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ORIGINAL ARTICLE

Accumulation of gold nano-rods in the failing heart of transgenic mice with the cardiac-specific expression of TNF-α

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Abstract

Gold nano-rods, rod-shaped gold nanoparticles, act as contrast agents for in vivo bioimaging, drug delivery vehicles and thermal converters for photothermal therapy. Pro-inflammatory cytokines play critical roles in the development of heart failure. We examined the delivery of GNRs into the failing heart of a transgenic (TG) mouse model of inflammatory cardiomyopathy with the cardiac-specific overexpression of TNF-α. We modified GNRs with polyethylene glycol (PEG) to avoid cytotoxicity and reduce the rapid clearance of nanoparticles from blood. PEG-modified GNRs (4.5 mM as gold atoms, 200 μL) were administered intravenously to TG ($n = 7$) and wild-type (WT) mice ($n = 5$). These were killed 24 h later, and the heart, lung, liver, kidney and spleen were excised. A quantitative analysis of gold was performed using inductively coupled plasma mass or optical emission spectrometry. The amount of gold (ng) in the TG heart (3.24 ± 1.56 ng/mg heart weight) was significantly greater than that in the WT heart (1.01 ± 0.19; $p < 0.05$). No significant differences were observed among the other organs of TG and WT mice. The amount of gold in the TG heart was significantly and positively correlated with the ratio of the ventricular weight to body weight, which is known to be an index of ventricular hypertrophy. In conclusion, PEG-modified GNRs accumulated in the inflammatory TG heart in proportion with the severity of ventricular hypertrophy.

Keywords Gold nano-rod · Heart failure · Inflammation · TNF-α

Introduction

Inflammation is an initial and common symptom of the pathogenesis of various conditions, including heart failure. The development and progression of heart failure have been recognized to be associated with an inflammatory process because of the associated activation of pro-inflammatory cytokines [1–3]. Although heart failure is associated with an inflammatory process, we do not have imaging technologies or drug delivery systems to target inflammatory lesions in the heart at the molecular or cellular levels.

Gold nano-rods (GNRs) are rod-shaped gold nanoparticles which have two distinctive different absorption bands derived from the transverse and longitudinal surface plasmon resonance of free electrons in the visible and near-infrared regions, respectively. GNRs absorb near-infrared light, which is suitable for in vivo applications such as imaging and for photoradiation therapy, because of the maximal penetration of light into tissues [4]. GNRs have a photothermal effect (i.e., the absorbed light energy is converted into heat). Thus, GNRs are expected to have an application as contrast agents for near-infrared imaging and exothermic nanodevices for photothermal therapy. To apply GNRs as medical nanodevices, biocompatible GNRs have been prepared by coating GNRs with phosphatidylcholine [5], or by modifying GNRs with polyethylene glycol (PEG) [6]. PEG-modified GNRs show high dispersion stability and high circulation stability in the blood after intravenous injection into mice [6]. PEG-modified GNRs have been applied to the near-infrared imaging and photodynamic/photothermal

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therapy of tumors [4, 7–9]. The accumulation of GNRs in tumors was mediated by the enhanced permeability and the retention effect [10, 11]. They also have potential applications as a drug delivery system because the surface of GNRs is easily modified to attach small molecules or deoxy nucleotide acid, which are released to the targeted organ on exposure to light irradiation [12].

A recent study reported the successful targeted delivery of GNRs to inflammatory articular tissues in a rat model of adjuvant-induced arthritis [13]. To our knowledge, the accumulation of GNRs in the inflammatory myocardium has not been reported. We developed transgenic TNF1.6 mice (TG mice), a mouse model of inflammatory cardiomyopathy, which exhibit the heart-specific overexpression of TNF-α [14]. The TG mice displayed cardiac hypertrophy, dilatation, and dysfunction, with myocardial infiltration leading to congestive heart failure with upregulated levels of pro-inflammatory myocardial cytokines (TNF-α and IL-1β) [15]. We examined the uptake of the PEG-modified GNRs in the failing TG heart in comparison to wild-type (WT) mice and further investigated the effect of the degree of ventricular hypertrophy on the accumulation of GNRs in the heart.

