

Inhibition of Kupffer Cell Autophagy Abrogates Nanoparticle-Induced Liver Injury

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The possible adverse effects of engineered nanomaterials on human health raise increasing concern as our research on nanosafety intensifies. Upon entry into a human body, whether intended for a theranostic purpose or through unintended exposure, nanomaterials tend to accumulate in the liver, leading to hepatic damage. A variety of nanoparticles, including rare earth upconversion nanoparticles (UCNs), have been reported to elicit hepatotoxicity, in most cases through inducing immune response or activating reactive oxygen species. Many of these nanoparticles also induce autophagy, and autophagy inhibition has been shown to decrease UCN-induced liver damage. Herein, using UCNs as a model engineered nanomaterial, this study uncovers a critical role for Kupffer cells in nanomaterial-induced liver toxicity, as depletion of Kupffer cells significantly exacerbates UCN-induced liver injury. Furthermore, UCN-induced prodeath autophagy in Kupffer cells, and inhibition of autophagy with 3-MA, a well-established chemical inhibitor of autophagy, enhances Kupffer cell survival and further abrogates UCN-induced liver toxicity. The results reveal the critical importance of Kupffer cell autophagy for nanoparticle-induced liver damage, and inhibition of autophagy may constitute a novel strategy for abrogating nanomaterial-elicited liver toxicity.

1. Introduction

The twenty-first century has witnessed remarkable progress in nanotechnology and its related products. Accompanying this trend is the rising concern on nanosafety, which centers on the possible adverse effects engineered nanomaterials may have on human health.^[1,2] Even though a majority of the engineered

nanomaterials are not intended for human use, their mass production and wide applications in various industries inevitably lead to increasing human exposure, mostly through skin, respiratory system, and digestive tract.^[3] On the other hand, thanks to their unique and oftentimes superior physicochemical properties, engineered nanomaterials have shown tremendous potential in diverse biomedical applications, including bioimaging, drug delivery, tissue repair and regeneration, and cancer therapy.^[4] Some of the engineered nanoparticles or devices have reached clinical use or entered clinical trials, while a large number of them are still in pre-clinical development.^[5] These nanomaterials, intended for either diagnostic or therapeutic purpose, are administered to human body via different delivery routes. Regardless of the intention and the means of getting into the body, the nanomaterials would gain access to the blood circulation and eventually reach the various organs

and tissues. An interesting observation is that many nanomaterials preferentially accumulate in the liver.^[6] While this feature has been exploited for designing nanomaterials that specifically target liver and treat liver disease such as liver fibrosis,^[7,8] hepatocellular carcinoma,^[9–11] and viral hepatitis,^[12–14] it also raises the issue of liver toxicity. Indeed, previous studies have reported that a variety of nanoparticles, such as SiO₂, TiO₂, Ag, GO,

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UCP, CdSe/ZnS core–shell quantum dots, Poly-amidoamine (PAMAM) dendrimers, and so on, induced hepatic injury.^[15–22] In detail, these injuries were shown to be dose dependent, with the size, charge and modification of nanoparticles all playing important roles for their hepatotoxicity.^[15,19,20,23–25] Thus, a thorough understanding on how engineered nanomaterials provoke hepatotoxicity would be greatly beneficial for designing safer and more effective theranostic nanoparticles.

Liver is rich in blood vessels. Blood enters liver through the portal vein and hepatic arteries, arrives at sinusoids, then accumulates in the central veins and leaves the liver.^[26] As the major organ for metabolism and immune defense, liver consists of parenchymal cells (hepatocytes and cholangiocytes), nonparenchymal cells (immune cells, liver sinusoidal endothelial cells, and hepatic stellate cells).^[27] Hepatocytes are the most abundant cells in liver, performing the function of metabolism, endocrine, and secretory.^[26] Kupffer cells are specialized macrophages, which reside in liver and are critically important for clearance of substances such as nanoparticles, gut-derived bacteria, and bacterial toxins.^[28] To date, several studies revealed that nanomaterials could induce liver injury by triggering immune response and elevating reactive oxygen species (ROS) level, leading to liver cell death.^[17,29–31] With the ability to secret cytokines, activate ROS, process and present both soluble and particulate antigens, Kupffer cells represent the first defense against nanomaterials in liver and are likely to play critical roles in nanomaterial-induced liver toxicity.^[32]

Autophagy is a lysosome-based evolutionarily conserved and dynamic intracellular process, in which cytoplasmic constituents are engulfed by autophagosomes and delivered to lysosomes for degradation.^[33] Autophagy is critical for cellular homeostasis, and basal level of autophagy participates in differentiation, development, homeostasis and tissue remodeling in various organisms.^[34,35] Autophagy also plays a significant role in maintaining liver function, and dysfunction of autophagy is reported to be a notable player in the pathogenesis of liver diseases. In most circumstances, autophagy plays a protective role in liver, such as in nonalcoholic and alcoholic fatty liver, drug induced liver injury, protein conformational liver diseases, viral hepatitis, and liver ischemia reperfusion injury.^[36] On the other hand, in diseases like liver fibrosis, autophagy has an destructive effect.^[37] Furthermore, the autophagy in Kupffer cells is also involved in liver diseases. Generally, impaired autophagy in Kupffer cells exacerbated liver injury in liver fibrosis, toxin-induced liver injury and alcoholic liver disease.^[38–40]

Nanomaterials are considered to be a novel class of autophagy activator. A series of nanomaterials, including quantum dots,^[41] metal and alloy nanoparticles,^[42–44] metal oxides nanomaterials,^[45] SiO₂,^[46] carbon-based nanostructures,^[47,48] polystyrene particles,^[49] etc., are known to trigger autophagy. Notably, rare earth oxide nanoparticles and upconversion nanoparticles (UCNs) have been reported to induce prodeath autophagy, and autophagy inhibition significantly decreased UCN-induced liver toxicity.^[22] However, how autophagy contributes to nanomaterial-induced liver toxicity and whether Kupffer cells play a role in this process is unclear. In this work, using UCN as a model-engineered nanomaterial, we have uncovered a critical role for Kupffer cells in nanomaterial-induced liver toxicity, as depletion of Kupffer cells significantly exacerbated UCN-induced liver

injury. Furthermore, UCNs induced prodeath autophagy in Kupffer cells, and inhibition of autophagy with 3-MA, a well-established chemical inhibitor of autophagy, prevents Kupffer cells death and further abrogates UCN-induced liver toxicity.

