Electronic Supplementary Information

Size Macrophage Responses and Toxicological Effects of Ag Nanoparticles

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Method

Synthesis and characterization of nano-Ag. 4 and 20 nm nano-Ag were prepared by using the procedures described below. 3 g of polyvinylpyrrolidone (PVP) and 0.5 g and D-glucose were added to 20 mL of pure water. The mixture was heated to 98°C with stirring. Meanwhile, 170 mg of $Ag(NO_3)_3$ was rapidly added and reacted for 5 min. The mixture was cooled in ice rapidly. The nanoparticles were separated by adding acetone to the mixture solution followed by centrifugation. The nanoparticles were dispersed in deionized water thoroughly. By using 100,000 Da cut-off filters, 4 nm nano-Ag and 20 nm nano-Ag were separated. 70 nm PVP-coated nano-Ag were prepared in a manner similar to that used for 4 nm nano-Ag and 20 nm nano-Ag. A mixture solution of PVP and D-glucose were reacted for 3 h after $Ag(NO_3)_3$ addition and the nano-Ag were dispersed in pure water. Stock concentrations were made at 0.5–1.0 mg/mL for 4 nm nano-Ag, 20–22 mg/mL for 20 nm nano-Ag, and 5–6 mg/mL for 70 nm particles. All nano-Ag were stably dispersed for two weeks by storing at 4°C. Characteristics and size profiles of the synthesized nano-Ag were analyzed by using dynamic light scattering (DLS, Zetasizer Nano,

Malvern Instruments, UK) and transmission electron microscope (TEM, JEM-2100, JEOL, Japan).

Cell culture. A human monocytic cell line (U937) was purchased from ATCC (Manassas, VA, U.S.A.) and cultured in RPMI-1640 medium with 10 % fetal bovine serum, streptomycin (1 mg/ml) and penicillin (1,000 units/ml) at 37 °C in a moisturized 5 % CO_2 incubator. The media were replaced with fresh culture media every 2-3 d to maintain cell densities of *ca.* 2 x 10^6 cells/mL. During each experiment the endotoxin neutralizer polymyxin B (InvivoGen, San Diego, CA) was added to every nano-Ag containing medium at the concentration of 10 ng/mL.

Cytotoxicity assay. Cell viability was tested by using a colorimetric cell counting kit 8 (CCK8) (Dojindo Laboratories, Kyoto, Japan). CCK8 is based on the colorimetric assays with the highly water soluble tetrazolium salt, WST-8[2-(2-methyxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-^{2}H-tetrazolium, monosodium salt]. Cells were plated in 24-well plates at densities of 2 x 10^5 cells in 200 μL growth medium per well. The wells were treated with 200 μL of nano-Ag solutions in growth medium. After 24 h, 15 μL of CCK8 was added to each well followed by incubation at 37°C for 2 h. Each culture medium was centrifuged and the 100 μL of supernatant was transferred to 96-well microtiter plates. Optical densities (O.D.) were measured at 490 nm to avoid the optical interference by nano-Ag.

The mechanism study of nano-Ag induced cell death by staining with annexin V-fluorescein (FITC) and propidium iodide. U937 cells were plated in 12-well plates at a concentration of 5 x 10^5 cells per well in 1 mL of culture media and rested for overnight. Next, 250 μL nano-Ag containing medium were added and the resulting solutions were incubated for 15 h. Cells were harvested and washed once with PBS. ApoTarget (Biosource, Camarillo, CA) was used to detect apoptotic cells by using flow cytometry. After washing with PBS, cells were suspended in 200 μL of Annexin-V binding buffer and 5 μL of Annexin-V-FITC and 10 μL of propidium iodide buffer were added. The cells were then incubated for 30 min in the dark and washed. From each sample, a total of 10,000 cells were analyzed by using the FACSCalibur flow cytometer and CELL-Quest software (Becton-Dickinson, Franklin Lakes, NJ).

Screening of cytokines using cytokine array. U937 cells were plated in six-well plates at the density of 2 x 10^6 cells per well in 2 mL of RPMI-1640 containing 5% FBS. Nano-Ag were added to each well and incubated for 24 h. The cell culture supernatants were collected by

centrifugation. Proteome Profiler^{TM} antibody arrays (R&D Systems Inc., Minneapolis, MN) were used to screen for cytokines.

Enzyme linked immunosorbent assay (ELISA) of interleukin (IL)-8. U937 cells were plated in 24-well plates at the density of 2.5 x 10^5 cells per well in 200 μL of RPMI-1640 containing 5% FBS. Nano-Ag in media were added to each well, making the final volume of 400 μL per well. After 18 h, the cell culture supernatants were collected and stored at -80°C and ELISA was performed with a human cytokine IL-8 assay kit (BD Biosciences, San Jose, CA).

