Supporting Information

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SI Materials and Methods

Mice. Wild-type C57BL/6 mice were purchased from Taconic. Mice carrying the floxed *Fcgr2b* allele (*Fcgr2b*^{fl}) were generated from B6 ES cells using a previously described *Fcgr2b* conditional knockout targeting vector (1). *Fcgr2b*$^{fl/fl}$*Mb1Cre*$^{+}$, *Fcgr2b*$^{fl/fl}$*CD11cCre*$^{+}$, and *Fcgr2b*$^{fl/fl}$*LysMCre*$^{+}$ mice were generated by crossing mice carrying *Fcgr2b*^{fl} alleles to Mb1-Cre mice (2), CD11c-Cre mice (3), and LysM-Cre mice (4), respectively, which have been backcrossed to the B6 background at least 10 times. *Fcgr2b* homozygous knockout mice (*Fcgr2b*$^{-/-}$) were generated by crossing mice carrying floxed *Fcgr2b* alleles to Cag-Cre mice on a pure B6 background (5). *Fcgr2b* homozygous knockout mice backcrossed to a BALB/c background have been previously described (6). *Cd40*$^{-/-}$ mice (B6.129P2-Cd40tm1Kik/J) were obtained from The Jackson Laboratory, and were crossed to human (*h*)*FCGR2B* transgenic mice (1) to generate *Cd40*$^{-}$*hFCGR2B*$^{+}$ and *Cd40*$^{+}$*hFCGR2B*$^{-}$ mice. *Ship* knockout mice have been described previously (7). All mice were maintained in The Rockefeller University 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. Agonistic anti-mouse CD40 antibodies, including 1C10 and 1C10-derived αCD40:mIgG1, αCD40:mIgG1(D265A), and αCD40:hIgG1(S267E); agonistic αDR5 antibody clone MD5-1 have all been described previously (8, 9). Agonistic αFas antibody (clone Jo2) was purchased from BD Biosciences. Ly17.2 and 2B6 have been described previously (10, 11). Other antibodies used for flow cytometry analysis were purchased from BD Biosciences or BioLegend.

Agonistic αCD40 Antibody-Induced B-Cell Activation. *CD45.1*$^{+}$ 5-(6)-carboxyfluorescein diacetate succinimidyl diester (CFSE)-labeled WT or *CD45.2*$^{+}$ *Fcgr2b*$^{-/-}$ splenocytes were adoptively transferred into WT C57BL/6 or *Fcgr2b*$^{-/-}$ recipient mice that express different CD45 congenic markers on day −1. Recipient mice were then treated with 30 μg of agonistic αCD40 antibody (αCD40:mIgG1) or inactive control αCD40 antibody (αCD40:D265A) on day 0. On day 3, blood cells were stained with fluorescence-conjugated antibodies against CD45.2 (104), CD45.1 (A20), B220 (RA3-6B2), or CD80 (16-10A1). CD80 levels were analyzed in host and transferred B cells (B220^{+}) identified based on CFSE and CD45 expression. On day 5, CFSE levels were analyzed in transferred B cells in spleen.

OVA-Specific T-Cell Response. Mice were i.p. injected with 5 μg of DEC-OVA in the presence of 30 μg of αCD40 antibodies (clone 1C10) or 1C10-derived αCD40 antibody αCD40:hIgG1(S267E). Seven days later, blood cells or splenocytes were depleted for erythrocytes and analyzed for OVA-specific CD8^{+} T cells by OVA peptide SIINFEKL H-2^{b} tetramer staining as described previously (8). Briefly, cells resuspended in FACS buffer (PBS with 0.5% FBS, 2 mM EDTA, and 0.1% NaN_3) were stained with FITC-conjugated αCD4 (RM4-5), allophycocyanin–conjugated αCD8α (53.67) antibodies, and phycoerythrin-conjugated OVA peptide SIINFEKL H-2^{b} tetramer (tet-OVA; Beckman Coulter) on ice for 1 h, and analyzed by FACSCalibur after a wash. 7AAD was included to exclude dead cells. In the bone marrow chimeric experiment, 10^6 bone marrow cells isolated from *Cd40*$^{+}$*hFCGR2B*$^{+}$, *Cd40*$^{+}$*hFCGR2B*$^{-}$, or *Cd40*$^{-}$*hFCGR2B*$^{+}$ mice or 10^6 1:1 mixed *Cd40*$^{+}$*hFCGR2B*$^{-}$ and *Cd40*$^{-}$*hFCGR2B*$^{+}$ bone marrow cells were transferred by i.v. injection into lethally irradiated (900 rad) WT C57BL/6 recipient mice. Three months later, mice were i.p. injected with 5 μg of DEC-OVA and 30 μg of αDR5:hIgG1 (S267E). Seven days later, blood cells were depleted for erythrocytes and analyzed for OVA-specific CD8^{+} T cells by OVA peptide SIINFEKL H-2^{b} tetramer staining.

