Ultrasound Molecular Imaging as a Potential Non-invasive Diagnosis to Detect the Margin of Hepatocarcinoma via CSF-1R Targeting

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Through radiofrequency ablation (RFA) is considered to be an effective treatment for hepatocellular carcinoma (HCC), but more than 30% of patients may suffer insufficient RFA (IRFA), which can promote more aggressive of the residual tumor. One possible method to counter this is to accurately identify the margin of the HCC. Colony-stimulating factor 1 receptor (CSF-1R) has been found to be restrictively expressed by tumor associated macrophages (TAMs) and monocytes which more prefer to locate at the boundary of HCC. Using biotinylation method, we developed a CSF-1R-conjugated nanobubble CSF-1R (NBCSF−1R) using a thin-film hydration method for margin detection of HCC. CSF-1R expression was higher in macrophages than in HCC cell lines. Furthermore, immunofluorescence showed that CSF-1R were largely located in the margin of xenograft tumor and IFRA models. In vitro, NBCSF−1R was stable and provided a clear ultrasound image even after being stored for 6 months. In co-culture, NBCSF−1R adhered to macrophages significantly better than HCC cells (p = 0.05). In vivo contrast-enhanced ultrasound imaging, the washout half-time of the NBCSF−1R was significantly greater than that of NBCTRL and Sonovue® (p = 0.05). The signal intensity of the tumor periphery was higher than the tumor center or non-tumor region after NBCSF−1R injection. Taken together, NBCSF−1R may potentially be used as a non-invasive diagnostic modality in the margin detection of HCC, thereby improving the efficiency of RFA. This platform may also serve as a complement method to detect residual HCC after RFA; and may also be used for targeted delivery of therapeutic drugs or genes.

Keywords: ultrasound imaging, HCC tumor margin, non-invasive tumor margin detection, CSF-1R targeting, macrophage
INTRODUCTION

Hepatocellular carcinoma (HCC), is the third leading cause of cancer death in China (Chen et al., 2016). Radiofrequency ablation (RFA) which considered to be a valid local treatment method with curative intent and shows a comparable overall outcome to that of liver resection when patients with HCCs smaller than 3 cm in diameter (N’Kontchou et al., 2009; Kang et al., 2015). However, one major cause of insufficient RFA (IRFA) is the uncertain ablation margin, which may lead to local recurrence with a more aggressive phenotype and worse prognosis (Kim et al., 2010; Wang et al., 2013; Liu et al., 2015; Shady et al., 2016; Sotirchos et al., 2016; Dai et al., 2017; Zhang et al., 2019).

Some researchers found that colony-stimulating factor 1 receptor (CSF-1R) expression and tumor associated macrophage (TAM) density (CSF-1 receptor, CSF-1R or CD68) in the adjacent liver tissues are associated with patient survival after resection of HCC (Zhu et al., 2008; Jia et al., 2010; Kong et al., 2013). CSF-1R is highly expressed by monocytes (precursors of macrophages) and TAMs which support tumor cell proliferation, motility, and drug resistance (Lewis and Pollard, 2006; Pyonteck et al., 2013). CSF-1R and macrophages are the front line of defense to prevent tumor growth. The peritumoral liver tissue, which possessed of abundant CSF-1R, plays an opposite role in anti-tumor effect by providing a fertile environment for metastasis (Qian and Pollard, 2010). A high density of CSF-1R in peritumoral liver tissue, but not in tumor tissue, was associated with poor survival and a high incidence of metastasis after resection of the primary tumor (Zhu et al., 2008; Nandi et al., 2013). Leftin et al., 2019 confirmed that macrophage-targeted inhibition of CSF-1R by immunotherapy inhibits macrophage accumulation and slows mammary tumor growth in vivo. Thus, CSF-1R might be a feasible target for molecular imaging of HCC.

