Supporting Information

Deep tumor penetration of drug-loaded nanoparticles by click reaction-assisted immune cell targeting strategy

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Experimental Section

Characterization of MSNs-Tz and anti-CD11b-TCO. Transmission electron microscopy (TEM) was performed by using a JEOL JEM-2100F (JEOL) microscope at an acceleration voltage of 200 kV. Fourier-transform infrared (FT-IR) analysis was conducted on a VERTEX80v FT-IR spectrophotometer (Bruker, Germany). Sinapinic acid (Sigma-Aldrich) was used as the MALDI matrix, which was prepared by dissolving 1 mg in 0.1 mL 70:30 acetonitrile/water with 0.1% trifluoroacetic acid. Unmodified and TCO-modified antibodies were diluted to 150 µg mL^{-1} with PBS, combined at a 2:1 ratio with a sinapinic acid solution and 1 µl was dried onto a MALDI sample plate. MALDI-TOF mass spectrometry was performed on a Voyager-DETM STR Biospectrometry Workstation manufactured by Applied Biosystems Inc. in National Center for Inter-University Research Facilities. Hydrodynamic sizes and zeta potentials were measured by dynamic light scattering (Malvern).

Drug loading into MSNs-Tz. For loading of anticancer drug molecules, MSNs-Tz were dispersed in 0.5 ml of methanolic doxorubicin (DOX) solution (4 mg ml^{-1}), and the mixture was shaken overnight in the dark. After removing unreacted DOX by centrifugation and extensive washing with methanol and PBS, the supernatant was collected for UV-Vis absorption measurement (JASCO V-550) in order to determine the adsorbed amount of DOX. Loading amount of DOX was calculated from the peak intensity at 480 nm.

Cell culture and animals. Mouse mammary gland cancer cell line transfected with the firefly luciferase gene (4T1-luc2) was purchased from Perkin-Elmer. The cells were cultured in a
Roswell Park Memorial Institute 1640 medium (RPMI1640; Gibco-BRL, USA) containing 10% fetal bovine serum (FBS; Gibco-BRL), 1% penicillin-streptomycin (10,000 U mL\(^{-1}\) of penicillin and 10,000 µg mL\(^{-1}\) of streptomycin, Gibco-BRL). The cells were incubated at 37 °C under a humidified atmosphere of 5% CO\(_2\). Balb/c nude mice (age: 6 weeks) were obtained from the Experimental Animal Center, Chuncheon Center, Korea Basic Science Institute (KBSI), Chuncheon, South Korea. These animals were individually housed in transparent plastic cages with wire grid covers under controlled temperatures (22-24 ºC with the 12 hr light/dark cycle (lights on from 08:00 to 20:00). All animal procedures were by the Guide for the Care and Use of Laboratory Animals issued by the Laboratory Animal Resources Commission of KBSI. The Institutional Animal Care and Use Committee at the KBSI (KBSI-AEC 1724) reviewed and approved this study. All of the experiments were conducted to minimize the number of animals used and the suffering caused by the procedures used in the current study.

**In vitro viability assay.** Cell viability was assessed using MTS cell proliferation assay (G3582, Promega) to examine the toxicity of DOX-loaded MSNs-Tz to CD11b\(^+\) cells. After RAW cells were incubated with various concentrations of anti-CD11b-TCO and DOX-loaded MSNs-Tz for 24 hr, MTS solution was added to media, and the absorbance was measured at 490 nm using a microplate reader (VICTOR, Perkin-Elmer).

