Supplementary Information

EXPERIMENTAL PROCEDURES

Cell culture

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). 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).

Antibody

The following antibodies were used in this study: anti-CD20 hIgG1 mAb (Ofatumumab, Novartis), FITC-labelled anti-hIgG-Fc Fragment Ab (BETHYL).

Procedures for Trp-targeted conjugation of folate-PEGn-ABNO to mAb.

Folate-PEGn-ABNO was prepared according to our previous report.[1] Anti-CD20 mAb (1.92 mg, 13.1 nmol), folate-PEG4-ABNO (112 μg, 131 nmol, 29.3 μL of 4.48 mM stock solution), $NaNO_2$ (5.43 μg, 78.8 nmol, 5.43 μL of 1 mg/mL stock solution) and AcOH (6.56 μL) were dissolved in H_2O (657 μL) and the resulting mixture was stirred at room temperature for 3 h. The reaction mixture was transferred into 20 mM PBS (pH 7.4, 200 μL) to quench the reaction. The small molecules were removed by ultrafiltration (Amicon Ultra, 30 K) (10 times, 200 μL of PBS was added in each time). UV/Vis spectrum of the sample was measured and the FA-to-antibody ratio was determined (Figure S1-S7). Other conjugates with different PEGn (n = 8, 12) were prepared by the same procedures.

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 mAb-FA conjugate (100 nM) or mAb (100 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 mAb-FA conjugates. Then cells were washed twice with 100 μL of folate-free RPMI-1640 containing 1% FBS and then incubated with folate-free RPMI-1640 containing 1% FBS and 1% FITC anti-hIgG-Fc Fragment Ab (x 100). 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 cells were re-suspended in 2% FBS/PBS(-) to a density of 5×10^5 cells/mL. mAb-FA conjugate (final concentration: 0.001-1000 nM) was added and the cells were further incubated for 30 min on ice. After two washes with 2% FBS/PBS (-), cells were incubated for 1 h on ice with 2% FBS/PBS(-) and 1% FITC anti-hIgG-Fc Fragment Ab (x 100). After two washes with 2% FBS/PBS (-), cells were resuspended in 200 µL of 2% FBS/PBS (-) and analyzed using Cytoflex (Beckman coulter). The data was fitted using GraphPad Prism.

ADCC assay

IGROV-1 cells in tissue culture dishes (Greiner Bio-One) were washed in 2 mL of DPBS, detached with Accutase, and washed with folate-free RPMI-1640 medium containing 1% FBS. After cell counting, 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). mAb-FA conjugate (50 µL, final concentration: 10 nM) was 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 lactate dehydrogenase (LDH) Assay Kit-WST (Dojindo) according to the manufacturer's instructions. The absorbance at 490 nm was measured using the Infinite® 200 PRO M Plex (TECAN). The percentage of cytotoxicity was calculated using the following formula:

% cytotoxicity = (Abs490 sample - Abs490 effector spontaneous - Abs490 target spontaneous) ×100/(Abs490 target max - Abs490 target spontaneous)

For competition experiments, folic acid (1 µM) was added simultaneously with mAb-FA.

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.

Statistical analysis

Statistically significant differences were tested using two-tailed Student's t-test or Welch's t-test depending on the result of F-test. The symbols *, **, and *** indicate P values less than 0.05, 0.01, and 0.005, respectively; N.S., not significant.

Reference

(1) Maruyama, K., Malawska, K. J., Konoue, N., Oisaki, K., Kanai, M. (2020) Synthesis of Tryptophan–Folate Conjugates. *Synlett.* DOI: 10.1055/s-0039-1691735

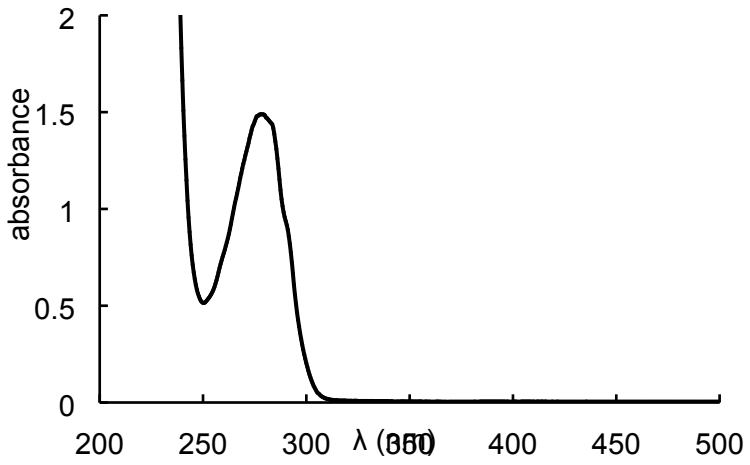

Figure S1. Absorption spectrum of anti-CD20 mAb (ca. 14 μM in H_2O).