Ultrasound molecular imaging can provide high specificity and sensitivity imaging as it combines the advantages of ultrasound contrast agents (UCAs). UCAs can targeted with ligands such as antibodies or other proteins to detect expression of cancer-specific molecular markers (Jiang et al., 2016; Li et al., 2018; Wang et al., 2018). Unfortunately, traditional UCAs composed of microbubbles with a diameter about several micrometers, which cannot penetrate through the vasculature and have the short circulation time, which has constrained the advancement of ultrasound molecular imaging (Krupka et al., 2010; Wang et al., 2010). Nanobubbles (NBs, <1000 nm) were then introduced as a contrast agent enhancer in ultrasound imaging. However, NBs may decrease the echogenicity under clinical ultrasound (Sheeran et al., 2013). So it extremely challenging to fabricate not only small, highly echogenic particles but also can provide new, paradigm shifting applications of ultrasound agents in diagnosis and therapy (theranostics; Guvener et al., 2017; Tang et al., 2017; Liu et al., 2019).

Herein, to address the above shortcomings, we designed a novel CSF-1R targeted nanobubble (NBCSF−1R) and characterized its properties in vitro and in vivo. We also investigated the specificity and efficacy of the nanobubbles (NBCTRL and NBCSF−1R) against HCC xenograft tumors and IRFA models to evaluate the feasibility of using NBCSF−1R in the clinical diagnosis of HCC margin (Scheme 1).

MATERIALS AND METHODS

Materials

1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, United States). Polyethylene glycol (PEG4000) was 
The following primers were used: human CSF-1R: forward (5'- > 3') AGCGATAGGTCCCCGTGTTTT, reverse (5'- > 3') CAGGGTCCAAGGCTCCAGTGG, reverse (5'- > 3') TGTTGTTAGAGCGGCTGAAA. Macrophages and SMMC-7721 cells were seeded into six-well plates at 5 x 10^5 cells/well in 2 mL of medium for 12 h. Cells were collected and each sample was divided into two tubes. One tube was incubated with anti-CSF-1R antibodies for 30 min and then rinsed with phosphate-buffered saline (PBS) one time. Then, the samples were incubated with PE-conjugated anti-mouse IgG for 20 min and rinsed with PBS. The fluorescence intensity in the macrophages and SMMC-7721 cells was calculated by Flow Cytometry (Beckman Coulter, Fullerton, CA, United States).

**Expression of CSF-1 in vivo**

Immunohistochemistry (IHC) analysis of human liver cancer tissue and peritumor tissue adjacent to tumor (about 10 mm) was performed. Procedures for IHC analysis of CSF-1R (anti-CSF-1R antibody, 1:200 dilution, Novus International, Inc., United States) were performed. Procedures for IHC analysis of CSF-1R (anti-CSF-1R antibody, ab215441, 1:100 dilution, Abcam, Cambridge, MA, United States) were performed. Procedures for IHC analysis of CSF-1R (anti-CSF-1R antibody, ab215441, 1:100 dilution, Abcam, Cambridge, MA, United States) were performed in two random fields in tumor tissue and peritumor tissue for each slide. The quantification of stained cells was analyzed by Image-Pro Plus. The slides were observed by using a light microscope (ECLIPSE 80i, Nikon, Japan).

**Preparation of the Nanobubbles**

Nanobubbles were prepared according to our previous studies (Jiang et al., 2016; Zhou et al., 2019). Briefly, a homogenous mixture containing DSPE-PEG_2000-biotin, DSPE-PEG_2000-DSPC, and DPPE at a mole ratio of 2.5:2.5:3:10 was mixed in 15 mL chloroform. The mixture was stirred for 1 h, then vacuum dried for 2 h at 60°C using a rotary evaporator (EYELA, Tokyo, Japan). The resulting film was rehydrated with PBS and agitated for 2 h. The size of the resulting liposomes was reduced by sonication, and then C_3F_8 gas was injected to replace the air over the fluid to generate NBs. The bubbles were purified by centrifugation and collected according to our previous research. Then, NBs were resuspended in PBS and stored at 4°C. For the development of fluorescent NBs, DiI-encapsulated NBs were prepared through the same method, with the addition of DiI in the initial mixture of phospholipids in chloroform. DiI-encapsulated NBs were observed by an inverted fluorescence microscope (Olympus IX73, Japan) and Western Blot. Excitation wavelength of DiI is 549 nm and the emission wavelength is 565 nm.