2. Results

2.1. UCNs Deplete Kupffer Cells and Induce Hepatotoxicity in a Dose-Dependent Manner

Liver stands in the forefront of nanomaterials defense. Many published studies report that a variety of nanoparticles in liver lead to hepatic injury.^[15–22] As potential imaging nanomaterial, the lanthanide-based upconversion nanoparticles (UCNs) are used in this study. UCNs, whose chemical composition is NaYF₄:18%Yb, 2%Er, are prepared, and their average size is of 40 nm, as visualized by transmission electron microscopy (TEM) (Figure 1a,b).^[50] What is more, two emission peaks at 521 and 539 nm were observed in UCNs fluorescence spectra while excited by 980 nm near-infrared laser, indicating that the UCNs used here occupied strong green fluorescence (Figure 1c).^[50] As previously reports, nanomaterial-induced hepatotoxicity was mainly dose dependent. Thus, we intravenously injected different dosage of UCNs to mice to test the liver injury. Haematoxylin and eosin staining (HE staining) results showed that 150 mg kg⁻¹ UCNs led to extensive infiltration of inflammatory cells, whereas it was not observed using lower dosage (Figure 2a). Additionally, high dose of UCNs significantly increased alanine aminotransferase (ALT) level (Figure 2b). Moreover, the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) digoxigenin nick-end labeling (TUNEL) assay revealed that there were more apoptotic cells in high dose UCN-treated mice than the lower dosage group (Figure 2c). Collectively, the hepatotoxicity of UCNs was dose dependent.

As the most abundant macrophages in liver, Kupffer cells are involved in both nanoparticles clearance and desposition.^[26] To explore the impact of nanoparticles on Kupffer cells, F4/80 was used as the marker of kupffer cells in immunofluorescence.^[51] As shown in Figure 2d, F4/80 positive Kupffer cells were observed in liver of 10 mg kg⁻¹ UCNs injected mice, although the morphology of kupffer cells was slightly changed. However, F4/80 positive kupffer cells were depleted after higher dose of UCNs treatment and 40 mg kg⁻¹ was the threshold dose to deplete kupffer cells without causing liver injury. These results also suggested that liver injury was observed in mice-depleting Kupffer cells. Thus, Kupffer cells could be involved in nano-hepatotoxicity.

2.2. Kupffer Cells Promote Liver Resistance to UCN-Induced Hepatotoxicity

To prove the speculations above, clodronate liposome (CLL) (Figure S1, Supporting Information), a common reagent for Kupffer cells depletion, was used here.^[52] After the intravenous injection of CLL, Kupffer cells were depleted in vivo (Figure 3a). Next, we administrated CLL-pretreated mice or control mice

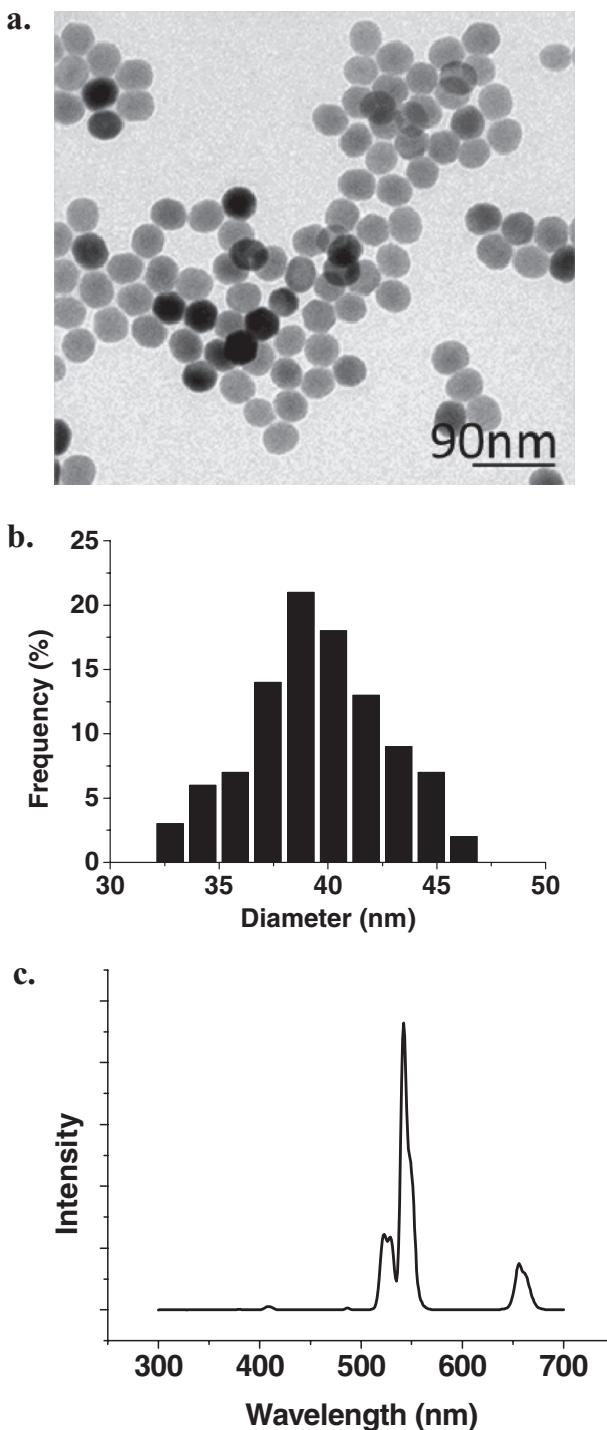


Figure 1. Characterization of UCNs. a) TEM photos of UCNs. b) Size distribution of UCNs, 400 particles from the TEM photos were measured by NANO Measurer software. c) Upconversion fluorescence spectrum of UCNs under excitation of an NIR laser (980 nm).

with 40 mg kg^{-1} UCNs. Normal liver functions were observed in control mice receiving UCNs. Surprisingly, pathologic change and elevation of ALT level were observed in CLL-pre-treated mice (Figure 3b,c), indicating the liver injury induced by UCNs. Consistent with the above results, TUNEL assay

showed obvious liver cell apoptosis in Kupffer cells depleted mice (Figure 3d). These results revealed that Kupffer cells could protect the mice from UCN-induced liver injury.

