

5 nm Silver Nanoparticles Amplify Clinical Features of Atopic Dermatitis in Mice by Activating Mast Cells

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The triggering effect of silver nanoparticles (NPs) on the induction of allergic reactions is evaluated, by studying the activation of mast cells and the clinical features of atopic dermatitis in a mouse model. Granule release is induced in RBL-2H3 mast cells by 5 nm, but not 100 nm silver NPs. Increases in the levels of reactive oxygen species (hydrogen peroxide and mitochondrial superoxide) and intracellular Ca^{++} in mast cells are induced by 5 nm silver NPs. In a mouse model of atopic dermatitis induced by a mite allergen, the skin lesions are more severe and appear earlier in mice treated simultaneously with 5 nm silver NPs and allergen compared with mice treated with allergen alone or 100 nm silver NPs and allergen. The histological findings reveal that number of tryptase-positive mast cells and total IgE levels in the serum increase in mice treated with 5 nm silver NPs and allergen. The results in this study indicate that cotreatment with 5 nm silver NPs stimulates mast cell degranulation and induces earlier and more severe clinical alterations in allergy-prone individuals.

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1. Introduction

The stimulatory effect of metal nanoparticles (NPs) on immune cells is dependent on the chemical characteristics and size of the particles, and on the production of reactive oxygen species (ROS).^[1–4] Since the use of silver NPs is widening to include antimicrobial films for food preservation^[5] and medical applications such as wound dressings,^[6–8] the chance of exposure to silver NPs via the dermal route is increasing. With regard to the immune response, our in vitro studies indicate that low doses of silver NPs stimulate interleukin (IL)-1 β production through inflammasome formation^[9] and IL-8 secretion in peripheral blood and mononuclear cells. However, when exposed to higher doses, the phagocytic function of alveolar macrophages is reduced.^[10] Intravenous administration of high doses of silver NPs resulted in the complete suppression of natural killer cell activity and decreased production of interferon- γ , IL-6, IL-10, and tumor necrosis factor- α , whereas IL-1 β production, serum levels of IgM and IgE, and the number of blood neutrophils increased.^[11] Silver NPs stimulate macrophages to release cytokines,^[2,12] and inhalation studies in particular have revealed the activation of alveolar macrophages.^[13–15] These results indicate

that silver NPs involve multiple immune systems and induce diverse effects. Recently, an in vitro study reported that silver NPs induce mast cell degranulation and may impact allergic disease.^[16] Another study reported that inhalation of silver NPs triggers significant allergic responses (elevated IgE levels) in mice.^[17] Meanwhile, however, other studies have demonstrated an inhibitory effect for NPs on allergic reactions. Researchers previously showed that ZnO NPs markedly inhibit both histamine and β -hexosaminidase release in rat mast cells and mouse bone marrow-derived mast cells: the same study group found that TiO₂ NPs failed to inhibit the allergic response.^[18] Also, preincubation of C60 fullerene was shown to inhibit IgE-induced elevation of cytoplasmic ROS levels in human mast cells and basophils.^[19] These findings suggest that while some NPs may enhance allergic reactions, others may exert an inhibitory effect applicable in immunological diseases. However, the exact mechanism for this difference has not yet been clarified.

Since mast cells are principle effectors of the allergic response and play an important role in asthma, atopic dermatitis, and other allergic reactions,^[20] we focused on the activation of mast cells (in vitro) and atopic animal models (in vivo) by silver NPs. We found that 5 nm silver NPs, which do not trigger clinical symptoms when used on their own, activate mast cells in vitro and induce clinical symptoms earlier and in a more severe way in mice with atopic dermatitis. Therefore, our study indicates that silver NPs can amplify an allergic immune response in allergy-prone individuals.

2. Results and Discussion

2.1. Cytotoxicity of Silver NPs and Granule Release in Mast Cells

The transmission electron microscopy (TEM) images of silver NPs (5 and 100 nm) showed that they are relatively uniform in size (Figure S1A, Supporting Information) and stable against aggregation in culture medium. Dynamic light scattering (DLS) analysis by number shows that the mean sizes were 5.1 nm for 5 nm silver NPs and 74.2 nm for 100 nm NPs (Figure S1B, Supporting Information). Although the endotoxin level was negative (endotoxin < 0.01 U mL⁻¹), polymyxin B was added to the culture medium as a neutralizer of endotoxin. Since mast cells express the endotoxin receptor toll-like receptor 4,^[21,22] the activation of mast cells, even to minute levels of endotoxin, may compromise the biological results following silver NPs exposure. Cell death was initiated by 1.2 μ g mL⁻¹ of 5 nm silver NPs, but not by the same concentration of 100 nm silver NPs (Figure 1A). The LC₅₀ of 5 nm silver NPs was 1.96 μ g mL⁻¹ and cell death was not induced by 100 nm silver NPs at up to 4 μ g mL⁻¹. Therefore, cytotoxicity was dependent on the particles size of the NPs. Cell death was completely inhibited by *N*-acetyl cysteine (NAC) (Figure 1A), a ROS scavenger, which indicates that ROS produced by 5 nm silver NPs are the main cause of cell death.

The cardinal pathway of granule release is induced by the attachment of IgE-bound allergens to high-affinity IgE

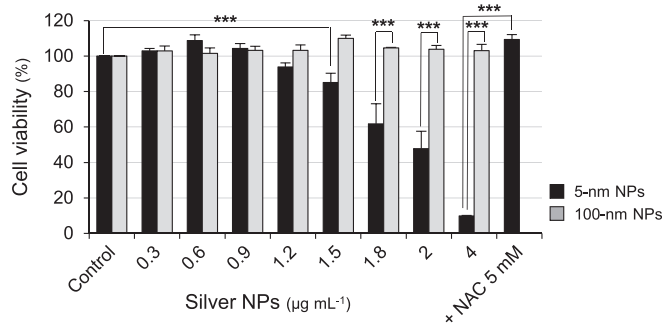
(Fc ϵ RI) receptors on mast cells and the subsequent cross-linking of Fc ϵ RI receptors. However, other factors, for instance, toxins such as delta toxins of *Staphylococcus*^[23] as well as hemolytic lipid toxin of group B *Streptococcus*,^[24] can elicit mast cell activation directly. In addition, IgE-mediated mast cell degranulation can be enhanced by neuropeptides such as substance P and calcitonin gene-related peptide.^[25] Since the degranulation of mast cells has been correlated with the severity of atopic dermatitis,^[26] we determined if silver NPs induce granule release from mast cells. Therefore, in this study we estimated the release of β -hexosaminidase, the representative component of mast cell granules.^[27] β -hexosaminidase was released by 5 nm silver NPs in the range of 18.9% at 0.15 μ g mL⁻¹ to 76.9% at 0.6 μ g mL⁻¹ (Figure 1B), but not by 100 nm silver NPs at the same concentration range. Phorbol 12-myristate 13-acetate (PMA)/ionomycin was used as the positive control of granule release. In the same cytotoxicity assay, the granule release in RBL-2H3 cells was inhibited by NAC (Figure 1C).

The immediate increase of cytoplasmic Ca⁺⁺ levels is also important for mast cell activation,^[28,29] as shown by the 5 nm silver NPs, but not by the 100 nm NPs (Figure 1D). In our study, Ca⁺⁺-free medium was used. Therefore, the results suggest that Ca⁺⁺ influx induced by 5 nm silver NPs depends on Ca⁺⁺ release from intracellular stores. The calcium signal is an essential mechanism for degranulation in mast cells after IgE cross-linking by allergens,^[30] and our data show that it is also influenced by silver NPs. Although there have not been many studies on NPs and mast cells, some reports have shown that Ca⁺⁺ influx into the cytosol is induced by TiO₂ NPs in mast cells,^[31] and by silica NPs in monocytes.^[32] This calcium release is related to mitochondrial disruption and ROS.^[33] These reports support our opinion that silver NPs initiate the activation of mast cells without specific IgE engagement to increase cytoplasmic Ca⁺⁺ levels and ultimately to induce degranulation.

