Supporting Information

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SI Materials and Methods

Mice. WT C57BL/6 mice were purchased from Taconic. The FcγRIIB-deficient (*Fcgr2b*$^{-/-}$) and human *FCGR2B (huFCGR2B)* BAC (RP11-474I16) transgenic mice were generated on a pure B6 genetic background (1). Fc receptor (FcR) common γ-chain deficient mice (*Fcer1g*$^{-/-}$, deficient for all activating FcγRs) and *Fcgr2b*$^{-/-}$*Fcer1g*$^{-/-}$ mice have been described previously (2, 3). Mice carrying the *huFCGR2B* transgene were crossed to *Fcgr2b*$^{-/-}$*Fcer1g*$^{-/-}$ or a previously generated *Fcgr2b*$^{-/-}$ line after extensive backcrossing to B6 mice (2, 4) to generate *Fcgr2b*$^{-/-}$*Fcer1g*$^{-/-}$*huFCGR2B*$^{+}$ and *Fcgr2b*$^{-/-}$*huFCGR2B*$^{+}$ mice on the B6 background. To generate *Fcgr2b*$^{-/-}$*huFCGR2B*$^{+}$ mice on the BALB/c background, *Fcgr2b*$^{-/-}$*huFCGR2B*$^{+}$ mice on the B6 background were backcrossed twice to *Fcgr2b*$^{-/-}$ mice on the BALB/c background (5). All mice were maintained in The Rockefeller University's Comparative Bioscience Center. All experiments were performed in compliance with federal laws and institutional guidelines and had been approved by The Rockefeller University Institutional Animal Care and Use Committee.

Antibodies. MD5-1 antibodies were either purified from MD5-1 culture supernatant by protein G Sepharose 4 Fast Flow (GE Healthcare) or purchased from Bio X Cell. To make MD5-1–derived anti-DR5 antibodies with human or mouse IgG Fc, the heavy and light chain variable region genes were cloned with the 5′ RACE system (Invitrogen) according to the manufacturer's instructions and cloned into vectors with human IgG1 Fc or its N297A or S267E variant, or mouse IgG2a Fc using protocols described previously (2). The following primers were used for 5′ RACE:

MD51_HC_GSP1: 5′-GCTCACGTCCACCACCACACATGT-3′ (for V_H cloning)
MD51_HC_GSP2: 5′-GAAATAGCCCTTGACCAGGCATCC-3′ (for V_H cloning)
MD51-LC-GSP1: 5′-CTAACACTCATTCCTGTTCAGGGT-CTTG-3′ (for V_L cloning)
MD51-LC-GSP2: 5′-GCTGCTCAGGCTGTAGGTGCTGTC-3′ (for V_L cloning).

MD5-1–derived anti-DR5 antibodies were produced in 293T cells by transient transfection and purified by protein G Sepharose 4 Fast Flow (GE Healthcare). The 2.4G2 was purchased from Bio-XCell. Control hamster, human, and mouse IgG were purchased from Jackson ImmunoResearch.

Tumor Models. MC38 colon carcinoma, MC38-cFLIP (MC38 cells transfected with cFLIP), and 4T1.2 breast carcinoma lines (kindly provided by Dr. Mark J. Smyth, University of Melbourne, Melbourne, Australia) have been described previously (6). All of these lines were cultured in DMEM with 10% (vol/vol) FBS and 1% (vol/vol) pen/strep (Invitrogen). To establish MC38 or MC38-cFLIP tumors, 10^6 cells were inoculated s.c. After 5∼7 d, mice with palpable tumors were treated with 11, 33, or 100 μg/mouse of control IgG (hamster, mouse, or human IgG) or anti-DR5 antibodies of various Fcs [MD5-1, αDR5:hIgG1, αDR5:hIgG1(N297A), αDR5:hIgG1(S267E), and αDR5:mIgG2a] i.v. as described in *Results*. When MD5-1, αDR5:mIgG2a, hamster, and mouse control IgG were used, another two dosages were injected at 4-d intervals. Tumor areas were measured once every 2∼4 d and calculated as $\pi ab/4$, where a and b are width and length, respectively. In the 4T1.2 metastasis model, *Fcgr2b*$^{-/-}$ and *Fcgr2b*$^{-/-}$*huFCGR2B*$^{+}$ littermates on the BALB/c background were inoculated with 10^6 tumor cells i.v. and treated with 33 μg/mouse of human control IgG, αDR5:hIgG1, or αDR5:hIgG1 (S267E) through i.v. injection 4 d later, and monitored daily for survival.

