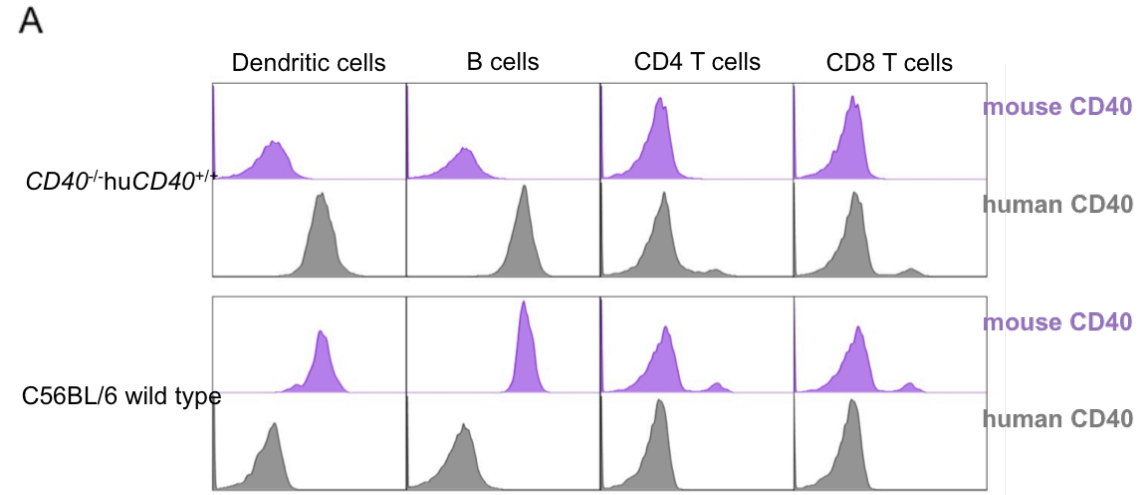
Supplemental Data



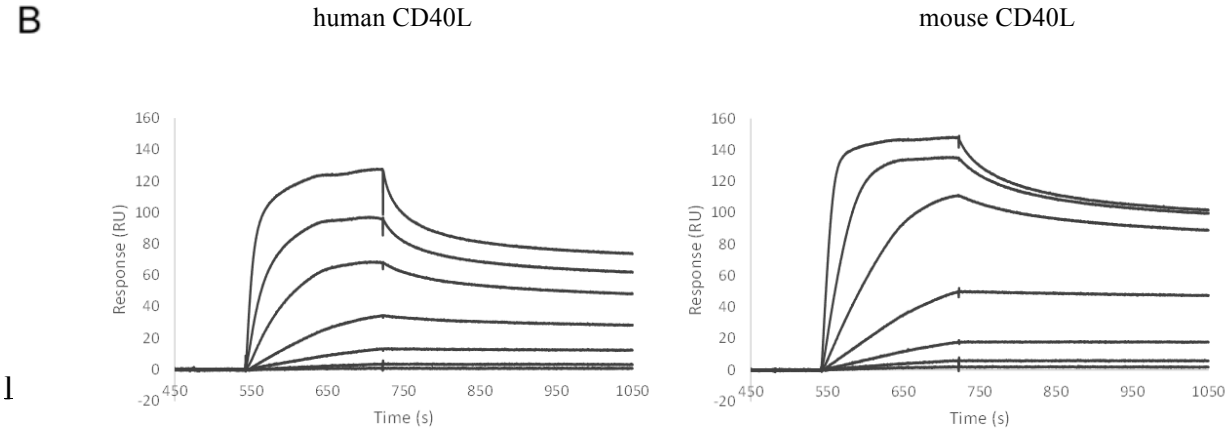


Figure S1, Related to Figure 1. Binding of hCD40 to mouse and human CD40L.