2.3. Inhibition of Autophagy Promotes Kupffer Cell Survival

Nanomaterials have emerged as a new class of autophagy inducers. Most of them promote cell death, whereas some play an opposite role. LC3 is autophagy marker protein, and it will be cleaved from LC3 I into a lower molecular weight LC3 II during autophagy induction. To test the autophagy-inducing effect of UCNs, 3-methyladenine (3-MA), a kind of autophagy inhibitor, was employed here.^[53] Kupffer cells were isolated (Figure S2, Supporting Information) and treated with UCNs in presence or absence of 3-MA. As shown in Figure 4a, UCNs increased LC3 II level, indicating the induction of autophagy, while 3-MA decreased the accumulation of LC3 II through inhibiting autophagy. Further, Hoechst/PtdIns staining showed that 3-MA decreased UCN-induced Kupffer cells death (Figure 4b). What is more, the ratio of dead cells was quantified, and combination of UCN and 3-MA led to a significantly decrease in cell death as compared to UCN alone (Figure 4c). These results illustrated that autophagy promotes UCN-induced Kupffer cell death.

2.4. Inhibition of Kupffer Cell Autophagy Abrogates UCN-Triggered Hepatotoxicity

To further investigate the detrimental role of autophagy, in vivo study was carried out. 3-MA was encapsulated by liposomes (Figure S1, Supporting Information), which could be specifically engulfed by Kupffer cells to inhibit autophagy. In mice liver, Kupffer cells were depleted by UCNs, which was similar to CLL. Meanwhile, 3-MA liposomes significantly increased the number of survival Kupffer cells per field (Figure 5a,b). Moreover, the function of mice liver was detected. Inhibition of autophagy in Kupffer cells by 3-MA liposomes prevented infiltration of inflammatory cells (Figure 5c) and elevation of ALT levels (Figure 5d). In addition, 3-MA liposomes treatment also abrogated the liver cells death (Figure 5e). Taken together, inhibition of Kupffer cells autophagy promotes their resistance to UCNs and then diminishes nanoparticles-induced liver injury.

2.5. Kupffer Cell Prevents UCNs Distribution to Hepatocyte

As one kind of nonparenchymal cells, Kupffer cells are located on the wall of the sinusoids. Nanoparticles in the blood-stream delivered to hepatocyte must go through the sinusoids. Albumin is synthesized in hepatocyte and widely used as a hepatocyte marker.^[54] Under a 980 nm laser, UCNs are excited and generated a green fluorescence thus let us be able to track them in liver. Using this technique, we observed that vast amount of UCNs located in sinusoids. Interestingly, in Kupffer cells depleted mice liver, UCNs were largely observed in hepatocytes (Figure 6a,b). In this case, the escaped UCNs may damage the hepatocytes, thereby inducing liver injury. And our in vitro test proved the harmful effect of UCNs on