Materials and methods

Animals

Twelve- and 24-week-old male TG mice and their WT littermates were used for the investigation. TG mice displayed a variation in the time course of the development of heart failure, with marked cardiac hypertrophy at 12 weeks of age and marked ventricular dilation with congestive heart failure at older ages. Approximately, 50% of the mice died from congestive (decompensated) heart failure by 24 weeks of age [16]. The over-expression of the transgene (murine TNF-α) is driven by the α-myosin heavy chain (α-MHC) promoter, which restricts the expression of the protein to cardiac myocytes. The TG mice were identified by a PCR with a sense primer (5′-CCA CAT TCT TCA GGA TTC TCT-3′) specific to the α-MHC promoter exon 2 and an antisense primer (5′-CAG CCT TGT CCC TTG AAG AGA-3′) specific to the TNF-α cDNA nucleotides 579–599 [17]. All mice were of the same FVB genetic background. The experimental protocol conformed to the eighth edition of the Guide for the Care and Use of Laboratory Animals published by National Research Council and was approved by the Ethics Committee on Animal Experiments of Kyushu University Graduate School of Medical Sciences.

Preparation of PEG-coated GNRs

GNRs were kindly provided by Dai Nippon Toryo Co. Ltd. (Tokyo, Japan). The length and width of the obtained GNRs were approximately 50 nm and 10 nm, respectively. A suspension of GNRs containing the cationic detergent 1-hexadecyltrimethylammonium chloride (cetyltrimethylammonium chloride; CTAB) was stored at a constant temperature (37 °C). GNRs were modified with PEG chains. Briefly, 18 mL of the GNRs in suspension (1.6 mM as gold atoms) were centrifuged at 9170×*g* for 15 min at 37 °C, decanted, and re-suspended in water to remove excess CTAB. Thiol-terminated mPEG solution (molecular weight ~ 5000 Da; NOF Corporation, Tokyo, Japan) was added to the GNRs suspension at a PEG:gold molar ratio of 1.0. The mixture of PEG solution and GNRs suspension was stirred for 24 h at room temperature. The mixture was then centrifuged twice at 9170×*g* for 15 min at 37 °C, decanted, and re-suspended in water to remove the remaining CTAB and excess PEG reagent. Before injection into mice, the suspension was concentrated to 4.5 mM (as gold atoms). GNRs and the PEG layer on the GNRs were imaged with a transmission electron microscope (JEM-1400Plus, JEOL, Tokyo, Japan) after staining the PEG layer with 1% phosphotungstic acid. The zeta potentials of GNRs in water were evaluated using a Malvern Zetasizer Nano ZS (Malvern, Worcestershire, UK). The dispersal of GNRs in solution was confirmed from the absorbance spectrum using a V-670 spectrophotometer (JASCO, Tokyo, Japan).

Study protocol and tissue preparation

PEG-modified GNRs were injected intravenously via the tail vein into TG and WT mice. The dose of PEG-modified GNRs was fixed at 177 μg of gold in 200 μL of 5% glucose solution. Mice were killed at 24 h after injection and the major organs (liver, lung, heart, spleen and kidney) were excised. Freshly isolated mouse hearts were fixed with 4% paraformaldehyde and 0.5% glutaraldehyde. The fixed samples were sliced, stained with hematoxylin and eosin and observed under an optical microscope (Olympus BX41). For transmission electron microscope observation, the fixed samples were re-fixed with 1% osmium (VIII) oxide, embedded in Epon 812, cut, and stained as described previously [18], and observed under a transmission electron microscope (JEM-1400Plus, JEOL, Tokyo, Japan).

The quantitative analysis of gold

The samples were divided into appropriate sizes, and were added to approximately 4 mL of aqua regia, then heated overnight at 80–90 °C. After further heating to 130–140 °C for 2 h, the organic compounds were completely oxidized, the gold was ionized and the samples were completely dried. The dried residues were dissolved in 1 mL of 0.5 M HCl. A quantitative analysis of gold was performed by inductively coupled plasma mass spectrometry (ICP-MS) using an iCAP RQ ICP-MS (ThermoFisher Scientific, Waltham, MA, USA) for hearts and inductively coupled optical emission spectrometry (ICP-OES) using an iCAP 7400 ICP-OES (ThermoFisher Scientific, Waltham, MA, USA) for other organs.

