Supporting online material

Materials and methods

Mice: Wild-type C57BL/6 and BALB/c mice were purchased from Taconic. The FcγRIIB-deficient (*Fcgr2b*$^{-/-}$) mice and the Fc receptor common γ-chain deficient mice (*Fcer1g*$^{-/-}$) were generated in our laboratory as described previously and backcrossed to C57BL/6 for more than 12 generations (*24-25*). These two lines were crossed to generate double knockout mice (*Fcgr2b*$^{-/-}$ *Fcer1g*$^{-/-}$), which are deficient for all mouse Fcγ receptors and referred to as "FcγR$^{-/-}$" mice in this study. Human *FCGR2A* (*hFCGR2A*) transgenic mice have been described previously and backcrossed to C57BL/6. Human *FCGR2B (hFCGR2B)* BAC transgenic mice were generated on a pure B6 genetic background in our laboratory by pronuclear injection of linearized RP11-474I16 BAC DNA (http://bacpac.chori.org/)(*13*). Mice carrying *hFCGR2A* or *hFCGR2B* trangenes were bred to FcγR$^{-/-}$ or *Fcgr2b*$^{-/-}$ mice to generate FcγR$^{-/-}$*hFCGR2A*$^{+}$, FcγR$^{-/-}$ *hFCGR2B*$^{+}$, FcγR$^{-/-}$*hFCGR2A*$^{+}$*hFCGR2B*$^{+}$, and *Fcgr2b*$^{-/-}$*hFCGR2B*$^{+}$ mice. A new FcγRIIB knockout strain on a pure B6 genetic background (P. Smith and J.V.R., unpublished) was also used, and no difference was observed between the backcrossed *Fcgr2b*$^{-/-}$ mice and pure B6 *Fcgr2b*$^{-/-}$ mice in this study. T cell receptor knockout mice (B6.129P2-*Tcrb*^{tm1Mom} *Tcrd*$^{tm1Mom/J}$, referred to as "*Tcrb*$^{-/-}$*Tcrd*$^{-/-}$") and CD40-knockout mice (B6.129P2-Cd40<tm1Kik>/J, referred to as "*Cd40*$^{-/-}$") were purchased from the Jackson Laboratory. 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 IACUC.

DEC-OVA(hIgG1) and DEC-OVA(hIgG1)N297A: DEC-OVA(hIgG1) and DEC-OVA(hIgG1)N297A (the N297A variant of DEC-OVA(hIgG1), null for FcγR binding) were produced and purified as previously described (*26*). In order to make DEC-OVA(hIgG1), the DEC-mIgG1(D265A)-OVA heavy and light chain constructs described previously (*26*) were

modified so the coding sequences of mouse IgG1(D265A) heavy and kappa light chain constant regions were replaced with those of human IgG1 heavy and kappa light chain constant regions, respectively (*3*). Coding sequences for human IgG1 constant region and its N297A variant were cloned from 6A6-hIgG1(wt) and 6A6-hIgG1(N297A) as previously described (*27*). More specifically, the variable and constant regions were separately cloned in frame by PCR and ligated together by overlapping PCR using standard protocols (*28*). Full-length *Ig* coding sequences were digested with EcoRI/NheI and subcloned into the original DEC-mIgG1(D265A)-OVA heavy and light chain vectors. The following primers were used for the cloning:

DEC-OVA(hIgG1) and DEC-OVA(hIgG1)N297A heavy chains:

DEC_VH_F 5'CCTCGGTTCTATCGATTGAATTCCACCATGGGATGGTCATG3'

DEC_VH_R_hIgG1 5'CTTGGTGGAGGCTGAGGAGACTGTGACCATGACTCC3'

DEC_hIgG1_F 5'GGTCACAGTCTCCTCAGCCTCCACCAAGGGCCCATC3'

DEC_hIgG1_R 5'CTTGGCCATGTCGCTAGCTTTACCCGGAGACAGGGAGAGGC3'

DEC-OVA (hIgG1) and DEC-OVA(hIgG1)N297A light chains:

DEC_VL_F 5'CCTCGGTTCTATCGATTGAATTCCACCATGGGATGGTCATG3'

DEC_VL_R 5'CAGCCACAGTTCGTTTCAATTCCAGCTTGGTGCCTCC3'

DEC_hIgκ_F 5'GCTGGAATTGAAACGAACTGTGGCTGCACCATCTG 3'

DEC_hIgκ_R 5'CAAGCTTGGGAGCGGCCGCCTAACACTCTCCCCTGTTGAAGCTCTTTG3'

DEC-OVA(hIgG1) and DEC-OVA(hIgG1)N297A proteins were produced in 293T cells by transient transfection and purified by protein G Sepharose 4 Fast Flow (GE Healthcare) as previously described (*26*). LPS contamination was analyzed by Limulus Amebocyte Lysate Assay (Associates of Cape Cod, Inc.), and removed by TritonX-114 (Sigma) to keep endotoxin levels less than 0.01ng/μg.

CD40 antibodies: 1C10 was purified from culture supernatant by protein G Sepharose 4 Fast Flow (GE Healthcare). In order to make 1C10-derived CD40 antibodies with mouse or human IgG Fc's (mouse IgG1 or its D265A variant, mouse IgG2a, human IgG1 or its N297A or S267E variants) the heavy and light chain variable region genes were cloned by 5' RACE system according to manufacturer's instructions (Invitrogen), for which the following oligonucleotides were used: HC-GSP1 5'ACAAGGATTGCATTCCCTTGG3' and HC-GSP2 5'CTTGTCCACCT TGGTGCTGCT3' for heavy chain variable region gene cloning; LC-GSP1 5'CTCATTC CTGTTGAAGCTCTTGACGAC3' and LC-GSP2 5'GGGTGAGGATGATGTCTTATGAA CA3' for light chain variable region gene cloning.

To obtain full-length mouse and human Ig heavy and light chain coding sequences, the heavy and light chain variable region coding sequences were cloned in frame with signal peptide by one PCR, constant region coding sequences were cloned by another PCR, and full-length *Ig* sequences were obtained by overlapping PCR using standard protocols (*28*). Full-length *Ig* coding sequences were then digested with EcoRI/NotI and subcloned into the expression vector used by DEC-mIgG1(D265A)-OVA (*26*). The following primers were used to obtain full-length *Ig* coding sequences:

Anti-CD40 mouse IgG1 heavy chain and its D265A variant:

1C10_VH_F 5'CGATTGAATTCCACCATGGACATCAGGCTCAGCTTGGTT3';

1C10_VH_R_mIgG1 5'GCCCTTGGTGGTGGCTGAGGAGACTGTGACCATGACTC3';

1C10_mIgG1_F 5'GTCACAGTCTCCTCAGCCACCACCAAGGGCCCATCTGTC 3';

1C10_mIgG1_R 5'CTTGGGAGCGGCCGCTCATTTACCAGGAGAGTGGGAGAGGCTC3'.

