

Exposure to silver nanoparticles primes mast cells for enhanced activation through the high-affinity IgE receptor

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ABSTRACT

Mast cells are a key effector cell in type I allergic reactions. It has been shown that environmental exposures such as diesel exhaust and heavy metals exacerbate mast cell degranulation and activation. Today, the use of engineered nanomaterials (ENMs) is rapidly expanding and silver nanoparticles (AgNP) are one of the mostly widely utilized ENMs, primarily for their antimicrobial properties, and are being incorporated into many consumer and biomedical products. We assessed whether pre-exposure of bone marrow-derived mast cells (BMMCs) to 20 nm AgNPs enhanced degranulation and activation to an allergen (dinitrophenol-conjugated human serum albumin) by measuring β -hexosaminidase release, LTB₄ and IL-6 production. In addition, we assessed reactive oxygen species (ROS) generation, cell oxidative stress and toxicity as well as total and individual protein tyrosine phosphorylation (p-Tyr). We found that pre-exposure of BMMCs to AgNPs results in exacerbated allergen-mediated mast cell degranulation, LTB₄ production and IL-6 release. Exposure of BMMCs to AgNPs exacerbated allergen-induced ROS generation, however, this was not associated with oxidative stress nor cell death. Finally, pre-exposure to AgNPs enhanced allergen-mediated global p-Tyr as well as individual proteins including Syk, PLC γ and LAT. Our findings indicate that pre-exposure to AgNPs exacerbates mast cell allergen-mediated phosphorylation of Fc ϵ R1-linked tyrosine kinases and ROS generation resulting in amplified early and late-phase responses. These findings suggest that exposure to AgNPs has the potential to prime mast cells to allergic immune responses, which could be of particular concern to atopic populations as the use of AgNPs in consumer and biomedical products rapidly increases.

1. Introduction

Allergic diseases including asthma, rhinitis and food allergies are reaching epidemic levels within the last century in the developed and developing world (Holgate and Polosa, 2008). These diseases are multicellular and involve complex interactions between genetic and environmental components (Holgate and Polosa, 2008). Mast cells are key effector immune cells in allergic and inflammatory reactions (Brown et al., 2008). They are found in large numbers in close proximity to tissue-environment interfaces (e.g. mucosa and skin) and their activation is implicated in several allergic conditions such as asthma, allergic rhinitis, atopic dermatitis, allergic eye disease and anaphylaxis (Brown

et al., 2008). Allergen-induced mast cell activation is mediated by crosslinking (aggregation) of IgE-bound to the high affinity IgE receptor (Fc ϵ R1) leading to subsequent phosphorylation of protein tyrosine kinases (PTK) and mobilization of calcium, which eventually results in cell degranulation (exocytosis of preformed granule content, e.g. histamine, heparin, serotonin and neutral proteases) and synthesis/release of lipid mediators (e.g. prostaglandins and leukotrienes) and cytokines (e.g. TNF α , IL-1 β , IL-2, IL-4, IL-6 and IL-13) (Gilfillan and Tkaczyk, 2006). Activated mast cells rapidly recruit other effector immune cells (e.g. eosinophils) and modulate the microenvironment including cellular and non-cellular components thereby influencing the pathophysiology of the underlying allergic disease (Metz and Maurer, 2007).

Abbreviations: Ag⁺, Silver ions; AgNP, Silver nanoparticle; BMMC, Bone marrow derived mast cell; DAG, Diacylglycerol; DNP-HSA, Dinitrophenol-conjugated human serum albumin; DNPH, Dinitrophenylhydrazine; ENM, Engineered nanomaterial; FBS, Fetal bovine serum; Fc ϵ R1, High affinity IgE receptor 1; GPx, Glutathione peroxidase; GSH, Glutathione; HEPES, N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid; HO-1, Hemoxygenase-1; IgE, Immunoglobulin E; IP₃, Inositol triphosphate; LAT, Transmembrane adaptor molecule; MT-1, Metallothionein-1; NQO1, NAD(P)H Quinone Dehydrogenase 1; p-Tyr, Protein tyrosine phosphorylation; PIP₂, Phosphatidylinositol bisphosphate; PLC, Phospholipase C gamma; PNAG, *p*-nitrophenyl-*N*-acetyl- β -D-glucopyranoside; PTK, protein tyrosine kinase; ROS, Reactive oxygen species; Syk, Spleen tyrosine kinase

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Importantly, it has been recognized that certain environmental exposures such as diesel exhaust particles, heavy metals and environmental estrogens can activate and/or exacerbate mast cell IgE-mediated activation (Diaz-Sanchez et al., 2000; Narita et al., 2007; Walczak-Drzewiecka et al., 2003). Furthermore, previous evidence demonstrated a positive correlation between exposure to particulate matter and worsening of asthmatic patient symptoms (Donaldson et al., 2000; Gavett and Koren, 2001). Indeed, exposure to metal and transition metal ions alone or with allergen-mediated stimulation (co-exposure), including aluminum, nickel, strontium and cadmium, as part of particulate matter, has been shown to result in enhanced allergen-mediated mast cell degranulation (up to 10–20% greater response) and mediator release (up to 100% greater response) (Walczak-Drzewiecka et al., 2003). Moreover, heavy metals such as mercury, silver and gold have been shown to induce mast cell activation (Dastyeh et al., 1999; Schedle et al., 1998; Suzuki et al., 2001). However, whether pre-exposure to such metals could influence allergen-mediated mast cell activation is unknown.

Nanotechnology is rapidly growing across multiple disciplines such as materials science, physics, engineering, and pharmaceutical sciences. Nanomaterials are materials with a size between 1 and 100 nm (1 billionth of a meter) in at least one dimension. Engineered nanomaterials (ENM)—precisely designed and synthesized materials at the nano scale—are utilized for a vast range of applications including electronics, health care, biotechnology, and drug delivery (Buzea et al., 2007). The number of nano-enabled consumer products is greatly expanding, and this will inevitably lead to increased human and environmental exposure (Boxall et al., 2007; Colvin, 2003; Vance et al., 2015). Importantly, immunomodulation and immunotoxicity have been shown to be a detrimental outcome of ENM exposure (Alsaleh and Brown, 2018). The immune system can largely dictate ENM adverse outcomes (e.g. tissue accumulation *versus* clearance). Therefore, understanding immune responses to ENMs at the cellular and molecular levels is important in the assessment of ENM safety. We and others have demonstrated mast cell activation *in vivo* and *in vitro* in response to ENM exposure of different compositions including carbon-, silica- and metal-based ENMs (Aldossari et al., 2015; Chen et al., 2012; Katwa et al., 2012; Zhuravskii et al., 2016). However, the cellular and molecular mechanisms driving mast cell responses to ENMs are still poorly understood. One of the most widely utilized ENMs is silver nanoparticles (AgNPs) primarily for their antimicrobial properties, which are often mediated through release of silver ions (Vance et al., 2015). However, recent research has shown that in addition to ionic silver, AgNPs in their particulate form can mediate distinct biological responses (Boudreau et al., 2016; Kim et al., 2009; Recordati et al., 2016; Xiu et al., 2011). Indeed, we have previously shown that exposure to AgNPs induced robust mast cell degranulation which was dependent on key physicochemical properties, but was independent of silver ions (*i.e.* the particulate form of AgNPs was required for degranulation) (Aldossari et al., 2015). Recently, we have demonstrated that 20 nm AgNP-induced mast cell degranulation is mediated through a non-IgE mechanism that involves activation of signal transduction and calcium signaling (Alsaleh et al., 2016; Johnson et al., 2018). However, whether pre-exposure to AgNPs influences mast cell response to allergen-mediated activation has yet to be examined. Therefore, we sought to investigate whether pre-exposure to AgNPs would prime mast cells leading to exacerbation of allergen-mediated activation.

2. Materials and methods

2.1. Nanoparticle characterization

Size and shape of AgNPs (BioPure, 20 nm citrate-coated, 1 mg/ml stock concentration, NanoComposix, San Diego, CA) was assessed by transmission electron microscopy (TEM). AgNP hydrodynamic size, zeta potential and polydispersity index (PDI) were measured using a

Table 1

Characterization of silver nanoparticles by dynamic light scattering (DLS).

Vehicle	Hydrodynamic size (nm)	Zeta potential (mV)	PDI
DI water	35.57 ± 4.17	−31.93 ± 0.32	0.22 ± 0.02
Cell culture media	443.63 ± 72.03	−16.40 ± 0.86	0.25 ± 0.02

20 nm silver nanoparticles (AgNPs) hydrodynamic size (nm), ξ -potential (mV) and polydispersity index (PDI) were measured using Malvern Nanosizer in DI water and cell culture media. Values are expressed as mean ± SEM ($N \geq 3$).

