Supplementary Information for

Fc-binding antibody-recruiting molecules exploit endogenous antibodies for anti-tumor immune responses

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Experimental

Cell culture

A549 human lung carcinoma cells were purchased from Riken BioResource Research Center. IGROV-1 human ovarian carcinoma cells were kindly provided by Dr. T. Matsuyama (Kagoshima University). KHYG-1/CD16a-158 V cells were kindly provided by Dr. Y. Mishima (Japanese Foundation for Cancer Research).[1] A549 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Fujifilm Wako). IGROV-1 cells were cultured in folate-free RPMI-1640 culture medium (Invitrogen). KHYG-1/CD16a-158 V cells were cultured in RPMI-1640 medium (Nacalai Tesque) containing 10 ng mL^{-1} recombinant human IL-2 (Peprotech). All of the media were supplemented with 10% heat inactivated fetal bovine serum (FBS, Gibco), 100 U mL^{-1} penicillin, 100 µg mL^{-1} streptomycin, and 0.25 µg mL^{-1} amphotericin B (Thermo Fisher Scientific). Cells were cultured in a humidified atmosphere containing 5% CO_2 and 95% air at 37 °C. The cell lines were checked for mycoplasma contamination using a MycoAlert mycoplasma detection kit (Lonza).

Antibodies

The following antibodies were used in this study: anti-CD20 hIgG1 mAb (Ofatumumab, Novartis), anti-EGFR chimeric IgG1 mAb (Cetuximab, Bristol-Myers Squibb), FITC-labeled IgG from human serum (Sigma–Aldrich), Alexa Fluor 647-labeled mouse anti-CD107a (H4A3, Biolegend), affinity-purified normal human IgG from serum (Fujifilm Wako), affinity-purified normal mouse IgG from serum (Fujifilm Wako), Venoglobulin IH (pooled hIgGs, Japan Blood Products Organization).

Surface plasmon resonance

A Biacore T-200 (GE Healthcare) was used for SPR measurements. Trastuzumab was immobilized on a CM5 chip (GE healthcare) via amine coupling for 2000 RU according to the manufacturer's instructions. Fc-ARM1 or Fc-ARM2 were analyzed at a flow rate of 50 µL/min in running buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.05% surfactant P20, pH 7.4). To solubilize Fc-ARMs in the running buffer, a maximum of 0.001% of DMSO was added. The chip was exposed to 10 mM Gly-HCl (pH 2.0) for 5 seconds for regeneration between each measurement. Non-linear kinetic analysis was conducted.

Synthesis of Fc-ARMs

Fc-ARM1 was synthesized as described previously.[2] For the synthesis of sulfo-cyanine 7-labeled Fc-ARM1 (Fc-ARM-Cy7), a lysine was introduced in between a FA and an ethylene glycol linker. Sulfo-cyanine 7 NHS ester (3 eq.; Lumiprobe) in dry DMSO and DIEA was added to the precursor of Fc-ARM-Cy7 and reacted overnight at room temperature. Fc-ARM2 precursor (without FA moiety) was chemically synthesized by Genscript to >90% purity. FA-NHS ester was synthesized similarly to a previous report.[3] N,N'-di-isopropylcarbodiimide was used as a condensation reagent, and N,N-dimethyl-4-aminopyridine was used instead of trimethylamine. The N-terminal amine of the Fc-ARM2 precursor was reacted with 4 eq. of FA-NHS ester overnight at room temperature. The reactant was purified by RP-HPLC (HITACHI, ELITE LaChrom or Chromaster) using a C18 column (Waters). The mobile phases comprised 0.1% TFA/water (mobile phase A) and 0.1% TFA/CH_3CN (mobile phase B). MALDI-TOF-MS analysis was performed for the identification of Fc-ARMs using

Autoflex III (Bruker Daltonics). Alpha-cyano-4-hydroxycinnamic acid (Sigma–Aldrich) was used as a matrix.

Preparation of Fc-fragment from hIgG1

Anti-CD20 hIgG1 mAb was dialyzed with PBS (-) overnight before papain digestion. Then, 1 mg/mL of Ab solution was mixed with 0.1 mg/mL of papain (Wako) and incubated for 3 h in the presence of EDTA (2 mM) and L-cysteine (20 mM) at 37 °C. The reaction was quenched by iodeacetamide, and purified using a KANEKA KanCapA prepacked column (Wako) according to the manufacturer's instructions. After ultrafiltration using Vivaspin (Sartorius, 10 K MWCO), SDS-polyacrylamide gel electrophoresis (PAGE) was performed on a freshly prepared 12% gel without the reduction of proteins by DTT, onto which 1 μg of each protein was loaded. After electrophoresis, gels were stained with InstantBlue (Expedeon) according to the manufacturer's instructions. Gel images were acquired with E-BOX (Vilber). The Ab solution was stored at 4 °C.

Fluorescent labeling of antibodies

Obtained Fc fragment and anti-EGFR mAb were labeled with fluorescein NHS ester and purified with Sephadex G-25 in a PD-10 desalting column (GE Healthcare) according to the manufacturer's instructions. The concentration of fluorescein in the final product was quantified by the absorbance at 494 nm, using a molar extinction coefficient of 70000 ($L \cdot mol^{-1} \cdot cm^{-1}$). The concentration of Ab was measured using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

Fluorescent microscopy

IGROV-1 cells were seeded at 1×10^4 cells/well in a folate-free RPMI-1640 medium onto a 96-well glass bottom microplate (Greiner Bio-One) and incubated for 24 h. The cells were washed twice with 100 μL of PBS (-), and then incubated with Fc-ARM2 (100 nM) and FITC-labeled hIgG (IgG-FITC) or the fluorescein-labeled Fc fragment of anti-CD20 mAb (Fc-fluo) (500 nM) in folate-free RPMI-1640 medium containing 1% FBS for 30 min at 4 °C. For competition experiments, folic acid (100 μM) was added simultaneously with Abs and Fc-ARM2. After washing, cells were stained with Hoechst 33342 (Life Technologies) and analyzed using a BZ-8000 fluorescent microscope (Keyence).