Hepatotoxicity. To study the hepatotoxic effects of MD5-1 antibody, mice were treated with one or four 300 μg-doses of MD5-1 antibodies i.v., as described in *Results*, and then monitored for survival over 2 mo. At 7 or 14 d after the initial MD5-1 treatment, serum aspartate aminotransferase and alanine aminotransferase levels were analyzed using the MaxDiscovery Aspartate Transaminase Enzymatic Assay Kit and MaxDiscovery Alanine Transaminase Enzymatic Assay Kit (Bioo Scientific) as described in *Results*. Serum samples of *Fcer1g*$^{-/-}$ mice shown in Fig. 2*A* were analyzed 9 d after the initial MD5-1 treatment because of their poor condition. Intact and dissected WT or *Fcgr2b*$^{-/-}$ mice were examined and photographed at 39 d after the initial MD5-1 treatment. To study the effect of 2.4G2 blockade on MD5-1–induced hepatotoxicity, 150 μg of 2.4G2 was injected i.v. along with the MD5-1. Hepatotoxic effects of MD5-1–derived anti-DR5 antibodies were analyzed in *Fcgr2b*$^{-/-}$*huFCGR2B*$^{+}$ mice treated with 300 μg of control human IgG, αDR5:hIgG1, αDR5:hIgG1 (N297A), or αDR5:hIgG1(S267E) and analyzed for serum aspartate aminotransferase and alanine aminotransferase 7 d later.

In Vitro Proapoptotic Activity Analysis. To analyze proapoptotic activity of anti-DR5 antibodies, ∼70% confluent MC38 or 4T1.2 cells were split into flat 96-well tissue culture plates (BD Biosciences; catalog no. 353072) with 4×10^4 cells in 200 μL of culture media (DMEM + 10% FBS + 1% Pen/strep) per well. After overnight culture at 37 °C, the culture media was gently aspirated from each well by pipetting to avoid disturbing cells, and 100 μL of fresh media with or without 10^6 erythrocyte-depleted splenocytes prepared from WT, *Fcgr2b*$^{-/-}$, *Fcer1g*$^{-/-}$, *Fcer1g*$^{-/-}$*Fcgr2b*$^{-/-}$, or *Fcer1g*$^{-/-}$*Fcgr2b*$^{-/-}$*huFCGR2B*$^{+}$ mice (all on the B6 background) were gently added, followed by 100 μL of fresh media or fresh media containing 1 μg/mL of control IgG, MD5-1, αDR5:hIgG1, αDR5:hIgG1(N297A), or αDR5:hIgG1 (S267E) or 1 μg/mL of MD5-1 and 1 μg/mL of 2.4G2 antibodies. At 4 h later, cells were placed on ice and harvested by pipetting. After washing in cold PBS, cells were fixed, permeabilized, and stained for intracellular active caspase-3 (clone C92-605; BD Biosciences). Samples were analyzed with a FACSCalibur flow cytometer (BD Biosciences). MC38 and 4T1.2 cells were gated using forward and side scatters and analyzed for expression of active caspase-3.

