Determining the Cytotoxicity of Rare Earth Element Nanoparticles in Macrophages and the Involvement of Membrane Damage

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S Supporting Information

ABSTRACT: Rare earth element nanomaterials (REE NPs) hold considerable promise, with high availability and potential applications as superconductors, imaging agents, glass additives, fertilizers additives and feed additives. These results in potential REE NP exposure to humans and the environment through different routes and adverse effects induced by biological application of these materials are becoming an increasing concern. This study investigates the cytotoxicity of REE NPs: nLa_2O_3 , nEu_2O_3 , nDy_2O_3 and nYb_2O_3 from 2.5 to 80 $\mu\text{g/mL}$, in macrophages. A significant difference was observed in the extent of cytotoxicity induced in macrophages by differential REE NPs. The high-atomic number materials (i.e., nYb_2O_3) tending to be no toxic whereas low-atomic number materials (nLa_2O_3 and nEu_2O_3 and nDy_2O_3) induced 75.1%, 53.6% and 20.7% dead cells. With nLa_2O_3 as the representative material, we demonstrated that nLa_2O_3 induced cellular membrane permeabilization, through the sequestration of phosphates from membrane. The further mechanistic investigation established that membrane damage induced intracellular calcium increased to 3.0- to 7.3-fold compared to control cells. This caused the sustained overload of mitochondrial calcium by approximately 2.4-fold, which regulated cell necrosis. In addition, the injury of cellular membrane led to the release of cathepsins into cytosol which also contributed to cell death. This detailed investigation of signaling pathways driving REE NP-induced toxicity to macrophages is essential for better understanding of their potential health risks to humans and the environment.

INTRODUCTION

Owing to their unique physical-chemical properties, rare earth elements (REEs) have been extensively applied as superconductors, imaging agents, glass additives, fertilizers additives and feed additives, etc.^{1–3} For instance, addition of Eu_2O_3 to ZnO films could improve the optical and structural properties and intense luminescence.⁴ Yb_2O_3 has been reported as a novel long circulation material as an in vivo CT imaging agent.⁵ Yb_2O_3 could be applied for novel additive material for concentrator solar cells, as a the solar energy converter.⁶ Dy_2O_3 nanoparticles mixed gadolinium–dysprosium oxide nanoparticles showed promising characteristics as a MRI contrast agent for imaging.⁷ It has been reported that fertilizers contain approximate 30% of La_2O_3 . Commercial feed additives for poultry containing 50% REEs and La_2O_3 accounts for 20% of REE oxides.⁸

With the increase in application of REEs worldwide, thousands of tons of REEs enriched fertilizers released into

the agricultural soil, resulting in the large scale of contamination of REEs in soil and water near the mining sites REEs.^{3,10–14} For instance, because of the large REE reserves, China is the largest worldwide producer and supplier of REEs.⁹ It has been evaluated in 2002 that REE containing fertilizers discharged into the cultivated soil are above 5000 tons in China.³ Therefore, application of REEs has enhanced the large scale contamination of REEs, displaying high accumulative in contaminated environmental compartments, and been considered persistence in environment and bioaccumulation in biota.¹⁵ REEs containing food products, industrial products or medicinal agents could be intake through oral, intravenous injection and skin exposure.¹⁶ The exposed REEs could

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accumulate in blood, brain and bone after introducing to human body,¹⁷ leading to a great concern for human health.^{18–21} The inhalation of air-borne particles of REEs oxides is the most typical accidental exposure route.²² Because of the chronic ingestion of REEs, the population living in high-REE-background showed abnormality in activities of some digestive enzymes.²³ As various nanomaterials containing REEs have been developed as probes for imaging,^{24–28} these direct administrations of these REEs containing nanomaterials, therefore, may also result in a potential risk to human health. It has been reported fibrosis and renal disorders in patients when Gd elements (9–25 mmol) used as an imaging agent.^{18,29–31} At the nanoscale, REE nanoparticles (REE NPs) present remarkably different biological activities in comparison to their larger-scale counterparts, with the higher surface area significantly altering cellular uptake and biodistribution in vivo, resulting in altered biological effects.³¹ Therefore, improved understanding of the impacts on biological systems induced by these materials is highly beneficial for the prevention of side effects in biomedical applications.³²

Macrophages are considered a vital sentinel cell, forming a defensive line to combat invading pathogens and they also take a prominent role in the clearance of nanomaterials from circulatory systems and local organs.²⁶ Because of the instinct role of macrophage, the high accumulation level of environmental particles in these cells and the resulted negative effects are considered the dominant risk upon particle exposure. Disordered macrophages not only lose their defense activity, but also have been found to trigger various pathologies such as autoimmune diseases,³³ inflammation and pneumonitis.³⁴ For instance, atmospheric particles could be cleared by macrophages located in lung and the accumulation of particle in macrophages leads to sustainable inflammation, and the resulted in lung diseases.^{35,36} The environmental exposure of atmospheric particle has been demonstrated to trigger lung cancer due to the inflammation initiated by lung macrophages.^{35,36} Exposure to REE NPs has been associated with nephrogenic systemic fibrosis in patients,¹⁸ because macrophage internalized REE NPs such as La_2O_3 and Eu_2O_3 cause the transfer of phosphates from lipids, leading to membrane damage and injury to lysosomal cell membranes, promoting activation of the multiprotein oligomer NLRP3 inflammasome.^{37–43} However, as a lack of complete understanding of the mechanisms underlying REE NP effect on macrophages, the impact of REE NPs on cell survival and viability is at present still unclear. Therefore, the investigation on the toxicity of REE NPs with macrophage as cell model is of help to evaluate their risks under the exposure REE NPs by various routes.