Anti-CD40 mouse IgG2a heavy chain:

1C10_VH_F 5'CGATTGAATTCCACCATGGACATCAGGCTCAGCTTGGTT3';

1C10_VH_R_mIgG2a 5'GGCTGTTGTTTTGGCTGAGGAGACTGTGACCATGACTC3';

1C10_mIgG2a_F 5'GTCACAGTCTCCTCAGCCAAAACAACAGCCCCATCGGTC3';

1C10_mIgG2a_R 5'CTTGGGAGCGGCCGCTCATTTACCCAGAGACCGGGAGATGGTC 3'.

Anti-CD40 human IgG1 heavy chain and its N297A or S267E variants:

1C10_VH_F 5’CGATTGAATTCCACCATGGACATCAGGCTCAGCTTGGTT3’;

1C10_VH_R_hIgG1 5’GCCCTTGGTGGAGGCTGAGGAGACTGTGACCATGACTC3’

1C10_hIgG1_F 5’GTCACAGTCTCCTCAGCCTCCACCAAGGGCCCATCGGTC3’

1C10_hIgG1_R 5’CTTGGGAGCGGCCGCTCATTTACCCGGAGACAGGGAGAGGCTC3’.

Anti-CD40 mouse Igκ light chain:

1C10_VL_F 5’CGATTGAATTCCACCATGGAGACAGACAGACTCCTGCTA3’

1C10_VL_R_mIgκ 5’GCAGCATCAGCCCGTGAGGAGACTGTGACCATGACTCC3’

1C10_mIgκ_F 5’CAAGCTGGAATTGAAACGGGCTGATGCTGCACCAACTGTA3’

1C10_mIgκ_R 5’CTTGGGAGCGGCCGCTCAACACTCATTCCTGTTGAAGCTCTTG3’.

Anti-CD40 human Igκ light chain:

1C10_VL_F 5’CGATTGAATTCCACCATGGAGACAGACAGACTCCTGCTA3’

1C10_VL_R_hIgκ 5’GCAGCCACAGTTCGTGAGGAGACTGTGACCATGACTCC3’

1C10_hIgκ_F 5’CAAGCTGGAATTGAAACGAACTGTGGCTGCACCATCTGTC3’

1C10_hIgκ_R 5’CTTGGGAGCGGCCGCTCAACACTCTCCCCTGTTGAAGCTCTTTG3’

Mouse IgG1 and IgG2a constant region coding sequences were cloned from constructs described previously (*7*). Mouse IgG1 constant region coding sequences with the D265A mutation were cloned from the DEC-mIgG1(D265A)-OVA heavy chain construct by PCR using primers 1C10_mIgG1_F and 1C10_mIgG1_R. Human IgG1 constant region sequences with the N297A mutation were cloned from the 6A6-hIgG1(N297A) heavy chain construct (*27*) using primers 1C10_hIgG1_F and 1C10_hIgG1_R. Human IgG1 constant region sequences with the S267E mutation were generated by mutagenesis using the following primers:

S267E_F 5’GTGGTGGACGTGGAACACGAAGACCCT3’

S267E_R 5’AGGGTCTTCGTGTTCCACGTCCACCAC3’

CD40 antibodies were produced in 293T cells by transient transfection and purified by protein G Sepharose 4 Fast Flow (GE Healthcare). LPS contamination was analyzed by Limulus Amebocyte Lysate Assay (Associates of Cape Cod, Inc.), and removed by TritonX-114 (Sigma) to keep LPS less than 0.01ng/μg.

$F(ab')_2$ fragment of 1C10 was made using $F(ab')_2$ preparation kit (Pierce) following manufacturer's instructions. 1C10, 1C10 $F(ab')_2$ fragment, and CD40 antibodies with mouse or human IgG Fc's were examined by SDS-PAGE (NuPAGE, 4-12% Bis-Tris Mini Gels, Invitrogen) under non-reducing conditions.

In order to prepare deglycosylated 1C10, 1C10 was treated with endoS (IgGZERO, Genovis) following manufacturer's instructions, and purified by protein G Sepharose 4 Fast Flow (GE Healthcare). The efficiency of endoS treatment was examined by *Lens culinaris* agglutinin (LCA, Vector Laboratory) lectin blot as previously described (*27*).

FGK45 and 3/23 were purchased from Enzo Life Sciences and BD Biosciences, respectively.

OVA-specific T cell response: Sex matched mice, two to three months of age were immunized through *i.p.* injection with 5μg of DEC-OVA(hIgG1) or DEC-OVA(hIgG1)N297A, in the presence or absence of 30μg of CD40 antibodies with one of the various Fc's (rat IgG2a Fc of 1C10, deglycosylated rat IgG2a Fc of deglycosylated 1C10, mouse IgG1 Fc or its D265A variant, mouse IgG2a Fc, human IgG1 Fc or its N297A or S267E variants), or 20μg of 1C10 $F(ab')_2$ fragment. In the experiment presented in fig. S3, 100μg of 2.4G2 or rat IgG2b isotype control antibodies (BD biosciences) were injected *i.p.* with DEC-OVA(hIgG1)N297A and 1C10. In the experiment presented in fig. S4, 30μg of 3/23 or FGK45 were injected *i.p.* with DEC-OVA(hIgG1)N297A. In the experiment presented in fig. S10, 100μg of NK cell depleting antibodies (αNK1.1, clone PK136) were injected intravenously 1h before the injection of DEC-OVA(hIgG1)N297A and CD40 antibodies. Seven days later, spleens were harvested to prepare single cell suspension, and in some experiments, peripheral blood was collected instead. After

lysing red blood cells, tetramer staining and intracellular IFN-γ staining were exploited to quantify $CD8^+$ OVA-specific T cells. For tetramer staining, spleen or blood cells resuspended in FACS buffer (PBS with 0.5% FBS, 2mM EDTA and 0.1%NaN_3) were stained with FITC-conjugated anti-CD4 and APC-conjugated anti-CD8α antibodies (BD Biosciences), and PE-conjugated OVA peptide SIINFEKL H-2^b tetramer (tet-OVA, Beckman Coulter), and analyzed on FACScalibur or BD LSRII with 7AAD included to exclude dead cells. For intracellular IFN-γ staining, spleen cells were cultured in media (RPMI with 10% FBS, 1% Pen Strep, 10mM HEPES, 50μM 2-Mercaptoethanol) with 1μg/ml anti-CD28 antibody and 1μg/ml OVA peptide SIINFEKL for 6 hours at 37°C with 5% CO_2. Brefeldin A was added 1 hour after the culture started to a final concentration of 10μg/ml. Cultured spleen cells were stained for surface CD4 and CD8α with FITC-conjugated anti-CD4 and PE-conjugated CD8α antibodies, then intracellular IFN-γ with APC-conjugated anti-IFN-γ following manufacturer's protocol (BD biosciences) before analysis on FACScalibur or BD LSRII.

