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10 nm Silver Nanoparticles Alter Neutrophil Function, Viability, and Adherence

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ABSTRACT

Neutrophils are circulating leukocytes found in the blood that migrate into tissues during inflammation. They are highly phagocytic, and due to the presence of cytotoxic granules, they are known to be very important microbicidal cells. Since it has been demonstrated that tissue-resident macrophages secrete pro-inflammatory cytokines following exposure to silver nanoparticles (AgNP), which activate the inflammatory response, we wanted to determine how the neutrophils would respond to the presence of AgNPs since they will likely migrate into exposed tissues. 10 nm PVP-coated AgNPs were incubated with primary neutrophils isolated from human blood, and it was noted using microscopy that at doses of AgNP greater than 10 micrograms per milliliter (µg/mL), the neutrophils were completely dead within an hour. Even a 1 hour incubation with 10 µg/mL resulted in a 20% reduction in neutrophil cell viability as determined with an MTS colormetric assay. Neutrophil activity was assessed at sub-toxic doses to determine the full extent at which these cells are being effected by AgNPs. At 1 and 10 µg/mL doses with 1 hour of exposure, a significant decrease in myeloperoxidase activity was observed, which suggests a defect in bactericidal activity of these cells. Microscopic observation demonstrates that the larger doses of AgNPs tend to form larger aggregates and these aggregates are likely taken up by these active phagocytes, which impacts gene expression and enzyme function. Since neutrophils are an essential cell in the clearance of microbial pathogens, it is important to assess whether the use of AgNPs as antimicrobial agents will impact the removal of infectious agents.

Keywords

Silver nanoparticles (AgNPs), Neutrophils, Nanotoxicology, Cellular viability. Immune response.

Introduction

Silver nanoparticles (AgNP) are capable of causing cellular dysfunction and apoptosis in many cell lines [1-3]. They are also known to augment cellular responses even at sub-toxic doses [2]. They have been shown in many mammalian cell lines to cause cytotoxic effects at high doses and activate or suppress transcription and translation at lower doses [2,4,5]. For these reasons it is important to assess the fate of important cell lines that may come into contact early during AgNP exposure.

One of the first cellular encounters that occurs when AgNPs enter the body is with the phagocytes of the innate immune system. Macrophages are localized in all tissues of the body, and following stimulation, secrete a pro-inflammatory response to recruit other phagocytes to aid in removal of foreign entities [6]. One of the most highly phagocytic cells that is recruited during the inflammatory process is the neutrophil [7]. Neutrophils are known for their fast phagocytic and high bactericidal activities, and they are well-equipped to remove foreign entities from the host [7]. The down-side to neutrophils is that during their killing activity, they often release some of the toxic products onto the host tissues contributing to some of the pathology observed during infections [7]. Therefore, an overactivation of neutrophils with a persistent agent that cannot be killed has the potential to be very dangerous to the body. Alternatively, a lack of activation of neutrophils by a foreign agent may leave the host susceptible to infectious agents.

Previous research has demonstrated that exposure of macrophages to AgNPs results in the production and release of interleukin-8, which will lead to the recruitment and extravasation of additional leukocytes, like neutrophils, to the exposed area [8]. Since neutrophils are the most numerous and most active phagocyte in the blood, and can lead to increased pathobiology when dysregulated, here we wanted to examine the impact of AgNP exposure on primary human neutrophils. Neutrophils are challenging to grow in culture since they have a very short life-span, and the neutrophil immortalized cell lines do not tend to respond similarly to primary cells [9]. Therefore, we isolated neutrophils from whole human blood and began all assays within an hour of isolation to ensure that the cells were still highly functional for these experiments. This study examined the impact of AgNPs on cell viability of primary neutrophils followed by an examination of enzymatic activity and gene expression of neutrophils following a one-hour exposure to sub-lethal concentrations of AgNPs.

