COMPARATIVE ANALYSIS OF ENDOCYTOSIS: UPTAKE MECHANISMS OF BIOLOGICAL AND ARTIFICIAL PARTICLES BY EARTHWORM AND HUMAN IMMUNOCYTES

PhD Thesis

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1. Summary

Due to evolutionary conserved innate immune mechanisms between invertebrates and vertebrates, invertebrate immunocytes are hypothesized to be functionally similar to human macrophages. Hence, in our research, we aimed to compare the endocytosis pathways used by invertebrate macrophages (earthworm coelomocytes) and vertebrate phagocytes (human THP-1 cells) for uptake of particles of biological (bacteria) and synthetic (nanoparticles) origin.

In the first part of the thesis we identify and compare endocytosis pathways involved in *Escherichia coli* and *Staphylococcus aureus* uptake by human and earthworm immunocytes applying various endocytosis inhibitors. Moreover, we investigated the mRNA expressions of immune receptor-related molecules and bacteria/lysosomes colocalization after uptake inhibition. We found that during bacterial uptake both earthworm and human cells share the evolutionary conserved actin-dependent phagocytosis mechanism, which was inhibited by preincubation of cells with cytochalasins B and D. Decreased numbers of colocalized lysosomes/bacteria supported these findings. Our data strengthen the hypothesis about the conserved phagocyte-like functions of earthworm coelomocytes as possible functional similarities of human myeloid cells.

In the second part of the thesis, we characterize the endocytosis mechanisms for the uptake of 75 nm silver nanoparticles (AgNPs) by earthworm coelomocytes, human THP-1 monocytes, and macrophages. Subsequently, we analyze the intracellular localization, metabolic, epigenetic, and immune receptor-related effects upon the inhibition of AgNP uptake. According to our results, microtubule-dependent, scavenger-receptor, and PI3K signaling-mediated macropinocytosis is utilized by human THP-1 and PMA-differentiated THP-1 for AgNPs internalization. In contrast, earthworm coelomocytes rely on actin-dependent phagocytosis, similar to bacterial uptake. In all cell types, AgNPs localized in the cytoplasm, within endo-/lysosomes. The uptake is TLR/MyD88-dependent, in the case of human immunocytes BPI is also involved. These findings contribute to NPs trafficking that might be applied in the nanomedicine field (nano-carriers), as well as provide more data about ecotoxicological consequences of nanoparticle exposure.

2. General introduction

Various endocytic pathways are differentiated based on the size and origin of the engulfed particle, and the mechanism of formation of an endocytic vesicle. These mechanisms were developed for the delivery of various macromolecules, nutrients, and particles into the cells and generally classified as phagocytosis (for large particles >0.5 μm) and pinocytosis (for

liquids and soluble molecules). The last one is further divided into macropinocytosis, clathrin-mediated endocytosis (CME), caveolae-mediated endocytosis (CvME), and clathrin/caveolin-independent endocytosis[1]. One of the methods to study endocytosis pathways is using pharmacological inhibitors to block the aforementioned uptake pathways.

Due to the evolutionary conservation of endocytosis[2], various animal models are also applied, in particular, earthworms are promising animal research models for evolutionary immunological studies[3]. The two subpopulations of earthworm immune cells (coelomocytes) include amoebocytes and eleocytes, which possess a variety of immune functions similar to those carried out by vertebrate immunocytes. While eleocytes produce antimicrobial, cytotoxic, opsonizing, and agglutinating molecules[4], amoebocytes are capable of phagocytosis, and encapsulation[5]. Hence, in our research, we focus on amoebocyte subpopulation, since they were proved to be a major player during uptake of foreign particles by earthworm immunocytes[5].

Hypothesizing that earthworm amoebocytes resemble vertebrate myeloid cells, we investigate the similarities and differences between endocytic uptake pathways used by invertebrate and vertebrate immunocytes and apply three different cell types, such as earthworm coelomocytes, human THP-1 monocytes, and PMA-differentiated THP-1 macrophage-like cells. We compare the uptake pathways between invertebrate macrophages (earthworm coelomocytes) and vertebrate phagocytes (human THP-1 cells) towards bioparticles by assessing the endocytosis mechanisms against both Gram-positive and Gram-negative bacteria. Second, we characterize the corresponding uptake routes of engineered nanomaterials (silver nanoparticles) in earthworm coelomocytes and human THP-1 monocytic cells.