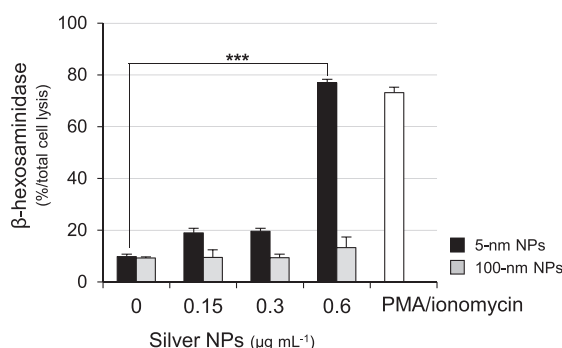
2.2. Generation of Total ROS and Mitochondrial Superoxide, and Disturbance of Mitochondrial Membrane Integrity

The production of total ROS was increased by 4.9-fold in a dose-dependent manner by 5 nm silver NPs compared with the control at 4 μ g mL⁻¹ (Figure 2A). The level of mitochondrial superoxide was increased by twofold in a dose-dependent manner by 5 nm silver NPs compared with the control at 4 μ g mL⁻¹ (Figure 2B). An increase in the total ROS (Figure 2A) and mitochondrial superoxide levels (Figure 2B) was not induced by 100 nm silver NPs up to 4 μ g mL⁻¹. Since several papers suggest that ROS cause mitochondrial damage and cytotoxicity,^[34–36] we assessed perturbation of the mitochondrial membrane. JC-1, a lipophilic cation, is used to measure mitochondrial membrane integrity.^[9] The JC-1 fluorescence intensity, which was dose-dependent, increased by 4.1-fold at 4 μ g mL⁻¹ in the presence of 5 nm silver NPs compared with the control, whereas the 100 nm silver NPs did not increase JC-1 fluorescence intensity up to 4 μ g mL⁻¹ (Figure 2C). These data confirm the dose-dependency of ROS production and the disruption of

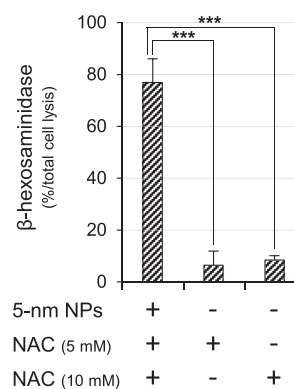
(A) Cytotoxicity



(B) β -hexosaminidase release



(C) Effect of ROS scavenger



(D) Intracellular Ca²⁺ level

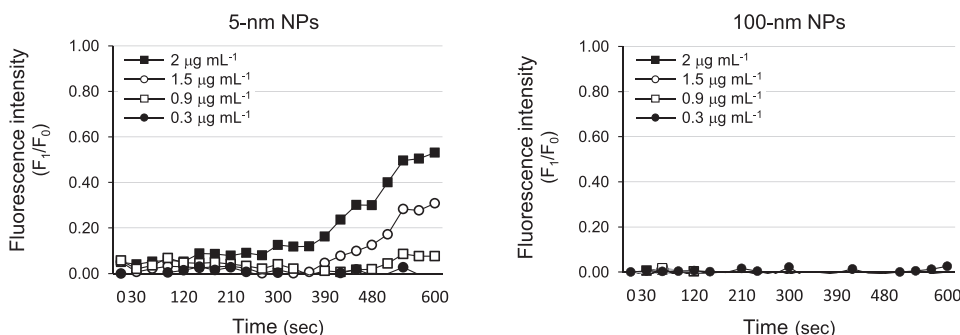


Figure 1. Cytotoxicity of silver NPs and granule release in mast cells. A) RBL-2H3 cells were treated with 5 or 100 nm silver NPs for 24 h and cytotoxicity was determined by CCK-8 assay. Cells were treated with NAC for 30 min before treatment with silver NPs. The LC50 of 5 nm silver NPs was $\approx 1.96 \mu\text{g mL}^{-1}$. B) RBL-2H3 cells were treated with silver NPs for 30 min and granule release was determined by β -hexosaminidase assay. PMA/ionomycin-treated cells were used as a positive control. C) Cells were pretreated with NAC for 30 min before treatment with silver NPs ($0.6 \mu\text{g mL}^{-1}$). D) RBL-2H3 cells were stained with Fluo-4 AM for 30 min and diluted with HBSS without Ca^{2+} at 37°C in the dark. Ca^{2+} release was monitored using a Victor X4 multilabel plate reader at 36°C and measured every 5 s for 600 s. Data represent the mean $\pm$ SD. The statistical comparison was performed by two-way analysis of variance (ANOVA). *** $P < 0.001$.

mitochondrial membrane integrity following exposure to 5 nm silver NPs. Therefore, ROS production by 5 nm silver NPs is the critical cause of cytotoxicity as well as degranulation in mast cells (Figure 1).

Regarding nanoparticle toxicity, smaller nanoparticles of a given concentration have been known to show greater toxicity due to surface effects, as reported for TiO_2 ^[37] and ZnO ^[38] Although surface area is indicated as an effective metric with which to measure the toxicity of nanoparticles,

our in vitro study showed that a 20 times higher concentration of 100 nm silver NPs, which is comparable to the same surface area of the 5 nm NPs, did not induce cell death (Figure S1C, Supporting Information). Owing to their surface effect, small-sized NPs tend to dissolve more quickly and to a greater extent than larger particles of the same chemical nature.^[39] This dissolution difference between 5 and 100 nm silver NPs might be one reason for the observed toxicity of the 5 nm silver NPs in our study.

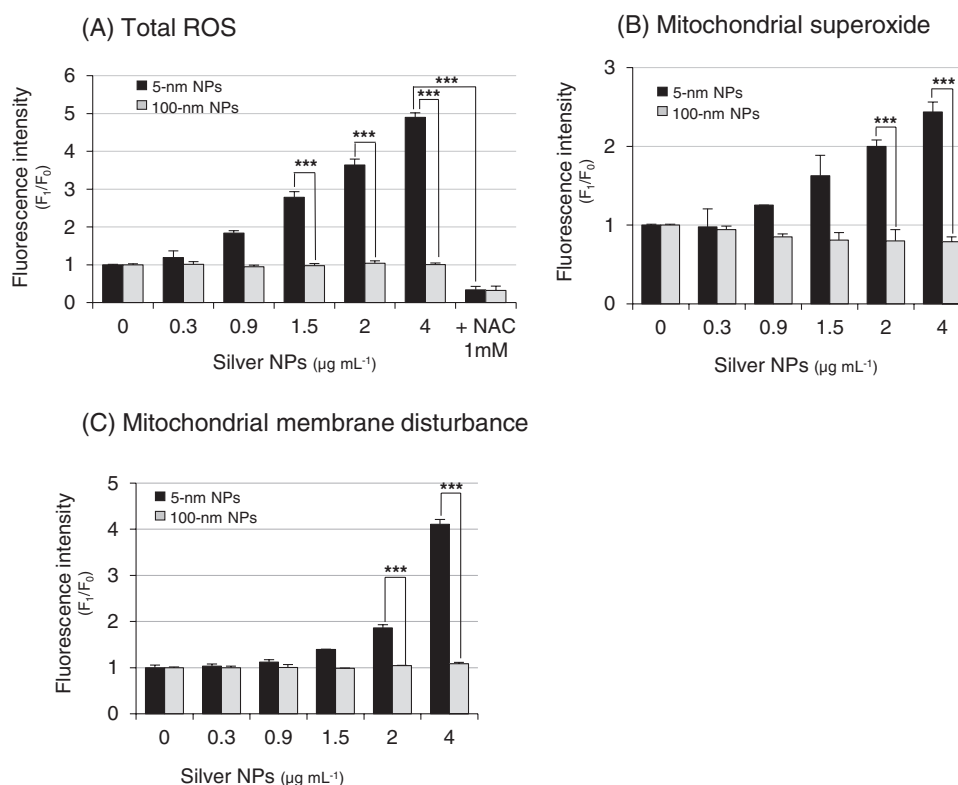


Figure 2. Generation of total ROS and mitochondrial superoxide, and disturbance of mitochondrial membrane integrity. To detect A) total ROS or B) mitochondrial superoxide RBL-2H3 cells were treated with silver NPs for 30 min and stained with CM-H2DCFDA for 30 min or with MitoSOX for 15 min. The fluorescence intensity was determined using a Victor X4 multilabel plate reader. C) RBL-2H3 cells were treated with silver NPs for 1 h. JC-1 staining was performed to evaluate disturbance of mitochondrial membranes. Data represent the mean $\pm$ SD. The statistical comparison was performed by two-way analysis of variance (ANOVA). *** $P < 0.001$.