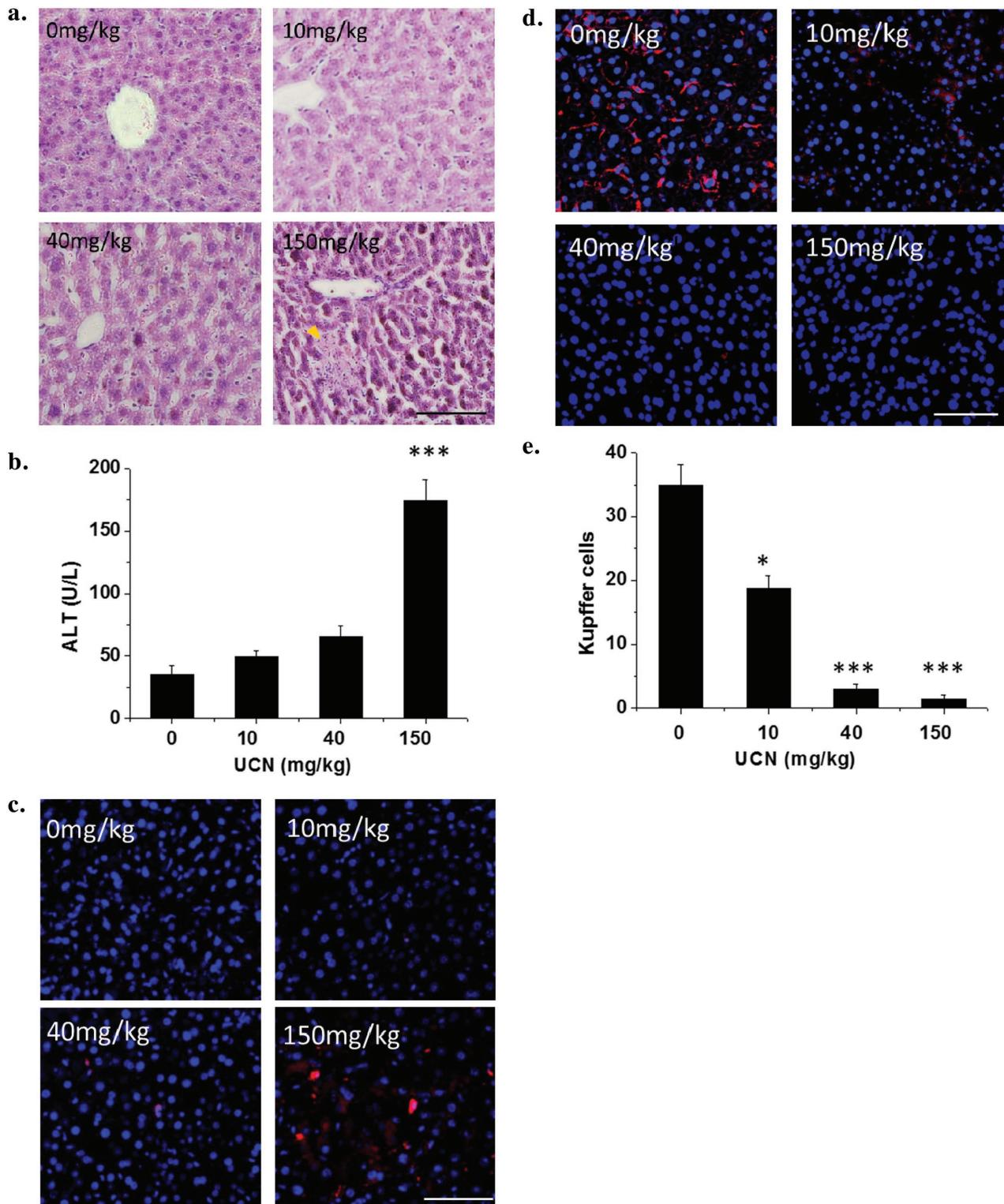


Figure 2. UCNs deplete Kupffer cells and induce hepatotoxicity. Wild-type C57BL/6 mice were intravenously injected with PBS or UCN (10, 40, 150 mg kg⁻¹) for 24 h. a) Representative images of mice liver section for HE staining. Scale bar = 100 µm. b) Blood ALT level of mice was detected. c) TUNEL staining of liver section. Scale bar = 100 µm. d) Liver sections were immunostained with anti-F4/80 antibody, nucleus were stained with Hoechst. Scale bar = 100 µm. e) Statistical result of the number of Kupffer cells per field. (Mean ± S.E.M., n = 4–6, *p < 0.05, ***p < 0.001).

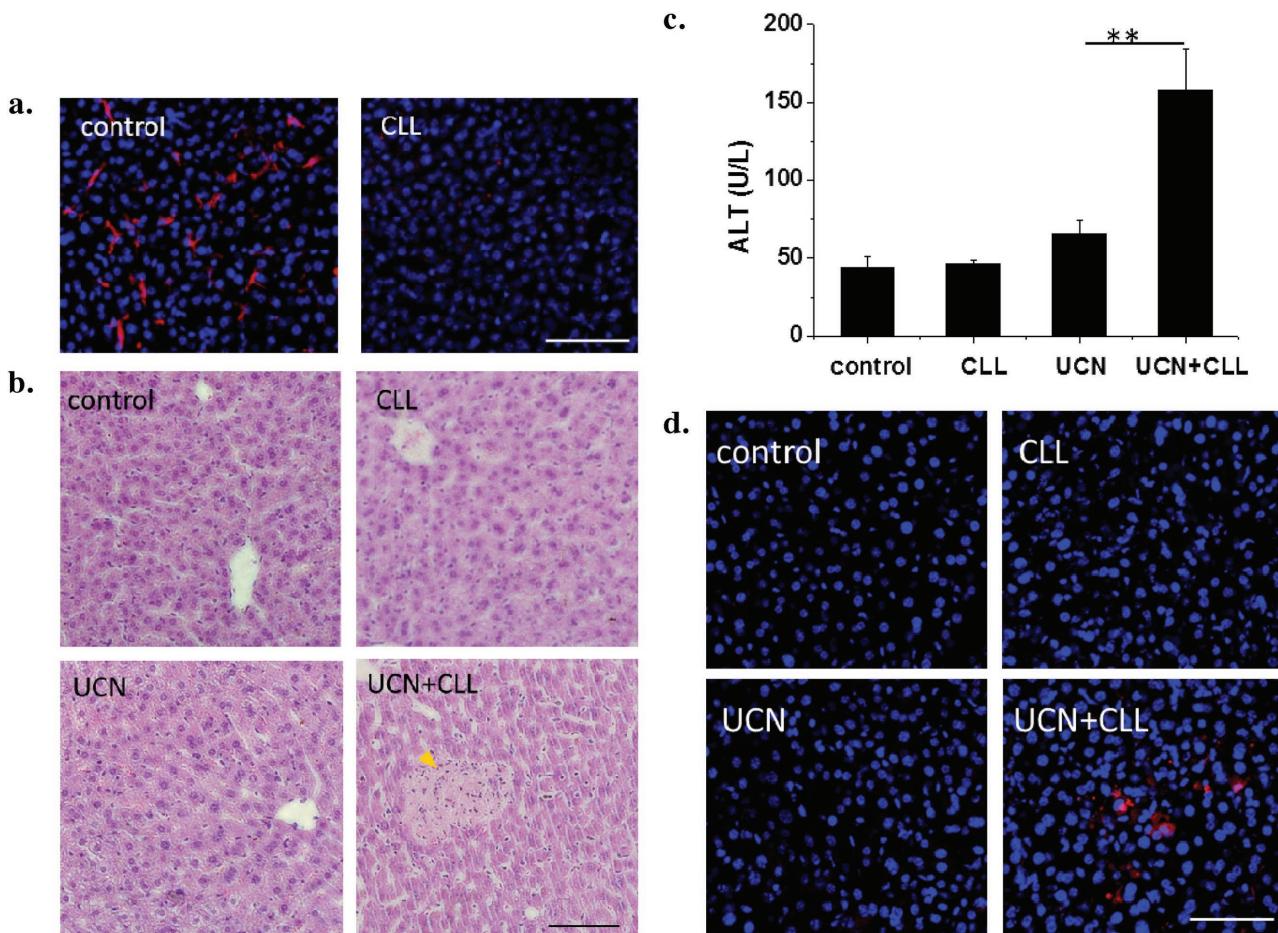


Figure 3. Depletion of Kupffer cells induces liver injury under UCNs treatment. Wild-type C57BL/6 mice were intravenously preinjected with CLL to deplete Kupffer cells. Control mice or Kupffer cells predepleted mice were injected with UCNs (40 mg kg^{-1}) for 24 h. a) Liver sections were immunostained with anti-F4/80 antibody, nucleus were stained with Hoechst. Scale bar = $100 \mu\text{m}$. b) HE staining images of liver section. Scale bar = $100 \mu\text{m}$. c) Blood ALT level of mice. d) TUNEL staining of liver section. Scale bar = $100 \mu\text{m}$. (Mean \pm S.E.M., $n = 4\text{--}6$, $*p < 0.05$, $^{***}p < 0.001$).

hepatocytes (Figures S3 and S4, Supporting Information). Previously reported, 3-MA was certified to promote Kupffer cells survival by inhibiting the autophagy, and we further found that 3-MA led to a 17% decrease in uptake of UCNs by hepatocytes (Figure 6c,d), thus UCN-induced liver injury was ameliorated. According to the above results, large dose of UCNs killed Kupffer cells and escaped out of sinusoids (Figure 6e,f). As a consequence, uptake of excess UCNs by hepatocytes further caused liver injury. Hereto, the theory of this study has been schematically shown in Figure 7.

