Grapefruit-Derived Nanovectors Use an Activated Leukocyte Trafficking Pathway to Deliver Therapeutic Agents to Inflammatory Tumor Sites

Qilong Wang1,2, Yi Ren3, Jingyao Mu4, Nejat K. Egilmez2, Xiaoyin Zhuang4, Zhongbin Deng5, Lifeng Zhang4, Jun Yan5, Donald Miller4, and Huang-Ge Zhang1,2

Abstract

Inflammation is a hallmark of cancer. Activated immune cells are intrinsically capable of homing to inflammatory sites. Using three inflammatory-driven disease mouse models, we show that grapefruit-derived nanovectors (GNV) coated with inflammatory-related receptor enriched membranes of activated leukocytes (IGNVs) are enhanced for homing to inflammatory tumor tissues. Blocking LFA-1 or CXCR1 and CXCR2 on the IGNVs significantly inhibits IGNV homing to the inflammatory tissue. The therapeutic potential of IGNVs was further demonstrated by enhancing the chemotherapeutic effect as shown by inhibition of tumor growth in two tumor models and inhibiting the inflammatory effects of dextran sulfate sodium-induced mouse colitis. The fact that IGNVs are capable of homing to inflammatory tissue and that chemokines are overexpressed in diseased human tissue provides the rationale for using IGNVs to more directly deliver therapeutic agents to inflammatory tumor sites and the rationale for the use of IGNVs as treatment for certain cancers in personalized medicine.

Introduction

Inflammation is a hallmark of most diseases, including cancer, autoimmune disease, and infectious disease. The development of target-specific delivery systems to inflammatory sites is needed urgently. The attraction of leukocytes, including T cells, to sites of inflammation and infection is an essential component of the host response to disease, including autoimmune and chronic inflammatory diseases as well as infectious disease and cancer. Recruitment of circulating T cells to sites of pathogen entry or inflammation involves at least two separate migration processes, termed extravasation and chemotaxis. Adhesion to the luminal side of blood vessels, trans-endothelial migration, and subsequent chemotaxis of leukocytes are highly complex processes (1, 2). Chemokines and their receptors play a coordinating role in both the homeostatic circulation of T cells, as well as their movement to sites of inflammation or injury (1–4). Once T cells are within inflammatory tissue, their response can be affected by the many inflammatory chemokines that are overexpressed and have broad target cell selectivity (5–9). The fact that there is a redundancy within the chemokine network with respect to ligand–receptor binding and that an array of chemokines is overexpressed by a variety of cells in inflammatory tissues makes the use of chemokines a potential component for the development of therapeutic targeting.

For a therapeutic agent to exert its desired effect, it needs to (1) reach the desired site and (2) be in physical contact with its target. The development of target-specific delivery systems has not yet been broadly successful. Despite many potential advantages for using nanoparticles (10) and liposomes, hurdles to their use include cytotoxicity, induction of chronic inflammation, host immune responses, difficulties of large scale production at affordable prices, and potential biohazards to the environment (11, 12). Unlike the situation with artificially synthesized nanoparticles, naturally released nano-sized exosomes derived from many different types of mammalian cells play an important role in intercellular communication. Nano-sized exosomes released from mammalian cells have been utilized for encapsulating drugs Stat3 inhibitor JSI-124 (12.5 pmol) and curcumin (1.5 nmol; ref. 13) and siRNA (14) to treat diseases in mouse disease models without side effects. Although this approach is promising, production of large quantities of mammalian cell nanoparticles and evaluation of their potential biohazards have been challenging. We have identified recently exosome-like nanoparticles from the tissue of edible plants, including grapefruit, grapes, and tomatoes, and produced them in large quantities (15–17). As with mammalian exosomes, we have demonstrated that exosome-like nanoparticles from grapes naturally encapsulate small RNAs, proteins, and lipids. Using both in vitro cell culture models as well as mouse models, we have shown that grapefruit-derived nanovectors (GNV) are highly efficient for delivering a variety of therapeutic agents, including drugs, DNA expression vectors, siRNA, and antibody (18). In this study, we set out to determine whether the binding of inflammatory cell-derived membranes on grapefruit GNVs would be an efficient strategy to take...
advantage of the unlimited availability of GNVs and to generate personalized delivery vectors that would target inflammatory sites in diseases we are investigating (Fig. 1A). Our hypothetical model (Fig. 1A) has two advanced features that we sought to prove in this study: (i) plasma membrane from activated leukocytes is easily and preferentially bound on the microvesicles made of fruit nanoparticles lipids and (ii) the resultant microvesicles are safe and can be successfully used for targeted delivery of therapeutic agents to inflammatory sites. As proof of concept, we used GNVs as an example since the data from our previous publication (18) suggested that GNVs are nontoxic and are capable of carrying a number of different types of therapeutic agents. Also, because multiple factors are involved in the attraction of leukocytes, including T cells, to sites of inflammation, and chemokines/chemokine receptors play an important role in the last step of activated leukocyte homing to inflammatory sites, the role of IGNVs chemokines/chemokine receptors for targeted delivery was investigated as a proof of concept.

