Internalization of Garlic-Derived Nanovesicles on Liver Cells is Triggered by Interaction With CD98

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ABSTRACT: The mechanism of how plant-derived nanovesicles are uptaken by cells remains unknown. In this study, the garlic-derived nanovesicles (GDVs) were isolated and digested with trypsin to remove all surface proteins. Digested GDVs showed less uptake compared to undigested GDVs, confirming that the surface proteins played a role in the endocytosis. On the cell side (HepG2), interestingly, blocking the CD98 receptors significantly reduced the uptake of GDVs. During the cellular internalization of GDVs, we observed that some surface proteins of GDVs were co-localized with CD98. A total lysate of the GDV surface showed a high presence of a mannos-specific binding protein, II lectin. Blocking GDV II lectin (using mannose preincubation) highly reduced the GDV internalization, which supports that direct interaction between II lectin and CD98 plays an important role in internalization. The GDVs also exhibited in vitro anti-inflammatory effect by downregulating proinflammatory factors on the HepG2 cells. This work contributes to understanding a part of the GDV internalization process and the cellular anti-inflammatory effects of garlic.

INTRODUCTION

Recently, plant-derived nanovesicles were isolated from several edible plants such as grapefruits, gingers, carrots, apples, and lemons.1−4 Those plant-derived nanovesicles resembled their mammalian exosome counterparts in that they carried the functional cargos such as small molecules, proteins, mRNA, and miRNA, which can modulate the fate of recipient cells.5 Several plant-derived nanovesicles have been reported, which have been used for cancer therapy, changing the gut microbiota composition, and protecting the mice from dextran sulfate sodium (DSS)-induced colitis and inflammatory bowel disease.5,6,7−9 These reports indicate that plant-derived nanovesicles are involved in the interspecies communication for their biological effect.8 Although plant-derived nanovesicles are promising in anti-inflammatory and cancer therapy, the uptake mechanism of the plant-derived nanovesicles is still unknown.

Understanding the mechanisms of the mammalian exosomes can elucidate the potential mechanisms of plant-derived nanovesicles. The main uptake mechanisms for mammalian exosomes include phagocytosis, clathrin-dependent, or clathrin-independent pathways.7,9−10 Heparan sulfate proteoglycans (HSPG) on cell surfaces could be a possible receptor for exosome endocytosis, but it has been reported that the HSPG on the surface of exosomes is not involved in the internalization of exosomes.10 Another study revealed that fibronectins on the surface of myeloma cell-derived exosomes have interactions with HSPG on the target cells.11 Also, proteins on both the surface of ovarian cancer cell-derived exosomes and ovarian cancer cells are important for uptake.12 CD98 is a transmembrane, glycoprotein heterodimer, which consists of the CD98 heavy chain (CD98hc) and several light chains (CD98lc) such as large amino acid transporter 1 (LAT 1).13 The glycan on CD98hc is consist of tetra-antennary with terminal fucosylation and mannose oligosaccharides (oligo-mannose).13,14 CD98 has various functions, which include mediating cell proliferation through activating the integrin-β pathway, importing amino acid to support cell growth, and regulating cell fusion in several types of cell lines (BeWo cells, peripheral blood mononuclear cells, and L929 cells).13,13,15−18 CD98 has been reported to play an important role in the internalization of human β-defensin 3 and endocytosis of mature vaccinia virus particles, and recently a study has shown that the LAT 1 may be involved in the uptake of nanoparticles.17−19 CD98 was used as a targeted protein to enhance the internalization of therapeutic nanoparticles as it is overexpressed in tumor and inflammatory tissues.20 These reports indicated that CD98 played an important role in the uptake process. CD98 has also been linked to the inflammatory bowel disease (IBD) and plays a potential role in chronic liver disease, e.g., nonalcoholic fatty liver disease (NAFLD).21 Thus, CD98 can be a promising target for both therapeutic and drug delivery.

Lectin, a type of protein that has specific binding affinity to saccharides, has played an important role in the interspecies
recognition. For example, the innate immune recognition of coronaviruses was mediated by the interaction of certain lectins on cell membranes and the oligomannose from coronaviruses. A lectin from Sparassis latifolia (a mushroom) has shown antibacterial activity against Escherichia coli and other drug-resistant strains. Inspired by those lectin-carbohydrate-mediated interspecies communications, we hypothesized that the interaction between plant-derived nanovesicles and mammalian cells was mediated by the interaction of lectins and saccharides.

Several dietary lectins have been found to exert their health benefits through inducing apoptosis after binding to the membrane. Among those edible plants, garlic is enriched in the lectins (1 mg of lectins per 1 g of garlic) and has well-documented health benefits in many epidemiology studies. A lectin from Sparassis latifolia (a mushroom) has shown antibacterial activity against Escherichia coli and other drug-resistant strains. Inspired by those lectin-carbohydrate-mediated interspecies communications, we hypothesized that the interaction between plant-derived nanovesicles and mammalian cells was mediated by the interaction of lectins and saccharides. Several dietary lectins have been found to exert their health benefits through inducing apoptosis after binding to the membrane. Among those edible plants, garlic is enriched in the lectins (1 mg of lectins per 1 g of garlic) and has well-documented health benefits in many epidemiology studies. A lectin from Sparassis latifolia (a mushroom) has shown antibacterial activity against Escherichia coli and other drug-resistant strains. Inspired by those lectin-carbohydrate-mediated interspecies communications, we hypothesized that the interaction between plant-derived nanovesicles and mammalian cells was mediated by the interaction of lectins and saccharides.