3. Discussion

Nanomaterials are promising in biomedical use, but their safety issues should be concerned. In this study, we evaluated the biosafety of UCN, a potential imaging nanomaterial. Although UCN is reported to be a relative safe material,^[55] it surprisingly excited acute toxicity in mice liver at a high dose.^[56] The threshold dose of UCNs in liver was 40 mg kg^{-1} , Kupffer cells were depleted but overall liver function was intact at this particular dosage. Inspired by one study that Kupffer cells

mediated silica nanoparticles induced hepatic injury,^[32] we predepleted Kupffer cells and treated mice with the critical value dose of UCNs. Obvious liver injury was detected, which demonstrated the protective role of Kupffer cells in liver resistance to nanoparticles. In summary, we proposed a conjecture that high dose of UCNs treatment depleted Kupffer cells and decreased liver resistance to nanoparticles.

Most previous studies illustrated the role of Kupffer cells in inducing nano-hepatotoxicity by regulating cytokine production or ROS. In our work, we demonstrated that the location of Kupffer cell in liver was vitally important for liver resistance to nanoparticles. Like a security guard, Kupffer cells constantly intercepted and captured nanoparticles in sinusoid, consequently prevented nanoparticles diffusion to hepatocyte, which avoided the harmful effects of nanoparticles on hepatocytes. Predepletion of Kupffer cells or overwhelming their tolerance by administration of high-dose nanoparticles would exposed hepatocytes to excessive nanoparticles. However, in poly lactic-co-glycolic acid (PLGA)-nanoparticles treated mice liver, predepletion of Kupffer cells increased nanoparticles retention in liver sinusoidal endothelial cells, but not in hepatocytes.^[27] Notably, the molarity of PLGA-nanoparticles was lower than

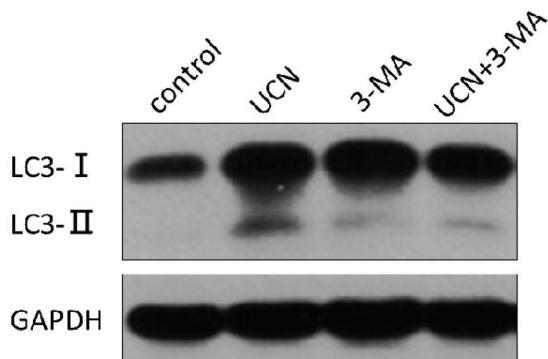
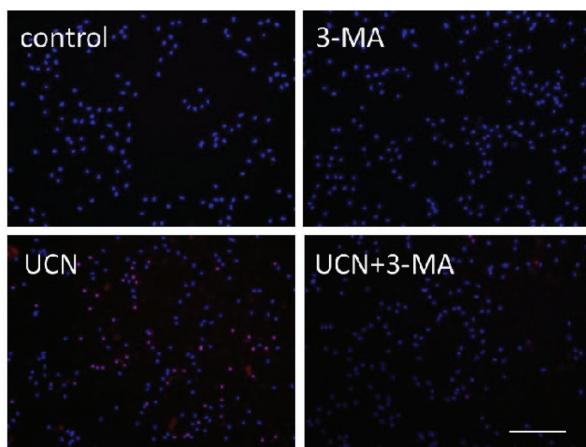
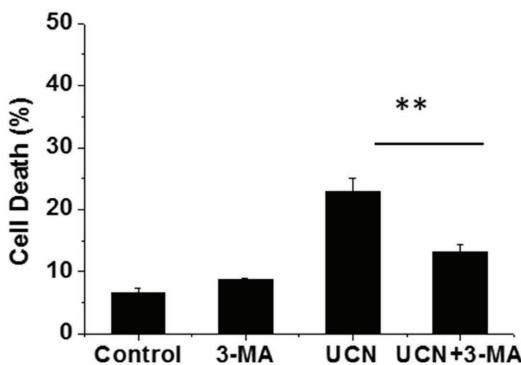
a.**b.****c.**

Figure 4. Inhibition of autophagy promotes Kupffer cell survival in vitro. Isolated Kupffer cells were treated with PBS or UCNs (1 mg mL^{-1}) in the presence or absence of $25 \times 10^{-3} \text{ M}$ 3-MA for 24 h. a) Western blot results of LC3 and GAPDH. b) Representative fluorescence pictures and c) the cell death rate of Kupffer cells. Cell death was assessed by Hoechst/PI staining and expressed as the percentage of PI-stained cells. Scale bar = $100 \mu\text{m}$. (Mean \pm S.E.M., $n = 3$, ** $p < 0.01$).

UCNs in our work. Moreover, sinusoidal endothelial cells were closer to sinusoid compared to hepatocytes. Thus, few PLGA-nanoparticles go through sinusoidal endothelial cells to hepatocytes and no significant differences in hepatic retention were reasonable. Furthermore, the content of UCNs in sinusoidal endothelial cells and the role of sinusoidal endothelial cells in nano-hepatotoxicity remained to be determined.

Autophagy, an important biological process, can be induced by UCNs in Kupffer cells. In most circumstances, nanomaterial-triggered autophagy is detrimental to cells, which is

also proved in Kupffer cells here. Inhibition of autophagy by 3-MA promotes Kupffer cells survival and abrogates the nano-hepatotoxicity, revealing the protective role of Kupffer cells. In addition, our previous study showed incomplete autophagy induced by UCNs, which caused harmful effects on hepatocytes by leaving giant autolysosomes, and this could also happen to Kupffer cells (Figures S5 and S6, Supporting Information).^[57] However, autophagy would not be the only factor contributing to the death of the Kupffer cells with the treatment of nano-materials (Figure S7, Supporting Information). Moreover, it is reported that Kupffer cells inhibited the increase of several cytokines, such as IL-10 and tumor necrosis factor (TNF)- α in silver nanoparticles treated mice liver.^[28] Thus, in our case, Kupffer cells are likely to mediate UCN-induced hepatotoxicity by regulating cytokine productions as well, which has yet to be testified.

4. Conclusion

UCNs can deplete Kupffer cells in vivo and induce liver injury in a dose-dependent manner. The threshold dose of UCNs in liver is quantified as 40 mg kg^{-1} . Moreover, Kupffer cells are proved to be protective in liver resistance to UCN-induced hepatotoxicity. And inhibiting the autophagy of Kupffer cells by 3-MA promotes Kupffer cells survival and abrogates UCN-induced liver injury. Further, Kupffer cells in sinusoid prevent intrusion of nanoparticles to hepatocytes, greatly help liver to evade nanotoxicity.