Zetasizer Nano (Malvern, Westborough, MA). Measurements were made with AgNPs in both DI water and cell culture medium. Fig. S1 shows a representative TEM image of 20 nm AgNPs demonstrating size and shape. Table 1 shows hydrodynamic size, zeta potential and PDI of AgNPs in DI water (AgNP stock vehicle) and in cell culture media. Lastly, the hydrodynamic size distribution is provided in Fig. S1B.

2.2. Cell culture

Bone marrow derived mast cells (BMMC) were cultured from femoral bone marrow obtained from C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) at the age of 6–8 weeks. BMMCs were cultured at 37 °C and 5% CO₂ for 4–6 weeks and cell maturity *i.e.* Fc ϵ R1 and cKIT surface expression was assessed before use (~95–98%). Cells were cultured in RPMI 1640 medium (Corning, Manassas, VA), supplemented with 10% FBS (Corning, Manassas, VA), 25 mM HEPES (Corning, Manassas, VA), 1.0 mM sodium pyruvate (Sigma-Aldrich, St. Louis, MO), 1.0 mM non-essential amino acids (Sigma-Aldrich, St. Louis, MO), 100 U/ml penicillin, 100 μ g/ml streptomycin (Life Technologies, Carlsbad, CA), 100 μ g/ml Primocin™ (Invitrogen, San Diego, CA), 0.0035% β -mercaptoethanol and 30 ng/ml recombinant murine IL-3 (PeproTech, Rocky Hill, NJ). All animal procedures were conducted in accordance with the National Institutes of Health guidelines and were approved by the University of Colorado Institutional Animal Care and Use Committee. Mice were maintained in individually ventilated cages under 12-h light-dark cycles and fed *ad libitum*. Experiments were conducted from at least 3 sets of BMMCs (each set was isolated from femur bones obtained from 2 mice).

2.3. Cell viability

Cell viability was assessed based on staining with propidium iodide (PI) (for necrotic cell death) (Invitrogen, San Diego, CA) and Annexin V (for apoptotic cell death) (BD Biosciences, San Jose, CA). Briefly, BMMCs at 2×10^5 cells per sample were exposed to AgNPs (25 μ g/ml) for the indicated time points. Then cells were washed two times with PBS buffer and resuspended into PI-containing binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl₂, pH = 7.4) at a final concentration of 3 μ M. 2.5 μ l of Annexin V was then added to each sample. Samples were incubated at room temperature protected from light for 15 min and then fluorescence was measured by a flow cytometer (BD Accuri™ C6, BD Biosciences, San Jose, CA).

2.4. Reactive oxygen species measurement

Reactive species formation was measured by flow cytometry using H2DCFDA (Invitrogen, San Diego, CA). Briefly, BMMCs at 2×10^5 cells per sample were exposed to AgNPs (25 μ g/ml) for the indicated time points. Then cells were washed two times with PBS and resuspended into H2DCFDA-containing PBS (5 μ M) and incubated at 37 °C protected from light for 30 min. Fluorescence was measured by a flow cytometer (BD Accuri™ C6, BD Biosciences, San Jose, CA).

2.5. Mast cell degranulation

Mast cell degranulation was measured based on the release of β -hexosaminidase as previously described (Alsaleh et al., 2016). Briefly, BMMCs were first pre-incubated (sensitized) with anti-DNP IgE (100 ng/ml) (Sigma–Aldrich, St. Louis, MO) overnight. Cells were then plated at 1×10^6 cells per ml and then exposed to AgNPs (25 μ g/ml) for 24–72 h. Cells were then washed two times and resuspended in warm HEPES buffer and plated at 5×10^4 cells per well per 96-well plate. Cells were then treated with dinitrophenol-conjugated human serum albumin (DNP-HSA or DNP for short) (100 ng/ml) (allergen-mediated mast cell activation) (Sigma–Aldrich, St. Louis, MO), and incubated at 37 °C for 30 min. After, plate was centrifuged at $300 \times g$ for 5 min and 50 μ l of supernatant was transferred into a new 96-well plate. 150 μ l of Triton-X was used to lyse cell pellet, of which 50 μ l was transferred into a new 96-well plate. 100 μ l of PNAG (p-nitrophenyl-N-acetyl-b-D-glucopyranoside) (Sigma–Aldrich, St. Louis, MO), a chromogenic substrate, was added to supernatant and lysed cells and incubated at 37 °C for 90 min. Reaction was stopped by adding 100 μ l of glycine and optical density was read at 405 nm. Percentage of degranulation was calculated as follows: $[(\text{supernatant} \times 2)/(\text{lysate} \times 4)] \times 100$ (dilution factors).

2.6. Protein expression by Western blot

BMMCs were first pre-incubated (sensitized) with anti-DNP IgE (100 ng/ml) overnight. BMMCs were plated at 1×10^6 cells per sample then exposed to AgNPs (25 μ g/ml) for the indicated time points and then washed and treated with DNP (100 ng/ml) for 5 min. Cells were washed two times with cold PBS and lysed with cold Tris-HCL based lysis buffer containing 1% SDS, protease (1:100) and phosphatase (1:100) inhibitors on ice for 45 min. Cells were briefly sonicated and then centrifuged at $15,000 \times g/4$ °C for 10 min. Pellet was discarded (insoluble debris) and supernatant was collected into a new 1.5 ml eppendorf tube and protein was assayed using the Bradford method (Bio-Rad, Hercules, CA). Cell lysate was kept at -20 °C until processing. When ready to run, lysate was thawed on ice and then brought to boil for 5 min with 5% β -mercaptoethanol-containing $4 \times$ Laemmli sample buffer. 20 μ g of protein was electrophoretically separated by molecular weight using 12% SDS-polyacrylamide gels then blotted into nitrocellulose membranes overnight (16 h) at 10 V/4 °C. The following day,¹ membranes (on a rocker) were blocked with 5% BSA in TBS-containing 0.1% tween-20 (TBS-T) for an hour and then incubated with primary antibody: p-Tyr, p-PLC $_{\gamma 1}$, t-PLC $_{\gamma 1}$, p-Syk, t-Syk, p-LAT, t-LAT and β -Actin (Cell Signaling, Beverly, MA) overnight (16 h) at 4 °C. Subsequently, membranes were washed 3 times with TBS-T followed by incubation with horseradish peroxidase-linked-secondary antibody (Cell Signaling, Beverly, MA) for 1 h at room temperature. Membranes were then washed and developed using Pierce™ enhanced chemiluminescence (ECL) substrate (ThermoFisher Scientific, Waltham, MA) and Bio-Rad ChemiDoc™ Imaging System (Bio-Rad, Hercules, CA). Relative density was calculated using Image Lab™ Software (Bio-Rad, Hercules, CA).

2.7. Cytokine release by enzyme-linked immunosorbent assay (ELISA)

BMMCs were first pre-incubated (sensitized) with anti-DNP IgE (100 ng/ml) overnight. BMMCs were then plated at 1×10^6 cells per sample and pre-exposed to AgNPs (25 μ g/ml) for the indicated time points and then treated with DNP (100 ng/ml) for 24 h. Cells were centrifuged at $200 \times g$ at 4 °C for 5 min and supernatants were collected

and stored at -80 °C until further processing. Cytokine release was measured by DuoSet® ELISA Development Systems (R&D Systems, Minneapolis, MN) according to the manufacturer instructions. Briefly, Fisherbrand adsorption immunoassay 96-well plate was incubated with capture antibody at room temperature overnight. The next day, the plate was washed with 0.05% tween-20 containing PBS at least four times and then was blocked with 1% BSA in PBS (0.22 μ m filtered) for 1 h. After, the plate was washed and then incubated with samples or standard (serial dilutions) for a minimum of 2 h, after which it was washed and then incubated with detection antibody for 2 h. After wash, the plate was incubated with HRP-conjugate for 30 min protected from light, and then washed and incubated with TMB substrate for 20–30 min protected from light (until color develops). Reaction was stopped with 2 N H₂SO₄ and optical density was measured spectrophotometrically at 405 nm. Sample concentrations were determined based on known standard concentration. *Note: LTB4 ELISA Kit (Cayman, Ann Arbor, Michigan) was carried out according to the manufacturer instructions.

