Gold nanorod-mediated photothermolysis induces apoptosis of macrophages via damage of mitochondria

**Aims:** Induction of apoptosis or necrosis in activated macrophages by gold nanorod-mediated photothermolysis is demonstrated and the mechanisms underlying the processes are investigated. **Materials & methods:** Gold nanorods were functionalized with cysteine-octaarginine peptides (R8-NRs). Uptake of R8-NRs by activated macrophages was monitored by two-photon luminescence imaging. The laser irradiation conditions were controlled to induce apoptosis or necrosis to R8-NR-integrated macrophages. Mitochondrial damage and reactive oxygen species overproduction during photothermolysis was investigated by confocal fluorescence microscopy and transmission-electron microscopy. **Results:** Activated macrophages efficiently uptake R8-NRs both in vitro and in live animals. Laser irradiation of internalized nanorods with controlled power density induces apoptosis of macrophages via intracellular perturbation and subsequent injury of mitochondria. **Conclusions:** Gold nanorod-mediated photothermolysis provides one promising way to eliminate activated macrophages in autoimmune and inflammatory diseases.

Macrophages constitute a major component of the mononuclear phagocyte system and play an important role in tissue homeostasis and inflammation. However, when macrophages become activated unnecessarily, they could contribute to many autoimmune and inflammatory diseases, such as atherosclerosis [1], rheumatoid arthritis [2] and sarcoidosis [3]. During the development of these diseases, activated macrophages may release cytokines, chemokines, digestive enzymes and prostanandins to aggravate or accelerate damage to the normal tissues [4]. Therefore, detection and eradication of the macrophages in the injurious states provide an efficacious way to cure those diseases [5,6]. The distinctive function of macrophages to rapidly recognize and uptake particulate matter offers a preference to deliver nano-sized contrast agents (e.g., paramagnetic iron oxide nanocrystals [7]) and drug carriers (e.g., liposomes [8]) to macrophages. However, traditional drug carriers such as liposomes may induce considerable toxicity to healthy organs based on the biodistribution study [9]. A photomedicine method for selective eradication of activated macrophages with minimal toxicity to healthy organs would be attractive.

Optical hyperthermia has been used to eradicate diseased cells and tissues in a noninvasive manner. The photoactivated therapy could be localized and intensified by targeting exogenous agents with large absorption cross-sections to the area of interest and confining damage with minimal collateral effects. Gold nanospheres [10–13], nanoshells [14,15] and nanocages [15,16] have been successfully used in ablation of tumor cells and tissues. Recently, gold nanorods have become an ideal probe for theragnosis (combined therapy and diagnosis) owing to their unique properties. They can be easily synthesized [18,19] and modified with various surface modifications including antibody [20–23], surfactant [24], folic acid [25,26], phospholipid [27], DNA [28], peptide [29] and polymer [24,30–32]. Unlike gold nanospheres, the longitudinal plasmon resonance mode of nanorods could be tuned to the near-infrared region, a spectral window permitting relatively deep tissue penetration of photons. Besides a strong plasmon scattering [33,34], it has recently been shown that gold nanorods emit a bright two-photon luminescence (TPL) [35,36], allowing 3D imaging of nanorod-labeled cells [23,24]. Meanwhile, more than 96% of the absorbed photons in nanorods are converted into heat by the ultra-fast nonradiative electron relaxation dynamics [37], at a higher efficiency than gold nanospheres and nanoshells [38–40]. These properties make gold nanorods an attractive agent for gene delivery [28,41], cell imaging [22–24,42] and photothermalysis of tumor cells [25,26,39,43], macrophage cell lines [44] and pathogens [45,46].

In nearly all the photothermal injury studies, cell death was determined by checking the cell membrane permeability with fluorescent
indicators, such as trypan blue [39,43], calcine AM [26] and ethidium bromide [25,26]. No control of cell death in either the necrosis or apoptosis pathway has been considered. However, apoptosis is preferred for in vivo applications because necrosis might induce serious secondary inflammation. In addition, despite the report of membrane damage by irradiation of nanoparticles [10,12] and gold nanorods [26], the mechanisms underlying intracellular injury remain under-investigated.