3. Aims

Cells always communicate with their outer environment through different routes and – consequently - several processes have been evolved to engulf the environmental compounds. These endocytic pathways have high importance in the life of different cells, which can explain the conservation of these mechanisms in the course of evolution. In addition, the cells also face several threats by the invading pathogens eliminated by phagocytosis. In the light of these thoughts, the thesis is structured into two main parts, which are closely connected since they deal with the experimental data about the comparative aspects of endocytosis towards various environmental particles.

1. We characterize the conserved endocytosis mechanisms against bioparticles by:

A) identifying the **uptake mechanisms** towards Gram-positive and Gram-negative bacterial strains **in earthworm coelomocytes and human** (normal and PMA differentiated) **THP-1 monocytic cells** applying different pharmacological inhibitors;

B) determining the **co-localization rate of engulfed bacteria/lysosomes** upon endocytosis inhibition;

C) observing the **mRNA expression of conserved pattern recognition receptors (PRRs)** following endocytosis inhibition.

2. We characterize the engulfment processes towards silver nanoparticles (AgNPs) by:

A) identifying the **AgNP uptake mechanisms of earthworm coelomocytes and**, **human** (normal and PMA differentiated) **THP-1 monocytic cells** applying different pharmacological endocytosis inhibitors;

B) **ultrastructural localization of AgNPs** upon endocytosis inhibition;

C) observing the **variation in mRNA expression of conserved PRRs** following the inhibition of AgNP uptake;

D) **evaluation of global DNA methylation (5-mC) pattern** upon endocytosis inhibition of AgNP engulfment;

E) **assessment of cellular respiration** after AgNP exposure**.**

4. Bacterial uptake mechanisms share functional similarities between earthworm and human immunocytes

4.1. Introduction

Earthworms are living in a microbe-rich milieu[6], yet the cellular uptake routes for microbes elimination are rather unexplored in earthworms, as well as in other invertebrate models. Hence, applying pharmacological inhibitors of different endocytosis pathways, we aimed to compare Gram-negative (*Escherichia coli*) and Gram-positive (*Staphylococcus aureus*) bacterial endocytosis mechanisms utilized by *Eisenia andrei* earthworm coelomocytes and their human counterpart THP-1 monocytes and diff. THP-1 macrophage-like cells.

The knowledge about the functional aspects of PRRs (regarding TLR) in earthworms is relatively limited[7]. *Eisenia andrei* TLR (*Ea*TLR) and its role in antibacterial response were recently characterized[8], but its downstream effector molecules in earthworms have not been yet determined. To keep the comparative approach, in our studies we choose only those immune-

related genes (*TLR*, *MyD88*, and *LBP/BPI*) that we can test in both invertebrate and vertebrate models.

Hence, in this part, we discuss the potential endocytosis mechanisms, as well as the receptors, that are involved in Gram-negative and Gram-positive bacteria-induced responses in earthworm and human immune cells.

4.2. Materials and methods

4.2.1. Cell culturing, earthworm husbandry, and coelomocyte isolation

4.2.2. Treatment with endocytosis inhibitors and bacterial challenge

4.2.3. Flow cytometry measurements

4.2.4. F-actin and lysosome labeling, confocal laser scanning microscopy (CLSM), and quantification of co-localization

4.2.5. RNA isolation, cDNA synthesis, and real-time PCR

4.2.6. Statistical analysis and data presentation

4.3. Results

Time-dependent analysis of FITC-conjugated heat-inactivated *E. coli* and *S. aureus* uptake in THP-1 cells showed rapid bacterial internalization by THP-1 cells already from the first hour of incubation and up to 90% until the 24 h. Pretreatment of cells with different compounds to target the endocytosis pathways indicated that actin-depolymerizing agent cytochalasin B significantly inhibited only the engulfment of *S. aureus*, while cytochalasin D significantly blocked the uptake of both Gram-positive and Gram-negative strains in THP-1 cells compared to controls.