Nevertheless, in this study, we included 100 nm silver NPs as a control for the same weight of silver rather than to compare the surface effect of differently sized nanoparticles. Therefore, we fixed the concentration of NPs at weight base for further in vivo studies.

2.3. Penetration of Silver NPs into Mice Skin

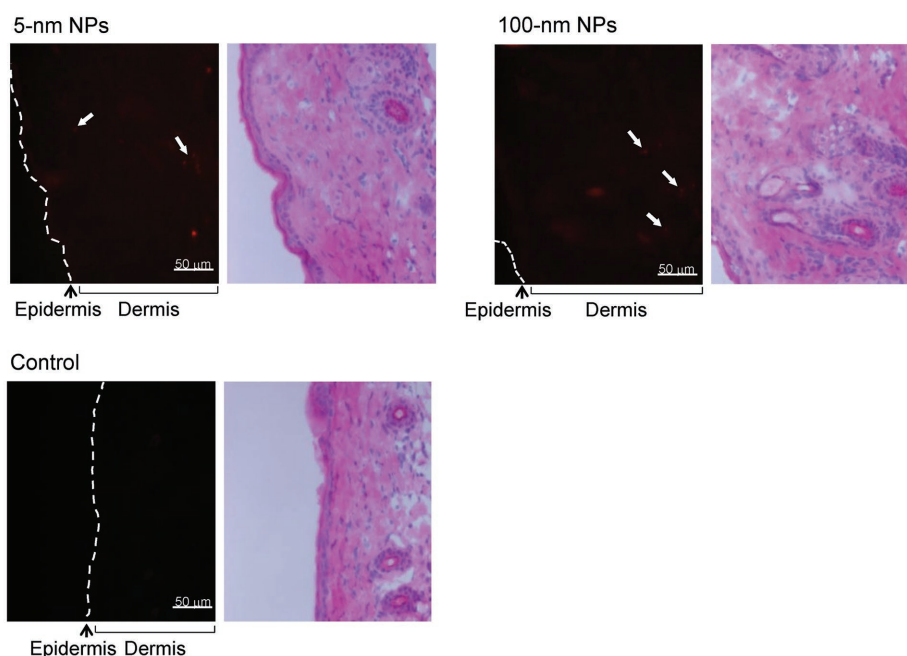
Before using the atopic dermatitis mouse model, the penetration of silver NPs into the skin of the mice was assessed using rhodamine-conjugated NPs. The dorsal skin of the mice was prepared in a way similar to that adopted for the atopic dermatitis model. As shown in **Figure 3A**, 5 and 100 nm NPs were detected in the mice skin dermis 3 h after local administration of 40 μg of each type of NP onto a 12.5 cm^2 area of the back skin, whereas no signal was detected in the control skin. Under dark-field microscopy, silver NPs were detected as orange spots in the dermis of skin samples treated with both 5 and 100 nm NPs at the third week in the dermal regions, mainly around the hair follicles (**Figure 3B**). A recent study describing the dermal penetration of TiO_2 NPs showed that NPs are detected in the dermis, surrounding the hair follicle,^[40] which was quite similar to our results. However, we assume that a small amount of NPs might penetrate the dermis and affect mast cells, because our in vitro results

showed that a sub-lethal dose of silver NPs could activate mast cells.

2.4. Clinical Features of Skin Lesions and Serum IgE Levels in Mice with Atopic Dermatitis

We speculated that the exposure concentration of NPs in daily lives is not high enough to induce serious damage, so an animal model with a background of allergic disease was adopted. We chose the atopic dermatitis model because it is easy to evaluate clinical symptoms and signs with the naked eye using such a model. NC/Nga mice have been used for the atopic dermatitis model,^[41] and they developed typical skin lesions 4–6 weeks after the application of house dust mite allergen. In our study, we focused on early developing skin lesions; therefore, the clinical score of the atopic dermatitis-like skin lesions was measured once per week for three weeks. From the third week, owing to severe skin lesions and scar formation, the allergen treatment and the insufficient dispersal of the NPs might have hindered the study results. At the second week the severity score increased in mice treated with allergen + 5 nm silver NPs (9.4 ± 0.9) compared with the allergen-only group (7.8 ± 2.2) or the allergen + 100 nm NPs group (7.3 ± 0.9) (**Figure 4A**). At the third week, the severity score increased in mice treated with

(A) Rhodamine-conjugated silver NPs in dermis of skin samples from healthy mice



(B) Silver NPs in dermis of skin samples from atopic dermatitis mice

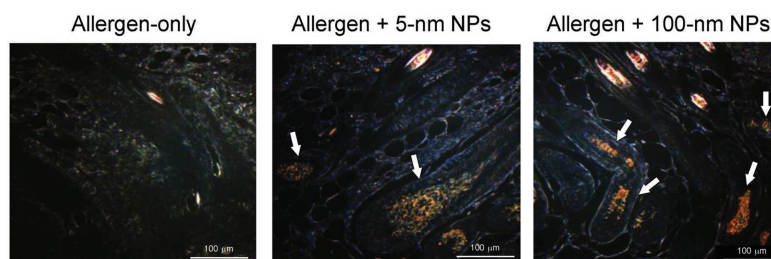


Figure 3. Penetration of silver NPs into skin. A) Rhodamine B-conjugated silver NPs (40 μ g) comprising 5 or 100 nm NPs were applied to the dorsal skin of C57BL/6N mice for 3 h. A skin biopsy was then performed and samples were prepared by cryosection. The presence of silver NPs was determined by fluorescence microscopy. Hematoxylin and eosin (H&E) staining was performed for histological observation. White arrows indicate rhodamine B-conjugated silver NPs. B) Silver NPs without rhodamine were detected by dark-field microscopy in dermal skin from the third week in atopic dermatitis mice.

allergen + 5 nm silver NPs (8.0 ± 3.0) compared with the allergen-only group (6.8 ± 3.0), or the allergen + 100 nm NPs group (4.6 ± 1.5) (Figure 4A). Representative skin pictures at the second and the third weeks are shown in Figure 4B. When 100 nm silver NPs were used, the dermatitis symptoms recovered a little and hair even grew in the center of the skin lesion (Figure 4B). For this reason, we cautiously suggest that 100 nm NPs induce very low levels of oxidative stress, which activate cells rather than causing toxicity, because ROS possibly acts as a secondary messenger to induce cellular activation and proliferation.^[42] Since ZnO NPs^[18] and C60 NPs^[19] have been reported to inhibit mast cell activation and allergic symptoms, the possibility of immunoinhibitory effect of 100 nm silver NPs at this concentration could be considered.

Moreover, the total IgE levels in the sera increased in mice treated with allergen + 5 nm silver NPs (Figure 4C).

From the second week, total IgE levels in the sera increased in mice treated with allergen + 5 nm silver NPs versus allergen alone. Owing to the wide range of IgE titers among individuals, we did not obtain highly significant results. Nevertheless, our data for the second and fourth weeks showed that treatment with allergen + 5 nm silver NPs induced the production of more IgE than allergen alone. The levels of IgE are shown in Table S1 (Supporting Information). When 5 or 100 nm silver NPs were used alone as treatments, the total IgE levels in the sera were indistinguishable from those in healthy mice (Figure S5, Supporting Information). Immunologically, atopic dermatitis is associated with high levels of total serum IgE^[43] as well as allergen-specific IgE. Accordingly, our data strongly suggest that 5 nm silver NPs, not 100 nm NPs, enhance IgE production and aggravate the clinical symptoms of atopic dermatitis. Moreover, a recent population cohort study of children reported that atopic individuals with high serum