2.8. Gene expression by real time PCR

BMMCs were first pre-incubated (sensitized) with anti-DNP IgE (100 ng/ml) overnight. BMMCs were then plated at 1×10^6 cells per sample and exposed to AgNPs (25 μ g/ml) for the indicated time points. Cell were lysed with TRI Reagent® (Sigma–Aldrich, St. Louis, MO) and then kept at -80 °C until further processing. RNA was isolated from lysed cells using Direct-20L™ RNA MiniPrep kit (Zymo Research, Irvine, CA). The quality of RNA was measured using NanoDrop™ 2000 (ThermoFisher Scientific, Waltham, MA). RNA was reverse transcribed into cDNA using iScript™ cDNA Synthesis kit (Bio-Rad, Hercules, CA) and a thermocycler (Eppendorf, Hauppauge, NY). cDNA was mixed with SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA) and QuantiTect primer sets (Qiagen, Germantown, MD). Real time PCR was carried out in triplicate for each sample using the real time PCR cycler Applied Biosystems StepOnePlus™ (ThermoFisher Scientific, Waltham, MA). Gene expression was measured using the $\Delta\Delta C_T$ method relative to GAPDH control (housekeeping gene).

2.9. Glutathione measurement by HPLC

BMMCs were first pre-incubated (sensitized) with anti-DNP IgE (100 ng/ml) overnight. BMMCs were then plated at 1×10^6 cells per sample and exposed to AgNPs (25 μ g/ml) for the indicated time points. Cells were processed and GSH measurement by High performance liquid chromatography (HPLC) was carried out as previously described (Jones and Liang, 2009). Briefly, cells were washed two times with cold PBS and cell pellet was resuspended in 500 μ l of 5% perchloric acid, 0.2 M boric acid, 10 μ M γ -glutamylglutamate (internal standard) and then sonicated. Samples were centrifuged at $13,000 \times g/4$ °C for 2 min and 300 μ l of supernatant was collected into a new tube. Pellet was used to assay protein content. Samples were treated with 9.3 mg/ml iodoacetic acid (60 μ l per sample) (to alkylate free thiols) and then pH was adjusted to 8.8–9.2 for each sample followed by a 20 min of incubation at room temperature. Samples were derivatized with 300 μ l dansyl chloride (20 mg/ml) (to fluorescently tag amino groups) followed by an overnight incubation protected from light. 500 μ l of chloroform was added per sample (to extract unreacted dansyl chloride) and then samples were vortexed and centrifuged at $13,000 \times g/4$ °C for 2 min to separate the aqueous (top) and organic layers. HPLC was performed using Supelcosil™ (LC-NH₂ 25-cm x 4.6-mm, 5 μ m) column (Sigma Aldrich, St. Louis, MO) on Agilent 1200 series instrumentation. The concentrations of GSH, GSSG, Cys (cysteine), and CySS (cystine) were calculated relative to internal standard. The Nernst equation (E_h (mV) = ($E_0 + RT/nF$) $\ln([\text{oxidized}]/[\text{reduced}])$) was used to calculate the redox potential (E_h).

¹ For OxiSelect™ Protein Carbonyl Immunoblot Kit (Cell BioLabs, San Diego, CA), membranes were derivatized with dinitrophenylhydrazine (DNPH) then blocked with 5% non-fat milk according to the manufacturer instructions.

2.10. Statistical analysis

GraphPad Prism 5 software (San Diego, CA) was used to generate figures and analyze the data. Data are presented as mean $\pm$ standard error mean (SEM). One-way ANOVA with Bonferroni post-hoc testing was utilized to test for significant differences between multiple treatment groups. Significant differences are at $p < 0.05$.

3. Results

3.1. Pre-exposure to silver nanoparticles primes mast cells for enhanced allergen-mediated early-phase activation

Mast cell activation by allergen crosslinking of Fc ϵ R1 leads to early-phase response (degranulation) and late-phase responses (lipid mediator and cytokine release). To test whether pre-exposure to AgNPs primes mast cells to allergen-mediated degranulation, we first exposed mast cells to AgNPs (0.25, 2.5 or 25 μ g/ml) for 24 h (or at 25 μ g/ml for two additional time points *i.e.* 48 and 72 h), washed and then treated cells with 100 ng/ml allergen (DNP - dinitrophenol conjugated human serum albumin, DNP-HSA) for 30 min and then assessed release of β -hexosaminidase as a measurement of mast cell degranulation. Our results showed that pre-exposure to AgNPs resulted in enhanced allergen-mediated mast cell degranulation (Fig. 1). Exposure to AgNPs by itself appeared to induce (although not statistically significant) minimal mast cell degranulation. Indeed, the observed enhancement of allergen-induced mast cell degranulation with AgNP pre-exposure is more than what is induced by AgNPs alone suggesting that the enhancement in allergen response is not solely due to an additive effect (Fig. 1). In addition, a dose response study revealed that exposure to AgNPs as low as 2.5 μ g/ml resulted in priming of mast cells for increased allergen-mediated degranulation (Fig. S2).

3.2. Pre-exposure to silver nanoparticles primes mast cells for enhanced allergen-mediated late-phase activation

In addition, to early phase responses, we characterized the effect of AgNP pre-exposure on allergen driven mast cell late-phase activation *i.e.* lipid mediator and cytokine release. Our results showed that pre-exposure to AgNPs (25 μ g/ml) followed by treatment with DNP (100 ng/ml) for 1 h resulted in enhanced allergen-mediated leukotriene B₄ (LTB₄) release ($> 100\%$ increase) (Fig. 2A). The same pattern was observed with cytokine release, where pre-exposure to AgNPs (25 μ g/ml) for 24 h followed by treatment with DNP (100 ng/ml) for 24 h resulted in exacerbated IL-6 release ($\sim 100\%$ increase) (Fig. 2B). As shown in Supplemental Fig. 3, pre-exposure to lower doses of AgNPs (0.25 and 2.5 μ g/ml) did not exacerbate IL-6 release following allergen stimulation. Together, these results suggest that pre-exposure to AgNPs (25 μ g/ml) primes mast cells for enhanced allergen-mediated late-phase activation.

3.3. Pre-exposure to silver nanoparticles primes mast cells for enhanced allergen-mediated ROS generation

Reactive oxygen species (ROS) formation has been established as an important component during the process of mast cell activation in response to IgE and non-IgE stimuli (Swindle and Metcalfe, 2007). We have measured ROS generation in response to allergen-mediated stimulation following pre-exposure to AgNPs. Consistent with the observed exacerbation of allergen-mediated early and late-phase activation, our results showed that pre-exposure to AgNPs for 24 h at 25 μ g/ml resulted in exacerbated ROS generation ($\sim 100\%$ increase) in response to allergen-mediated stimulation (100 ng/ml, 15 min) (Fig. 3A).

ENMs have a large surface-to-volume ratio and formation of ROS has been previously demonstrated following exposure to ENMs in many cell models (Manke et al., 2013). However, ROS burst during mast cell

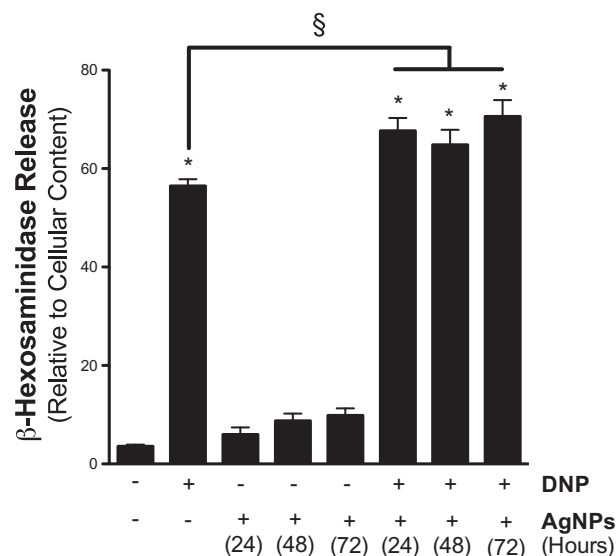


Fig. 1. Allergen-mediated mast cell degranulation in the presence or absence of silver nanoparticle pre-exposure. Bone marrow derived mast cell (BMMC) degranulation was assessed based on the release of β -hexosaminidase in supernatants relative to its cellular content. BMMCs were first treated (sensitized) with IgE anti-dinitrophenyl (DNP) overnight and then washed and exposed to silver nanoparticles (AgNPs) at 25 μ g/ml for 24, 48 or 72 h, after which they were washed and then challenged with DNP (100 ng/ml) for 30 min. Values are expressed as mean $\pm$ SEM ($N \geq 3$). * = $p \leq 0.05$ from control group. § = $p \leq 0.05$ from DNP group. $N \geq 3$ indicates that a minimum of 3 independent cultures of mast cells were isolated from C57BL/6 mice (2 mice per batch).

activation is typically transient, whereas in the case of ENMs, it is typically persistent and overwhelms the cell antioxidant system resulting in cellular death (Manke et al., 2013). In an attempt to explain the observed exacerbation of mast cell allergen-mediated activation following AgNP pre-exposure, we sought to measure ROS generation in response to AgNP exposure over time. We speculated that persistent ROS generation in response to AgNP exposure could be an underlying factor in priming mast cells to allergen-mediated stimulation. Our data showed that mast cell exposure to AgNPs (25 μ g/ml) resulted in a rapid burst of ROS formation (within 15 min and up to 60 min) (Fig. 3B). Although there appears to be another burst of ROS at 6 h, ROS levels appeared to subside over a course of 24 h post AgNP exposure, yet not quite to baseline levels (Fig. 3B).