Kinetic analysis of bacterial engulfment by PMA-differentiated THP-1 cells (diff. THP-1) revealed that the uptake was mostly analogous to THP-1 monocytes and the level of *E. coli* internalization reached approximately 90% and somewhat less for *S. aureus* at 24 h of incubation. Just like in the case of THP-1 monocytes, the strongest uptake inhibition was evoked by cytochalasin D, however, cytochalasin B also decreased the rate of *S. aureus* engulfment in the exposed diff. THP-1 cells. Other inhibitors did not influence the bacterial uptake. Imaging of the cytochalasin D exposed cells has confirmed the data of bacterial uptake inhibition measured by flow cytometry.

Evaluating the kinetics of bacterial uptake by coelomocytes (amoebocytes), we detected a slower uptake rate in comparison to the human counterpart. It begins to gradually increase at

4 h and reaches about 70% by 48 h. Endocytosis by earthworm coelomocytes was blocked by cytochalasin D similarly to THP-1 and diff. THP-1 cells. Interestingly, cytochalasin B also effectively hindered the uptake of Gram-negative bacteria, as opposed to human immunocytes.

With CLSM we detected the colocalization of FITC-conjugated bacteria and labeled actin filaments as well as compromised bacterial engulfment upon application of cytochalasin D, which confirms the flow cytometry data. Applying LysoTracker, we detected that pretreatment of cells with cytochalasin D reduced bacterial uptake, and, consequently, it significantly decreased the amount of colocalized signals from bacteria and lysosomes in all three cell types. This data was statistically validated by Manders' colocalization coefficient value analysis.

With regard to mRNA expression levels of immune-related genes, there was no significant increase in *TLR*, *MyD88*, or *BPI* levels stimulated by bacterial uptake in THP-1 cells, however, *TLR* expression was elevated upon cytochalasin D pretreatment. In diff. THP-1 cells the exposure to *E. coli* decreased *TLR* mRNA expression compared to control levels. We did not detect significant changes in *MyD88* expression, however, *BPI* mRNA level was increased after *S. aureus* exposure and downregulated after application of cytochalasin D. The *TLR* mRNA expression in coelomocytes was strongly upregulated only after *S. aureus* exposure and neither of the cytochalasins affected this parameter. *LBP/BPI* levels were significantly upregulated in response to *E. coli* and downregulated by cytochalasin D inhibition in each condition.

4.4. Conclusions

In the case of mammalian cells, it is generally accepted that actin polymerization is needed for the completion of phagocytosis and macropinocytosis, but not essential for other uptake pathways[9]. Our results underline the bacterial phagocytosis involving actin cytoskeleton not only in humans but also in earthworm immunocytes. Because of the essential role of actin cytoskeletal machinery in phagosome formation, an F-actin depolymerizing agent cytochalasin D turned out to be a universal inhibitor for the uptake of both Gram-negative and Gram-positive bacteria in all three studied cell types, indicating that both *E. coli* and *S. aureus* are engulfed *via* phagocytosis. Even though some differences in intracellular signaling exist, the actin-dependent phagocytosis suggests conserved functional similarities of bacterial uptake by human and earthworm immunocytes.

5. Endocytosis uptake routes of AgNPs are different between earthworm and human immunocytes

5.1. Introduction

In recent decades nanoparticles (NPs) among many other exploitations have been used by industry and healthcare as catalysts, wastewaters treatment agents[10], and, importantly, as drug delivery systems[11]. Nanosilver is the most widely used commercial product among engineered NPs[12] and the innate immune cells, particularly monocytes and macrophages stand on the front line of response to AgNPs and experience the related consequences. AgNPs often end up in the soil and are able to enter into the food chain causing a hazard for terrestrial organisms[13], including invertebrates, which are one of the first to encounter with NPs when they are released into the environment. Earlier we reported that AgNPs exposure in *E. andrei* earthworm leads to oxidative stress, DNA damage, and biased immune-related gene expressions[14–16].