Figure 1. Physicochemical properties of garlic-derived nanovesicles (GDVs). (A) Intact GDVs and (B) GDVs in which the surface proteins have been trypsin-digested for 4 h (T4 GDVs) were visualized by transmission electron microscopy (TEM). Atomic force microscopy images of (C) GDVs and (D) T4 GDVs were captured. The average size was measured by the dynamic light scattering (DLS) method as well as the ζ-potential. (E) Size distribution of GDVs by intensity (red) and number (blue). (F) Size distribution of T4 GDVs by intensity (red) and number (blue), (G) ζ-potential distribution of GDVs, and (H) ζ-potential distribution of T4 GDVs.

| Table 1. The analysis of the size curves after trypsin digestion |  |
|---|---|
| |  |
| **Size and ζ-potential** | **Size and ζ-potential** |
| **Intensity** | **Number** |
| GDVs | T4 GDVs |
| 70-200 nm | 70-200 nm |
| 178.6 nm | 191.8 nm |
| 31 mV | 23 mV |

The size curves showed a narrow size distribution as listed in Table 1. The analysis of the size curves after trypsin digestion showed that T4 GDVs had slightly decreased size. The DLS showed that the sizes were homogeneous. The ζ-potential analysis showed that the ζ-potential of digested or undigested GDVs was negative and around −31 mV (Figure 1G,H). It has been reported that the particles derived from the edible plants such as carrot, grape, grapefruit, and ginger were quite stable in both stomach- and intestine-like solutions. Moreover, plant-derived nanovesicles showed intact biological effects after oral
Expression of CD98 on the HepG2 Cells.

Uptake of GDVs is dependent on the GDV surface protein integrity and CD98 expression level in the HepG2 cells. (A) 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) cell viability assay on the HepG2 cells after incubation with GDVs for 24 h (the concentration of GDVs was 100 μg/mL; the values represent means ± standard deviation (SD). Data are representative of n = 6 determinations). Uptake of GDVs was (B) concentration-dependent and (C) time-dependent for the first hour and reached a plateau after 2 h. For (B) GDVs were incubated with the HepG2 cells for 6 h and for (C) HepG2 cells were incubated with GDVs at a concentration of 100 μg/mL of GDVs (more than 98% of the viable cells). Hence, GDV uptake within 24 h of exposure was used to track GDV endocytosis.

The uptake of GDVs by the HepG2 cells was concentration- and time-dependent. As shown in Figure 2B, we investigated the uptake of GDVs by the HepG2 cells by flow cytometry. We tagged GDVs with coumarin-6. We studied the kinetics uptake at the range of concentration of coumarin-6-tagged GDVs of 25–150 μg/mL for 6 h. GDVs were incubated with the HepG2 cells for different times (1–6 h at 100 μg/mL of GDVs). The uptake was measured by flow cytometry (Figure 2C) and it showed that the GDV uptake was fast and reached a plateau after 1 h of incubation.

Our study also investigated the kinetics uptake of GDVs on cells (HepG2) expressing the differential levels of expression of CD98. Many groups have found that LPS stimulation increased the expression level of CD98 mRNA. In this study, the three differential expression levels of CD98 proteins were accessed by LPS stimulation (upregulation, regular condition, and CD98 siRNA (downregulation). Both immunofluorescent images and Western blot successfully confirmed that CD98 express at different levels on the HepG2 cell surface. The basal level (HepG2 cells under regular culture condition) was set as control (Figure 3B). A higher CD98 expression level was observed by supplementing the cell medium with LPS (Figure 3A), while a lower level was found by transfecting the cells with the CD98 siRNA (Figure 3C). The Western blot (Figures 3D, and S1A) obviously verified that we were able to generate differential CD98 expression with the HepG2 cells (control, LPS, siRNA). Since the CD98 is already highly overexpressed in the liver cancer cells, the changes in the amount of CD98 are further investigated by the quantification of the Western blot films. The quantification of Western blot (Figure S1B) showed that LPS can increase the expression of CD98 by 10% when compared with the control group (HepG2 cell under regular culture condition). In contrast, CD98 siRNA significantly decreased CD98 expression by 37%.