Surface Plasmon Resonance. All surface plasmon resonance (SPR) analyses were performed with a Biacore T100 SPR system (GE Healthcare) at 25 °C in HBS EP+ buffer [10 mM Hepes (pH 7.4), 150 mM NaCl, 3.4 mM EDTA, 0.005% surfactant P20]. His-tagged soluble mouse FcγR extracellular domains (Sino Biological) were immobilized on CM5 chips by amine coupling at pH 4.5, resulting in a density of ∼2,000 response unites (RUs). Serially diluted (by twofold) MD5-1 or other anti-DR5 antibody samples were injected through flow cells for 3 min at a flow rate of 30 μL/min for association, followed by a 5-min dissociation phase. The concentrations of MD5-1 or other anti-DR5 antibody samples ranged from 4,000 nM to 15.63 nM for FcγRIIB and FcγRIII binding analyses and from 1,000 nM to 1.95 nM for FcγRI and FcγRIV binding analyses. After each assay cycle, the sensor surface was regenerated with a 30-s injection of NaOH of

optimized concentration at a flow rate of 50 μL/min. Background binding to blank immobilized flow cells was subtracted, and affinity constant K_D values were calculated using the 1:1 binding kinetics model in the Biacore T100 evaluation software (version 1.1) (GE Healthcare).

Statistics. All statistical analyses were performed using Prism 5 for Windows, version 5.04 (GraphPad). The two-tailed *t* test was used for comparisons of two groups. Comparisons of more than two groups were done using one-way ANOVA with a Tukey post hoc test or a Dunnett post hoc test, or the log-rank test.

1. Smith P, DiLillo DJ, Bournazos S, Li F, Ravetch JV (2012) Mouse model recapitulating human FcγR structural and functional diversity. *Proc Natl Acad Sci USA* 109:6181–6186.
2. Li F, Ravetch JV (2011) Inhibitory Fcγ receptor engagement drives adjuvant and anti-tumor activities of agonistic CD40 antibodies. *Science* 333:1030–1034.
3. Takai T, Li M, Sylvestre D, Clynes R, Ravetch JV (1994) FcR gamma chain deletion results in pleiotrophic effector cell defects. *Cell* 76:519–529.
4. Takai T, Ono M, Hikida M, Ohmori H, Ravetch JV (1996) Augmented humoral and anaphylactic responses in Fc gamma RII-deficient mice. *Nature* 379:346–349.
5. Bolland S, Ravetch JV (2000) Spontaneous autoimmune disease in Fc(gamma)RIIB-deficient mice results from strain-specific epistasis. *Immunity* 13:277–285.
6. Haynes NM, et al. (2010) CD11c$^+$ dendritic cells and B cells contribute to the tumoricidal activity of anti-DR5 antibody therapy in established tumors. *J Immunol* 185:532–541.