Physicochemical properties such as elemental composition, shape, size and surface groups largely dictate the toxicity profiles of nanomaterials.²⁸ Therefore, different REE NPs are therefore assumed to induce distinct biological effects. In the present study, we investigated the cytotoxicity of REE NPs on the survival of macrophages, establishing potential mechanisms responsible for the cytotoxicity induced by these materials. These findings show a safer pathway for the design of REE-containing nanomaterials, for application in biomedical systems.

MATERIALS AND METHODS

Chemicals and Reagents. Dulbecco's Modified Eagle's Medium (DMEM), penicillin/streptomycin and L-glutamine were all purchased from Invitrogen (Carlsbad, USA). Fetal

bovine serum (FBS) was obtained from Atlanta Biologicals Inc. (Lawrenceville, USA). All horseradish peroxidase-conjugated secondary antibodies were obtained from GE Healthcare Life Sciences (Pittsburgh, USA). The mammalian protein extraction reagent and ECL reagent were purchased from Thermo Fisher Scientific (Waltham, USA). The protein inhibitor cocktail, 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), rapamycin and tetramethylrhodamine methyl ester (TMRM) were purchased from Sigma-Aldrich (Shanghai, China). The Bradford reagents for protein determination were obtained from Bio-Rad (Shanghai, China). The following antibodies (Abs) were used: anti-Bid Ab from R&D Systems (Minneapolis, USA), anti-LC-3 Ab from Novagen (Medison, USA), whereas anti-GAPDH and anti-Caspase 3 Ab were purchased from Cell Signaling Technology (Shanghai, China). 4-(6-Acetoxy-methoxy-2,7-dichloro-3-oxo-9-xanthonyl)-4'-methyl-2,2'-(ethylenedioxy)dianiline-*N,N,N',N'*-tetraacetic acid tetrakis (acetoxy-methyl) ester (Fluo-3/AM), 9-[4-[bis[2-[(acetyloxy)-methoxy]-2-oxoethyl]amino]-3-[2-[2-[bis[2-[(acetyloxy)-methoxy]-2-oxoethyl]amino]-5-methylphenoxy]ethoxy]-phenyl]-3,6-bis(dimethylamino)xanthylium bromide (Rhod-2), E-64d, Pepstatin A, CA-074 methyl ester (CA-074Me), Cyclosporin A (CsA), Cytochalasin D, ethylenediaminetetraacetic acid (EDTA) and Z-VAD-fmk were purchased from MedChem Express (Monmouth Junction, USA).

Nanomaterials and Characterization. All REE NPs including La_2O_3 NP (nLa_2O_3), nEu_2O_3 , nDy_2O_3 and nYb_2O_3 (sources of REE NPs are shown in Table S1) were prepared in endotoxin-free water forming stock solutions of 5 mg/mL. Before characterization or exposure, REE NPs were sonicated at 32 W for 30 s. High-resolution transmission electron microscopy (HRTEM) samples were prepared by depositing of REE NP suspension (20 $\mu\text{g}/\text{mL}$) onto a carbon-coated copper electron microscopy grid (Beijing Zhongjingkeyi Technology Co., Ltd., China) and dried under ambient conditions overnight before analysis using a HRTEM (JEOL-2100F, Japan). The ζ -potential and hydrodynamic diameters of REE NPs in DMEM medium were determined using a Zetasizer (Malvern Nano series, Malvern, U.K.). Fourier transform infrared spectroscopy (FT-IR) was performed using a Nicolet 6700 FT-IR spectrophotometer (Thermo Fisher Scientific Inc., USA), according to the KBr pellet method. X-ray photoelectron spectroscopy (XPS) determination of REE NPs was performed using sample deposits on silicon wafers, where samples were air-dried prior to analysis.

Cell Culture, Cellular Treatment and Toxicity Assays. J774A.1 cells and Hep1–6 cells were purchased from the Shanghai Cell Bank of Type Culture Collection of China. Following treatment at 2.5, 10, 20, 40 and 80 $\mu\text{g}/\text{mL}$, cells were stained using 50 $\mu\text{g}/\text{mL}$ propidium iodide (PI) in phosphate-buffered saline (PBS), with dead cells analyzed by flow cytometric analysis.^{38,44–46} The CellTox Green Cytotoxicity assay was used to determine cytotoxicity according to manufacturer instructions (Promega, Shanghai, China).

J774A.1 cells were preincubated with 1 μM cytochalasin D, 50 μM EDTA or 2 nM rapamycin for 2 h, respectively, followed by exposure to 20 $\mu\text{g}/\text{mL}$ nLa_2O_3 with or without cytochalasin D, EDTA or rapamycin. Cell death was then evaluated using PI staining after 24 h.

RAW-Difluo mLC3 cells (invivogen) were cultured in DMEM containing 10% (v/v) FBS, 50 U/mL penicillin, 50 mg/mL streptomycin, 100 mg/mL Normocin and 2 mM L-glutamine. Before the exposure experiment, the culture medium

was changed with DMEM containing heat-inactivated FBS (30 min at 56 °C), 50 U/mL penicillin, 50 mg/mL streptomycin, 2 mM L-glutamine and 4.5 g/L glucose, 10% (v/v). After 24 h, RAW-Difluo mLC3 cells were treated with nLa₂O₃ for 6 h followed by the observation with confocal microscopy.

Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) Analysis for Cellular Uptake of Nanomaterials. Cells were cultured in 10 cm plates and treated with 20 µg/mL REE NPs for 6 h. Cells were washed 3 times with PBS. Then we collected and digested the cells by combining with RIPA buffer containing 1% protease cocktail (Sigma-Aldrich) on ice for 30 min. After centrifugation at 15,000g for 30 min at 4 °C, the protein concentrations of the supernatant of cellular lysates were determined using the Bradford method. The suspension of cell lysates post centrifugation ICP-MS or the pellets of cellular lysates were digested for ICP-MS determination. In brief, 200 µL of supernatants or pellets was digested with 200 µL of nitric acid-H₂O₂ (3:1) at 75 °C for 12 h, respectively. The digested solutions were diluted to 10 mL with the acid concentration below 3% (v/v) and subjected to ICP-MS analysis to determine the quantity of particulate REEs (La, Eu, Dy and Yb). All analyses were performed in triplicates using the external calibration method. The standard mixture solution for REE determination was purchased from Guobiao (Beijing) Testing & Certification Co., Ltd. (Beijing, China). The calibration curve is shown in Figure S1 ([Supporting Information](#)). Linear regressions showed that *R* was above 0.99 (Figure S1). The signal intensity repeatability (RSD) of standard was 9.4%. Each group includes at least 3 parallel samples, and the average value was calculated. The sum of the dissolved REE and particulate REE content is defined as the total intracellular REE concentration. The intracellular total REEs concentration was expressed as REE content (ng) versus intracellular protein level (mg).

Transformation of REE NPs in Phagolysosomal Simulant Fluid (PSF). Nanoparticles were dissolved in 1 mL of PSF solution (142 mg/L Na₂HPO₄, 6.65 g/L NaCl, 62 mg/L Na₂SO₄, 29 mg/L CaCl₂·H₂O, 250 mg/L glycine, 8.09 g/L potassium phthalate, pH 4.5) at a concentration of 50 µg/mL and sonicated at 32 W for 30 s. Samples were incubated at 37 °C for either 0.25, 0.5, 1, 3 or 6 h, prior to centrifugation at 15,000g for 30 min. Pellets were analyzed by inductively coupled plasma-optical emission spectrometry (ICP-OES) to assess the concentrations of phosphorus and REE ions, whereas suspensions were digested and REE ion concentrations were determined by ICP-MS.³⁸

Assessment of Lysosomal Membrane Permeabilization (LMP). Regarding concentration selection, for the investigation of the cytotoxicity of nLa₂O₃ as the representative material, 20 µg/mL was frequently selected because the concentration higher than 20 µg/mL (40 or 80 µg/mL) possess dramatic toxicity and could induced cell detached from the tissue culture dish. As a result, some experiments such as confocal fluorescence observation could not performed under the exposure concentration higher than 20 µg/mL. After exposure to 20 µg/mL nLa₂O₃ in DMEM for 6 h, J774A.1 cells were washed with PBS and then stained with Magic Red Cathepsin B (ImmunoChemistry Technologies, Bloomington, USA) for 2 h according to manufacturers' instructions. The plasma membrane was stained using a membrane labeling kit (Sigma-Aldrich, Shanghai, China) and nuclei were stained with Hoechst 33342 (Thermo Fisher Scientific, Waltham, USA), at room temperature for 1 h. Finally, cells were visualized using a

TCS SP5 laser scanning confocal microscope (Leica, Germany).

Calcium Flux Analysis and Mitochondrial Potential Assessment. Alterations to concentrations of intracellular Ca²⁺ and mitochondrial Ca²⁺ were monitored using Fluo-3/AM and Rhod-2 fluorescence probes, respectively. Briefly, after treatment nLa₂O₃ at 20 µg/mL 1 × 10⁵ cells were incubated at 37 °C in the dark with 4 µM Fluo-3/AM and 4 µM Rhod-2 for 20 min. Cells were then washed 3 times and resuspended in Hank's Balanced Salt Solution without Ca²⁺ and Mg²⁺. Cells treated with REE NPs were additionally stained with 20 nM TMRM, then analyzed by flow cytometry (FACS) for mitochondrial membrane assessment. FACS analysis was performed using an LSR II flow cytometer (Becton Dickinson, Mountain View, USA) equipped with a single 488 nm argon laser. Fluo-3/AM fluorescence was assessed using the fluorescein isothiocyanate (FITC) channel, whereas PI, TMRM and Rhod-2 fluorescence were examined using the PE channel; both forward and side scatters were used to eliminate cellular fragments. Following treatment, a portion of cells were also fixed with 10% PBS-buffered formaldehyde with 0.1% Triton X-100 for 10 min, then stained with 4',6-diamidino-2-phenylindole (DAPI) (blue) and Fluo-3/AM (green) or Rhod-2 (red). Fluorescent images were visualized through confocal laser scanning microscopy as described previously.⁴⁰

TEM Observation on Cell Samples. After treatment with REE NPs at 20 µg/mL for 6 h, cells were harvested and fixed in 2.5% glutaraldehyde for 2 h, then washed with PBS buffer. Staining was performed using 1% OsO₄ for 1 h, prior to dehydration of fixed and stained cells using alcohol (40, 50, 70, 80, 90, 95 or 100% ethanol). Cells were then submerged in propylene oxide for two 30 min cycled and were then embedded in beam capsules containing pure resin. Blocks were hardened at 70 °C for a 2-day period, then 70 nm sections were cut and stained with 1% lead citrate and 0.5% uranyl acetate, prior to examination using a JEOL JEM 2010F high-resolution transmission electron microscope (Hitachi Scientific Instruments, Japan).