**Materials and Methods**

**Cell culture**

The mouse T-lymphoma EL4 cells, mouse 4T1, 4TO7 breast cancer cell lines, mouse NMuMG mammary gland epithelial cells, CT26 colon cancer, and human umbilical vein endothelial cells (HUVEC) were purchased from ATCC. CT26 cells were cultured in RPMI-1640 media; EL4, 4T1, and 4TO7 cells were maintained in DMEM media supplemented with 10% heat-inactivated FBS. HUVECs were cultured in complete endothelial cell growth medium (ECGM; Promocell #C-22010). NMuMG cells were cultured in complete DMEM media supplemented with 10 μg/mL insulin. All cells were maintained in a humidified CO₂ incubator at 37°C.
Plasma membrane isolation and purification

Plasma membranes were isolated and purified using a method as described (10). In brief, mouse lymphoma cell line EL4 cells were cultured with/without phorbol 12-myristate 13-acetate (PMA) stimulation (100 ng/mL) for 12 hours. Cells (2 × 10^7) were then collected and centrifuged at 500 g for 10 minutes at 4°C. The cell pellets were resuspended in 1 mL of homogenization buffer at a final concentration of 10 mmol/L Tris-HCl, 25 mmol/L D-sucrose, 1 mmol/L MgCl_2, 1 mmol/L KCl, 10 μg/mL RNase, 10 μg/mL DNase, and 1 × proteinase inhibitor cocktail. The cell suspension was homogenized on ice by 100 passes using a hand-held Dounce homogenizer. The supernatant was collected and centrifuged at 500 g for 10 minutes. For further purification, collected supernatants were subjected to a discontinuous sucrose density gradient centrifugation at 28,000 g for 45 minutes. The cell pellets were resuspended in 1 mL of homogenization buffer at a final concentration of 10 mmol/L Tris-HCl, 25 mmol/L D-sucrose, 1 mmol/L MgCl_2, 1 mmol/L KCl, 10 μg/mL RNase, 10 μg/mL DNase, and 1 × proteinase inhibitor cocktail. The cell suspension was homogenized on ice by 100 passes using a hand-held Dounce homogenizer. The supernatant was collected and centrifuged at 500 g for 10 minutes at 4°C. Leukocytes were collected and the red blood cells were lysed by incubation with ACK lysis buffer (NH_4Cl 8.024 g/L, KHCO_3 1 g/L, EDTA-2Na 3.722 mg/L) for 5 minutes at 22°C. The procedure for lysis of red blood cells was repeated once. The leukocytes were then cultured in RPMI-1640 with lipopolysaccharide (LPS, 100 ng/mL) for 12 hours before the plasma membranes from leukocytes of mice or human peripheral blood, anticoagulant-treated peripheral blood was collected and centrifuged at 3000 g for 10 minutes at 4°C. Leukocytes were collected and the red blood cells were lysed by incubation with ACK lysis buffer (NH_4Cl 8.024 g/L, KHCO_3 1 g/L, EDTA-2Na 3.722 mg/L) for 5 minutes at 22°C. The procedure for lysis of red blood cells was repeated once. The leukocytes were then cultured in RPMI-1640 with lipopolysaccharide (LPS, 100 ng/mL) for 12 hours before the plasma membranes from leukocytes of mice or human peripheral blood, anticoagulant-treated peripheral blood was collected and centrifuged at 3000 g for 10 minutes at 4°C. Leukocytes were collected and the red blood cells were lysed by incubation with ACK lysis buffer (NH_4Cl 8.024 g/L, KHCO_3 1 g/L, EDTA-2Na 3.722 mg/L) for 5 minutes at 22°C. The procedure for lysis of red blood cells was repeated once. The leukocytes were then cultured in RPMI-1640 with lipopolysaccharide (LPS, 100 ng/mL) for 12 hours before the plasma membranes from leukocytes of mice or human peripheral blood, anticoagulant-treated peripheral blood was collected and centrifuged at 3000 g for 10 minutes at 4°C.

