Supplementary Material

Nanosilver pathophysiology in earthworms: Transcriptional profiling of secretory proteins and the implication for the protein corona

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Number of pages (including the cover page): 11
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Supplemental description of methods

MTT and NR assays

Two types of well-established plate assays were employed, namely MTT and neutral red (NR) assays according to procedures described in Beer et al. (2012) and Repetto et al. (2008), respectively. Briefly, 5×10^4 cells were exposed in quadruplicate to a concentration range of 0.06-1.00 µg/ml ($AgNO_3$) and 1-20 µg/ml (NM-300K) for 24 h. In MTT assays, the cells were spun down (the supernatant removed) and incubated in 500 µg/ml MTT working solution (in CCM without FBS) for 4 h, before the formazan precipitates were completely dissolved in dimethyl sulfoxide (DMSO) for the colorimetric measurement (absorbance at 570 nm, reference at 650 nm). In NR assays, the cells were incubated in 40 µg/ml NR working solution (prepared in CCM) for 3 h. The cells were then spun down (the supernatant removed) and washed in R-PBS, before they were treated with a destain solution (50% ethanol and 1% glacial acetic acid in water) for the colorimetric measurement (absorbance at 540 nm). Formation of NR crystals was not observed under the incubation condition used, and thus the NR extract should correspond to the amount accumulated in the lysosomal compartment. No nanoparticle- or Ag-specific measurement interference was observed at the highest concentration, and the absorption values in the treatments were compared against the control after cell-free background subtraction.

UV-visible spectrophotometry and nanoparticle tracking analysis

The localized surface plasmon resonance peak of NM-300K (at 10 µg/ml) was scanned on a UV-visible spectrophotometer (UV-vis, UV-3600, Shimadzu, Japan). The hydrodynamic radius was determined by nanoparticle tracking analysis (NTA, NanoSight LM10-HS, NanoSight Ltd., UK). Following incubation at 2 µg/ml (the low-cytotoxic concentration), each sample was diluted 1:100 – 1:1000 in a sterile-filtered solution (Milli-Q water or CCM without FBS) in order to have a working concentration of ca. 10^8 nanoparticles/ml. Due to this procedure, the samples to be analyzed contains a very low amount of free proteins (although a substantial amount of proteins may be present in the form of protein coronas around nanoparticles). Signal-to-noise ratio was high enough to confidently differentiate light scattering of AgNPs from that of proteins and, if any, protein aggregates. We did not measure a blank sample of CCM because under this calibration it is not possible to obtain meaningful results from a blank sample, as nothing apart from background noise would be detected. It should also be noted that due to dilution of the samples the protein environment at the time of measurements may be different, however, the UV-vis results support our observations that there were some nanoparticle agglomerates. Therefore, we believe the NTA results are valid even after dilution. We have also attempted to perform another type of analysis to study the velocity of Brownian motion, namely dynamic light scattering. However, we could not obtain results with confidence due to weak light scatter as compared to light absorption, as well as the presence of some agglomerates (greatly influences the measurement because of much higher scattering of the light).

Quantification of dissolved silver and cellular silver burdens

Soluble silver fraction (dissolved silver complexes and free Ag^+) of NM-300K under the exposure condition (2 µg/ml, 4 h and 24 h in CCM) was determined using an ultracentrifugation method (for details see Beer et al., 2012, Foldbjerg et al., 2012, Hayashi et al., 2012). Briefly, following incubation, NM-300K was ultracentrifuged for 30 min at 4 °C at 50 000 rpm (corresponding to 70 000-135 000 × *g*) and the supernatant was digested in 69% HNO_3 (tracemetal grade, Sigma-Aldrich). The analyte was heated (stepwise, 80-100-120-130 °C) to dry and redissolved in 0.2% HNO_3. Total Ag was determined on a PerkinElmer Analyst 4100 atomic absorption spectrophotometry mounted with a HGA700 graphite oven (GFAAS) and a silver lumina hollow cathode lamp (PerkinElmer, Denmark) within the linear detection range (Ag limit of quantification

