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Supplemental Information

Route of 41BB/41BBL Costimulation Determines Effector Function of B7-H3-CAR.CD28ζ T Cells

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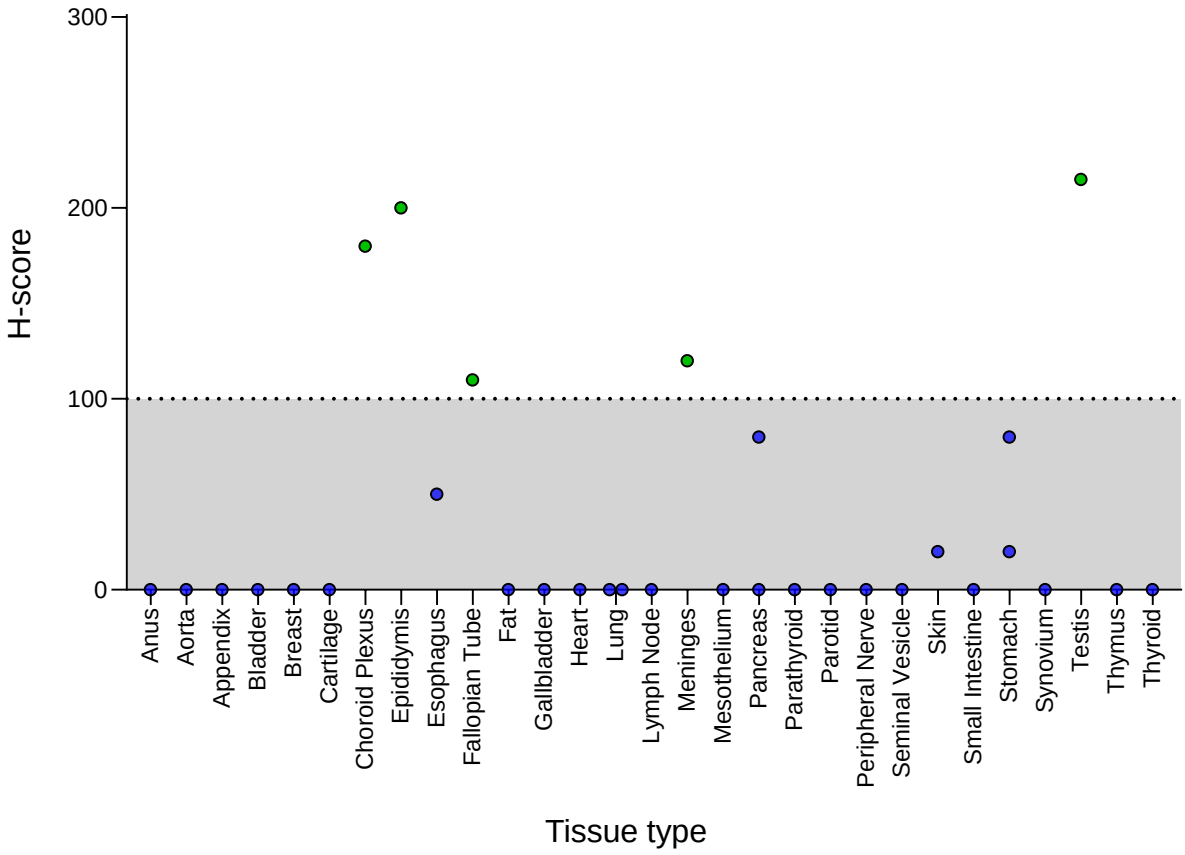

Supplementary Figure 1. Normal tissue staining by IHC. An adult normal tissue microarray was stained for B7-H3 by IHC. H-scores for normal tissue specimens with less than 3 samples available are depicted.