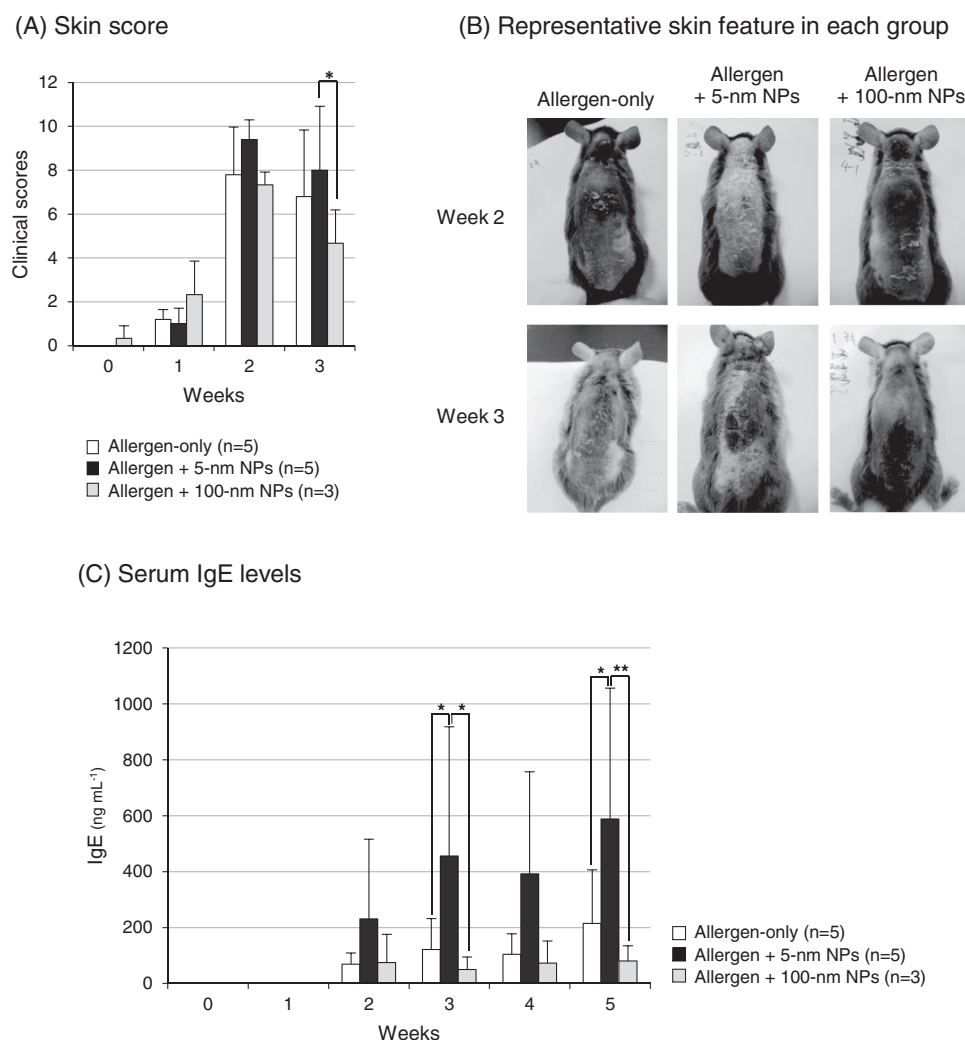


Figure 4. Clinical skin scores and serum IgE levels in mice with atopic dermatitis. A) The score is the sum of individual scores graded as 0 (absence), 1 (mild), 2 (moderate), and 3 (severe) for each of four signs and symptoms (erythema/hemorrhage, scaling/dryness, edema, and excoriation/erosion). B) Representative skin features for the second or third week are shown. C) The amount of IgE in the sera was determined using a mouse IgE-specific ELISA kit. Samples were diluted at 1:100 and 1:1000. Data represent the mean $\pm$ SD. The statistical comparison was performed by two-way analysis of variance (ANOVA). * $P < 0.05$, ** $P < 0.005$.

IgE levels have a tendency to develop other allergic diseases such as asthma.^[44] Therefore, dermal exposure to 5 nm silver NPs might trigger other allergic diseases as well as atopic dermatitis.

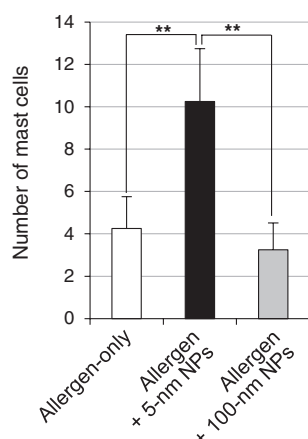
Most studies to detect dermal toxicity have involved treatment with silver NPs alone rather than with disease-prone animals. One study showed that there was no acute dermal toxicity in rats treated with 10 nm silver NPs up to 2000 mg kg⁻¹ once daily for 15 d.^[45] In another study, when silver NPs (<100 nm) were used to treat the dorsal skin of guinea pigs once daily, an inflammatory response was detected in the skin, corresponding to an increase in the levels of Langerhans cells.^[46] The changes were detected at 100 μ g/lesion and became more severe at 1000 μ g/lesion. Similar results were reported in an acute toxicity study (14 d) and a subchronic toxicity study (13 weeks). In our study, when 5 or 100 nm silver NPs were administered alone, no skin symptoms developed for up to six weeks (data not shown). No significant body weight differences

were observed between mice treated with allergen alone and those treated with allergen + silver NPs (Figure S4, Supporting Information).

2.5. Histopathological Findings in Skin from Mice with Atopic Dermatitis

In addition to the clinical symptoms, pathological findings such as the infiltration of mast cells supported the evidence of allergic response in skin from mice treated with allergen + 5 nm silver NPs. The major enzymes in mast cell granules are tryptase and chymase. These enzymes are related to atopic dermatitis and are released during the degranulation of mast cells when allergic reaction occurs.^[47,48] In human skin atopic dermatitis, numerous tryptase-positive mast cells are found close to the dermal-epidermal border.^[49] Moreover, tryptase induces an itching sensation in atopic dermatitis.^[50] Our study showed that the number of tryptase-positive mast cells in the

(A) Mast cell counting



(B) Histopathological findings

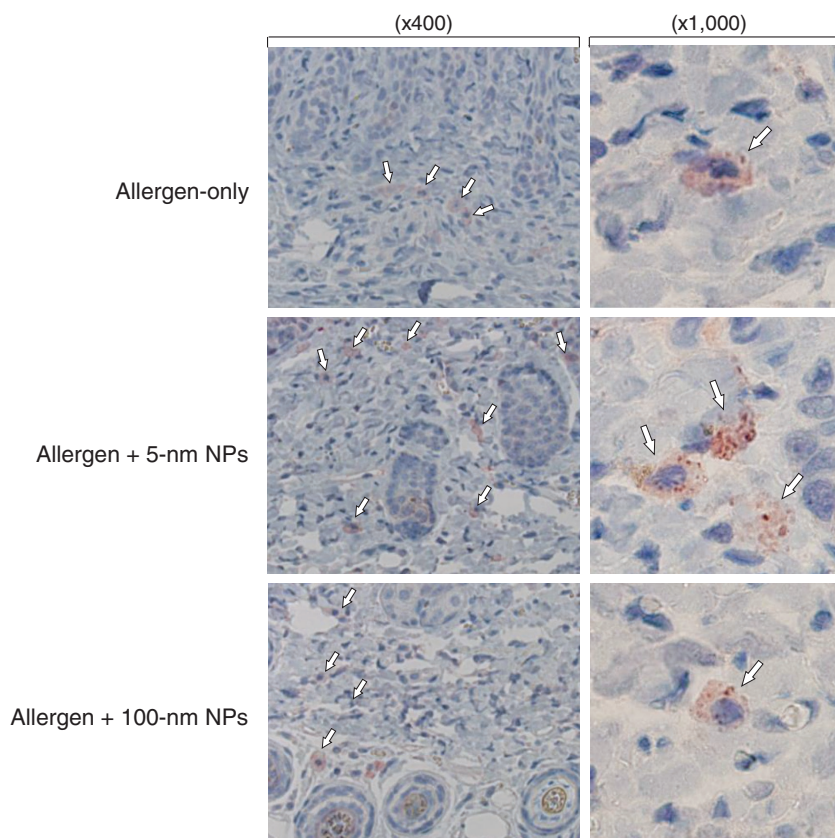


Figure 5. Mast cells in skin lesions. A) The number of mast cells in the dermis was assessed by counting tryptase-positive cells at four regions (each 50 000 μm^2) per section at a magnification of 400 $\times$. Skin samples at the second week were examined. The statistical comparison was performed by Student's *t*-test. $**P < 0.005$. B) White arrows indicate tryptase-positive cells in a histopathological preparation of each sample.

skin lesion (50 000 μm^2 area) increased in mice treated with allergen + 5 nm silver NPs (10.0 ± 1.3) compared with the allergen-only group (4.6 ± 1.8) at the second week (**Figure 5A**). In the group with allergen + 100 nm silver NPs, the number of tryptase-positive mast cells remained at 2.5 ± 1.0 (Figure 5A), similar to the allergen-only group. The histopathological

examination clearly shows that significantly more mast cells were detected in the allergen + 5 nm NPs group compared with the allergen-only or allergen + 100 nm NPs groups (Figure 5B).