**Cell migration assay and cellular uptake assay.** Cell migration assay was performed using transwell plates of 8 µm pore size (Corning). 4T1-conditioned media, which was obtained after 24 hr incubation with 4T1 cells, was located in the lower compartment. After pre-incubation
with anti-CD11b-TCO (0.1 mg ml\(^{-1}\)) and MSNs-Tz (0.4 mg ml\(^{-1}\)) for 2 hr each, RAW cells were incubated in the upper compartment for 24 hr. Then, the upper chamber was removed, and the migrated cells in the under compartment were observed using microscopy. The relative migration activity was calculated by counting the cells on each group. The ratio of internalized NPs was calculated via trypan blue quenching method. First, RAW cells were incubated with anti-CD11b-TCO and RITC-MSNs-Tz for 2 hr each. After 24 hr, RAW cells were washed with PBS several times, and 0.4 mg ml\(^{-1}\) of trypan blue solution was added. Trypan blue solution was removed after 1 min, and the fluorescence intensity of RITC was measured using a microplate reader before and after trypan blue addition. The percentages of the NPs on the cell surface were measured by the ratio of fluorescence intensity differences.

**Bone marrow-derived cells labeling by click chemistry.** Bone-marrow cells were obtained from femurs and tibias, and red blood cells were removed using ammonium chloride lysis buffer. One million bone marrow cells were cultured in RPMI 1640 medium supplemented with 10% FBS in the presence of 30% v/v 4T1 tumor-conditioned media. Cells (1×10^6/sample) were labeled with anti-CD11b-TCO (25 μg ml\(^{-1}\)) in 0.1 ml PBS for 30 min at room temperature. Following centrifugation and aspiration of the antibody solution, antibody-labeled cells were directly re-suspended in MSNs-Tz (0.8 mg ml\(^{-1}\)), incubated for 30 min at room temperature. After washing twice by centrifugation with PBS, the samples were analyzed using FACS Calibur flow cytometer (BD Biosciences) and Flowjo software. Debris was excluded in an FSC/SSC dot plot, and the gate was applied to all samples. The fluorescence intensity of aCD11b-FITC was shown in the figure, while that of the negative control group was 1.89%. For confocal microscopy studies, labeled cells were attached to glass slides with removable
chamber wells (Corning). Rhodamine B in MSNs-Tz and fluorescein in anti-CD11b-TCO were imaged using a multichannel upright laser-scanning confocal microscope (LSM 780 NLO; Carl Zeiss) with a 40× water immersion objective lens. Images were analyzed using ZEN software (Carl Zeiss).

**Cellular distribution of the MSNs-Tz in the tumor by using flow cytometry.** Balb/C nude mice were inoculated with $1 \times 10^6$ 4T1-luc2 cells at mammary gland, and 4T1 tumors were harvested in 10 days post-implantation. Resected tumors first cut into small pieces and incubated in enzyme mix (Tumor Dissociation Kit for mouse, Miltenyi Biotech) or RPMI 1640 medium containing 0.2 mg ml$^{-1}$ collagenase type I (Worthington Biochemical Corporation). The enzymatic digestion process was conducted by GentleMACS™ Octo Dissociators with Heaters (Miltenyi Biotech) manually. Digested tumors were filtered through a 70 μm cell strainer (BD Falcon) to remove remaining tissue and cellular aggregates. The resulting single-cell suspension was washed in PBS with 0.5% BSA and followed by removal of red blood cells. Red blood cell lysis buffer (Sigma-Aldrich) was added to the cell pellet and incubated on ice for 5 min, followed by successive washing procedure. Cell labeling was performed with appropriate antibodies as indicated below for 45 min at 4°C: Tumor cells (PE-CD326 EPCAM monoclonal antibody, Invitrogen, Clone: G8.8), macrophages (APC/Cy7 anti-mouse F4/80, BioLegend, Clone: BM8), and CD11b$^+$ cells (APC/Cy7 anti-mouse/human CD11b, BioLegend, Clone: M1/70). CD16/32 monoclonal antibody (Invitrogen) was used to block non-specific antibody labeling. After antibody labeling, the cells were stained with DAPI (0.1 mg/mL) in PBS and subsequently fixed by 4% paraformaldehyde. The cell samples were analyzed using flow cytometry (FACS Aria, BD Biosciences) and Flowjo software.
Pre-conjugation of MSNs-Tz and anti-CD11b-TCO (TCO::Tz complex). After the synthesis of anti-CD11b-TCO, the antibodies (1 mg ml⁻¹) were mixed with MSNs-Tz (4 mg ml⁻¹) for 1 hr at room temperature. After the conjugation, the products were purified several times by centrifugation using PBS. The absorption of the TCO::Tz complex was measured with UV-Vis absorption spectroscopy (Spectro V-550, Jasco), and hydrodynamic size was characterized by dynamic light scattering (Malvern).