MO4 tumor model: MO4 is an OVA-expressing melanoma cell line that has been described previously (*3*) and maintained in DMEM with 10% FBS, 1% Pen Strep, and 0.4mg/ml Geneticin (Invitrogen). B6, $FcγR^{-/-}$ and $FcγR^{-/-}$*hFCGR2B*$^+$ mice were inoculated with 10^7 MO4 cells in 50μl PBS subcutaneously. Seven to twelve days later (after tumors were established), mice were treated with 5μg DEC-OVA(hIgG1)N297A and 30μg of CD40 antibodies with one of the various mouse or human IgG Fc's (mouse IgG1 Fc or its D265A variant, mouse IgG2a Fc, human IgG1 Fc or its N297A or S267E variants), or control IgG (ChromPure mouse or human IgG, Jackson ImmunoResearch). Tumor growth was monitored after treatment, and tumor area values were calculated as $\pi d^2/4$ where "d" was the diameter of the tumors. In the MO4 experiment presented in Fig S11 A, WT and *Fcgr2b*$^{-/-}$ mice were treated with 5μg of DEC-OVA(hIgG1)N297A and 30μg of CD40 antibodies with different mouse IgG Fc's (mouse IgG1 Fc or its D265A variant, or

mouse IgG2a Fc) or control mouse IgG one month before 10^7 MO4 cells were inoculated subcutaneously.

A20 tumor model: A20 cells were maintained in RPMI with 10% FBS, 1% Pen Strep, 1mM Sodium Pyruvate, 10mM HEPES, and 50μM 2-Mercaptoethanol (Invitrogen). BALB/c mice were injected intravenously with either 200μg of mouse control IgG, or CD40 antibodies with different mouse IgG Fc's (mouse IgG1 Fc or its D265A variant, or mouse IgG2a Fc). One hour later, $2x10^7$ A20 cells were inoculated subcutaneously. Tumor growth was monitored and tumor area values were calculated as $\pi d^2/4$ where "d" was the diameter of the tumors.

B6BL and B6BL-CD40 models: Spontaneous B cell lymphomas in mice with p53 deficiency have been described previously (*15*). B6BL is a spontaneous B cell lymphoma isolated from $p53^{fl/fl}CD19Cre^+$ mice on pure B6 genetic background (Nussenzweig laboratory, the Rockefeller University). B6BL cells were maintained in RPMI with 10% FBS, 1% Pen Strep, 1mM Sodium Pyruvate, 10mM HEPES, and 50μM 2-Mercaptoethanol (Invitrogen). FACS analysis of B6BL surface phenotype showed that B6BL cells were $CD19^+Fc\gamma RIIB^+IgM^-CD40^-$, therefore represented a pro/pre-B cell lymphoma line. In order to generate CD40-expressing B6BL (B6BL-CD40), CD40 isoform1 (Genbank Acc#: NM_011611) cDNA was cloned from wild-type C57BL/6 spleen RNA by SuperScript® III one-Step RT-PCR System with Platinum® Taq High Fidelity (Invitrogen), into pFB-neo retroviral vector (Stratagene). Retroviral particles were produced in 293T cells and used to transduce B6BL cells. Transduced B6BL cells were selected by 1mg/ml Geneticin for 2 weeks and sorted for $CD40^+$ cells as shown in fig. S12. Sorted $CD40^+$ cells, referred to as "B6BL-CD40" in this study, were maintained in RPMI with 10% FBS, 1% Pen Strep, 1mM Sodium Pyruvate, 10mM HEPES, 50μM 2-Mercaptoethanol, and 0.4mg/ml Geneticin (Invitrogen). The following primers were used to clone CD40 isoform1 cDNA:

mCD40_F 5'AATTGTCGACCACCATGGTGTCTTTGCCTCGGCTGTGC3';

mCD40_R 5'AATTGCGGCCGCTCAGACCAGGGGCCTCAAGGCTATG3'.

In B6BL and B6BL-CD40 models, B6, *Tcrb*$^{-/-}$*Tcrd*$^{-/-}$, *Fcgr2b*$^{-/-}$, *Fcgr2b*$^{-/-}$*hFCGR2B*$^{+}$, *Fcer1g*$^{-/-}$ mice were inoculated with 2.5X10^{7} tumor cells intravenously on day 0, and treated with a first dose of control IgG or CD40 antibodies with one of the various mouse or human IgG Fc's (mouse IgG1 Fc or its D265A variant, mouse IgG2a Fc, human IgG1 Fc or its N297A or S267E variants) on day 3 and the second dose on day 4 or 6 by *i.v.* injection. Each treatment used 200μg mouse or human control IgG, or CD40 antibodies except where lower dosage treatments (40μg αCD40:hIgG1 or αCD40:hIgG1(S267E) per dose) were studied (Fig. 3E). Only one dose was used to treat mice studied in Fig. 3D. In the study presented in Fig. 3D and Fig. 4B, 200μg/dose of αCD4 and αCD8 depleting antibodies (clone GK1.5 and 2.43, respectively, BioXcell) were also included in the treatment. Mice were monitored daily for survival. In the experiment presented in Fig. 3E, long-time survivors were re-challenged with 2.5X10^{7} B6BL cells 10 weeks after the initial B6BL inoculation.

CD40 antibodies induced ADCC and CD8^{+} T cell expansion: WT mice were treated with 200μg control mouse IgG, or CD40 antibodies with different mouse IgG Fc's (moue IgG1 Fc or its D265A variant, or mouse IgG2a Fc). Six days later, peripheral blood samples were collected and treated with red blood cell lysing buffer (BD biosciences).The percentage of CD40^{+} cells and ratio of CD8^{+} to CD4^{+} T cells were analyzed by FACS using fluorescence-labeled anti-CD4, anti-CD8α, and anti-CD40 (1C10). 7AAD was added to exclude dead cells.

Flow cytometry

For surface staining, about 1~5 x 10^{6} cells were resuspended in 50μl staining buffer (1xPBS + 0.5% FBS + 2mM EDTA + 0.1% NaN_3) containing staining reagents (antibodies and tetramer, etc) and incubated on ice for 15 minutes. When tetramer was used, the incubation time was extended to 60 minutes. Cells were then washed twice with staining buffer, followed by resuspension of cells in staining buffer with 7AAD (BD Biosciences) or DAPI (Invitrogen) for analysis, or a second staining step, or an additional intracellular IFN-γ staining using BD

Cytofix/Cytoperm™ Fixation/Permeabilization Solution Kit (BD BioSciences). Data were acquired on FACScalibur or BD LSRII, and analyzed in Flowjo (Version 7.5 for Windows)

For Fig. 2D, Spleen cells were prepared by liberase digestion and stained with fluorescence labeled CD11c, CD80, and CD86 antibodies. 7AAD was added to samples before analysis to exclude dead cells.