Detection of intercellular ROS and treatment of N-acetyl-cysteine (NAC). Cells were plated in 12-well plates at initial density of 5 x 10^5 cells per well in 1 mL of culture medium and allowed to stand overnight. Then, 250 μL of nano-Ag containing medium was added. After 1 h, chloromethyl-2`,7`-dichlorodihydrofluorescein diacetate acetyl ester (CM-H_2DCFDA) (Invitrogen, Carlsbad, CA) was added. Cells were incubated at 37°C in the dark for 30 min. Cells incubated with 25 μM H_2O_2 served as positive control. A total of 10,000 cells were analyzed by using FACSCalibur flow cytometers and CELL-Quest software. The results were further analyzed by using WinMDI Ver 2.9 software (Joseph Trotter, Scripps Research Institute, San Diego, CA). As a ROS scavenger, 1 mM N-acetyl-cysteine (Sigma-Aldrich, St. Louis, MI) was added 30 min before adding nano-Ag. The toxicity of NAC to U937 cells was tested.

Statistical analysis. All experiments were performed at least three times. Data were expressed as the mean ± S.D. One-way analysis of variance (ANOVA) was used for the analysis between control and study groups, and differences with $p < 0.05$ were considered as significant.

Analysis of physical characteristics of nano-Ag

The sizes of synthesized nanoparticles were averaged by measuring over 200 nanoparticles in TEM images. They were 4 nm (σ = 9%), 20 nm (σ = 4%), 70 nm (σ = 10%), respectively (Fig. S1). Nano-Ag were coated with PVP and their hydrodynamic size and surface charge were measured by using DLS (Malvern, UK). Deionized water was used as the diluent for the DLS analysis. Results were consistent with those of TEM (Fig. S2). In addition, nano-Ag were well dispersed in deionized water and no noticeable aggregation was observed to take place in the cell culture medium during the culture process. All nano-Ag showed a negative surface charge of -10 mV (Table S1).

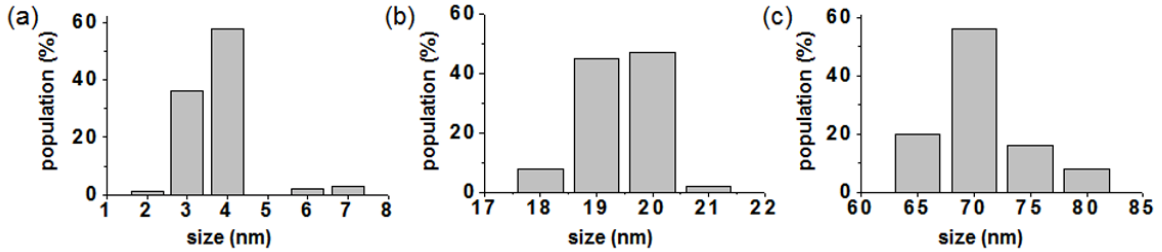

Fig. S1 The size distribution of Ag nanoparticles. (a) 4 nm, (b) 20 nm and (c) 70 nm nano-Ag.

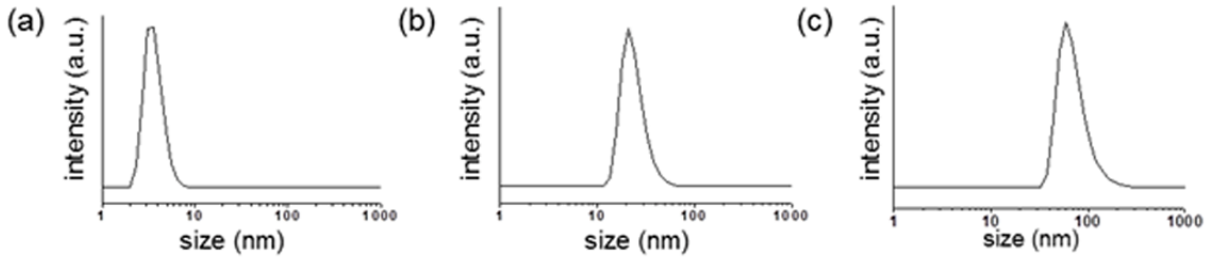

Fig. S2 The hydrodynamic size of nano-Ag. (a) 4 nm, (b) 20 nm and (c) 70 nm nano-Ag.

Table S1. Surface charge of nano-Ag.