5. Experimental Section

Materials: Ultrapure water (pH 6.7; Milli-Q, Bedford, MA, USA) was used in all situations throughout the experiments. Anti-LC3 antibody (NB100-2220, 1:2000 dilution for WB, 1:100 dilution for IF) was purchased from Novus Biologicals (Colorado, USA). Antialbumin antibody (66051-1-Ig, 1:100 dilution) was purchased from Proteintech Group (Wuhan, China). Anti-GAPDH antibody (AB9132, 1:10 000 dilution) was from Merck Millipore (Darmstadt, Germany). PE antimouse F4/80 Antibody (123109) was purchased from Biologend (San Diego, USA). HRP-conjugated antirabbit antibody (W4011) and HRP-conjugated antimouse antibody (W4021) were purchased from Promega (Madison, USA). Goat antirabbit IgG-FITC antibody (SC2-12, 1:100 dilution), goat antimouse IgG-R (SC-2092, 1:100 dilution) were purchased from Santa Cruz Biotechnology (Texas, USA). Enhanced chemiluminescence (ECL) kits were from Biological Industries (Kibbutz beit Haemek, Israel). Hoechst 33342 (B2261), CLL (22560-50-5), 3-methyladenine (M9281), dioleoylphosphatidylethanolamine (DOPE) (42490), and cholesterol (C8667) were purchased from Sigma Aldrich (St. Louis, MO). TUNEL Bright Red Apoptosis Detection Kit (A113-01) was purchased from Vazyme (Nanjing, China). Serum ALT level was determined using a kit from Rongsheng (Shanghai, China). NH_4F and methylalcohol were purchased from Sinopharm Chemical Reagent (Shanghai, China). YCl_3 , YbCl_3 , and ErYCl_3 were purchased from Sigma-Aldrich. Oleic acid and 1-octadecene were purchased from Aladdin (Shanghai, China).

Nanoparticles Preparation: UCN was prepared as described previously.^[50] The morphology of the nanoparticles was characterized by transmission electron microscope (JEOL-2010), upconversion fluorescence spectrum of UCN was detected by fluorescence spectrophotometer (RF-5301 PC, Shimadzu, Japan) with an IR laser (MDL-980 nm 1 W, Changchun New Industries Optoelectronics Tech. Co., Ltd, China).

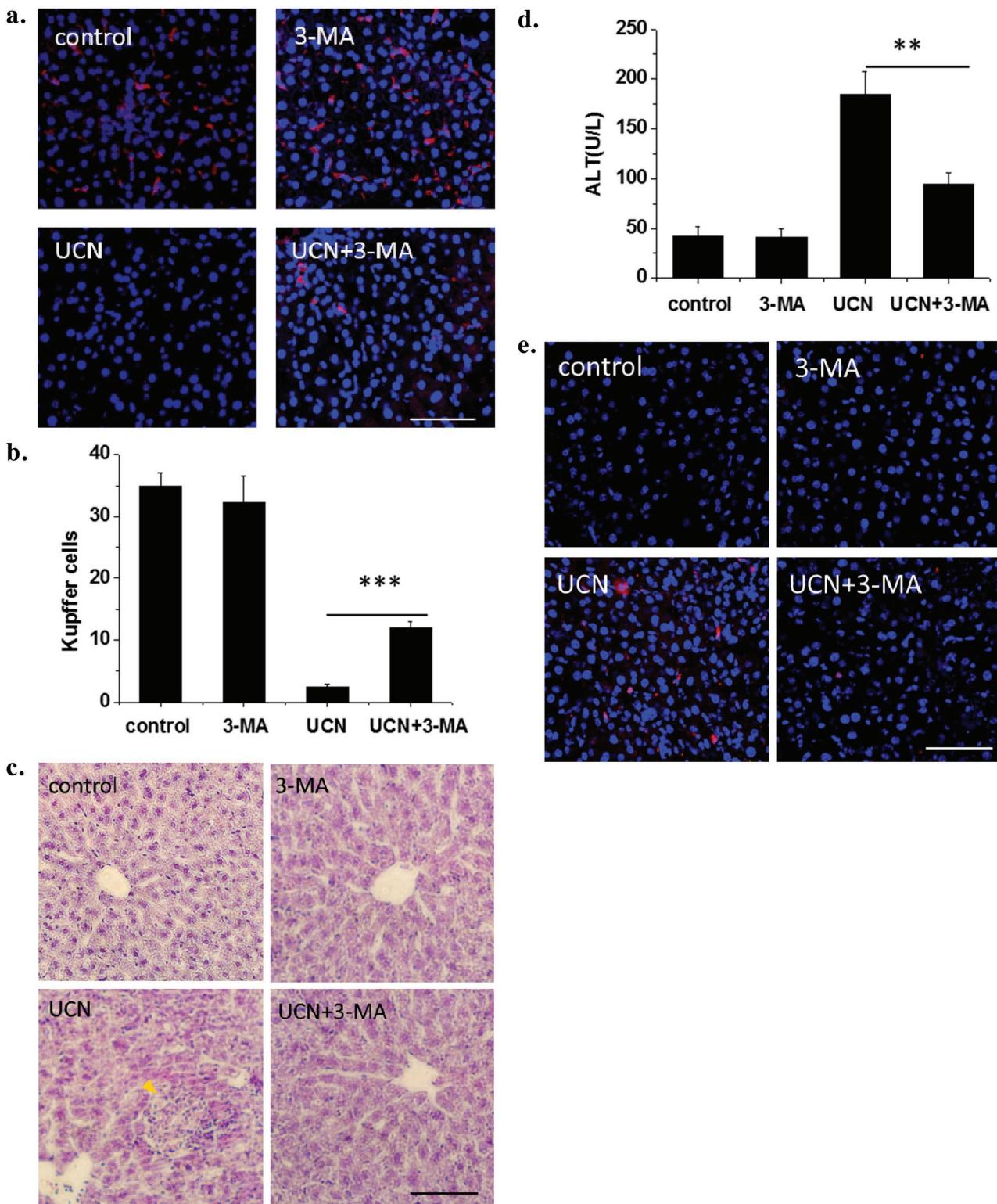


Figure 5. Inhibiting the autophagy of Kupffer cell abrogates UCN-triggered hepatotoxicity. Wild-type C57BL/6 mice were intravenously preinjected with 3-MA liposomes to inhibit the autophagy of Kupffer cells. Control mice or autophagy-inhibited mice were injected with UCNs (150 mg kg^{-1}) for 24 h. a) Liver sections were immunostained with anti-F4/80 antibody, nucleus were stained with Hoechst. Scale bar = 100 μm . b) Statistical result of the number of Kupffer cells per field. c) Typical images of liver section HE staining. Scale bar = 100 μm . d) Blood ALT level was quantified. e) TUNEL staining of liver section. Scale bar = 100 μm . (Mean \pm S.E.M., n = 4–6, **p < 0.01, ***p < 0.001).