In this article, we demonstrate the use of nanorod-mediated optical hyperthermia for elimination of primary activated macrophages. We show that gold nanorods functionalized with arginine-rich peptides could be selectively and efficiently internalized by activated macrophages in vitro and in vivo. Moreover, we demonstrate that controlled laser irradiation of internalized nanorods could lead to apoptosis by internal damage of mitochondria or acute necrosis by compromising the plasma membrane integrity.

Materials & methods

Preparation & characterization of R8-NRs

Gold nanorods were synthesized in micellar surfactant (cetyl trimethyltrimethylammonium bromide [CTAB]) solution using seeded growth method with the presence of AgNO₃ [18,47]. The growth was initiated 10–15 min after seed injection, carefully monitored by an UV-visible spectrophotometer (DU-530, Beckman, CA, USA) for the next 5–30 min and quenched at the desired wavelength with 15 min treatment of Na₂S [47]. The nanorods were then centrifuged (10,500 rpm for 10 min) to remove residual CTAB and sulfide and redispersed in milli-Q water, resulting in an optical density (O.D.) of 1.5–2.0. Particle size analysis by transmission-electron microscopy (TEM) indicated a mean length and aspect ratio of 45.5 nm and 3.8, respectively.

R8-NRs were prepared by conjugation of cysteine-octaarginine peptides (GenScript, NJ, USA) through an Au–S bond. Briefly, gold nanorod solution was centrifuged at 10,000 rpm for 6 min to remove unassociated CTAB and sulfide and redispersed in milli-Q water. A total of 2.5-ml nanorod solution (O.D. ~1.8) was treated with a dropwise addition of 200 µl aqueous solution containing 0.5 mg peptides. The mixture was kept at room temperature overnight and dialyzed (MWCO 6000–8000, Fisher Scientific, PA, USA) for 12 h to remove CTAB and unconjugated peptides. This procedure yielded a stable dispersion of R8-NRs with a plasmon resonance peak at 788 nm and a final O.D. close to 1.3. The extinction spectra of the nanorods were not affected by surface modification, as shown in Figure 1B. The nanorod concentration was estimated using the measured extinction coefficient (ε = 4.6 ± 0.6 × 10⁶ M⁻¹ cm⁻¹) [48], to be 0.28 and 0.22 nM for O.D. of 1.3 and 1.0, respectively. To determine the binding efficiency of peptide on the nanorod surface, we performed an amino acid analysis to measure the amount of peptides conjugated to gold nanorods. The analysis gave 73 nmol peptides for 3.94 pmol nanorods, corresponding to 1.85 × 10⁴ peptides per nanorod. PEGylated nanorods (mPEG-NRs) were prepared by a procedure similar to R8-NRs, with the addition of 3 µmol of mPEG₅K-SH (Rapp Polymere GmbH, Tübingen, Germany; aqueous solution, pH = 9) to 4 ml of nanorod solution.