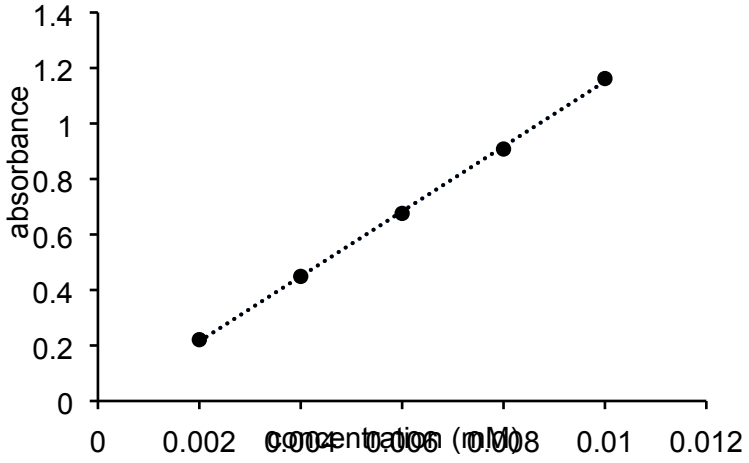

Figure S2. Calibration line (278 nm) of IgG in 20 mM PBS (pH 7.4). Molar extinction coefficients for each IgG were determined by UV-Vis spectrophotometer; ε(anti-CD20 mAb, 278 nm) = 206.6 mM^{-1} cm^{-1}.

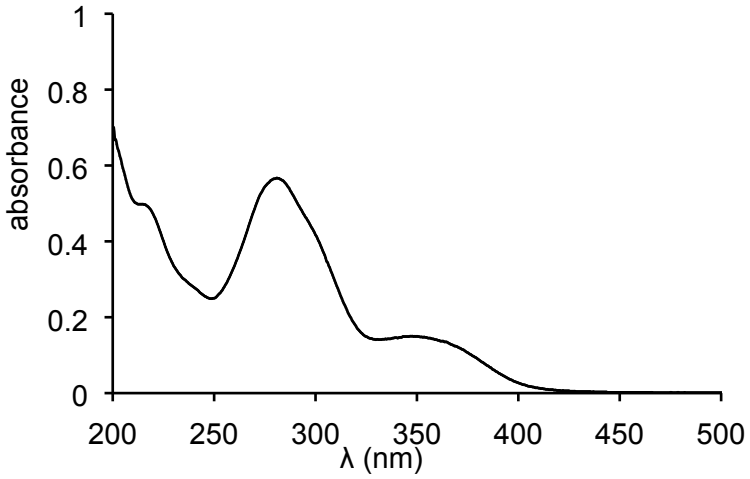

Figure S3. Absorption spectrum of folic acid (0.05 mM in 20 mM PBS, pH 7.4).