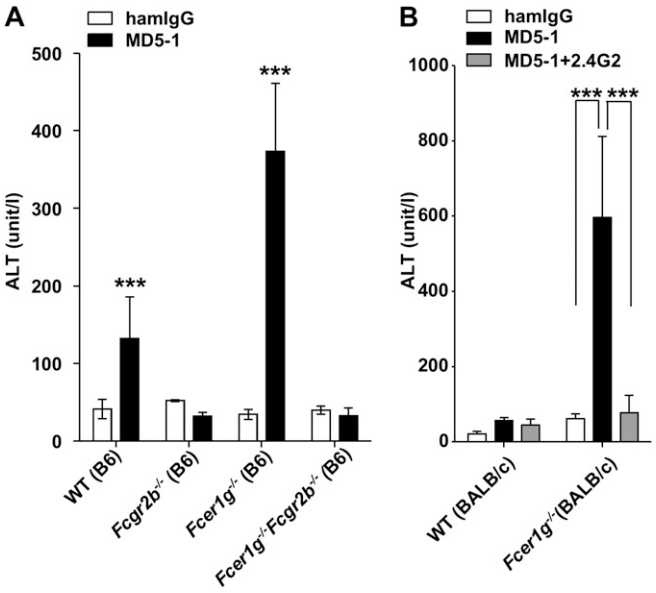

Fig. S1. MD5-1 uniquely depends on FcγRIIB for its hepatotoxic effect. (*A*) WT, *Fcgr2b*$^{-/-}$, *Fcer1g*$^{-/-}$, and *Fcer1g*$^{-/-}$*Fcgr2b*$^{-/-}$ mice on the B6 background were treated with high dose of MD5-1 or hamster control IgG (300 μg/mouse repeated at 3-d intervals, for a total of 1.2 mg/mouse), and analyzed for serum ALT levels at 14 d after the first treatment. (*B*) WT and *Fcer1g*$^{-/-}$ mice on the BALB/c background were treated with 300 μg/mouse of MD5-1 or hamster control IgG in the presence or absence of 150 μg of 2.4G2, and analyzed for serum ALT levels 7 d later. ***$P < 0.001$. Error bars represent SD.

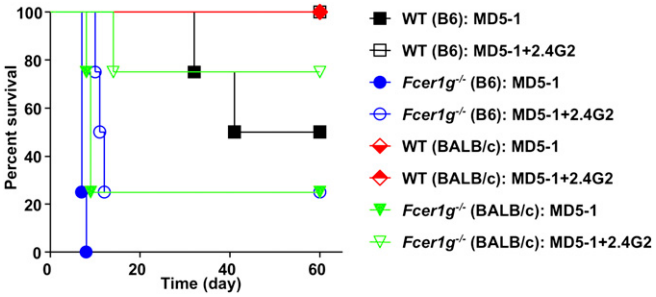

Fig. S2. Treatment with 2.4G2 attenuates MD5-1–induced hepatotoxicity. WT and *Fcer1g*$^{-/-}$ mice on the B6 or BALB/c background were treated with 300 μg/mouse of hamster control IgG or MD5-1 in the presence or absence of 150 μg/mouse of 2.4G2 antibodies, and monitored for survival over 2 mo. Survival curves are shown.

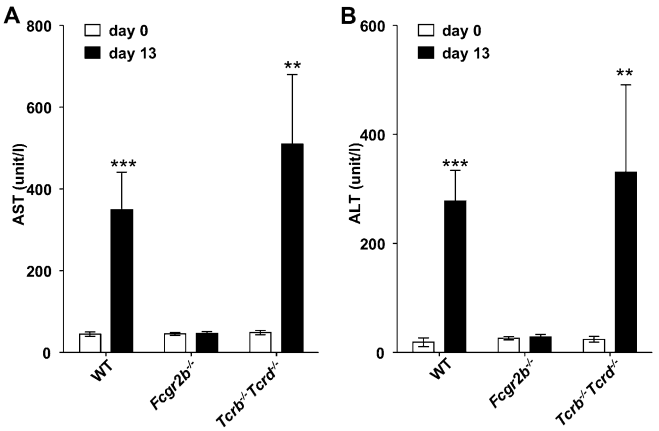

Fig. S3. T cells are not required for MD5-1–induced hepatotoxicity. WT, *Fcgr2b*$^{-/-}$, and T-cell receptor KO mice (*Tcrb*$^{-/-}$*Tcrd*$^{-/-}$) on the B6 background were treated with high-dose MD5-1 (300 μg/mouse repeated at 3-d intervals for a total of 1.2 mg/mouse), and analyzed for serum AST (*A*) and ALT (*B*) before and 13 d after the first treatment. $^{**}P < 0.01$; $^{***}P < 0.001$. Error bars represent SD.