MC38 Tumor Model. MC38 colon carcinoma cells (kindly provided by Mark J. Smyth, Peter MacCallum Cancer Centre, East Melbourne) have been described previously (9) and were cultured in DMEM with 10% (vol/vol) FBS and 1% pen/strep (Invitrogen). To establish MC38, 10^6 cells were implanted s.c. After 5–7 d, mice with palpable tumors were treated with 100 μg per mouse of control hamster IgG or αDR5 antibodies (clone MD5-1) i.v. three times at 4-d intervals as described previously (9). Tumor areas were measured once every 4 d and calculated as πab/4, where "a" and "b" are width and length, respectively.

IIA1.6 and IIA1.6-Derived B-Cell Lymphoma Models. IIA1.6 and IIA1.6-derived cells that express either unmutated FcγRIIB (IIA1.6-FcγRIIB) or mutant FcγRIIB that contains a Y→F mutation in the immunoreceptor tyrosine-based inhibitory motif (ITIM) [IIA1.6-FcγRIIB(Y→F)] were described previously (12). FcγRIIB expression was verified on the protein level by FACS using ly17.2 antibody and on the mRNA level by cDNA sequencing. In order to test the antitumor activity of agonistic αCD40 antibody (clone 1C10), 2.5×10^7 IIA1.6 or IIA1.6-derived cells were injected i.v. into WT or *Fcgr2b*-deficient (*Fcgr2b*$^{-/-}$) BALB/c mice, which were treated with 200 μg of 1C10 on days 7 and 10, and monitored for survival.

EL4 and EL4-Derived T-Cell Lymphoma Models. EL4 cells were obtained from ATCC and cultured in RPMI with 10% FBS and 1% pen/strep (Invitrogen). To generate EL4 cells expressing unmutated or truncated human FcγRIIB, human FcγRIIB cDNA (isoform 1) was cloned from hFCGR2B transgenic mice, and a pFB-Neo retroviral vector (Stratagene) expressing unmutated human FcγRIIB was constructed. A mutated version of this retroviral vector was constructed by mutagenesis using primers 5′-GTA GTG GCC TTG ATC TAG TGC AGG AAA AAG CGG-3′ and 5′-CCG CTT TTT CCT GCA CTA GAT CAA GGC CAC TAC-3′ to introduce a nonsense mutation at position Y246 of human FcγRIIB. EL4 cells were transduced with retroviruses carrying these constructs and selected with 0.2 mg/mL of Geneticin (Invitrogen). The resulting Geneticin-resistant cells that express WT and truncated hFcγRIIB are referred to as EL4-hFcγRIIB and EL4-hFcγRIIB(ΔC), respectively. In order to test the antitumor activity of human FcγRIIB-enhanced agonistic αCD40 antibody αCD40:hIgG1(S267E), ∼1.5×10^7 EL4, EL4-hFcγRIIB, or EL4-hFcγRIIB(ΔC) cells were injected i.v. into WT C57BL/6 mice, which were then treated with 200 μg of αCD40:hIgG1(S267E) or human control IgG on days 3 and 5, and monitored for survival.

Hepatotoxicity. To study the hepatotoxic effects of MD5-1 antibody, mice were treated with four doses of 300 μg of MD5-1 antibodies i.v. at 3-d intervals, and monitored for survival over 2 mo. Before and 13 d after the initial MD5-1 treatment, serum alanine aminotransferase (ALT) levels were analyzed using the MaxDiscovery Enzymatic Assay Kit (Bioo Scientific) following the manufacturer's instructions. To study agonistic αFas antibodies, WT C57BL/6, *Fcgr2b*$^{-/-}$, *Fcgr2b*$^{+/-}$, and *Ship*$^{-/-}$ mice were treated with 10 μg of αFas antibodies (clone Jo2) and monitored for survival.