The results of our study shed light on mast cell activation by 5 nm silver NPs and amplification of clinical symptoms in atopy-prone mice. The activation of mast cells in our study was not derived via the typical immunological pathway, such as cross-linking of IgE receptors. Rather, ROS production following minimal cellular insult induces mast cell activation. Silver NPs can either damage cell membranes or enter cells by diffusion for some cell types, including endothelial cells^[51] or hepatocytes,^[52] and by endocytosis in phagocytic cells.^[53,54] The K^+ efflux followed by membrane damage may trigger histamine release from mast cells directly^[55] or indirectly via the production of intracellular ROS.^[56,57] Given the results of our *in vitro* study that a ROS scavenger, NAC, inhibited mast cell activation and the production of total ROS (Figures 1C and 2A,B), we assumed that the intracellular ROS produced by silver NPs was likely the major factor leading to the mast cell degranulation. By contrast, internalized silver NPs can destroy the cytoplasmic granule membranes of mast cells via similar mechanisms used to disturb the lysosomal membrane or mitochondrial membrane in macrophages.^[9] Deterioration of the mitochondrial membrane was also observed (Figure 2C). Collectively, this ROS production and granule membrane disruption may lead to the release of enzymes and bioactive amines such as histamine. A high concentration of silver NPs alone may elicit mast cell activation and the release of enzymes and bioactive amines. However, our study focused only on the amplification of allergic symptoms in allergy-prone animals at relatively low/nontoxic concentrations. Herein, clinical symptoms appeared earlier and as severer forms when nontoxic levels of 5 nm silver nanoparticles were treated concomitantly with house dust mite allergen. Also, the histological findings, as well as elevated serum

IgE levels, in our study suggest that 5 nm silver NPs at low concentrations can amplify allergy induction while not eliciting allergic symptoms. To date, there has been no report on the allergy-enhancing effect of silver NPs. Very recently, it was reported that when Der p 1, an allergen closely related to that used in our study, was bound to nanogold,

IgE-dependent basophil degranulation and the proteolytic activity were increased.^[58]

3. Conclusions

To assess the effects of NPs on the immune system, specialized experimental animal models should be considered to precisely mimic real-life exposure scenarios such as subtoxic-level exposure. In this study, we adopted an animal model of atopic dermatitis to confirm the impact of the *in vitro* results. The *in vitro* study showed that 5 nm silver NPs induced granule release, and an increase in the level of intracellular Ca^{++} via the generation of oxidative stress (total ROS and mitochondrial superoxide) at subtoxic concentrations. In mice with atopic dermatitis, co-exposure to 5 nm silver NPs and allergens induced earlier and more severe clinical symptoms and higher levels of serum IgE, as supported by the histopathological observation of increased numbers of tryptase-positive mast cells in the skin. Our results clearly indicate that sublethal doses of 5 nm, but not 100 nm silver NPs, stimulate mast cells to release granule components and amplify atopic dermatitis in allergy-prone animals.

4. Experimental Section

Silver NPs: Silver NPs in water suspension were provided by I&C (5 nm, Seoul, Korea) or were purchased from ABC Nanotech (100 nm, Daejeon, Korea). All silver NPs were round and coated with polyvinylpyrrolidone (PVP). The diameter was determined by TEM (model JEM-1011, JEOL, Tokyo, Japan). The TEM images of the silver NPs (5 nm and 100 nm) showed their relatively uniform size (Figure S1A, Supporting Information). DLS analysis by number showed that the mean size of the 5 nm silver NPs was 5.1 nm and that of the 100 nm NPs was 74.2 nm (Figure S1B, Supporting Information). Agglomeration of silver NPs at $1 \mu\text{g mL}^{-1}$ in 10% fetal bovine serum–minimum essential media (FBS-MEM) was investigated by DLS (Novato, CA, USA). The particle dispersion was similar between 5 and 100 nm particles in the DLS assay (Figure S1B, Supporting Information). The silver NPs used in this study were prepared by steric stabilization using high molecular weight PVP; therefore, they do not easily aggregate. Endotoxin contamination was tested by the pyrogen recombinant factor C assay (Cambrex Bioscience, Walkersville, MD, USA) and was found to be negative (endotoxin $< 0.01 \text{ U mL}^{-1}$). For cell culture, the silver NPs were prepared in MEM with L-glutamine ($2 \times 10^{-3} \text{ M}$) supplemented with 10% FBS, penicillin (100 U mL^{-1}), and streptomycin (100 U mL^{-1}). To detect skin penetration, 5 or 100 nm silver NPs were conjugated with rhodamine B. Briefly, rhodamine B solution (1.5 mL , 0.6 mg mL^{-1} , R6626, Sigma-Aldrich, St. Louis, MO, USA) was added to silver NPs solution (10 mL , 0.025 mg mL^{-1}) and stirred (1.5 d , room temperature). Silver NPs conjugated with rhodamine B by this method are stable for at least 20 d in the pH range of 2–11.^[59] To isolate silver NPs, NPs were centrifuged for 30 min at $100\,000 \times g$ for 5 nm NPs and 15 min at $16\,500 \times g$ for 100 nm NPs. The concentration of silver NPs was measured using inductively coupled plasma optical emission spectrometry (ICP-OES) (OPTIMA 8300, PerkinElmer, Waltham, MA, USA). The

rhodamine-conjugated silver NPs were internalized by RBL-2H3 mast cells and appeared as red spots under fluorescence microscopy (Figure S2A, Supporting Information). The silver NPs without rhodamine appeared as orange spots under dark-field microscopy (Figure S2B, Supporting Information).

Cell Culture: The RBL-2H3 rat mast cells (ATCC, Manassas, VA, USA) were cultured in 10% FBS-MEM in a 5% CO_2 incubator at 37°C . Although endotoxin was not detected in the silver NPs, polymyxin B (10 ng mL^{-1} , InvivoGen, San Diego, CA, USA) was added as an endotoxin neutralizer.

Cytotoxicity Assay: Cell viability was assessed using the colorimetric cell counting kit-8 (CCK-8) (Dojindo Laboratories, Kyoto, Japan). Cells were plated onto 24-well plates at 4×10^5 cells in 10% FBS-MEM ($500 \mu\text{L}$) and cultured in a 5% CO_2 incubator at 37°C . After incubating overnight, the cells were treated with silver NPs diluted with 10% FBS-MEM. After 24 h, CCK-8 reagent ($15 \mu\text{L}$) was added to each well followed by incubation at 37°C for 2 h. After centrifugation, the supernatant ($100 \mu\text{L}$) was transferred to 96-well microtiter plates and the optical density (O.D.) was measured at 450 nm. Cells were pretreated with NAC (Sigma-Aldrich) for 30 min prior to treatment with the silver NPs.

Granule Release Assay: RBL-2H3 cells were plated onto 24-well plates at 4×10^5 cells in 10% FBS-MEM ($500 \mu\text{L}$), and cultured in a 5% CO_2 incubator at 37°C . After overnight incubation, the cells were treated with silver NPs ($200 \mu\text{L}$) diluted with Hanks' balanced salt solution (HBSS) for 30 min. The release of β -hexosaminidase from cells was measured by incubating the supernatant ($25 \mu\text{L}$), or the cell pellet lysed with 5% Triton-100, with *p*-nitrophenyl *N*-acetyl- β -D-glucosaminide ($25 \mu\text{L}$) on a 96-well plate (Nunc, Roskilde, Denmark) for 2 h at 37°C . The reaction was stopped by adding glycine (pH = 10.6, $250 \mu\text{L}$, 0.2 M) and the O.D. was read at 405 nm. A combination of PMA (50 ng mL^{-1}) and ionomycin ($1 \times 10^{-6} \text{ M}$) was used as a positive control.