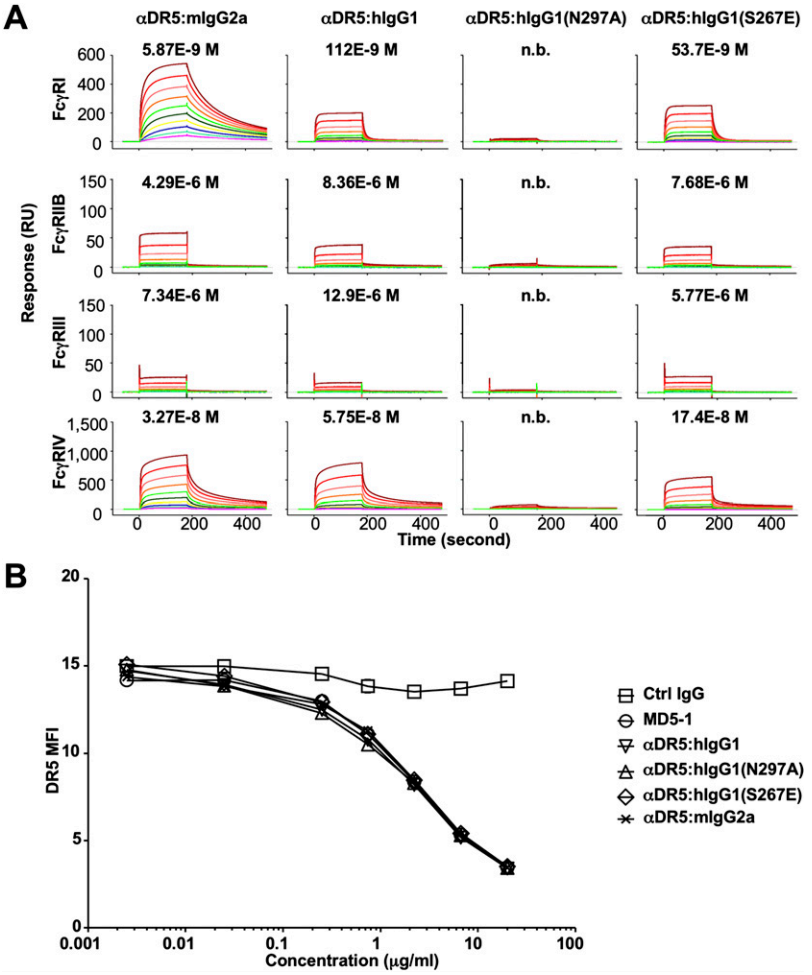

Fig. S4. Binding properties of MD5-1–derived anti-DR5 antibodies. (*A*) SPR analysis of the binding of MD5-1–derived anti-DR5 antibodies to mouse FcγRs. Real-time sensorgrams with affinity constants (K_D) are shown. (*B*) MD5-1–derived anti-DR5 antibodies have the same binding specificity and affinity to DR5 as MD5-1. MC38 cells were stained with 2 μg/mL of Alexa Fluor 647-conjugated MD5-1 in the presence of serially diluted unlabeled MD5-1 or the other indicated anti-DR5 antibodies, or control IgG. DR5-staining on the MC38 cells is expressed as mean fluorescence intensity (MFI) and plotted against the concentration of the control IgG or competing anti-DR5 antibodies. Although the control IgG had no effect on the staining of DR5, MD5-1 and MD5-1–derived anti-DR5 antibodies all competed with Alexa Fluor 647-conjugated MD5-1 with the same efficiency.

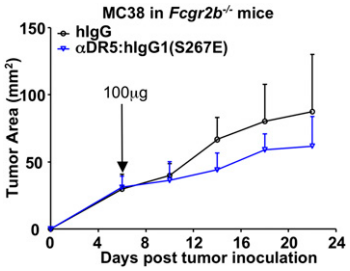

Fig. S5. The enhanced tumoricidal effects of αDR5:hIgG1(S267E) depend on the *huFCGR2B* transgene. *Fcgr2b*$^{-/-}$ mice on the B6 background were inoculated with MC38 cells s.c. and treated with the indicated amount of control or αDR5:hIgG1(S267E) antibodies at the indicated times. Tumor growth curves are presented. Error bars represent SD.