*In vivo whole-body fluorescence imaging.* Balb/C nude mice (n = 4 animals per group) were inoculated with 1 × 10⁶ 4T1-luc2 cells at mammary gland and then received treatment of the CRAIT strategy. The subcutaneous 4T1 tumor model mice have received i.v. injection of anti-CD11b-TCO (200 μg per mouse). After 24 hr, MSNs-Tz (25mg kg⁻¹) was injected intravenously, and whole-body fluorescence images were obtained over time. The *in vivo* fluorescence tissue imaging was conducted to track the location of cells by using whole-body imaging system (IVIS200; Xenogen Corporation, Perkin-Elmer). All animals were anesthetized in an induction chamber with 2.5% isoflurane in 100% oxygen at a flow rate of 1.0 L min⁻¹ for 10 min. For analyses, mice were imaged simultaneously for 1 min using the IVIS system with a 2.0% mixture at 0.5 L min⁻¹, and the regions of interest were quantified with photon flux (p s⁻¹) using Living Image software 4.5 (Perkin-Elmer). The data represent fluorescent signals from an individual mouse, combined from at least two independent studies. For the *ex vivo* analyses, each organ (heart, lung, liver, spleen, kidney, and tumor) was dissected after completing euthanasia.

Quantification of MSNs-Tz accumulation in organs. For biodistribution of MSNs-Tz, the
4T1 tumor model mice \((n = 3\) animals per group\) were sacrificed 24 hr after the intravenous injection of anti-CD11b-TCO (200 μg per mouse) and followed by injection of MSNs-Tz. Tumors and organs (heart, lung, liver, spleen, and kidney) were first digested in HF to extract Si contents in the tissue. Upon digestion, the samples were passed through a 0.2 μm filter to eliminate large debris. The concentrations of Si were measured by inductively coupled plasma mass spectrometry (ICP-MS, 7900, Agilent).

**Pharmacokinetics study.** To evaluate and compare the circulation half-life of CRAIT strategy probe, anti-CD11b-TCO (200 μg per mouse) were injected into the tail vein of the orthotopic 4T1 tumor model mice \((n = 3\) animals per group\). After 24 hr, MSNs-Tz (25mg kg\(^{-1}\)) containing Cy5 was injected intravenously, and 30 μl of blood from the eye socket were collected at 5 min, and 1,2,3,5,12 and 24 hours following the injection. The same dose of MSNs-Tz without anti-CD11b-TCO (PBS / MSNs-Tz) and MSNs-Tz pre-conjugated with anti-CD11b-TCO (TCO::Tz complex) was also tested in parallel as controls. The intensity of fluorescence in the collected blood was determined by Cy5 fluorescence spectrum of each sample. Pharmacokinetics parameters were calculated to fit a one-compartment model.

**In vivo toxicity evaluation of MSNs-Tz.** For *in vivo* toxicity studies, major organs including heart, lung, liver, spleen, kidney, and blood were extracted from mice at 7 days after intravenous administration of MSNs-Tz \((n = 5\) animals per group\). Organ toxicities were investigated by using hematoxylin and eosin (HHS16, HT110180, Sigma Aldrich) staining. After collecting 1 ml blood samples from mice heart, serum biochemistry was examined to assess liver function (ALP, AST, and ALT), and kidney function (BUN, and CREA) by using
blood chemical analyzer (BS400; Mindray).