Flow cytometry

IGROV-1 cells were harvested with Accutase (PAN-Biotech) and washed with PBS (-) containing 2% FBS. After cell counting, the IGROV-1 cells were re-suspended in 2% FBS/PBS (-) to a density of 5×10^5 cells/mL. Fc-ARMs (final concentration ~ 10 nM) and fluorescently labeled antibodies (final concentration ~ 1000 nM) were added and the cells were further incubated for 30 min on ice. After two washes with 2% FBS/PBS (-), cells were resuspended in 200 μL of 2% FBS/PBS (-) and analyzed using an EC800 cell analyzer (Sony).

Antibody-dependent cell-mediated cytotoxicity assay

IGROV-1 cells or A549 cells in tissue culture dishes (Greiner Bio-One) were washed in 2 mL of PBS (-), detached with Accutase, and washed with folate-free RPMI-1640 medium containing 1% FBS. After cell counting, IGROV-1 cells were re-suspended in the folate-free RPMI-1640 medium containing 1% FBS diluted to 1×10^5 cells per mL.

This suspension was seeded into 96-well U-bottom plates (Greiner Bio-One) at 5000 cells per well (50 µL per well). Antibodies and Fc-ARMs (50 µL, final concentration of each reagent is indicated in the figures or figure legends) were added to the wells. Subsequently, KHYG-1/CD16a-158V was added (100 µL/well at the indicated effector/target ratio) and the plates were centrifuged (200 × g, 5 min). After incubation at 37 °C under 5% CO_2 for ~16 h, the plates were centrifuged and 100 µL of the supernatant was transferred to a new 96-well F-bottom plate. ADCC was evaluated using the Cytotoxicity LDH Assay Kit-WST (Dojindo) according to the manufacturer's instructions. The absorbance at 490 nm was measured using the Wallac 1420 ARVOsx (Perkin Elmer). The percentage of cytotoxicity was calculated using the following formula:
% cytotoxicity = (Abs_{490} sample - Abs_{490} effector spontaneous - Abs_{490} target spontaneous) ×100/(Abs_{490} target max - Abs_{490} target spontaneous)
For competition experiments, folic acid (10 µM) was added simultaneously with Abs and Fc-ARM.

Enzyme-linked immunosorbent assay
A sample of cell supernatant (20 µL) from the ADCC assay was collected and stored at -30 °C until use. IFN-γ was quantified using the Human IFN-γ ELISA MAX Deluxe kit (BioLegend) according to the manufacturer's instructions.

CD107a mobilization assay
IGROV-1 cells and KHYG-1/CD16a-158V cells were mixed in folate-free RPMI-1640 medium containing 1% FBS (1×10^6 cells/mL each, effector/target = 1). Fc-ARM2 (10 nM), anti-CD20 hIgG1 mAb (100 nM), Alexa Fluor 647-labeled mouse anti-CD107a (1:100 dilution, H4A3, Biolegend), and monensin (2 µM) were added and cells were centrifuged (200 × g, 5 min). After 6 h of incubation, cells were washed with PBS (-) containing 2% FBS and placed on ice until flow cytometric measurements using the CytoFLEX (Beckman Coulter). KHYG-1/CD16a-158V cells were gated as $ZsGreen^+$ cells as reported previously,[1] and were analyzed for surface expression of CD107a. Dead cells were excluded by propidium iodide staining (2 µg/mL).

IGROV-1 tumor inoculation and treatment
Female BALB/cAJcl-nu/nu mice, aged 4 to 8 weeks, were obtained from CLEA Japan. Mice were administered a folate-deficient diet (Research Diet, A09073002N) for at least one week prior to tumor inoculation. Mice were randomized into experimental groups before tumor inoculation. A total of 1×10^6 IGROV-1 cells suspended in 50 µL of PBS (-) were injected intradermally into the left side of the back of each mouse. Mice received 1 g/kg of Venoglobulin intraperitoneally on days 0 and 9 after tumor inoculation. Mice separately received 4 mg/kg of Fc-ARM2 intraperitoneally 0, 3, 6, 9, 12, and 15 days after tumor inoculation. On days 0 and 9, Fc-ARM2 was injected about 2 h post Venoglobulin injection. Tumors were measured with a digital caliper and volumes were calculated as follows: V = 4/3 × 3.14 × depth/2 × width/2 × height/2. A tumor volume over 500 mm^3, active ulceration, or more than 10% body weight loss compared with day 0 after treatment were set as humanitarian endpoints. Experiments were performed with approval from the ethics committee for animal experiments of Kyushu University and in accordance with the guidelines of the animal care and use committee of Kyushu University.

Bio-distribution of fluorescently labeled Fc-ARM1 (Fc-ARM-Cy7)

When the volume of IGROV-1 tumors reached around 300 mm^3, mice were intraperitoneally injected with 20 mg of Venoglobulin. After 2 h, mice received 5 nmol of Fc-ARM-Cy7. At the indicated time points, fluorescent signals derived from Fc-ARM-Cy7 were detected using the IVIS Lumina II (Xenogen). 24 h after intraperitoneal injection of Fc-ARM-Cy7, the tumor, heart, lung, liver, kidney, and spleen were dissected and imaged *ex vivo*. The following conditions were applied to IVIS imaging for both the mouse body and the dissected organs: exposure time, 20 seconds; binning, medium; f/stop, 2; excitation filter, 745; emission filter, ICG; lamp level, high.

Statistical analysis

Prism software (v8, GraphPad) was used for analyzing statistically significant differences among experimental controls. One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used. The similarity of data variance between groups was confirmed by a Brown–Forsythe test. Two-tailed Welch's t-test was used for single comparisons. The symbols * and ** indicate P values less than 0.05 and 0.01, respectively; N.S., not significant.