Statistical analyses

Student's *t* test was used to compare continuous variables. Correlations between 2 parameters were assessed using simple linear regression. Pearson's correlation coefficient (*r*) was then obtained. The results are presented as the mean ± standard deviation. The analyses were performed using the SPSS software package for Windows (version 11.5). *p* values of < 0.05 were considered to indicate statistical significance.

Results

PEG-modified GNRs

As shown in Fig. 1, the PEG layer (stained with 1% phosphotungstic acid) on the GNRs was shown as a gray shadow on a transmission electron microscopy image, which contributed to the high dispersion stability and high circulation stability in the blood that was observed after intravenous injection. The zeta potentials of the GNRs was almost zero (data not shown), indicating that PEG layer had fully coated the surface of the GNRs, as was previously reported by our group [6].

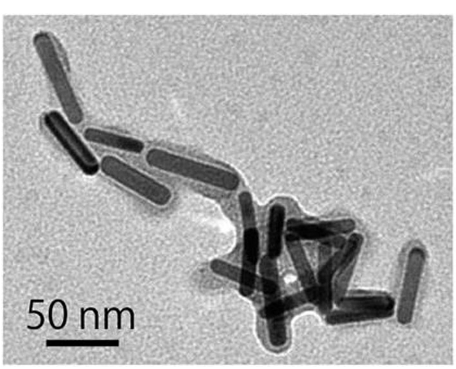

Fig. 1 TEM image of PEG-modified GNRs. Samples were stained with 1% phosphotungstic acid, and then images were taken. Scale bars = 50 nm. *TEM* transmission electron microscopy, *PEG* polyethylene glycol, *GNRs* gold nano-rods

GNRs accumulated in the TG myocardium

Hematoxylin and eosin staining showed the diffuse interstitial infiltration of the myocardium of TG mice (Fig. 2a) in comparison to their WT littermates (Fig. 2b). These findings were consistent with the findings of a previous paper which showed that myocarditis was induced by the overexpression of TNF-α in the TG myocardium [17]. GNRs scattered in the TG myocardium were detected by transmission electron microscopy (Fig. 2c).

The amount of GNRs in the TG and WT heart

The amount of gold in the heart at 24 h after intravenous administration, as measured by ICP-MS, was 3.24 ± 1.56 and 1.01 ± 0.19 (ng/mg heart, $p < 0.05$) in the TG ($n = 7$) and WT ($n = 5$) mice, respectively; thus, approximately 3 times as much gold was detected in the TG heart as in the WT heart (Fig. 3a). Figure 3b shows a significant positive correlation between the ratio of ventricular weight to body weight (VW/BW) and the gold concentration in the TG ($r = 0.809$, $p = 0.028$; $n = 7$) but not WT heart ($r = -0.807$, $p = 0.098$; $n = 5$). We previously reported that the VW/BW is an index of ventricular hypertrophy [17]. These results indicate that PEG-modified GNRs are able to accumulate in the inflammatory heart in proportion with the severity of ventricular hypertrophy.

The biodistribution of GNRs in mouse organs

In Fig. 4a, biodistribution of PEG-modified GNRs in the organs of TG and WT mice is presented as the % injection dose per organ. It has been already shown that at 24 h after the injection of PEG-modified GNRs, the amount of GNRs in the blood is small and that most GNRs are distributed to organs [6, 11]. These accumulated GNRs are probably cleared from these organs by the reticuloendothelial system [19]. The results show that the highest levels are found in the liver, followed by spleen, kidney, heart, and lung, and that with the exception of the heart, there were no significant differences in the amounts of GNRs in these organs between TG and WT mice. The % injected GNRs in the TG heart (0.40%) was significantly higher than that in the WT heart (0.06%; $p < 0.05$). In contrast, when we expressed the gold content in tissues (absolute amount of gold, ng) as per mg of organ, the highest exposure was observed in the spleen, followed by the liver (Fig. 4b). The bar graph of the heart presented in Fig. 4b is the same as that in Fig. 3a.