For fig. S7A and B, WT and $CD40^{-/-}$ splenocytes were stained with unlabeled CD40 antibodies (αCD40:mIgG1, αCD40:mIgG1(D265A), αCD40:mIgG2a, αCD40:hIgG1, αCD40:hIgG1(N297A), or αCD40:hIgG1(S267E), or isotype controls (mouse IgG1, IgG2a, or human IgG1) in the first step, which were detected in the second step by FITC-conjugated goat anti-mouse IgG-Fc (Sigma), rat anti-mouse IgG1, rat anti-mouse IgG2a (BD Biosciences), or mouse anti-human IgG (Zymed). Cells were also stained with PE-conjugated 1C10 (eBioscience) as positive control. 7AAD was added to samples before analysis to exclude dead cells.

For fig. S7 C, 10^6 $Fc\gamma R^{-/-}$ splenocytes in 50μl were stained with 2.2μg/ml PE-conjugated 1C10 in the presence of fixed amount of FITC conjugated anti-CD19 (5.5μg/ml) and 0.25ng/ml~20μg/ml unlabeled 1C10, CD40 antibodies with mouse or human IgG Fc's, or control IgG (1:1:1:1 mix of rat IgG2a, mouse IgG1, mouse IgG2a and human IgG1; its concentration refers to the concentration of every component). 7AAD was added to samples before analysis to exclude dead cells.

For fig. S8, splenocytes of $Fc\gamma R^{-/-}$, $Fc\gamma R^{-/-}$*hFCGR2A*$^+$, $Fc\gamma R^{-/-}$*hFCGR2B*$^+$, $Fc\gamma R^{-/-}$ *hFCGR2A*$^+$*hFCGR2B*$^+$ mice were prepared by liberase digestion, and stained with fluorescence conjugated anti-human FcgRIIA/B (clone FL18. 26) and an antibody cocktail of anti-B220, anti-CD3ε, anti-CD11c and DX5, or a cocktail of anti-CD11b, anti-Gr1 and anti-CD115 (BD BioSceiences, eBioscience). DAPI (Invitrogen) was added before analysis to exclude dead cells. Data were acquired on BD LSR II.

For fig. S12, B6BL, transduced B6BL, sorted B6BL-CD40 and A20 cells were stained with fluorescence labeled anti-CD19, 2.4G2, ly17.2 and its mouse IgG2a isotype control, IgM, anti-CD40 (1C10) and rat IgG2a isotype control (BD BioSciences, and eBioscience).

Statistical analysis: Graphpad Prism (version 5.04, for windows) was used for statistical analysis. One way ANOVA with Dunnett post hoc test was performed to compare: all groups to the control groups ("Untreated" in Fig. 2D, "mIgG" in Fig. 3A, "hIgG" in Fig. 3B, "mIgG" in Fig. 4A, "mIgG" in Fig. 4C, and "Tg^+:hIgG" in fig. S11, respectively); "αCD40:hIgG1" to "αCD40:hIgG1(S267E)" in Fig. 3B; "aCD40:mIgG1" with "aCD40:mIgG2a" in Fig. 4C. Logrank test of survival curves was used to compare: all groups to the control groups ("mIgG" in Fig. 3C, 4B and 4D, "WT: mIgG" in Fig. 3D, Tg^+:hIgG(400μg) in Fig.3E, respectively); between Tg^+ mice treated with same doses of αCD40:hIgG1 and αCD40:hIgG1(S267E). Two tailed *t* test was used to compare

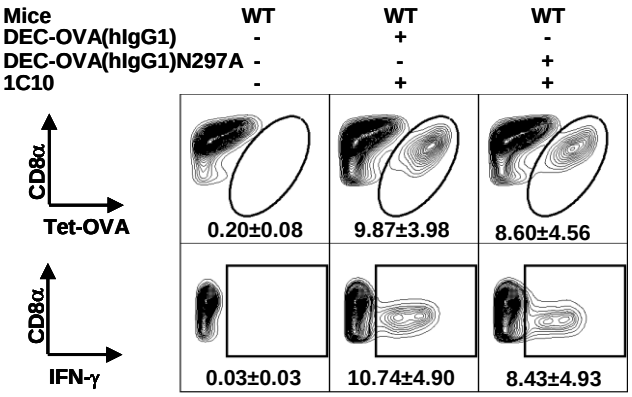

Fig. S1. FcγR-engagement is not required for DEC-OVA(hIgG1) to induce OVA-specific T cell response in the presence of agonistic CD40 antibodies. WT mice were immunized with 5μg of DEC-OVA(hIgG1) or DEC-OVA(hIgG1)N297A (the N297A variant of DEC-OVA(hIgG1), null for FcγR binding) in the presence or absence of 30μg of 1C10, and analyzed for splenic OVA-specific CD8 T cells as in Fig. 1 (and in "Materials and methods"). Shown are representative 2% contour plots gated on $CD4^{-}CD8\alpha^{+}$ cells, with gate and percentage (mean ± S.D) of tet-OVA^{+} or IFN-g^{+} cells, of two or more independent experiments with similar results.

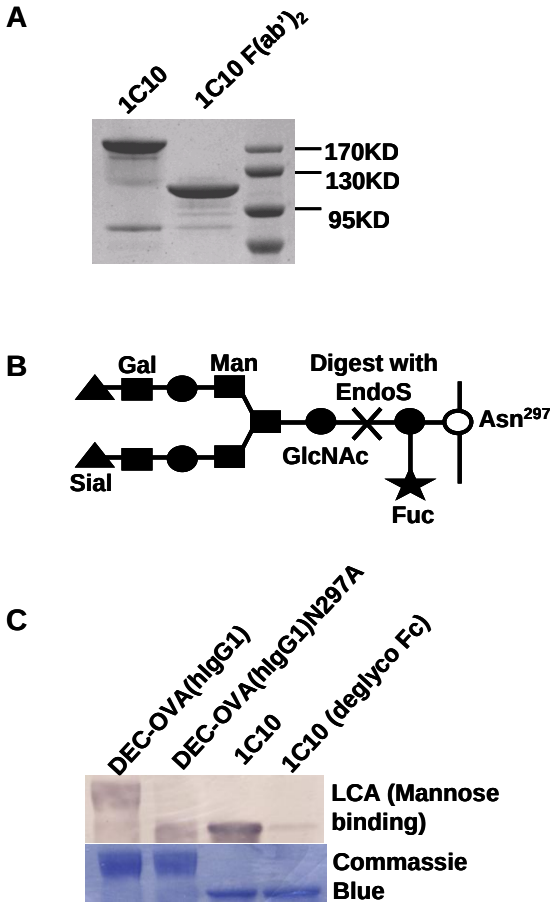

Fig. S2. Preparation of 1C10 F(ab')2 fragment and deglycosylated 1C10. (A) Shown are 1C10 and 1C10 F(ab')2 fragment examined on SDS-PAGE gel under non-reducing conditions. (B) Diagram shows the sugar moiety at Asn297 of human IgG1 heavy chain and the cleavage site of endoS. GlcNAc, N-acetylglucosamine (GlcNAc); Fuc, fucose; Man, mannose; Gal, galactose; Sial, N-acetylneuraminic acid. (C) 1C10 and endoS treated 1C10 (1C10 deglyco Fc) were analyzed by LCA lectin blotting and Coomassie staining. DEC-OVA(hIgG1) and its N297A variant (DEC-OVA(hIgG1)N297A were included as positive and negative controls, respectively, for LCA lectin blotting.