(A) Representative flow cytometry staining of hCD40 expression on the indicated splenic cell populations in *CD40*$^{-/-}$hu*CD40*$^{+/+}$ and wild type mice

(B) SPR analysis with immobilized human CD40 and soluble human/mouse CD40L titrated from 33 to 0.5nM.

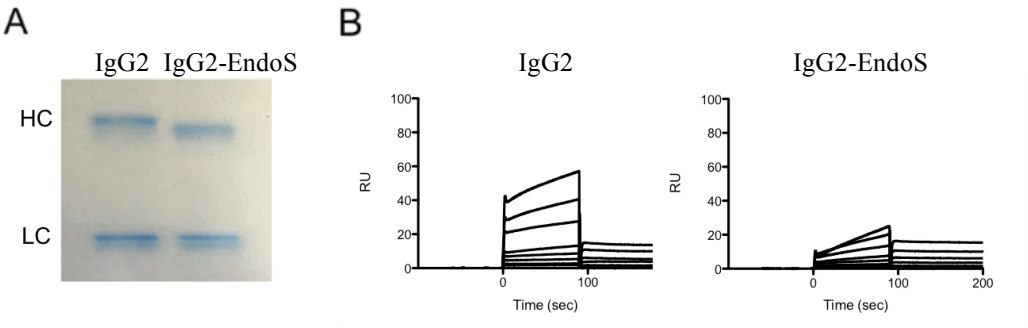


Figure S2, Related to Figures S. Characterization of deglycosylated CP-870,893-IgG2.

(A) SDS-PAGE characterization of IgG2 and IgG2 treated with EndoS. Reduced molecular weight size of the heavy chain of the EndoS-treated IgG2 compared to wild type implies on its deglycolisation. HC, heavy vhain; LC, light chain.

(B) SPR analysis of the binding of CP-870,893 IgG2 and deglycosylated-IgG2 to human FcγRIIB.