By comparing Figure 3A–C, we observed the effect of CD98 cell expression on the uptake of GDVs. The intensity of uptake was detected using the CD98-silenced HepG2 cells (Figure

### Table 1. Summary of GDVs’ and Trypsin-Digested GDVs’ Size and ζ-Potentials

| sample          | size (d, nm) | PDI       | ζ (mV)  |
|-----------------|--------------|-----------|---------|
| GDVs            | 191.8 ± 2.0  | 0.217 ± 0.011 | −32.3 ± 0.86  |
| T1 GDVs         | 178.6 ± 1.8  | 0.171 ± 0.017 | −31.5 ± 1.2   |
| T2 GDVs         | 175.7 ± 4.2  | 0.199 ± 0.008 | −34.9 ± 0.60   |
| T3 GDVs         | 170.8 ± 1.6  | 0.170 ± 0.015 | −33.2 ± 0.70   |
| T4 GDVs         | 175.2 ± 1.7  | 0.188 ± 0.018 | −30.8 ± 1.0    |
| Trypsin-Digested GDVs' | 175.2 ± 1.7  | 0.188 ± 0.018 | −30.8 ± 1.0  |

administration, indicating their stability in vivo. This latter observation is interesting as such particles (size of 200 nm such as GDVs) are suitable for both active and passive internalizations. Altogether, these observations established that the deletion of surface membrane proteins on GDVs.

Uptake Kinetics of GDVs is Correlated to the Surface Expression of CD98 on the HepG2 Cells. The global incidence of nonalcoholic fatty liver disease (NAFLD) is 25%, which induces a strong burden to the society. In the United States, this increased incidence of NAFLD is associated with increased liver disease mortality. The goal of this paper is to develop a preliminary understanding of the uptake mechanisms of the GDVs and their potential therapeutic effects to apply them further as a nanotherapeutics for inflammatory and liver disease. Thus, the HepG2 cell was used in this study. As shown in Figure 2A, we studied the cytotoxicity of GDVs on the HepG2 cells and found that both the undigested and digested GDVs were not cytotoxic in the HepG2 cells after 24 h incubation with 100 μg/mL of GDVs (more than 98% of the viable cells). Hence, GDV uptake within 24 h of exposure was used to track GDV endocytosis.

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![Figure 2. Uptake of GDVs is dependent on the GDV surface protein integrity and CD98 expression level in the HepG2 cells.](https://dx.doi.org/10.1021/acsomega.0c02893)
2D), untreated HepG2 (Figure 2E) cells, and LPS-stimulated HepG2 cells (Figure 2F). As shown in Figure 2D-F, the GDV uptake was proportionally increased with the expression of CD98 as shown by the position of the peak maximum and the area under the curve of each flow cytometry experiment. Comparing each line within the same plot, we showed that for any CD98 expression, the surface protein digestion reduced the uptakes of GDVs. Similarly, in the literature, proteins on the membranes of exosomes were digested by protease K, which inhibited the uptake by the cells.12 Trypsin-digested GDV uptake was significantly decreased compared to native GDVs, demonstrating the importance of the ligands present on their surface. Also, comparing each curve of the same color in the three different experiments, we showed that for the same GDV surface protein profile, the uptake of GDVs was correlated to CD98 expression. This result was further demonstrated by investigating the uptake of GDVs with differential CD98 expression by fluorescent microscopy (Figure 4).

The intensity of uptake was also quantified by the geometric mean, as shown in Figure 3E. Intake of T4 GDVs was lesser than that of intact GDVs by the HepG2 cells. For the uptake of GDVs, it was shown that CD98 silencing significantly decreased the uptake of GDVs by 33.85% compared with that in the HepG2 cells under regular culture condition. In contrast, LPS-stimulated HepG2 increased the uptake of GDVs by 17.16%. These results together showed that the uptake of GDVs by the HepG2 cells was correlated to the level of expression of CD98 on the HepG2 cells.

Figure 3. CD98 expression level is significantly involved in GDV uptake. Differential levels of expression of surface CD98 (red) were stained by stimulating the HepG2 cells with 10 μg/mL of LPS for 24 h (A) (the highest expression of CD98), by regular cell culture of HepG2 (B) (basal CD98 expression), and by treating the HepG2 cells with CD98 siRNA using oligofectamine (C) (the lowest expression of CD98). The CD98 was stained in red and the nucleus was stained in blue (4′,6-diamidino-2-phenylindole (DAPI)). (D) Western blot showed the levels of CD98 after different treatments (LPS, control, and siRNA). (E) CD98 expression is directly correlated with GDV uptake. The uptake was monitored by a flow cytometer and the result was calculated relatively to the uptake without treatment, considered as 1.0.