Statistics. All statistical analyses were performed in GraphPad Prism 5 for Windows. When two groups were compared (hamIgG vs. MD5-1 in Fig. 4 *B*–*F*), a two-tailed *t* test was used. When more than two groups were compared, a one-way ANOVA with a Dunnett post hoc test was used in Fig. 4*A* to compare all groups with the *Fcgr2b*$^{fl/fl}$ control group; a one-way ANOVA with a Tukey post hoc test was used in Figs. 1 *E* and *F*, 2*A*, and 5*A*. A log-rank test was used in Fig. 3.

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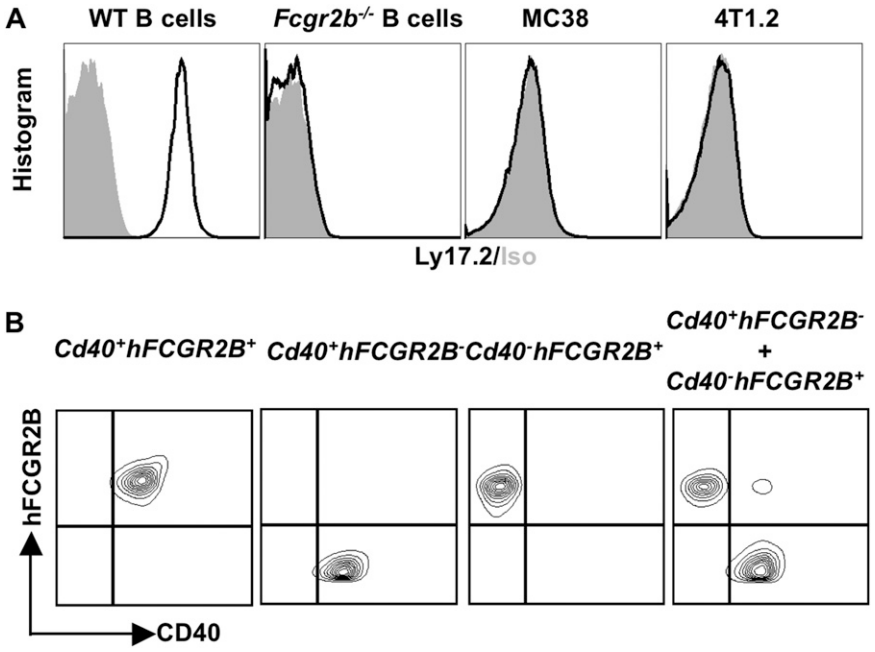

Fig. S1. (*A*) MC38 and 4T1.2 tumor cells do not express FcγRIIB. To analyze FcγRIIB expression levels, cells were stained with fluorescence-conjugated Ly17.2 or isotype control antibodies and analyzed by FACS. Presented are histogram profiles. (*B*) Expression of CD40 and human FcγRIIB in B cells of WT C57BL/6 recipient mice reconstituted with bone marrow cells isolated from either *Cd40*$^{+}$*hFCGR2B*$^{+}$, *Cd40*$^{+}$*hFCGR2B*$^{-}$, or *Cd40*$^{-}$*hFCGR2B*$^{+}$ mice or 1:1 mixed *Cd40*$^{+}$*hFCGR2B*$^{-}$ and *Cd40*$^{-}$*hFCGR2B*$^{+}$ mice.