Assays for Ca^{++} Release, Total ROS, Mitochondrial Superoxide, and Mitochondrial Membrane Integrity: To detect Ca^{++} release, cells were stained with Fluo-4 AM (Invitrogen) for 30 min and diluted with HBSS without Ca^{++} at 37°C in the dark. After treatment with fluorescent dyes, cells were treated with silver NPs and Ca^{++} release was monitored using a Victor X4 multilabel plate reader (PerkinElmer, Norwalk, CT, USA) at a constant temperature of 36°C , measured every 5 s for 600 s. To detect ROS, cells were treated with silver NPs for 30 min. Cells were then stained with CM- H_2DCFDA ($2 \times 10^{-6} \text{ M}$, Invitrogen, Carlsbad, CA, USA) for 30 min to detect reactive hydrogen peroxide and MitoSOX ($2 \times 10^{-6} \text{ M}$, Invitrogen) for 15 min to detect mitochondrial superoxide at 37°C in the dark, respectively. To determine mitochondrial membrane integrity, cells were treated with silver NPs for 30 min and stained with JC-1 ($2 \times 10^{-6} \text{ M}$, Invitrogen) for 15 min at 37°C in the dark. O.D. was read at 590 nm, using a Victor X4 multilabel plate reader.

Evaluation of Rhodamine-Conjugated Silver NPs Penetration into Mice Skin: Rhodamine-conjugated silver NPs ($40 \mu\text{g}$) were applied to the dorsal skin of ten-week-old C57BL/6NJ mice. After 3 h, a skin biopsy was performed and skin samples were prepared by cryosection. The presence of silver NPs was detected by fluorescence microscopy. Hematoxylin and eosin (H&E) staining was performed to compare histological observations.

Induction of Atopic Dermatitis in Mice: Six-week-old female NC/Nga mice weighing $22 \pm 2 \text{ g}$ (Central Lab. Animal Inc., Seoul, Korea) were maintained for one week prior to the experiment. All

animal experiments were performed in compliance with the Yonsei University Institutional Animal Care guidelines, and protocols were reviewed and approved by the Institutional Animal Care and Use Committees of the Laboratory Animal Research Center at Yonsei University Health System (Permit No. 2013-0176-2). All mice were maintained in the SPF facility of the Yonsei Laboratory Animal Research Center. To induce atopic dermatitis, the hair on the back ($\approx 12.5 \text{ cm}^2$) was shaved with depilatory cream (Veet, Oxy Reckitt Benckiser Ltd., France) 1 d before the experiment (Figure S3A, Supporting Information). On the next day, sodium dodecyl sulfate (150 μL , 4% w/v) was applied to the shaved dorsal skin for barrier disruption. SDS is a highly effective surfactant and not toxic at low levels. In the atopic dermatitis model, SDS is used as a standard protocol to facilitate penetration and dispersion of house dust mite allergens in skin lesions. After 2 h, 5 or 100 nm silver NPs (100 μL , 400 $\mu\text{g mL}^{-1}$) were applied to the same regions and left for 2 h. Then, as an allergen, Biostir AD ointment (50 mg, Biostir, Kobe, Japan), including mite allergens derived from *Dermatophagoides farinae*, was applied. The skin treatment was performed twice per week for five weeks (Figure S3B, Supporting Information). Clinical scores were assessed until the third week because after that severe scars formed, which might have affected the scoring accuracy. However, serum IgE levels were evaluated until the fifth week. The mice were allocated to three groups: an allergen-only group (100 μL distilled water + allergen); a 40 μg of 5 nm silver NPs group (100 μL , 400 $\mu\text{g mL}^{-1}$ + allergen); and a 40 μg of 100 nm silver NPs group (100 μL , 400 $\mu\text{g mL}^{-1}$ + allergen). To assess the effect of silver NPs without allergens, 5 or 100 nm silver NPs were used without allergens.

Measurement of Clinical Skin Score and Mast Cell Numbers, Serum IgE Levels, and Detection of Silver NPs in Skin: The clinical skin score is the sum of individual scores graded as 0 (absence), 1 (mild), 2 (moderate), and 3 (severe) for each of four signs and symptoms (erythema/hemorrhage, scaling/dryness, edema, excoriation/erosion). The scoring was performed as a blind test and mean values were calculated from the results of two independent examinations. For immunohistochemistry, atopic dermatitis-like skin lesions were selected and 2 mm punch biopsies were performed. Skin samples were fixed with 10% neutral formalin, embedded in paraffin, and sectioned at a depth of 4 μm . Sera were obtained by submandibular collection, separated by centrifugation at 13 000 rpm for 5 min, and stored at -80°C until required for determination of serum IgE levels. The serum IgE levels were investigated using a mouse IgE enzyme-linked immunosorbent assay (ELISA) kit (432402; BioLegend, San Diego, CA, USA). The microplates (Nunc) were coated with buffer, and IgE antibody capture was allowed to proceed overnight at 4°C . After blocking with 10% FBS in phosphate-buffered saline (PBS) for 1 h at room temperature, serially diluted serum samples (1:100 to 1:1000) were added and incubated for 2 h at room temperature. The plates were then washed with PBS plus Tween 20 (0.05%) and incubated for 2 h with horseradish peroxidase-conjugated rat antimouse IgE antibodies. After washing with PBS plus Tween 20 (0.05%), the reaction color was developed with 3,3',5,5'-tetramethylbenzidine and stopped with H_2SO_4 (1 N). Absorbance was read at 450 nm and IgE levels were determined by comparing O.D. values with those of standard IgE. Tryptase staining was performed to count mast cells in skin regions. Briefly, tryptase antigen was retrieved in a high-pressure cooker using citrate buffer (pH 6.0). Endogenous peroxidase

activity was blocked by H_2O_2 for 10 min. As primary antibody, rabbit antimouse rat and human mast cell tryptase (sc-32889; Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibody was applied at a ratio of 1:100, and incubated at room temperature for 1 h and at 4°C overnight. The secondary antibody, peroxidase-labeled goat antirabbit antibody (474-1516, KPL, Gaithersburg, MA, USA), was then applied at a ratio of 1:200, and incubated for 30 min at room temperature; 3-amino-9-ethylcarbazole (Abcam, Cambridge, UK) was then applied as chromogen. Counter staining was carried out using hematoxylin. The positively stained mast cells in the dermis were counted at four areas (each 50 000 μm^2) per skin sample, under a microscope with twin CCD cameras (Olympus, Tokyo, Japan) at 400 $\times$ magnification. Microscopic images of silver NPs were obtained using an upright optical microscope (DM5000B, Leica) equipped with a dark-field condenser (D 0.80–0.95, Leica). The hollow cone light from the dark-field condenser was focused into the sample. Only scattered light from the sample entered the objective lens (HCX PL FLUOTAR 20 $\times$, N.A. 0.5, Leica) and was then directed to the digital microscope camera with a 5 megapixel CCD sensor (DFC425C Leica).

