Toxicity of an Fc engineered anti-CD40 antibody is abrogated by intratumoral injection and results in durable anti-tumor immunity

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Supporting Information

Materials and Methods

Antibodies

Human antibodies were generated as previously described[1]. The IgG2 and N297A variants of 2141 were made by site-directed mutagenesis using specific primers using the QuikChange site-directed mutagenesis Kit II (Agilent Technologies) according to the manufacturer's instructions. 2141-V11 contains the variable heavy and light regions of CP-870,893, with the Fc-domain of human IgG1 containing 5 point mutations (G237D/P238D/H268D/P271G/A330R). Plasmid sequences were validated by direct sequencing (Genewiz). PD-1 blocking antibody (RPM1-14 N297A) was generated and used as previously described[2]. All antibodies were generated by transfection of Expi293T cells (ATCC), purified using Protein G Sepharose 4 Fast Flow (GE Healthcare), dialyzed in PBS, and sterile filtered (0.22 mm), as previously described[1]. Purity was assessed by SDS-PAGE and Coomassie staining, and was estimated to be >90%. Antibodies used for in vivo experiments were quantified for endotoxin (lipopolysaccharide) contamination by the Limulus amebocyte lysate assay and verified to have levels <0.1 EU/mg.

Flow Cytometry

Peripheral blood, spleen, and LN tissues were collected as previously described[2]. For intratumoral analysis, tumors were surgically resected and processed using a Tumor Dissociation Kit (Miltenyi Biotec). Cells were first stained with LiveDead Aqua (Invitrogen) prior to surface staining. Surface antigens were stained using the following clones: CD45 (30-F11), CD3 (17A2), CD4 (RM4-4), CD8 (53-6.7), NK1.1 (PK13), MHC-II (AF6-120.1), F4/80 (BM8), CD11b (M1/70), Ly6C (HK1.4). Human Fc receptors were stained using the following clones: CD64 BV421 (Clone 10.1, Biolegend), CD32A FITC (clone IV.3, Stem Cell Technologies), CD32B AF647 (clone 2B6 labeled using AF647 labeling kit from Invitrogen), CD16A/B APC-Cy7 (clone 3G8, Biolegend), and CD16b PE (clone REA589 from Miltenyi Biotec). Ova-specific CD8 T cells were stained for with PE-conjugated anti-CD4, APC-conjugated anti- CD8a, and PE-conjugated OVA peptide SIINFEKL H-2^b tetramer (Tet-OVA, Beckman Coulter). All samples were analyzed on an LSR Fortessa (BD Biosciences).

Depletion studies

Anti-mouse CD8a (clone 2.43), anti-mouse CD4 (clone GK1.5), anti-mouse NK cell (clone PK13), and anti-CSF-1R (clone AFS98) antibodies were purchased from BioXCell. Clodronate liposomes were purchased from ClondronateLiposomes.org. Anti-CD4 and anti-CD8 mAbs were injected 2 days and 1 day before therapy, on the day therapy was begun, and at 5, 8, and 19 days after the beginning of therapy at a dose of 0.5 mg per injection. For macrophage depletion using clodronate, liposomes were injected intravenously through lateral tail veins on day -2 and then repeated every 4 days. For macrophage depletion using CSF-1R antibodies, mice received injections of 300 ug every other day. The depletion conditions were validated by flow cytometry of blood showing specific depletion of more than 95% of each respective cell subset.