**Western Blot Analysis**

In order to determine the success of CSF-1R onto NBs surface, SDS-PAGE and Western blot were used. An 8% SDS-polyacrylamide gel was loaded with NB_CTRL, NB_CSRF-1R, and CSF-1R_mAb (Novus International, Inc., United States) and electrophoresed under reducing condition for 2 h at 60 mV and for an additional 180 min at 300 mA. The gel was then transferred to a membrane and blocked using 5% skim milk. After blocking, the membranes were horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:2000 dilution; Santa
Cruz Biotechnology, Santa Cruz, CA, United States) was used as the secondary antibody. Protein signals were detected using a chemiluminescence system (New Life Science Products, Boston, MA, United States).

**Preparation of NB_{CSF-1R}**

*In vitro* CSF-1R_{mAbs} was biotinylated using the EZLink NHS-Biotin Kit (Muralidhara et al., 2019; Wang et al., 2019). Biotinylated CSF-1R_{mAbs} was bound to the NBs (NB_{CTRL}) by linking the biotin groups of CSF-1R_{mAbs} and DSPE-PEG2000-biotin on the NBs with Streptavidin. Briefly, nanobubbles was mixed with biotinylated CSF-1R_{mAbs} using a DSPE-PEG2000-biotin:CSF-1R_{mAbs}; Streptavidin molar ratio of 30:1:15, then incubated at 4°C for 8 h (NB_{CSF-1R}). To remove the excess free CSF-1R_{mAbs}, the upper layer of the suspension was centrifuged three times (1000 rpm, 5 min) and stored at 4°C. To determine the success of conjugation, the CSF-1R_{mAbs} was labeled with fluorescein isothiocyanate (FITC) and co-localization of the CSF-1R_{mAbs} with CSF-1R were confirmed by fluorescence microscope.

**Characterization of NB_{CTRL} and NB_{CSF-1R}**

*Size, Zeta, Concentration, TEM, and Stability Test*

The mean diameter and Zeta potential of NB_{CTRL} and NB_{CSF-1R} were measured using a Malvern Zetasizer Nano (Malvern Instruments, Ltd., United Kingdom). Their morphology was detected by scanning electron microscopy (SEM, SU8020, Hitachi, Japan). The concentration of NBs was measured with a Coulter counter (Multisizer 4e, United States) according to Liu et al. (2019).

The long-term stability test of NB_{CSF-1R} were confirmed by using a Vevo 2100 small animal imaging device with a frequency of 20 MHz, in a static state. NB_{CSF-1R} was diluted from 100 to 10,000 times. The contrast imaging was then observed for each sample. To determine the long-term stability of NB_{CSF-1R}, the above experiments were repeated in samples that had been stored for 1, 3, or 6 months at 4°C. As a control, Sonovue® was suspended at the same concentration.

**Cytotoxicity Analysis**

Macrophages were induced from THP-1 cells. Approximately $5 \times 10^6$ cells were cultured with 100 ng/ml PMA for 24 h at 37°C with 5% CO2. SMMC-7721 cells and macrophages were separately inoculated into 96-well plates at 3000 cells/well for 12 h. The same volume of fresh media with various concentrations NB_{CSF-1R} were incubated with the cells for an additional 24 h, the concentration of NB_{CSF-1R} ranging from $2 \times 10^3$ to $2 \times 10^6$ bubbles/ml. Then, 10 μL CCK-8 reagent in 100 μL fresh medium replaced, and incubated for an additional 2 h. The plates were gently shook for 5 min, and Infinite F200 multimode plate reader (Spectra Max M5, Molecular Devices) was used to test the absorbance of each well at 450 nm. All experiments were conducted in triplicate.