was 0.25 ng/ml). GFAAS was performed with recommended matrix modifiers (palladium and magnesium; Sigma-Aldrich) on a standard protocol for Ag. Each reading was duplicated and quantified Ag concentration was subtracted with a method blank. Recovery of Ag from the reference $AgNO_3$ solution (Reag. Ph. Eur., Sigma-Aldrich) and reference AgNPs (BioPure 75 nm OECD reference nanomaterial silver, NanoComposix, CA, USA) was 91-111% and 80-95%, respectively. Cellular accumulation of silver was quantified following the procedure described previously (Hayashi et al., 2013, Jiang et al., 2013). Briefly, coelomocytes (10^6 cells/sample) were exposed for 4 h or 24 h to the low-cytotoxic concentration of $AgNO_3$ or NM-300K, and subsequently they were washed twice in R-PBS. Small aliquots of each cell suspension were stained with trypan-blue and examined using an automated cell counter (Countess, Invitrogen). Remaining cells were pelleted and digested in 69% HNO_3. Controls included were unexposed cells and cell-free background samples (for each silver treatment). GFAAS was performed in the same manner as described above, and analytical spike recovery (spiked/unspiked pair) of unexposed cell samples was 86-87%. Data from each treatment was subtracted with the corresponding cell-free background value and normalized by the number of cells. Three independent assays were conducted.

Flow cytometry

Coelomocytes (10^6 cells/sample) were exposed to the low-cytotoxic concentration of $AgNO_3$ or NM-300K for 2, 4, 8 and 24 h. A positive control (100 µM H_2O_2; Sigma-Aldrich) was included for this study. Following exposure, the cells were dissociated using Accutase (Gibco), half of which was analyzed for the intracellular reactive oxygen species (ROS) level (Hayashi et al., 2012) while the remaining half was for the basal intracellular calcium level (Opper et al., 2010). To measure the intracellular ROS level, the cells were loaded with 40 µM of the fluorescent marker 2´,7´-dichlorodihydrofluorescein diacetate (H_2DCF-DA; Sigma-Aldrich) in CCM (without FBS) for 20 min. The cells were then washed and resuspended with sterile-filtered 0.1% w/v bovine serum albumin (BSA; Sigma-Aldrich) in R-PBS, kept on ice, and immediately analyzed by flow cytometry (Gallios, Beckman Coulter). The 488 nm laser was used for excitation and DCF was detected in FL1 (525/50 BP filter). For each sample, the time-of-flight analysis of forward scatter (FS) was used to exclude cell duplets, and a total of minimum 15 000 cells was gated for analysis in Kaluza (Beckman Coulter). As described previously (Hayashi et al., 2013), two major populations of coelomocytes were separated based on the level of autofluorescence (detected in FL4, 695/30 BP filter). The population with non- or less autofluorescence was assumed as amoebocytes and studied for the level of intracellular ROS. At the low-cytotoxic concentration, leakage of the dyes from the stained amoebocytes was minimal as membrane damage determined by YO-PRO-1 iodide (Molecular Probes) was <10% at 24 h. To measure the intracellular calcium level, the cells were loaded with 20 µM Fluo-3 AM (Molecular Probes) in CCM for 20 min, and further 20 min in CCM to allow complete de-esterification of intracellular AM esters. The cells were then washed and resuspended with sterile-filtered 0.1% w/v BSA in R-PBS, kept on ice, and immediately analyzed by flow cytometry in the same manner as described above (with the FL1 channel). Three independent assays were performed.

Gene expression profiling

Coelomocytes (10^6 cells/sample) were exposed in duplicate (and repeated as an independent assay; i.e. the total $n = 4$) to the low-cytotoxic concentration of $AgNO_3$ or NM-300K for 2, 4, 8 and 24 h. The cells were subsequently washed in R-PBS and stored at -80 °C in RNAlater (Ambion). Total RNA was extracted using a NucleoSpin RNA kit (Macherey-Nagel, Germany) according to the manufacturer's instructions. RNA concentrations were determined by spectrophotometry using a NanoPhotometer (Implen) and RNA integrity was verified by 1% agarose gel electrophoresis. cDNA was synthesized from 68 ng total RNA using an Omniscript Reverse Transcriptase kit (Qiagen), RNaseOUT Recombinant Ribonuclease Inhibitor (Invitrogen) and Anchored Oligo(dT)