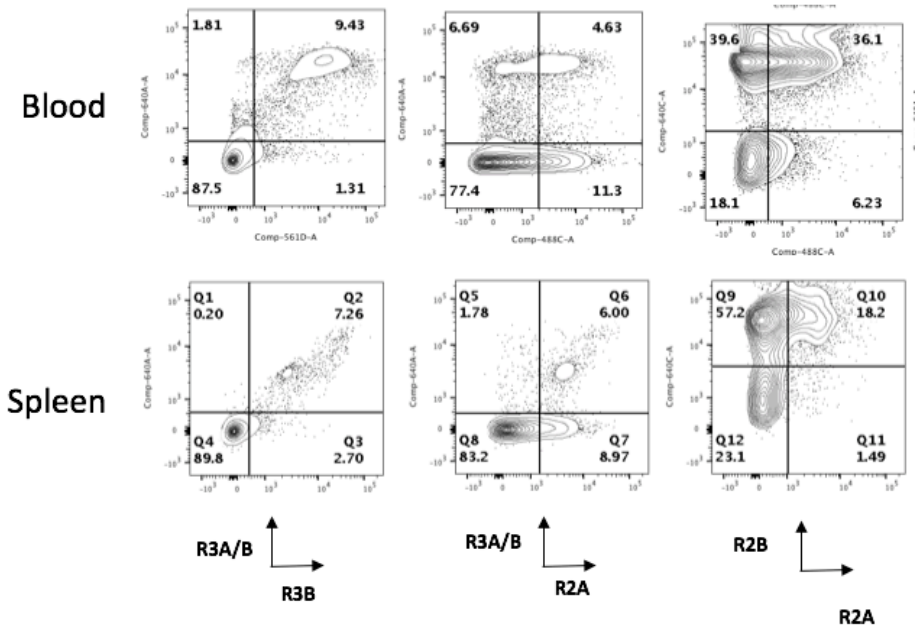


Fig. S1. Human Fc receptor expression in blood and spleen of humanized mice.
Humanized CD40/FcR mice were implanted with MC38 tumor cells and allowed to engraft for 2 weeks. Peripheral blood and spleen were then collected for analysis of hematopoietic cells expressing different human Fc receptors. Cells were gated to exclude doublets followed by gating on live murine hematopoietic cells (Live/Dead Aqua negative/mCD45+). Fc receptors were then evaluated on all infiltrating leukocytes.