**In vitro Targeting Ability of NB_{CSF-1R}**

SMMC-7721 and macrophages were seeded into confocal dishes at $1 \times 10^5$ cells/dish and grown for 24 h at 37°C with 5% CO2. The cells were then rinsed gently with PBS three times at room temperature, 4% paraformaldehyde was added for 5 min, then cells were gently rinsed again with PBS three times. Then, 1 ml of PBS containing 0.5% Triton X-100 was added for 5 min and rinsed with PBS three times. The remaining steps were performed in the dark: added 100 μL of diluted phalloidin solution (5 μL of phalloidin solution to 200 μL of PBS containing 0.1% BSA) to cover the cells in the center of the confocal dish; incubated for 30 min; added 200 μL Dil labeled NB_{CSF-1R} or NB_{CTRL} to the center of the confocal dish and incubated for 2 h at 37°C with 5% CO2; added 200 μL of 100 ng/ml DAPI solution and incubated for 5 min; gently rinsed 5 times with PBS to remove the unbound CSF-1R. The cells were observed under a laser confocal microscope to observe the fluorescence distribution of the cytoskeleton and the NB_{CSF-1R}, and the specific targeting of the NB_{CSF-1R} to the CSF-1 was observed.

**In vivo Contrast-Enhanced Imaging**

To generate tumors, approximately $1 \times 10^7$ SMMC-7721 cells in 100 μL of single-cell suspension was injected into 5−6-week-old male BALB/c nude mice ($n = 30$, five animals/group) in the right hind legs, subcutaneously (s.c.). The mean maximum tumor size at ultrasound was about 10 mm. In this experiment, the mice were divided into six groups ($n = 30$). Group 1 = NB_{CTRL}, Group 2 = NB_{CSF-1R}, Group 3 = Sonovue, Group 4 = NB_{CTRL} + IRFA, Group 5 = NB_{CSF-1R} + IRFA, Group 6 = Sonovue® + IRFA. During imaging, Mice were kept warm using a heated stage and a heat lamp, and anesthesia at 2% isoflurane in oxygen at 2 L/min during imaging. Mechanically, the contrast enhanced imaging can only generated while enveloped bubbles undergo compression and expansion. In this experiment, negative blank (PBS) was not included as PBS was unable to generate ultrasound intensity. Three groups received radiofrequency ablation to simulate IRFA models, which was performed using a bipolar RFA device (Radionics, INC, Burlington, MA, United States), radiofrequency energy about 30 watts for 30 s. One group of the xenograft tumor models and one group of the residual cancer models received NB_{CSF-1R}. Mice were anesthetized with isoflurane by full anesthesia machine and placed on a warm pad. Approximately, $4 \times 10^7$ NB_{CSF-1R} was injected through caudal veins. The ultrasound contrast parameters were: (Visual Sonics, Vevo 2100) Transducer: MS-250; Frequency: 20 MHz; Imaging Mode: Non-linear Contrast Mode; Dynamic Range: 30 dB; Overall Contrast Gain: 45 dB; Output Power: 4%. NB_{CTRL} and Sonovue® were injected through caudal veins similarly. VevoCQ software was used to export the image of ultrasound molecular imaging (USMI) signal, and then observe the differential targeted enhancement distribution in the region of interest (green contour).

**Statistical Analysis**

For data analysis, Statistical Package for the Social Sciences (SPSS) version-21 (SPSS, Inc., Chicago, IL, United States) was
used. GraphPad Prism version 5.00 (GraphPad Software, Inc., San Diego, CA, United States) was used to generate figures. \( p < 0.05 \) was considered statistically significant. Data from the experiments was expressed as mean \( \pm \) SD for technical replicates and the mean \( \pm \) SEM for biological replicates. ANOVA was performed to compare differences between multiple groups and Differences in continuous variables were analyzed by Student’s \( t \)-test to compare two groups. A non-parametric test of two paired samples was analyzed by Wilcoxon Signed Rank Test.