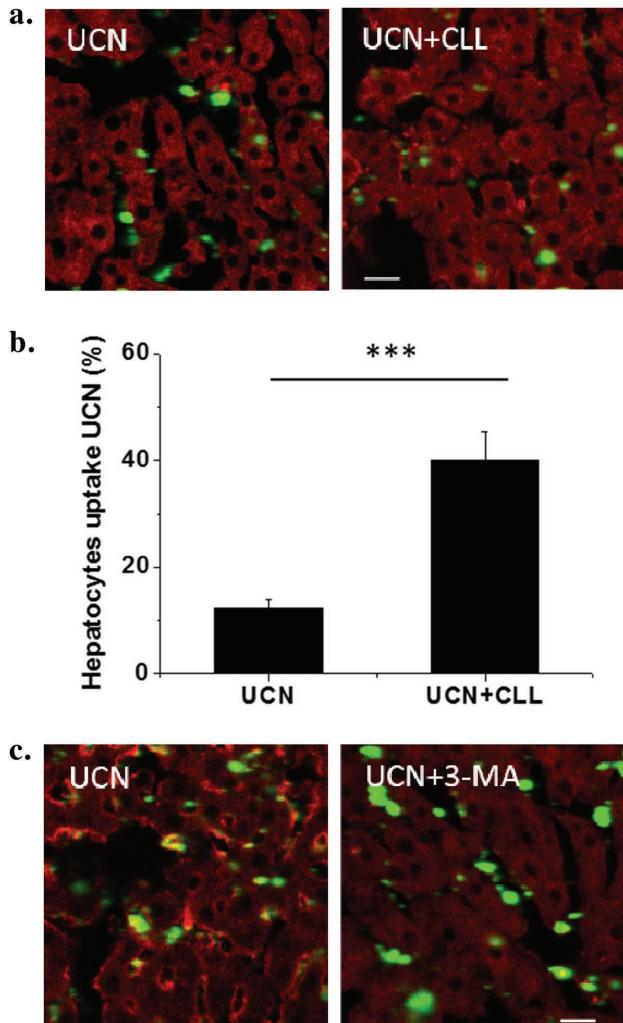


Figure 6. Relative location of UCNs in liver. Two-photon microscopy images of UCNs and immunofluorescence of albumin. a) Control mice or Kupffer cell predepleted mice were injected with UCN (40 mg kg^{-1}) for 24 h. c) Control mice or 3-MA liposomes pretreated mice were injected with UCNs (150 mg kg^{-1}) for 24 h. e) Mice were injected with UCNs 40 or 150 mg kg^{-1} for 24 h. Liver sections were immunostained with antialbumin antibody and observed under 543 nm laser or under a NIR laser (980 nm). Scale bar = $20 \mu\text{m}$. b,d,f) Statistical result of percentage of hepatocytes uptake UCNs.

Cell Culture: Cells were cultured at 37°C and 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS).

Immunofluorescence: Mice liver cryosection was washed by 0.05% tris buffered saline tween (TBST) for 5 min twice, permeabilized with 0.1% Triton X-100 for another 10 min, washed by 0.1% TBST for 5 min twice, then was blocked with 1% FBS for 1 h. Liver cryosection was incubated with primary antibodies overnight at 4°C and labeled with secondary antibody at 37°C for 1 h. Images were acquired by Zeiss LSM710 confocal microscope.

Western Blotting: The treated cells were harvested and lysed with sample buffer and boiled for 10 min. Proteins were separated by electrophoresis on a sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred to polyvinylidene fluoride (PVDF) membrane (IPVH00010, Millipore, Darmstadt, Germany). After blocking with 5% nonfat dry milk for 1 h, PVDF membrane was incubated with a primary antibody overnight at 4°C , extensively washed, incubated with a

HRP-conjugated secondary antibody (1:10 000 dilution) for 1 h, and then visualized with an ECL kit.

Isolation of Mouse Hepatocytes and Kupffer Cells: A mouse was anaesthetized and its feet were fixed with needles to operating board, the fur was wet with 70% ethanol to sterilize the abdominal area. Then the abdominal cavity was opened and intestines were pushed to the right side in order to expose the portal vein, the portal vein was cannulated using an I.V. catheter, and inferior vena cava was cut out. Next, the liver was perfused with prewarm $0.5 \times 10^{-3} \text{ M}$ ethylene glycol tetraacetic acid (EGTA) solution (in 37°C water bath) for 5 min at a flow rate of 5 mL min^{-1} then it was changed to prewarm perfusion medium (DMEM with 0.075% collagenase type I for hepatocyte or collagenase type II for Kupffer cells in 37°C water bath) for 5 min. During the perfusion, it was necessary to press inferior vena cava with iris curved forceps 30–40 s for two to three times in order to keep enough flow pressure in the liver. The liver was removed and placed in a sterile $60 \times 15 \text{ mm}^2$ tissue culture dish and the liver was cut with