Figure 4. Cross study of the modulation of GDV endocytosis by the HepG2 cells by tuning the expression level of CD98 on the HepG2 cells and the integrity of the surface proteins of GDVs. The HepG2 cells were incubated with 100 μg/mL GDVs for 6 h except column A (noted phosphate-buffered saline (PBS)), which represents the HepG2 cells without GDVs. The GDVs were labeled with coumarin-6 (green), the HepG2 cells were stained for actin with phalloidin (red), and the nuclei were stained with DAPI (blue). Vertically, other columns (B–F) represent a different “type” of GDV treatment. (B)–(F) columns represent, respectively, intact GDVs (GDVs in B), 1 h trypsin-digested GDVs (T1 GDVs in C), 2 h trypsin-digested GDVs (T2 GDVs in D), 3 h trypsin-digested GDVs (T3 GDVs in E), and 4 h trypsin-digested GDVs (T4 GDVs in F). Horizontally, line 1 (noted LPS) represents the LPS-stimulated HepG2 cells (overexpressing surface CD98 on the HepG2 cells). The second line (noted HepG2) represents the basal expression of CD98 on the HepG2 cells. Finally, line 3 (noted CD98 siRNA) shows the HepG2 cells with lower expression of CD98 (blocked by pretreatment with CD98 siRNA).
The uptake of GDVs was visualized by fluorescence microscopy (Figure 4). Figure 4 showed the uptake of GDVs (i) by the LPS-stimulated HepG2 cells (first panel), (ii) by the regular HepG2 cells (second panel), and (iii) by the HepG2 cells treated with CD98 siRNA (third panel). As expected, there is no coumarin-6 signal (GDVs) in the cells without GDVs (PBS column in Figure 4). The fluorescent study showed clearly that intake of the intact GDV group was significantly more than that of the trypsin-digested GDV groups. Within the GDV-supplemented cell column, we also observed that the kinetics of GDV uptake by the HepG2 cells was directly correlated with the CD98 expression levels. CD98 downregulation (line 3 in Figure 4) significantly showed a decrease in the endocytosis of GDVs. These indicated that CD98 expression in the HepG2 cell played an important role in the recognition and uptake process of GDVs.

**Important Presence of II Lectin on the GDV Surface.**

After establishing that CD98 expression in the HepG2 cells is a key factor for GDV uptake, a proteomics study was carried out to investigate which proteins on the GDVs’ surface might interact with CD98.

GDVs’ surface proteins were isolated using a standard protocol of membrane isolation for the cells. GDVs’ surface proteins were digested using trypsin (0.25%) and separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (4–15%, 135 V, 80 min). As shown in Figure 5A, the channels from left to right are protein markers, undigested plasma proteins from GDVs (undigested), and GDVs’ plasma proteins digested by trypsin for 4 h (digested). While fewer bands in the GDVs’ plasma membranes digested by the trypsin channel indicated that most proteins were degraded, the “undigested” plasma membrane proteins showed interesting significant bands (corresponding to the dominant presence surface proteins). As shown in Figure 5A, the bands between 110, 55, 25–37, 20–25, and 15 kDa were observed. These bands were cut off the gel and analyzed by mass spectroscopy (MS/MS), as described in the supplemental method section. The result of protein identification is shown in Figure 5B (table section). Some proteins, abundant in garlic, were found from protein identification, such as allinase, ribulose bisphosphate carboxylase large chain, and ATP synthase subunit β. Interestingly, a mannose high-affinity lectin, i.e., II lectin, was found. Because II lectin had been investigated before for specific interaction with the certain surface cell receptors, we decided to study further the specific interaction.

**Specific Interaction between the GDV Surface Ligands (II Lectin) and CD98.** The mechanism of extracellular vesicle endocytosis is diverse, including membrane fusions, protein interactions, and lipid raft-mediated internalization. As shown in Figure 6A, a significant “left” shift of the fluorescent channel peak was observed when the GDVs were exposed to the cells (2 h) at 4 °C versus 37 °C. This observation supports the fact that the uptake of GDVs by the mammalian cells was significantly inhibited under 4 °C, indicating that a predominant part of the uptake of GDVs by the cells was energy-dependent. In Figure 6B, the co-localization study of CD98 (stained in red) and undigested GDVs (stained in green) showed significant co-localization clusters visualized in yellow. To confirm that plasma membrane proteins were playing a key role in endocytosis, we conducted the same experiments (6 h incubation, 37 °C) after 1 h (Figure 6C) and 4 h (Figure 6D) digestion of GDVs’ plasma membrane proteins by trypsin. The results of this fluorescent microscopy study showed clearly that T1 GDVs (1 h trypsin digestion, Figure 6C) and T4 GDVs (4 h trypsin digestion, Figure 6D) showed significantly less co-localization signals than the undigested GDVs (Figure 6D). Combining the scientific literature with the results of the flow cytometry, mass spectroscopy, and the fluorescent microscopy experiments, we concluded that lectin family proteins present on GDVs are playing the major role in the endocytosis of GDVs mediated by CD98 interactions. A reminder, lectins are a type of proteins that can bind to glycoproteins, including CD98 glycoprotein, and lectins are found in mammals, bacteria, and plants. Animal lectins such as galectin-3 have been proved to be a ligand of CD98 and plant lectins such as GSL-IB4 have been reported to have similar carbohydrate-binding specificities with galectin-3.

Based on the literature and our corroborating results, we deduced that the interaction between a ligand (II lectin) on GDVs and CD98 glycoprotein determines the uptake of GDVs in the HepG2 cells. The next step aims to demonstrate that CD98 is the receptor involved on the side of the HepG2 and that the specific ligand on GDVs is the II lectin significantly found on the surface of GDVs by MS/MS.

