Glycyrrhizic Acid Nanoparticles as Antiviral and Anti-inflammatory Agents for COVID-19 Treatment

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ABSTRACT: COVID-19 has been diffusely pandemic around the world, characterized by massive morbidity and mortality. One of the remarkable threats associated with mortality may be the uncontrolled inflammatory processes, which were induced by SARS-CoV-2 in infected patients. As there are no specific drugs, exploiting safe and effective treatment strategies is an instant requirement to dwindle viral damage and relieve uncontrolled inflammation simultaneously. Here, highly biocompatible glycyrrhizic acid (GA) nanoparticles (GANPs) were synthesized based on GA. In vitro investigations revealed that GANPs inhibit the proliferation of the murine coronavirus MHV-A59 and reduce proinflammatory cytokine production caused by MHV-A59 or the N protein of SARS-CoV-2. In an MHV-A59-induced surrogate mouse model of COVID-19, GANPs specifically target areas with severe inflammation, such as the lungs, which appeared to improve the accumulation of GANPs and enhance the effectiveness of the treatment. Further, GANPs also exert antiviral and anti-inflammatory effects, relieving organ damage and conferring a significant survival advantage to infected mice. Such a novel therapeutic agent can be readily manufactured into feasible treatment for COVID-19.

KEYWORDS: SARS-CoV-2, hyperinflammation, glycyrrhizic acid nanoparticles, antiviral, anti-inflammatory

1. INTRODUCTION

Coronavirus disease 2019 (COVID-19) has been diffusely pandemic worldwide,1 with considerable impacts on the global economy and health.2 Excessive host inflammation3 in severe COVID-19 patients is likely to further progress into acute respiratory distress syndrome (ARDS)4 and multiorgan failure,5 eventually leading to death.6 However, current management is supportive,7 without specific drugs8 against COVID-19.9 Therefore, safe and effective treatment strategies to simultaneously reduce viral damage and relieve uncontrolled immune response10 characteristic of COVID-19 are urgently needed.11

Traditional Chinese medicines have shown great potential in the treatment of many diseases because of their antioxidant, anti-inflammatory,12 antiviral, immunoregulatory, and anti-tumor effects.13 Glycyrrhizic acid (GA), also called glycyrrhizin,14 a common ingredient in the Chinese herb licorice,15 has been used for liver disease treatment16 (including viral hepatitis)17 and specific inflammatory disorders of the skin18 (such as atopic dermatitis).19 GA has an antiviral effect against different viruses,20 including SARS-related21 coronaviruses.22 Based on its characteristics,23 GA is considered as one promising novel drug candidate to fight off SARS-CoV-2 by testing alone or combining with other drugs.24 However, its cytotoxicity and poor solubility in water and biological fluids25 limit its further clinical application,26 as it may result in low bioavailability.27

The popularity of nanoparticles (NPs) has opened up a new cross-disciplinary direction for medical research,28 such as bioimaging,29 biosensing, biolabeling, photodynamic therapy,30 and drug delivery, due to their unique properties of water solubility, biocompatibility,31 cost-effective synthesis, and low toxicity.32 There have been investigations into the practical integration of nanotechnology with small molecule-based nanomaterials to decrease the toxic side effects of raw materials,33 enhance raw material efficacy,34 and deliver drugs in a targeted manner via the enhanced permeability and retention (EPR) effect.35 For instance, synthesized silver NPs36 and selenium NPs37 could improve the biocompatibility of raw materials. Squalene-based nanoparticles had relatively low toxicity and could target inflamed tissues in multiple...
murine models. Rosmarinic acid-derived nanoparticles (RANPs) showed relatively good water solubility and bioavailability. Recently, functionalized quantum dots based on GA synthesized by the hydrothermal method had shown a relatively low toxicity.

Therefore, to protect against COVID-19, highly biocompatible glycyrrhizic acid nanoparticles (GANPs) based on the active component of GA were synthesized. The results showed that GANPs had significant anti-viral, anti-inflammatory, and antioxidant effects in vitro and in vivo (Figure 1). Such a novel approach may provide an effective therapeutic solution for the pandemic, as well as treatment of hyperinflammation of other diseases.