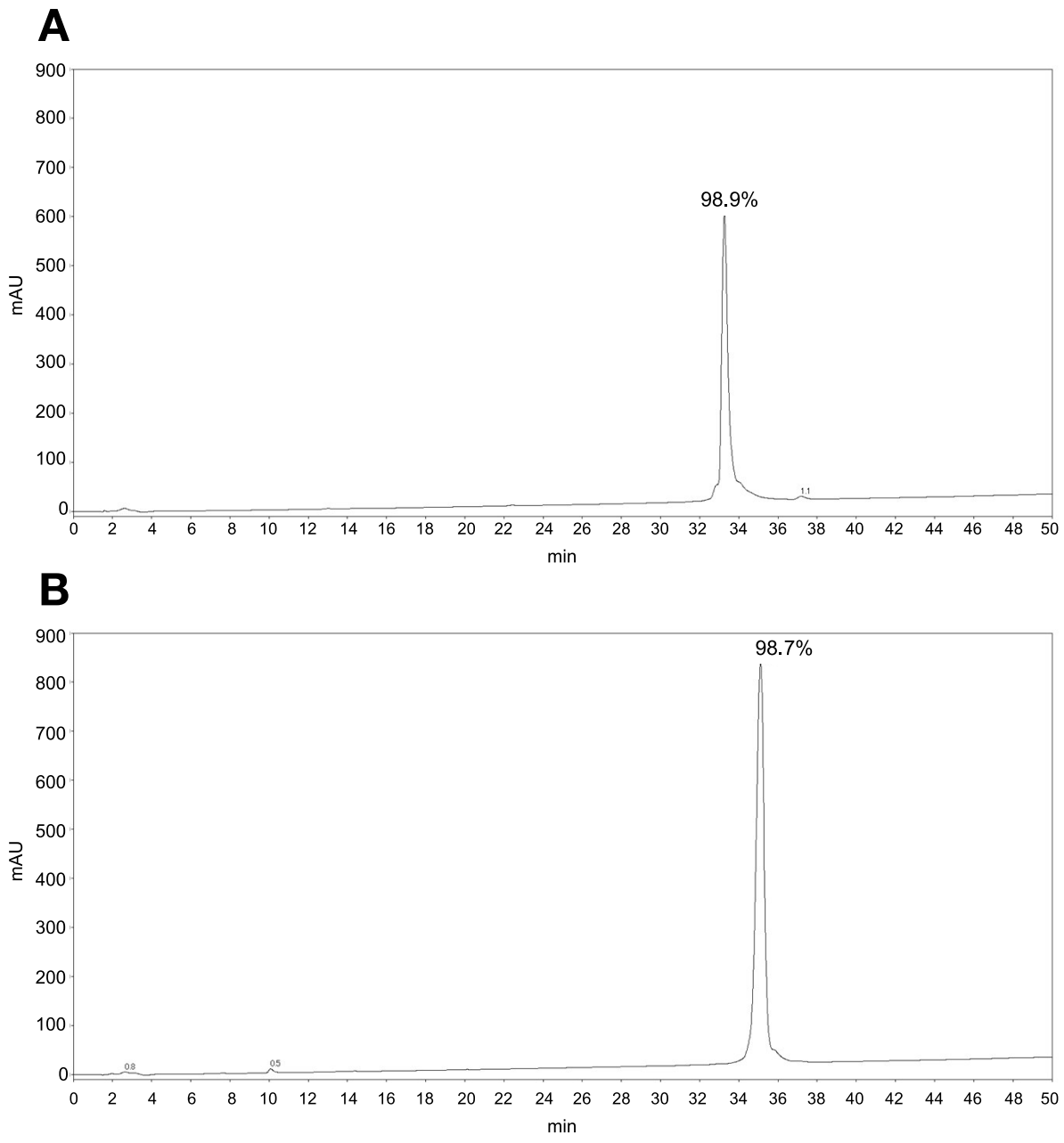

Fig. S1. Purity test of Fc-ARMs by RP-HPLC.
Purity test of Fc-ARM1 (A) and Fc-ARM2 (B) by RP-HPLC. The system was set to run at 1 mL/min at room temperature. The mobile phases comprised 0.1% TFA/water (mobile phase A) and 0.1% TFA/CH_3CN (mobile phase B). The gradient was started at 5% of mobile phase B, and increased to 55% (1%/min). The x axis represents the retention time, and the y axis represents the absorbance at 220 nm.

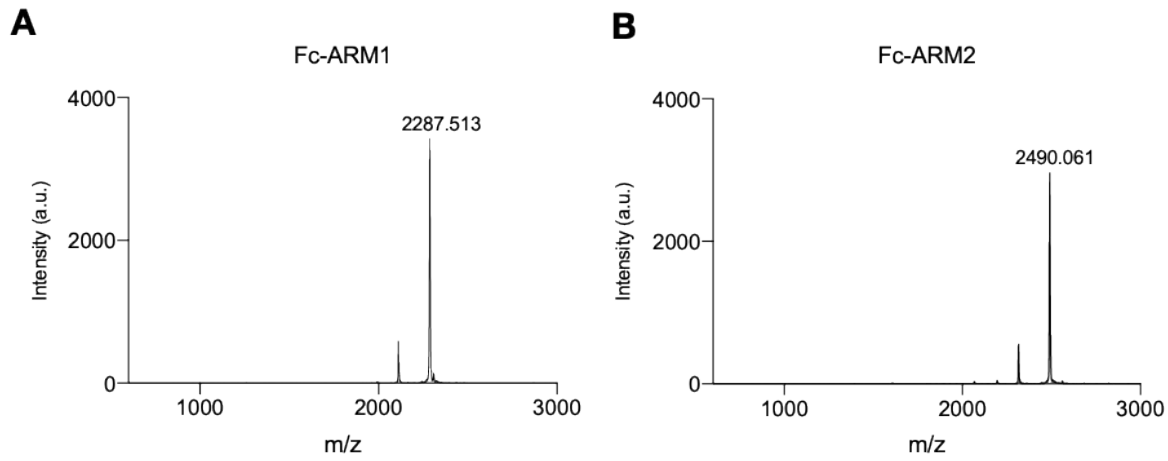

Fig. S2. Identification of Fc-ARM1 and Fc-ARM2 by MALDI-TOF MS.
(A) Fc-ARM1 and (B) Fc-ARM2 were analyzed by MALDI-TOF MS. The x axis represents the mass to charge ratio (m/z), and the y axis represents the intensity of the detected ions. The calculated exact mass of Fc-ARM1 ($C_{103}H_{142}N_{26}O_{30}S_2$) and Fc-ARM2 ($C_{109}H_{150}N_{28}O_{32}S_4$) were 2286.983 and 2490.985, respectively.