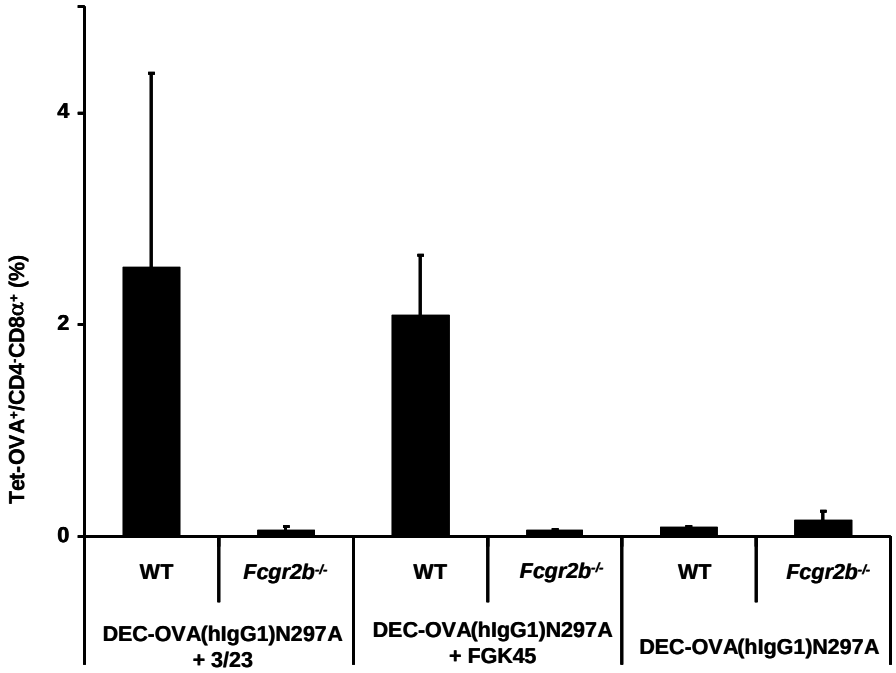

Fig. S3. FGK45 and 3/23, two rat IgG2a agonistic CD40 antibodies, also require FcγRIIB for their adjuvant effects. WT and *Fcgr2b*$^{-/-}$ mice (3~5 mice per group) were immunized with 5μg of DEC-OVA(hIgG1)N297A in the presence or absence of 30μg of 3/23 or FGK45. Seven days later, OVA-specific T cells were analyzed in the peripheral blood by OVA peptide SIINFEKL H-2^{b} tetramer (tet-OVA) staining as in Fig. 1 (and as described in "Materials and methods"). Shown is a bar-graph presenting the percentages of tet-OVA^{+} cells in CD4^{-}CD8α^{+} cells. Error bars are S.D.

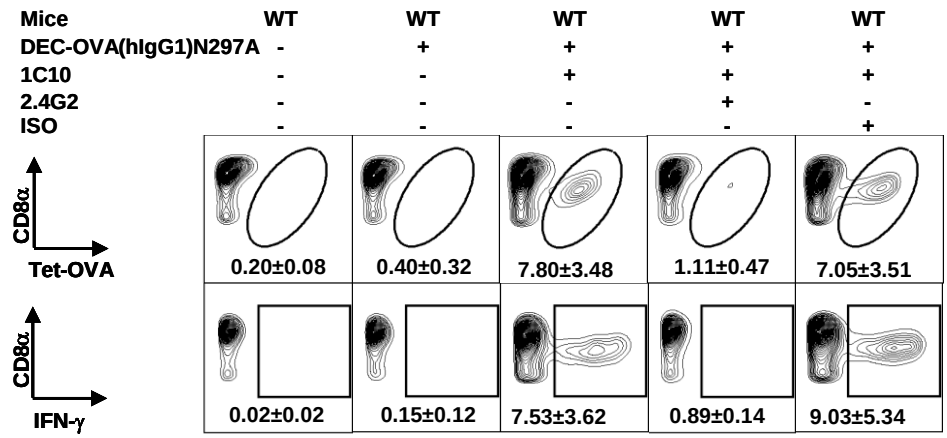

Fig. S4. DEC-OVA(hIgG1)N297A and 1C10 induced OVA-specific T cell responses can be blocked by 2.4G2. WT mice were immunized with 5μg of DEC-OVA(hIgG1)N297A with or without 30μg of 1C10, in the presence or absence of 100μg of 2.4G2 or isotype control antibodies (ISO). Seven days later, OVA-specific splenic T cells were analyzed as in Fig. 1 (and as described in "Materials and methods"). Shown are representative 2% contour plots gated on $CD4^-$ $CD8^+$ cells, with gate and percentage (mean ± S.D) of tet-OVA^+ or $IFN\gamma^+$ cells, of two or more independent experiments with similar results.

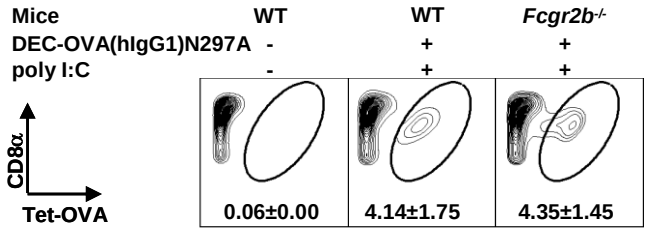

Fig. S5. DEC-OVA(hIgG1)N297A and poly I:C can induce OVA-specific CD8 T cell responses in both WT and *Fcgr2b*$^{-/-}$ mice. WT and *Fcgr2b*$^{-/-}$ mice were immunized with 5μg of DEC-OVA(hIgG1)N297A plus 150μg of poly I:C, and analyzed for splenic OVA-specific CD8 T cell responses 7 days later as in Fig. 1 (and as described in "Materials and methods"). Shown are representative 2% contour plots gated on $CD4^{-}CD8^{+}$ cells, with gate and percentage (mean ± S.D) of tet-OVA^{+} or $IFN\gamma^{+}$ cells, of two or more independent experiments with similar results.