Supplementary Figure 1

RED: Leader Sequence
BLUE: scFv MGA271 vH
GREY: scFv linker
BLUE: scFv MGA271 vL
ORANGE: Hinge/Transmembrane
PINK: Costimulatory Domain
BLACK: CD3ζ

CD8α/CD28

MDWIWRILFLVGAATGAHSEVQLVESGGGLVQPGGSLRLSCAASGFTFSSFGMHWVRQAPGKGLEWVAYISSDSSAIY
YADTVKGRFTISRDNAKNSLYLQMNSLRDEDTAVYYCGRGRENIYYGSRLDYWGQGTTVTVSSGGGGSGGGGSGGGGS
DIQLTQSPSFLSASVGDRVTITCKASQNVDTNVAWYQQKPGKAPKALIYSASYRYSGVPSRFSGSGSGTDFTLTISSL
QPEDFATYYCQQYNNYPFTFGQGTKLEIKTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIW
APLAGTCGVLLLSLVITLYCRSKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRSRVKFSRSADAPAYQQGQ
NQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLST
ATKDTYDALHMQALPPR

CD8α/41BB

MDWIWRILFLVGAATGAHSEVQLVESGGGLVQPGGSLRLSCAASGFTFSSFGMHWVRQAPGKGLEWVAYISSDSSAIY
YADTVKGRFTISRDNAKNSLYLQMNSLRDEDTAVYYCGRGRENIYYGSRLDYWGQGTTVTVSSGGGGSGGGGSGGGGS
DIQLTQSPSFLSASVGDRVTITCKASQNVDTNVAWYQQKPGKAPKALIYSASYRYSGVPSRFSGSGSGTDFTLTISSL
QPEDFATYYCQQYNNYPFTFGQGTKLEIKTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIW
APLAGTCGVLLLSLVITLYCKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSADAPAYQQG
QNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLS
TATKDTYDALHMQALPPR

CD28/CD28

MDWIWRILFLVGAATGAHSEVQLVESGGGLVQPGGSLRLSCAASGFTFSSFGMHWVRQAPGKGLEWVAYISSDSSAIY
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DIQLTQSPSFLSASVGDRVTITCKASQNVDTNVAWYQQKPGKAPKALIYSASYRYSGVPSRFSGSGSGTDFTLTISSL
QPEDFATYYCQQYNNYPFTFGQGTKLEIKIEVMYPPPYLDNEKSNGTIIHVKGKHLCPSPLFPGPSKPFWVLVVVGGV
LACYSLLVTVAFIIFWVRSKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRSRVKFSRSADAPAYQQGQNQL
YNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATK
DTYDALHMQALPPR