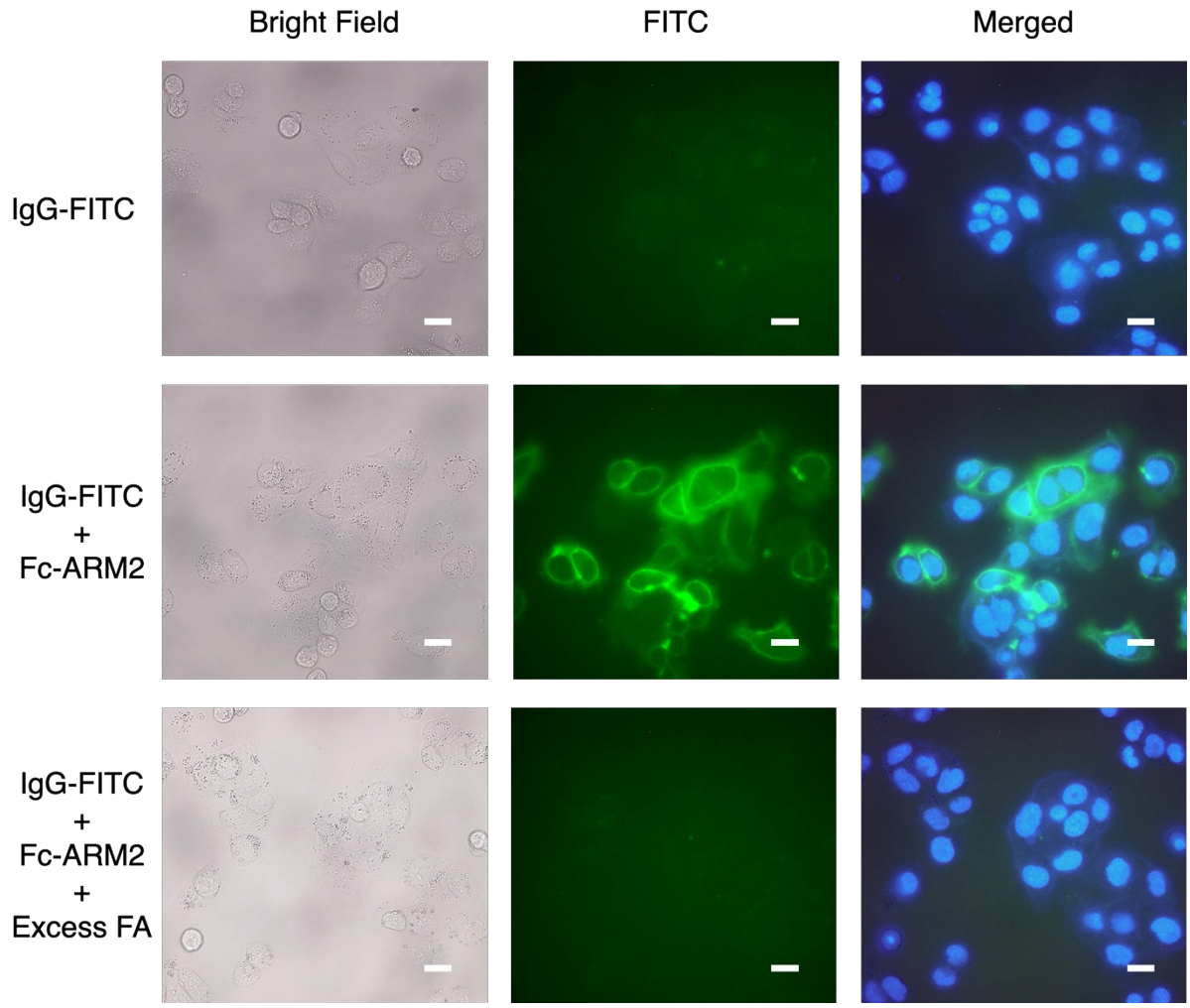

Fig. S3. Fc-ARM2 recruits IgG to FR-α^+ IGROV-1 cells.
IGROV-1 cells were seeded at 1 × 10^4 cells/well in RPMI-1640 medium (FA (-)) and were incubated overnight. After incubation with 100 nM of Fc-ARM2 and 500 nM of IgG-FITC from serum, the cells were washed and stained with Hoechst 33342. Excess FA (100 μM) was used as a competitive inhibitor of Fc-ARM2. Representative fluorescent microscopic images are shown. Scale bar = 20 μm. Two experimental repeats were performed.

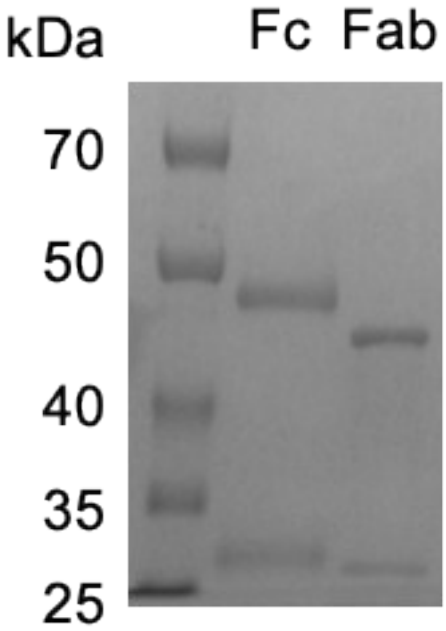

Fig. S4. SDS-PAGE analysis of anti-CD20 IgG1 mAb after papain digestion and purification by protein A affinity chromatography.
After separation by protein A affinity chromatography, the Fab and Fc fragments of anti-CD20 hIgG1 mAb were analyzed by SDS-PAGE without DTT reduction. The gel was stained with Coomassie dye.