Preparation of plasma membrane-coated GNVs

Grapefruit lipid-derived nanoparticles (GNV) were prepared according to the protocol as described in our previous publication (17). To prepare the plasma membrane-coated GNVs, 400 nmol of GNVs were mixed with plasma membrane-derived vesicles (from ~5 × 10^7 cells) and extruded 20 times through a 200-nm polycarbonate porous membrane using an Avanti mini extruder (Avanti Polar Lipids).

Preparation of plasma membrane-derived vesicles

The plasma membranes purified from EL4 cells and mice or human peripheral blood leukocytes were sonicated in a glass vial with 200 μL ddH_2O for 10 minutes using a FS30D bath sonicator (Fisher Scientific). The resulting vesicles were subsequently extruded through 100 nm polycarbonate porous membranes using an Avanti mini extruder (Avanti Polar Lipids).

Statistical analysis

One-way ANOVA followed by Turkey post hoc tests was used to determine the differences occurred between groups, and t test was used to determine the difference between two groups (*, P < 0.05; **, P < 0.01; and ***, P < 0.001).

Results

Characterization of GNVs coated with inflammatory chemokine receptor enriched membrane fraction of activated T cells (IGNVs)

IGNVs were generated by binding membranes derived from the PMA-activated EL4 T-cell line to GNVs. Morphology (Supplementary Fig. S1A) and quantity of chemokine receptors (Supplementary Fig. S1B) of EL4 T cells stimulated with/without PMA were analyzed. Vesicles from the purified middle band of a sucrose gradient (Supplementary Fig. S2A) were prepared by extrusion and subsequently bound with GNVs. The Zeta potential and size distribution of IGNVs were then analyzed (Fig. 1B). Transmission electron microscopy imaging (Fig. 1C) indicated that IGNVs had a similar morphology of GNVs. IGNVs were internalized by CT26 colon cancer cells in a similar manner as GNVs (Fig. 1D), and colocalized with EL4 membrane (Fig. 1D, last column). Simple mixing of GNVs with EL4 cell-derived membrane vesicles did not result in colocalization in transfect ed 4T1 cells (Supplementary Fig. S2B). Fluorescence resonance energy transfer (FRET) analysis further confirmed that more than 83 ± 2.2% of the GNVs were coated with plasma membrane from activated EL4 T cells (IGNVs, Fig. 1E). After preparation and through 5 days at 22°C (Supplementary Fig. S2C) or 25 hours at 37°C (Supplementary Fig. S2D), the IGNVs were stable without significant changes in size or charge.

IGNVs home to inflammatory sites

Next, to determine whether IGNVs would leave the peripheral circulation and home to inflammatory tissues, three inflammatory mouse models were tested. We first sought to determine whether IGNVs transmigrated through a HUVEC monolayer at a higher efficiency than GNVs by using an in vitro Transwell assay. The data demonstrated that much higher numbers of IGNVs-transfected HUVECs (DAPI PKH26; data not shown) migrated to the bottom of the Transwell (right) than did GNVs (left) over a 48-hour period (Fig. 2A). Addition of chemokines (CXCL1/2/9/10, CCL2/5) in the bottom of the Transwell further enhanced the efficiency of IGNV transmigration (Fig. 2A, right; 4th column, ***, P < 0.01). Enhanced homing of IGNVs to inflammatory sites when compared with GNVs was further confirmed in different inflammatory models, two acute inflammation models [a LPS-induced skin inflammation (Fig. 2B) and dextran sulfate sodium (DSS)-induced colitis model (Fig. 2C; **, P < 0.01)] and two chronic inflammation cancer models [the CT26 colon cancer (Fig. 2D; ***, P < 0.001) and 4T1 breast cancer models (Fig. 2E; ***, P < 0.001).