20 primers (Invitrogen) following the manufacturers' instructions. Two cDNA-negative controls (reactions without reverse transcriptase) were prepared from representative samples. The cDNA templates were diluted to a concentration equivalent of 0.8 ng total RNA/µl and stored at -20 °C. To analyze expression of 8 target genes (see Table 1 for the putative functions), quantitative real-time PCR (qPCR) was performed on a Stratagene MX3005P (Agilent Technologies) using Brilliant III Ultra-Fast SYBR qPCR Master Mix (Agilent Technologies). The target genes were chosen based on their putative roles in immune responses including those encoding secretory proteins. Primer sequences of the target genes and the amplification efficiencies are listed in Table S1. Primers were designed using Primer3 and synthesized by Eurofins MWG Operon. Each reaction was run in duplicate and contained 5 µl of cDNA template (equivalent to 4 ng total RNA) along with 100 nM primers in a final volume of 20 µl. The amplification was performed under the recommended conditions for the SYBR master mix: 95 °C for 3 min to activate the DNA polymerase, then 40 cycles of 95 °C for 10 s and 60 °C for 20 s. Melting curves were visually inspected to verify a single amplification product with no primer-dimers. The cDNA-negative controls showed no or very little amplification. Data analysis for real-time PCR (DART-PCR; Peirson et al., 2003) was used to analyze the amplification kinetics and expression values. Target gene expression was then normalized with NORMA-Gene (Heckmann et al., 2011). The normalized expression values were set relative to the mean control values at corresponding time points (i.e. differential expression in the treatment at each time point was studied).

Protein secretion

Secretion of proteins, in particular of lysenin, was examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in a time-resolved manner as in the qPCR study. Coelomocytes (2.5×10^5 cells/sample) were exposed to the low-cytotoxic concentration of $AgNO_3$ or NM-300K for 2, 4, 8 and 24 h. Subsequently, the supernatant was collected in a Protein LoBind tube (Eppendorf) and centrifuged for 5 min at 500 × *g* to pellet the cells. The cell-free supernatant was mixed with concentrated SDS-loading buffer (with 100 mM dithiothreitol as a reducing agent; 5X Lane Marker Reducing Sample Buffer, ThermoScientific) and stored at -20 °C. Prior to SDS-PAGE, the samples were boiled for 5 min to denature the proteins and strip off the protein coronas from the nanoparticles in the supernatant, after which the nanoparticles were spun down at 16 000 × *g* for 20 min at 4 °C. The protein samples (ca. 12 µg) were separated by 4-20% gradient SDS-PAGE (Precise Protein Gels, ThermoScientific) along with a PageRuler Unstained Protein Ladder (ThermoScientific) as the molecular weight standard (10-200 kDa). Protein bands in the gels were detected by Coomassie Brilliant Blue staining (Imperial Protein Stain; ThermoScientific) following the manufacturer's instructions. The stained gels were scanned on a Gel Doc EZ system (Bio-Rad) and the images were processed with the Image Lab 4.0.1 software (Bio-Rad). Three independent assays were conducted.

Primer sequences and amplification efficiencies (Table S1)

Table S1. GenBank Accession numbers and primers used for qPCR analysis

Gene name (gene symbol)	GenBank accession #	Primers (5´-3´)[a]	Amplicon size (bp)	Amplification efficiency (*E*)[b]
MEK kinase 1 (MEKK1)	EH672240[c]	CAAAGGTGCAAACATTGTGG ATGAATGGGATCGTTCCTTG	120	0.923 ± 0.045
Cadmium-metallothionein (MT)	AJ236886	CTTGTTGCTGCACAAACTGC TTTCCACATTTGCCCTTCTC	97	0.924 ± 0.037
Lysenin (Lysenin)	D85846	TGAATGCTGACGTTGGTGGA TAGCCCGACTTGTGATGACC	108	0.924 ± 0.050
Lysozyme (Lyz)	KC493575	TGAGAGCCAGATCGGAAAGT GGTTCCTTGATTTGGAATGG	72	0.915 ± 0.043
LPS binding protein / Bactericidal permeability-increasing protein (LBP/BPI)	JQ407018[d]	ATTTCCATCGGCGGTGATTG GAGACATCGTGAAGCTTCCG	82	0.919 ± 0.035
Ferritin (Ferritin)	HO001288[c]	CGGTCGACAGTTACTCATCCA AAGAGAGTCGGACCAGGAATG	70	0.929 ± 0.026
Coelomic cytolytic factor 1 (CCF1)	AF030028	TCTTCGACGCCAGAGGAAAC GCGCTTGTAGACTCGGATGT	92	0.951 ± 0.026
Toll-like receptor (TLR)	JX898685[d]	CCTGGAGAGCATCGACAACA TGCCATCGTCAGCTCAAAGT	94	0.949 ± 0.029