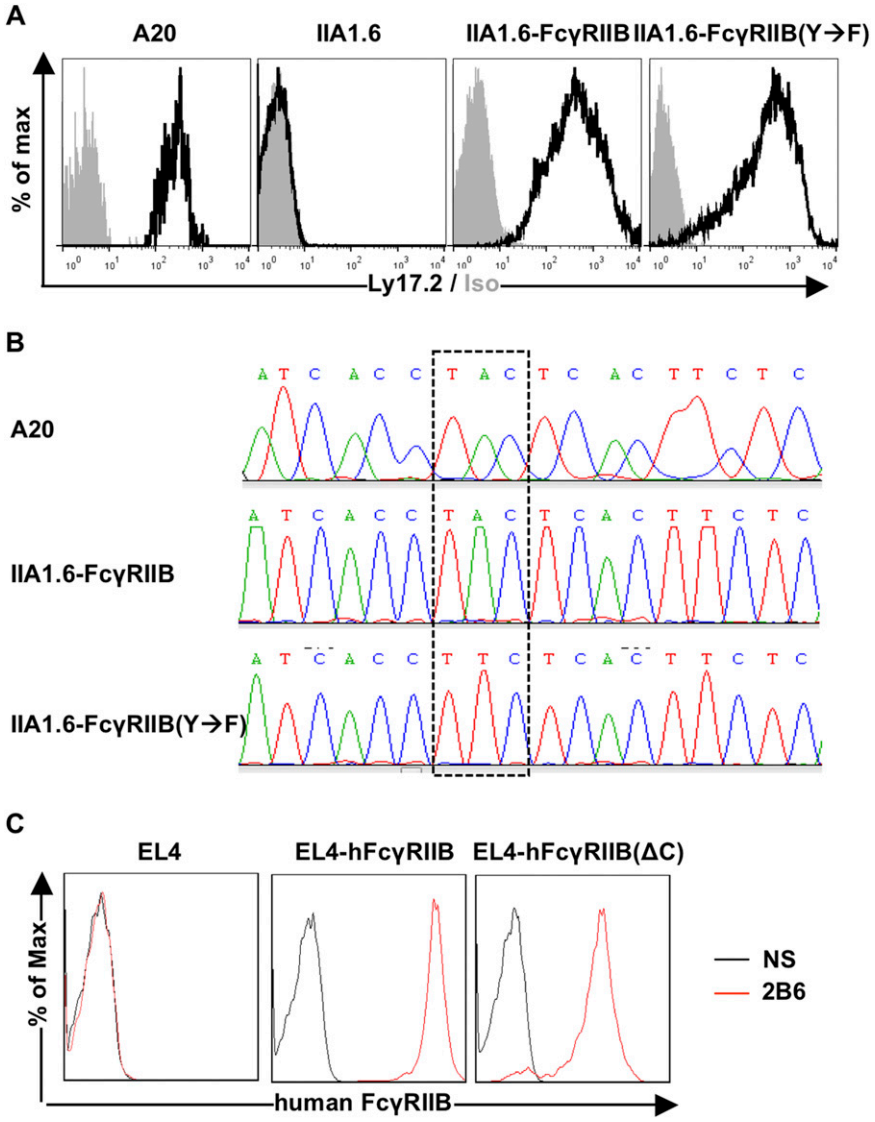

Fig. S2. (*A* and *B*) Expression of FcγRIIB in IIA1.6-derived cell lines. (*A*) Histogram profiles showing the expression of FcγRIIB analyzed by FACS using Ly17.2 antibodies in A20 (the FcγRIIB$^+$ parental cell line of IIA1.6), IIA1.6, IIA1.6-FcγRIIB (IIA1.6 cells expressing unmutated FcγRIIB), and IIA1.6-FcγRIIB(Y→F) cells (IIA1.6 cells expressing FcγRIIB with a Y→F mutation in the ITIM). (*B*) Sequencing results of *Fcgr2b* cDNA prepared from the indicated cells. The coding sequences for the conserved ITIM tyrosine are boxed. (*C*) Expression of human FcγRIIB in EL4-derived cell lines. Human FcγRIIB expression was analyzed in EL4, EL4-hFcγRIIB (EL4 cells expressing human FcγRIIB), and EL4-FcγRIIB(ΔC) cells (EL4 cells expressing truncated human FcγRIIB without the cytoplasmic domain) by FACS using 2B6 antibodies (mouse anti-human FcγRIIB). Histogram profiles are presented. NS, no staining.