**Histological analysis.** The animals were anesthetized with pentobarbital sodium and subsequently perfused transcardially with PBS followed by 4% paraformaldehyde solution. The tumor tissues were removed and post-fixed in the same fixative for 6 hr. The tumor tissues were cryoprotected by infiltration with 30% sucrose overnight. After that, frozen tissues were serially sectioned on a cryostat (CM1850, Leica, Germany) into 5 μm coronal sections, and they were then mounted on slide glasses. These sliced tissues were stained with hematoxylin and eosin (HHS16, HT110180, Sigma Aldrich, USA) followed by the standard histochemical procedures. After dehydration, the stained tissues were mounted with Canada Balsam (Kanto Chemical Co., Inc., Japan). Images were taken through a light microscope and were processed with CaseViewer 2.1 (3DHISTECH Ltd., Hungary). The sections were sequentially treated with 5% normal goat serum in PBS for 30 min. For immunohistochemical staining, the sections were next incubated with diluted FITC anti-mouse/human CD11b antibody (1:200, BioLegend, USA) overnight at 4°C. The tissues were exposed to Hoechst 33342 (1:2000, Thermo Fisher Scientific, USA), and sections were mounted with Fluoromount-G (SouthernBiotech, USA). The tumor tissue slides were observed under an LSM 780 NLO microscope (Carl Zeiss, Germany, Institute for Basic Science, Korea).
Figure S1. Anti-CD11b antibodies target 4T1 tumor microenvironment. (a) Subcutaneous 4T1 tumor mouse models were i.v. injected with αCD11b, αLy6C, and αLy6G antibodies that were functionalized with Alexa Fluor 680 dye molecule (one dye molecule per antibody). After 24 hr, the biodistribution of administered antibodies was confirmed by (i) in vivo whole-body and (ii) ex vivo fluorescence imaging. CD11b antibodies showed enhanced accumulation in a tumor compared to Ly6C or Ly6G antibodies. (b) Ex vivo immunohistochemistry analysis of 4T1 tumor slices shows CD11b⁺ cells are uniformly distributed from tumor periphery to interior regions. Data are from one experiment representative of two independent experiments (a, b).
Figure S2. Surface functionalization of MSNs. (a) Schematic illustration of synthetic procedure of MSNs-Tz. After synthesis of MSNs, cetyltrimethylammonium chloride (CTAC) molecules that were used as a porogen were extracted by ammonium nitrate (60 mg/ml in methanol). Amine-functionalized MSNs were obtained by reaction with (3-aminopropyl)triethoxysilane (APTES). The resulting MSNs were further reacted with two different chain-length N-hydroxysuccinimide PEG (NHS-PEG), including fluorenylmethoxycarbonyl (Fmoc)-PEG5k-NHS and PEG2k-NHS. After deprotection of Fmoc moiety with piperidine, the Tz-NHS molecules were reacted with the amine group at the terminal end of the long-chain PEG5k, which provides easy access to TCO-containing antibodies. (b, c) Zeta potentials (b) and FT-IR spectra (c) of MSNs-Tz for each synthetic step demonstrate successful surface functionalization. (d) Normalized FCS curves of free rhodamine B dyes and MSNs-Tz 24 hr after incubation in PBS and 10% FBS cell media. MSNs-Tz incubated for 24hr in PBS and FBS exhibited the same FCS curves, demonstrating excellent colloidal stability of MSNs-Tz. Data are from one experiment representative of three independent experiments (b-d).
Figure S3. Characterization and optical properties of MSNs-Tz. (a) Quantitative analysis of the number of tetrazine (Tz) molecules on the surface of MSNs. UV-Vis absorption spectroscopy shows that the characteristic peak of Tz at 544 nm increases as the concentration of Tz increases. The inset shows a linear relationship between absorbance at 544 nm and a surface concentration of Tz molecules on the MSNs-Tz. (b) Absorption spectra of MSNs after reaction with the excess amount of TCO molecules labeled with Cy5 dye. Compared to the control group that lacks Tz molecules on its surface, the MSNs-Tz exhibited prominent absorption peak at 653 nm, which is the characteristic absorption peak of Cy5. This assay demonstrates chemo-selective click reaction between TCO and Tz. (c, d) Photoluminescence (c) and absorption spectra (d) of free rhodamine B and rhodamine B-incorporated MSNs-Tz. Incorporation of rhodamine B into MSN did not change the fluorescent properties of rhodamine B, but the absorption and emission spectra were red-shifted slightly. Data are from one experiment representative of three independent experiments (a-d).