**CD98 is the Main Receptor of GDVs via II Lectin-Coated GDVs.** As we showed previously in this study (Figure 3E), the downregulation of CD98 by its specific siRNA significantly inhibited the uptake of GDVs by 33% and decreased the co-localization of CD98 and GDVs, demonstrating that the uptake was highly related with CD98 on the side of the HepG2 membrane. To further confirm this, we preincubated the HepG2 cells with CD98 antibody before conducting the flow cytometry uptake assay. As observed in Figure 6E, we noticed a significant left shift of the fluorescent signal related to endocytosis of GDVs by the HepG2 cells. Based on Figure 6E, the uptake of GDVs after blocking the

**Figure 5. II Lectin presence on the surface of the intact GDVs.** (A) Gel analyses have shown that the surface proteins of GDVs were isolated using a standard protocol of membrane isolation for the cells. GDVs’ surface proteins were digested using trypsin (0.25%) and separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (4–15%, 135 V, 80 min). As shown in Figure 5A, the channels from left to right are protein markers, undigested plasma proteins from GDVs (undigested), and GDVs’ plasma proteins digested by trypsin for 4 h (digested). While fewer bands in the GDVs’ plasma membranes digested by the trypsin channel indicated that most proteins were degraded, the “undigested” plasma membrane proteins showed interesting significant bands (corresponding to the dominant presence surface proteins). As shown in Figure 5A, the bands between 110, 55, 25–37, 20–25, and 15 kDa were observed. These bands were cut off the gel and analyzed by mass spectroscopy (MS/MS), as described in the supplemental method section. The result of protein identification is shown in Figure 5B (table section). Some proteins, abundant in garlic, were found from protein identification, such as allinase, ribulose bisphosphate carboxylase large chain, and ATP synthase subunit β. Interestingly, a mannose high-affinity lectin, i.e., II lectin, was found. Because II lectin had been investigated before for specific interaction with the certain surface cell receptors, we decided to study further the specific interaction.

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CD98 receptors on the HepG2 cells with a specific CD98 antibody (10 μg/mL, 1 h) decreased as much as 47.01%. These results combined with the ones previously described in this study definitively set CD98 as the main receptor on the HepG2 cells involved in this endocytosis. Thus, we aimed to demonstrate that it was lectin protein that was the ligand of CD98. To confirm this hypothesis based on the MS/MS results and scientific literature, we deduced that if our hypothesis was right, the uptake of GDVs could be inhibited by preincubated GDVs with free mannose highly binding lectin. Our strategy was to block lectin via free mannose and observe the potential effects on the endocytosis of GDVs by the HepG2 cells. To complete the study, we decided to experiment on the different types of carbohydrates (mono- or disaccharides). The choice of free carbohydrates was defined by literature search, where it is clearly shown that lectins are a family of proteins that have high affinity to carbohydrates such as glucose, mannose, galactose, fructose, N-acetylgalactosamine (noted GalNac), lactose, and maltose. It has been demonstrated that the II lectin has a high affinity for the mannose compared with other sugars. The uptake of GDVs after preincubation with free carbohydrates (150 mM) was investigated using flow cytometry (Figure 6F). The GDVs without incubation with carbohydrates (noted control) were normalized as 100%. As shown in Figure 6F, the highest relative decrease (−44% compared to control) of the uptake of GDVs by the HepG2 cells was caused by the preincubation of GDVs with the mannose. Notably, galactose preincubation decreased uptake by 30%, while glucose did not affect the uptake.

Figure 6. CD98 is the main receptor of GDVs via II lectin-coated GDVs. (A) Uptake of GDVs by the HepG2 cells was temperature sensitive as shown by the endocytosis studied by flow cytometry at 4 °C versus 37 °C. The “left-shift” signal of fluorescence at 4 °C showed a decrease of intact GDV uptake by the HepG2 cells compared to 37 °C. (B) Co-localization of intact GDVs (green) with CD98 expressed on the HepG2 cells (red). (C) Co-localization of 1 h trypsin-digested GDVs (T1 GDVs in green) with CD98 expressed on the HepG2 cells (red). The HepG2 cells were incubated with 100 μg/mL GDVs for 6 h. The GDVs were labeled with coumarin-6 (green), CD98 was stained in red, and cell nuclei were stained with DAPI (blue). The co-localization of GDVs-CD98 appears in yellow. (E) Uptake of GDVs by the HepG2 cells was CD98 sensitive as shown by the endocytosis studied by flow cytometry with a preincubation of the cells with CD98 antibody (CD98 Ab) versus no blocking. The left-shift signal of fluorescence with the CD98 Ab showed a decrease of intact GDV uptake by the HepG2 cells compared to the cells without Ab. (F) Flow cytometry quantification of the GDV uptake by the HepG2 cells after preincubation of GDVs with 150 mM of different saccharides, glucose, mannose, galactose, fructose, N-acetylgalactosamine (noted GalNac), lactose, and maltose. Relative uptake was calculated and standardized to the uptake of GDVs by the HepG2 cells without treatment considered as 100%. The results are displayed in relative percentages ± SD. The results shown are representative of three independent experiments performed in duplicate.