CXCR2 and LFA-1 play a key role in IGNV homing

Leukocyte recruitment into inflamed tissue follows a well-defined cascade of events, beginning with capturing of free flowing leukocytes to the vessel wall, followed by rolling, adhesion to endothelial cells, post-adhesion strengthening, crawling, and finally transmigration through endothelial junctions into sites of inflammation. During these steps, chemokines/chemokine receptors play a key role in the last step, transmigration. We therefore analyzed the profiles of chemokines from the extracts of inflammatory tissues and the types of chemokine receptors coated on the IGNVs. Chemokine array data (Fig. 3A) indicated that chemokines identified are in much higher concentrations in the extracts of the inflammation models we tested than the extracts from noninflamed mammary gland (Fig. 3A, normal). We also noticed, in general, that stronger chemokine signals were detected in the extracts from 4T1 breast tumor than from the skin of LPS-induced skin inflammation or CT26 colon tumor. FACS analysis data indicated that corresponding receptors of chemokines are detected on the IGNVs (Fig. 3B). To determine which...
The effects of chemokines on IGNVs transmigration were then confirmed by the results generated from a neutralizing chemokine assay. Although preincubation with recombinant chemokine against each chemokine receptor partially attenuated the migration of IGNVs, neutralizing all six chemokine receptors as listed or preincubation with 4T1 tumor extract led to a maximum reduction of IGNVs transmigration (Fig. 3C; *P < 0.05; **P < 0.01; and ***P < 0.001). This reduction was also confirmed in a LPS-induced skin inflammation model in mice where the mice were i.v. injected with DiR dye-labeled IGNVs that had been preincubated with 4T1 tumor extract (Fig. 3D; **P < 0.01).

The data generated from a 24-hour (time course was determined based on the data from Fig. 3D) in vivo imaging analysis further confirmed that although CXCL1, CCL2, and CCL5 have an effect, CXCL2 plays a key role in homing IGNVs into inflammatory sites, including LPS-induced acute inflammation in skin (Fig. 3E; *, P < 0.05; **P < 0.01; and ***P < 0.001) and the CT26 colon cancer model (Fig. 3F; *, P < 0.05; **P < 0.01; and ***P < 0.001).

Integrins, including LFA-1 and α4β7, have multiple functions in the process of leukocyte recruitment from initially adhering to vessel walls to crawling before the final step, transmigration. FACS data indicated that although no α4β7 was detected, LFA-1 was highly expressed on both PMA-activated EL4 cells (Supplementary Fig. S4A) and present on IGNVs (Supplementary Fig. S4B). Transmigration of IGNVs was dramatically decreased in vitro (Fig. 3H; ***, P < 0.001) and in vivo (Fig. 3I; *, P < 0.05) when LFA-1 on IGNVs was neutralized. Because multiple factors, including LFA-1, play a role in the process of leukocytes and chemokine(s) in the inflammatory tissues play a causative role in recruiting IGNVs into the inflammatory tissue, an in vitro Transwell assay was conducted. The results indicated that the transmigration of IGNVs was affected in a remarkable way by addition of chemokines, CXCL1/2/9/10/CCL2/5, whereas there was no change in GNV migration with the addition of chemokines. Distribution of DiR dye-labeled IGNVs in: LPS-induced skin acute inflammatory mouse model (B); DSS-induced colitis mice (C); CT26 tumor model (D); and 4T1 tumor model (E). Live-mouse images (left) were collected 6 and 24 hours after i.v. injection of DiR dye-labeled IGNVs. Skin, colon, and tumor tissues were removed 24 hours after the injection and scanned for DiR dye signals. A representative image from each group of mice is shown (left) and followed by graphical figures (right) presented as the mean net intensity (sum Intensity/area, n = 5). *P < 0.01 and **P < 0.001. Data are representative of at least five independent experiments.
nanoparticles (10) homing to inflammatory sites, and chemokines/chemokine receptors have been known to play important roles in the last step (transmigration) of leukocyte homing to inflammatory tissue in general, for proof of concept in this study, chemokine-related assays were used as a primary determinant of function without further analysis of the role of IGNV LFA-1.