Western Blot Analysis. The supernatant of cellular lysates was (20 µg) subjected to both sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting, according to established protocols, as previously described.⁴⁰ Western blotting signals were detected using a BIO-RAD ChemiDoc XRS chemiluminescence system (Bio-Rad Inc., USA).

Statistical Analysis. One-way analysis of variance (ANOVA) was utilized for the analysis of mean differences between two or more treated groups and the untreated control group, whereas the differences between the two groups were determined using the two-tailed Student's *t* test. Data are shown in mean ± standard deviation (SD), and *p* < 0.05 was considered statistically significant.

■ RESULT AND DISCUSSION

Characterization of REE NPs. To compare the cytotoxicity, REE NPs with varying elements were selected, including nLa₂O₃, nEu₂O₃, nDy₂O₃ and nYb₂O₃. The detailed physicochemical characterization of the selected REE NPs (Figure S2 and Table S2) showed that average primary REE NP diameters ranged from 21.3 to 58.8 nm. These materials were dissolved in DMEM for cellular treatment and the hydrodynamic diameters of REE NPs were also assayed in

Figure 1. (A) Representative flow cytometrical analysis of J774A.1 macrophages. Percentages of dead cells in J774A.1 cells. (B) Dynamic monitoring of the transformation process of REE NPs suspended in PSF at 40 μg/mL for 0.25, 0.5, 1, 3 or 6 h. Ratios of between phosphates concentration and REE levels. (C) Cell death was evaluated with PI staining 24 h after 20 μg/mL nLa₂O₃ treatment with or without PSF. *n* = 3 per group; * versus ctrl group, # versus nLa₂O₃ group, \$ versus nEu₂O₃, & versus nDy₂O₃ group. *, #, \$ or & represents *p* < 0.05.

DMEM, showing that most nanoparticles formed aggregates with hydrodynamic diameters of 299.1–544.9 nm (Table S2). XPS analysis confirmed the chemical properties of nLa₂O₃, nEu₂O₃, nDy₂O₃ and nYb₂O₃ (Figure S3), whereas REE NP surface group analysis was also performed using FT-IR spectra. As shown in Figure S4, the FT-IR spectra demonstrated that the selected REE NPs possessed similar surface characteristics, as characterized by the common peak positions observed for O–H groups (3430 cm⁻¹), C–H groups (2914 cm⁻¹), C=O groups (1730 cm⁻¹) and C–O groups (1105 cm⁻¹).^{47,48} The presence of these functional groups on the surface of REE NPs, confirmed the formation of hydroxides and carbonates due to the reaction of REE NPs with ambient water and/or carbon dioxide.⁴⁹

Sequestering of Phosphates from Cellular Biomolecules Involves the Cell Death. As shown in Figure S5, using PI as a cell membrane impermeable indicator,⁵⁰ we first screened the cytotoxicity of REE NPs on J774A.1 macrophages and Hep1–6 hepatocytes as representative cell models.³⁴ nLa₂O₃, nEu₂O₃ and nDy₂O₃ triggered dramatic high population of death cells of macrophages, whereas displayed no toxicity on hepatocytes. Previous studies have demonstrated that liver is a major deposition site for nanomaterials,³⁸ due to the high ability to remove toxin or particles from blood flow through the liver. Specialized macrophages such as Kupffer cells and hepatocytes are the two dominant cell types in the liver. The findings of the present study, show that nLa₂O₃, nEu₂O₃ and nDy₂O₃ showed cytotoxicity to J774A.1 macrophages but displays no effect on the survival of hepatocytes.

We therefore further focused our investigation on the cytotoxicity of REE NPs on macrophages. As shown in Figure 1A, there was a significant difference observed in the cytotoxicity caused by the four different REE NPs, with the most toxic material being nLa₂O₃, which induced 75.1% cell death at 80 μg/mL, as compared to the untreated control (*p* < 0.05). nEu₂O₃ and nDy₂O₃ displayed significantly less toxicity, inducing 53.5% and 20.7% cell death respectively, at the highest exposure dose (Figure 1B, *p* < 0.05). Conversely, nYb₂O₃ exerted only a negligible effect on cell survival (Figure 1A). To investigate these varying cytotoxic effects induced by different REE NPs, cell death was also assessed using the CellTox assay,⁴⁵ where dead cells were stained with a green fluorescent dye once the permeability of its cell membrane is sufficiently increased. As shown in Figure S6, a significant dose- and time-dependent increase in the intracellular fluorescence intensity of J774A.1 cell was observed (*p* < 0.05). After 12 h exposure, 80 μg/mL nLa₂O₃ induced up to a 4-fold increase in relative fluorescent intensity as compared to control cells, showing the highest level of cytotoxicity (Figure S6, *p* < 0.05). nEu₂O₃ and nDy₂O₃ induced an approximately 2-fold and 1-fold increase in intracellular fluorescence intensity, respectively (Figure S6, *p* < 0.01). Conversely, nYb₂O₃ did not induce any increase in intracellular fluorescence intensity, even at the highest concentration (Figure S6, *p* > 0.05). The findings of the present study, show that cytotoxicity is induced by toxic REE NPs including nLa₂O₃, nEu₂O₃ and nDy₂O₃ on J774A.1 macrophages, and significant variations in cytotoxicity can be seen among REE NPs containing different REEs.