Methods

Isolation of Primary Neutrophils

Whole blood samples were obtained from anonymous donors. The blood samples were transferred as soon as possible to individual 15 mL centrifuge tubes and were brought up to 10 mL with sterile phosphate-buffered saline (PBS). 4 mL of Ficoll-Paque PREMIUM 1.073 (GE Healthcare, Chicago, IL) was gently underlaid beneath the blood:PBS mixtures. The tubes were centrifuged at 400 x g for 20 minutes in an Eppendorf 5810R (Hamburg, Germany) with a swinging bucket rotor. The supernatant was removed and the erythrocyte/granulocyte pellets were each resuspended in 12 mL of cold 1X red blood cell (RBC) lysis solution. (BioLegend, San Diego, CA). The tubes were rocked for 5 minutes at room temperature and then centrifuged at 4°C for 5 minutes at 250 x g in a Beckman Coulter Microfuge 22R (Pasadena, CA). The supernatant was removed and this process was repeated to ensure that a majority of the erythrocytes were lysed. Then the cell pellet was washed by adding 10 ml cold phosphate-buffered saline supplemented with magnesium and calcium (PBS +/+). The cells were given one last spin at 4°C for 5 minutes at 250 x g and the supernatant was discarded. The final cell pellet was resuspended in 1.5 mL of PBS +/+ and 5 microliters of the cell suspension was placed on a slide and stained with the Easy III blood stain kit (Azer Scientific, Morgantown, PA) to ensure a high percentage of viable neutrophils (data not shown). The samples were then pooled and the cells were enumerated and immediately used.

Silver Nanoparticle Exposure

Biopure 10 nm silver nanoparticles (AgNP) coated with polyvinylpyrrolidone (PVP) (nanoComposix, San Diego, CA) were diluted in Dulbecco's Modified Eagle Medium (DMEM; VWR, Radnor, PA) at various concentrations and were incubated with 100,000 neutrophils at 37°C for 1 hour with rotation. The resulting suspensions were viewed using an Olympus 2K inverted microscope (Olympus, Tokyo, Japan) and imaged with a Moticam 2000 microscope mount camera (Schertz, TX) to confirm the presence of neutrophils.

Cell Viability

Neutrophils were isolated and exposed to AgNPs at 0, 1, or 10 µg/mL for one hour as described above and the viability of the cells was determined using both an MTS cell viability assay (Promega, Madison, WI) and a live/dead cell stain (Enzo Life Sciences, Farmingdale, NY) as per the manufacturer's protocols.

Neutrophil Activity

Myeloperoxidase (MPO) was used as a marker to assess neutrophil function following nanoparticle exposure. 10,000 freshly isolated cells were seeded into 40 wells of a 96-well plate. A DMEM control, PVP, or PVP-coated AgNPs at 1 or 10 µg/mL were added to 10 wells of neutrophils each. The reactions were assessed for MPO activity 60-minutes post-treatment by removing 10 µL of sample and adding it to another plate with 80 µL of 0.75 mM hydrogen peroxide and 10 µL of 3,3',5,5'-tetramethylbenzidine (TMB; Alfa Aesar, Haverhill, MA) and incubating at 37°C for 5 minutes [10]. 50 µL of stop solution (2N sulfuric acid) was added and the absorbance was obtained at a wavelength of 450 nm in the Biotek ELx800 plate spectrophotometer (Winooski, VT).

Gene Expression

Neutrophils were exposed to AgNPs as described above at 0, 1, or 10 µg/mL (n = 6) for 1 hour at 37°C in 5% CO_2. After incubation, RNA was extracted from the cells using an RNeasy kit (Qiagen, Hilden, Germany) with optional DNase step. Extracted RNA concentrations were measured with a Qubit RNA high-sensitivity Assay Kit (Thermo Fisher Scientific, Waltham, MA) and 50 ng of RNA was used for cDNA synthesis (iScript cDNA Synthesis Kit, Biorad, Hercules, CA). The cDNA was added to a 10 µL PCR reaction using iTaq universal SYBR green supermix (Biorad, Hercules, CA) for real-time amplification of 14 different primer combinations listed in Table 1 in a 384-well plate using the Roche LightCycler 480 (Basel, Switzerland). Gene expression was determined using the $2^{-\Delta\Delta Ct}$ method described elsewhere using GAPDH expression as a control [11].