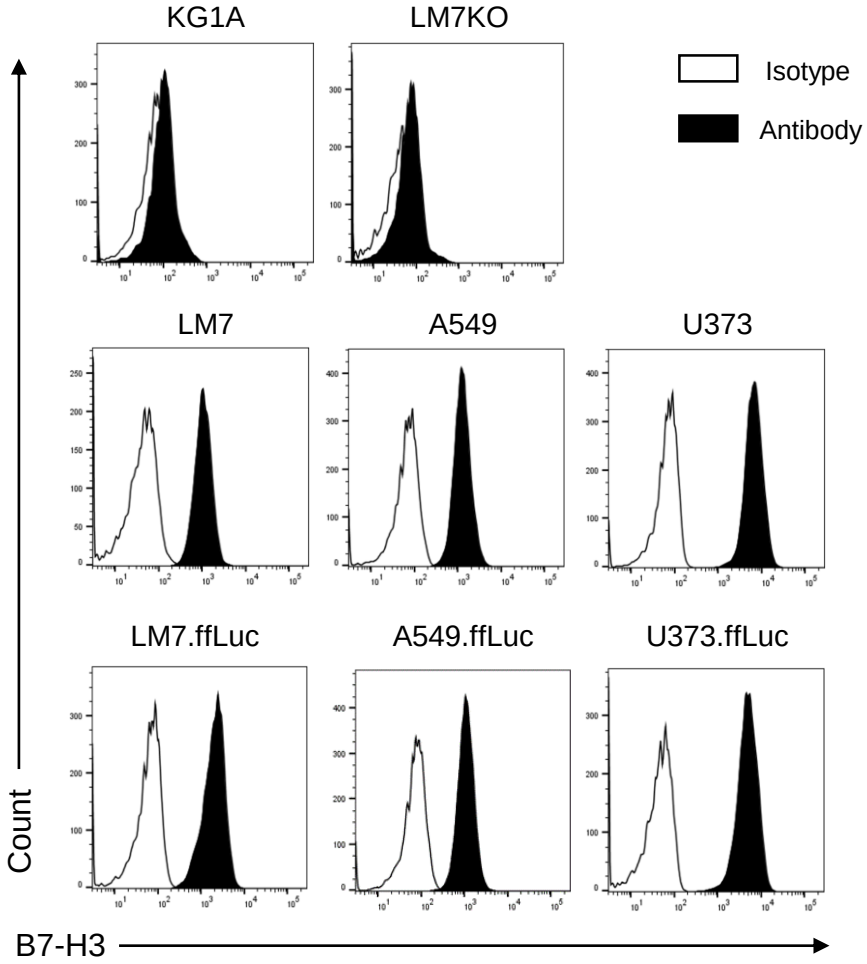
CD28/41BB

MDWIWRILFLVGAATGAHSEVQLVESGGGLVQPGGSLRLSCAASGFTFSSFGMHWVRQAPGKGLEWVAYISSDSSAIY
YADTVKGRFTISRDNAKNSLYLQMNSLRDEDTAVYYCGRGRENIYYGSRLDYWGQGTTVTVSSGGGGSGGGGSGGGGS
DIQLTQSPSFLSASVGDRVTITCKASQNVDTNVAWYQQKPGKAPKALIYSASYRYSGVPSRFSGSGSGTDFTLTISSL
QPEDFATYYCQQYNNYPFTFGQGTKLEIKIEVMYPPPYLDNEKSNGTIIHVKGKHLCPSPLFPGPSKPFWVLVVVGGV
LACYSLLVTVAFIIFWVKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSADAPAYQQGQNQ
LYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTAT
KDTYDALHMQALPPR