Figure 2. Cellular uptake of REE NPs and their dissolution within cells. (A) Total intracellular RE levels in cells after treatment with REE NPs at 20 $\mu\text{g/mL}$ for 6 h. Cells were lysed and the total intracellular RE concentrations were determined by ICP-MS. (B) Concentrations of dissolved RE in cells. Cells were lysed and dissolved REE ions in the suspensions were measured using ICP-MS. (C) Rate of released dissolved REE ions by REE NPs after PSF incubation. (D) PI-positive cells for J774A.1 cells 24 h after nLa_2O_3 treatment at 20 $\mu\text{g/mL}$ with or without EDTA preincubation. (E) Cell death was determined with PI staining 24 h after nLa_2O_3 treatment at 20 $\mu\text{g/mL}$ with or without cytochalasin D. $n = 3$ per group; * versus ctrl group, # versus nLa_2O_3 group, \$ versus nEu_2O_3 , & versus nDy_2O_3 group. *, #, \$ or & represents $p < 0.05$.

The interaction of REE NPs with phosphates in plasma membrane are considered to be one of the causes for biological membranes damage.^{9,13} In order to investigate the ability to sequester cellular phosphates, the amount of phosphates adsorbed on the REE NPs was compared among different REE NPs. We incubated REE NPs with PSF to evaluate the ability to form REE NP-phosphates complex.⁴⁴ We then characterized the molar ratio of particulate associated P/REE (mol/mol) for each REE NPs. As shown in Figure 1B, nLa_2O_3 , nEu_2O_3 and nDy_2O_3 appeared to sequester phosphates in PSF, but presented with different P/REE dynamics upon exposure to PSF. nLa_2O_3 displayed the highest ability to sequester phosphates after 15 min, with the significantly highest P/REE ratios (mol/mol) of 1.07 and 1.26, at 15 min and 6 h, respectively. nEu_2O_3 presented a weaker affinity to react with phosphates, with the initial P/REE ratio of 0.44 at 15 min, increasing to 1.31 at 6 h. The P/REE ratio established for nDy_2O_3 was 0.2 at 15 min, elevating to 0.72 at 6 h, notably lower than that of nLa_2O_3 and nEu_2O_3 . The P/REE ratio of nYb_2O_3 was initially around 0.08 and remained constant for the 6 h exposure period. Therefore, the ranking of ability to form P-REE complexes is $\text{La} > \text{Eu} > \text{Dy} > \text{Yb}$, with nYb_2O_3 showing a very limited ability to sequester phosphates. The findings of these in vitro incubation experiments further confirm the ability of toxic REEs and phosphates to form complexes and the dephosphorylation of cell membrane may involve the membrane damage. To establish the possible correlation of the activity in P-REE complexes formation and cytotoxicity, we performed regression analysis (Figure S7A). Positive trends were observed for all the REE NPs ($R = 0.928$, $p < 0.05$). Therefore, it may be assumed that the ability in REE NPs to

form complex with phosphates decreased with higher REE atomic number, and the ability of REE NPs to complex phosphates decreased with higher REE atomic number.

To assess the role of REE NP-induced dephosphorylation on cell death, nLa_2O_3 was selected as representative REE NPs. For the investigation of the cytotoxicity of nLa_2O_3 as the representative material, 20 $\mu\text{g/mL}$ was selected because the concentration higher than 20 $\mu\text{g/mL}$ (40 or 80 $\mu\text{g/mL}$) possess dramatic toxicity which could induce cell detached from the tissue culture dish. Therefore, 20 $\mu\text{g/mL}$ nLa_2O_3 was incubated in 10 mM PSF (pH 7.4) at 37 $^\circ\text{C}$ for 24 h, resulting in cytotoxicity being reduced by 60%, as compared to treated cells without PSF preincubation (Figure 1C, $p < 0.05$). These results together suggest the sequestration of phosphates induces cell membrane damage cell death.

Cell Uptake of Toxic REE NPs Involves the Cell Death.

We then identified whether cellular uptake of REE NPs was responsible for the observed cytotoxicity. As shown in Figure 2A, ICP-MS results showed that intracellular particulate REE mass ranged from 45.1 to 52.3 $\mu\text{g/mg}$ protein, with no significant difference detectable in total REE mass among Dy, Eu, La and Yb materials ($p > 0.05$). To identify the effects induced by extracellular dissolved RE ions released by REE NPs, the mass of dissolved REE ions in DMEM medium were determined, showing approximately an average of 2.7% dissolution after 24 h incubation (Figure S8). This concentration of dissolved ions was then applied to toxicity assays to assess cell death. According to the dissolved ion concentrations, J774A.1 cells were treated with dissolved $\text{La}(\text{NO}_3)_3$, $\text{Eu}(\text{NO}_3)_3$, $\text{Dy}(\text{NO}_3)_3$ or $\text{Yb}(\text{NO}_3)_3$, containing dissolved La (0.44 $\mu\text{g/mL}$), Eu (0.55 $\mu\text{g/mL}$), Dy (0.56 $\mu\text{g/mL}$) or Yb (0.28 $\mu\text{g/mL}$)