2. EXPERIMENTS AND METHODS

2.1. Materials. Glycyrrhizic acid (75%) was obtained from Aladdin Chemistry Co., Ltd. Ultrapure distilled water (DNase and RNase-free) was acquired from InVitrogen. A Reactive Oxygen Species Assay Kit was provided by Beyotime Biotecnology. A lipopolysaccharide (LPS) was provided by Sigma-Aldrich. Species Assay Kit was provided by Beyotime Biotechnology. A Reactive Oxygen Species Assay Kit was provided by Beyotime Biotecnology. A lipopolysaccharide (LPS) was provided by Sigma-Aldrich. Glycyrrhizic acid (10 mg/mL) and LPS (1 mg/mL) were stimulated for 24 h, and the cell supernatant and the cell precipitation were collected. Inflammatory cytokine production was evaluated using RT-qPCR assay and ELISA.

2.2. Preparation of GANPs and PEG-Cy5-Coated GANPs. Glycyrrhizic acid nanoparticles (GANPs) were synthesized by using a hydrothermal method. Briefly, glycyrrhizic acid (10 mg/mL) was dissolved in deionized water (pH = 9.0 ± 0.2), and the mixture was subsequently incubated at 185 °C for 2 h. The supernatant was collected. After 1 h of incubation in a 37 °C cell incubator, the original culture supernatant was replaced with 0.40 mg/mL GANPs and reincubated for 24 h. Plaque assay and RT-qPCR were used to evaluate the antiviral effect of GANPs on MHV-A59 infection.

2.3. Antiviral Experiments. Antiviral-related experiments were referred to the previous description. In brief, L929 cells were incubated with 0.40 mg/mL GANPs for 2 h, and then, the supernatant was discarded and replaced with pretreated MHV-A59 (multiplicity of infection (MOI) = 1), which was incubated with 0.40 mg/mL GANPs at 4 °C for 1 h in advance. After 1 h of incubation in a 37 °C cell incubator, the original culture supernatant was replaced with 0.40 mg/mL GANPs and reincubated for 24 h. Plaque assay and RT-qPCR were used to evaluate the antiviral effect of GANPs on MHV-A59 infection.

Figure 1. Schematic diagram of GANPs for COVID-19 treatment.

2.4. Evaluation of Proinflammatory Cytokine Production. After MHV-A59, the N proteins of SARS-CoV-2 (1 mg/mL) or LPS (1 mg/mL) were stimulated for 24 h, and the cell supernatant and the cell precipitation were collected. Inflammatory cytokine production was evaluated using RT-qPCR assay and a precoated ELISA kit as per manufacturer’s instructions (Dekewei, China).

2.5. Establishment of a Surrogate Model of COVID-19 and In Vivo Therapeutic Effects. Six-week-old female BALB/c mice were intranasally (i.n.) inoculated with the MHV-A59 virus of 1.5 × 106 plaque forming units (PFU), which were given sodium pentobarbital and chloral hydrate to abdominal anesthesia in advance. Meanwhile, the same amount of PBS was inoculated intranasally into control mice. The health status of these mice was monitored every day. MHV-A59-infected mice and uninfected control mice were given GANPs (24 mg/kg) via the tail vein at 2, 4, and 6 days after infection. After the lungs and livers of different groups of mice were dissected, they were washed several times, one part of the tissues was subsequently taken for hematoxylin and eosin (H&E) staining, the other part was ground, and the expressions of MHV-A59 and inflammatory cytokines were detected by RT-qPCR assay and ELISA.

2.6. Establishment of an Animal Model of Excessive Inflammation and In Vivo Therapeutic Effects. Six-week-old female BALB/c mice were intraperitoneally with LPS (20 mg/kg), while the same amount of PBS was inoculated intranasally into control mice. The mice were monitored hourly for health conditions. Thirty minutes after LPS injection, infected mice and uninfected control mice were given GANPs (24 mg/kg) via the tail vein. After the lungs and livers of different groups of mice were dissected, they were washed several times, one part of these tissues was taken for H&E staining accordingly, the other part was ground, and the expressions of inflammatory cytokines and chemokines were detected by RT-qPCR assay and ELISA.