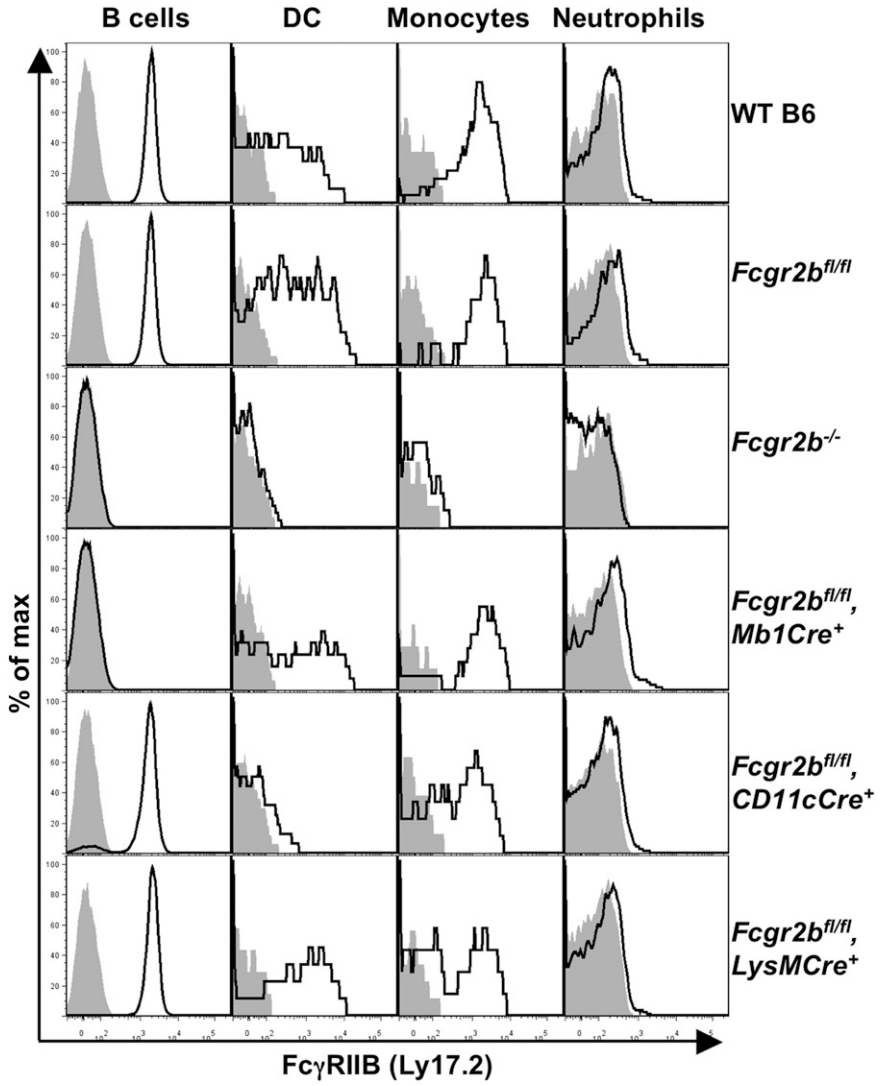

Fig. S3. Expression profiles of FcγRIIB in WT and mutant mice with germ-line or conditional knockout of *Fcgr2b*. Histogram profiles showing the expression of FcγRIIB in the indicated cell types of the indicated mice. FcγRIIB levels were analyzed in B cells ($CD19^+$), monocytes ($CD11b^+NK1.1^-Gr1^{low/-}SSC^{low}$), and neutrophils ($CD11b^+NK1.1^-Gr1^{high}SSC^{int}$) in the peripheral blood and dendritic cells (DC; $CD11c^{high}$) in spleen.

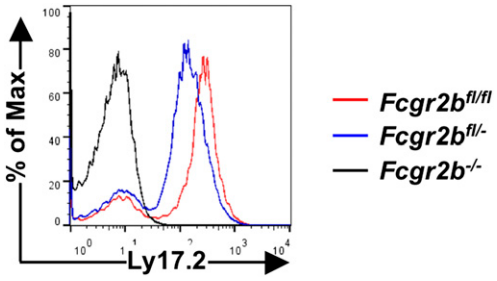

Fig. S4. Reduced expression of FcγRIIB in heterozygous *Fcgr2b* knockout mice. Histogram profiles showing the expression of FcγRIIB in splenocytes isolated from wild-type mice and heterozygous and homozygous *Fcgr2b* knockout mice, analyzed by FACS using Ly17.2 antibodies.