3.4. Mast cell exposure to silver nanoparticles is not associated with oxidative stress or cell death

We sought to examine whether exposure to AgNPs induces oxidative stress and/or cell death to exclude cell toxicity as a mechanism contributing to mast cell priming to allergen-mediated stimulation following extended exposure to AgNPs. Our data demonstrated that exposure to AgNPs (25 μ g/ml) induced NQO1 mRNA expression but not glutathione peroxidase (GPx) (Fig. S4). Exposure to AgNPs (25 μ g/ml) was also associated with induction of hemoxygenase-1 (HO-1) and metallothionein-1 mRNA levels (MT-1) as well as HO-1 protein levels (Fig. S5). Furthermore, exposure to AgNPs at 25 μ g/ml and up to 24 h post exposure was not associated with reduced glutathione (GSH) levels, altered redox potential (Fig. 4A) or protein oxidation (Fig. 4B) suggesting that exposure to AgNPs at 25 μ g/ml is not associated with oxidative stress. In fact, there appears to be an increase in GSH levels and enhanced redox potential indicating a possible protective role for AgNPs (Fig. 4A). Finally, our data revealed that neither apoptotic nor necrotic cell death was associated with AgNP exposure at 25 μ g/ml and up to 72 h (Fig. S6) confirming a lack of significant cell toxicity

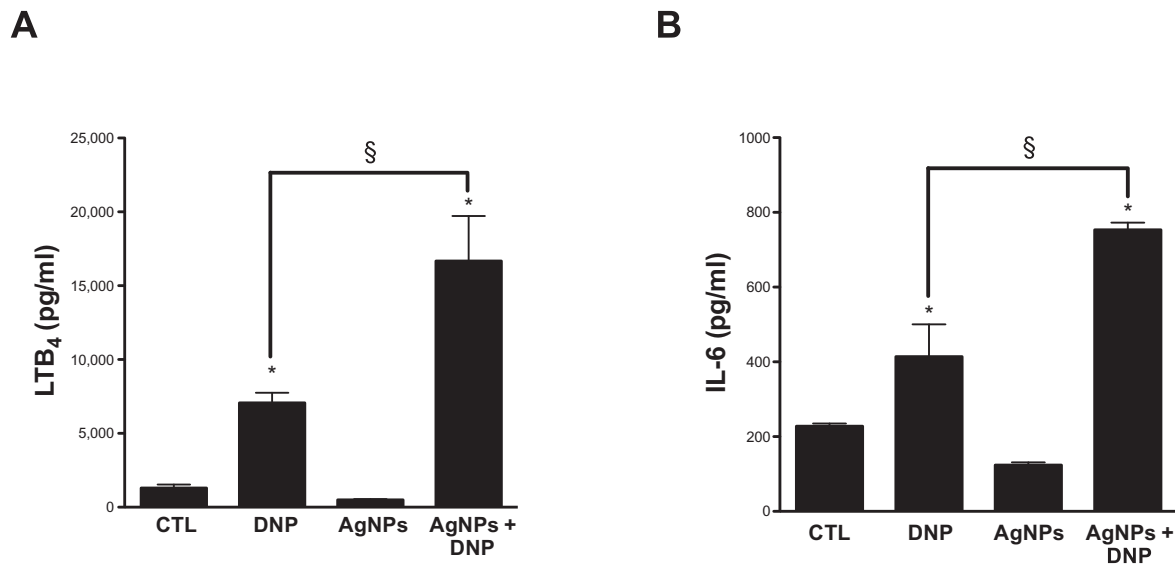


Fig. 2. Allergen-mediated mast cell late-phase activation in the presence or absence of silver nanoparticle pre-exposure. Bone marrow derived mast cell (BMMC) late-phase activation was assessed based on lipid mediator and cytokine release. BMMCs were first treated (sensitized) with IgE anti-dinitrophenyl (DNP) overnight and then washed and exposed to silver nanoparticles (AgNPs) at 25 μ g/ml for 24 h, after which they were washed and then challenged with DNP (100 ng/ml) for 1 h (A) or 24 h (B) and release of LTB₄ (A) or IL-6 (B) were measured using ELISA. Values are expressed as mean $\pm$ SEM ($N \geq 3$). * = $p \leq 0.05$ from control group. § = $p \leq 0.05$ from DNP group. $N \geq 3$ indicates that a minimum of 3 independent cultures of mast cells were isolated from C57BL/6 mice (2 mice per batch).

following exposure to AgNPs.

3.5. Pre-exposure to silver nanoparticles primes mast cells for enhanced allergen-mediated tyrosine phosphorylation

Rapid phosphorylation of protein tyrosine kinases (PTK) following crosslinking of Fc ϵ R1 represents the initial signal for a plethora of downstream events including degranulation, lipid and cytokine synthesis/release (Gilfillan and Tkaczuk, 2006). To gain some insights into the phosphorylation of Fc ϵ R1-linked protein tyrosine kinases, we sought to compare the phosphorylation status of protein tyrosine phosphorylation (p-Tyr) in response to DNP in the presence or absence of AgNP pre-exposure. To do so, we pre-exposed mast cells to AgNPs (25 μ g/ml) for 24, 48 or 72 h then washed and treated cells with DNP (100 ng/ml) for 5 min and assessed total p-Tyr. Interestingly, our results overall demonstrated that exposure to AgNPs may result in persistent protein tyrosine phosphorylation (p-Tyr) of a number of proteins (*i.e.*

24, 48 or 72 h). Challenging with DNP (100 ng/ml) in cells that were pre-exposed to AgNPs resulted in enhanced protein p-Tyr compared to DNP treatment alone, more prominent at ~25–37 kDa (Fig. S7). Furthermore, the results also suggest that exposure to AgNPs alone appeared to enhance p-Tyr of a number of proteins at a molecular weight range of ~40–50 and ~60–75 kDa.

To confirm these results, we assessed phosphorylation of individual proteins including Syk (spleen tyrosine kinase, 72 kDa), PLC γ (phospholipase C gamma, 150 kDa) and LAT (a transmembrane adaptor molecule, 36 kDa). Consistent with p-Tyr data, pre-exposure of BMMCs to AgNPs resulted in enhanced phosphorylation of PLC γ 1 and Syk in response to DNP treatment in (Fig. 5). In contrast, we did not observe an enhancement in LAT phosphorylation by pre-exposure to AgNPs. In accordance with the p-Tyr blot (Fig. 5), exposure to AgNPs not only enhanced DNP-induced Syk phosphorylation, but also showed a trend of enhanced Syk phosphorylation. This indeed matched the increased phosphorylation of p-Tyr at the molecular range of 60–75 kDa (Fig. S7).