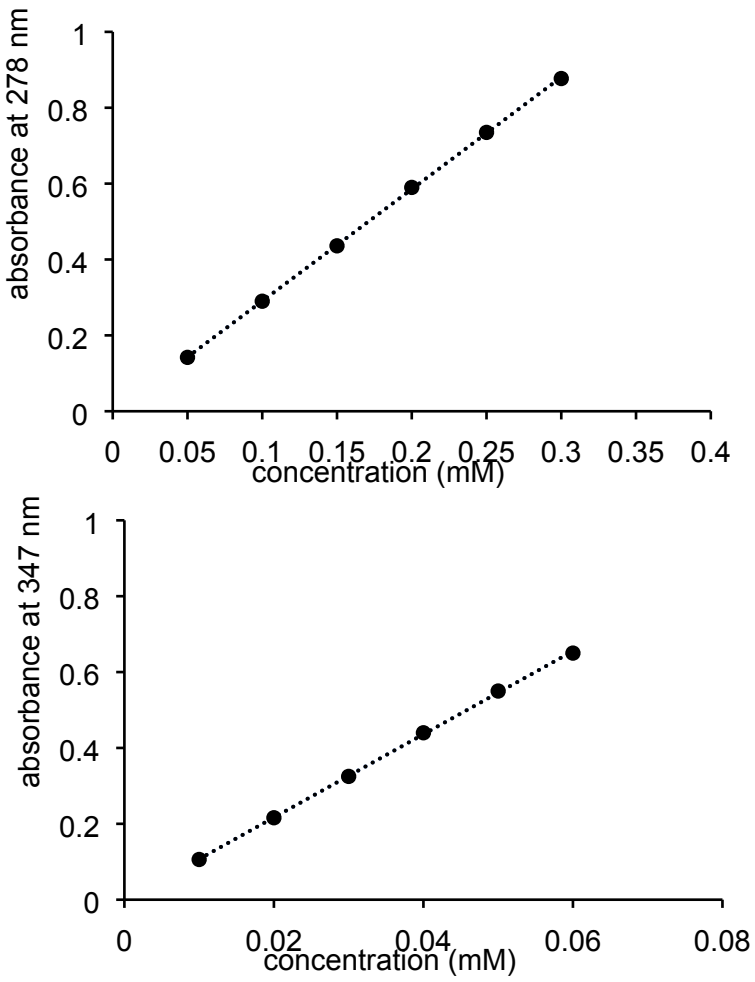

Figure S4. Calibration line (278, 347 nm) of folic acid in 20 mM PBS (pH 7.4). Molar extinction coefficient for folic acid in 278 nm and 347 nm were determined by UV-Vis spectrophotometer; ε(FA, 278 nm) = 21.926 mM^{-1} cm^{-1}, ε(FA, 347 nm) = 5.9018 mM^{-1} cm^{-1}.

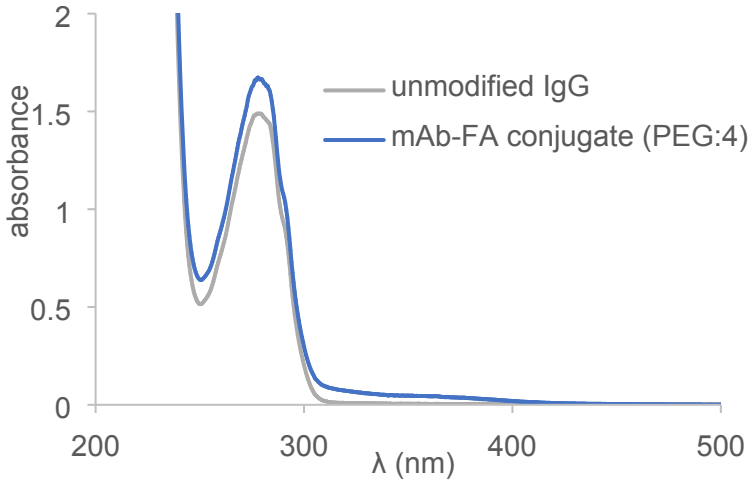

Figure S5. Absorption spectra of unmodified anti-CD20 mAb (gray) and anti-CD20-FA conjugate (PEG:4, blue) in 20 mM PBS (pH 7.4).

347 nm: $0.047 = 5.9018 * C_{folate} * 0.5$

278 nm: $1.647 = (21.926 * C_{folate} + 234.1 * C_{mAb}) * 0.5$

$C_{folate} = 0.0159$ mM, $C_{mAb} = 0.0126$ mM

$N = C_{folate}/C_{mAb} = 1.26$

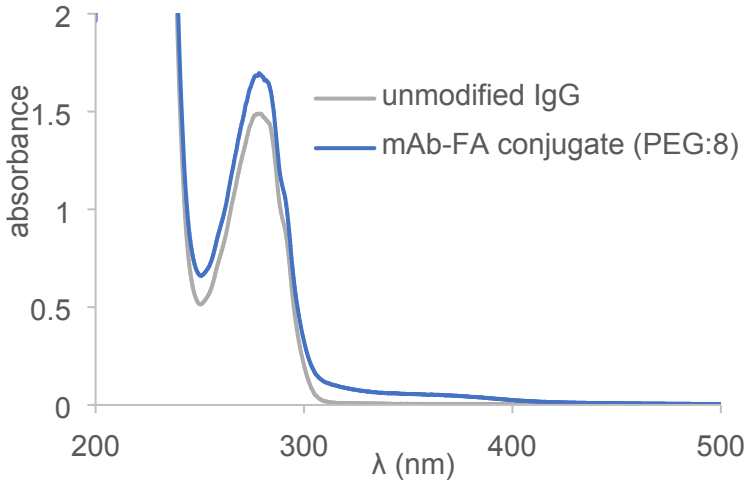

Figure S6. Absorption spectra of unmodified anti-CD20 (gray) and anti-CD20-FA conjugate (PEG:8, blue) in 20 mM PBS (pH 7.4).