Recruitment of peritoneal macrophages & cellular uptake of NRs

Dehydrated Brewer thioglycollate medium powder (Sigma-Aldrich, MO, USA) was dissolved in water (29.8 g/l) and autoclaved for 20 min at 15 lbs pressure (121°C). The autoclaved medium was kept in the dark under sterile conditions at room temperature for 3 months before use. Primary peritoneal macrophages were elicited by intraperitoneal injection of 1.5 ml of the sterile thioglycollate medium into BALB/c mice. On the fourth day, after euthanizing the mouse by CO₂, macrophages were collected by peritoneal lavage using 8 ml phosphate-buffered saline (PBS), plated into the glass-bottomed Petri dishes (MatTek, MA, USA) in RPMI 1640 medium (Invitrogen, CA, USA) with 10% fetal bovine serum (FBS; Sigma-Aldrich) and 1% penicillin–streptomycin (Invitrogen) for 2 h and washed with PBS three times to remove nonadherent cells. Then the macrophages were treated with an aliquot of R8-NRs (100 µl, 0.22 nM) or CTAB-coated nanorods (CTAB-NRs, 100 µl, 0.22 nM) or mPEG-NRs (100 µl, 0.22 nM), and incubated with periodic monitoring. For in vivo uptake experiments, 300 µl of R8-NR solution (0.22 nM) was injected into the peritoneal cavity of a mouse prestimulated with thioglycollate medium. The mouse was then euthanized and the peritoneal macrophages were collected at 0.5 h after injection, centrifuged at 1000 rpm for 10 min to remove free R8-NRs and plated on the Petri dish. Tri-color-labeled F4/80 antibody (1:200, Invitrogen) was used to mark macrophages.
Figure 1. In vitro uptake of R8-NRs by macrophages. (A) Structure of R8-NR. (B) Normalized extinction spectra of gold nanorods before and after conjugation with peptides. (C) R8-NRs (red) were efficiently internalized by primary activated macrophages. (D) Only a few mPEG-NRs entered activated macrophages. (E) Only a few R8-NRs entered KB cells. Scale bar: 10 µm. (F) TEM image (285000×) of R8-NRs inside a macrophage. For (C–F), macrophages or KB cells were incubated with 100-µl R8-NR solution for 3 h at 37°C. a.u.: Arbitrary unit; CTAB-NR: Cetyl trimethyltrimethylammonium bromide gold nanorod; mPEG-NR: PEGylated gold nanorod; N: Nucleus; R8-NR: Cysteine-octaarginine-conjugated gold nanorod; TEM: Transmission-electron microscopy.
Cell culture
KB cells (a carcinoma cell line) and Chinese hamster ovary (CHO) cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂ and grown continuously in folate-deficient RPMI 1640 medium containing 10% FBS and 1% penicillin–streptomycin. In a typical experiment, a 1-ml suspension of KB cells or CHO cells (10⁵/ml) was plated onto a glass-bottomed Petri dish, grown for 2 days, then treated with an aliquot of R8-NRs (100 µl, 0.22 nM) and maintained at 37°C with periodic monitoring.

TPL & TEM imaging
A femtosecond (fs) Ti:sapphire laser (MaiTai HP, Spectra-Physics, CA, USA) with a pulse width of 130 fs and a repetition rate of 80 MHz was used for TPL imaging of nanorods. The laser beam was directed into a scanning confocal microscope (FV1000/IX81, Olympus America Inc., CA, USA) equipped with a 60× water-immersion objective (NA = 1.2). The excitation power used was 0.7 mW at the sample. TEM images were acquired at Purdue University Life Science Microscopy facility (IN, USA) following standard procedures.

Controlled laser irradiation of macrophages loaded with R8-NRs
Macrophages incubated with R8-NRs were rinsed with fresh RPMI 1640 medium and irradiated with the Ti:sapphire laser beam with an expanded diameter of 5 mm at 788 nm for 15 min. The laser power was increased from 0.43 W (2.18 W/cm²) to 0.86 W (4.36 W/cm²). The macrophages were kept at 37°C during the irradiation to mimic the physiological environment. Control experiments were performed at the same condition with macrophages without R8-NRs.