Figure 7. Intact GDVs had a biological effect on reducing the expression of an anti-inflammatory gene (interferon γ noted IFN-γ and interleukin 6 noted IL-6). The HepG2 cells were incubated without (control noted control), with GDVs (100 μg/mL), or with T4 GDVs (100 μg/mL) for 24 h and total RNAs of (A) interferon γ noted IFN-γ and (B) interleukin 6 noted IL-6 were isolated by using the RNA extraction kits. The CD98 expression was regulated by LPS, CD98 siRNA, and without any treatment (regular). The HepG2 cell without any treatments was set as 1. The values represent means ± SD. Data are representative of n = 3 determinations, *p < 0.05, ***p < 0.001. Normalization of the results has been done over a gene known as stable expression (housekeeping gene) gene glyceraldehyde 3-phosphate dehydrogenase (GAPGH).
efficiency. Lactose, GalNac, maltose, and fructose preincubations did not significantly modify the uptake of GDVs by HepG2 (less than 5% change). The data indicated that the uptake of GDVs was highly inhibited by mannose preincubation, strongly supporting the role of the lectins on GDV surfaces. Taken together, we can conclude that CD98 is a key receptor for GDV uptake and that the mannose preincubation coupled with the MS/MS results strongly supports that this interaction with CD98 is lectin-dependent on the GDV surface side.

**GDVs Uptake by the HepG2 Cells Reduced the LPS-Induced Inflammation.** Knowing the biological effect of GDVs on the liver cells could help understand the benefit of garlic in the human diet. The IFN-γ and IL-6 have been reported to play an important role in many pathological conditions, such as inflammation, cancer, and infection.\(^\text{54,55}\)

The in vivo amount of LPS was majorly produced by the gut Gram-negative bacteria and the LPS was considered as the inducer of hepatic inflammation and the colitis (gut–liver axis).\(^\text{53,55}\) LPS mainly stimulated the liver cell by interacting with the different toll-like receptors. The induction of IL-6 by LPS is through the MyD88-dependent signaling pathway, while the generation of IFN-γ was in the MyD88-independent way.\(^\text{56}\) As shown in Figure 7, the anti-inflammatory effects of GDVs or T4 GDVs were tested on the HepG2 cells with different expression levels of CD98. The regular HepG2 cell control group was treated with PBS only and their relative expressions of IFN-γ (Figure 7A) and IL-6 (Figure 7B) mRNA were used as the baseline and set to 1. After the treatment of GDVs or T4 GDVs, the expression levels of IFN-γ were reduced by 42% (p < 0.001) and 29% (p < 0.05), respectively. A similar result was observed in the IL-6 mRNA expression. Moreover, more inhibition on the expression of IFN-γ and IL-6 was found in the GDV-treated groups. The LPS stimulation increased the expression level of IFN-γ and IL-6.\(^\text{57}\) The expression of IFN-γ and IL-6 mRNA on the LPS-treated HepG2 cells was significantly inhibited by applying GDVs or T4 GDVs. Strikingly, the expression level of IFN-γ mRNA in the GDV-treated LPS-stimulated HepG2 cells was significantly lower than that induced by its counterpart (T4 GDVs). The stronger anti-inflammatory effects were observed on the GDV-treated groups rather than the T4 GDV-treated groups, confirming the higher internalization of GDVs than T4 GDVs by the HepG2 cells. After the treatment of GDVs, the level of IFN-γ and IL-6 mRNA was close to the baseline, indicating the strong anti-inflammatory effect of GDVs. Silencing CD98 decreased the expression of IFN-γ and IL-6. Thus, even on being treated with GDVs or T4 GDVs, no significant difference was found in the expression level of IFN-γ and IL-6. Recently, our group reported that CD98 could be an inducer receptor in nonalcoholic fatty liver disease (NAFLD), and the CD98 siRNA-loaded nanoparticles were shown to have therapeutic potential for this disease. These nanoparticles downregulated the expression of CD98 both in vitro and in vivo, decreasing the level of NAFLD-related proinflammatory cytokines such as TNF-α, IFN-γ, and IL-1 beta.\(^\text{58}\) These results associated with the previous conclusions suggested that GDVs showed broad anti-inflammatory potentials against the LPS-induced inflammation on the liver cells. Taken together, GDVs could be a promising strategy to reduce the anti-inflammatory process in pathologies like fibrosis or colitis.

**CONCLUSIONS**

In summary, this study demonstrated that the CD98 glycoprotein (rich of mannose motifs) on the HepG2 cells was involved in the internalization of GDVs via binding with lectin-type proteins. As CD98 is highly expressed in many types of cancers, intestinal inflammation, and nonalcoholic fatty liver disease, we could investigate the potential therapeutic interest of oral administration of native GDVs in different disease models. GDVs are great therapeutic potential to target CD98 based on their significant anti-inflammatory effect and high nontoxicity specifically. GDVs may open a novel therapeutic platform for acute or chronic diseases via oral administration.