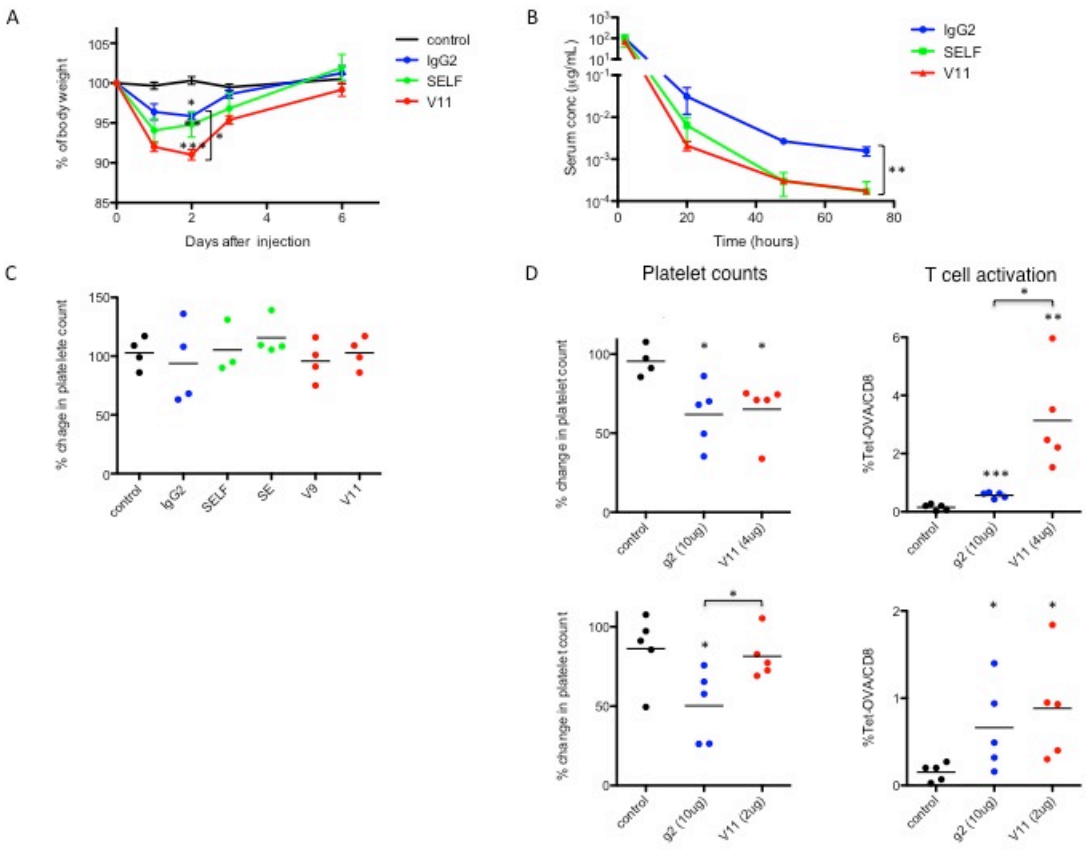


Figure S3, Related to Figures 3. Increased activity of CP-870,893 anti-CD40 human IgG by Fc-engineering for selective FcγRIIB enhancement.

A) Change in total body weight over time after single injection of CP-870,893 Fc variant into humanized FcγR/CD40 mice. Data represented as mean +/- SEM. n=4.

B) Serum concentrations over time after single injection of of CP-870,893 IgG2, IgG1-SELF, and IgG1-V11 Fc variants. Data are represented as mean ± SEM. n=3.

C) Platelet counts in humanized CD40/ FcγR mice blood 7 days after administration of CP-870,893 anti-CD40 Fc variants. Each dot represents individual mouse.

D) 2.5-fold (upper panel) and 5-fold (lower panel) reduced dose of CP-870,893-V11 compared to CP-870,893-IgG2 was injected to hCD40/FcγR mice combined with DEC-OVA. Relative platelet counts after 24 hours (left) and percentages of OVA^+ of CD8 T cell at day 7 (right) are shown. Each dot represents individual mouse.