**RESULTS**

**CSF-1R Expression in vitro and in vivo**

To verify the expression of CSF-1R *in vitro*, qRT-PCR, Flow Cytometry, and Western blot were carried out. As seen in Figure 1A, qRT-PCR analysis revealed that the expression of CSF-1R mRNA is significantly higher in macrophages as compared to H22, SMMC-7721, HepG2, Hepa1-6, and THP-1 (\( p = 0.05 \)). We then proceed to select a mouse originated cell line SMMC-7721 for consideration of *in vivo* experiments. Western blot analysis also confirmed that the protein level of CSF-1R is overexpressed in macrophages and THP-1, while minimally expressed in SMMC-7721 cells (Figure 1B). Comparison of CSF-1R intensity showed a significantly greater extent of expression within macrophages (macrophage: intensity of 143.75 \( \pm \) 4.2 a.u.; THP-1: 103.02 \( \pm \) 3.4 a.u.; SMMC-7721: 78.36 \( \pm \) 3.4 a.u.; \( p < 0.001 \); Figure 1C). Quantification analysis using FACS indicated that 97.57% of macrophages are CSF-1R positive compared to 9.32% of SMMC-7721 cells (Figure 1D).

Immunohistochemistry analysis was carried out to confirm the expression of CSF-1R in HCC patients. As seen in Figure 1E, CSF-1R deposits were detected in the peritumoral tissues of carcinoma *in situ* in human HCC (Figure 1E). The counts of
positive CSF-1R differed significantly between the normal tissue and the margin \((p < 0.05; \text{Figure 1F})\).

### NPs Synthesis and Characterization

**Particle Surface Modification**

Figure 2A shows the schematic illustration of NB\(_{\text{CSF-1R}}\) fabrication through Streptavidin/biotin interaction. Western blot showed that the band intensity of CSF-1R attached on NB\(_{\text{CSF-1R}}\) was similar with CSF-1R input, while NB\(_{\text{CTRL}}\) showed no indication of CSF-1R band (Figure 2B), indicating that CSF-1R successfully conjugated with the NB\(_{\text{CSF-1R}}\) specifically \((p < 0.005; \text{Figure 2C})\).

Figure 3A depicted the two NBs synthesized, the non-targeted NB\(_{\text{CTRL}}\) and the targeted NB\(_{\text{CSF-1R}}\). The morphologies of NB\(_{\text{CTRL}}\) and NB\(_{\text{CSF-1R}}\) were observed by SEM. As shown in Figures 3B,C, NB\(_{\text{CTRL}}\) and NB\(_{\text{CSF-1R}}\) were spherical, uniform in size and had distinct shell structures. The physical properties of NB\(_{\text{CTRL}}\) and NB\(_{\text{CSF-1R}}\) are summarized in Figure 3D. Dynamic laser scattering (DLS) analysis indicated that the average hydrodynamic size of NB\(_{\text{CTRL}}\) and NB\(_{\text{CSF-1R}}\) was \(408.0 \pm 17.5\) nm and \(428.0 \pm 12.47\) nm, respectively. Zeta potential values showed that NB\(_{\text{CTRL}}\) was with charge of \(-4.03 \pm 0.23\) mV, and NB\(_{\text{CSF-1R}}\) was \(-4.42 \pm 0.51\) mV. The concentrations of NB\(_{\text{CTRL}}\) and NB\(_{\text{CSF-1R}}\) were \((5.99 \pm 0.08) \times 10^8\) bubbles/mL \((n = 5)\) and \((4.24 \pm 0.07) \times 10^8\) bubbles/mL \((n = 5)\), respectively.

### CSF-1R-Binding Efficiency to the NBs

To illustrate the \textit{in vitro} binding efficacy and co-localization of NB\(_{\text{CSF-1R}}\) with CSF-1R, we synthesized DiI-labeled NB\(_{\text{CTRL}}\) while CSF-1R\(_{\text{mAb}}\) were labeled with FITC. After co-incubation, the cells were observed under microscope. The green light of the FITC-labeled antibody (Figure 4A) and the red light of the DiI-labeled nanobubbles (Figure 4B) merged perfectly (Figure 4C), indicating that CSF-1R\(_{\text{mAb}}\) were successfully attached to the NBs, and could specifically target CSF-1R.