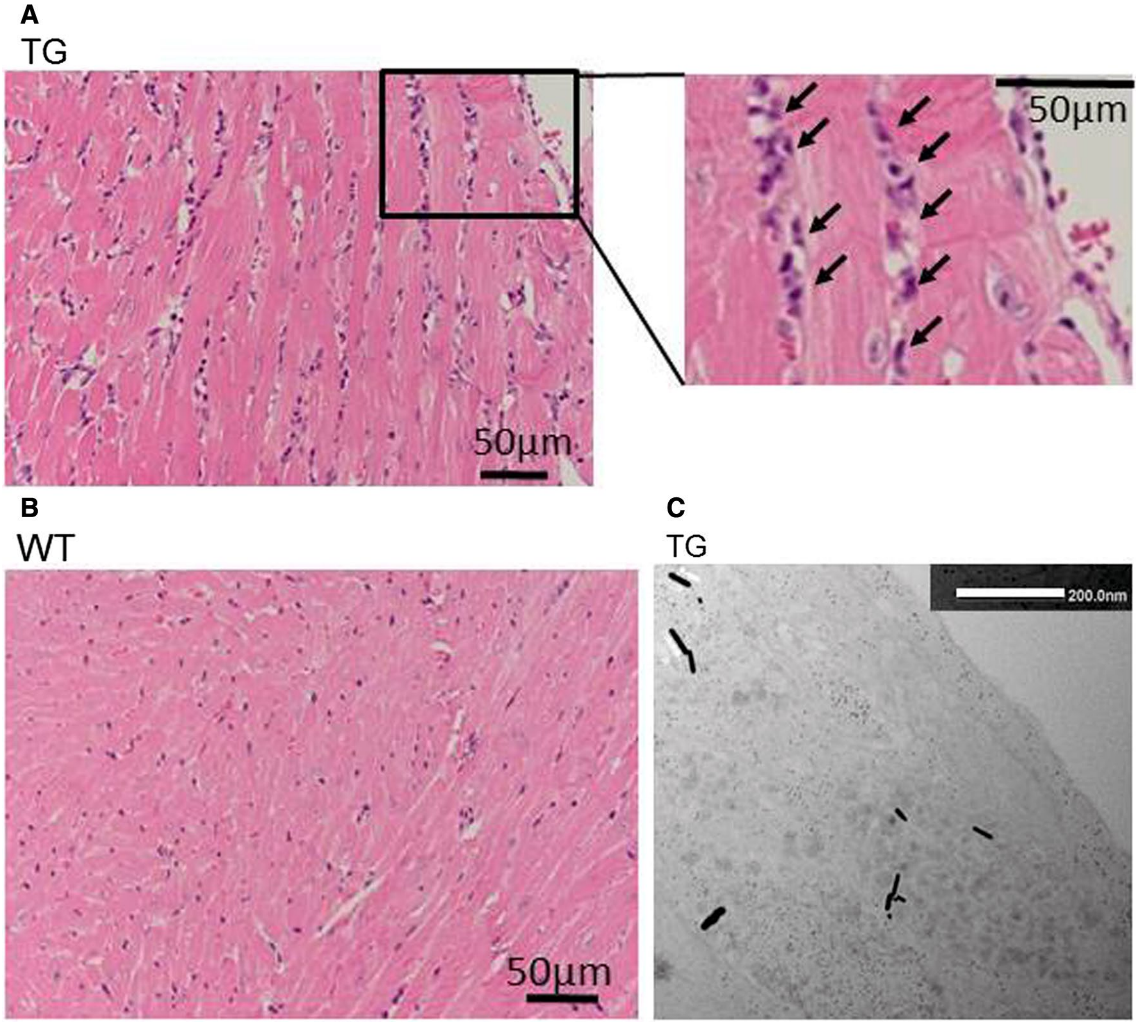

Fig. 2 H/E staining and TEM images of the TG myocardium. Representative H/E images of the **a** TG and **b** WT myocardium were obtained from three each mice, with five slices per mouse. The scale bar indicates 50 µm. The inset at a higher magnification shows the perivascular inflammatory infiltrate (arrows). **c** Representative TEM images of PEG-modified GNRs accumulated in the TG myocardium were obtained from three mice, with five slices per mouse. The scale bar indicates 200 nm. *H/E* hematoxylin and eosin, *TEM* transmission electron microscopy, *TG* transgenic mouse, *WT* wild-type mouse, *PEG* polyethylene glycol, *GNRs* gold nano-rods