3. RESULTS AND DISCUSSION

3.1. Synthesis and Characterization of GANPs. A hydrothermal process was used to synthesize GANPs. As shown in Figure 2A, GA was dissolved in an aqueous solution (pH = 9 ± 0.2) followed by heating in a 185 °C reector for 6 h. Then, purified GANPs were obtained for subsequent analysis. Transmission electron microscopy (TEM) was used to characterize the surface properties of GANPs (Figure 2B). GANPs were round in shape with uniform particle size and good distribution. In addition, dynamic light scattering (DLS) results (Figure 2C) are consistent with TEM, the hydrodynamic diameter distribution of GANPs is relatively narrow, and the calculated average particle size is 70.65 nm. The average ζ potential measurements revealed that GANPs had a negative surface charge of −32.7 mV (Figure 2D). The UV–vis absorption spectrum of GANPs has a relatively small absorption peak at 267 nm, which is caused by the surface effect and quantum size effect, and an absorption peak for GA at 256 nm (Figure S1A), so the similarity of the absorption peaks of GANPs and GA further confirmed the formation of GANPs (Figure 2E). In the fluorescence spectrum analysis, the maximum excitation wavelength of GANPs is 356 nm, and the maximum emission wavelength is 438 nm (Figure 2F). Fourier transform infrared spectroscopy (FT-IR) results illustrated that...
there were five distinct absorption peaks at 1072, 1396, 1621, 2944, and 3421 cm\(^{-1}\) in the GANP spectra, corresponding to C−O, C≡O, C≡C, C−H, and O−H, respectively. The results showed that −OH and −COOH are abundant on the surface of GANPs. Compared with the results of GA, it was found that partial functional groups of GA were present in GANPs (Figure S1B). These physical properties of GANPs are consistent with our expectations.

### 3.2. The Biocompatibility of GANPs

Then, we assessed the biocompatibility of GANPs and the raw material GA in vitro (Figure S2). In these experiments and subsequent experiments, L929 cells (mouse fibroblasts), RAW264.7 cells (mouse mononuclear macrophages), THP-1 cells (human mononuclear cells), and human peripheral blood mononuclear cells (hPBMCs) were evaluated. As the concentration of GANPs gradually increased from 0.2 to 1.2 mg/mL, the survival rate of all cells was almost 100%, while cell viability was approximately 60% at 1.5 mg/mL and 30% at 2 mg/mL of GANPs, indicating that 1.2 mg/mL is a physiologically safe concentration of GANPs. In contrast, as the GA concentration was increased, the viability of all cells decreased. THP-1 cell viability was even less than 30% when the concentration of GA was 0.6 mg/mL, and the cell viabilities of the other three types of cells were approximately 50% when the concentration of GA was 1.2 mg/mL. At concentrations as high as 2 mg/mL of GA, the cell viabilities were only about 10%. These data suggested that compared with that of GA, the biocompatibility of GANPs has indeed observably improved.

### 3.3. GANPs Inhibit the Proliferation of MHV

The anticoronavirus activity of GA has been reported previously.\(^{21}\) Here, the coronavirus mouse hepatitis virus A59 (MHV-A59) was used to evaluate antiviral activity of GANPs. MHV-A59 is a greatly crucial member of the hepatitis B virus 2A subgroup,\(^{39}\) which is closely related to the current pandemic human coronavirus SARS-CoV-2 in the same subgroup.\(^{40}\) There is considerable evidence that MHV-A59 infection is associated with various pathological conditions, including hepatitis, autoimmune hepatitis-like diseases, thymic degeneration, hyperglobulinaemia, and transient demyelination.\(^{41}\) The respiratory tract and lung tissues can also be infected with MHV-A59, resulting in severe pathological damage to the respiratory tract and lungs similar to that of SARS-CoV, leading to acute inflammation.\(^{42}\) Several studies suggested that MHV-A59 is expected to be used as a surrogate model of SARS-CoV-2.\(^{43}\) Therefore, we planned to construct a surrogate SARS-CoV-2 infection model. First, a cell model was established in vitro. Figure 3A–C shows that the addition of GANPs notably weakened MHV-A59 in intracellular and cell supernatants and also decreased RNA expression of MHV-A59 genes in the cells. In the supernatant of the cells, GANPs reduced the titers by a maximum of 10\(^5\) times. Additionally, we found that the inhibitory effect of GANPs on MHV-A59 showed a dose-dependent trend, and the inhibition effect reached almost 80% when the concentration of GANPs was 0.4 mg/mL. Then, the addition of GANPs was found to significantly reduce MHV-A59 mRNA expression at 12, 24, 36,
and 48 h post infection (hpi) (Figure 3D), further supporting the potent antiviral effect of GANPs. The above results showed that GANPs could significantly inhibit the multiplication of MHV-A59.