Figure 3. REE NPs induced cell death through LMP. (A) Particle uptake in J774A.1 cells were assessed after 6 h treatment with 20 µg/mL nLa₂O₃ through TEM. Yellow arrows denote enlarged lysosomes. Red arrows denote transformed nLa₂O₃. (B) After incubation for 6 h, cells were stained with Magic Red to characterize Cathepsin B location through confocal microscopy. (C) Cell death was evaluated by PI staining after 20 µg/mL nLa₂O₃ treatment for 24 h with or without the preincubation of cathepsins inhibitors. *n* = 3 per group; * versus ctrl group. * represents *p* < 0.05.

ions in medium. Following 24 h of incubation, no cell death was observed in any REE NP exposure group (data not shown), allowing toxicity of extracellular REEs to be discounted as a cause of cytotoxicity.⁴⁵

It has been documented that intracellular metal ion release upon exposure to and accumulation of nanomaterials largely dictates the differing biological effects observed among and their extracellular ions.^{44,51} Therefore, the amount of dissolved REE ions were measured from supernatants from the digested total cell lysates after REE NP exposure, then analyzed using ICP-MS (Figure 2B). The relative abundance of the intracellular dissolved ions of nLa₂O₃, nEu₂O₃ and nDy₂O₃ were 11.5% (6.26 µg/mg protein), 9.7% (5.4 µg/mg protein) and 6.2% (3.6 µg/mg protein), respectively. It is of note that nYb₂O₃ showed only minor levels of dissolution, releasing 0.7% of ions intracellularly (0.3 µg/mg protein) (Figure 2B).

To determine whether the concentration of intracellular dissolved REEs was driven by interaction between REE NPs with membrane phosphates, we incubated REE NPs with PSF and monitored the dissolved REE ion concentrations by determination of REEs in suspensions following PSF incubation with REE NPs. nLa₂O₃, nEu₂O₃ and nDy₂O₃ were found to increase the levels of dissolved REE ions in PSF (Figure 2C). The proportion of dissolved REEs released in PSF

from nLa₂O₃, nEu₂O₃ and nDy₂O₃ were 17.3%, 6.4% and 5.2% at 15 min, increasing to 34.1%, 26.3% and 15.1% at 6 h, respectively. Conversely, nYb₂O₃ released only 0.8% dissolved ions, showing a significantly lower ion release potential. Therefore, the order of dissolved rates of REEs was La > Eu > Dy > Yb, with a similar trend to the ability to for P-REEs complexity. These results indicate the release of dissolved ions from REE NPs is because the interaction between REEs and phosphates. To further evaluate the relation between cell death and dissolved ions levels, the regression analysis was performed. The positive trend was observed between the cell death percentages and the rate of dissolved REEs (Figure S7B, *R* = 0.987, *p* < 0.05). To further define the effects of dissolved La ions on cell death, nLa₂O₃ was selected to expose J774A.1 cells with or without EDTA, as a chelating agent for dissolved La ions (stability constant = 15.46) and therefore inhibit their biological reaction with phosphates. As shown in Figure 2D, 50 µM EDTA reduced cell death by 30%, compared to treated cells without EDTA pretreatment (*p* < 0.05). These findings together demonstrate the intracellular dissolved toxic REE ions plays a role in the cell death. To investigate the involvement of cellular uptake in REE NP toxicity, cells were preincubated with cytochalasin D, an inhibitor of actin polymerization, for 2 h prior to nLa₂O₃ exposure. Cell death induced by nLa₂O₃ was

Figure 4. Mitochondrial and autophagic flux inhibition contributed to REE NP-induced cell death. After 2, 6 and 24 h of incubation with 20 μg/mL nLa₂O₃, cells were stained by Fluo-3/AM (A) or Rhod-2 (B), followed by FACS. (C) Cellular staining with Fluo-3/AM, Rhod-2 and DAPI (blue) in cells upon nLa₂O₃ treatment at 20 μg/mL for 6 h. Cells were examined with confocal microscopy. Two colors are shown individually or merged together. (D) After 2, 6 and 24 h of incubation with 20 μg/mL REE NPs, alteration in membrane potential was evaluated by TMRM staining followed by FACS. (E) Cell death was assessed with PI staining at 24 h after 20 μg/mL nLa₂O₃ treatment with or without CsA. (F) Western blot analysis of caspase 3 activation in J774A.1 cells with nLa₂O₃ treatment at 20 μg/mL for 12 h. (G) Cell death 24 h after 20 μg/mL nLa₂O₃ treatment with or without Z-VAD-fmk. *n* = 3 per group; * versus ctrl group. * represents *p* < 0.05.

mitigated due to the presence of the inhibitor, whereas cytochalasin D itself showed no significant effect on cell viability, demonstrating that REE NP-induced cell death was dependent on cellular REE NP uptake (Figure 2E, *p* < 0.05).