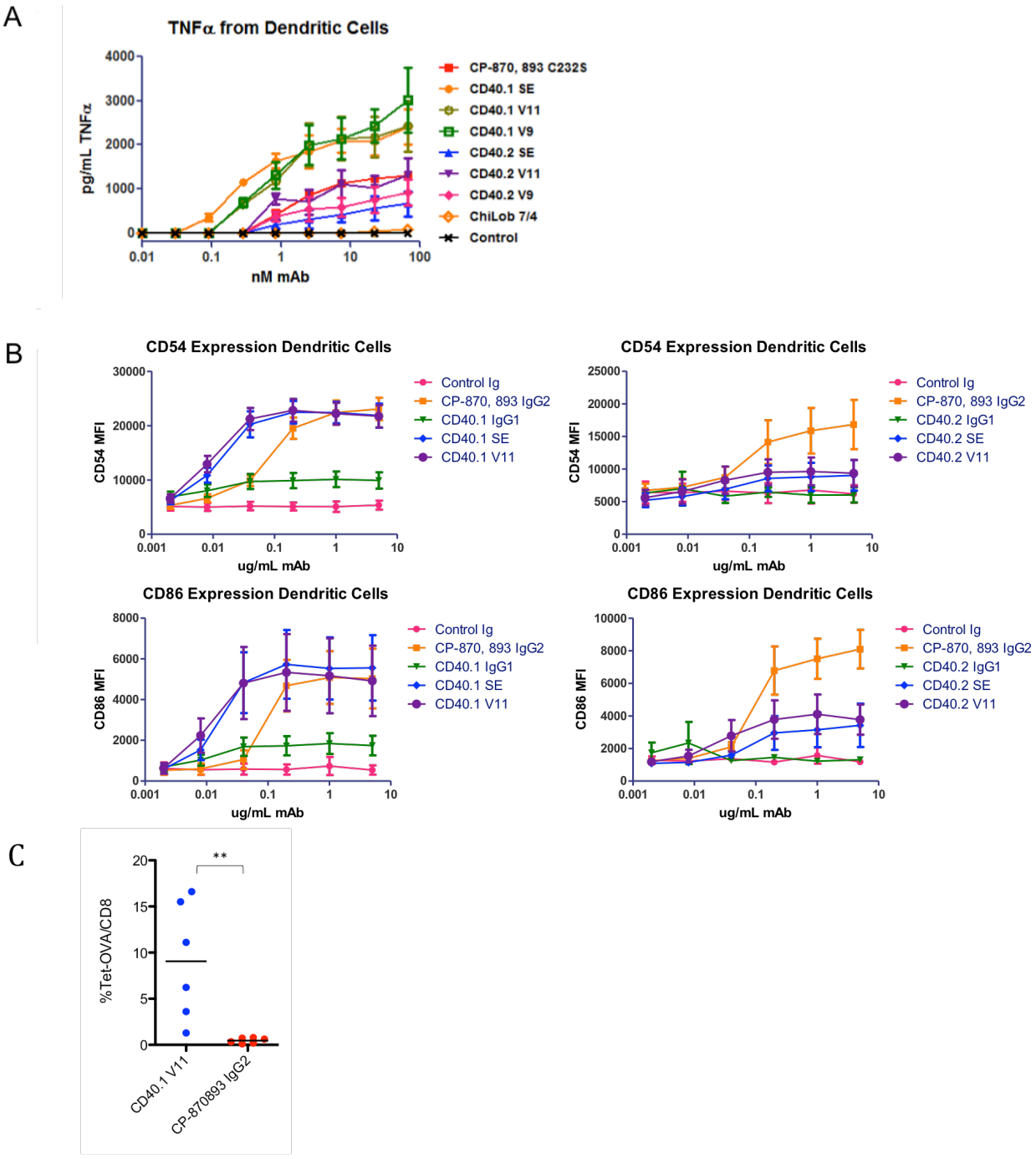


Figure S4, Related to Figure 4. Multiple agonistic, anti-CD40 antibodies demonstrate FcγRIIB-mediated enhancement, regardless of the epitopes they recognize.

(A) Immature dendritic cells were incubated in vitro with the indicated anti-human CD40 mAbs and were analyzed for secretion of TNF-α. Data from 2-3 donors were averaged and SEM is shown.

(B) Immature dendritic cells were incubated in vitro with the indicated anti-human CD40 mAbs and were analyzed in flow cytometrty for their upregulation of the activation markers CD54 and CD86. Data from 3 donors were averaged and SEM is shown.

(C) The indicated CD40 Abs were injected to hCD40/FcγR mice in together with DEC-OVA. Percentages of OVA^+ of CD8 T cell at day 7 are shown. Each dot represents individual