Subsequently, we analyzed the effect of GANPs on the proliferation of MHV-A59 during various stages of adsorption, invasion, replication, and release to explore the possible mechanism of the antiviral properties of GANPs. The first test was to see if GANPs could directly inactivate MHV-A59. Plaque experiments showed that the number of MHV-A59 was reduced by about 20 times after GANP treatment, suggesting that GANPs had the ability to directly inactivate MHV-A59 (Figure 3E). Furthermore, MHV-A59 tagged with QD605 was used to infect L929 cells for inactivation analysis, and the results of laser confocal microscopy showed that compared with no GANP treatment, the GANP treatment group showed a noteworthy decrease in the red fluorescence of MHV-A59 (Figure 3F), which further indicated the direct inactivation of GANPs. In the process of the adsorption of MHV-A59, the plaque test revealed that the GANP treatment group had a significant inhibitory effect on the adsorption of MHV-A59 (Figure 3G). In the process of the invasion of MHV-A59, GANP treatment reduced the infected titers of the virus by approximately 10^3-fold compared with no treatment (Figure 3H). We performed RT-qPCR to test the viral RNA level of MHV-A59 and evaluate the effect of GANPs on viral replication (Figure 3I). GANPs reduced the MHV-A59 RNA level by nearly 10 times, indicating that GANPs had a certain influence on MHV-A59 during the replication stage. In addition, the effect of GANPs on the release of MHV-A59 was studied. Compared with the untreated group, the titer of MHV-A59 did not significantly decrease after GANP treatment (the data was not shown), indicating that GANPs had no ability to inhibit the release of newly generated MHV-A59 in the offspring. In summary, GANPs suppressed the proliferation of MHV-A59 through targeting invasion, adsorption, and replication, and statistical analysis showed that GANPs had a definite direct inactivation of the virus, but it could not inhibit the release of the progeny virus.

3.4. GANPs Exhibit Anti-inflammatory and Antioxidant Activities In Vitro. Studies have shown that severe COVID-19 patients may have hyperinflammatory syndrome. Therefore, methods to reduce excessive inflammation to decrease the mortality rate are urgently needed. Our data showed that GANPs could prominently suppress the proliferation of MHV-A59, but whether GANPs can relieve the excessive inflammation caused by MHV-A59 simultaneously remained to be determined. Here, MHV-A59 was used

Figure 3. Antiviral effects of GANPs in vitro. (A) Relative intracellular MHV-A59N mRNA expression in L929 cells incubated with GANPs at different concentrations for 24 h measured through RT-qPCR. (B,C) Titors of supernatant (B) and cell lysate (C) of MHV-A59 treated with GANPs at different concentrations for 24 h determined by plaque assay. (D) Relative intracellular MHV-A59N mRNA expression in L929 cells incubated with 0.4 mg/mL GANPs at 12, 24, 36, 48 hpi. (E) Effect of GANPs on direct inactivation of MHV-A59 determined by plaque assay. (F) Confocal laser scanning microscopy (CLSM) images for MHV-A59 inactivation analysis, QD605-labeled MHV-A59-infected L929 cells treated or untreated with 0.4 mg/mL GANPs (scale bar: 20 μm). (G–I) Effect of GANPs on the proliferation of MHV-A59 at various stages including adsorption (G), invasion (H), and replication (I). The data are presented as the mean ± SEM and are representative of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, and ns means nonsignificant difference, compared with the indicated group by the t test.
to stimulate mouse monocyte RAW 264.7 cells. The results in Figure 4A revealed that compared with normal RAW 264.7 cells (the CTL group), GANP treatment only (GANP group) showed similar cytokine levels, revealing that GANPs could not stimulate the inflammation. The mRNA expression levels of interleukin (IL)-1α, IL-1β, IL-6, and IL-12 were significantly increased after MHV-A59 stimulation (MHV group), which are all proinflammatory cytokines in physiological processes, indicating that the model of cellular inflammation was successfully constructed. After GANP treatment (MHV +GANP group), the mRNA expression levels of these cytokines were all observably diminished. Subsequently, ELISA results revealed that the protein expressions of IL-1β (Figure 4B) and IL-6 (Figure 4C) also exhibited the same inhibitory effects. These data suggested that GANPs have antiviral properties and alleviate the hyperinflammation caused by MHV-A59.