Analysis of cell death
Cell death was determined using a standard apoptosis kit (Invitrogen) including Alexa Fluor 488 annexin V to indicate early apoptosis and propidium iodide (PI) to label necrosis. A total of 5 µl of Alexa Fluor 488 annexin V and 1 µl of PI (100 µg/ml) were added to macrophages in 100 µl annexin-binding buffer and incubated at room temperature for 15 min. As a positive control, paclitaxel (PTX)-induced apoptosis was performed by treatment of macrophages with 1 µM PTX for 6 h at 37°C. For quantitative analysis of cell death at different laser powers, the macrophages were plated in a 96-well plate with 100 µl RPMI medium in each well and treated with the R8-NR solution (0.22 nM, 10 µl for each well). The percentage of necrotic or apoptotic cells was determined by dividing the number of macrophages labeled with both PI and Alexa Fluor 488 annexin V or Alexa Fluor 488 annexin V alone, with the total number of macrophages counted in the well (>1300). A MTT assay was also performed to quantify the cell death. After laser irradiation, 10 µl MTT solution (5 mg/ml in PBS) was added to each well and incubated at 37°C for 3 h. After removing the medium, 200 µl dimethyl sulfoxide was added to each well and
Mechanism study
To examine the plasma membrane integrity and Ca^{2+} influx in apoptosis induced at lower laser power density and necrosis induced at higher laser power density, Oregon Green BAPTA 2 (Invitrogen) was added to macrophages with a final concentration of 4 μM and washed with medium before laser irradiation. For the mitochondria damage study, Mito Tracker Red CMXRos (membrane potential dependent, Invitrogen) or Mito Tracker Green FM (membrane potential independent, Invitrogen) was added to macrophages with a final concentration of 0.12 μM. To detect the level of reactive oxygen species, carboxy-H_{2}DCFDA (Invitrogen) was added to macrophages after irradiation with a final concentration of 25 μM and incubated at 37°C for 30 min. Fluorescence imaging was carried out on a confocal microscope; 488-nm Ar laser and 543-nm He/Ne laser were used for excitation with 10.0 and 9.5 μW at the sample, respectively. Fluorescence intensities of Mito Tracker Red or Oregon Green BAPTA 2 within more than 100 randomly selected macrophages were measured for statistical analysis.
Results & discussion

Gold nanorods with a length of 45.5 nm and aspect ratio of 3.8 were synthesized in micellar surfactant (CTAB) solution using the seeded growth method in the presence of AgNO₃ [18,47]. To replace the cytotoxic surfactant CTAB on the nanorod surface and promote the cellular internalization, gold nanorods were conjugated with cell-penetrating peptides, cysteine-octaarginine, to produce R8-NRs (Figure 1A). The surface modification with a coating of 1.85 x 10⁴ peptides per nanorod yielded a stable dispersion of gold nanorods without any change of the extinction spectra profile and the...

Figure 4. Examination of Ca²⁺ influx in apoptotic and necrotic macrophages under low and high power density. Macrophages were incubated with Ca²⁺ indicator Oregon Green BAPTA 2 (4 µM) for 25 min and washed before irradiation. (A) Control: macrophages after irradiation at 2.18 W/cm². (B) Apoptosis: nanorod (NR)-internalized macrophages after irradiation at 2.18 W/cm². (C) Necrosis: NR-internalized macrophages after irradiation at 4.36 W/cm². Necrosis was indicated by propidium iodide (red). (D) Statistical analysis of fluorescent intensity of intracellular Oregon Green at different conditions. Scale bar: 10 µm. a.u.: Arbitrary unit.
plasmon resonance peak at 788 nm (Figure 1B). Monitored by TPL imaging, the R8-NRs were found inside primary macrophages after 0.5 h incubation and the cellular uptake increased with time (Supplementary Figure 1A–C) (see online www.futuremedicine.com/toc/nnm/4/3). The efficiency was much higher than CTAB-NRs (Supplementary Figure 1D). After 3 h incubation, a high density of R8-NRs was found in the cytoplasm (Figure 1C). By contrast, few mPEG-NRs were observed after 3 h incubation (Figure 1D). Additionally, the uptake of R8-NRs in macrophages was much faster than other cell types. Only a few R8-NRs were found binding to KB cells (Figure 1E) or CHO cells (not shown) after 3 h incubation. The inefficient internalization in KB or CHO cells was likely due to the large size of the functionalized gold nanorods compared with clarhin-coated pits, which slowed down the uptake. On the other hand, the cationic arginine peptides could conceivably bind to the anionic components on the macrophage surface (e.g., cysteine-rich domain of mannose receptors) and trigger phagocytic uptake of nanorods. The phagocytic internalization of gold nanorods in macrophages was supported by TEM (Figure 1F), where an accumulation of nanorods was found in the cytoplasmic vesicles. The cytotoxicity of R8-NRs in macrophages was examined by using calcein AM to indicate the viability and ethidium bromide staining of nuclei to indicate increased membrane permeability. After 32 h incubation with R8-NRs, the macrophages were strongly labeled with calcein AM, whereas the ethidium bromide signal was undetectable, showing little cytotoxicity of nanorods (Supplementary Figure 2).