Table 1: Gene targets and primers for qRT-PCR. These primers were designed from gene coding sequences obtained from the National Center for Biotechnology Information using Primer3 software.

Target	Forward primer	Reverse primer
MPO	catgcgtgtgtgttgtctga	aacccaggcagtctagttcc
Elastase	actgcgtggcgaatgtaaac	cgttgagctggagaatcacg
Alpha defensins	gtcaccctgcctagctagag	ccatgcaagggaaacaacca
Lactoferrin	gcacagtgtttgaggacctg	tcctccttgccattcacact
Il-1	ggagaatgacctgagcacct	ggaggtggagagctttcagt
Il-6	agtcctgatccagttcctgc	aagctgcgcagaatgagatg
TNFalpha	aggaccagctaagagggaga	cccggatcatgctttcagtg
ICAM-1	aagatcaaatggggctggga	aatgtatgtgggtggggagg

Statistical analysis

The one-way analysis of variance was performed to determine statistical significance with a Kruskal-Wallis multiple comparison test for individual treatments compared to control. GraphPad Prism 10.6.1 was used for all graphing and statistics. Differences were considered significant when $P < 0.05$.

Results

Cell Viability of Neutrophils

Neutrophils isolated from human blood were immediately exposed to PVP-coated AgNPs at 1, 10, 25, or 50 micrograms per milliliter (µg/mL), or to 0.01% polyvinylpyrrolidone (PVP) to control for the effect of the capping agent alone. All solutions were diluted in DMEM for exposure to the cells, and a DMEM control was used. After 60 minutes of exposure, a significant decline in cell viability was observed with the 10 µg/mL dose ($P = 0.0243$) as compared to the control DMEM only cells (Figure 1). We found it odd that the cell viability was back to 100% with the 25 and 50 µg/mL doses (Figure 1), so we decided that it would be important to use microscopy to visualize the interaction between the AgNPs and neutrophils at the higher concentrations.

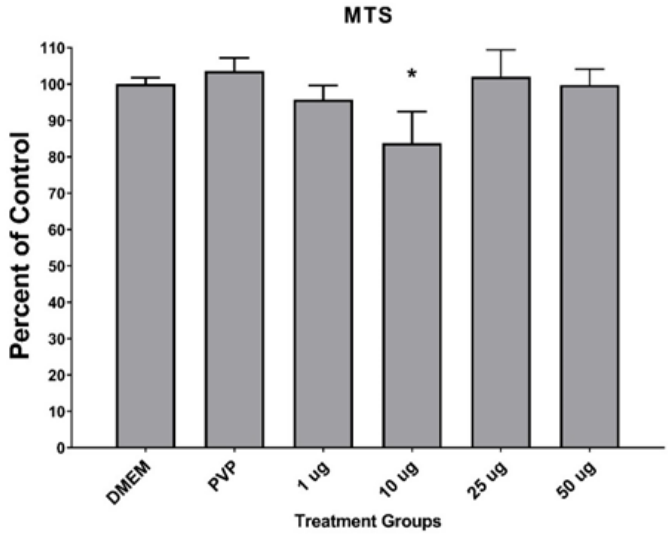

Figure 1: Cell Viability. An MTS assay was used to determine cell viability of neutrophils following a 1-hour incubation with AgNPs at various doses (or a mock exposure with just DMEM). The graph represents values based on percent of control (0 µg/mL). *denotes a significant reduction in cell number ($P < 0.05$).