	4 nm	20 nm	70 nm
ζ-potential (mV)	-10	-10	-10

X-ray diffractometric (XRD) analysis was performed to confirm the presence of nano-Ag. As shown in supplementary Fig. S3, X-ray diffraction patterns matched with the literature values of Ag (JCPDS #: 04–0783). In the case of 4 nm nano-Ag, peak broadening was observed because of their small size and broadened (111) and (200) peaks were overlapped in the XRD data where (200) peak was shown as a shoulder.

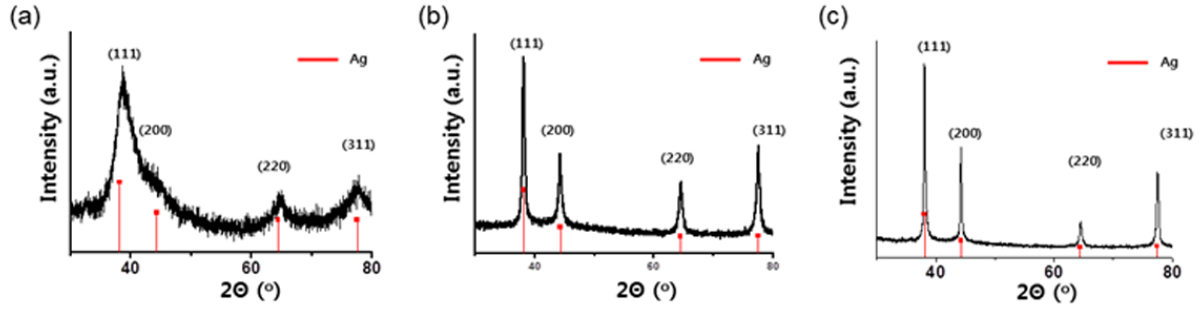

Fig. S3 X-ray diffraction of (a) 4 nm, (b) 20 nm and (c) 80 nm nano-Ag. All nanoparticles were highly crystalline and had face centered cubic structures (JCPDS card #: 04–0783).

The mechanism study of nano-Ag induced cell death by staining with annexin V-fluorescein (FITC) and propidium iodide.

Nano-Ag were treated on macrophage cells and their mechanism of cell death were studied by using annexin V-fluorescein and propidium iodide. The four different regions of graph from left bottom to right bottom, right top, and left top represent the population of healthy cells and cells in early apoptosis, late apoptosis, and necrosis, respectively. 4 nm nano-Ag showed toxicity from the concentration of 6 μg/ml and late apoptosis was observed. Similarly, 20 nm nano-Ag treated cell were under late apoptosis with the concentration of 30 μg/ml. However, they showed toxic effect at higher concentration compared to 4 nm particles.

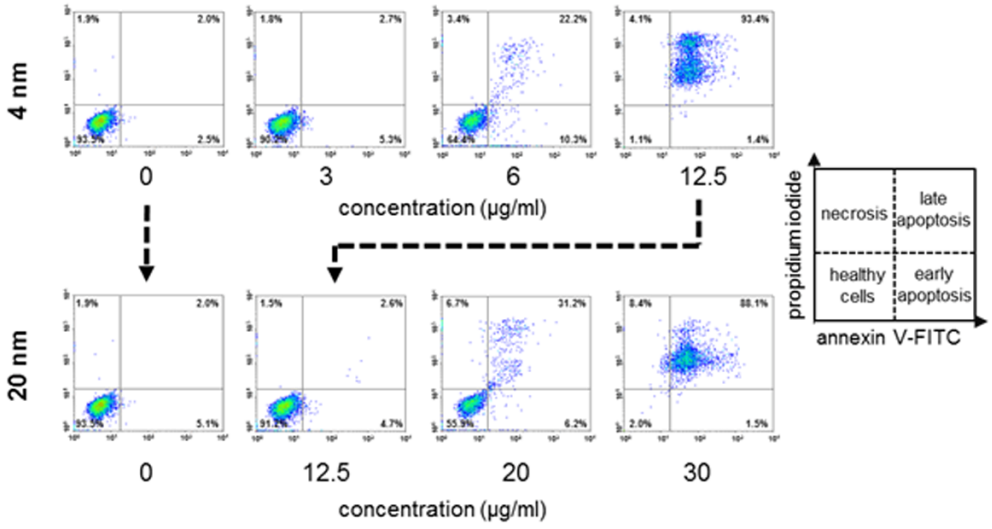

Fig. S4 The mechanism study of cell death under nano-Ag treatment. U937 cells were treated with nano-Ag for 15 h and stained with Annexin V-FITC and propidium iodide. Samples were

analyzed by using a FACSCalibur flow cytometer and CELL-Quest software.