Lysosomal Enzyme Release into Cytosol Involves the Cell Death. To further characterize the intracellular accumulation of REE NPs, TEM imaging was performed to characterize intracellular REE NP localization. nLa₂O₃, nEu₂O₃, nDy₂O₃ and nYb₂O₃ particles were predominantly localized in lysosomes (Figure S9). With nLa₂O₃ as a representative material, a sea urchin-like formation was observed in lysosome earlier than 6 h (Figure 3A), suggesting REE NP transformation in lysosomes, in agreement with previously reported results.⁵² Energy dispersive X-ray (EDX) data confirmed the REEs within lysosome (Figure 3A, Figure S9). nLa₂O₃ accumulation resulted in the apparent enlargement of lysosomes, suggesting lysosomal injury had occurred. Conversely, control cells showed no significant disturbance to lysosomal size (Figure 3A, Figure S9). Lysosomes are acidic organelles containing a wide range of hydrolytic enzymes that have significant roles in intracellular protein recycling. Cathepsins are dominant and highly active hydrolytic enzymes,³⁸ with various cathepsins been classified as cysteine

proteases.⁵³ Once cathepsins released into the cytosolic compartment, they remain active for some time under neutral pH conditions, triggering downstream signaling pathways.⁵⁴ LMP-led cytosolic translocation has been linked to the cell death,⁵⁵ and the degree of LMP induced by REE NPs was assessed using the cathepsin substrate Magic Red, which is cleaved by cathepsin B.^{53,56,57} Therefore, to determine whether the LMP take place, we monitored the localization of cathepsin B at 6 h, and as shown in Figure 3B, concentrated red dots appeared within the cytosol, suggesting of the presence of cathepsins in lysosomes. The intensity of red color in cytosolic compartment in nLa₂O₃-treated cells was quantitatively greater than that in control cells, indicating that nLa₂O₃ has a greater capability to disrupt cell membrane integrity than the other REE NPs assessed. To assess the implication of LMP and the consequential release of cathepsins on cell viability, J774A.1 cells were exposed to nLa₂O₃ in the presence or absence of a range of cathepsin inhibitors, including CA-074Me, E64-d and pepstatin A. As shown in Figure 3C, cell death was significantly inhibited by approximately 50.0% of cathepsin inhibitors assessed, suggesting an important role of cytosolic cathepsins in REE NP induced cell death. The observed toxicity was highly

dependent on the ability of REE NPs to sequester phosphates from lysosomal membranes and the resulting degree of LMP.

Autophagy is a major mechanism used by cells to clear damaged organelles from intracellular compartments, transporting them to lysosomes for digestion.^{58–60} The ratio of cytosolic form of LC3/LC3-phosphatidylethanolamine conjugate (LC3-II/LC3-I) was assessed using Western blot analysis and as shown in Figure S10A, a significant 3.7-fold increased ratio of LC3-II/LC3-I was observed in cells treated with nLa_2O_3 , as compared to the control group. This indicates that the impaired autophagy flux occurs due to disruption to the fusion between autosome and lysosome.^{44,61} With a reporter cell (RAW-Difluo mLC3 Cells, invivogen), we confirmed the blockage of autophagic flux by nLa_2O_3 (Figure S10B). Therefore, these results suggest that the attenuated autophagic flux was another side effect of LMP. Given the importance of clearance of aged and damaged cellular components to the survival of cells, the attenuated autophagic flux may enhance the cell death.⁴⁴ We then test whether rapamycin, as an agent to stimulate autophagy, could rescue the cell death of J774A.1 cells. As shown in Figure S10C, the promotion of autophagic flux showed no effect on cell death, suggesting autophagy-associated cell death is not involved in cell death.

Role of Intracellular Calcium in REE NP-Induced Cell Death. Intracellular Ca^{2+} flux was one of important regulator for cell death.⁶² As shown in Figure 4A, increased intensity of Fluo-3A within cells suggests that the concentration of $[\text{Ca}^{2+}]_i$ significantly increased in cells exposed to nLa_2O_3 . We used Rhod-2 fluorescence as an indicator for mitochondrial calcium ($[\text{Ca}^{2+}]_m$), and found that nLa_2O_3 induced a significant 1.5-fold increase in Rhod-2 fluorescence intensity as compared to the control group (Figure 4B, $p < 0.05$) at both 6 and 24 h. The increase in $[\text{Ca}^{2+}]_i$ and $[\text{Ca}^{2+}]_m$ was also confirmed by confocal microscopy, observing enhanced intensity of both intracellular Fluo-3/AM and Rhod-2 (Figure 4C). We then evaluated the mitochondrial potential ($\Delta\Psi_m$) with TMRM to reflect the health status of mitochondria. As shown in Figure 4D, comparing with control cells, the proportion of cells with TMRM^{low} intensity increased to 6.8%, 24.7% and 19.6% at 2 h, 6 and 24 h ($p < 0.05$), respectively, suggesting a reduction in $\Delta\Psi_m$ due to nLa_2O_3 exposure. Mitochondria provides buffering against the overload of intracellular $[\text{Ca}^{2+}]_i$. The elevation of $[\text{Ca}^{2+}]_m$ is a key regulator of mitochondrial function and is known to cause permeability transition pore (PTP) formation.^{38,63–65} To assess the occurrence of this, before REE NP exposure, cells were treated with CsA, which inhibits PTP by binding to Ca^{2+} . As shown in Figure 4E, pretreatment with CsA reduced cell death from 19.4% to 6.9%, indicating that CsA can successfully inhibit the cytotoxicity of nLa_2O_3 . Because mitochondria could regulate cell death through both apoptosis and necrosis, we then evaluated the cleavage of caspase 3 as a representative caspase to regulate apoptosis to determine whether cell apoptosis is involved. Following nLa_2O_3 exposure, Western blot analysis showed no evidence of caspase 3 cleavage observed (Figure 4F). To further confirm this observation, we selected Z-VAD-fmk as the pan-caspase inhibitor, which could inhibit all types of caspases, and was frequently used for evaluation on the cell apoptosis. We found that Z-VAD-fmk did not inhibit nLa_2O_3 -induced cell death (Figure 4G), indicating that cell death was due to necrosis but not caspase dependent apoptosis.