Generally, the properties of NPs and their surrounding environment, such as particle size, shape, coating, surface charge, exposure time, etc., define particles' uptake routes and - consequently- their toxicological effects[17]. However, while data on toxicological consequences of AgNPs exposure is quite abundant, there is limited information about the exact cellular uptake mechanisms to internalize NPs, especially for invertebrates.

Since *Eisenia andrei* earthworm was recommended by OECD as a model organism for toxicity testing in soil[18], coelomocytes-mediated AgNPs uptake mechanisms deserve special attention. The endocytosis mechanisms by human immunocytes are also crucial from the angle of the improvement of NP-related drug delivery systems. In particular, changing particles' physico-chemical properties can be used for their targeting and intracellular trafficking.

Therefore, in our study, using endocytosis inhibitors we fill the gap in understanding the endocytosis processes of 75 nm AgNPs in three functionally similar cell types of invertebrates and vertebrates. Besides, we assess the changes caused by AgNPs on a molecular level, including changes in immune-related genes and respiratory profiles.

5.2. Materials and methods

5.2.1. Physico-chemical characterization of nanoparticles

5.2.2. Cell culturing, earthworm husbandry, and coelomocyte isolation

5.2.3. AgNPs dose-response studies

5.2.4. Endocytosis inhibitors treatments

5.2.5. Flow cytometry measurement of the internal complexity (SSC) parameters

5.2.6. Transmission electron microscopy (TEM) for intracellular AgNP localization

5.2.7. Quantification of intracellular silver content using ICP-MS

5.2.8. RNA-isolation, cDNA synthesis, and real-time PCR

5.2.9. 5-methylcytosine immunocytochemistry and quantification of DNA methylation levels

5.2.10. Bioenergetic analysis of cellular respiration upon AgNP exposure

5.2.11. Statistical analysis and data presentation

5.3. Results

Based on the obtained survival rates, the AgNPs EC_{20} values were the following: 3.1 μg/mL for THP-1 cells 3.6 μg/mL for diff. THP-1 cells and 38.9 μg/mL for coelomocytes, indicating that earthworm coelomocytes are more resistant to high AgNPs doses than human immunocytes. The established low cytotoxic concentrations were used in the further experiments.

Using pharmacological inhibitors, AgNPs uptake by THP-1, diff. THP-1 cells and coelomocytes were evaluated with flow cytometry-based assays according to the side scatter (SSC) properties (SSC increase upon AgNP exposure). From two main cytoskeleton components, actin and microtubules, the last one that was blocked by colchicine treatment and was shown to be essential for AgNPs endocytosis by human immunocytes. The microtubules are the necessary tracks for endocytic trafficking in all types of pinocytosis, hence, this endocytic mechanism is used to take up AgNPs. From ten tested inhibitors, three (colchicine, poly(I), and wortmannin) could effectively hinder AgNPs uptake by both THP-1 monocytes and diff. THP-1 macrophage-like cells. Another two pharmacological inhibitors that effectively hindered AgNPs uptake by THP-1 and diff. THP-1 cells are poly(I) and wortmannin, which suggests that AgNPs uptake by these cell types is mediated by scavenger receptors and involves PI3K signaling pathway.

The endocytosis inhibitors which was proved to effectively block AgNPs uptake in THP-1 and diff. THP-1 cells did not show any effect on earthworm coelomocytes. In turn, the AgNPs endocytosis in this cell type was blocked by actin depolymerization chemicals cytochalasin B and cytochalasin D. These results show the variations between immunocytes of different evolutionary stages. From two main elements of cytoskeletal machinery, microtubules seem to be used for AgNPs uptake by human immunocytes, while earthworm coelomocytes depend on actin filaments in this process.

Despite the different endocytic mechanisms, no major differences were found in the cellular localization of AgNPs between the studied cell types. We observed AgNPs localization in the cytoplasm closer to the periphery of the cell, allocated within the endosomes or phagolysosomes. In the inhibitors-treated cells, only seldom AgNPs were found inside the vesicles.