The progressive progression of the inflammatory process is associated with the disturbance of the redox balance. Reactive oxygen species (ROS) take vital effects on the occurrence and development of inflammatory diseases, bacterial infections, and other physiological diseases. Reducing excessive ROS levels can inhibit viral proliferation. Therefore, it is necessary to relieve severe oxidative stress in addition to anti-inflammation and antivirus. Briefly, after 2 h of MHV-A59 stimulation, GANPs with gradient concentrations were added into RAW264.7 cells to incubate for 12 h, and ROS represented by green fluorescence were detected by flow cytometry. Figure 4D,E reveals that MHV-A59-stimulated cells without GANP treatment displayed a considerably high ROS level. After GANP treatment, the ROS levels were significantly reduced, and the inhibitory effect of the GANPs tended to be dose-dependent. The above data suggested that GANPs could relieve the overproduction of ROS induced by MHV-A59.

To verify that GANPs have direct anti-inflammatory and antioxidative properties in vitro, not just a decrease in inflammation levels caused by a drop in viral titer, we next used a lipopolysaccharide (LPS) as a non-viral inflammatory stimulator to develop in vitro models of hyperinflammation and hyperoxidation. LPS stimulation of RAW264.7 macrophages causes overproduction of proinflammatory cytokines and ROS.5 The results (Figures S3 and S4) showed that LPS-stimulated cells without GANP treatment displayed increased expression of proinflammatory cytokines and a considerably high ROS level. After GANP treatment, the expression levels of these cytokines were all prominently reduced; meanwhile, the ROS levels were significantly decreased. These results imply that GANPs have direct immunoregulatory and antioxidant abilities in vitro.

3.5. In Vivo Targeting and Systematic Therapeutic Effects of GANPs. We then systemically estimated the biocompatibility of GANPs in vivo. Ten female healthy BALB/c mice aged 6 weeks were randomly divided into two groups: a GANP group or a control group, in which these healthy mice were given GANPs (24 mg/kg) or an equal amount of PBS via the tail vein, respectively. Seven days after injection, the main organs of these mice were harvested for sectioning and staining with H&E. Figure 5A shows that no significant histological abnormalities or lesions were present, indicating that GANPs are safe and nontoxic to the major organs of mice and could be used in subsequent animal experiments, further suggesting that GANPs have great potential for clinical application.

To verify the in vivo effects of GANPs in a relatively safe environment, we used MHV-A59 intranasal infection to construct a surrogate mouse model of COVID-19, forming similar hyperinflammation and pathological damage caused by...
SARS-CoV-2; this model has previously been reported as a surrogate mouse model of severe pneumonia induced by SARS-CoV or Middle East respiratory syndrome coronavirus (MERS-CoV) infection. Then, Cy5-labeled PEG was attached to the surface of GANPs (GANPs-Cy5) (Figure S5) by an amide bond to study distribution and metabolism in vivo. Ten female healthy BALB/c mice aged 6 weeks were randomly divided into two groups: GANPs-Cy5 (24 mg/kg) was injected into both uninfected and MHV-A59-infected mice via the tail vein. Twelve hours after injection, the main organs of these mice in the different experimental groups were imaged using a Xenogen IVIS Spectrum. As we can see in Figure 5B,C, Cy5 fluorescence in the uninfected group (MHV− group) was mainly found in the livers and kidneys, suggesting that GANPs were metabolized through hepatobiliary and kidney systems. Cy5 fluorescence in the infected group (MHV+ group) was mainly found in the lungs, livers, and kidneys, and the lungs and livers showed a stronger fluorescence than that in the uninfected group. As the MHV-infected mice suffered extensive inflammatory damage in lung and liver tissues, exhibiting enhanced vascular permeability and blood perfusion, GANPs with longer circulation time in blood were thought to have a higher likelihood of preferentially locating to these tissues. These above results indicated that GANPs may have the ability to target sites of severe inflammation through the EPR effect to further exert antiviral and anti-inflammatory effects.