Environmental Implication. The extensive application of REEs is already a great concern for the effects on eco-

environmental safety and human health due to the exposure to the human body through various exposure route. As the important role for clear the introduced nanoparticles by various routes, macrophages is more sensitive than the other cell type and responsible for the series side effects. With macrophage as a cell model, a dramatic variation was observed in the degree of cytotoxicity induced by different REE NPs, which was highly dependent on the types of REEs. The activity in phosphates sequestration induced by REE NPs is responsible the cell death which could induce both cell membrane damage and LMP (Figure 5). Previous studies have reported that the damage of

Figure 5. Schematic deciphering REE NP-induced cytotoxicity. The toxic REE NPs including nLa_2O_3 , nEu_2O_3 and nDy_2O_3 induce the damage of cell membrane and leads to the increase in intracellular $[\text{Ca}^{2+}]_i$ and $[\text{Ca}^{2+}]_m$, and the resultant cell necrosis. Accumulated REE NPs in lysosome triggered LMP, leading to the release of cathepsins into cytosol, which also contributes cell death.

cell membrane could trigger the influx of intracellular calcium ($[\text{Ca}^{2+}]_i$).⁶² LMP has also been demonstrated to govern the storage of intracellular calcium ($[\text{Ca}^{2+}]_i$) and act as a regulator of cell death, triggering cell death through the release of Ca^{2+} from lysosome to cytosol compartment.^{63–66} Our results indicated that cell membrane or lysosomal membrane disruption could result in the elevation of $[\text{Ca}^{2+}]_i$ and consequentially induce cell death. Despite mitochondrial responses to sequester $[\text{Ca}^{2+}]_i$, the sustained high Ca^{2+} concentrations result in PTP which promotes cell necrosis. Although the mechanism for REE NPs warrant further investigation, the elevation of $[\text{Ca}^{2+}]_i$ and $[\text{Ca}^{2+}]_m$ upon exposure to REE NPs has been unveiled in the current study. Caspase-3 was a predominant enzyme to regulate cell apoptosis, which has been frequently applied for the marker to evaluate the possible apoptosis of cells. We first applied this marker to determine whether macrophages undergo apoptosis upon La_2O_3 treatment. We also applied Z-VAD-fmk as an inhibitor to inactivate all kinds of caspases to confirm the conclusion that caspase-apoptosis was not involved in the cell death. We discussed this in detail in the manuscript. The fact that caspase inhibitor failed to rescue cell death suggested the cytotoxicity was not due to apoptosis. Upon normal autophagic flux, LC3-I is conjugated to LC3-II and delivered to autophagosomal membranes. Autophagosomes then fuse with lysosomes to form autolysosomes to degrade intra-autophagosomal components by lysosomal enzymes. Meanwhile, LC3-II in the autolysosomal lumen is degraded. Thus, the accumu-

lation of LC3-II and LC3-I accumulation is applied as a surrogate for autophagy flux to elucidate the possible role of lysosomes in REE NP-induced cell death.⁶¹ In the current study, although the disruption of autophagic flux has been found, it showed no contribution to cell death. Therefore, our data together demonstrate that cell death of macrophages induced by REE NPs is because of necrosis, but not caspase dependent apoptosis or autophagy-associated cell death. These findings provide an in-depth insight into the mechanisms of cytotoxicity induced by exposure to a range of REE NPs and that element composition-associated toxicity profiles should be considered during the selection of REEs for use in the design of nanomaterials, such as with upconversion nanoparticles. Our findings also suggested the coating of REE NPs with phosphates could block the activity of REE NPs, providing higher safety of these materials. This method is also helpful for the treatment of waste REE NPs, through the blockage of surface activity with phosphates treatment. Also, upon incidental exposure to REE NPs such as inhalation of these materials in miners, suitable EDTA administration could relieve the toxic of the inhaled REE NPs. As the widely application of REE NPs, our results would be useful for the risk assessment of the environmental safety and health upon exposure different types of REE NPs.

■ ASSOCIATED CONTENT

■ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.7b04231.

Linearity of La, Eu, Dy and Yb using ICP-MS; representative HRTEM image of the 4 kinds of REE NPs in the current study; XPS spectra of the selected REE NPs in the current study; FT-IR spectra of the selected REE NPs materials; percentages of dead cells in J774A.1 cells or Hep1–6 cells; heat map showing the relative intensity of intracellular fluorescence of J774A.1 cells after REE NPs treatment using the CellTox assay; relations between ratio of P/REE and cell death or relations between dissolved REE ions and cell death; dissolution of rare earth ions in DMEM medium; particle uptake in J774A.1 cells were assessed after 6 h treatment with REE NPs through TEM and the energy dispersive X-ray (EDX) data; autophagic flux analysis upon nLa₂O₃ treatment (PDF)

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