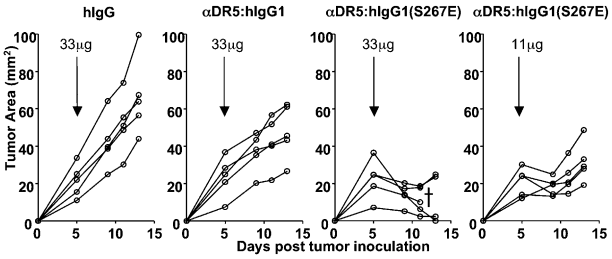

Fig. S6. The tumoricidal effect of agonistic anti-DR5 antibodies can be enhanced by FcγRIIB-targeted Fc engineering. *Fcgr2b*$^{-/-}$*huFCGR2B*$^{+}$ mice on the B6 background were implanted with MC38 cells s.c. and treated with the indicated amount of control or anti-DR5 antibodies at the indicated times. Tumor growth curves of individual mice are presented. A cross represents mortality.

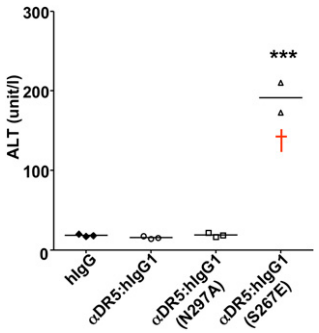

Fig. S7. *Fcgr2b*$^{-/-}$*huFCGR2B*$^{+}$ on the B6 background were treated with 300 μg of the indicated control or anti-DR5 antibodies and analyzed for serum ALT 7 d later. ***$P < 0.001$. The cross represents mortality. Error bars represent SD.

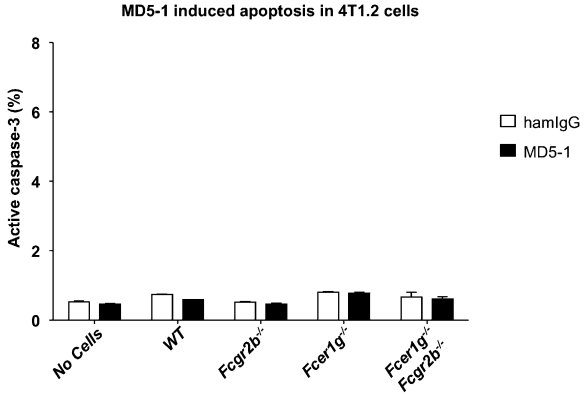

Fig. S8. The 4T1.2 cells are not sensitive to proapoptotic activity of MD5-1. The percentage of 4T1.2 cells with active caspase-3 is shown; 4T1.2 cells were treated with hamster control IgG or MD5-1 in the absence or presence of splenocytes isolated from the indicated mice. Error bars represent SD.

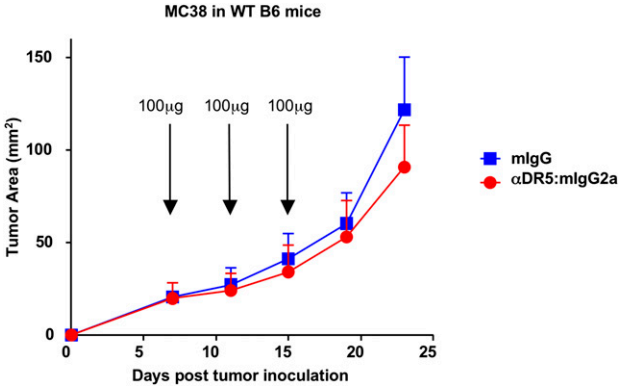

Fig. S9. αDR5:mIgG2a does not have strong antitumor activity in the MC38 model. MC38 cells were inoculated s.c. into WT B6 mice, which were then treated with αDR5:mIgG2a or mouse control IgG antibodies at the indicated doses at the time points indicated by arrows. Representative tumor growth curves of five mice are shown. Error bars represent SD.