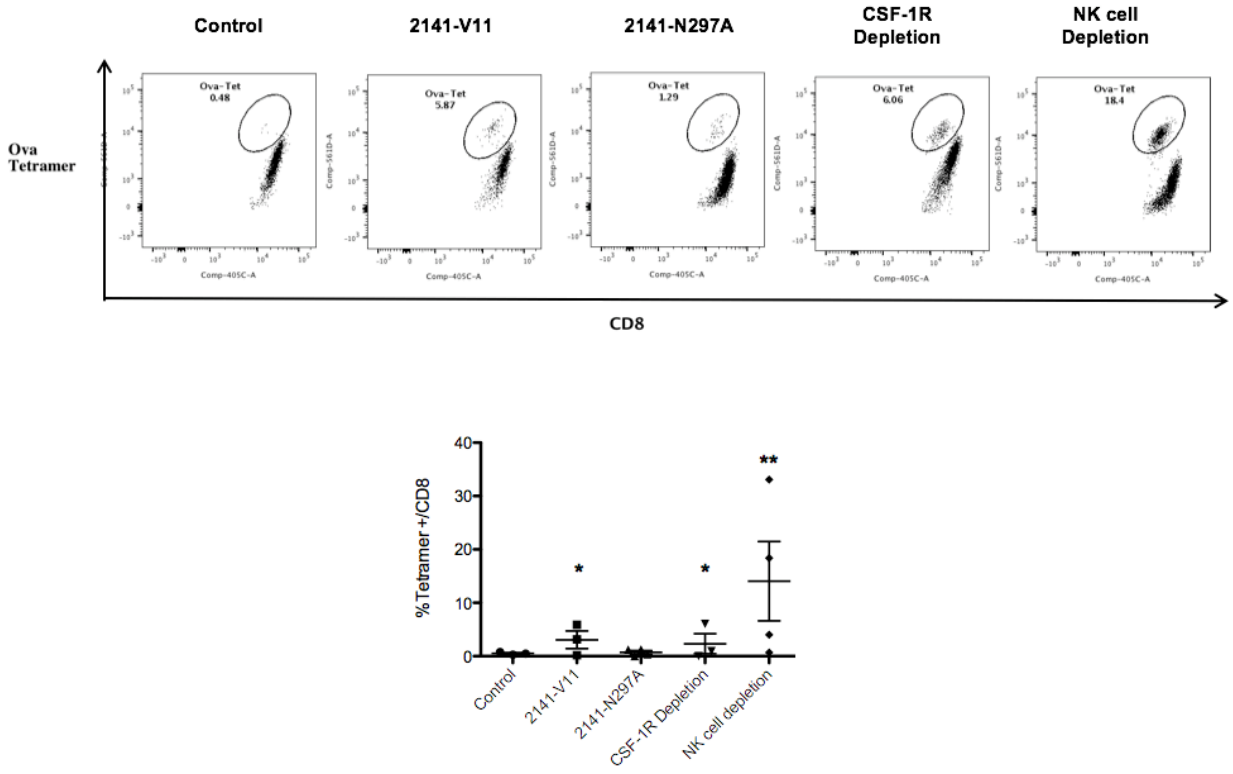


Fig. S2. Expansion of antigen-specific CD8 T cells in treated mice. Mice were evaluated for the expansion of ovalbumin-specific T cells by flow cytometry. In response to treatment with 2141-V11, mice depleted of macrophages (using anti-CSF-1R antibody) or NK cells (using anti-NK cell antibody) showed significant expansion of tetramer positive cells compared to control or 2141-N297A treated mice, p value *<0.05 or **<0.01. 2141-V11 therapy significantly expands ova-specific CD8 T cells in the periphery of mice and is not affected by macrophage depletion. Ova-specific CD8 T cells are absent in mice receiving antibodies depleting CD8 T cells. Mouse peripheral blood T cells were gated on live CD3 T cells expressing CD4 or CD8. CD8 T cells were then gated for evaluation of ovalbumin-tetramer^{+} cells.