Figure 5. Mitochondria damage during apoptosis. The laser irradiation was at 2.18 W/cm² for 15 min in all experiments. (A–C) Decrease of mitochondrial membrane potential. Mitochondria were labeled by Mito Tracker Red CMXRos (0.12 µM). Apoptotic macrophages were indicated by Alexa Fluor 488 annexin V (green). (A) Macrophages without R8-NRs did not undergo apoptosis and were strongly labeled with Mito Tracker Red. (B) R8-NR-internalized macrophages were apoptotic (green) and the labeling of Mito Tracker Red significantly reduced. (C) Fluorescent intensity of intracellular Mito Tracker Red dropped 70% in R8-NR-internalized macrophages and 16.8% in macrophages without NRs. (D) Mitochondria swelling (labeled with Mito Tracker Green FM) after laser irradiation. Scale bar: 10 µm. (E & F) Transmission-electron microscopy images before and after laser irradiation (×285000) also indicated mitochondria swelling. a.u.: Arbitrary unit; M: Mitochondria; N: Nucleus; NR: Nanorod; R8-NR: Cysteine-octaarginine-conjugated gold nanorod.
To test whether R8-NRs could target macrophages in vivo, 300 µl of R8-NR solution (0.22 nM) was directly injected into the peritoneal cavity of a mouse prestimulated with thioglycollate. After 0.5 h, the mouse was euthanized and peritoneal macrophages were collected by lavage. Tri-color-conjugated F4/80 antibody was used to mark the activated macrophages. After washing the macrophages with PBS, F4/80 labeling and R8-NR internalization were examined by sequential confocal fluorescence and TPL imaging. As shown in Figure 2A & B, only F4/80-positive cells showed R8-NR labeling. The small round cells (presumably neutrophils) did not uptake any R8-NRs. Compared with the in vitro results, higher in vivo uptake efficiency was found during the short 0.5 h incubation period (Figure 2C). The

Figure 6. Reactive oxygen species overproduction during nanorod-mediated photothermalysis. After laser irradiation at 2.18 W/cm² of macrophages with (A & B) or without (C & D) NRs, reactive oxygen species (ROS) indicator (carboxy-H₂DCFDA; green) was added and incubated for 30 min. (A & B) ROS was detected (green) in apoptotic macrophages with NRs after irradiation. (C & D) No ROS was detected in macrophages without NRs at the same condition. Scale bar: 10 µm.

NR: Nanorod.
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selective internalization allows subsequent photothermolysis of activated macrophages by laser irradiation of internalized nanorods.