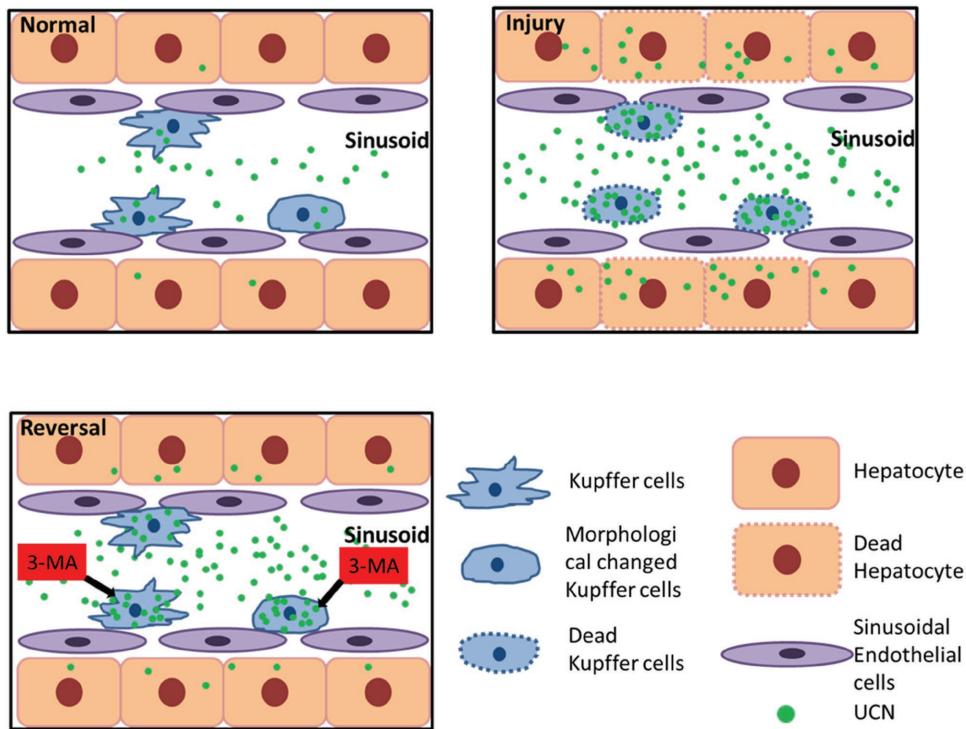


Figure 7. Kupffer cells prevent UCNs diffusion to hepatocytes and protect liver from nano-hepatotoxicity. Large dose of UCNs deplete Kupffer cells and escape out of sinusoids to hepatocytes, further induce hepatotoxicity. Inhibiting the autophagy of Kupffer cells promotes cells survival and diminishes the uptake of UCNs by hepatocytes, thereby abrogating the nano-hepatotoxicity.

iris scissors and then DMEM was added into tissue culture dish. The liver tissue solution was pipetted into a sterile 50 mL conical tube, it was made sure that the solution is up to 35 mL mark and then 5 mL perfusion medium was added. The tube was shaken at 150–210 rpm at 37 °C for 20–30 min, then cooled on ice. The cells were transferred and filtered through a 70 µm cell strainer into a clean 50 mL conical tube and centrifuged on 50 g for 5 min in room temperature. Cell pellets were resuspended in DMEM and centrifuged on 50 g for 5 min in room temperature.

For Hepatocyte Isolation: The above supernatants were discarded, cells were resuspended by adding 10 mL DMEM, and 15 mL 40% percoll was added underneath the existent cells suspension slowly. It was centrifuged on 400 g for 10 min at room temperature. Supernatants were discarded, cell pellets were resuspended in 50 mL 1× phosphate buffer solustion (PBS) and centrifuged on 50 g for 5 min in room temperature. Finally, supernatant was discarded, cell pellets were resuspended in 10 mL DMEM and counting the cells.

For Kupffer Cells Isolation: The above supernatants were discarded, cells were resuspended by adding 3 mL 20% percoll, and 3 mL 50% percoll was added underneath the existent cells suspension, adding slowly. It was centrifuged on 800 g for 15 min at room temperature. The middle layer was pipetted (Kuffer cell contained) to a new tube and topped up with DMEM, it was centrifuged on 500 g for 5 min at room temperature. Supernatant was discarded, cell pellets were resuspended in 1 mL DMEM and counting the cells. Then plated in noncollagen coated culture plate (60 mm dish or a six-well plate) for 20 min. Cells were washed with 1× PBS slowly to remove debris and unattached cells. To detach attached cells on the plate, cells were incubated with ethylene diamine tetraacetic acid (EDTA)–trypsin (0.25%) for 5 min at 37 °C. Cells were collected and washed with 1× PBS using 500 g for 10 min, twice. Cell number was counted (purity is over 90%) and cells were used as wanted.

Animal Experiments: Healthy male C57BL/ 6 mice, aged 6–8 weeks, ranging from 18 to 22 g were purchased from the SLAC Laboratory

Animal (Shanghai, China), animal welfare and experimental procedures were carried out in accordance with the Ethical Regulations on the Care and Use of Laboratory Animals of University of Science and Technology of China and were approved by the school committee for animal experiments. Mice were injected with liposomes and UCNs both in tail vein. CLL liposomes (25 mg mL⁻¹, 150 µL) or control liposomes were administrated 3 d before injection of saline or UCNs to deplete Kupffer cells. 3-MA liposomes (25 mg mL⁻¹, 200 µL) or control liposomes were given to the mice 30 min before the administration of saline or UCNs to inhibit the autophagy in Kupffer cells. After treatment, mice were sacrificed and mice liver were dissected, fixed in 4% PFA overnight. For HE staining, fixed mice liver were sent to the First Affiliated Hospital of Anhui Medical University to stain. For immunofluorescence or TUNEL assay, fixed mice liver were dehydrated in 30% sucrose overnight, and sliced to 8 µm cryosection by Cryostat Microtome (Leica CM1950), excess liver tissue were stored at -80 °C with OCT for further analysis. ALT level was determined with automatic biochemical analyzer (Rayto 240, Shenzhen, China) using a kit from Rongsheng (Shanghai, China).

Preparation of Clodronate Liposome and 3-MA Liposome: CLL was prepared in accordance with a previously published method. Briefly, DOPE and cholesterol were mixed in chloroform by the ratio of 7:3. Equal volume of methanol was added and dried the organic solvent to form a phospholipid film by Termovap Sample Concentrator with nitrogen. The phospholipid film was dispersed in PBS (for empty liposomes), 0.7 M clodronate solution (for clodronate liposomes) or 50 × 10⁻³ M 3-MA solution (for 3-MA liposomes) by vortex. Washed four to five times to remove nonencapsulated clodronate or 3-MA using centrifugal filter units (Millipore UFC801096). Finally, the drug loading of clodronate liposomes and 3-MA liposomes are defined as 55.36% and 21.78%, respectively.

Statistical Analysis: All data were expressed as mean ± SEM and analyzed by ANOVA. *P < 0.05, **P < 0.01, and ***P < 0.001 were considered statistically significant.

Supporting Information

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

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