We further determined whether the chemokines of interest were upregulated in human cancer tissues. The results from immunohistological staining of chemokines in human breast cancer (Supplementary Table S1) and colon cancer (Supplementary Table S2) suggest a much higher expression of CXCL1, CXCL10, CCL2, and CCL5 in tumor tissues than in adjacent nontumor tissues (Fig. 3E and Supplementary Tables S3 and S4).

To further demonstrate whether this approach can be applied for treatment of patients in a personalized manner, GNVs coated with the membrane of LPS stimulated leukocytes isolated from the peripheral blood of healthy human subjects (Supplementary Fig. S5A) or of mice (Supplementary Fig. S5B) were purified using a sucrose gradient. The chemokine receptors on the IGNVs were quantitatively analyzed (Fig. 4A and B). We then determined whether IGNVs are capable of homing to human tumor using the human colon SW620 tumor model because the chemokines of interest are overexpressed in human colon cancer, as well as breast tumor tissue, and are also released from SW620 colon cancer cells (Supplementary Fig. S5C, ***, *P < 0.001). The results from in vivo imaging analysis indicate that human SW620 tumor-bearing mice (Fig. 4C, ***, *P < 0.01) or mice locally challenged with LPS (Fig. 4D, ***, *P < 0.05 and ***, *P < 0.001) attracted more IGNVs than GNVs, and IGNVs coated with purified membranes of LPS-stimulated leukocytes have the highest fluorescence intensity day 5 after i.v. injection. The results from an in vitro Transwell assay (Fig. 3C) further stimulated us to further determine whether CXCXR2 plays a dominant role in IGNV homing to the inflammatory site. The results generated from in vivo imaging analysis indicate that IGNVs coated with the membrane of LPS-stimulated leukocytes isolated from the peripheral blood of CXCXR2 knockout mice had significantly attenuated the migration of IGNVs to the inflammatory site (Fig. 4E), suggesting that IGNV CXCR2 plays a key role in IGNV homing. In vivo therapeutic effects of drugs carried by IGNVs

Because no adverse side-effects had been observed with an i.v. injection of IGNVs (Supplementary Fig. S6), we tested whether IGNVs can be used as a therapeutic drug delivery vehicle. IGNVs are capable of being loaded with different drugs, including chemo drugs, such as doxorubicin, and anti-inflammatory agents like curcumin (Supplementary Fig. S7A). Both doxorubicin and curcumin-loaded IGNVs have a similar Zeta potential and size distribution (Supplementary Fig. S7B and S7C) although the loading capacity (Supplementary Fig. S7D) and releasing of an agent (Supplementary Fig. S7E) are different for different types of agents. Further analysis of the stability of IGNVs indicated that circulating IGNVs were stable and detectable until day 5 (Fig. 5A) after an i.v. injection, which in turn provides a longer time span for IGNVs to home to where inflammation is occurring. In addition, IGNV-DOX was stable without an increase in releasing doxorubicin until they were in a pH 5.5 (Fig. 5B, **, *P < 0.01). In contrast, a pH as low as 5.0 did not result in the increase of releasing doxorubicin from commercially available doxorubicin-loaded liposomes (Supplementary Fig. S7F), although there were no differences observed in the cell cytotoxicity (Supplementary Fig. S7G), in vivo induction of proinflammatory cytokines (Supplementary Fig. S7H), and the release of liver enzymes (Supplementary Fig. S7I) between IGNVs (Supplementary Fig. S6D) and control liposomes. The tissue distribution of doxorubicin encapsulated into IGNVs was determined next. The concentration of doxorubicin was higher in the tumor and lower in the liver of tumor-bearing mice i.v. injected with IGNV-DOX when compared with DOX-NP-treated mice (Fig. 5C, **, *P < 0.05). This result was further confirmed by the much stronger intensity of doxorubicin signals detected in the CT26 tumor as well as 4T1 breast tumor-bearing mice i.v. injected with IGNV-DOX when compared with mice treated with GNV-DOX or free doxorubicin (Fig. 5D). Injection of GNV-DOX mixed with EL4 cell-derived membrane vesicles had significantly lower levels of doxorubicin detected in the 4T1 tumor than IGNV-DOX (Supplementary Fig. S8A), suggesting that the extrusion of GNVs with the activated leukocyte membranes, which leads to formation of