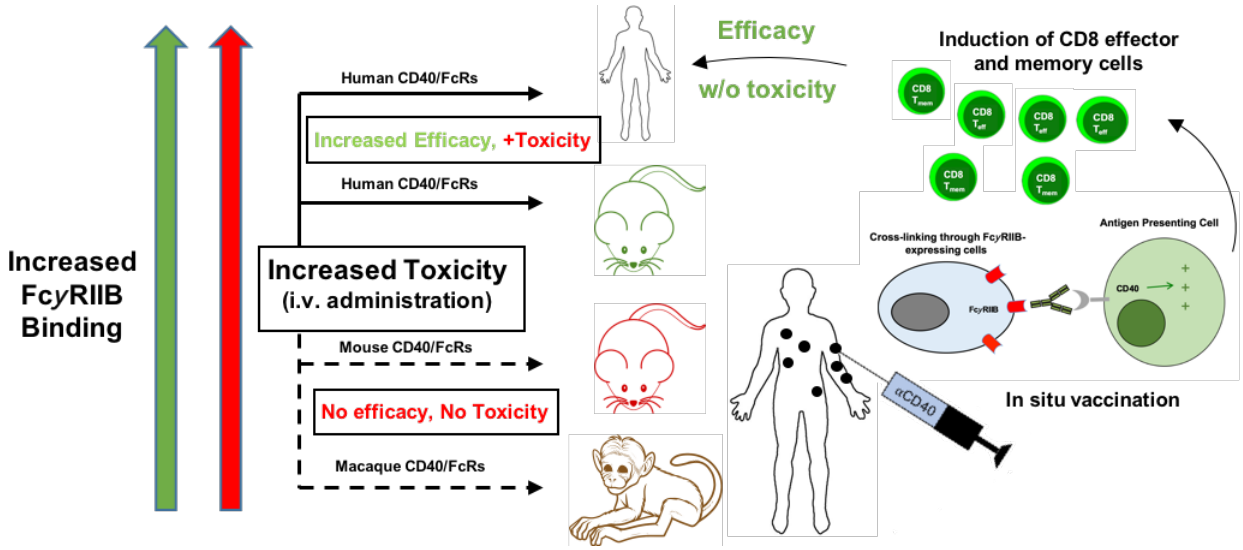
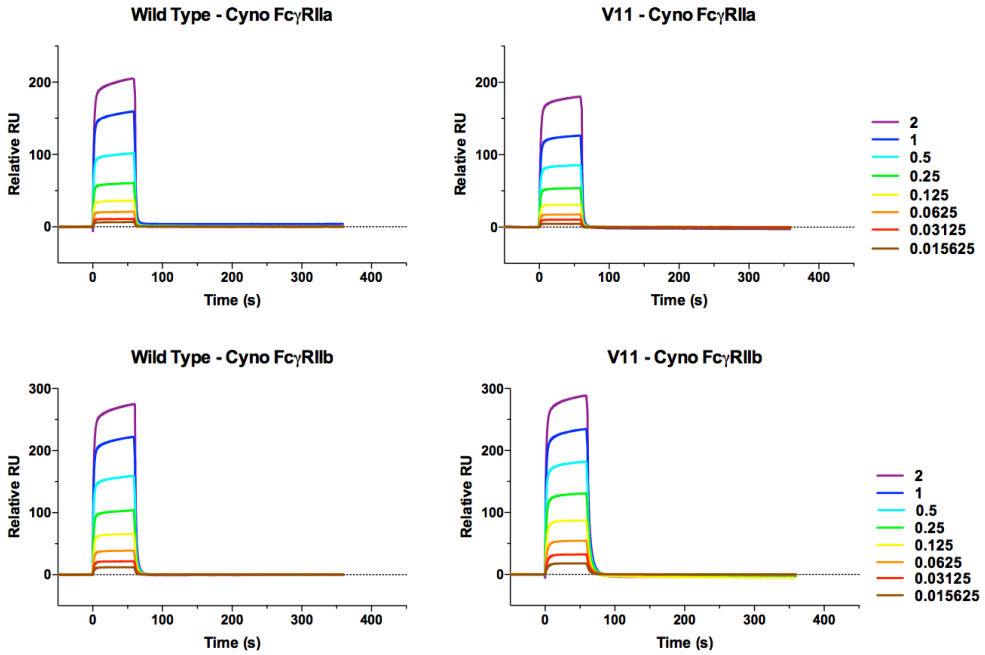
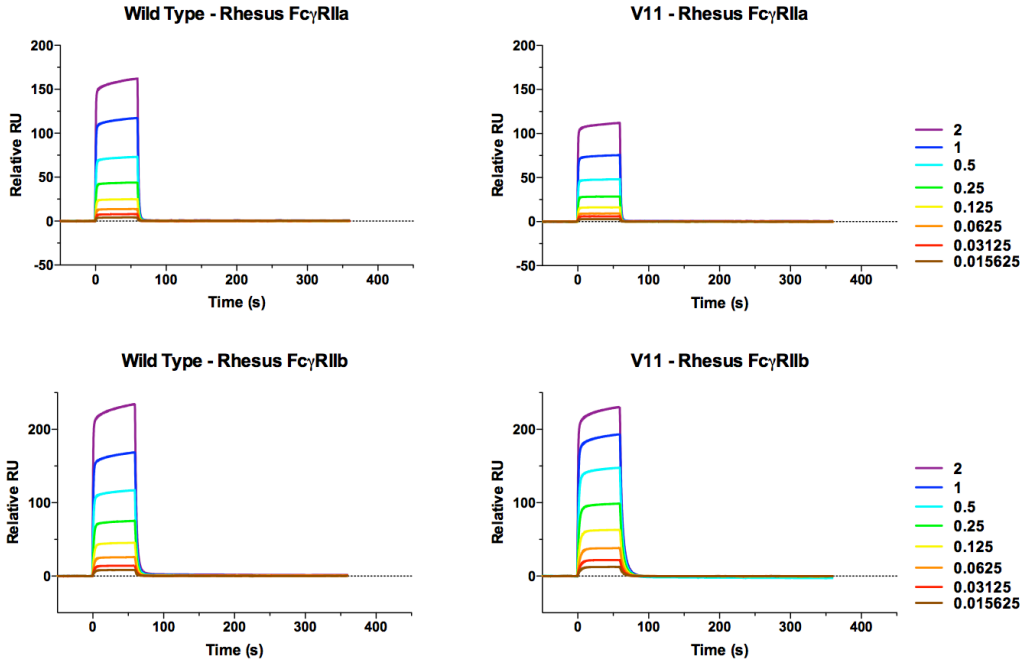