Visualization of Neutrophils Exposed to AgNPs

After observing that the MTS reagent was reduced with the 25 or 50 µg/mL dose but not with the 10 µg/mL dose, we wanted to visualize what the AgNPs were doing to the neutrophils. Using a phase contrast microscope at 40X, we imaged the neutrophils exposed to 0, 1, 5, 10, 25, and 50 µg/mL of AgNPs for 1 hour to examine changes in cell morphology and viability. After 1 h there were few detectable cells in the 25 or 50 µg/mL doses, suggesting that the nanoparticles alone, or enzymes released after interacting with the neutrophils as aggregates, were capable of reducing the MTS reagent at these concentrations. Noted here were the formation of large aggregates of AgNPs at these higher doses (Figure 2), which may have contributed to the rapid cell death. There was also a large reduction in cell population with the 10 µg/mL dose (Figure 2), but this correlated with the results of the MTS assay (Figure 1). The 5 µg/mL dose looks as though the cells are beginning to be impacted, but the 1 µg/mL dose looks very similar to control (Figure 2). The 1 and 10 µg/mL doses were selected for continued study to examine a "low" dose with minimal cytotoxicity and a "high" dose with notable cytotoxic effects.

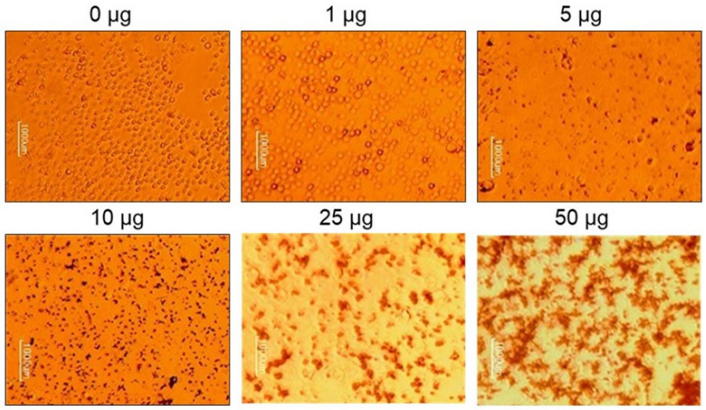

Figure 2: Brightfield Microscopy. Brightfield microscopy was used to determine how the AgNPs were aggregating and associating with human neutrophils. AgNPs were added to primary neutrophils at 0, 1, 5, 10, 25, or 50 micrograms per milliliter and incubated for 1 hour before imaging the cells.

Live/Dead Neutrophils

To confirm the observations of the first two figures, neutrophils were incubated in a 12 well plate and were exposed to 0, 1, or 10 µg/mL of AgNPs for 1 hour. They were then stained with a live (green) and dead (red) stain to assess cell viability (Figure 3). The control and 1 µg/mL exposures had mostly viable cells, but there was a percentage that were lost during the washing steps (Figure 3A, B). The 10 µg/mL dose had far more dead cells (red), but adhered better to the plate during the subsequent washing steps (Figure 3C).

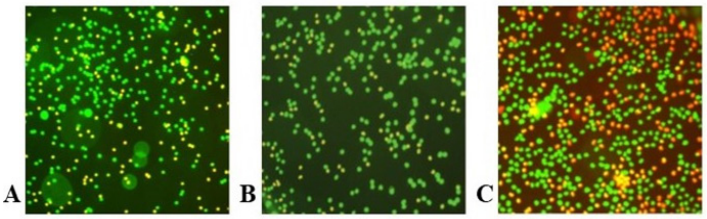

Figure 3: Live/dead Staining. Neutrophils were exposed to 0 (A), 1 (B), or 10 (C) micrograms per milliliter of AgNPs for 1 hour. Nonadherent nanomaterials were washed off and the cells were exposed to a live (green) v. dead (red) assay to confirm cell viability studies.