Figure 3. Chemokine-mediated pathways play a causative role in efficient targeted delivery of IGNVs to inflammatory sites. A, chemokine expression in normal skin (normal), LPS-induced acute inflammatory skin tissues (Skin-LPS), CT26 (CT26 tumor), and 4T1 (4T1 tumor) were determined using the Proteome Profiler immunoassay kit from R&D Systems. Each dot represents a chemokine detected by a capture antibody and printed in duplicate on the membrane. B, expression of chemokine receptors on the IGNVs was analyzed by FACS analysis of IGNVs coated on 4-μm diameter aldehyde/sulfate latex beads. A representative image (n = 5) from each sample is shown. C, in vitro transmigration of IGNVs. HUVEC cells (n = 3) were cultured in the fibronectin-coated upper chamber as a transmigration barrier. PHK26-labeled IGNVs (PHK26-IGNVs) were preincubated with recombinant chemokines as listed in the figure and then the extract from 4T1 tumor. After washing, preincubated PHK26-IGNVs were added to the upper chamber and cultured in the presence of recombinant chemokines (CXCL1/2/5/10 plus CCL2/5) in the lower chamber. After a 24-hour incubation, the intensity of PHK26 fluorescence of media in the lower chamber (n = 5) was measured and expressed as the percentage of Transwell efficiency of PHK26. IGNVs. D, images of IGNVs in LPS-induced acute skin inflammatory mice. DiR dye-labeled IGNVs were preincubated overnight at 4°C with (neutralized) or without (not neutralized) 4T1 extract before an i.v. injection. A representative image at 6 and 24 hours after the injection from each group of mice (n = 5) is shown (left) and followed by graphical figures (right) presented as the mean net intensity (sum intensity/area, n = 5). Data are representative of at least three independent experiments. E, IHC staining of chemokines (CCL2, CCL5, CXCX9, and CXCX10) expressed in human breast cancer, colon cancer tissues (bottom), and paired adjacent tissues (upper panels). A representative image (n = 20 for colon cancer; n = 21 for breast cancer) from each sample is shown. DiR dye-labeled IGNVs were preincubated at 4°C overnight with recombinant chemokines as listed in the figure and then i.v. injected into LPS-induced acute skin inflammatory mice (F) or CT26 tumor-bearing mice (G). DiR dye signals in skin and tumor tissues were determined 24 hours after the injection. H, transmigration of IGNVs with/without LFA-1 neutralization. PHK26-labeled IGNVs were preincubated overnight with anti-LFA-1 antibody at 4°C and then added into the apical chamber. The intensity of PHK26 fluorescence of the media in the lower chamber was measured after 24 and 48 hours of incubation and expressed as the percentage of Transwell efficiency of PHK26-IGNVs. I, IHC dye-labeled IGNVs were preincubated with functional anti-LFA-1 antibody at 4°C overnight, washed, i.v. injected into LPS-induced acute skin inflammatory mice and the DiR signals was detected after 24-hour injection. A representative image (F, G, and I) from each group of mice (n = 5) 24 hours after the injection is shown and graphical figures are presented as the mean net intensity (sum intensity/area, n = 5). ***, *P < 0.005; **, *P < 0.01; and ***, *P < 0.001. Data are the mean ± SEM. of at least three independent experiments.
IGNVs, is required for higher efficiency delivery of DOX to inflammatory sites. The biologic effects of IGNV-DOX on the CT26 colon tumor and 4T1 breast tumor models were also significant when compared with the other treatments. IGNV-DOX treatment led to significant inhibition in the growth of CT26 and 4T1 tumor (Fig. 5E; /C3, P < 0.05; /C3/C3, P < 0.01; and /C3/C3/C3, P < 0.001) and extended the survival of tumor-bearing mice (Fig. 5F; /C3, P < 0.05 and /C3/C3, P < 0.01). The results from IGNV-Cur treatment of DSS-induced mouse colitis also indicated that IGNVs carrying curcumin has a better therapeutic effect on the inhibition of colitis than GNVs carrying curcumin or curcumin alone. This conclusion is supported by the fact that there was less blood in stools (Supplementary Fig. S8B), there was less weight loss (Supplementary Fig. S8C), there was a significantly improved survival rate (Supplementary Fig. S8D) in DSS-induced colitis mice i.v. injected with IGNV-Cur than control groups as listed, and fewer leukocytes were observed infiltrating hematoxylin and eosin-stained mouse colon tissue (Supplementary Fig. S8E). These results were also consistent with higher concentrations of curcumin detected in the colon tissues of mice treated with IGNV-Cur (Supplementary Fig. S8F). ELISA analysis further indicated that significantly less TNFα, IL6 and IL1β were detected in the colon tissue extracts of DSS-induced colitis mice i.v. injected with IGNV-Cur than with PBS/DSS, free Cur, or GNV-Cur (Supplementary Fig. S8G).
Discussion