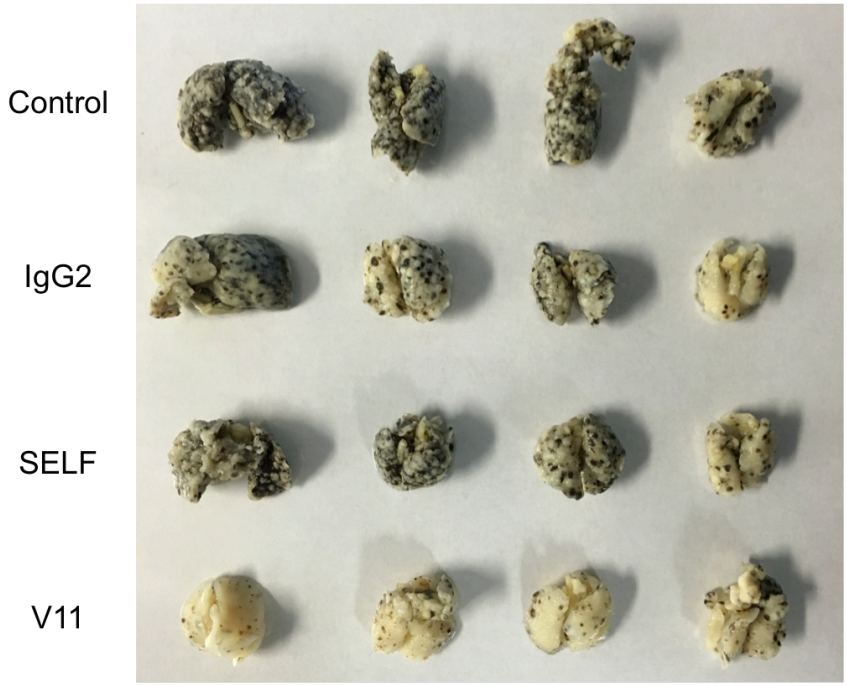


Figure S5, related to Figure 5. Superior anti-tumor activity of V11 Fc variant of CP-870,893.
Effect of CP-870,893 anti-CD40 Fc variants on the frequencies of lung metastases. Humanized CD40/FcγR mice were injected intravenously with B16 melanoma cells and treated with the indicated anti-CD40 Ab. Images are shoen for lung that were harvested at day 13 post injection.

Fc Variant	Ka (1/Ms)	Kd (1/s)	KD (M)
IgG1	$5.973 * 10^{4}$	$1.189 * 10^{-3}$	$1.991 * 10^{-8}$
IgG1-N297A	$5.234 * 10^{4}$	$1.161 * 10^{-3}$	$2.219 * 10^{-8}$
IgG1-S267E	$4.999 * 10^{4}$	$1.155 * 10^{-3}$	$2.309 * 10^{-8}$
IgG1-S267E/L328F	$4.232 * 10^{4}$	$1.159 * 10^{-3}$	$2.738 * 10^{-8}$
IgG1- G237D/P238D/P271G /A330R (V9)	$4.217 * 10^{4}$	$1.129 * 10^{-3}$	$2.678 * 10^{-8}$
IgG1- G237D/P238D/H268D /P271G/A330R (V11)	$4.1 * 10^{4}$	$1.125 * 10^{-3}$	$2.743 * 10^{-8}$
IgG2	$3.486 * 10^{4}$	$1.081 * 10^{-3}$	$3.101 * 10^{-8}$
IgG2- C232S (IgG2A)	$3.515 * 10^{4}$	$1.088 * 10^{-3}$	$3.095 * 10^{-8}$
IgG2- C127S (IgG2B)	$3.549 * 10^{4}$	$1.096 * 10^{-3}$	$3.088 * 10^{-8}$

Table S1. Affinities of 21.4.1 Fc variants to human CD40.
Binding constants were obtained by SPR analysis with immobilized IgGs and soluble CD40.

	Inhibitory		Activating					
Fc variant	hFcγRIIB		hFcγRI		hFcγRIIA^{R131}		hFcγRIIIA^{F158}	
	K_D (M)	Fold	K_D (M)	Fold	K_D (M)	Fold	K_D (M)	Fold
IgG1 wild type	3.01×10^{-6}	1	5.17×10^{-9}	1	1.16×10^{-6}	1	6.7×10^{-6}	1
N297A	n.d.b	NA	n.d.b	NA	n.d.b	NA	n.d.b	NA
SE	8.33×10^{-8}	30.2	2.6×10^{-9}	1.1	9.8×10^{-8}	15.8	n.d.b	NA
SELF	4.31×10^{-8}	69.8	3.68×10^{-9}	1.4	1.77×10^{-8}	65.5	n.d.b	NA
V9	9.39×10^{-8}	32	5.78×10^{-7}	0.009	4.11×10^{-6}	0.28	n.d.b	NA
V11	3.15×10^{-8}	95.6	2.3×10^{-7}	0.022	3.84×10^{-6}	0.3	n.d.b	NA
IgG2	$<10^{-5}$	NA	n.d.b	NA	$<10^{-5}$	NA	$<10^{-5}$	NA

Table S2. Affinities of 21.4.1 Fc variants to human FcγRs
Binding constants were obtained by SPR analysis.
Fold=K_D(IgG1)/K_D(Fc variant)
n.d.b, no detectable binding; NA, not applicable.
K_D values and fold changes compared to wild type IgG1 for the SE mutant are from references (smith et al and Chu et al 2008).