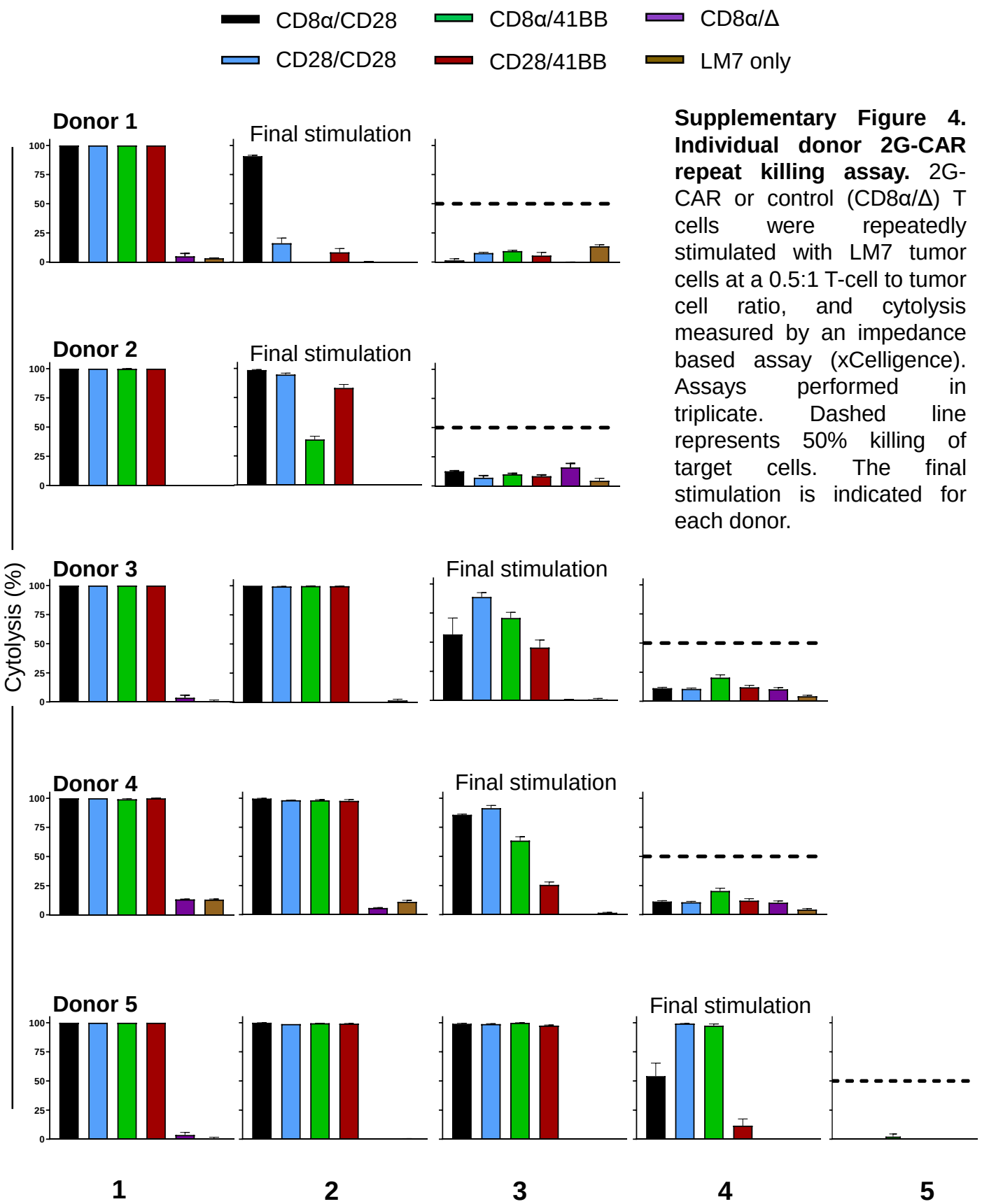
CD8α/Δ

MDWIWRILFLVGAATGAHSEVQLVESGGGLVQPGGSLRLSCAASGFTFSSFGMHWVRQAPGKGLEWVAYISSDSSAIY
YADTVKGRFTISRDNAKNSLYLQMNSLRDEDTAVYYCGRGRENIYYGSRLDYWGQGTTVTVSSGGGGSGGGGSGGGGS
DIQLTQSPSFLSASVGDRVTITCKASQNVDTNVAWYQQKPGKAPKALIYSASYRYSGVPSRFSGSGSGTDFTLTISSL
QPEDFATYYCQQYNNYPFTFGQGTKLEIKTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIW
APLAGTCGVLLLSLVITLYCKRGR

Supplementary Figure 2. Second generation B7-H3-CAR amino acid sequences.



Supplementary Figure 3. Flow cytometry for B7-H3 expression on tumor cell lines. A known B7-H3-negative cell line KG1A (acute myeloid leukemia) served as a negative control. B7-H3 antibody was used to determine B7-H3 expression on LM7KO, LM7, A549, U373 and ffLuc-expressing cell lines.



Supplementary Figure 4. Individual donor 2G-CAR repeat killing assay. 2G-CAR or control (CD8α/Δ) T cells were repeatedly stimulated with LM7 tumor cells at a 0.5:1 T-cell to tumor cell ratio, and cytolysis measured by an impedance based assay (xCelligence). Assays performed in triplicate. Dashed line represents 50% killing of target cells. The final stimulation is indicated for each donor.

Supplementary Figure 4

RED: Leader Sequence
BLUE: scFv MGA271 vH
GREY: scFv linker
BLUE: scFv MGA271 vL
ORANGE: CD8a Hinge/Transmembrane
PINK: CD28 Costimulatory Domain
PINK: 41BB Costimulatory Domain
BLACK: CD3ζ
GREEN: 41BB ligand
BROWN: GSG.P2A