Our study advances an approach for targeted delivery of therapeutic agents to inflammatory sites where the appropriate cells are targeted; thereby promoting much more substantial therapeutic benefits without inducing adverse side effects. We show that IGNVs can be an effective, personalized approach to potentially treat patients with a variety of inflammatory conditions. The use of IGNVs avoids several of the problems that have arisen with conventional therapy vectors, such as the lack of tissue targeting specificity, immunogenicity, difficulty in scalability and production, and the need for life-long monitoring for tumorigenesis and other adverse clinical outcomes.

Because IGNVs do not cause these concerns, they have great potential as targeted delivery vehicles, in particular, because production of GNVs is easily scaled up and the GNVs can be coated with leukocyte membranes from an individual making this approach personalized and economically feasible for treatment of patients in low and middle income countries where they do not have comprehensive facilities to make synthetic therapeutic vectors.
macromolecular drugs. This phenomenon is referred to as the “enhanced permeability and retention (EPR) effect” of molecules and lipids in solid tumors (19, 20). Therefore, the longer the vector is in the circulation, the greater opportunity it has to penetrate the tumor tissue by utilizing the EPR effect. Our previously published work shows that a nanovector made of lipids from grapefruit nanoparticles circulates in the peripheral blood more than 5 days in tumor-bearing mice (18). GNVs are stable in the circulatory system and thus have a greater opportunity to enter the tumor tissue. In addition, the critical concentration of grapefruit nanoparticle-derived lipids for GNV formation was 1.5 μmol/L (Supplementary Fig. S9), and GNVs do not increase in size with increasing concentrations of total lipids up to 5 μmol/L. The diameter of GNVs at 25 μmol/L to 50 μmol/L concentration and at 25°C is increased up to 200 nm, and retained at 200 nm, whereas lipid concentration was further increased up to 100 μmol/L. We believe that this finding not only provides a guideline for selecting concentrations of grapefruit nanoparticle-derived lipids to make desirable sized GNVs (50–200 nm), but also a broad range of concentrations of grapefruit nanoparticle-derived lipids can be selected for making GNVs without leading to a further increase of GNV size. The latter feature has an advantage in case that a large amount of grapefruit nanoparticle-derived lipids is required to make GNVs, which allows for higher concentrations of therapeutic agents to be encapsulated per GNV. However, this feature of GNVs alone may not be sufficient to deliver ample agent to the targeted tissue to have a therapeutic effect. Achieving specific and safe delivery of a drug across endothelial barriers from activated leukocytes. This modiﬁcation of IGNVs significantly enhanced their endothelial cell transmigration capability so the IGNVs could enter inflammatory tissues. As we demonstrated in this study, not only can this strategy be applied to breast cancer and colon cancer, but also the potential exist that the IGNVs could be used to treat many different types of diseases since the inflammatory process is a hallmark of many chronic diseases, including cancer, infectious diseases, and autoimmune diseases. As shown in this study, the chemotherapy drug doxorubicin and the anti-inﬂammatory agent curcumin can be delivered successfully by IGNVs to reach the desired inﬂammatory site and achieve a therapeutic effect through modification of GNVs with membranes of activated leukocytes from individuals. Speciﬁcally, we demonstrated that i.v. injection of IGNV-DOX or IGNV-Cur signiﬁcantly enhances the inhibition of breast tumor and colon tumor growth, and attenuates DSS-induced colitis, respectively. This response was due to improved recruitment of IGNVs into tumor as well as inﬂamed colon tissue. The membranes from activated leukocytes were required to obtain this beneﬁt, as it permitted IGNV delivery to the precise place where inﬂammation was occurring.