Figure S4. Characterization of anti-CD11b-TCO. (a) Schematic illustrating the synthetic procedure for functionalizing anti-CD11b antibodies with NIR dye (Alexa Fluor™ 680) and TCO. (b) MALDI-TOF analysis of anti-CD11b-TCO for each synthetic step. By comparing with unmodified CD11b antibody, the NIR dye and TCO valence on the antibody was determined. (c) Absorption spectra of anti-CD11b-TCO incubated with excess amounts of Cy3-labeled Tz molecules after extensive washing. Presence of absorption peak at 550 nm demonstrates a fast and selective click reaction between TCO and Tz. (d) Emission spectra of MSNs-Tz before and after the click reaction. Excitation at 550 nm produces low fluorescence for MSNs-Tz because the emission of rhodamine B is partially quenched by Tz molecules. After the click reaction with anti-CD11b-TCO, the emission intensity was recovered due to loss of conjugated π systems. Data are from one experiment representative of three independent experiments (b–d).
Figure S5. Fluorescence cross-correlation spectroscopy of MSNs-Tz and anti-CD11b-TCO. (a) Cross-correlation curve between anti-CD11b-TCO and MSN-NH$_3$$^+$ in the absence of Tz molecules on the MSNs, the amine-functionalized MSNs were unable to react with anti-CD11b-TCO, and non-specific interactions between MSNs and antibodies were not detected. (b) Changes in the cross-correlation curve between anti-CD11b-TCO and MSNs-Tz in a time-dependent manner. (c) Time changes of relative cross-correlation amplitude (RCA) value obtained from cross-correlation curves between MSNs-Tz and anti-CD11b-TCO. Depending on the concentration of anti-CD11b-TCO, the RCA value showed kinetic rates of the click reaction between MSNs-Tz and anti-CD11b-TCO. (d) The corresponding time-dependent conjugation efficiency as determined by RCA values. Click reaction was fast and completed within 40 min. Data are from one experiment representative of two independent experiments (a-d).
Figure S6. Evaluation of CRAIT strategy in vitro. (a) Flow cytometry was used to determine the total percentage of RAW 264.7 cells with CD11b expression on their surfaces. (b) Evaluation of in vitro cytotoxicity of DOX-loaded MSNs-Tz on RAW cells using MTS assay. Negligible toxicity was seen for up to 2 μg ml⁻¹ DOX (n = 4 per group). Error bars are S.D. from three independent experiments. (c) Quantification of cellular uptake of MSNs-Tz in RAW cells as determined by flow cytometry. The RAW cells firstly incubated with PBS, CD11b, and CD11b-TCO antibodies for 2 hr, followed by several washing steps and incubation with MSNs-Tz for 2 hr. After excessive washing, the cellular uptake of MSNs-Tz was quantified based on the fluorescence intensity of the MSNs-Tz (n = 3 per group). (d) Evaluation of transmigration capability of RAW cells tagged with anti-CD11b-TCO and MSNs-Tz using transwell chamber. 4T1 tumor cell-conditioned media in the lower chamber stimulate migration of RAW cells in the upper chamber, and the migration capability did not change significantly even after conjugation with anti-CD11b-TCO and MSNs-Tz (MSN-conjugated). The number of cells in each group is normalized to the number of cells in the unconjugated group. All the data (a-d) represent the average ± S.D. and *P < 0.05 by one-way ANOVA with Tukey post-hoc test, n.s., not significant,  P = 0.684
Figure S7. Chemo-selective click reaction *in vitro*. (a) Confocal microscopy images of CD11b\(^+\) cells incubated with PBS, followed by MSNs-Tz. There were no nonspecific interactions between CD11b\(^+\) cells and MSNs-Tz and no endocytosis of MSNs-Tz into the CD11b\(^+\) cells. (b) Confocal microscopy images of CD11b\(^+\) cells incubated with anti-CD11b and MSNs-Tz as a control group. Without TCO molecules on the antibody, bioorthogonal click reaction did not occur. Green channel, anti-CD11b-TCO labeled with AF 488; red, MSNs-Tz incorporating rhodamine B dye; and blue, DAPI nuclear stain. Data are from one experiment representative of three independent experiments (a-b).