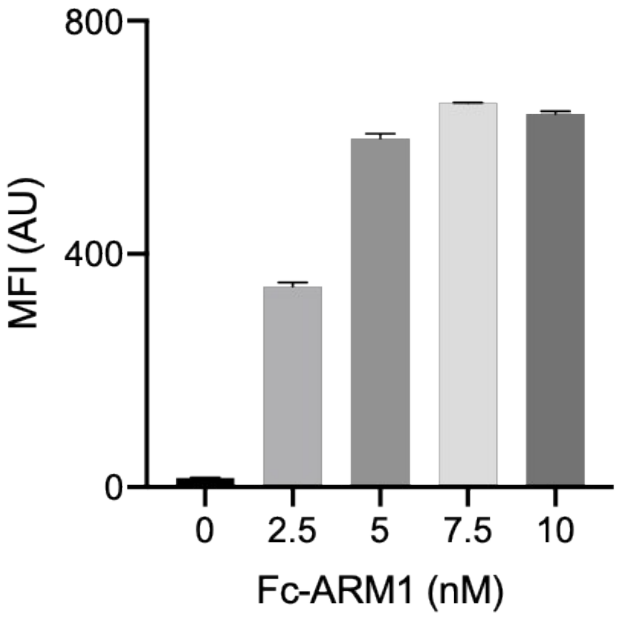

Fig. S5. 10 nM of Fc-ARM1 is sufficient for antibody redirection to IGROV-1 cells. Increasing concentrations of Fc-ARM1 and a fixed concentration of IgG-FITC (1000 nM) was added to IGROV-1 cells (5 × 10^5 cells/mL) and incubated for 30 min on ice. After washing, the fluorescence intensity derived from IgG-FITC was analyzed by flow cytometry (n = 3, mean ± SEM). Two experimental repeats were performed.

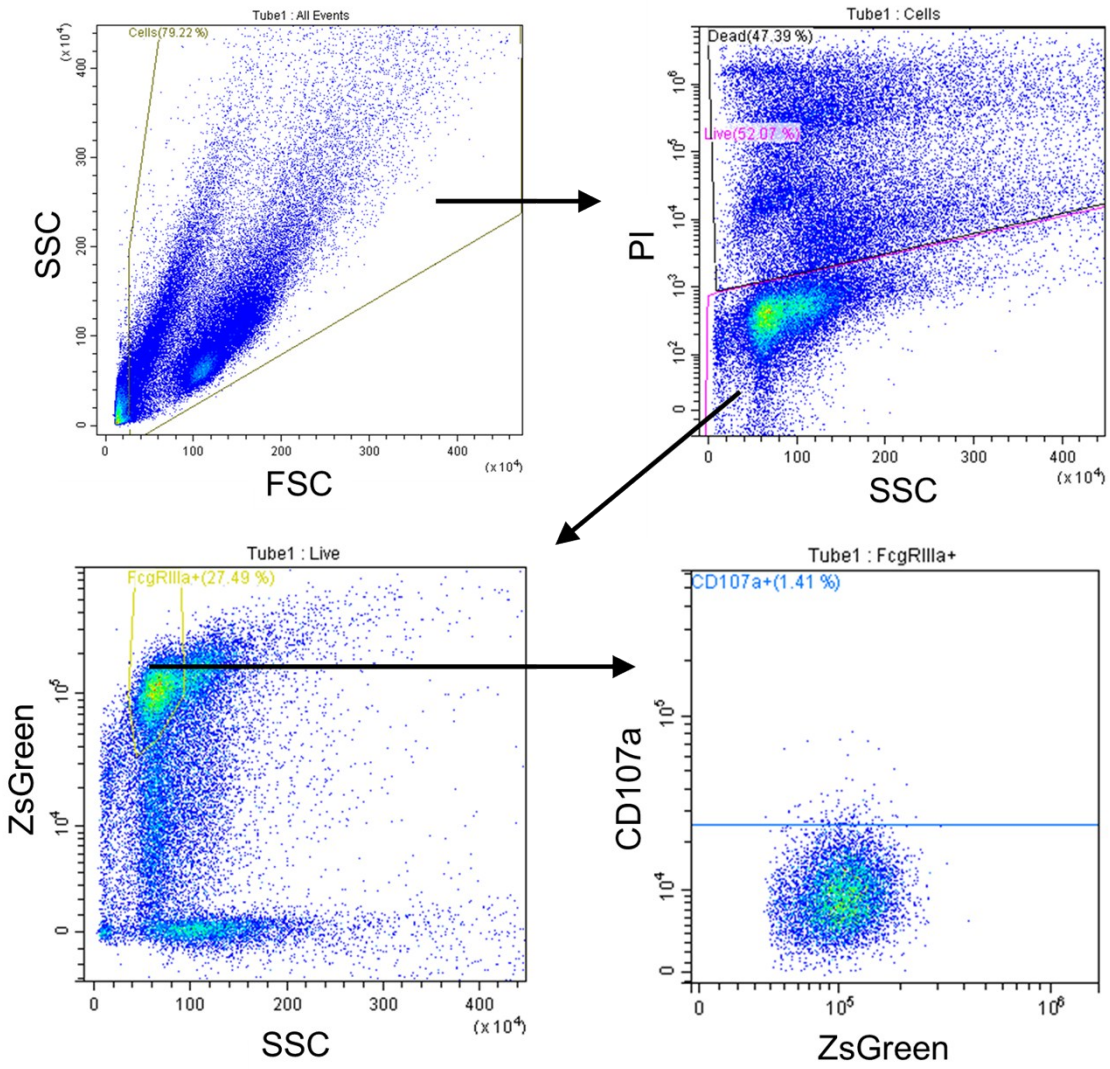

Fig. S6. Gating strategy for the CD107a mobilization assay in Fig. 3C.
The gating strategy is shown. A cell mixture of IGROV-1 and KHYG-1/CD16a-158V was used. Briefly, we excluded debris (Cells), dead cells (Live), and ZsGreen$^-$ cells (FcgRIIIa$^+$), and analyzed the percentage of CD107a$^+$ cells.