Next, we validated the therapeutic properties of GANPs in vivo. Several healthy female BALB/c mice aged 6 weeks were randomly divided into four groups (n = 12 per group): GANPs (24 mg/kg) or PBS was injected into the uninfected mice (named CTL or GANP group) or MHV-A59-infected mice (named MHV or MHV+GANP group) via the tail vein. The mice from the MHV group had a decreased appetite and less weight gain as compared to the CTL group, while the mice from the MHV+GANP group gradually regained their body weight (Figure 5D). Mice from the MHV group began dying on the third day, while GANP treatment conferred a significant survival advantage to infected mice (Figure 5E). The raised serum levels of IL-6 in the mouse serum measured by ELISA. The data are presented as the mean ± SEM and are representative of three independent experiments. **P < 0.01 and ***P < 0.001, compared with the indicated group by the t test.

Figure 5. In vivo safety, targeting, and systematic therapeutic effects of GANPs. (A) Representative H&E staining images of the main organs from mice injected with GANPs or PBS (scale bar, 100 μm). (B,C) Representative NIRF images (B) and fluorescence quantification analysis (C) of mouse organs 12 h after intravenous injection of fluorescent GANPs-Cy5 into BALB/c mice intranasally infected with MHV-A59 or uninfected. (D) Daily weight changes in normal BALB/c mice treated with PBS (CTL) and in BALB/c mice intranasally infected with MHV-A59 treated with PBS (MHV) or GANPs (MHV+GANPs). (E) Survival analysis of BALB/c mice given the different treatments described above, n = 12 mice per group. For survival evaluation, the log-rank (Mantel–Cox) test was used, giving a P value of P = 0.0488 (*P < 0.05). (F) Concentrations of IL-6 in the mouse serum measured by ELISA. The data are presented as the mean ± SEM and are representative of three independent experiments. **P < 0.01 and ***P < 0.001, compared with the indicated group by the t test.
the IL-6 expression level in the serum was quantitatively detected by ELISA (Figure 5F). Compared with the CTL group, the GANP group showed similar cytokine levels, revealing that GANPs could not stimulate systemic inflammation, implying the favorable concealment of GANPs in the mouse immune system. Furthermore, the results showed that GANPs could significantly reduce IL-6 expression levels compared with the MHV-infected group, suggesting that GANPs may be able to relieve systemic inflammation.

3.6. In Vivo Therapeutic Effects of GANPs in the Lungs. More importantly, lung histopathological alterations were analyzed and compared among differently treated lungs that were collected after 7 days post treatment (Figure 6A). Typically, severe lung damage, including edema, diffuse alveolar damage, and inflammatory leukocyte infiltration, was observed in mice from the MHV group. Meanwhile, most of the mice from the MHV+GANP group exhibited less lung injury. Furthermore, the pulmonary computed tomography (CT) results of the MHV group showed severe ground-glass opacity, indicating severe inflammatory infiltration and exudation, which are the main CT changes in critical COVID-19 patients. Meanwhile, GANP treatment significantly relieved lung damage (Figure 6B). Moreover, the viral titers of MHV-A59 and cytokines were detected in the lung tissues. Figure 6C shows that compared with those in the MHV-A59-infected group, the viral load of MHV-A59 in the MHV +GANP group was significantly decreased, suggesting that GANPs could also alleviate MHV-A59 infection in vivo. These
results confirm the ability of GANPs to repress the proliferation of MHV-A59 in the lungs. Lung tissues were taken to test the expression levels of proinflammatory cytokines and chemokines. The IL-1β (Figure 6D), IL-6 (Figure 6E), IFN-γ (Figure 6F), TGF-β (Figure 6G), and MCP-1 (Figure S6A) expression levels in the lungs were quantitatively detected by ELISA, and the results prompted that GANPs could notably fall off the expression levels of these cytokines and chemokines. The mRNA expression levels of proinflammatory cytokines IL-6, IFN-γ, and TNF-α and chemokines IP-10, G-SCF, and MCP-1 in the MHV+GANP groups were also significantly decreased compared to the MHV group (Figure S6B), suggesting that GANPs could relieve lung inflammatory injury in MHV-A59-infected mice.

3.7. In Vivo Therapeutic Effects of GANPs in the Livers. Hepatic dysfunction has been reported in COVID-19 patients, particularly in those with severe disease. Acute liver injury cases are associated with higher mortality. So, we also analyzed the viral load and inflammatory damage in the livers. Similar to the lung results, GANPs could lessen pathological damage (Figure 7A) and dwindle the viral load (Figure 7B) and inflammation levels (Figure 7C–H) in the livers. Compared with the MHV group, biochemical indexes of liver function alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were prominently diminished in the MHV+GANP group (Figure 7I). To sum up, these above data indicated that GANPs have very characteristic antiviral activity in vivo; meanwhile, GANPs played a crucial role in reducing hyperinflammation and alleviating lung and liver injuries in vivo.