### In vitro Cytotoxicity and Stability of NB\(_{\text{CTRL}}\) and NB\(_{\text{CSF-1R}}\)

After the induction of THP-1 cells into macrophages by 100 ng/ml PMA, the cells changed from suspension state to adherent state, and some of the cells became spindle-like, which confirmed that successful induction of THP-1 cells into macrophages. The cytotoxicity of NB\(_{\text{CTRL}}\) and NB\(_{\text{CSF-1R}}\) was evaluated using SMMC-7721 and macrophages incubated with NB\(_{\text{CTRL}}\) at five concentrations between \(10^6\) and \(10^3\)/mL for 24 h (Figure 5A). Both SMMC-7721 and macrophages incubated with NB\(_{\text{CTRL}}\) did not show significant changes in cell viability in all concentration after 24 h of incubation. The cell viability of both SMMC-7721 cells and macrophages remained (85%) after incubation with either type of NB\(_{\text{CTRL}}\), indicating they were minimally cytotoxic. These results show that NB\(_{\text{CTRL}}\) and NB\(_{\text{CSF-1R}}\) have good biocompatibility and cause minimal harm to the tested cells.

The echogenic properties of NB\(_{\text{CSF-1R}}\) were investigated in agarose gel phantom in comparison to Sonovue\textsuperscript{®} \textit{in vitro}, using a Vevo 2100 small animal imaging device with a frequency of 20 MHz. The signal enhancements of NB\(_{\text{CSF-1R}}\) stored at 4°C for different periods of time (0, 30, 90, 120, and 180 days) were investigated. As indicated in Figure 5B, echogram result of NBs at Day-180 indicated no significant difference between NBs and Sonovue\textsuperscript{®} at Day-0 indicating that the NB\(_{\text{CSF-1R}}\) was stable.

The capability of NB\(_{\text{CSF-1R}}\) was also assessed \textit{in vitro} using a Vevo 2100 small animal imaging device with a frequency of 20 MHz at various concentrations. Different concentrations of NB\(_{\text{CSF-1R}}\) nanoparticles (approximately...
$1 \times 10^4 \sim 6.0 \times 10^5$/bubbles of same volume, 100 µL) were evaluated in this experiment. The signal intensity decreased with the decreasing concentrations of NB_{CSF-1R} (Figure 6A). However, even when the NB_{CSF-1R} were diluted 2000 times, the signal intensity remained relatively strong.

To determine the binding ability of NB_{CTRL} and NB_{CSF-1R} in SMMC-7721 cells and macrophages, we carried out confocal laser scanning microscopy (CLSM) assay. The cytoskeletons with FITC phalloidin were green and the NBs labeled with Dil were red. As seen in Figure 6B, the red fluorescence intensity of macrophages
FIGURE 4 | Fluorescence microscopy image of target NBs. (A) CSF-1RmAb (FITC showed green fluorescence), (B) Dil-dyed (nanobubbles showed red fluorescence), and (C) their co-localization (merge) under fluorescence microscope, with a sale bar of 200 µm.

FIGURE 5 | (A) Cell viability test for NBCSF−1R determined through CCK-8. In vitro cytotoxicity assays using macrophage (high CSF-1 expression) and SMMC-7721 cells (low CSF-1 expression) incubated with NBCSF−1R for 24 h; there was no significant difference in the viability of macrophages and SMMC-7721. (B) In vitro ultrasound images of NBCSF−1R stored for 0, 30, 90, 120, 180 days, and the Sonovue® stored for 0 days as a control. Ultrasound frequency, 20 MHz.

treated with NBCSF−1R was much higher than SMMC-7721 cells treated with NBCSF−1R, NBCTRL and macrophages treated with NBCTRL, while minimal attachment of NBCSF−1R and NBCTRL were seen in SMMC-7721. This result indicates that more NBCSF−1R adhered to macrophages, and demonstrated its excellent targeting ability.