We expect that other factors in addition to chemokines/chemokine receptors may also play a role in IGNVs homing to an inﬂammatory site. The results published by another group suggest that LFA-1 plays a role in the nanoparticle homing to an inﬂamed site (10). We used LFA-1 (CD11a–CD18) as an example. Our data also showed that CXCR2 and LFA-1 played a role in the transmigration of IGNVs as demonstrated in an in vitro Transwell blocking assay and in vivo skin inﬂammatory mouse model. Therefore, the role of IGNV chemokine receptors as demonstrated in this study does not exclude a number of other IGNV factors, such as CXCR2 and LFA-1, which also plays a role in the process of IGNV recruitment into inﬂamed tissue. This is also the reason that we believe that IGNVs coated with total leukocyte membranes may have a greater potential for being applied to personalized medicine for targeted delivery to inﬂammatory sites than the use of individual chemokine receptor coated IGNVs. It is conceivable that membrane-associated chemokine and integrin proﬁles of circulating inﬂammatory cells from patients with different chronic inﬂammatory diseases may be different in their makeup. Furthermore, individual chemokine receptor-coated GNVs may be potentially difﬁcult to optimize in combinations or as a customized set or group of chemokine receptors that are most suitable for targeted delivery for an individual patient. In addition, there may be a higher cost for production of recombinant chemokine receptors and recombinant production would require FDA approval for clinical use. Finally, potential biosafety issues could arise due to using synthesized recombinant chemokine receptors.

An ideal delivery vehicle should be susceptible to manipulation so delivered therapeutic drugs can be released within the targeted tissue. Evidence accumulated over the past decades has shown that the pH in electrode-evaluated human tumor is on average lower than the pH of normal tissues (21–24). Our results presented in this study show that doxorubicin encapsulated in the IGNVs is stable until the pH drops to 6.0 or below. This feature of IGNVs allows the encapsulated drug to selectively be released in tumor tissue, and therefore reduces the side effects seen when chemotherapy treatment nondiscriminately affects healthy organs and tissues, which is one of major obstacles for chemotherapy for treatment of patients with cancer.

In the past decades, substantial experimental and clinical evidence supports the conclusion that one of the most important mechanisms operating in tumor progression involves chemokines and their receptors (25, 26). Chemokines and their receptors not only play a role in cancer-related inﬂammation, but also have been implicated in the invasiveness and metastasis of diverse cancers (25, 27, 28). We speculate that pseudo-inﬂammatory chemokine receptors delivered by GNVs may also act as soluble receptors to block the pathway(s) mediated by chemokine receptors expressed on the tumor cells or other tumor-associated cells. This assumption is currently under examination in our laboratories, and if this is a case, not only could this delivery strategy be applied to targeted delivery as demonstrated in this study, but also could prove useful in treating a number of chemokine/chemokine receptor-associated diseases.

Disclosure of Potential Conﬂicts of Interest

No potential conﬂicts of interest were disclosed.

Authors’ Contributions

Conception and design: Q. Wang, D. Miller, H.-G. Zhang
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Q. Wang, Y. Ren, H.-G. Zhang

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Q. Wang, Y. Ren, H.-G. Zhang

Writing, review, and/or revision of the manuscript: Q. Wang, N.K. Egilmez, H.-G. Zhang

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Q. Wang, J. Mu, X. Zhuang, Z. Deng, L. Zhang, H.-G. Zhang

Study supervision: H.-G. Zhang

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References

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Grapefruit-Derived Nanovectors Use an Activated Leukocyte Trafficking Pathway to Deliver Therapeutic Agents to Inflammatory Tumor Sites

Qilong Wang, Yi Ren, Jingyao Mu, et al.


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