Screening of cytokines using cytokine array

Cytokines are inflammatory mediators produced during immunological response. An increase of cytokine release represents a stimulation of the immune cells. We performed the cytokine protein array to assess the production of major group chemokines such as IL-8 and RANTES and pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α. As results, IL-8 was identified as the most noticeable among cytokines tested. 4 nm nano-Ag (12.5 μg/ml) stimulated the production of IL-8 more significantly when compared to those of 20 nm (50 μg/ml) or 70 nm (50 μg/ml) particles. The activation of other cytokines except IL-8 was not observed.

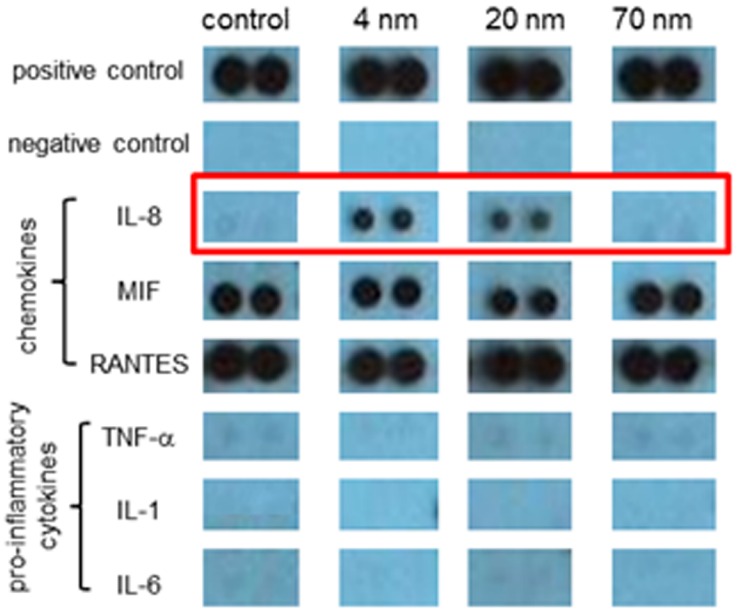

Fig. S5 Protein arrays were used to screen cytokines from U937 cells incubated with nano-Ag for 24 h. Among various cytokines, the activation of IL-8 by nano-Ag was clearly observed (red box).

Detection of intercellular ROS induced by nano-Ag

The ROS production was detected by a fluorogenic assay at 30 min after treatment of nano-Ag. ROS production in U937 cells was clearly observed when 12.5 μg/mL of 4 nm nano-Ag was applied and it reached a very high level at 25 μg/mL. In comparison, 20 nm nano-Ag (25 μg/mL)

promoted production of smaller amounts of ROS. 70 nm nano-Ag did not produce ROS even at 50 μg/ml.

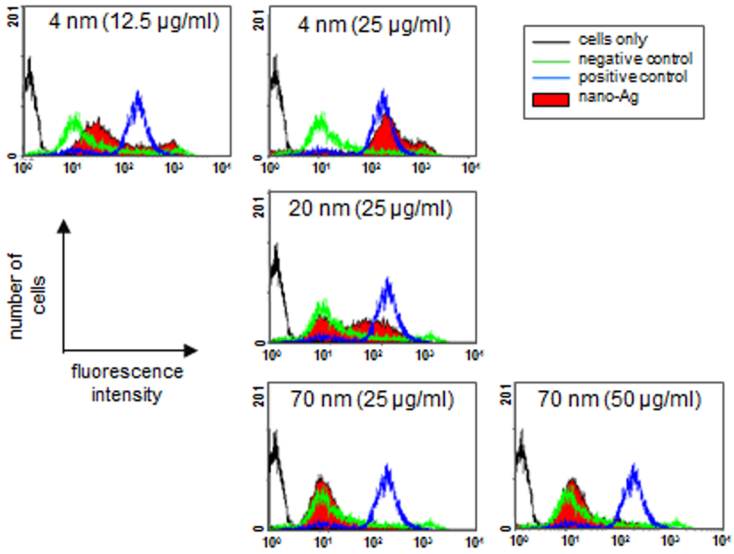

Fig. S6 Nano-Ag induced ROS production of immune cells. The fluorescence intensity of the ROS indicator, CM-H2DCFDA (5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester) was monitored following treatment of U937 cells with nano-Ag treatment. Higher fluorescence intensities indicate higher ROS levels. Negative control is cells only that were stained with CM-H2DCFDA and positive control is cells that treated with H_2O_2.