Stability and Ultrasound Sensitivity of the Targeted NBs in vivo

In vivo, NBCSF−1R, NBCTRL, and Sonovue® were tested in xenograft tumors and IRFA models which had been inoculated with SMMC-7721 cells (n = 30, 5 mice for each group). After examination, none of the mice exhibited apparent signs of distress in each group, under the same ultrasound conditions. Contrast-enhanced images of the tumors continuously exposed to ultrasound were taken at minutes 0, 5, 15, and 30 (Figures 7A,B). The peak intensity and washout half-time were compared between NBCSF−1R, NBCTRL, and Sonovue® in these models (Figures 7C,D). The peak intensity of NBCSF−1R, NBCTRL, and Sonovue® (Figure 7C) was 11.55 ± 1.397 a.u, 8.826 ± 1.348 a.u, 12.20 ± 1.974 a.u in the xenograft tumors, and 12.67 ± 3.126 a.u, 13.74 ± 2.878 a.u, 11.53 ± 4.401 a.u in the IRFA models (Figure 7C). There was no significant difference between the groups (Figures 7A,B). The washout half-time of NBCSF−1R, NBCTRL, and Sonovue® in the xenograft tumors was 29.17 ± 1.08 min, 15.87 ± 1.05 min, 3.35 ± 0.16 min (Figure 7D),
and 26.84 ± 0.44 min, 6.71 ± 0.07 min, 2.89 ± 0.44 min in IRFA models (Figure 7D). Therefore, in the xenograft tumors and IRFA models, the washout half-time (Figure 7D, p = 0.05) was significantly different between NBCSF−1R, NBCTRL, and Sonovue®. As shown in Figures 7A,B, even after 30 min, the NBCSF−1R contrast agent can still enhanced efficiently in xenograft tumors and IRFA models, which implied that it has a longer circulation time in vivo.

In the xenograft tumors, the echo signal intensity of NBCSF−1R, NBCTRL, and Sonovue® in the peritumoral tissues and tumor center are shown (Figure 8). The results indicated that the intensity of the peritumoral echo signal of NBCSF−1R was significantly higher than that of the central tissue (Figures 8A,B, p = 0.05) at the peak time, 5, and 15 min.

**Immunofluorescence Analysis of the Deposition of CSF-1**

Colony-stimulating factor 1 receptor deposits were detected at the boundary of the tumor (Figure 9A), and were also detected at boundaries of the residual tumor after IRFA (Figure 9C). However, there were few deposits detected at the center of the tumor tissue (Figure 9B) or the residual tumor tissue (Figure 9D). The fluorescence intensity at the peritumor was higher than the tumor center. Therefore, similar to the human HCC spatial infiltration profiles, CSF-1R expressed in murine HCC were also abundant at the outer margins of the tumors. These results support the potential of using CSF-1R as a cancer imaging biomarker of macrophages.
DISCUSSION

Researches have shown that RFA can lead to acute serologic elevation of active cytokines such as IL-6, nMDSC, and mMDSC, and a sustained high infiltration level of macrophages in the residual tumor (Shi et al., 2019; Sugimoto et al., 2019). In this study, CSF-1R was found highly expressed at the tumor boundary in patients with HCC, and also highly expressed in macrophages, but not tumor cells; making CSF-1R a feasible target. Frozen sections of the tumors revealed that macrophages were mostly located at the boundary of the xenograft tumors and residual tissue after performing IRFA.

Nanobubble CSF-1R had an average size of about 428 nm and were ultrasound-visible even at 20 MHz both in vitro and in vivo; imaging was still viable even when diluted 2000 times. Notably, NBs were administered at a low concentration compared with our previous research and other studies (Wischhusen et al., 2018), a technique which can be employed to reduce the level of background signal and modulate facilitate the comparison of heterogeneous tumor models (Wang et al., 2010, 2016).
FIGURE 8 | (A) The echo intensity fitting curve of the same area around or in the center of transplant tumor with NB
CSF−1R, NBCTRL, and Sonovue. The intensity of the peritumoral echo signal of the NBCSF−1R was significantly higher than that of the central tissue at the peak time, 5 and 15 min (p = 0.05). Data was shown in panel (B).

To gain the insight functions of NBCSF−1R, we explored the specificity and efficiency of targeting of NBs in SMMC-7721 cells and macrophages. The results confirmed that the CSF-1R antibody could bound onto NBs efficiently; and the resulting NBCSF−1R were stable and target specific. In an in vitro cell binding experiment, these NBCSF−1R were identified to aggregate selectively surrounding macrophages but not SMMC-7721 cells, implying that the attachment of NBs to CSF-1R-positive macrophages contributes to interactions between antigen and antibody. Moreover, unconjugated NBs did not bind to macrophages, suggesting that the CSF-1R antibodies conjugating on the surface of the NBs were able to specifically recognize and improve adhesion to macrophages with high CSF-1R expression. In vivo, non-invasive imaging modality can be applied in extra-vascular region once NBs penetrate deep into the tumor neovascularature with a feature of a maximum pore size of approximately 380—780; this is because a basement membrane and smooth muscle absent and the intercellular space expands in cancer vasculature (Maeda, 2015).