[a] Upper and lower sequences represent forward and reverse primers respectively.
[b] Values are mean ± SD (n = 62-64).
[c] Expressed sequence tag.
[d] Primers were designed based on the conserved domains (TIR, BPI) of mRNA sequence data from *E. andrei*, a very closely related species.

Estimated EC_x values (Table S2)

Table S2. Estimated EC_x values.

Assay type	Treatment	Hill slope	EC_{10} (µg/ml)	EC_{50} (µg/ml)
MTT	$AgNO_3$	-1.65 ± 0.24	0.16 ± 0.03	0.61 ± 0.09
	NM-300K	-2.12 ± 0.40	2.85 ± 0.35	8.86 ± 0.76
Neutral Red	$AgNO_3$	-0.98 ± 0.30	0.10 ± 0.06	0.78 ± 0.22*
	NM-300K	-1.02 ± 0.19	1.19 ± 0.54	8.84 ± 1.61
Average	$AgNO_3$	-	0.13	0.70
	NM-300K	-	2.02	8.85

Values are mean ± S.E. of three independent assays.
* An outlier was excluded (n = 2).

Concentration-response curves (Figure S1)

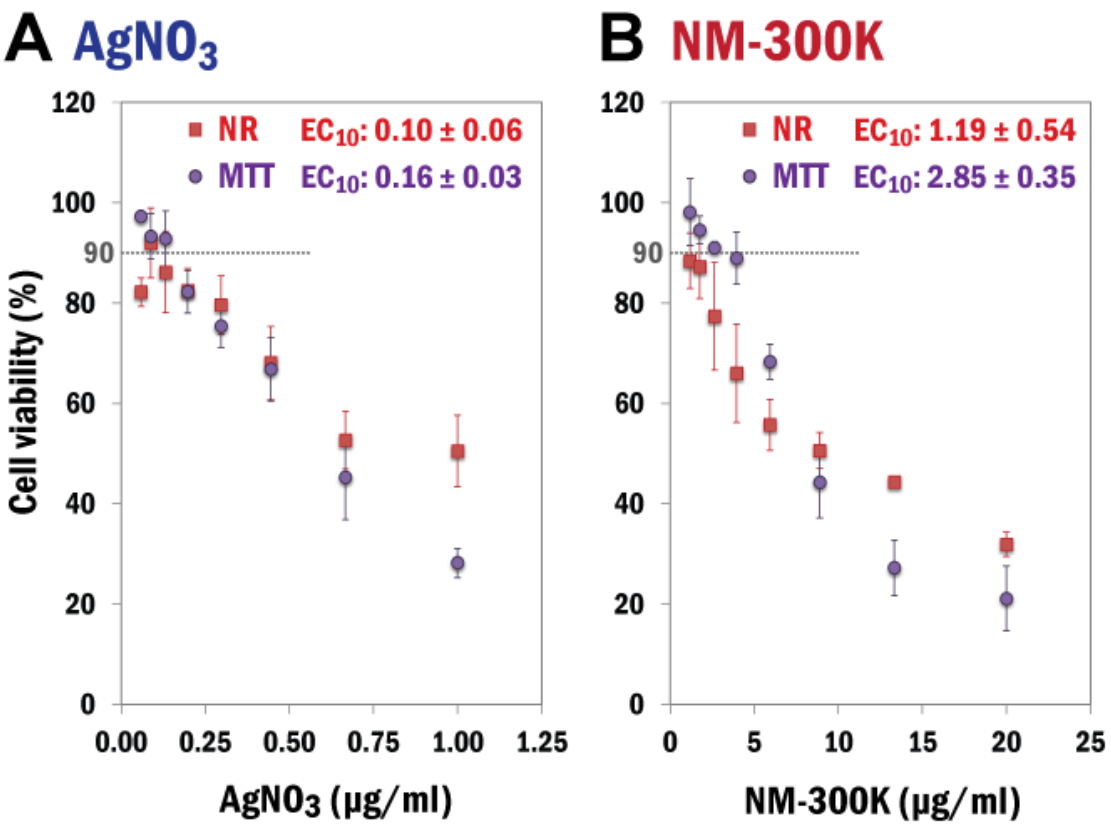

Figure S1. Concentration-response curves for (A) $AgNO_3$ and (B) NM-300K determined by MTT (circle) and NR (square) assays. EC_x values were estimated by individual curve-fitting (concentrations on a log scale) in each assay and shown are mean ± S.E. of three independent assays.

Particle characterization (Figure S2)

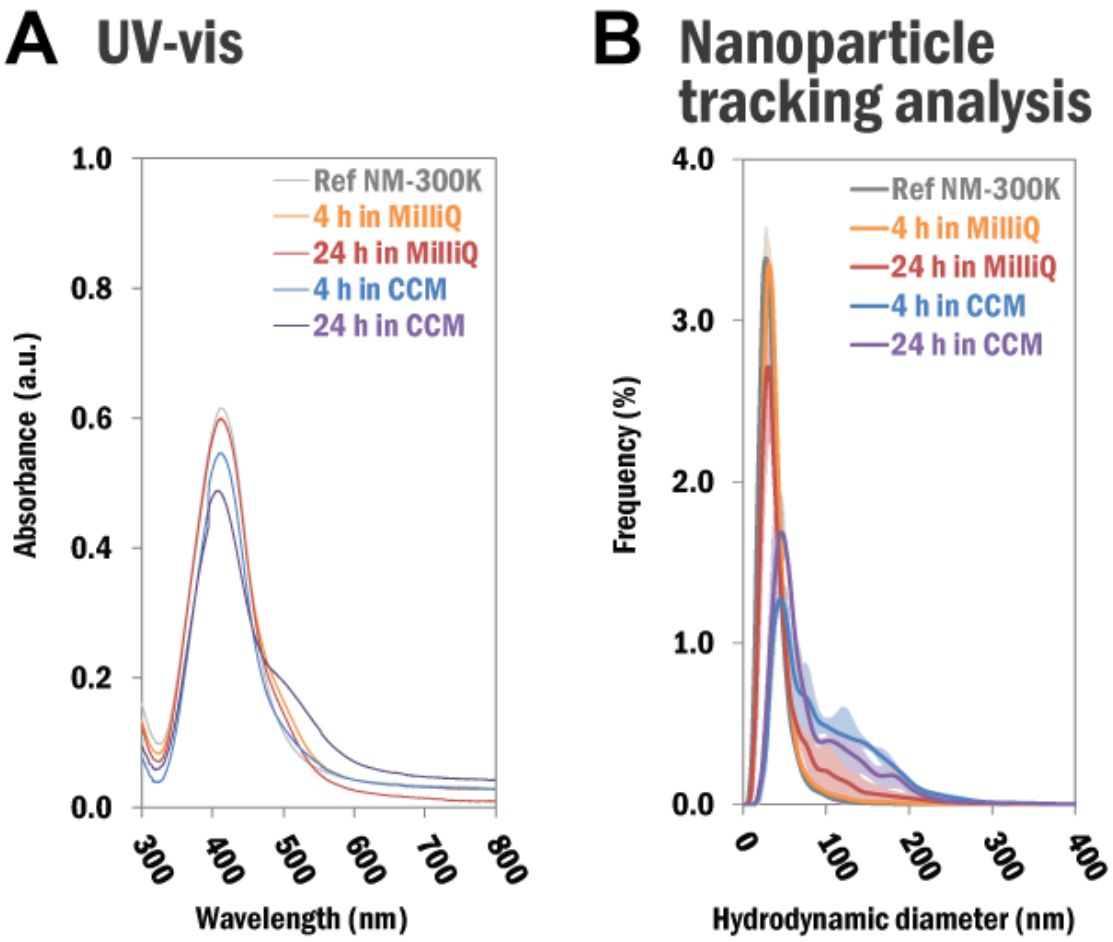

Figure S2. Colloidal stability of NM-300K under exposure conditions (dispersed in CCM, incubated at RT in darkness). (A) Localized surface plasmon resonance of NM-300K studied using UV-vis. (B) Nanoparticle tracking analysis to determine the agglomeration states.