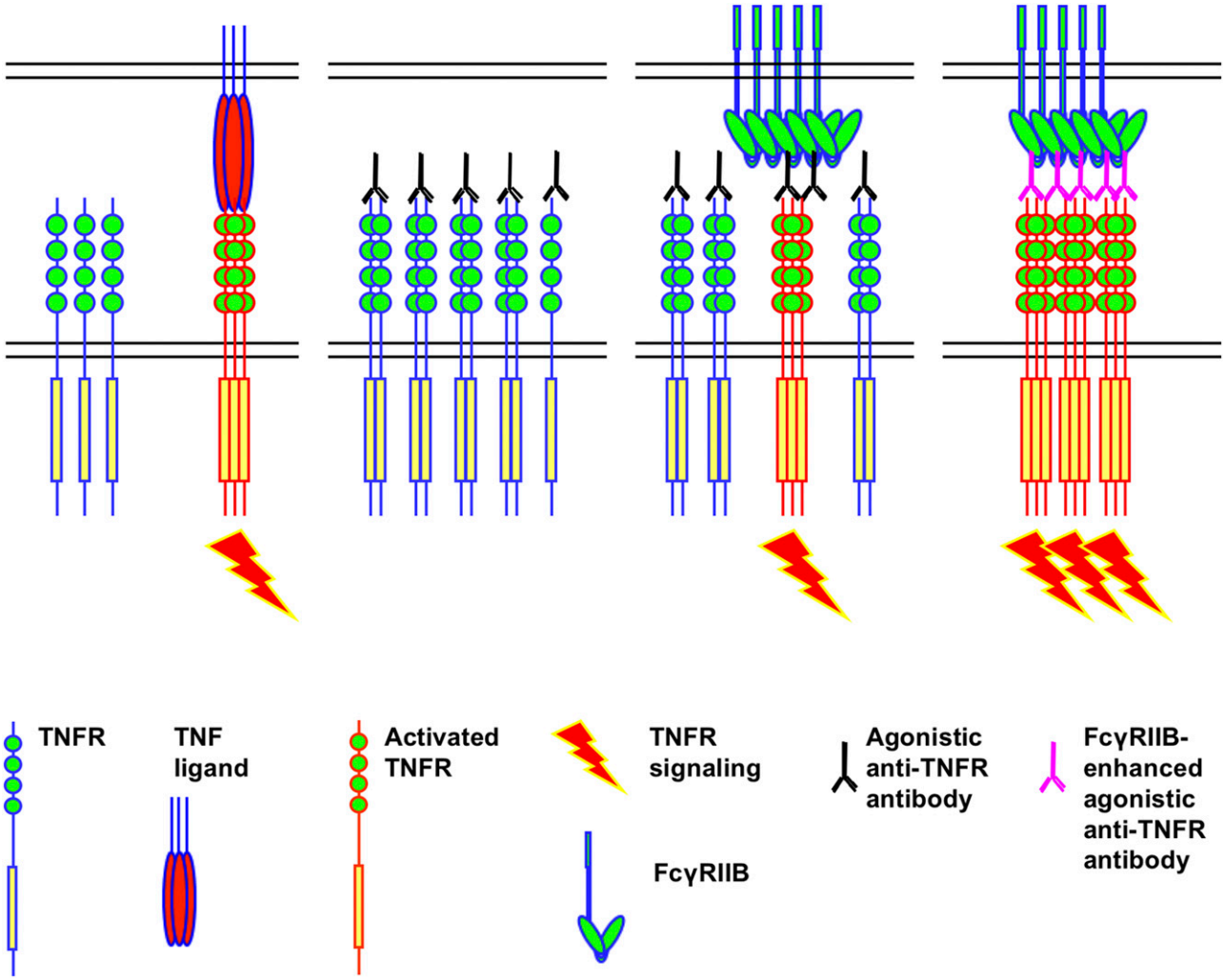

Fig. S5. Model of FcγRIIB-dependent activation of agonistic anti-TNF receptor (TNFR) antibodies. Endogenous multimeric TNF ligands can cluster TNFRs to trigger their downstream signaling. In the absence of FcγRIIB, clustering of TNFRs by the two arms of each single agonistic anti-TNFR antibody is insufficient to mimic the effect of multimeric TNF ligands. In contrast, agonistic anti-TNFR antibodies binding to TNFRs can be cross-linked by FcγRIIB in vivo, and result in effective TNFR clustering and activation, mimicking the effect of endogenous multimeric TNF ligands. In addition, when agonistic anti-TNFR antibodies are optimized for FcγRIIB binding, they can be more extensively cross-linked by FcγRIIB, and result in enhanced TNFR clustering and activation.