THP-1 and diff. THP-1 human immunocytes showed similar gene expression responses, in particular, we detected *MyD88* importance for AgNPs uptake and possible cross-talk between TLR, MyD88, and SRs complex. A significant increase in *BPI* expression upon incubation with AgNPs was shown for the first time in THP-1 and diff. THP-1 cells. Just like for human immune cells, *MyD88* in coelomocytes showed higher expression after applying AgNPs, and cytochalasins partly reduced *MyD88* expression to control levels. Surprisingly, compared to other earthworm species, *LBP/BPI* was not involved in coelomocytes' response to AgNPs, suggesting that not only different endocytosis pathways but also the uptake of various sized NPs is different between species.

While analyzing the global DNA methylation pattern, the AgNPs exposure in human immunocytes did not show significant changes in methylated cytosines (5-mC) levels, yet, we detected some effects related to endocytosis inhibitors. However, we observed the elevated DNA methylation levels in coelomocytes upon AgNPs exposure.

Measurements of the cellular metabolism mechanisms, such as oxygen consumption rate (OCR) and extracellular acidification rate (ECAR), showed the drop of OCR levels in both AgNPs-treated THP-1 and diff. THP-1 cells. Significantly differed the maximal respiration and spare respiratory capacity parameters which generally designates the weaker respiratory performance of human immunocytes in these stressed conditions. In the case of earthworm coelomocytes, while unexposed cells showed weak reliance on mitochondrial respiration, the AgNPs boosted the oxygen consumption rates in mitochondria. In particular, we observed significantly higher maximal respiration.

5.4. Conclusion

We observed that earthworm and human immunocytes use different endocytosis mechanisms, including different parts of the cytoskeleton and receptors for 75 nm AgNPs internalization. AgNPs uptake by human immunocytes is suggested to be performed through these endocytosis pathways simultaneously, and/or interchangeably with the involvement of scavenger receptors and microtubules. Unlike human cells, it seems that coelomocytes employ actin-dependent phagocytic mechanisms for both biological (Gram-positive and Gram-negative

bacteria) and engineered (AgNP) particle internalization. Besides, AgNPs have a dissimilar effect on immune-related genes and cellular metabolism of invertebrate and vertebrate immune cells.

6. Summary of new results

1. Among the tested endocytosis inhibitors, cytochalasin B attenuated the uptake of *S. aureus*, while cytochalasin D blocks the uptake of both *E. coli* and *S. aureus* in both THP-1 and diff. THP-1 cells.
2. Cytochalasins B and D effectively inhibited uptake of both *E. coli* and *S. aureus* in earthworm coelomocytes. These results suggest the conserved role of actin-dependent phagocytosis in bacterial uptake by human and earthworm immunocytes.
3. Engulfed bacteria are processed by lysosomes, while disruption of actin polymerization decreases the numbers of colocalized bacteria with lysosomes in all three cell types.
4. *TLR* expression in THP-1 cells increased after pretreatment with cytochalasin D, while it also resulted in dropped *LBP/BPI* expression in diff. THP-1 cells and coelomocytes.
5. SSC changes upon application of endocytosis inhibitors in THP-1 and diff. THP-1 cells revealed attenuation of AgNPs uptake by colchicine, poly(I), and wortmannin, indicating the importance of microtubules, scavenger receptors, and PI3K signaling in AgNPs internalization by human immunocytes.
6. In coelomocytes cytochalasins B and D inhibited AgNPs uptake, suggesting the involvement of actin-dependent phagocytosis.
7. TEM revealed that AgNPs are localized within the endo/lysosomes in the cytoplasm of the cells close to the cellular membrane, and do not enter the nucleus.
8. ICP-MS measurements detected decreased mass of silver in THP-1 and diff. THP-1 cells after preincubation with colchicine, poly(I), and wortmannin.
9. qPCR analysis suggests that AgNPs uptake in THP-1 and diff. THP-1 cells are mediated by BPI/TLR/MyD88 signaling, while in coelomocytes LBP/BPI does not take part in the process.
10. AgNPs uptake decreases the cell respiration characteristics, such as OCR and ECAR in human immunocytes, while coelomocytes exhibit enhanced respiratory parameters upon AgNPs internalization.