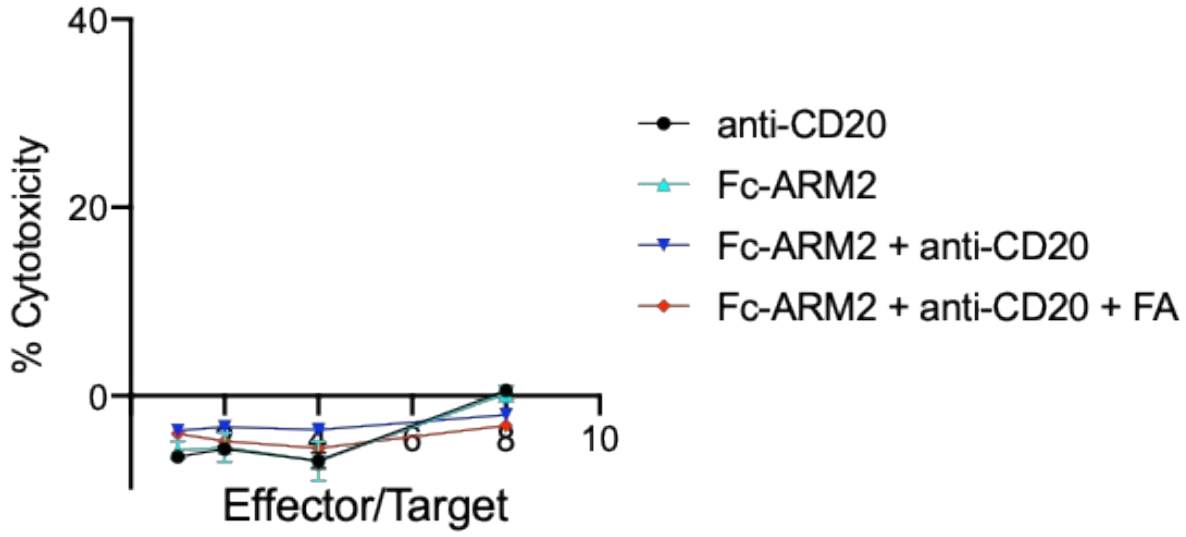

Fig. S7. Fc-ARM2 do not recruit anti-CD20 to induce ADCC against FR-α$^-$ A549 cells.

5000 cells/well of A549 cells were co-cultured with 5000–40000 cells/well of KHYG-1/CD16a-158V for 16 h in the presence of the indicated reagents. The percentage of cytotoxicity was determined by an LDH assay (n = 3, mean ± SEM). Two experimental repeats were performed.

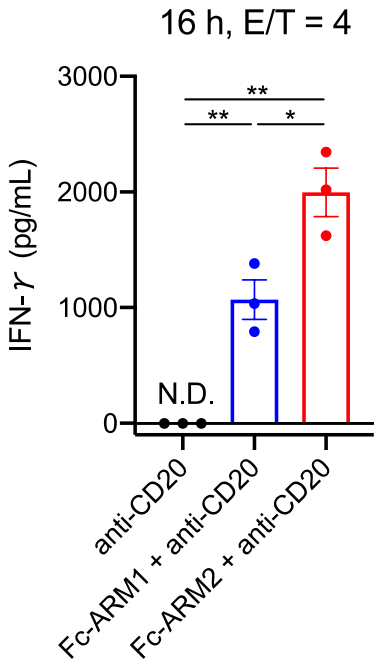

Fig. S8. The IFN-γ secretion from NK cells activated by the recruited antibodies depends on Fc affinity of Fc-ARM.
5000 cells/well of IGROV-1 cells were co-cultured with 20000 cells/well of KHYG-1/CD16a-158V for 16 h. The IFN-γ level in the supernatant was quantified by ELISA (mean ± SEM). N.D. = not detected. Two experimental repeats were performed. Statistical analyses were carried out using one-way ANOVA with Tukey's multiple comparison test. *p < 0.05; **p < 0.01.

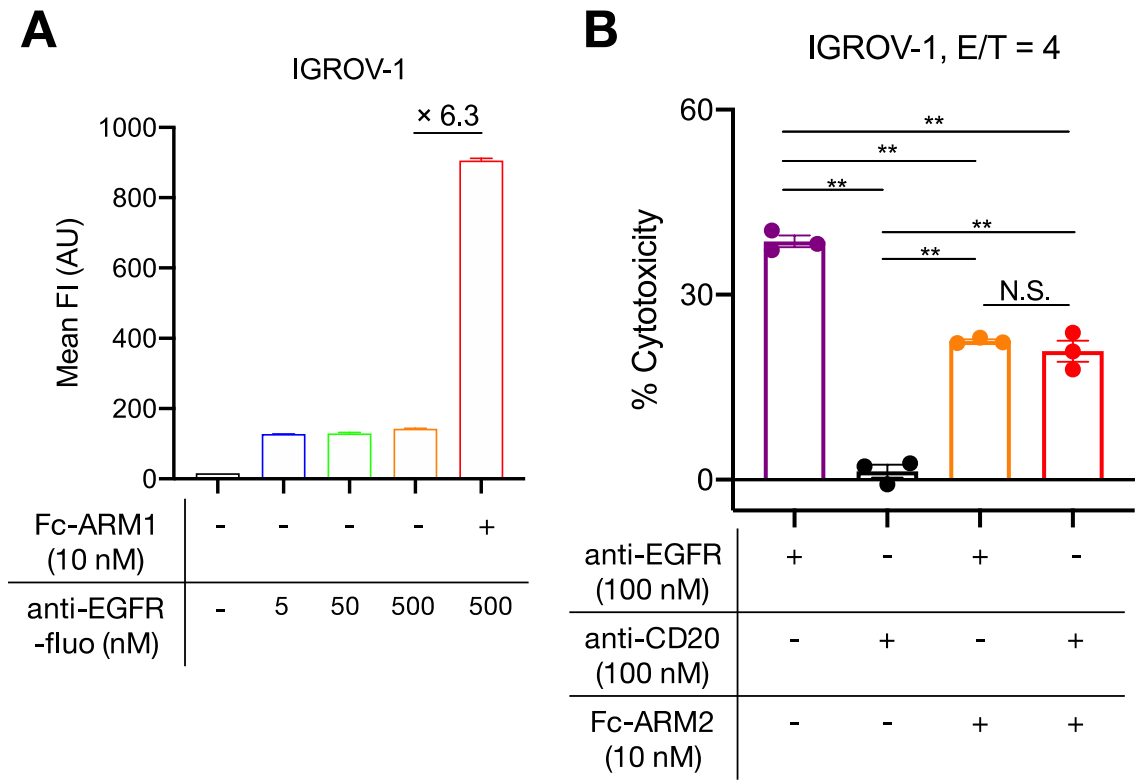

Fig. S9. Antagonistic effect of the ternary complex against the EGFR/anti-EGFR binary complex.
(A) IGROV-1 cells (5×10^5 cells/mL) were incubated with increasing concentrations of anti-EGFR mAb labeled with fluorescein and a fixed concentration of Fc-ARM1 (10 nM). Cells were analyzed by flow cytometry (n = 3, mean ± SD). (B) 5000 cells/well of IGROV-1 cells were co-cultured with 20000 cells/well of KHYG-1/CD16a-158V in the presence of the indicated reagents for 16 h. ADCC activity was quantified (mean ± SEM). Statistical analyses were carried out using one-way ANOVA with Tukey's multiple comparison test. **p < 0.01; N.S. = not significant.