**EXPERIMENTAL SECTION**

**Materials.** Garlic was purchased from Walmart. Penicillin and streptomycin, Dulbecco’s modified Eagle’s medium (DMEM), and trypsin were purchased from Corning (New York, NY). Fetal bovine serum (FBS), SpinSmart RNA mini purification kit, cDNA synthesis master mix, and SB-Green qPCR Master Kit were from the Denville Scientific (Metuchen, NJ). Oligofectamine was obtained from Invitrogen (Carlsbad, CA). Lipopolysaccharides (LPS), coumarin-6, bovine serum albumin (BSA), d-glucose (G8270), d-mannose (M8574), d-galactose (G-6404), d-fructose (F2543), N-acetylgalactosamine (A3286), lactose (L3750), and maltose (M9171) were purchased from Sigma-Aldrich (St Louis, MI). Primary antibodies CD98 (sc-9160), counmarin-6, bovine serum albumin (BSA), d-glucose (G8270), d-mannose (M8574), d-galactose (G-6404), d-fructose (F2543), N-acetylgalactosamine (A3286), lactose (L3750), and maltose (M9171) were purchased from Santa Cruz (Dallas, TX), the secondary antibody goat anti-Rabbit IgG (H + L) ads-HRP (Cat #4050-05) was from Southern Biotech (Birmingham, AL), and goat anti-Mouse IgG (H + L) HRP Conjugate (Cat #170-6516) was from BIORAD (Hercules, CA). Alexa Fluor 568 phalloidin and DAPI (4,’6-diamidino-2-phenylindole) were from ThermoFisher (Waltham, MA). The plasma membrane protein extraction kit was purchased from Abcam (Cambridge, MA).

**Cell Culture.** The HepG2 cell line was cultured in the 1:1 L-glutamine DMEM medium with 10% FBS, 5% penicillin, and streptomycin (100× Corning) at 5% CO\(_2\) and 37 °C. LPS stimulation was conducted by adding 10 μg/mL of LPS into the culture medium for 24 h.\(^\text{34}\) CD98 siRNA specific downregulation was performed following the oligofectamine transfection procedure. Briefly, serum-free medium (SFM) was gently mixed with oligofectamine and let for incubation at room temperature for 5 min. The diluted CD98 siRNA was combined to the mix oligofectamine/SFM and incubated for 15 min at room temperature to form a complex. The complex was then added into the cells for a 4 h incubation at 37 °C (the final concentration of siRNA is 100 nM). After the 4 h incubation, the reaction was stopped by adding concentrated FBS to reach a final concentration of 10% of FBS in the medium.

**Isolation of the Garlic-Derived Nanovesicles (GDVs).** Garlic was washed three times with 1× PBS buffer and then was homogenized using a blender with precooled PBS (4 °C). The collected juice was centrifuged according to a multistep procedure optimized in our laboratory to get GDVs. Briefly, the mixture was centrifuged at 5000g for 20 min three times, 10,000g for 1 h, and 120,000g for 70 min. The pellet was washed with cold PBS three times and resuspended in PBS. The size was detected using the dynamic light scattering...
(DLS) method, atomic force microscopy (AFM), and transmission electron microscopy (TEM). The ζ-potential was measured by Malvern nano ZS90.

**Digest the Surface Protein of GDVs.** The concentration of the GDVs was calculated by the total protein concentration using the detergent compatible (DC) assay (BIORAD, Hercules, CA). GDVs were incubated with trypsin (0.25%, Corning) at a mass ratio of 2:1 at 37 °C for 1, 2, 3, and 4 h. After digestion, the pellets were collected by ultracentrifugation at 120,000g for 2 h. The pellet was washed with cold PBS three times and resuspended with 1X PBS and labeled as T1, T2, T3, and T4 GDVs, respectively, for 1, 2, 3, and 4 h of enzymatic digestion.

**TEM Measurement.** See supplementary materials.

**AFM Measurement.** See supplementary materials.

**Western Blot.** The cells (treated or not) were lysed with radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0, Roche PhoSTOP, and EDTA-free protease tablets). The concentration of protein was analyzed using the DC assay. The samples were loaded into the 4−20% SDS-PAGE and transferred the samples to a nitrocellulose membrane. After 1 h blocking, the membrane was incubated with the primary antibody at room temperature for 2 h. The membrane was washed with TBST buffer for three times. The membrane was incubated with the secondary antibody for 1 h at room temperature. Primary antibodies CD98, β-actin, and GAPDH were diluted in 1:1000, and the secondary antibodies goat anti-Rabbit IgG (H + L) ads-HRP and goat anti-Mouse IgG (H + L) HRP conjugate were diluted at 1:5000. The films were developed using a SERIES 2000 A Processor 8 film developer.