Figure S8. Intravital confocal laser scanning microscopy for visualizing CRAIT strategy probes *in vivo*. (a) Experimental setup for intravital microscopic imaging of 4T1-tumor cells implanted in the mouse. Confocal laser microscope for imaging (left) and custom-made setup for intravital microscopy of cancer in a mouse model. 4T1 breast cancer cells expressing the firefly luciferase gene (4T1-Luc2) were implanted under the dorsal window chamber (middle). *In vivo* bioluminescence imaging of the 4T1-bearing mouse determines the exact position of tumor cells (right). (b) Intravital imaging of abnormal tumor blood vessels that were stained with MSNs-Tz (red). The tumor blood vessels exhibited tortuous and dilated morphology with a haphazard pattern of interconnection.1 (c) CD11b+ myeloid cells labeled with anti-CD11b-TCO (green) clustered adjacent to tumor vasculatures because CD11b+ myeloid cells are known to be critical mediators of tumor angiogenesis.2 Data are from one experiment representative of three independent experiments (b-c).
Figure S9. Optimization of injection interval and sequence. Three different combinations of injection method were tested to optimize the interval and sequence of MSNs-Tz. Orthotopic 4T1 mouse model ($n = 5$ per group) were treated with i) MSNs-Tz (25 mg kg$^{-1}$) firstly followed by anti-CD11b-TCO (200 µg per mouse) within 5 min (Tz (5 min) / TCO), ii) anti-CD11b-TCO firstly followed by MSNs-Tz within 5 min (TCO (5 min) / Tz), and iii) anti-CD11b-TCO firstly followed by MSNs-Tz after 24 hr. The fluorescent Cy5 probes incorporated within MSNs-Tz were utilized to compare tumor accumulation of nanoparticles in each group. Mice were sacrificed 24 hr after administration of the second injection, and the tumors were collected to confirm fluorescence intensity ex vivo. The fluorescence intensity was measured by using whole-body imaging system (IVIS200; Xenogen Corporation, Perkin-Elmer). The data represent the average ± S.D. and *$P < 0.05$ by one-way ANOVA with Tukey post-hoc test.
Figure S10. Intravital imaging 24 h after injection of MSNs-Tz in a 4T1 tumor mouse model. Injected MSNs-Tz (red) were undetectable in blood vessels, and accumulated in tumor interstitial and adjacent cells in the tumor microenvironment after 24 hr. Data are from one experiment representative of three independent experiments.
Figure S11. Gating strategy for quantifying the cellular distribution of the MSNs-Tz in the tumor microenvironment. Flow cytometry was used to quantify the total percentages of cells within the tumor that interacts with MSNs-Tz after 24 hours.³ (a) Single-cell suspension derived from the extracted tumor gated firstly for the non-debris population and (b) singlet population. (c) To exclude nonviable cells in the tumor, DAPI was used to confirm intact cells. (d) Cancer cells and TAMs were identified by anti-mouse EPCAM-PE and F4/80-APC, respectively. Double negative population was determined as “other cells.” (e) These cell populations were further analyzed to gate for the MSNs-Tz (Rhodamine B). All the data are from one experiment representative of three independent experiments (a-e). (f) Cellular distribution of MSNs-Tz in 4T1 tumor model (n = 3 per group). The data represent the average ± S.D. and *P < 0.05 by one-way ANOVA with Tukey post-hoc test.
Figure S12. Time-lapse images of an eruption event in a 4T1-bearimg mouse receiving anti-CD11b and subsequent MSNs-Tz. 4T1-bearing mouse model for intravital imaging received anti-CD11b without TCO molecules and MSNs-Tz after 24 h. Due to the lack of TCO on anti-CD11b, no co-localization of the signal from anti-CD11b (green) and MSNs-Tz (red) was observed. Instead, vascular burst followed by an eruption of the MSNs-Tz into interstitial space was observed (white arrow). It is previously reported that dynamic and stochastic eruptions are underlying mechanisms for enhanced extravasation of NPs from the tumor blood vessels by the EPR effect. Data are from one experiment representative of three independent experiments.
Figure S13. Characterization of pre-conjugated TCO::Tz complex. As a control group, anti-CD11-TCO and MSNs-Tz were conjugated via click reaction before administration. (a) Schematic illustration of preconjugated TCO::Tz complex. (b) TEM image shows no aggregation in the pre-conjugated TCO::Tz complex and the morphology is similar to MSNs-Tz. (c) Absorption spectra of MSNs-Tz and TCO::Tz complex. The MSNs-Tz were conjugated with anti-CD11b-TCO functionalized with AF 750, and the characteristic absorption peak at 750 nm was observed in the TCO::Tz complex, indicating successful conjugation. (d) DLS data shows that the hydrodynamic size of the TCO::Tz complex increased slightly after the conjugation. Data are from one experiment representative of three independent experiments (b-d).