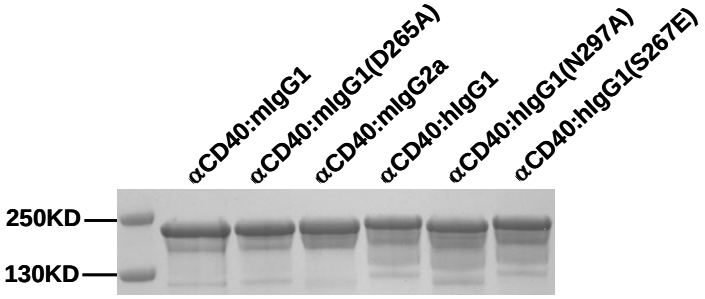

Fig. S6. Preparation of 1C10-derived anti-CD40 variants with the indicated mouse or human IgG Fc's. Shown is an SDS-PAGE gel examing purified antibodies under non-reducing conditions.

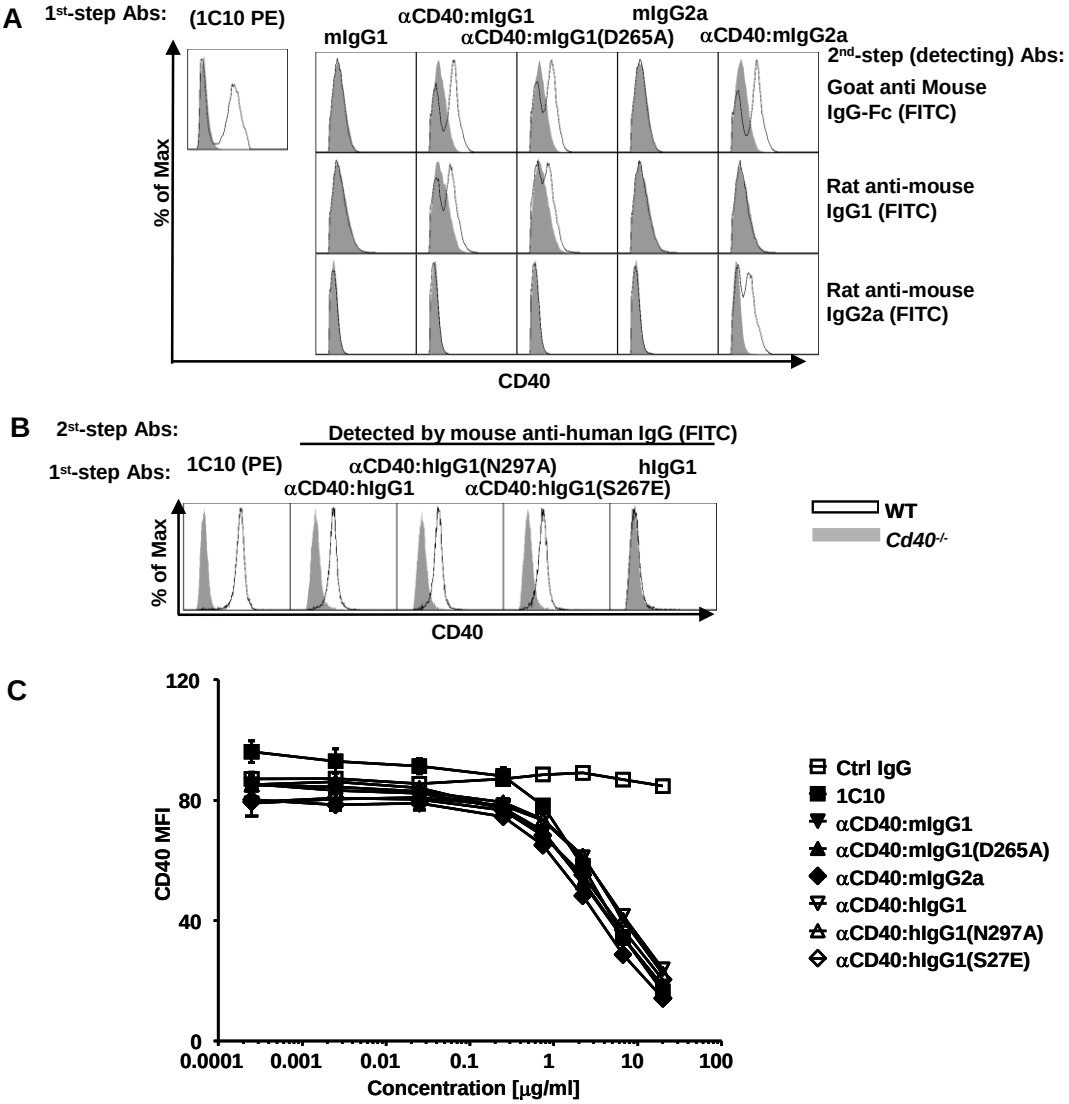

Fig. S7. 1C10-derived CD40 antibodies with mouse or human Fc's retain CD40 binding specificity and affinity. (A) Histogram profiles showing the CD40-expression on WT and *Cd40*$^{-/-}$ splenocytes detected by the indicated 1st step and 2nd step antibodies. Unlabeled αCD40:mIgG1, αCD40:mIgG1(D265A), or αCD40:mIgG2a used in the first step were detected in the second step by fluorescence labeled goat anti-mouse IgG-Fc, rat anti-mouse IgG1, or rat anti-mouse IgG2a. Negative control IgG (mouse IgG1 or mouse IgG2a) and positive control (PE-conjugated 1C10) were included. (B). CD40-expression was analyzed as in (A) with unlabeled αCD40:hIgG1, αCD40:hIgG1(N297A), or αCD40:hIgG1(S267E) in the first step and fluorescence labeled mouse anti-human IgG in the second step. In (A) and (B), only wild-type, not *Cd40*$^{-/-}$ cells were labeled by un-conjugated anti-CD40 variants, suggesting that these variants are specific for CD40. (C) FcγR$^{-/-}$ splenocytes were stained with 2.2μg/ml PE-conjugated 1C10 in the presence of fixed amount of FITC-conjugated anti-CD19 and various amounts of the indicated unlabeled anti-CD40 or control IgG (1:1:1:1 mix of rat IgG2a, mouse IgG1, mouse IgG2a and human IgG1). CD40-staining on viable CD19^{+} cells were expressed as mean fluorescence intensity (MFI) and plotted against the concentration of the competitive CD40 antibodies or control IgG. Control IgG did not show any effect on CD40 staining by PE-1C10, while anti-CD40 variants reduced CD40 MFI, with an efficiency similar to purified 1C10, suggesting these antibodies have similar affinity for CD40 as 1C10.