Discussion

We demonstrated that PEG-modified GNRs successfully accumulated in increased amounts in the failing heart in our mouse model of inflammatory cardiomyopathy in comparison to the normal heart. Our findings also showed that GNRs have the potential to accumulate in the failing heart in proportion with the severity of ventricular hypertrophy. We previously reported that the VW/BW is an index of ventricular hypertrophy [17] and well-correlated with the cardiac function estimated by echocardiography in TG mice [3]. In addition, the ablation of TNF receptor 1 or inhibition of NF-κB activity in TG mice was found to significantly improve the left ventricular systolic function and reduce the ventricular hypertrophy, as evidenced by a significant decrease in the VW/BW [3, 15]. Our study demonstrated a significant positive correlation between the VW/BW and the gold concentration in the TG heart ($p = 0.028$). These results suggest that PEG-modified GNRs may be able to accumulate in the TG heart in proportion with the severity of heart failure.

Recently, novel strategies for delivering small particles into the failing myocardium have been studied using nanoparticles such as nano-sized liposomal FK506 [20] and a nanoplatform called mesoporous silicon vector [21] in a rodent model of heart failure. They examined the tissue distribution of liposomal FK506 and mesoporous silicon vectors in organs by measuring [^{3}H] radioactivity and epifluorescence, respectively [20, 21]. With liposomal FK506, the accumulation of [^{3}H]-FK506 in the whole heart was

