vertebral leukocytes. In our experiments we tested whether mitogen induced calcium mobilization is an evolutionarily conserved process in the activation of invertebrate immune cells. Previous results have revealed that coelomocytes proliferate after mitogen stimuli. We have found that only PHA evoked calcium signal in the coelomocytes. Other mitogens, eg. LPS, PWM and ConA may lead to coelomocyte proliferation possibly through calcium independent pathways. fMLP caused transient increase of Ca^{2+} level in coelomocytes. This effect may play a role in early, but relatively weak activation of coelomocytes.

5.4. Effects of repeated cell isolations and mitogen stimuli on coelomocyte proliferation

Restoring coelomocyte numbers is essential in earthworm immunodefense. In our recent work we observed the increase of mitotically active coelomocytes using repeated cell isolations and resting periods. The highest cell numbers were detected after the second and third isolations. After the last, fourth coelomocyte harvesting we have found a drop in proliferating coelomocyte numbers.

We followed the short and long-term effects of mitogen stimuli on *E. fetida* and *A. caliginosa* coelomocyte proliferation. Using repeated mitogen stimuli we measured stronger ConA and PWM effects on *E. fetida* coelomocytes, whereas LPS treatment seemed to have a weaker effect on proliferation.

5.5. Lysenin expression of *Eisenia* coelomocytes and tissues

Cytotoxic and antimicrobial proteins play crucial role in innate immunity of earthworms. Previous studies have not reported clearly the expression pattern of lysenin in earthworms and its role in the immune response. According to our findings, lysenin is not produced by the sessile, central chloragocytes that are forming the chloragogenous tissue. We identified the free-floating chloragocytes/eleocytes as lysenin producing cells. The lysenin staining pattern was regardless to the applied polyclonal or monoclonal anti-lysenin antibody.

We can not exclude that central chloragocytes express lysenin only at mRNA level, thus mature, free chloragocytes are lysenin producing effector cells. To solve this contradiction more information is needed about the seasonal or maturational lysenin expression changes in coelomocytes.

5.6. *In vitro* bacterial challenge influences the lysenin production of coelomocytes

We have studied the role of lysenin in immunity using *E. coli* or *S. aureus* challenged coelomocytes. Our data indicates that Gram-positive bacteria can enhance lysenin expression using *in vitro* model system. Moreover, the lysenin expression decreased in *E. coli* challenged samples in agreement with previously published results.

Interestingly, Gram-positive or Gram-negative bacterial challenge modulates lysenin expression in different ways. For better understanding of the different lysenin expression pattern further studies are needed.

In summary, our experiments showed that lysenin is a multi-tasking protein for distinct purposes in earthworm immune system. Lysenin has not only cytotoxic, but also antibacterial functions.

6. PUBLICATIONS RELATED TO THE THESIS

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