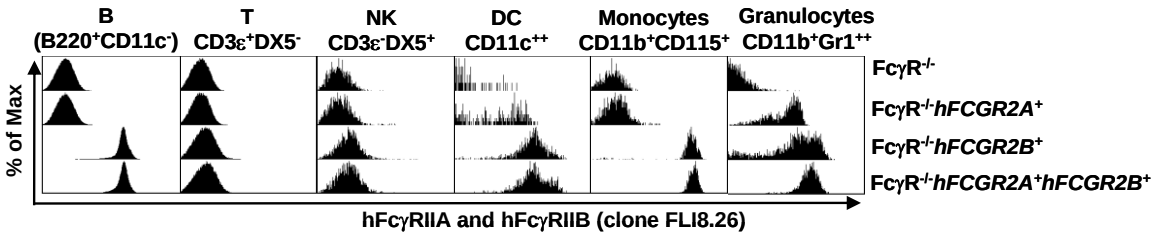

Fig. S8. Expression of *hFCGR2A* and *hFCGR2B* transgenes. Splenocytes of FcγR$^{-/-}$ mice without or with *hFCGR2A* or *hFCGR2B*, or both transgenes, were analyzed for surface expression of these transgenes using anti-human FcγRIIA and FcγRIIB (clone FL18. 26). Cell lineages were defined by indicated markers. Shown are representative histograms.

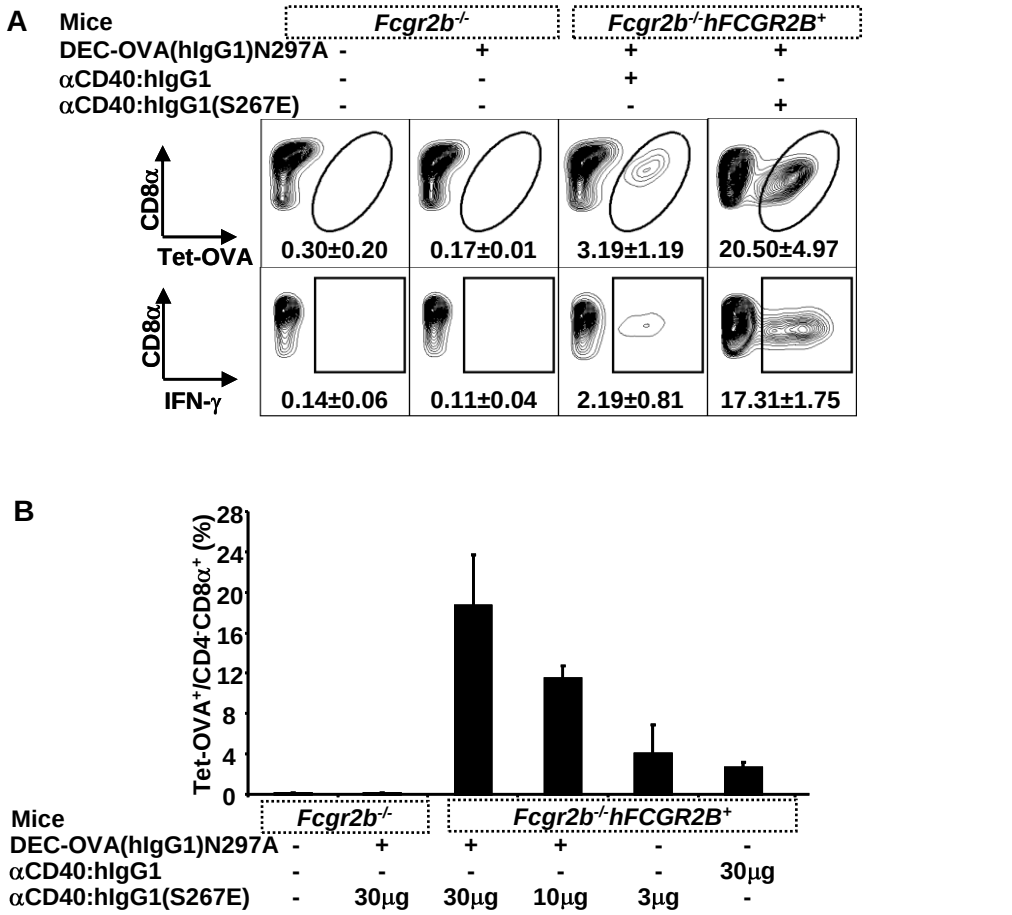

Fig. S9. CD40 antibodies with enhanced hFcγRIIB-binding affinities induce OVA-specific T cell response in an *hFCGR2B*-transgene and dose dependent manner. (A-B) *Fcgr2b*$^{-/-}$ and *Fcgr2b*$^{-/-}$ *hFCGR2B*$^{+}$ mice were immunized with 5μg of DEC-OVA(hIgG1)N297A with or without 30μg (or 10μg, or 3μg, as indicated otherwise) of the indicated anti-CD40. Seven days later, OVA-specific T cells were analyzed in spleen (A) or peripheral blood (B) as in Fig. 1 and Fig. 2A (and as described in "Materials and methods"). Shown are representative 2% contour plots gated on CD4^{-}CD8^{+} cells, with gate and percentage (mean ± S.D) of tet-OVA^{+} or IFNγ^{+} cells in (A), and a bar-graph presenting the percentage of tet-OVA^{+} cells in CD4^{-}CD8α^{+} cells (B). Error bars are S.D.

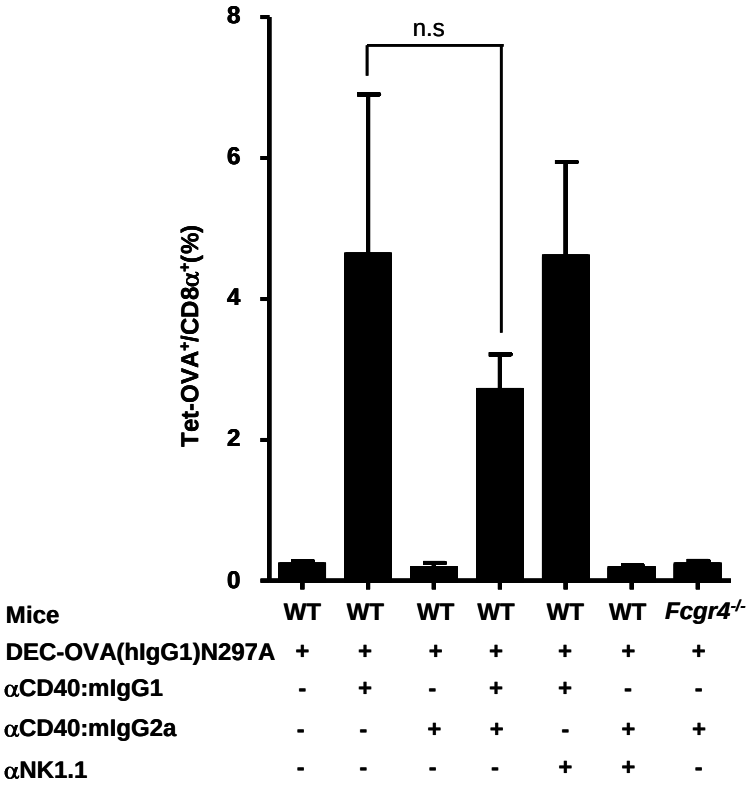

Fig. S10. αCD40:mIgG2a does not abrogate adjuvant activity of αCD40:mIgG1. WT and *Fcgr4*$^{-/-}$ mice (four mice per group) were injected *i.p.* with 5μg of DEC-OVA(hIgG1)N297A in the presence or absence of 30μg of the indicated anti-CD40, and/or 100μg of NK cell depleting antibodies, αNK1.1(clone PK136, injected intravenously). Spleen cells were analyzed 7 days later by OVA-tetramer (tet-OVA) staining and flow cytometry. Presented is a bar-graph showing percentages of tet-OVA^{+} cells in CD8α^{+} cells with S.D. n.s. not significant in two-tailed *t* test in Graphpad Prism.