Supplemental Experimental Procedures

Up-regulation of CD54 or CD86 in human dendritic cells

Human Monocytes ($CD14^+$) were isolated from healthy normal donors using plastic adherence or human CD14-micro beads (Miltenyi Biotec). Monocytes were cultured with 100 ng/mL GM-CSF (Miltenyi Biotec) and 100 ng/mL IL-4. (Miltenyi Biotec). Half of the medium was removed and replenished on day 2 and day 5. Immature dendritic cells were harvested at day 6-7. For up-regulation of CD54 and CD86, immature dendritic cells were plated at 80,000 cells/well in 96 well plates. Antibodies as indicated were added to wells and incubated overnight at 37º C. Cells were harvested and stained with the indicated markers.

Cytokine production by human dendritic cells

Immature DCs were derived as described above. DC (100,000 cells/well) were incubated with the indicated concentration of antibodies overnight at 37º C. Cell culture supernatants were collected and assayed for human-TNF-α production (CisBio).

SPR method for CD40-CD40L binding

Construction, expression and purification of the human CD40 Fc fusion protein (hCD40-Fc), and trimeric IZ-hCD40L and IZ-mCD40L proteins was described previously (Xie et al., 2014). mCD40-Fc was purchased from R&D Systems Catalog # 1215-CD. Surface Plasmon resonance (SPR) studies were performed on a Biacore T100 instrument (GE Healthcare) at 25oC in a running buffer of 10 mM NaPO4, 130 mM NaCl, 0.05% p20 (PBS-T) pH 7.1. Sensor chip surfaces were prepared by immobilizing protein A to densities of ~5000 RU on a CM5 sensor chip (GE Healthcare) using standard ethyl(dimethylaminopropyl) carbodiimide (EDC)/N-hydroxysuccinimide (NHS) chemistry, with ethanolamine blocking. hCD40-Fc or mCD40-Fc fusion proteins were captured via the Fc tag on these surfaces to densities of ~200-220 RU, followed by the binding of IZ-hCD40L or IZ-mCD40L (100-0.14 nM in 3:1 dilution series) using 180 s association and 360 s dissociation times at a flow rate of 30 ul/min. Surface regeneration between cycles was achieved using two 30s pulses of 10mm glycine pH 1.5 at 30ul/min.

PK study

hCD40/hFcγR mice were injected with 80 μg of CP-870,893 Fc variant antibodies and bled at indicated time points after dosing. Serum was stored at -80C until all time points were collected.
Antibody concentration in serum was determined using a standard colorimetric ELISA assay. Briefly, assay plates were coated with 1 mg/ml of recombinant human CD40 (R&D Systems) and incubates over night at 4C. Plates were washed three times with PBS-0.05% Tween 20, then blocked with 10% FBS in PBS. Serial dilutions of serum was added to the plates and incubated for 2h at RT then washed 3 times. Anti-human IgG HRP conjugate (Jacskon ImmunoResearch Laboratories) was used to detect anti-human CD40 antibodies. HRP substrate was added to the plates for 2-5 min; absorbance was measured at 405 nm.

Supplemental References

Xie, J. H., Yamniuk, A. P., Borowski, V., Kuhn, R., Susulic, V., Rex-Rabe, S., Yang, X., Zhou, X., Zhang, Y., Gillooly, K., *et al.* (2014). Engineering of a novel anti-CD40L domain antibody for treatment of autoimmune diseases. Journal of immunology *192*, 4083-4092.