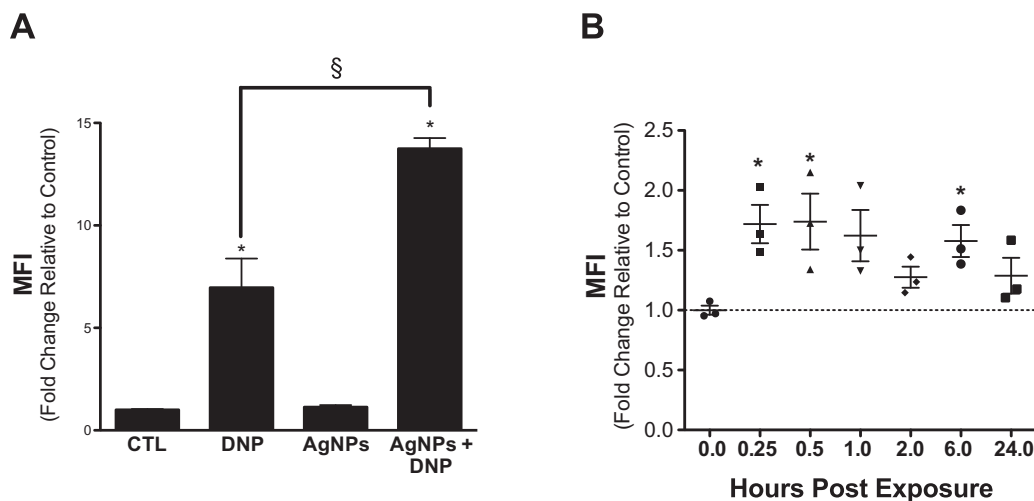


Fig. 3. Allergen-mediated mast cell ROS generation in the presence or absence of silver nanoparticle pre-exposure. Generation of reactive oxygen species (ROS) was measured in bone marrow derived mast cells (BMMCs) by H2DCFDA using a flow cytometer. (A) BMMCs were first treated (sensitized) with IgE anti-dinitrophenyl (DNP) overnight and then washed and exposed to silver nanoparticles (AgNPs) at 25 μ g/ml for 24 h, after which they were washed and challenged with DNP (100 ng/ml) for 15 min. (B) BMMCs were exposed to AgNPs at 25 μ g/ml for the indicated time points (X-axis). Fluorescence from cleaved H2DCFDA (by generated ROS) was measured by a flow cytometer. Values are expressed as mean $\pm$ SEM ($N \geq 3$). * = $p \leq 0.05$

from control group. § = $p \leq 0.05$ from DNP group. $N \geq 3$ indicates that a minimum of 3 independent cultures of mast cells were isolated from C57BL/6 mice (2 mice per batch).

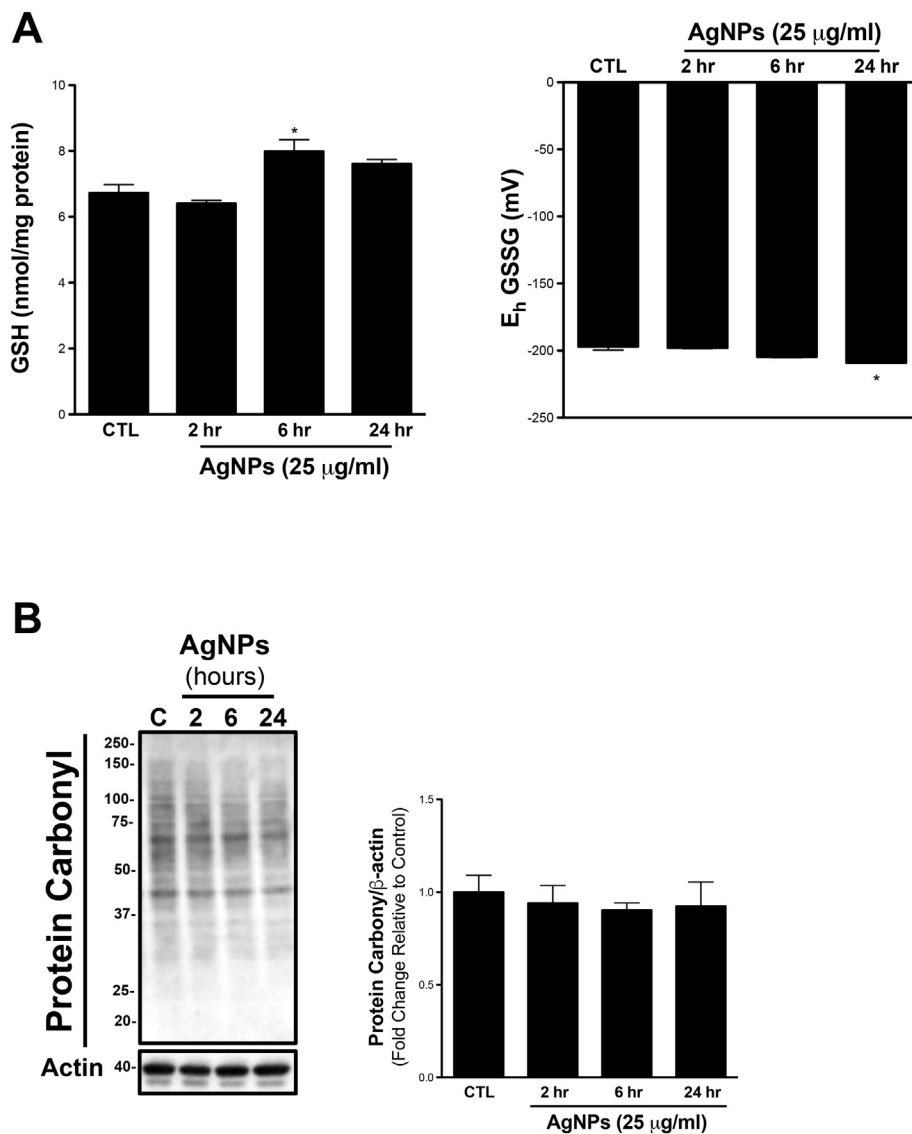


Fig. 4. Oxidative stress following exposure to silver nanoparticles. Oxidative stress was assessed in bone marrow derived mast cells (BMMCs) by measuring GSH levels, oxidative potential (E_h) (A) and protein carbonylation (carbonyls) (B). BMMCs were exposed to silver nanoparticles (AgNPs) at 25 µg/ml for 2, 6 or 24 h. GSH content and E_h (GSSG/GSH) were fluorescently detected using HPLC (A). Protein carbonylation (oxidation) was measured by Western immunoblot of derivatized dinitrophenylhydrazine (DNPH) (B). A representative immunoblot is shown with a quantification of immunoblots relative to β-actin expression. Values are expressed as mean ± SEM ($N \geq 3$). * = $p \leq 0.05$ from control group. $N \geq 3$ indicates that a minimum of 3 independent cultures of mast cells were isolated from C57BL/6 mice (2 mice per batch).

Whereas in PLC γ_1 and LAT, exposure to AgNPs alone for 24 h did not enhance their phosphorylation (Fig. 5), which was similar to the total p-Tyr results (Fig. S7).

4. Discussion

This study investigated the consequences of pre-exposure to 20 nm AgNPs on mast cell activation in response to allergen-mediated stimulation. Our study demonstrated that pre-exposure to 20 nm AgNPs resulted in exacerbated mast cell early- and late-phase activation in response to allergen-mediated stimulation through the high affinity IgE receptor (FcεR1). We utilized primary mast cells derived from bone marrow (BMMCs) and showed that such priming of mast cells by exposure to AgNPs was associated with exacerbated reactive oxygen species (ROS) generation and phosphorylation of FcεR1-linked signaling proteins in response to allergen-mediated stimulation. Such priming of mast cells by AgNPs was devoid of oxidative stress and cell death.

Today, silver nanoparticles (AgNPs) are one of the most widely utilized ENMs, primarily for their antimicrobial properties. They are found in a wide range of consumer products such as food packaging, health care products (sunscreen, creams, shampoos, toothpastes, deodorants, detergents, etc.), washers, air filters, paints, bluish toys and

athletic apparels as examples (Vance et al., 2015). AgNPs also have biomedical applications including medical devices, cavity fillers, catheters, and wound dressings (Chaloupka et al., 2010). Future applications could also include drug delivery and imaging (Dos Santos et al., 2014). Such broad exposure to AgNPs in day-to-day life raises concerns over any potential adverse responses. Our previous research and others have demonstrated that exposure to AgNPs triggers mast cell activation through a non-IgE pathway, which could worsen atopic symptoms (Aldossari et al., 2015; Alsaleh et al., 2016; Johnson et al., 2017; Kang et al., 2017). Along with our findings in this study, such wide utilization and exposure to AgNPs in consumer products could be of detrimental consequences for allergic and non-allergic forms of atopic diseases, which range from mild allergic response to severe anaphylaxis (Novak and Bieber, 2003). Recent *in vivo* evidence demonstrates the potential immunomodulatory and immunotoxicity properties of AgNPs (De Jong et al., 2013; Vandebruel and Tonk, 2014). Indeed, several *in vitro* reports of different immune cell types including macrophages, neutrophils and natural killer cells have been reported to undergo cell activation, functional modification, or cell death following exposure to AgNPs (Hamilton et al., 2014; Liz et al., 2015; Muller et al., 2018; Vallieres et al., 2016; Yang et al., 2012). Moreover, previous work has demonstrated that exposure to ENMs at subtoxic levels could influence cell function in response to known stimulants (Kodali et al., 2013).