We first optimized the laser irradiation conditions to induce apoptosis or necrosis to R8-NR-internalized macrophages by changing the power of an fs laser beam with a beam diameter of 3 mm. The laser wavelength was tuned to 788 nm, corresponding to the longitudinal plasmon peak of the nanorods used. Alexa Fluor 488 annexin V (green), a phosphatidylserine indicator, was used to label the plasma membrane of apoptotic cells and PI (red) was used to stain the nuclei of necrotic cells. We counted the macrophages that underwent apoptosis and necrosis at different laser powers (FIGURE 3D). When the laser power was as high as 0.86 W (power density 4.36 W/cm²), 98% of nanorod-internalized macrophages were damaged after 15 min irradiation, and 76% underwent necrosis, with both membranes (green) and nuclei (red) stained (FIGURE 3A). By reducing the laser power, the percentage of necrotic cells decreased. At the power density of 2.18 W/cm² (0.43 W), 73% of the macrophages were apoptotic and were only labeled by Alexa Fluor 488 annexin V on the membrane (green, FIGURE 3B). After further incubation at 37°C for 30 h, the macrophages underwent late apoptosis, stained with PI (SUPPLEMENTARY FIGURE 3A & B). The laser power density was lower than the one used in the earlier studies with gold nanorods (10 W/cm²) [39] and nanoshells (35 W/cm²) [44], possibly because of the efficient uptake of R8-NRs by macrophages and the use of an fs laser. The fs pulses were shown to be more effective than a continuous-wave laser in the optical-thermal transition for gold nanoparticles [26]. As a negative control, macrophages without nanorods were irradiated under the same condition. Little staining of Alexa Fluor 488 annexin V was observed (FIGURE 3C) and the macrophages remained viable after further incubation for 30 h. As a positive control, macrophages were treated with 1 μM PTX, a drug known to induce apoptosis [49], for 6 h. It was found that the treated cells could be labeled by Alexa Fluor 488 annexin V (SUPPLEMENTARY FIGURE 3C) in a similar way to FIGURE 3B. With the laser power further reduced, the cell death efficiency decreased to less than 50% (FIGURE 3D). MTT assay was also performed to quantify the cell death and the result is shown in FIGURE 3E. After laser irradiation at 2.18 W/cm², the O.D. at 570 nm was reduced by 54.8% for macrophages with nanorods, but only by 9.4% for the control group without nanorods. These results together confirmed that nanorod-mediated optical hyperthermia could effectively eradicate activated macrophages in the apoptosis or necrosis pathway by controlling laser irradiation condition, and we used 2.18 W/cm² (0.43 W) in our later study as the optimized condition to induce apoptosis.

We further investigated the mechanisms underlying the observed necrosis and apoptosis. In previous photothermal therapy studies, damage of the plasma membrane by irradiation of nanoparticles [10,12] and gold nanorods [26] was reported. Therefore, we first examined the plasma membrane integrity and Ca²⁺ influx into cells under the necrosis and apoptosis conditions. We incubated the macrophages with Oregon Green BAPTA 2, a common Ca²⁺ indicator, and washed the cells before laser irradiation. The control experiment was performed with macrophages without nanorods to estimate the percentage of photobleaching. After laser irradiation at 4.36 W/cm² for necrosis or 2.18 W/cm² for apoptosis, the intracellular intensity of Oregon Green BAPTA 2 was compared. As shown in FIGURE 4, the intensity level of Oregon Green was similar in the control and the apoptotic condition, while demonstrating an obvious increase during necrotic conditions. These results indicate that loss of plasma membrane integrity and Ca²⁺ influx occurred in necrosis but not in apoptosis during the nanorod-mediated photothermolysis.

We have studied the role of intracellular injury in the induction of apoptosis. At a molecular level there are two principal mechanisms for apoptotic cell death, extrinsic (mitochondria-independent apoptosis) and intrinsic (mitochondria-dependent apoptosis) [50]. Therefore, we examined whether mitochondria were damaged by nanorod-mediated optical hyperthermia. After laser irradiation of nanorod-internalized macrophages at 2.18 W/cm² for 15 min, Mito Tracker Red CMXRos and Alexa Fluor 488 annexin V were used to detect mitochondrial membrane potential change [51] and indicate apoptosis, respectively. In comparison with nonradiated macrophages, the fluorescent intensity of Mito Tracker Red decreased by 70% in apoptotic macrophages, but by only 16.8% in the control group without nanorod labeling (FIGURE 5A–C). Morphological change of mitochondria was also detected at the same condition with labeling of Mito Tracker green FM. As shown in FIGURE 5D, both mitochondrial fission and swelling were observed. Similar results were also found in the TEM images shown in FIGURE 5E & F. In addition, we also detected an overproduction of reactive oxygen species in the apoptotic macrophages after laser irradiation (FIGURE 6), a sign
of oxidative stress. These observations provide further evidence of mitochondrial damage [52]. Together, the results suggested a mitochondria-dependent apoptosis pathway for nanorod-mediated photothermolysis of macrophages.