347 nm: $0.059 = 5.9018 * C_{folate} * 0.5$

278 nm: $1.692 = (21.926 * C_{folate} + 234.1 * C_{mAb}) * 0.5$

$C_{folate} = 0.0200$ mM, $C_{mAb} = 0.0126$ mM

$N = C_{folate}/C_{mAb} = 1.58$

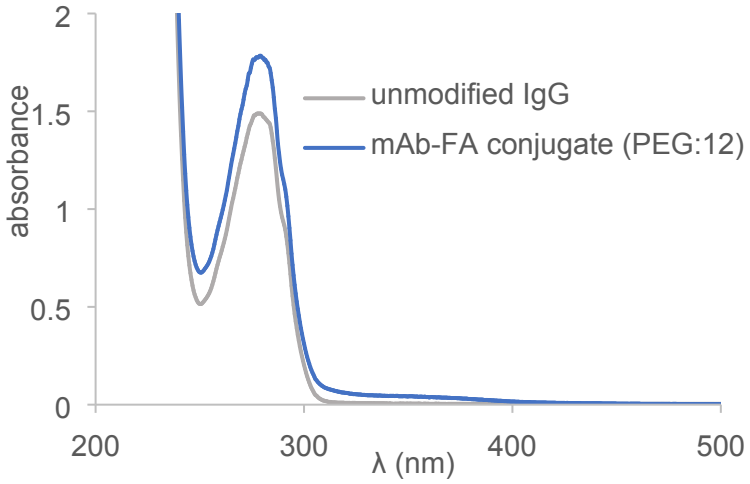

Figure S7. Absorption spectra of unmodified anti-CD20 (gray) and anti-CD20-FA conjugate (PEG:12, blue) in 20 mM PBS (pH 7.4).

347 nm: $0.044 = 5.9018 * C_{folate} * 0.5$

278 nm: $1.778 = (21.926 * C_{folate} + 234.1 * C_{mAb}) * 0.5$

$C_{folate} = 0.0149$ mM, $C_{mAb} = 0.0138$ mM

$N = C_{folate}/C_{mAb} = 1.08$

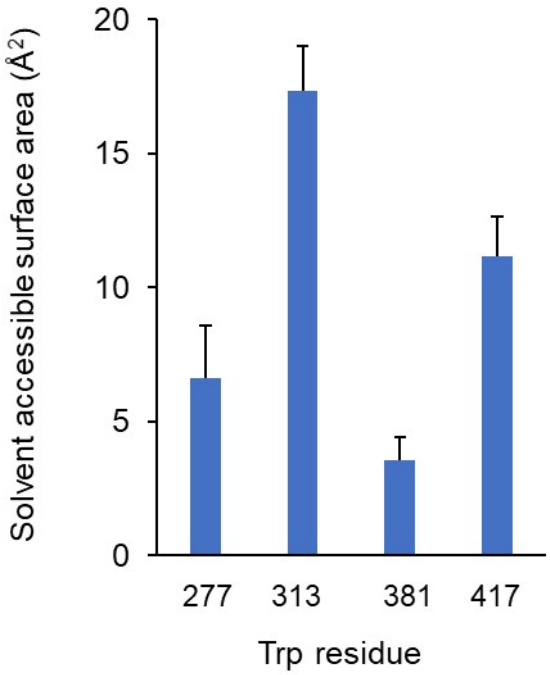

Figure S8. Solvent accessible surface area of each Trp residue in Fc region of IgG1 of several crystal structures (plot of Table S1).

Table S1. Solvent accessible surface area of each Trp residue in Fc region of IgG1 of several crystal structures (PDB ID: 1E4K, 5VU0, 3DNK).

chain	Trp residue	area (Å^2)		
		1E4K	5VU0	3DNK
A	277	3.496	6.278	7.302
	313	15.375	20.979	15.315
	381	3.331	1.914	3.201
	417	7.707	9.104	10.973
B	277	15.336	5.899	1.402
	313	23.289	16.77	12.463
	381	7.749	1.932	3.234
	417	17.498	8.576	13.032