Dissolved silver and cellular silver accumulation (Figure S3)

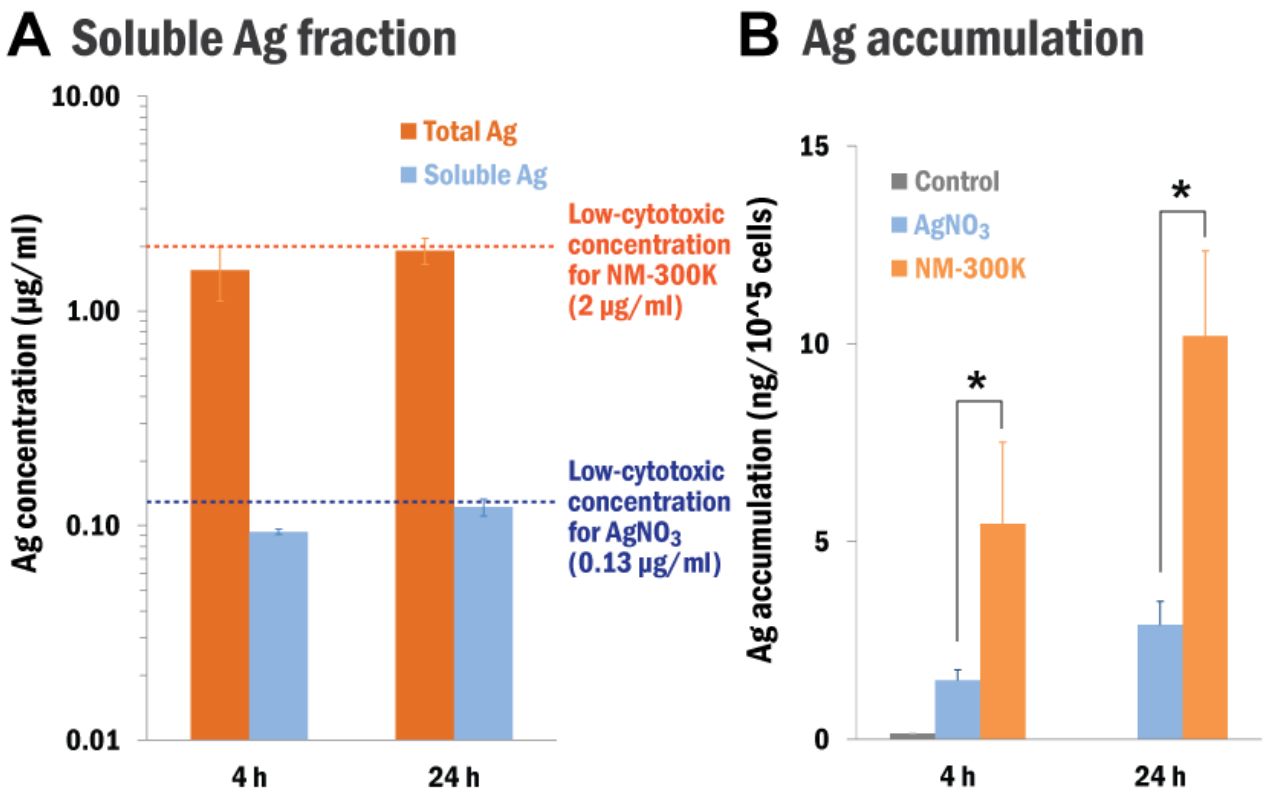

Figure S3. GFAAS quantification of (A) soluble Ag fraction and (B) cellular Ag accumulation. (A) Soluble silver fraction (dissolved silver complexes and free Ag^+) of NM-300K under the exposure condition (2 µg/ml, 4 h and 24 h in CCM) was determined using an ultracentrifugation method. Values are mean ± S.D. (n = 3-4). Dashed lines show the low-cytotoxic concentrations for $AgNO_3$ (0.13 µg/ml) and NM-300K (2 µg/ml). (B) Cellular accumulation of silver was quantified following 4 h or 24 h exposure to the low-cytotoxic concentrations of $AgNO_3$ (0.13 µg/ml) or NM-300K (2 µg/ml). Values are mean ± S.E. (n = 3). Asterisks (*) denote a significant difference between the treatments $AgNO_3$ and NM-300K (Student's t-test, $p < 0.05$).