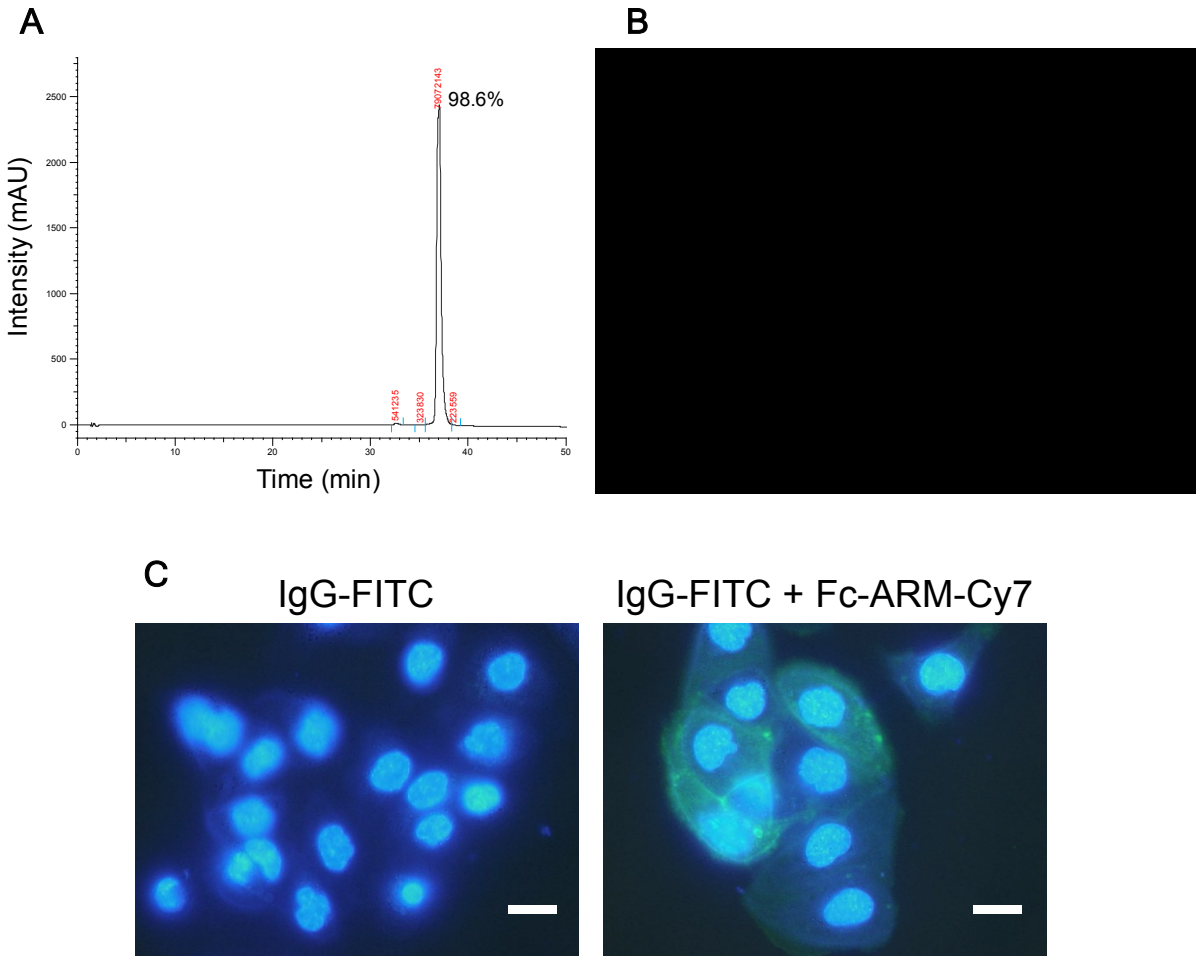

Fig. S10. Characterization of Fc-ARM-Cy7.
(A) Purity test of Fc-ARM-Cy7 by RP-HPLC. The system was set to run at 1 mL/min at room temperature. The mobile phases comprised 0.1% TFA/water (mobile phase A) and 0.1% TFA/CH_3CN (mobile phase B). The gradient was started at 5% of mobile phase B, and increased to 55% (1%/min). The x axis represents the retention time, and the y axis represents the absorbance at 750 nm. (B) Fc-ARM-Cy7 was analyzed by MALDI-TOF MS. The x axis represents the mass to charge ratio (m/z), and the y axis represents the intensity of the detected ions. Calculated exact mass of Fc-ARM-Cy7 ($C_{146}H_{196}N_{30}O_{38}S_4$) was 3105.321. (C) IGROV-1 cells were seeded at 1 × 10^4 cells/well in RPMI-1640 medium (FA (-)) and were incubated overnight. After incubation with 100 nM of Fc-ARM-Cy7 and 500 nM of IgG-FITC for 30 min, the cells were washed and stained with Hoechst 33342. Representative fluorescent microscopic images are shown. Scale bar = 20 μm. Two experimental repeats were performed.