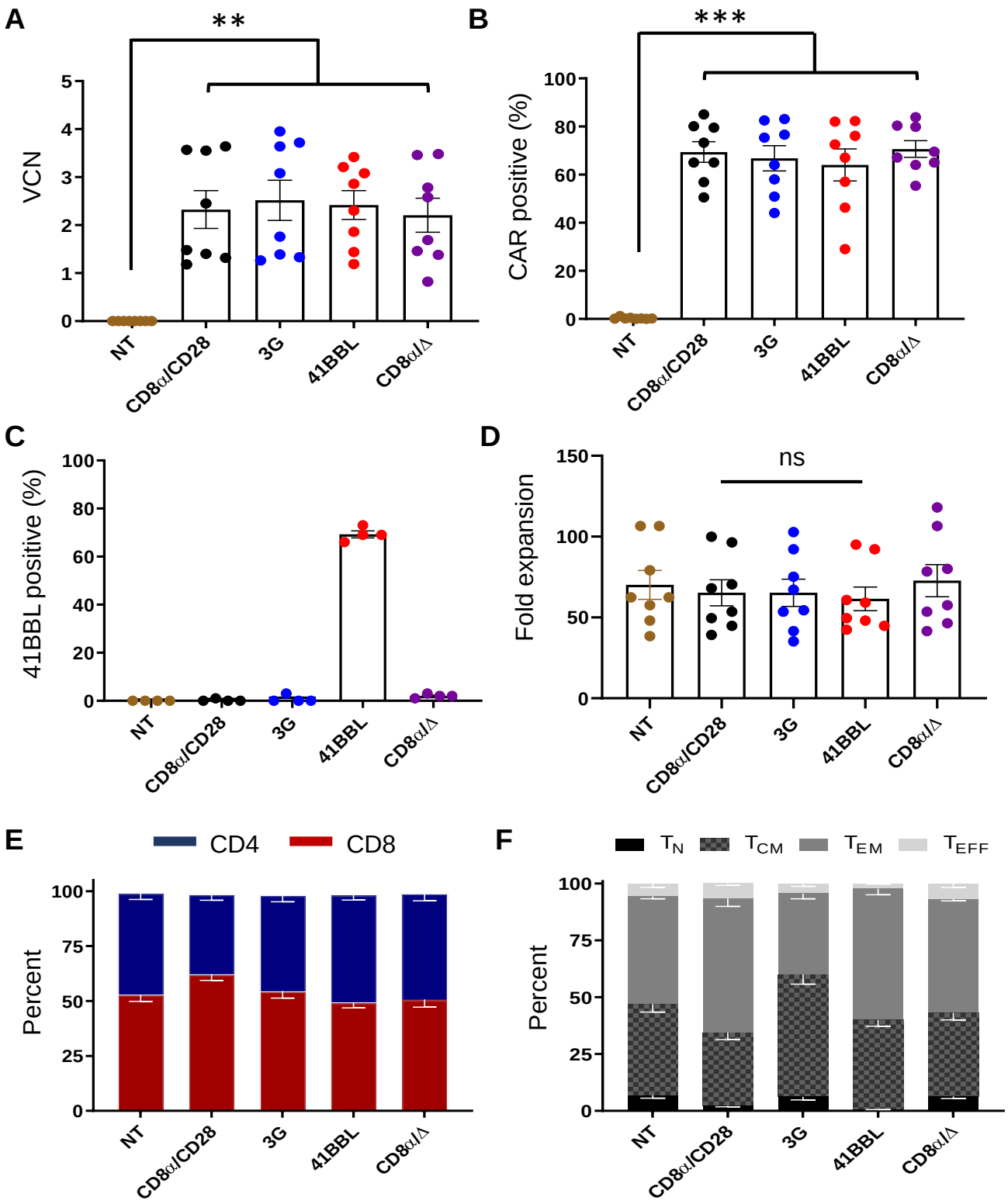
3G (CD8α/CD28.41BB)

MDWIWRILFLVGAATGAHSEVQLVESGGGLVQPGGSLRLSCAASGFTFSSFGMHWVRQAPGKGLEWVAYISSDSSAIY
YADTVKGRFTISRDNAKNSLYLQMNSLRDEDTAVYYCGRGRENIYYGSRLDYWGQGTTVTVSSGGGGSGGGGSGGGGS
DIQLTQSPSFLSASVGDRVTITCKASQNVDTNVAWYQQKPGKAPKALIYSASYRYSGVPSRFSGSGSGTDFTLTISSL
QPEDFATYYCQQYNNYPFTFGQGTKLEIKTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIW
APLAGTCGVLLLSLVITLYCRSKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRSKRGRKKLLYIFKQPFMR
PVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKN
PQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR

41BBL (41BBL.2A.CD8α/CD28)