Neutrophil Activity

To determine if the function of the neutrophils was at all impacted by AgNPs, myeloperoxidase (MPO) activity was measured after 60 minutes of exposure to DMEM, 0.01% PVP in DMEM, or AgNPs at 1 or 10 µg/mL diluted in DMEM (Figure 4). A significant reduction in MPO activity was observed when the neutrophils were exposed to 1 or 10 µg/mL AgNP for 60 minutes ($P = 0.0139$ and 0.0324, respectively). Quantitative PCR concludes that it is the activity of the MPO enzyme, and not the transcription of the gene itself, as the neutrophils stimulated with 10 µg/mL AgNPs had on average 6.5-fold greater MPO mRNA transcription than the

control or 1 µg/mL doses (P = 0.0299; Figure 5A). mRNA levels of elastase were also increased 5.3-fold in the 10 µg/mL AgNP dose (P = 0.0021) (Figure 5B), however, the 1 µg/mL AgNP dose led to a significant suppression in elastase mRNA transcription in neutrophils following a 1-hour exposure (P = 0.0006; Figure 5B). Lactoferrin transcription tended to be suppressed in neutrophils exposed to 10 µg/mL, but due to variability this was not significant (Figure 5C). Alpha defensin transcription was significantly upregulated in neutrophils exposed to both 1 and 10 µg/mL AgNPs for 1-hour 4.3 and 6.7-fold, respectively (P = 0.0130 and 0.0005, respectively) (Figure 5D).

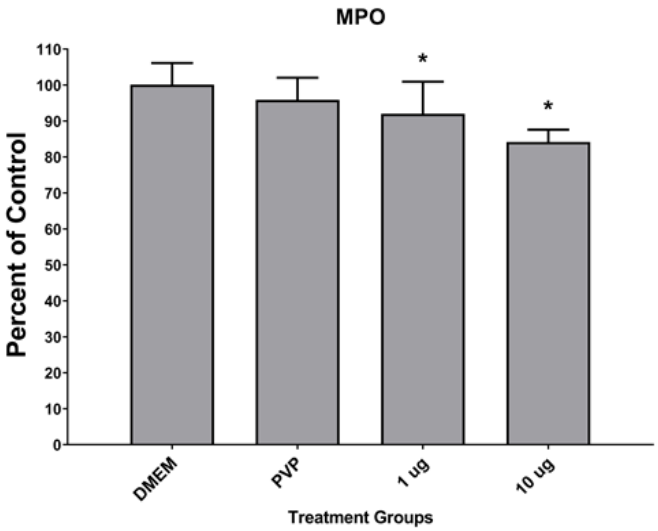

Figure 4: Myeloperoxidase Activity. Myeloperoxidase (MPO) activity (A) was selected as an enzyme to test neutrophil function after they were exposed to 0 ("DMEM"), 1, or 10 micrograms per milliliter of AgNPs for 1 hour. A dose of the PVP capping agent similar to the levels found in the 10 µg/mL dose was also used for control purposes. The graph represents values based on percent of control (0 µg/mL). *denotes a significant reduction in MPO activity (P < 0.05).