Oxidative stress and basal calcium levels (Figure S4)

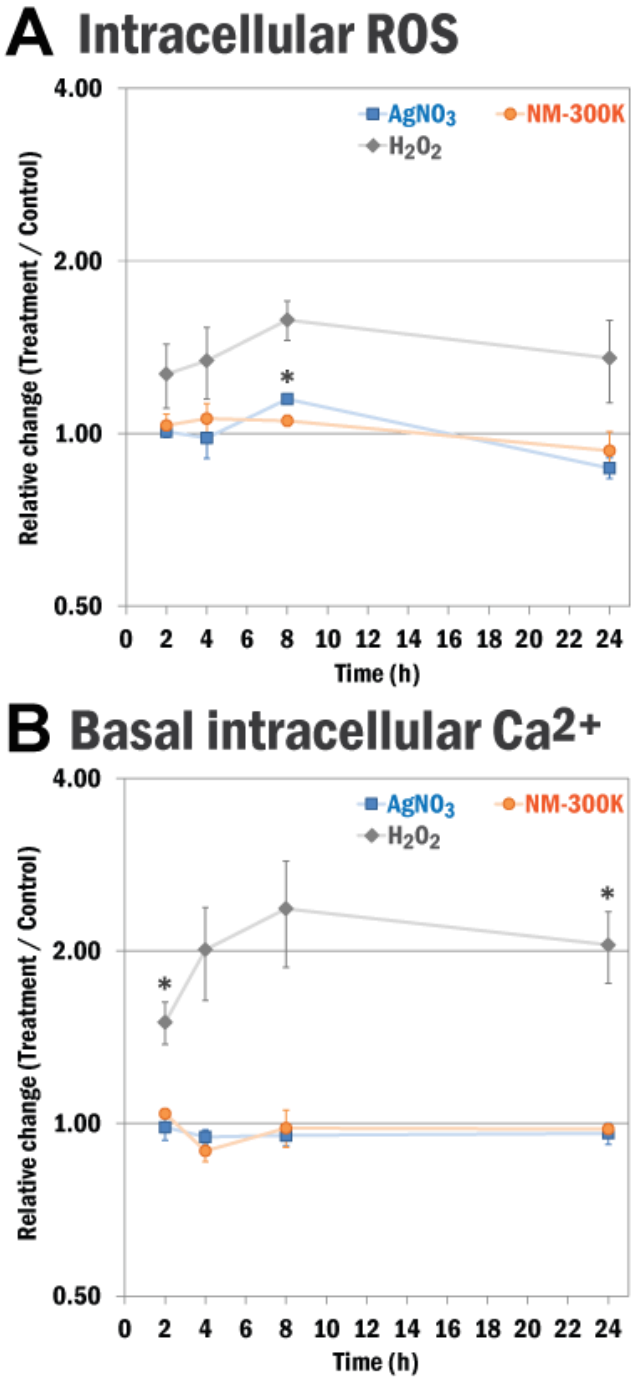

Figure S4. Intracellular ROS level as a hallmark of oxidative stress and basal intracellular calcium level as a signature of immune activation. The cells were exposed to low-cytotoxic concentrations of $AgNO_3$ (0.13 µg/ml) or NM-300K (2 µg/ml), or a positive control, 100 µM H_2O_2. (A) The fluorescent marker DCF (reacts with ROS) was used. (B) The calcium indicator Fluo-3 AM (binds Ca^{2+}) was used on the cells derived from the same treatment batch as in (A). Flow cytometry was applied to isolate the none- or less autofluorescence population (amoebocytes) and the fluorescence intensity was set relative to that of the corresponding control.Values are mean ± S.E. of three independent assays. Asterisks (*) denote a significant difference between the control and the treatment (Student's *t*-test, $p < 0.05$).

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