**MTT Assay.** To assess the potential toxicity of GDVs and digested GDVs, MTT assays were performed. The HepG2 cells were seeded in 96-well plates at a density of 5 × 10^4 cells/well and exposed to 100 μg/mL GDVs for 24 h. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to the wells for 4 h incubation and was reduced by the metabolically active cells to form the purple formazan dye crystals. The detergent solution was then added to the wells, solubilizing the crystals, so the absorbance can be read using a spectrophotometer. The wavelength for measuring the absorbance of the formazan product was 570 nm. Nontreated cells (controls) were set to 100% viability.

**Cell Uptake Assay.** GDVs (100 μg) were incubated with 1 μM coumarin-6 at room temperature for 30 min and then the pellets were collected by ultracentrifugation with three times of wash. The labeled GDVs were resuspended in cold PBS buffer and incubated with the HepG2 cells at 37 or 4 °C.

Uptake study was conducted by treating the HepG2 cells a concentration of 100 μg/mL labeled GDVs for 1 to 6 h. The blocking test was carried by preincubating HepG2 with 10 μg/mL CD98 antibody for 1 h before incubation with GDVs for 2 h.58,59 GDVs were incubated with carbohydrates for 30 min before the uptake assay.12 The carbohydrates were D-glucose, D-mannose, D-galactose, D-fructose, N-acetylglucosamine, lactose, and maltose. The results of uptake were visualized and analyzed by immunofluorescence microscopy and flow cytometry described below.

**Immunofluorescence Microscopy.** For CD98 staining, the cells were fixed with 4% paraformaldehyde for 15 min at room temperature. Then, the chamber slides were washed three times using the Dulbecco’s phosphate-buffered saline (DPBS). The cells were incubated with 5% BSA for 1 h at room temperature (blocking solution). Then, the cells were incubated with CD98 (1:1000 containing 1% BSA and 0.01% of Tween-20) antibody overnight at 4 °C. The cells were washed three times and incubated with the anti-rabbit IgG (1:4000) for 1 h at room temperature. After three washes with the DPBS, the cells were stained with DAPI for 5 min. Finally, the cells were washed with DPBS for three times and sealed with the Prolong Gold Antifade Reagent.

After the uptake incubation, the medium was removed and the cells were washed with DPBS three times. The cells were fixed with 4% paraformaldehyde for 15 min at room temperature. Then, the slides were washed three times and incubated with the Alexa Fluor 568 phalloidin (1:1000) for 1 h at room temperature. The cells were stained with DAPI for 5 min. Finally, the cells were washed with DPBS three times and sealed with the Prolong Gold Antifade Reagent for fluorescent microscopy.

**Flow Cytometry.** After the incubation with GDVs, the growth medium was removed and the cells were washed with DPBS for three times. The cells were fixed with 4% paraformaldehyde acid (PFA) for 10 min at room temperature. Then, the slides were washed three times and incubated with the Alexa Fluor 568 phalloidin (1:1000) for 1 h at room temperature. The cells were stained with DAPI for 5 min. Finally, the cells were washed with DPBS three times and sealed with the Prolong Gold Antifade Reagent for fluorescent microscopy.

**Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR).** Total RNA was extracted from the HepG2 cells using a SpinSmart RNA mini purification kit as per the manufacturer’s manual. RNA was quantified by a Synergy H1 plate reader (BioTek, Winooski). cDNA was synthesized from the total RNA using cDNA synthesis master mix according to the manufacturer’s instructions. Gene-specific cDNAs were synthesized by RT-qPCR using a SuperGreen qPCR Master Kit. The GAPDH gene was used as a housekeeping gene in RT-qPCR. Fold induction was calculated using the Ct method: ΔΔCt = (Cttarget gene − Cthousekeeping gene) treatment − (Cttarget gene − Cthousekeeping gene) nontreatment, and the result was calculated using the formula 2−ΔΔCt.

The primers used for qRT-PCR: IL-6 Fwd: 5′-CAATCTGGGTTACATTGAGAC-3′, 1L-6 Rv: 5′-CTCTGAGTTTGCCATGTACCTACTC-3′, IFN-γ Fwd: 5′-GCTTGAATCTAAATTATCAGTC-3′, IFN-γ Rv: 5′-GATTTTGACCCATACAGTG-3′.

**Statistical Analysis.** Data are presented as mean and standard deviation (SD). Two-way ANOVA analysis was performed to obtain statistical differences among the groups. The F-test was used for comparison between the two groups and the p-value less than 0.05 was considered as significant.

### ASSOCIATED CONTENT

**Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c02893.
In-gel digestion and MS experiments, AFM measurement, TEM measurement, the quantitative CD98 expression (Figure S1), and the secondary antibody only immunofluorescent image of the LPS-induced HepG2 cells (Figure S2) (PDF)

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**Author Contributions**

H.S. and H.L. designed, performed the experiments, and wrote the manuscript. B.S.B.C. performed some of the experiments. V.L.N. performed with H.S. the flow cytometry experiments. T.L.D. and P.G. helped corrected the manuscript and performed with H.S. the flow cytometry experiments.

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**Notes**

The authors declare no competing financial interest.

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