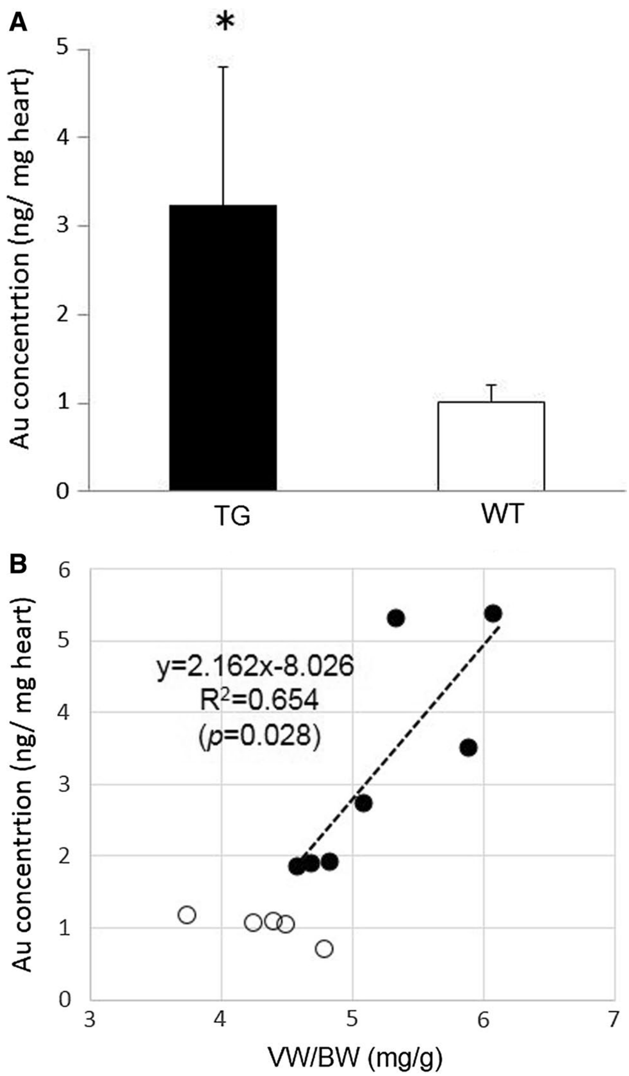

Fig. 3 The concentration of gold in the TG and WT heart. PEG-modified GNRs were intravenously injected into TG ($n=7$) and WT ($n=5$) mice. After 24 h, the amounts of gold (ng/mg heart) in the heart were evaluated by ICP-MS. **a** The amount of gold that accumulated in the TG heart was significantly increased in comparison to the WT heart. **b** A significant positive correlation was noted between the VW/BW and the concentration of gold in the heart in TG filled circle) but not WT (unfilled circle) mice. The data represent the mean value and the bars represent the standard deviation. *$p<0.05$, vs. WT. *TG* transgenic mouse, *WT* wild-type mouse, *PEG* polyethylene glycol, *GNRs* gold nano-rods, *ICP-MS* inductively coupled plasma mass spectrometry, *VW/BW* ventricular weight/body weight

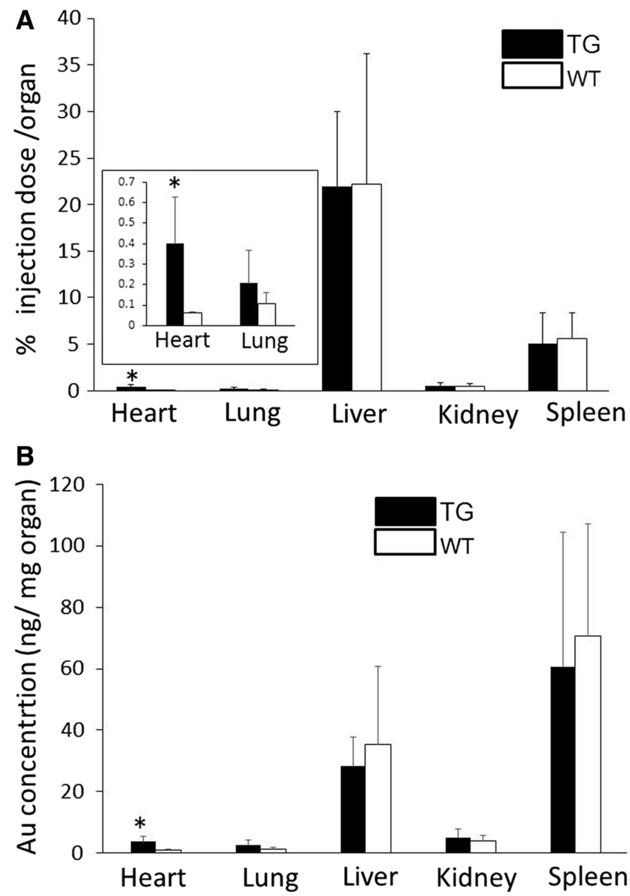

Fig. 4 The biodistribution of GNRs in the TG and WT mice. PEG-modified GNRs were intravenously injected into TG and WT mice. After 24 h, the amounts of gold in the heart, lung, liver, kidney and spleen were evaluated by ICP-MS (for the heart), and ICP-OES (for other organs). **a** The % injection dose of gold per organ (TG, $n=5$–7; WT, $n=5$). **b** The amount of gold (ng) as per mg of organ (TG, $n=5$–7; WT, $n=5$). The data represent the mean value and the bars represent the standard deviation. *$p<0.05$, vs. WT. *GNRs* gold nano-rods, *TG* transgenic mouse, *WT* wild-type mouse, *PEG* polyethylene glycol, *ICP-MS* inductively coupled plasma mass spectrometry, *ICP-OES* inductively coupled optical emission spectrometry

significantly increased (1.37-fold) in comparison to free FK506. A 14-fold increase in the uptake of mesoporous silicon vector was detected in the failing heart, which was imaged by high-content fluorescence microscopy. We measured the gold content in the organs by ICP-MS/OES. The determination of the gold content in the organs by ICP-MS/OES is a good measure of the tissue distribution of gold nanomaterials, because it directly measures the gold content in the tissues or serum samples [11]. In our study, the gold content in the TG heart was 3 times higher than that of the WT heart at 24 h after the injection of GNRs. The mechanism underlying the accumulation of GNRs in the TG myocardium is not known; however, it is most likely due to the ongoing inflammation and endothelial dysfunction that occurs within the local microenvironment of the inflammatory heart [22], and which is similar to the enhanced permeability and retention effect in the cancer vascular microenvironment [10, 11].