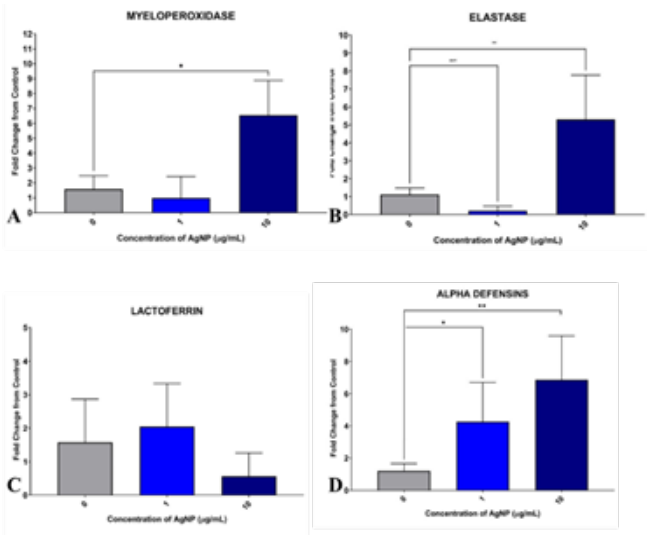

Figure 5: qRT-PCR. Neutrophil function. The mRNA levels of peptides and proteins found in neutrophil granules were measured using quantitative reverse transcriptase PCR. The RNA was obtained following a 1-hour exposure to 0, 1, or 10 micrograms per milliliter of AgNPs. qPCR was used to determine changes in (A) myeloperoxidase, (B) elastase, (C) lactoferrin, and (D) alpha defensin mRNA levels which were expressed as fold change from control (0 µg/mL). * and ** denotes a significant change from control levels (P < 0.05 or P < 0.01).

Neutrophil inflammatory production

Examining changes in the inflammatory properties of activated neutrophils, the mRNA isolated from neutrophils exposed to 0, 1, or 10 µg/mL AgNPs was also quantified for changes in interleukin-1β (IL-1β) or interleukin-6 (IL-6) transcription (Figure 6). There were no remarkable changes in IL-1β transcription after 1 hour (Figure 6A), but IL-6 transcription was increased 4.4-fold in the higher dose (P = 0.0042) (Figure 6B).

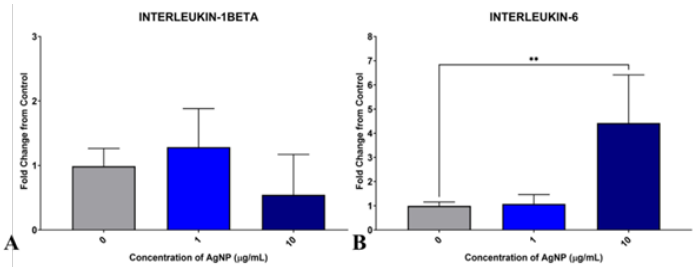

Figure 6: qRT-PCR. Inflammatory biomarkers. The mRNA levels of pro-inflammatory cytokines interleukin-1β (A) and interleukin- 6 (B) were measured using quantitative reverse transcriptase PCR. The RNA was obtained following a 1-hour exposure to 0, 1, or 10 micrograms per milliliter of AgNPs. mRNA levels were quantified and expressed as fold change from control (0 µg/mL). **denotes a significant change from control levels (P < 0.01).

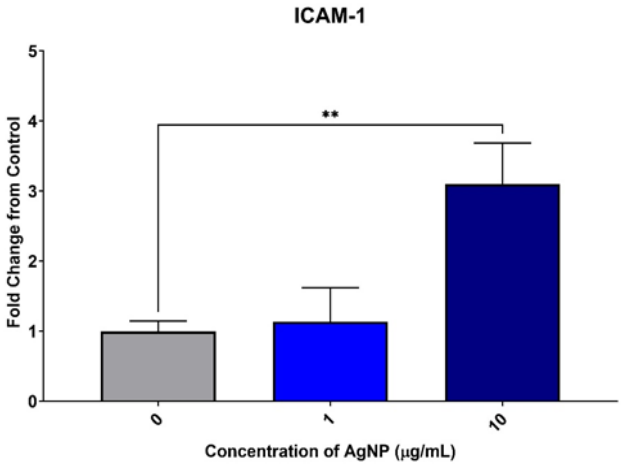

Figure 7: qRT-PCR. Cell adhesion. The mRNA levels of intracellular adhesion molecule-1 were measured using quantitative reverse transcriptase PCR. The RNA was obtained following a 1-hour exposure to 0, 1, or 10 micrograms per milliliter of AgNPs. mRNA levels were quantified and expressed as fold change from control (0 µg/mL). **denotes a significant change from control levels (P < 0.01).