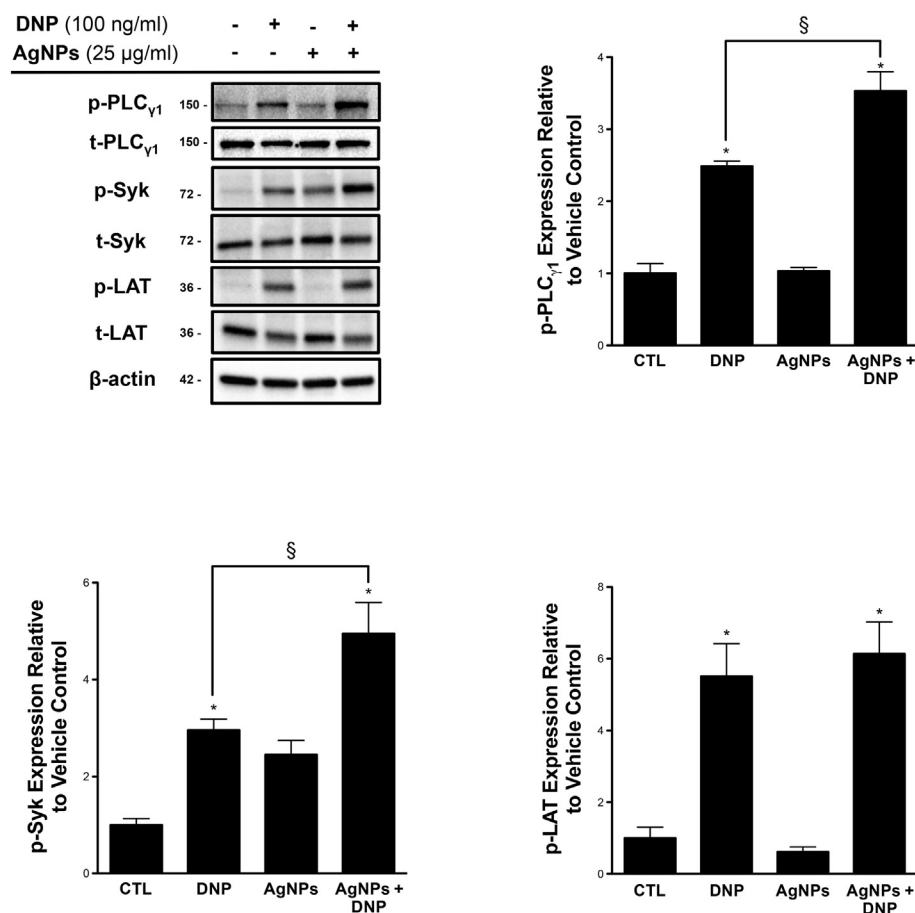


Fig. 5. Allergen-mediated phosphorylation of individual proteins in the presence or absence of silver nanoparticle pre-exposure. Allergen-mediated phosphorylation of individual proteins was assessed in bone marrow derived mast cells (BMMCs). BMMCs were first treated (sensitized) with IgE anti-dinitrophenyl (DNP) overnight and then washed and exposed to silver nanoparticles (AgNPs) at 25 μ g/ml for 24 after which the cells were washed and then challenged with DNP (100 ng/ml) for 5 min. p-PLC γ_1 , p-Syk and p-LAT were assessed in cell lysates by Western immunoblotting. Relative expression to control indicates quantifying phosphorylation of each protein of interest relative to its total expression, which then is presented relative to the loading control of each sample (β -actin). Representative immunoblots of p-PLC γ_1 , p-Syk and p-LAT are shown. * = $p \leq 0.05$ from control group. § = $p \leq 0.05$ from DNP group. $N \geq 3$ indicates that a minimum of 3 independent cultures of mast cells were isolated from C57BL/6 mice (2 mice per batch).

However, no previous work has investigated whether exposure to AgNPs (or any other ENMs) could influence mast cell responses to allergens particularly in regards to exacerbation of allergic symptoms. Thus, in this study, we sought to assess whether pre-exposure to AgNPs would influence mast cells degranulation and activation by an allergen.

Transient, low levels of ROS are critical in cell signaling and in the regulation of redox-sensitive molecules. For instance, hydrogen peroxide, a non-radical ROS, is recognized to play key roles in cell (redox) signaling e.g. hydrogen peroxide can directly oxidizes cysteine residues on protein tyrosine phosphatases rendering them temporarily inactive (Rhee, 2006). Formation of ROS has been demonstrated following exposure to a wide range of ENMs, which can be attributed to their large surface-to-mass ratio (Manke et al., 2013). Indeed, previous nanotoxicological studies have established generation of ROS following exposure to ENMs as a major mechanism of ENM toxicity and it represents the current paradigm for ENM toxicity (Carlson et al., 2008; Fu et al., 2014; Nel et al., 2006). Previous literature indicated that exposure to AgNPs is associated with persistent or increasing ROS generation over time (Eom and Choi, 2010; Foldbjerg et al., 2009; Kim et al., 2012). Therefore, we hypothesized that exposure to AgNPs would trigger ROS formation that would persist (or increase) over time thereby resulting in (or at least contributing to) AgNP-mediated priming of mast cells to allergen-mediated stimulation, possibly through a redox signaling mechanism. Our data showed that pre-exposure to AgNPs significantly exacerbated ROS generation in response to allergen-mediated stimulation. However, exposure to AgNPs alone resulted in a burst of ROS within the first hour and again at 6 h following exposure to AgNPs but which subsided over time. Based on these data and despite that AgNP-induced ROS formation was not persistent, the possibility of ROS being a driving factor for priming mast cells to allergen-mediated stimulation cannot be excluded, as even low levels of ROS could contribute

significantly to cell signaling events (Ray et al., 2012). Discerning the role of AgNP-induced ROS generation is complex, as the use of antioxidants, for instance, will also influence allergen-mediated mast cell activation. Importantly, a number of mechanisms have been previously proposed for ENM-induced ROS generation (Manke et al., 2013). However, the mechanism by which AgNPs mediates ROS generation in mast cells is yet to be explored. Elucidating such a mechanism could shed light into the underlying mechanism by which AgNPs prime mast cells to allergen-mediated stimulation.

Previous literature demonstrated in various experimental models that exposure to AgNPs induces molecular changes including but not limited to ROS formation, upregulation of stress-response genes, reduction in glutathione (GSH) levels, secretion of inflammatory cytokines, increase in mitochondrial membrane permeability, activation of cell stress response, which could ultimately result in oxidative stress (e.g. protein oxidation and lipid peroxidation) and induction of apoptotic cell death (Dubey et al., 2015). Importantly, cell toxicity and membrane damage could be an underlying factor that promotes leakage of granular content. Although exposure to AgNPs was not associated with persistent ROS generation, we sought to exclude any oxidative stress or cell death following extended exposure to AgNPs. While exposure to AgNPs induced up regulation of NQO1 mRNA levels and metal-sensing proteins including hemoxygenase-1 and metallothionein-1, extended exposure to AgNPs was neither associated with decreased GSH levels or protein oxidation (up to 24 h post-exposure), nor did it result in apoptotic or necrotic cell death (up to 72 h post-exposure) suggesting lack of major cell toxicity.

Mast cell activation by allergens through the high affinity IgE receptor (Fc ϵ R1) involves phosphorylation of tyrosine kinases downstream of Fc ϵ R1. Phosphorylation is rapid and transient yet results in downstream events which last for hours (Kalesnikoff and Galli, 2008).