7. References

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9. List of publications

First author publications connected to research topic:

Kokhanyuk, B., Bodó, K., Sétáló Jr, G., Németh, P., Engelmann, P., 2021. Bacterial Engulfment Mechanism Is Strongly Conserved in Evolution Between Earthworm and Human Immune Cells. *Front. Immunol.* 12:733541. *Independent citations: 0; IF: 7.56*

Kokhanyuk, B., Bagóné Vántus, V., Vámos, E., Radnai, B., Bélteki, A., Galbács, G., Telek, E., Mészáros, M., Deli, M.A., Németh, P., Engelmann, P. Earthworm and human immunocytes utilize different endocytosis mechanisms for AgNPs uptake (under preparation)

Co-author publications connected to research topic:

Bodó, K., Baranzini, N., Girardello, R., **Kokhanyuk, B.,** Németh, P., Hayashi, Y., Grimaldi, A., Engelmann, P., 2020. Nanomaterials and Annelid Immunity: A Comparative Survey to Reveal the Common Stress and Defense Responses of Two Sentinel Species to Nanomaterials in the Environment. *Biology* 9, 307. *Independent citations: 2; IF: 5.08*

Bodó, K., Hayashi, Y., Gerencsér, G., László, Z., Kéri, A., Galbács, G., Telek, E., Mészáros, M., Deli, M.A., **Kokhanyuk, B.,** Németh, P., Engelmann, P., 2020. Species-specific sensitivity of Eisenia earthworms towards noble metal nanoparticles: a multiparametric in vitro study. *Environ. Sci.: Nano* 7, 3509–3525. *Independent citations: 3; IF: 8.13*

Co-author publications not connected to research topic:

Bodó, K., Kellermayer, Z., László, Z., Boros, Á., **Kokhanyuk, B.**, Németh, P., Engelmann, P., 2021. Injury-Induced Innate Immune Response During Segment Regeneration of the Earthworm, *Eisenia andrei*. *Int. J. Mol. Sci. 22*, 2363. *Independent citations: 4; IF: 5.92*

Total number of independent citations: 9

Total IF: 26.69

10. Participation in conferences

Oral presentations (first-author):

Kokhanyuk B., Bodó K., Sétáló Jr. G., Németh P., Engelmann P. Evolutionary conserved uptake pathways are involved in bacterial engulfment mediated by invertebrate and vertebrate immunocytes. 2020. A Magyar Immunológiai Társaság 49. Vándorgyűlése

Kokhanyuk B., Nagy R., Bodó K., Németh P., Engelmann P. Phylogenesis of phagocytes: comparative investigations on invertebrate and vertebrate immune cells. 2019. Spring Wind Conference for young Hungarian researchers and PhD students, Debrecen

Poster presentations (first-author):

Kokhanyuk B., Bodó K., Radnai B., Bagóné Vántus V., Vámos E., Telek E., Mészáros M., Németh P., Engelmann P. Uptake routes of silver nanoparticles are different between invertebrate and vertebrate immunocytes. 2021. A Magyar Immunológiai Társaság 50. Vándorgyűlése

Kokhanyuk B., Bodó K., Sétáló Jr. G., Németh P., Engelmann P. Evolutionaly conserved uptake pathways are involved in the bacterial engulfment mediated by invertebrate and vertebrate immunocytes. 2020. 15th Spring School on Immunology, Ettal, Germany

Kokhanyuk B., Bodó K., Németh P., Engelmann P. 2019. Untangling the bacterial uptake mechanisms in invertebrate and vertebrate immunocytes. A Magyar Immunológiai Társaság 48. Vándorgyűlése

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Kokhanyuk B., Bodó K., Németh P., Engelmann P. Restoration kinetics of cellular immune components in earthworms following experimental depletion. 2019. Type-2 Immunity in Homeostasis and Disease, Bruges, Belgium

Kokhanyuk B., Nagy R., Bodó K., Németh P., Engelmann P. Comparative analyses of phagocytic activity in invertebrate and vertebrate immunocytes. 2019. XVI. János Szentágothai Multidisciplinary Conference and Student Competition

Kokhanyuk B., Bodó K., Gerencsér G., Telek E., Németh P., Engelmann P. Uncovering the intricate interactions of metal nanoparticles and *Eisenia andrei* immune cells. 2018. Medical Conference for PhD Students and Experts of Clinical Researches