Cell adhesion

After observing the change in adherence patterns following the wash steps in the live-dead stain (Figure 3), we decided to determine if the adhesion gene transcription was changing

in the neutrophils following AgNP exposure, and we used the transcription of Intercellular Adhesion Molecule 1 (ICAM-1) for assessment. As observed in Figure 3 of the live-dead micrographs, there is little change in 0 and 1 µg/mL exposure for adherence to the plate (Figure 3) or ICAM-1 transcription (Figure 7). However, after 1 hour of exposure to 10 µg/mL AgNPs, ICAM-1 had a 3.1-fold increase in mRNA transcription in primary human neutrophils (Figure 7) (P = 0.0049).

Discussion

The innate immune system functions to remove foreign particulates from our bodies. Macrophages stand resident awaiting exposures, and recruit necessary cells to aid in removal as needed. One of the most important cell types recruited early following exposure is the neutrophil. It has been shown that silver nanoparticles (AgNPs) will cause macrophages to secrete CXCL-8 (formerly IL-8), which is the chemokine responsible for recruitment of neutrophils to the site of exposure, making them an important cell to understand how they interact with AgNPs [12]. Due to their inability to restore granular contents after release, neutrophils often die shortly after pathogen exposure, thus making it imperative to understand if AgNPs can activate neutrophils in the absence of microbial stimulus, especially since they are believed by many to be an effective alternative antimicrobial [13,14].

Since neutrophils do die shortly after exposure, we wanted to first look at the cell viability following an acute exposure to AgNPs. We first tried for several hours (data not shown), but there were no visible cells, so we dramatically decreased the amount of time post-exposure for assessments. Even after 1 hour, there were few to no visible cells in the higher doses that we tried suggesting that the nanoparticles caused rapid cell death at 25 or 50 µg/mL (Figure 2). This is most likely due to the formation of larger aggregates, which would be more readily phagocytosed. Once neutrophils phagocytose bacteria, they release their granular contents, and then die by apoptosis. It would be interesting to see if these larger metallic aggregates behaved in a similar fashion. The lower concentrations of AgNPs did not appear to form large aggregates, as shown in Figure 2, which might indicate why there was more cell survival. Although through use of a live/dead cellular stain, it is apparent that a large proportion of neutrophils exposed to 10 µg/mL AgNPs have at least begun to die off (Figure 3), which is confirmed in the MTS cell viability assay (Figure 2). Unfortunately, with this particular live/dead stain, the mechanism of cell death still remains unclear, so we cannot conclude if it is normal apoptosis following engulfing of a foreign body, or if the AgNPs have a necrotic effect or induce NETosis as suggested by others [15]. AgNPs are known to induce reactive oxygen species (ROS), which in turn can stimulate the formation of a neutrophil extracellular trap (NET) [15]. This is likely a potential mechanism of cell death since we are observing a greater than 5-fold upregulation of elastase transcription following 10 µg/mL AgNP exposure (Figure 5B), which is required for NET formation [16]. In addition, there appeared to be a decline in lactoferrin transcription in some samples, but not others, suggesting it might be a gene to examine further (Figure 5C). Lactoferrin has anti-inflammatory properties and has been attributed to cell survival in neutrophils [17]. The decrease observed in the 10 µg/mL AgNP exposure could be contributing to the cell death observed [17].

To examine more about neutrophil function following AgNP exposure, we examined the expression of alpha defensin and myeloperoxidase (MPO) genes, as well as MPO activity (Figures 4 and 5). Alpha defensins, also known as human neutrophil peptides (HNP), are important for the destruction of phagocytosed pathogens as well as modulation of immune responses [18]. There was a nearly 7-fold increase in alpha defensin transcription following a one-hour exposure to 10 µg/mL AgNP (Figure 5D). There was also a significant upregulation observed when only 1 µg/mL AgNP were added to the neutrophils for one hour (Figure 5D). In addition to being helpful to combat bacterial pathogens, increased alpha defensin production has also been linked to several pathological conditions including increased endothelium permeability, edema, inflammation causing tissue injury, and progression of certain autoimmune diseases [19-21]. Myeloperoxidase (MPO) mRNA levels were also significantly increased in the 10 µg/mL AgNP exposure, but not the 1 µg/mL exposure (Figure 5A). This is the only enzyme we determined functionality above just increased transcription, and although there was more mRNA produced, the MPO function actually declined with increased dose (Figure 4). MPO is an enzyme that further converts the hydrogen peroxide generated by NADPH oxidase to hypochlorous acid, which is far more bactericidal [22]. MPO-deficient neutrophils cannot break down as many bacteria as normal neutrophils can [22]. The deficiency observed here may just have been that the azurophilic granules contents may not have been utilized against the metallic particle, or perhaps the particles themselves are not entering the neutrophil, but it is imperative to determine if this enzyme is indeed deficient of bactericidal activity following nanoparticle exposure if these materials are going to be used as antibacterial drug alternatives.