Following aggregation of FcεR1, a number of the Src family and Syk kinases are phosphorylated, which subsequently phosphorylates LAT (a transmembrane adaptor molecule) leading to the recruitment of a number of adaptor proteins and signaling molecules including phospholipase C_γ (PLC_γ), which mediates hydrolysis of phosphatidylinositol bisphosphate (PIP₂) in the plasma membrane into inositol triphosphate (IP₃) and diacylglycerol (DAG) that subsequently lead to calcium influx and cell activation (Gilfillan and Tkaczyk, 2006). To shed some light into the status of phosphorylation of these signaling molecules in response to DNP treatment in the presence or absence of AgNPs pre-exposure, we assessed total tyrosine phosphorylation (p-Tyr) following extended exposure to AgNPs (24–72 h). Consistent with the observed exacerbation of allergen-induced mast cell stimulation, our data suggest that pre-exposure to AgNPs results in enhanced allergen-mediated p-Tyr. Interestingly, exposure to AgNPs alone resulted in enhanced p-Tyr at a molecular size of 40–50 and 60–75 kDa, which could suggest a potential influence of AgNPs on the Src family and Syk kinases (Gilfillan and Tkaczyk, 2006). This is in accordance with our previous findings that showed pre-treatment with imatinib, a tyrosine kinase inhibitor, resulted in inhibition of AgNP-induced mast cell activation (Aldossari et al., 2015). We sought to confirm p-Tyr results by assessing phosphorylation of a number of key signaling proteins. Consistently, our results showed a trend of increase in Syk (72 kDa) phosphorylation by exposure to AgNPs alone and enhanced phosphorylation in response to allergen-mediated stimulation. Interestingly, pre-exposure to AgNPs enhanced PLC_γ (150 kDa) phosphorylation in response to allergen-mediated stimulation; however, PLC_γ phosphorylation was not induced by AgNPs alone. Since LAT phosphorylation in response to allergen-mediated stimulation was not significantly enhanced by pre-exposure to AgNPs, this could suggest that priming of mast cells to allergen-mediated stimulation by pre-exposure to AgNPs involves other non IgE-mediated mechanisms e.g. possibly G-protein coupled receptors or other tyrosine linked receptors by which the AgNPs are interacting thereby leading to an amplified allergen-mediated stimulation. However, such hypotheses are yet to be examined.

Due to the minimal uptake of AgNPs by mast cells shown in our previous report, we also hypothesize that AgNP-induced tyrosine phosphorylation is due to direct interaction with cell membrane proteins, possibly oxidation of exofacial thiols thereby modulating cell extracellular redox state leading to an enhanced allergen-mediated stimulation (Alsaleh et al., 2016). Mast cell extracellular redox state has yet to be investigated in response to IgE and non-IgE stimulation. Prior research on other cell models has shown that extracellular redox state influences cell differentiation, proliferation and responsiveness to stimuli and growth factors (Gelderman et al., 2006; Jonas et al., 2002; Noelle and Lawrence, 1981). AgNPs and silver ions (Ag⁺) have a high affinity to bind thiol groups (–SH). Indeed, one of the mechanisms by which AgNPs and Ag⁺ mediate their antimicrobial effect is by interacting with microbial vital thiol-containing enzymes (mostly cysteine residues) at the cell membrane level (forming –S–Ag groups) rendering them dysfunctional (Morones et al., 2005). Such hypotheses are being investigated in our laboratory. Indeed, our findings reported in this study certainly warrant further mechanistic studies to determine how AgNPs prime mast cells for enhanced allergen mediated activation.

Overall, with increased incidents of allergic diseases in industrialized countries, previous research has demonstrated that different environmental exposures including diesel exhaust particles and heavy metals (found in ambient air particulate matter) have the ability to activate mast cells and worsen atopic conditions (Diaz-Sanchez et al., 2000; Walczak-Drzewiecka et al., 2003; Morones et al., 2005; Gavett et al., 2003; Lambert et al., 2000; Schwarze et al., 2006). ENMs are being synthesized at a large scale to meet industrial demands that incorporate ENMs into consumer products and pharmaceuticals (Vance et al., 2015). As such, potential exposure to ENMs, whether intentional, environmental or occupational, raises concerns over associated health and environmental risks. Our findings described in this study indicate

that pre-exposure to 20 nm AgNPs exacerbates mast cell allergen-mediated phosphorylation of FcεR1-linked tyrosine kinases and ROS generation potentially resulting in amplified early and late-phase response. Such response is not associated with cell oxidative stress or cell death. Together, these findings suggest that exposure to AgNPs has the potential to prime mast cells to allergic immune responses, which could be of particular concern to atopic populations. These results underscore the importance of understanding ENM–immune cell interaction and undoubtedly necessitate further mechanistic studies to understand the nature of interaction between ENMs and cells at the cellular and molecular levels.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.taap.2019.114746>.

Ethics approval and consent to participate

All use of animals in this study was approved by the University of Colorado Anschutz Medical Campus Institutional Animal Care and Use Committee.

Consent for publication

All authors have read and consent for publication of this manuscript.

Availability of data and material

All data will be made available according to the National Institutes of Health policies.

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Authors' contributions

JMB conceived of the study and edited the manuscript; NBA performed the experiments and wrote the manuscript; RPM performed experiments.

Authors' information

Not applicable.

Declaration of Competing Interest

The authors have no competing interests in regards to this manuscript.