Figure S14. *In vivo* and *ex vivo* biodistribution of anti-CD11b-TCO and MSNs-Tz. (a) Time-gated whole-body fluorescence images of orthotopic 4T1 tumor-bearing mice injected with AF750-labeled anti-CD11b-TCO. (b-c) *Ex vivo* fluorescence imaging of anti-CD11b series (b) and MSNs-Tz (c) in major organs harvested from mice bearing subcutaneous 4T1 tumors 48 h after injection of anti-CD11b series and 24 h after injection of MSNs. Upper panel (left to right): heart, lung, and liver; lower panel (left to right): spleen, kidney, and tumor. Data are from one experiment representative of three independent experiments (a-b).
Figure S15. Quantitative analysis of MSNs-Tz in organs by ICP–MS. Biodistribution of nanoparticles (% Injected Dose/g) in tumors and organs harvested at 24 hr after intravenous injection of MSNs-Tz ($n = 3$ per group). The data represent the average ± S.D. The Si contents in the tumor are 5.24% and 5.66% for the nontargeted group (PBS / MSNs-Tz) and the CRAIT group (anti-CD11b-TCO / MSNs-Tz), respectively.
Figure S16. Pharmacokinetic analysis of MSNs-Tz. The blood circulation curves of MSNs-Tz in different groups (PBS / MSNs-Tz nontargeted group, TCO::Tz complex: pre-conjugated group, and anti-CD11b-TCO / MSNs-Tz: CRAIT group). Cy5-loaded MSNs-Tz were injected via the tail vein of the mice. At various time points, blood was collected intraorbitally, and the fluorescence was measured at 670 nm to evaluate the circulation half-life time of the MSNs-Tz ($n = 3$ per group). Blood autofluorescence was subtracted from the measured intensity of the sample. All the groups exhibited rapid clearance from blood circulation within 5 hr of injection followed by a slow elimination phase. The CRAIT and the nontargeted group had similar blood retention time, which was much longer than that of the pre-conjugated group up to 5 hr. Based on a one-compartment model, the elimination half-life was calculated as 1.55 and 1.56 hr for the both CRAIT and nontargeted group respectively, and 0.99 hr for the pre-conjugated group. After 5 hr, the CRAIT group have extended blood circulation time compared to other groups. The results expressed as the average ± S.D.
Figure S17. Labeling efficiency of CD11b antibody in the tumor microenvironment. Balb/C nude mice (n = 4 animals per group) were inoculated with $1 \times 10^6$ 4T1-luc2 cells at mammary gland and allowed to grow for 10 days. The animals were administered with CD11b antibody or control IgG antibody via tail vein injection. The animals were sacrificed 24 hr post-injection, and tumors were removed and dissembled into a single-cell suspension for subjecting to flow cytometry. Available CD11b integrins on the cell surface were confirmed by anti-mouse CD11b antibody (CD11b-APC-Cy7). For flow cytometry analysis, the single-cell suspension was first gated for (a) singlet population, (b) non-debris population, and (c) CD11b-positive cells. All the data are from one experiment representative of four independent experiments. (d) Total percentages of CD11b-positive cells in 4T1 tumor microenvironment after the single injection of antibodies. Compared to the control IgG group, ~78% of CD11b integrins were pre-saturated with CD11b antibodies. The data represent the average ± S.D. and **$P < 0.01$ by one-way ANOVA with Tukey post-hoc test.
Figure S18. Co-localization of MSNs-Tz and anti-CD11b-TCO in tumor tissues. (a,b) Ex vivo fluorescence images obtained from tumor tissues in the pre-saturation group (a) and CRAIT group (b). In the pre-saturation group, no obvious co-localization between MSNs-Tz (red) and anti-CD11b-TCO (green) was observed in the whole tumor region. White arrows indicate co-localization of anti-CD11b-TCO and MSNs-Tz in CRAIT group. Cell nuclei are stained with DAPI (blue). Data are from one experiment representative of three independent experiments (a-b).
Figure S19. *Ex vivo* accumulation profile analysis on the whole-tumor slide. Fluorescence intensity profiles of MSNs-Tz detected from the tumor surface to the central region marked by orange and red rectangles in Figure 5b.