3.8. Therapeutic Effect of GANPs on LPS-Induced Hyperinflammation In Vivo. In order to further verify the direct inflammatory regulation abilities of GANPs in vivo, LPS (20 mg/kg) was injected intraperitoneally to BALB/c mice, and GANPs (24 mg/kg) were injected into the tail vein 30 min later ($n = 12$ per group). After 12 h, blood was collected retro-orbitally, and liver and lung tissues were dissected to test the expression of inflammatory cytokines and chemokines by Q-
PCR and ELISA. The results (Figure S7) showed that GANPs could significantly reduce IL-6 expression levels compared with the LPS group, suggesting that GANPs may be able to relieve systemic inflammation. Results (Figures S8 and S9) from liver and lung tissues showed that GANPs significantly reduced the expression levels of proinflammatory cytokines and chemokines. To sum up, the above data suggested that GANPs played a crucial role in reducing hyperinflammation in vivo.

3.9. GANPs Inhibit the Production of Proinflammatory Cytokines Induced by the N Proteins of SARS-CoV-2.

To investigate whether GANPs effectively protect against hyperinflammation in human cells induced by SARS-CoV-2, an inflammatory injury cell model was first established by using human mononuclear THP-1 cells. In this experiment, we used the nucleocapsid (N) protein of SARS-CoV-2, a potent diagnostic and prophylactic target, to stimulate THP-1 cells; then, the expression of proinflammatory cytokines in the levels of mRNA and protein was detected by using RT-qPCR and ELISA, respectively. As we can see, Figure 8A depicts that after stimulation with the N protein of SARS-CoV-2, the mRNA expression levels of IL-1α, IL-1β, and IL-6 were observably increased, suggesting that we successfully constructed a cell model of inflammatory insult. Compared with N stimulation alone, GANP treatment observably dwindled the mRNA expression levels of IL-1α, IL-1β, and IL-6. ELISA results also showed the same anti-inflammatory effects (Figure 8B,C). These data suggested that GANPs were highly protective against uncontrolled inflammation in vitro.

To further approximate the real state of SARS-CoV-2 infection in humans, additionally, we used the N protein of SARS-CoV-2 to stimulate hPBMCs from healthy donors (n = 12). As shown in Figure 8D−F, after stimulation with the N protein, the mRNA expression levels of IL-1α, IL-1β, and IL-6 were markedly enhanced, and after GANP treatment, the expression levels of these three proinflammatory cytokines prominently lessened. ELISA results also showed the same anti-inflammatory effects (Figure 8G,H). These results revealed that GANPs also had a good role in regulating the inflammatory process of human PBMCs stimulated with the N protein of SARS-CoV-2. To sum up, these above results demonstrated that GANPs could also be used to alleviate the excessive inflammatory response of human cells induced by SARS-CoV-2.

4. CONCLUSIONS

In the present study, we have confirmed the antiviral effect and anti-inflammatory property of GANPs, which did not have
noticeable toxicity in vitro or in vivo. The hydrothermal synthesis of GANPs significantly improved the biocompatibility of the raw material GA, which provided a technical basis for extending the application range of GA. This study identified antiviral and anti-inflammatory management simultaneously, which produced relatively good and thorough effects that relieved the excessive inflammation caused by SARS-CoV-2. Additionally, GANPs could target areas of severe inflammation through the EPR effect in the surrogate mouse model of COVID-19, which appeared to improve the accumulation of GANPs in the lungs and livers, further increasing the effectiveness of the treatment. Our findings may provide some ideas for a new effective and low-toxicity pandemic therapeutic strategy and a potential treatment for hyperinflammation in general that can be readily manufactured.

■ ASSOCIATED CONTENT

1 Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsami.1c02755.

UV−vis absorption spectra and FT-IR spectra of the GA; cytotoxicity of GANPs and GA; concentrations and the relative mRNA expression of proinflammatory cytokines in RAW264.7; ROS levels in RAW264.7; TEM image, hydrodynamic size distribution, ζ potential, UV−vis absorption spectra, and fluorescence spectra of GANPs-Cy5; concentrations and relative mRNA expression of proinflammatory cytokines and chemokines in lung and liver tissues (PDF)

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Notes
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