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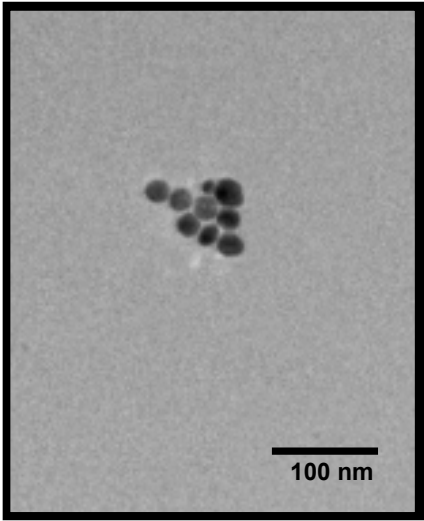
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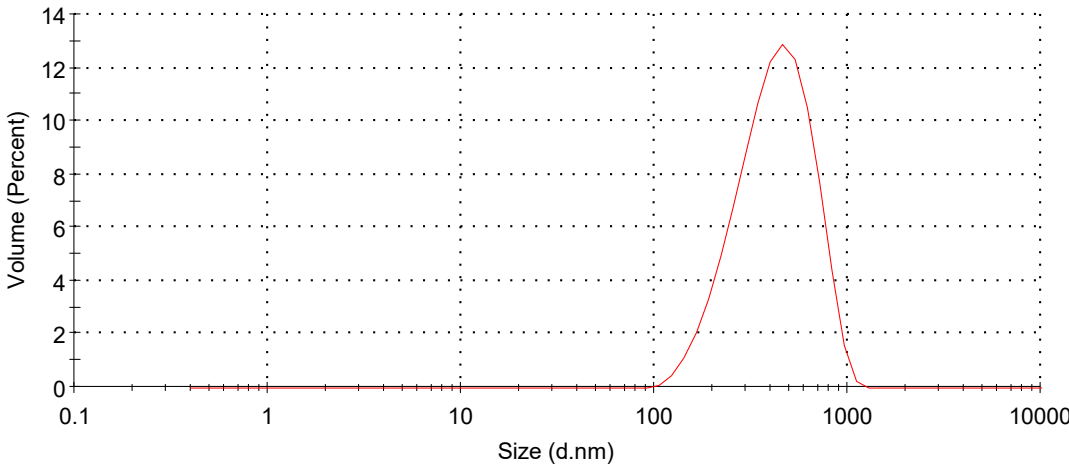
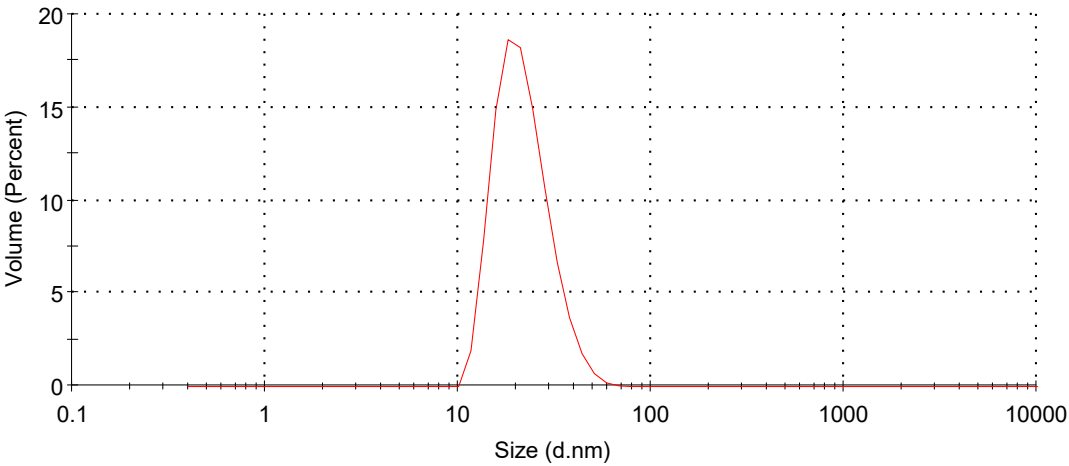
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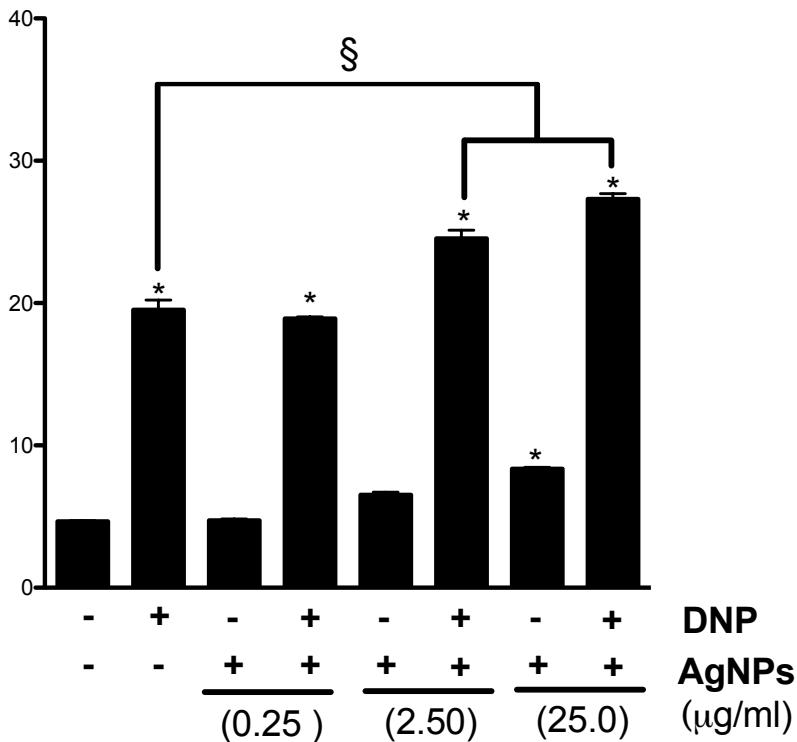
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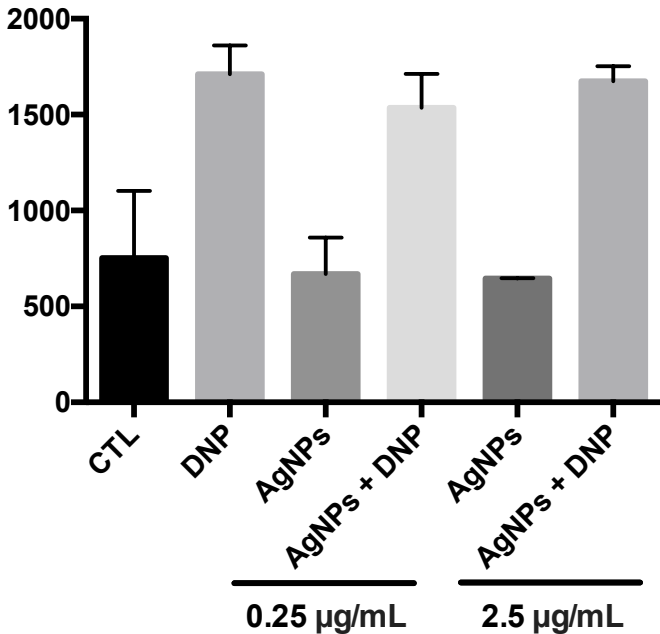
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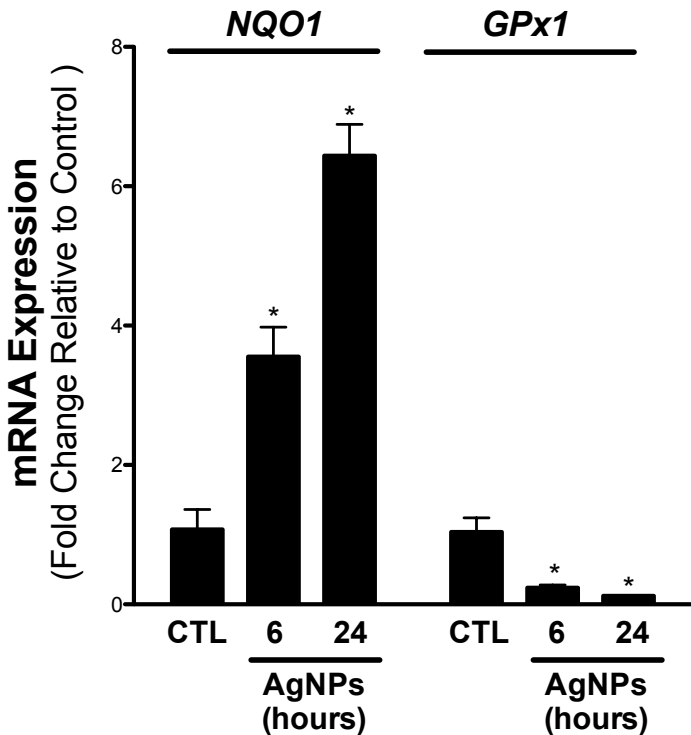


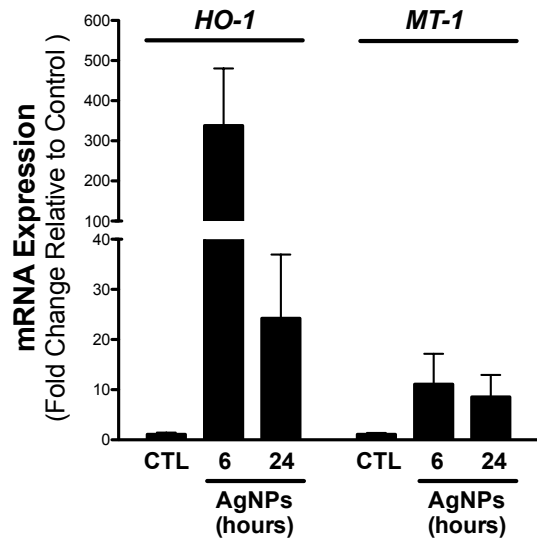
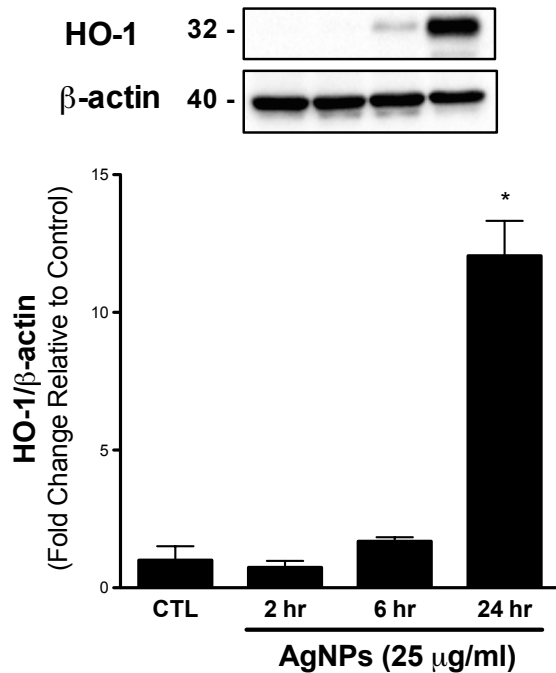
β -Hexosaminidase Release
(Relative to Cellular Content)

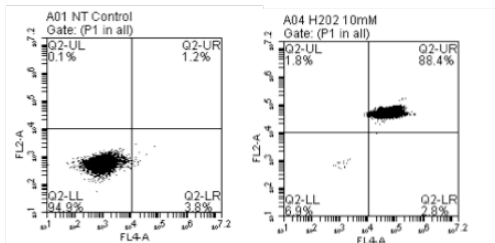
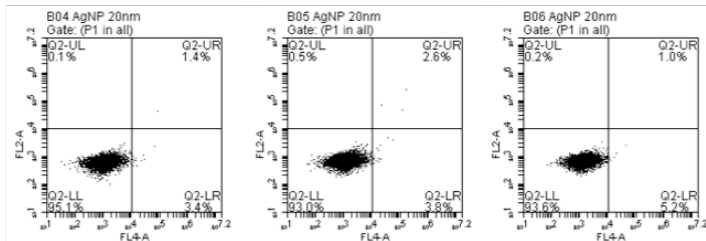


IL-6 (pg/mL)





A**B**

Control**H₂O₂****PI****AgNPs, 24h****AgNPs, 48h****AgNPs, 72h****Annexin V**