Figure S20. Co-localization of MSNs-Tz and anti-CD11b-TCO in the avascular region of the tumor. Representative fluorescence images obtained from the tumor tissues in the CRAIT group. CD31 staining (blue) and anti-CD11b-TCO (red) indicated that this tumor section contains infiltrated CD11b+ cells without mature blood vessels. The signal of MSNs-Tz (green) was co-localized with that of anti-CD11b-TCO, suggesting transport of MSNs-Tz by infiltrating CD11b+ cells. Data are from one experiment representative of three independent experiments.
Figure S21. Evaluation of kidney toxicity of CRAIT strategy probe. Healthy BALB/c nude mice ($n = 5$ animals per group) were injected intravenously with CRAIT probes, including anti-CD11b-TCO (200 $\mu$g) and MSNs-Tz (25 mg kg$^{-1}$). After 1 and 7 days, blood was collected and analyzed for kidney enzymes. a-b, Changes in serum blood urea nitrogen (BUN) (a) and creatinine (b). Yellow regions indicate the reference for healthy ranges. The results expressed as the average ± S.D.

Video S1 | Time-lapse movie demonstrating that CD11b$^+$ cell labeled by anti-CD11b-TCO (green) and MSNs-Tz (red) exhibited crawling behavior along the tumor blood vessels after 3 hr post-injection. 4T1-tumor implanted dorsal window chamber mouse model received two successive i.v. injections of anti-CD11b-TCO (200 $\mu$g) and MSNs-Tz (25 mg kg$^{-1}$) after 24 hr. The images were taken 3 hr after MSN-Tz i.v. injection.

Video S2 | Time-lapse movie demonstrating that CD11b$^+$ cell labeled by anti-CD11b-TCO (green) and MSNs-Tz (red) moved rapidly out of tumor blood vessels (labeled by MSNs-Tz) and flowed through the interstitial space. The images were taken after approximately 2.5 hr post-injection.

Video S3 | Time-lapse movie demonstrating that CD11b$^+$ cell labeled by anti-CD11b-TCO (green) and MSNs-Tz (red) moved and crawled through tumor blood vessels even after 24 hr post-injection. The injected MSNs-Tz were not observed flowing in blood vessels.

Video S4 | Time-lapse movie of MSNs-Tz (red) circulating in 4T1-tumors implanted in BALB/c nu/nu mouse. Control group are CD11b antibodies (green) lacking TCO molecules. The eruption event is seen in the left corner of the image. Eruption events are the underlying mechanism of the EPR effect. The images were taken 4 hr post-injection.
References