Observing that the nanoparticles are activating genes, we determine that it was important to examine impacts on inflammatory gene expression. Many studies have shown that AgNPs can activate inflammatory gene expression in monocytes and macrophages, including induction of the inflammasome [23,24]. Since uncontrolled inflammation can lead to a variety of disease states, we wanted to assess if the same phenomenon occurred in this neutrophil model. It does not appear as though the inflammasome is being activated due to the low levels of IL-1β compared to control (Figure 6A). If anything, it appears as though the higher dose of AgNPs may actually be suppressing IL-1β transcription (Figure 6A). IL-6, on the other hand, was highly upregulated after a one-hour exposure to 10 µg/mL AgNPs (Figure 6B). Although important for the normal functioning of many immunological responses, high IL-6 mRNA levels are concerning due to the systemic impact that this protein has on the body [25]. There are many diseases that IL-6 initiates or exacerbates, and thus high levels produced by external stimuli could be concerning [25].

An interesting observation occurred during the live/dead stain in

that there were more neutrophils remaining post-wash steps for the 10 µg/mL exposure of AgNPs (Figure 3). Therefore, we decided to also examine the production of adhesion proteins, specifically ICAM-1. As hypothesized, ICAM-1 transcription was increased following a one-hour exposure to 10 µg/mL AgNPs (Figure 7). ICAM-1 expression is important for mediating leukocyte-endothelial interactions to slow the neutrophils for extravasation from the blood into infected tissue [26]. It is generally induced during inflammatory reactions, and the increased production of IL-6 could be causing the upregulation seen here (Figures 6 and 7). Neutrophils that produce high levels of ICAM-1 also have increased phagocytic capabilities against bacterial pathogens [26]. Thus, induction of this adhesion protein by AgNPs could potentially promote bacterial clearance, even if MPO activity is somewhat inhibited. Additional studies examining how the neutrophils can respond to bacteria would likely provide more insight in to any potential advances or deficits observed from dysregulation of gene expression caused by 10 µg/mL AgNPs in primary human cell lines.

This study shows that very high doses (25 and 50 µg/mL) or long exposure periods (>1 hour; data not shown) can cause primary neutrophils to die in culture. Since neutrophils naturally undergo apoptosis, necrosis, or even NETosis following exposure to microbial pathogens, it was expected that they would die off, but the exposure times and doses are far lower than for any other human cell line. The neutrophils are starting to be impacted at 10 µg/mL around the one-hour time post-exposure, and it is evident by cell death, changes in gene expression, and decreases in enzymatic activity, all which could potentially have negative impacts on the overall health of persons exposed. However, the upregulation in the intercellular cell adhesion molecule, ICAM-1, could also suggest that the neutrophils could benefit from AgNP exposure. Although overall ICAM-1 function in human neutrophils is still not well understood, its adherence functions could help neutrophils combat infections more readily and recent evidence suggesting that it is involved in phagocytosis of bacteria could indicate that AgNP exposure to neutrophils could aid in pathogen removal, in addition to their demonstrated bactericidal activities [27]. Since neutrophils are such an important leukocyte in early responses to foreign materials, it is very important to better assess how they are going to respond to any material administered inside of the human body.

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