Statistical Analysis: Data are expressed as the mean $\pm$ SD. The statistical comparisons were performed by Student's *t*-test for two groups and analysis of variance (ANOVA) for more than two groups. Statistical analyses were performed using GraphPad Prism 5 software. $P < 0.05$ was considered statistically significant.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Supporting Information

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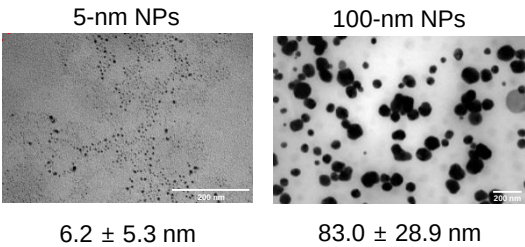
5 nm Silver Nanoparticles Amplify Clinical Features of Atopic Dermatitis in Mice by Activating Mast Cells

*HanGoo Kang, Seungjae Kim, Kwang Hoon Lee, Shan Jin, Seo Hyeong Kim, Kangtaek Lee, Hyungjoon Jeon, Young-Geon Song, Sang-Won Lee, Jinwon Seo, Soomin Park, and In-Hong Choi**

Supporting Information

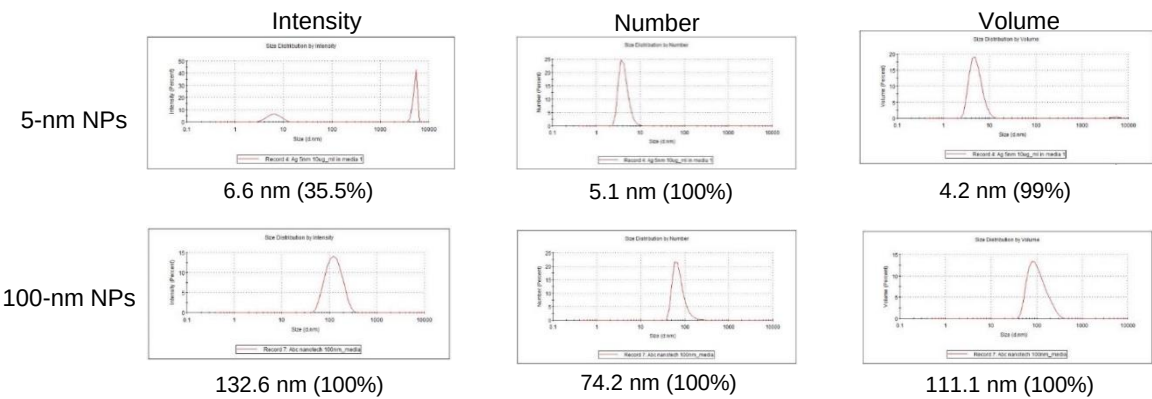
Figure S1

A. TEM image



B. DLS analysis

- A representative data of NPs in 10%-FBS MEM



- Size

Medium		Mean ± SD (nm)		
		Intensity	Number	Volume
5 nm	10% FBS-MEM	10.4 ± 1.6	5.1 ± 0.4	6.9 ± 1.0
	10% FBS-HBSS	17.2 ± 7.4	5.3 ± 0.2	7.0 ± 0.1
100 nm	10% FBS-MEM	106.0 ± 9.7	57.7 ± 6.3	81.7 ± 10.5
	10% FBS-HBSS	114.0 ± 18.1	64.0 ± 17.0	91.0 ± 23.0

- Percentage of NPs in each denoted size

Medium		Mean ± SD (%)		
		Intensity	Number	Volume
5 nm	10% FBS-MEM	46.9 ± 6.3	100.0 ± 0.0	99.8 ± 0.0
	10% FBS-HBSS	82.2 ± 25.2	100.0 ± 0.0	99.9 ± 0.1
100 nm	10% FBS-MEM	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
	10% FBS-HBSS	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0

Supporting Information
Figure S1

C. Cytotoxicity of silver NPs according to surface area

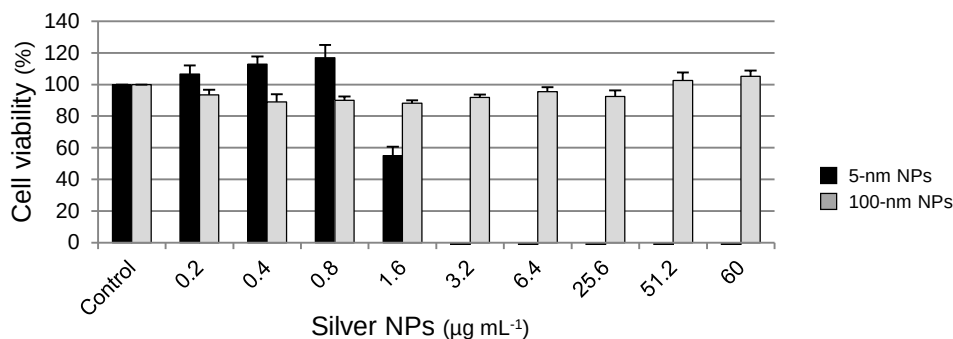


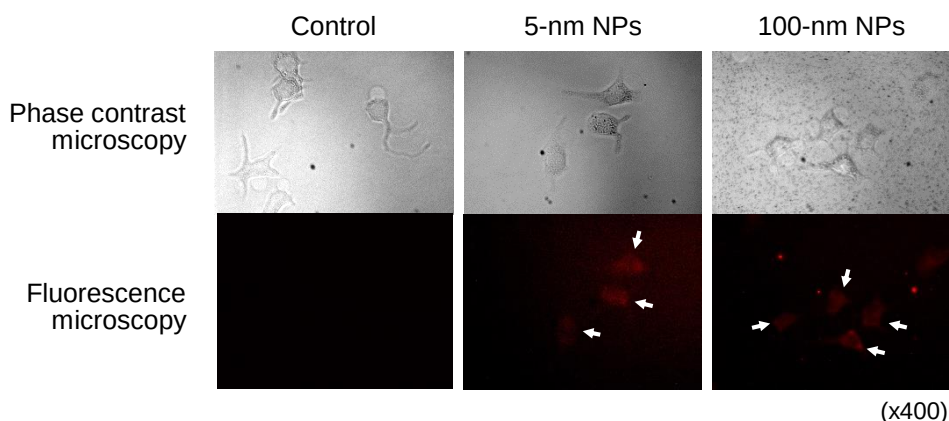
Figure S1. Characterization of silver NPs.

(A) TEM images of silver NPs show that the 5-nm and 100-nm NPs were relatively uniform in size. (B) A representative result of DLS analysis shows that the mean sizes were 5.1 nm for 5-nm silver NPs and 74.2 nm for 100-nm silver NPs by number. Silver NPs were dispersed in 10%-FBS MEM (n=10) or in 10%-FBS HBSS (n=3). The mean + SD of size or percentage in the denoted size are shown as tables (C) RBL-2H3 mast cells were treated with 5-nm or 100-nm silver NPs for 24 h and cytotoxicity was determined by CCK-8 assay.

Supporting Information

Figure S2

A. Fluorescence microscopic analysis of rhodamine-conjugated NPs in RBL-2H3 mast cells



B. Dark-field microscopic analysis of NPs without rhodamine

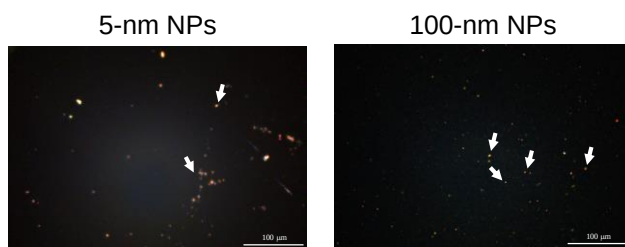
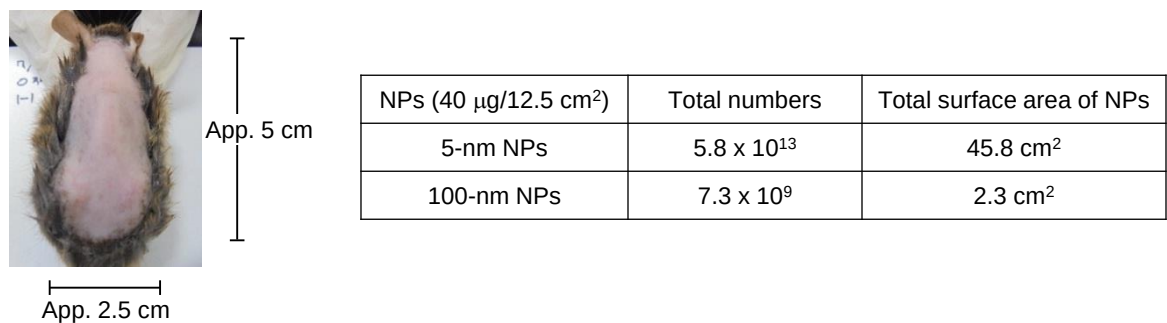


Figure S2. Imaging of silver NPs.