Reduction in the size of the MBs not only decreases its echogenicity under clinical ultrasound but also cause instability (Sheeran et al., 2013). In our in vivo imaging experiments,
FIGURE 9 | The distribution of CSF-1R in xenograft tumor observed by fluorescence immunoassay, higher fluorescence intensity was observed at the peritumor, lower fluorescence intensity was observed at the tumor center. ([A] boundary of the tumor, [B] central of tumor, [C] boundary of the residual tumor after IRFA, and [D] residual tumor after IRFA. With a scale bar of 200 μm. The yellow line showed the tumor margin).

however, showed that the peak intensity of NB_{CSF-1R}, NB_{CTRL}, and Sonovue had no statistical difference in the xenograft tumor models and IRFA models (Figure 7C). This is probably due to the fact that lipid contrast agents can produce preferable harmonic signal intensity (Postema and Schmitz, 2007), and nanoparticles could be accumulate within tumor tissue through the enhanced permeability and retention (EPR) effect and then were transformed into micro-sized echogenic bubbles (Min et al., 2016). These microbubbles at targeted tumor tissues could serve as new echogenic particles for cancer-targeting ultrasound imaging.

With the application of acoustic radiation forces (ARF) to upregulate contrast agent binding (Zhao et al., 2004), molecular ultrasound imaging is constantly improving. Frinking et al. (2012) manifested enhanced adhesion of targeted MB in vivo upon ARF performed in experimental models of cancer. In comparison with normal vessels, they found an increased binding of VEGFR2-targeted MB (BR55) in the vasculature of experiment (Frinking et al., 2012). In this study, in the xenograft tumor model and IRFA model, the washout half-time ratio of NB_{CSF-1R} to NBs was two times higher, and about nine times higher compared to Sonovue. Furthermore, being a nanoparticle, NB_{CTRL} and NB_{CSF-1R} could accumulate at the targeted tumor tissue via the EPR effect, and NB_{CSF-1R} can abound onto higher CSF-1 expression cells effectively. The adherent NB_{CSF-1R} maintained visible for a long time, contributing to a longer persistence of enhanced contrast compared to NB_{CTRL} and Sonovue. This result further verifies that the duration of contrast enhancement may be applied as an indicator for the investigation of targeted NBs enhanced imaging. The molecular imaging would also be helpful in finding the residual tumor after IRFA. With long-term stability, NB_{CSF-1R} could be used to evaluate the boundary of the tumor when performing RFA.

CONCLUSION

In this study, a uniform nano-sized lipid NBs was prepared, and could successfully combined the NBs with biotinylated
The NB\textsubscript{CSF−1R} which was small and stable as well as high specificity for the molecule that is overexpressed in macrophages. We demonstrated the high specificity of our NB\textsubscript{CSF−1R} on targeting CSF-1R overexpressing macrophages and HCC tumor margin. \textit{In vitro} and \textit{in vivo} studies demonstrated that NB\textsubscript{CSF−1R} exhibited effective ultrasound imaging capabilities in evaluating the RFA response, which can be used to detect the residual HCC after RFA, opening a possibility of clinical translation of a non-invasive diagnosis method for IRFA.

**DATA AVAILABILITY STATEMENT**

All datasets generated for this study are included in the article supplementary material.

**ETHICS STATEMENT**

The animal study was reviewed and approved by Ethics Committee of Sun Yat-sen Memorial Hospital and Ethics Committee of Zhongshan School of Medicine (ZSSOM) on Laboratory Animal Care, Sun Yat-sen University.

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**AUTHOR CONTRIBUTIONS**

HL and BZ performed animal imaging analysis. YK performed statistical analysis. PS and BL designed and oversaw all the experiments and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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