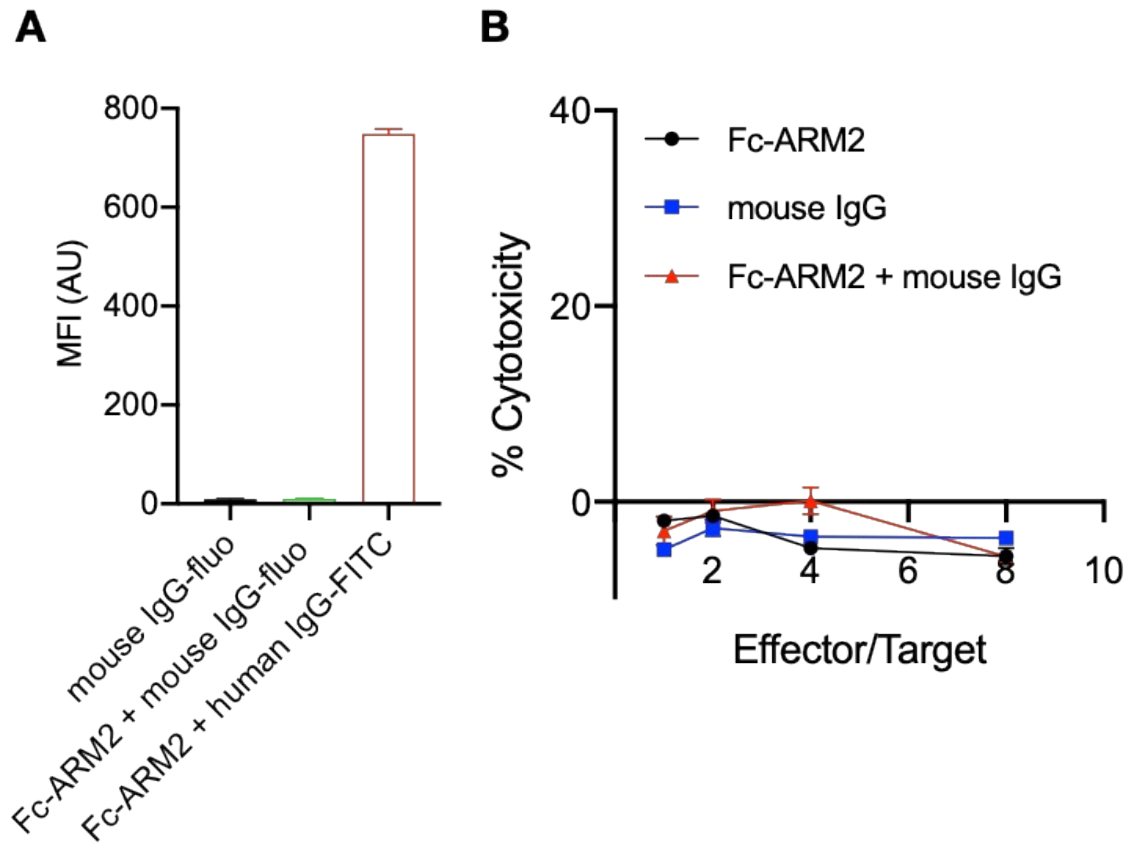

Fig. S11. Fc-ARM2 does not recruit mouse IgG to activate NK cells.
(A) 100 nM of fluorescently labeled IgG (human IgG-FITC or mouse IgG-Fluorescein (mouse IgG-fluo)) and 10 nM of Fc-ARM2 were added to 5 × 10^5 cells/mL of IGROV-1 cells (200 µL) and incubated for 30 min on ice. After washing, cells were analyzed by flow cytometry (n = 4, mean ± SEM). (B) 5000 cells/well of IGROV-1 cells were treated with Fc-ARM2 (10 nM), mouse IgG (100 nM), or Fc-ARM2 + mouse IgG and co-cultured with 5000–40000 cells/well of KHYG-1/CD16a-158V for 16 h. ADCC activity was quantified by an LDH assay (n = 3, mean ± SEM). Two experimental repeats were performed.

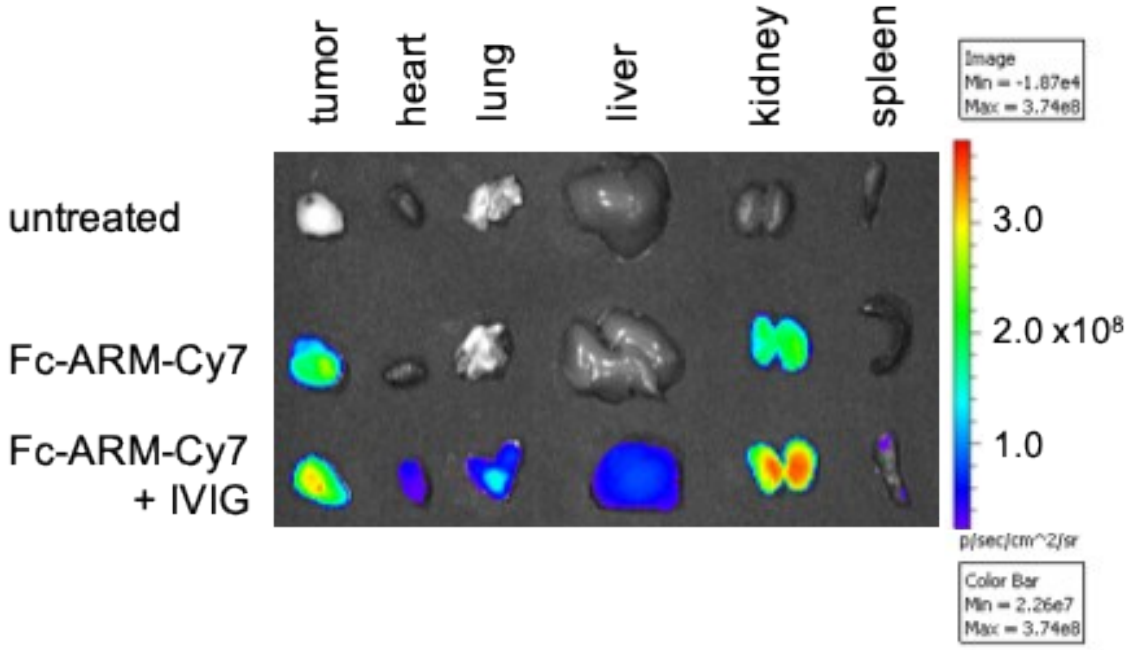

Fig. S12. Ex vivo imaging of organs dissected from a mouse used in the experiment shown in Fig. 5E.
24 h after Fc-ARM-Cy7 injection, organs were harvested and imaged *ex vivo*. A representative image of the organs is shown (n = 4 per group).

Supplemental References

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3. R.J. Lee, P.S. Low, J. Biol. Chem. 1994, **269**, 3198-3204.