(A) RBL-2H3 mast cells were treated with $50 \mu\text{g mL}^{-1}$ of silver NPs for 1 h. Mast cells treated with 100-nm NPs or 5-nm NPs appeared as red, which suggest NPs were internalized. (C) When NPs without rhodamine were observed by dark-field microscopy, orange spots were detected, which suggest silver NPs. Individual 100-nm NPs were visible easily, but only aggregated forms of 5-nm NPs, not individual NP, were visible.

Supporting Information
Figure S3

A. Skin area for silver NPs administration



B. Induction of atopic dermatitis and sample schedule

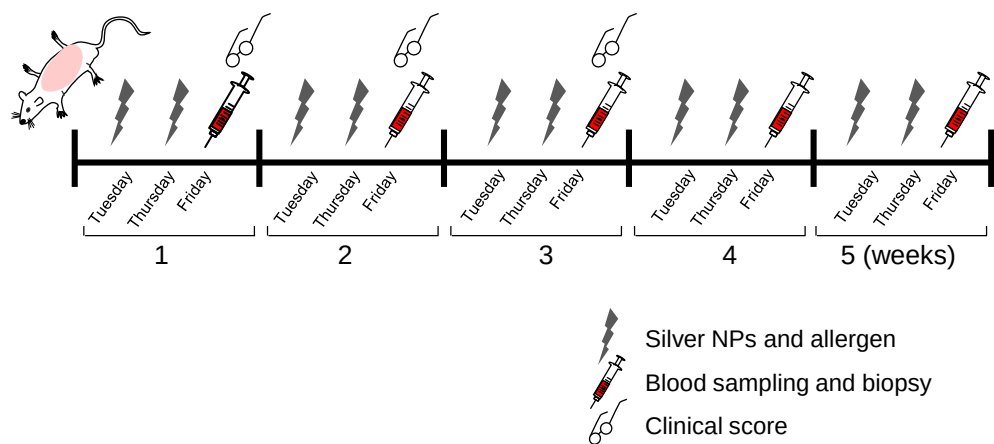


Figure S3. Induction of atopic dermatitis in mice.

(A) Skin to be treated with allergen and silver NPs. (B) Schedule for induction of atopic dermatitis and sampling of blood, measuring clinical scores and skin biopsy. To induce atopic dermatitis in NC/Nga mice, the hair on the back was shaved one day before the experiment. Next day, sodium dodecyl sulfate (150 µL, 4% w/v) was applied to the shaved dorsal skin for barrier disruption. After 2 h, 5-nm or 100-nm silver NPs (100 µL, 400 µg mL⁻¹) was applied to the same region and left for 2 h. Then, as allergen, Biostir AD ointment (50 mg) was applied. The skin treatment was performed twice per week for 5 weeks.

Supporting Information
Figure S4

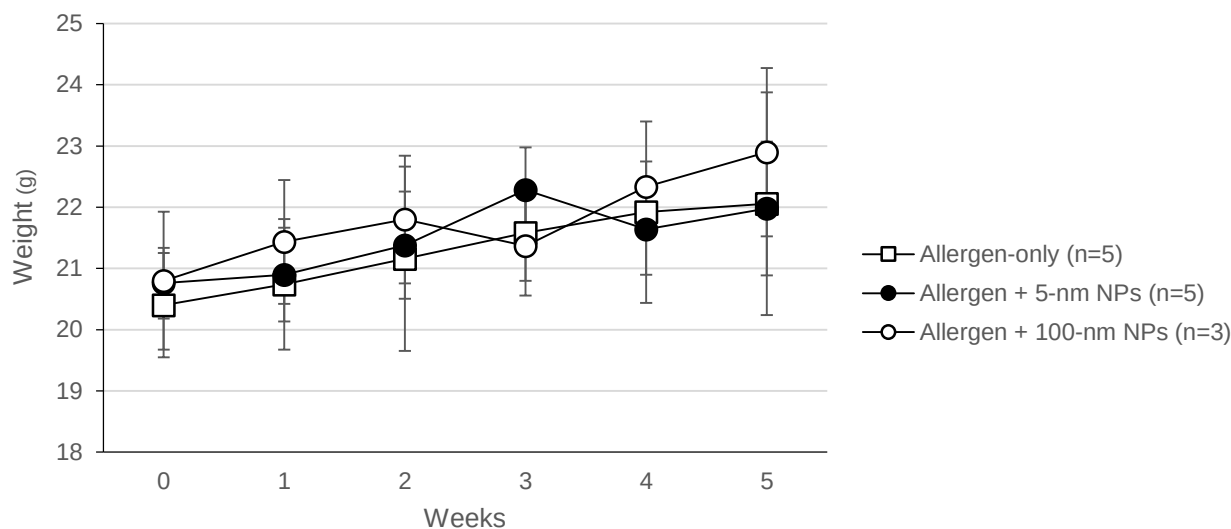


Figure S4. Body weight of mice.

Body weight was measured up to 5 weeks during atopic dermatitis induction, and showed no differences between the allergen-treated group and the groups co-treated with 5-nm or 100-nm silver NPs. The statistical analysis was performed by two-way analysis of variance (ANOVA). Data represent the mean $\pm$ SD.

Supporting Information

Figure S5

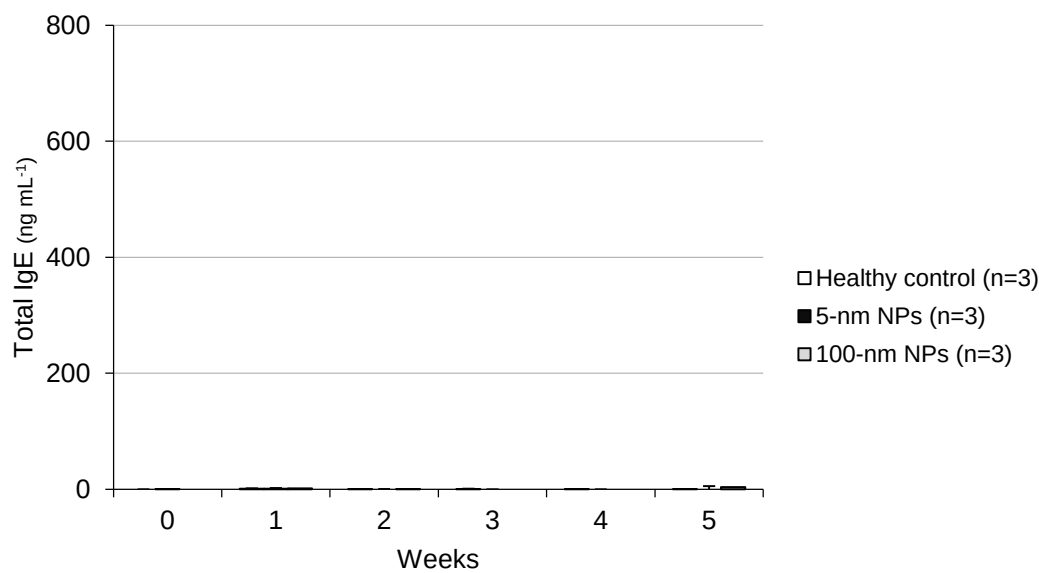


Figure S5. Serum IgE levels in mice treated with silver NPs without allergens.

The levels of IgE in the sera were determined using a mouse IgE-specific ELISA kit. Samples were diluted at 1:100 and 1:1,000. Data represent the mean ± SD.

Supporting Information

Table S1

Week	Total IgE (ng mL ⁻¹ , mean ± SD)		
	Allergen-only	Allergen + 5-nm NPs	Allergen + 100-nm NPs
0	0 ± 0	1 ± 2	0 ± 0
1	0 ± 0	1 ± 1	0 ± 0
2	69 ± 40	231 ± 285	75 ± 58
3	121 ± 110 [*]	456 ± 461	50 ± 25 [*]
4	105 ± 72	392 ± 365	73 ± 45
5	215 ± 191 [*]	589 ± 467	80 ± 31 ^{**}

Table S1. Numerical levels of serum IgE levels in atopic dermatitis mice.

The levels of IgE in the sera were determined using a mouse IgE-specific ELISA kit. Samples were diluted at 1:100 and 1:1,000. Data represent the mean ± SD. The statistical analysis was performed by two-way analysis of variance (ANOVA) with Bonferroni post-tests comparing the group with allergen + 5-nm NPs versus the other groups. **P* < 0.05, ***P* < 0.005.