The detailed pathway of mitochondrial damage is beyond the scope of the current paper. We anticipate that irradiation of gold nanorods and local energy deposition could generate heat and cavitation, in other words, bubble formation and collapse [12,53]. The localized heat and cavitation might cause cellular stress and disruption of some intracellular compartments such as the endoplasmic reticulum. These intracellular perturbations and signals released could lead to the formation of mitochondrial permeability transition pores [54], causing membrane potential decrease and mitochondrial swelling. Upon the permeability transition, apoptogenic factors (e.g., cytochrome c) could leak into cytoplasm and trigger a cascade of apoptotic cell death.

**Conclusion**

The current work demonstrated that gold nanorods conjugated with arginine-rich peptides could be selectively internalized by activated macrophages in vitro and in vivo without the assistance of antibody- or ligand-mediated targeting. Utilizing gold nanorods as efficient optothermal energy converters, apoptosis of macrophages was induced in a mitochondria-dependent pathway during nanorod-mediated optical hyperthermia under the optimized laser power density of 2.18 W/cm². As activated macrophages are becoming a valid pharmaceutical target of the autoimmune and inflammatory diseases, optical hyperthermia using gold nanorods provides a promising alternative to theragnostic activation of macrophages and to treat the related diseases. The controlled cell death and better understanding of the mechanism facilitate the translation of the laboratory innovation to the clinic.

**Future perspective**

With the development of catheter-based optical fiber, lasers have been used in clinical therapy to ablate atherosclerotic lesions. Taking the advantages of selective uptake and localized hyperthermia of macrophages mediated by gold nanorods, the efficiency and accuracy of laser surgery could be greatly improved. As demonstrated in our work, cell death could be induced in an apoptosis pathway to reduce the inflammatory effect caused by necrosis.

Several issues have to be considered when pushing the application to the in vivo level. First, the surface coating needs to be designed to ensure elongated circulation of gold nanorods in blood and sufficient accumulation in the lesion area. As shown in Figure 1, octaarginine peptides could increase the efficiency of internalization of gold nanorods in macrophages, while coating with polyethylene glycol (PEG) could reduce uptake by macrophages. Therefore, gold nanorods with both PEG protection and specific ligands to trigger efficient uptake by macrophages at the site of injury could be applicable. Second, although colloidal gold has been used in vivo since the 1950s [55], it remains unknown whether gold nanorods cause organ toxicity. The viability of cultured cells after long-term incubation of gold nanorods might not represent what will occur in the in vivo environment. These issues are being addressed by investigating the pharmacokinetic behavior and toxicity of functionalized gold nanorods in animal models in our laboratory.

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**Financial & competing interests disclosure**

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**Executive summary**

**Efficient uptake of cysteine-octaarginine-conjugated gold nanorods by activated macrophages**

- Conjugated with arginine-rich peptides, gold nanorods could be efficiently internalized by primary activated macrophages in vitro and in live animals.

- Cellular uptake of gold nanorods is monitored by two-photon luminescence.

**Apoptosis & necrosis induced by nanorod-mediated photothermolysis**

- Apoptosis or necrosis to nanorod-internalized macrophages could be induced by controlling the laser irradiation condition.

- The current study demonstrates a promising method to treat inflammatory diseases via photothermal elimination of activated macrophages.

**Mechanisms of photothermolysis**

- Apoptosis was induced by intracellular mitochondrial damage. Reactive oxygen species release, mitochondrial membrane potential drop, and mitochondrial swelling were observed.

- Necrosis was induced by disruption of plasma membrane integrity and subsequent Ca²⁺ influx.
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Ethical conduct of research
The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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