Fig. S3. Schematic model of the proposed mechanism by which 2141-V11 promotes systemic anti-tumor activity while limiting toxicity. By increasing the binding of anti-CD40 agonists for FcγRIIB there is improved activity which is accompanied by enhanced toxicity. However, this is only recapitulated in hosts expressing both human CD40 and human FcRs. Using an *in situ* vaccination approach, 2141-V11 leads to expansion of antigen-specific CD8 T cells capable of clearing both local and systemic disease, and protects from tumor rechallenge. Thus, this provides therapeutic rationale for testing agonistic antibodies in patients in order to avert dose-limiting toxicities.

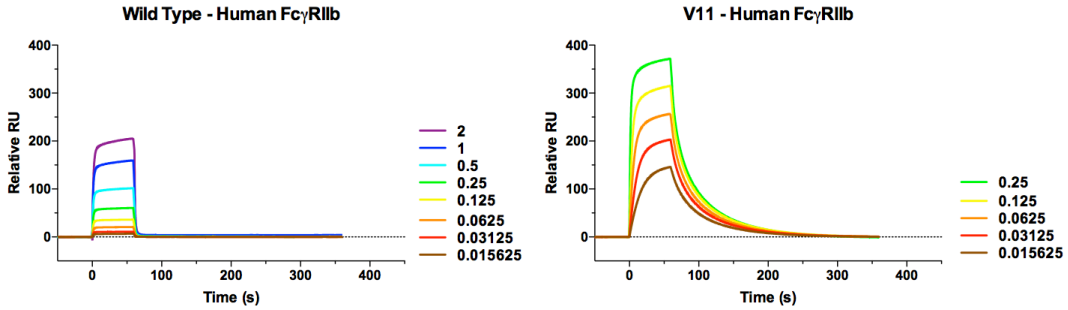
A


Wild Type - Cyno FcγRIIa
V11 - Cyno FcγRIIa
Wild Type - Cyno FcγRIIb
V11 - Cyno FcγRIIb
Relative RU
Time (s)
2
1
0.5
0.25
0.125
0.0625
0.03125
0.015625

B


Wild Type - Rhesus FcγRIIa
V11 - Rhesus FcγRIIa
Wild Type - Rhesus FcγRIIb
V11 - Rhesus FcγRIIb
Relative RU
Time (s)
2
1
0.5
0.25
0.125
0.0625
0.03125
0.015625

C



D

	WT	V11	Fold
Cyno RIIa	6.43E-06	6.89E-06	0.93
Cyno RIIb	4.81E-06	4.69E-06	1.03
Rhesus RIIa	9.65E-06	1.73E-05	0.56
Rhesus RIIb	6.20E-06	4.03E-06	1.54
Human RIIb	3.31E-06	5.92E-08	55.91

Fig. S4. Binding affinities of human wild type IgG1 (WT) and 2141-V11 (V11-Fc) variant to human and macaques FcγRs. Binding affinities were measured by Surface Plasmon Resonance (SPR). Binding of both IgG1 and 2141-V11 for A) cynomolgus or B) rhesus macaque FcγRIIA and FcγRIIB are shown and compared to the binding affinity of 2141-V11 for human FcγRIIB. D) Summary table of the various binding affinities of each antibody for FcγRIIB and fold enhancement for the V11 variant.

References

1 Bournazos, S. *et al.* Bispecific Anti-HIV-1 Antibodies with Enhanced Breadth and Potency. *Cell* **165**, 1609-1620, doi:10.1016/j.cell.2016.04.050 (2016).

2 Dahan, R. *et al.* Therapeutic Activity of Agonistic, Human Anti-CD40 Monoclonal Antibodies Requires Selective FcγR Engagement. *Cancer Cell* **0**, 3755-3766, doi:10.1016/j.ccell.2016.05.001 (2016).