We investigated the tissue distribution of PEG-modified GNRs in the TG and WT mice. We previously reported that 54% of the injected dose of gold was found in blood at 0.5 h after the injection of PEG-modified GNRs into mice and that only 8% of gold remained in the blood at 24 h after injection [6]. The amount of gold in the blood decreased in a time-dependent manner, with no gold observed at 72 h [6]. In contrast, 35% of the injected dose had accumulated in the liver at 72 h after injection. In the present study, injected PEG-modified GNRs mainly accumulated in the liver and spleen, which is consistent with our previous report [23]; a small amount of gold was found in other organs, and was probably cleared by the reticuloendothelial system. When we considered the biodistribution of gold as the % injection dose of gold per organ, the highest levels were in the liver followed by the spleen (Fig. 4a). The increase in the hepatic accumulation of gold may be due to the uptake by the hepatic parenchyma through hepatic sinusoidal endothelial fenestrations below 120 nm in diameter by modification with PEG on the GNRs used in the present study [11]. In contrast, when we expressed the gold content in the tissues (absolute amount of gold, ng) as per mg of organ (ng/mg organ), the highest exposure was observed in the spleen, followed by liver (Fig. 4b). In our previous report [11], we demonstrated that the uptake capacity of GNRs in the spleen was higher than in the liver. The liver uptake may be saturated, allowing more gold to be distributed to the other tissues, such as the spleen, at the level of the injection dose of gold (relatively high dose) used in the present study.

PEG-modified GNRs showed low cytotoxicity [6]. This is necessary for the medical application of GNRs. Considering that GNRs may be administered to humans, the assessment of their safety is getting more and more attention. PEG modification is an excellent candidate approach for stabilizing GNRs in physiological conditions, and enabling long-lasting circulation in blood, due to its 'stealth character' [6]. Yang et al. [24] evaluated the chronic cardiac toxicity of PEG-coated spherical gold nanoparticles (not rod-shaped but spherical particles) on the cardiac systolic function, hypertrophy, fibrosis and inflammation after repeated administration via the mouse tail vein. They found that chronic gold nanospheres exposure did not induce inflammatory cell infiltration in the mouse heart. Interestingly, PEG-coated gold nanospheres (50 nm) led to a significant decrease in the TNF-α and IL-1β protein levels and in the collagen I gene expression in the mouse heart at 12 weeks after the repeated administration of gold nanoparticles. They suspected that chronic 50-nm-gold-nanosphere exposure may serve as a strategy to inhibit the expression of inflammatory factors and collagen. Ruiz-Esparza et al. [21] also reported that cardiac cells are able to internalize and traffic nanoparticles to the perinuclear cellular regions, and that this process was non-toxic. We need to confirm the chronic cardiac toxicity of PEG-modified GNRs in TG mice.

In the present study, we used a transgenic mouse model of inflammatory cardiomyopathy. TG mice are useful for quantitating the amount of gold in the myocardium because of its reproducibility of diffuse interstitial inflammation in the myocardium. We believe that TG mice are the best model for investigating the accumulation of nanoparticle in heart and show utility for revealing the relationship between the gold content and ventricular hypertrophy. Different states of heart failure exist and the future applications of GNRs will have to be explored in other models of heart failure, such as mice with myocardial infarction constructed by coronary ligation [25], as the accumulation dynamics of GNRs in non-ischemic and ischemic states may differ according to important pathophysiological differences.

GNRs are a promising candidate nanoparticle, which have been expected to act as a contrast agent for in vivo bioimaging, as drug delivery system, and as a thermal converter for photothermal therapy [12]. The targeted delivery of nanoparticles to the inflammatory myocardium following systemic injection is important for future medical applications to develop nanotechnology-based therapeutics and diagnostics to better treat and manage heart failure associated with inflammation.

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Compliance with ethical standards

Ethical approval All procedures performed in this study involving animals were in accordance with the ethical standards of our institution.

Conflict of interest The authors declare no conflicts of interest in association with the present study.

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