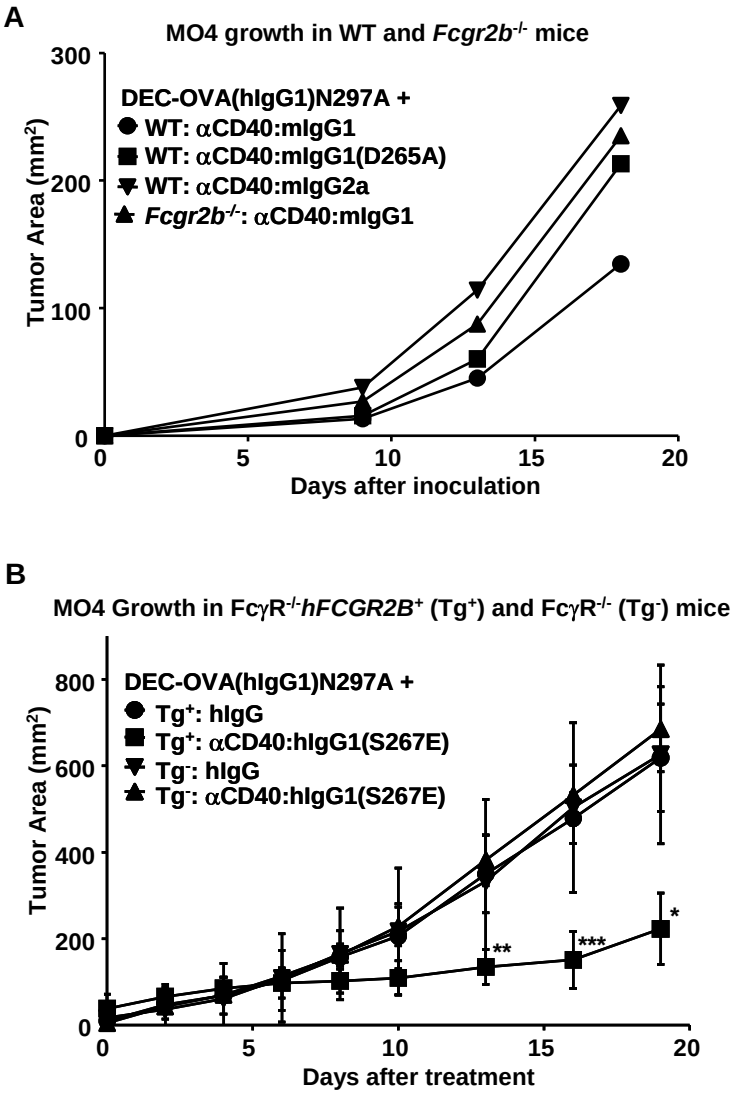

Fig. S11. Anti-tumor activities of agonistic CD40 antibodies require FcγRIIB. (A) WT and *Fcgr2b*$^{-/-}$ mice (5 mice per group) were immunized with 5μg of DEC-OVA(hIgG1)N297A plus 30μg of control mouse IgG or the indicated anti-CD40. One month later, 10^7 MO4 melanoma cells were implanted subcutaneously. (B) *Fcgr2b*$^{-/-}$ and FcγR$^{-/-}$*hFCGR2B*$^{+}$ mice (3 mice per group) were inoculated with 10^7 MO4 cells. Seven days later, mice were treated with 5μg of DEC-OVA(hIgG1)N297A plus 30μg of control human IgG or the indicated anti-CD40. Average tumor areas are presented in (A). Tumor areas with S.D. are presented in (B). *p<0.05, **p<0.01, ***$p<0.001$. One-way ANOVA with Dunnett post hoc test was used to compare: all groups to the "Tg^{+}:hIgG" control group in (B).

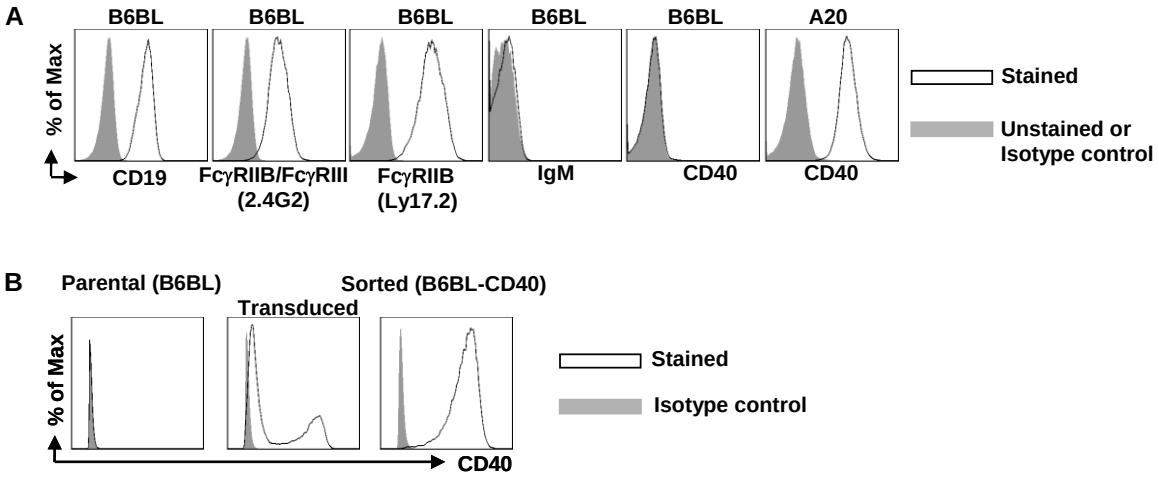

Fig. S12. Characterization of B6BL and B6BL-CD40 cells. (A) Histograms showing surface expression of CD19, FcγRIIB/FcγRIII, IgM, CD40 in B6BL cells. A20 cells were included as positive controls for CD40 expression. (B) Histograms showing surface CD40 expression in parental, transduced B6BL cells, and sorted cells (B6BL-CD40) based on CD40-expression.