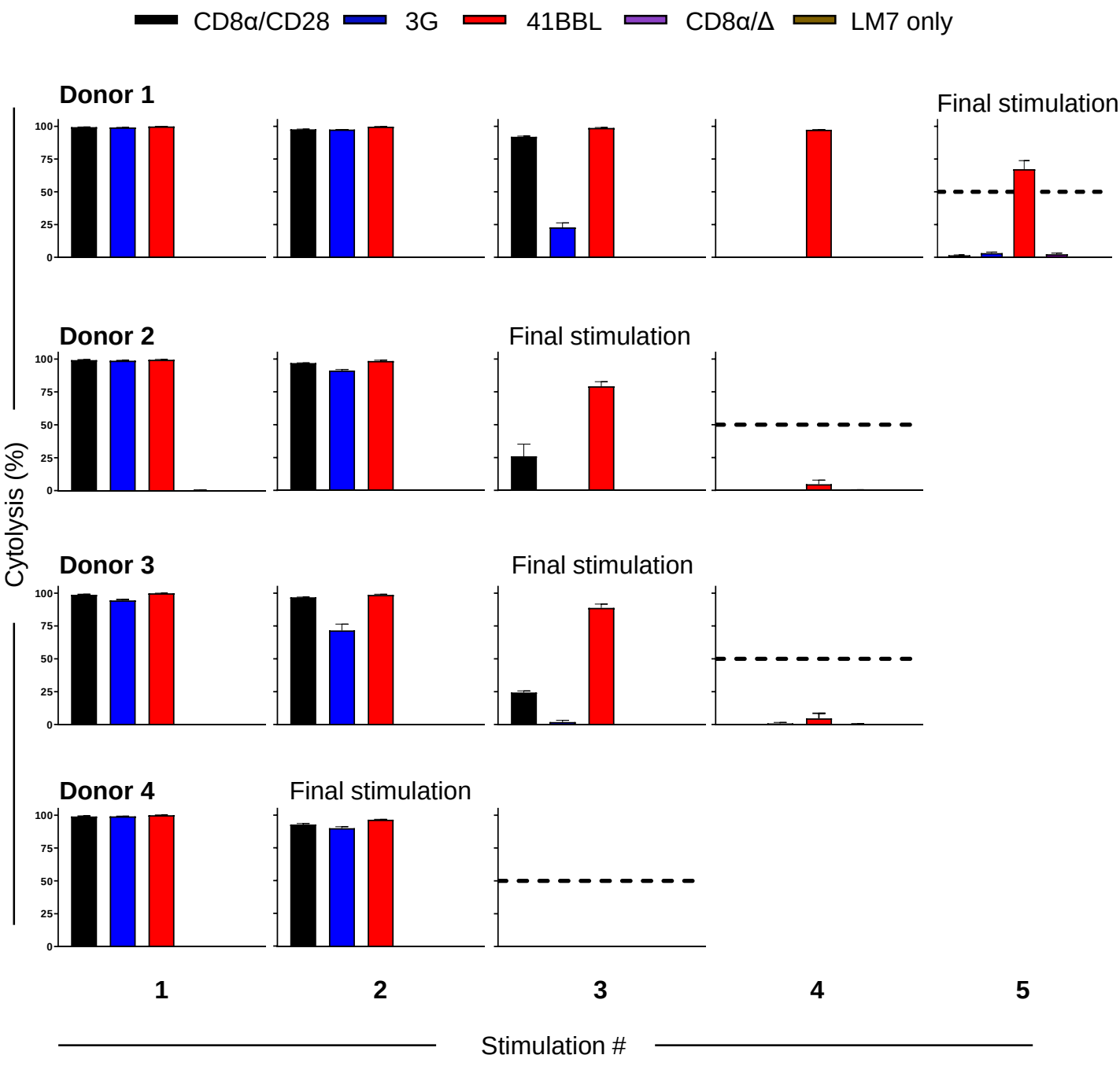
MEYASDASLDPEAPWPPAPRARACRVLPWALVAGLLLLLLLAAACAVFLACPWAVSGARASPGSAASPRLREGPELSP
DDPAGLLDLRQGMFAQLVAQNVLLIDGPLSWYSDPGLAGVSLTGGLSYKEDTKELVVAKAGVYYVFFQLELRRVVAGE
GSGSVSLALHLQPLRSAAGAAALALTVDLPPASSEARNSAFGFQGRLLHLSAGQRLGVHLHTEARARHAWQLTQGATV
LGLFRVTPEIPAGLPSPRSEGSGATNFSLLKQAGDVEENPGPMDWIWRILFLVGAATGAHSEVQLVESGGGLVQPGGS
LRLSCAASGFTFSSFGMHWVRQAPGKGLEWVAYISSDSSAIYYADTVKGRFTISRDNAKNSLYLQMNSLRDEDTAVYY
CGRGRENIYYGSRLDYWGQGTTVTVSSGGGGSGGGGSGGGGSDIQLTQSPSFLSASVGDRVTITCKASQNVDTNVAWY
QQKPGKAPKALIYSASYRYSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYNNYPFTFGQGTKLEIKTTTPAPR
PPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCRSKRSRLLHSDYMNMT
PRRPGPTRKHYQPYAPPRDFAAYRSRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKN
PQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR

Supplementary Figure 5. 3G and 41BBL B7-H3-CAR amino acid sequences.



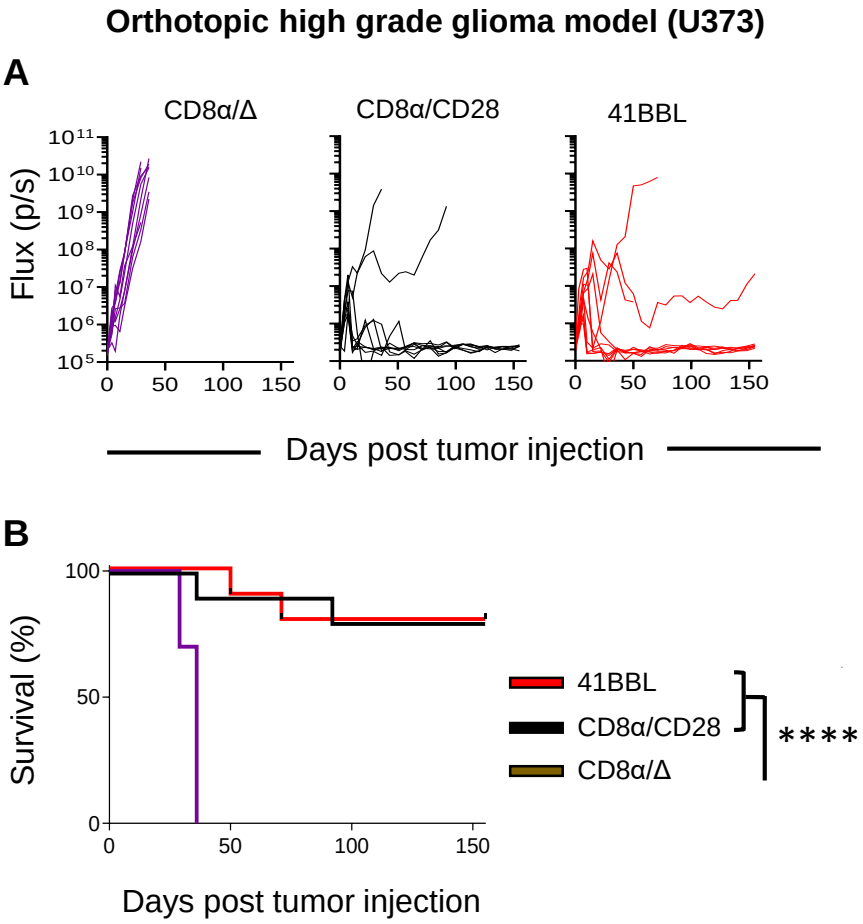
Supplementary Figure 6. CD8α/CD28-, 3G- and 41BBL-CAR transduction, expansion, and phenotype. Transduction was determined by (**A-B**) VCN and CAR surface expression (N=8, one-way ANOVA), and (**C**) 41BBL expression (N=4). (**D**) T cells were grown in media plus IL-7 and IL-15, and fold expansion determined on day 9/10 post-transduction (N=8, one-way ANOVA). (**E**) CD4/CD8 expression and (**F**) memory phenotype of CAR-positive T cells. Data, mean ± SEM; ** p<0.01, *** p<0.001, ns = non-significant.

Supplementary Figure 6



Supplementary Figure 7. Individual donor CD8α/CD28-, 3G- and 41BBL-CAR repeat killing assay. CAR or control (CD8α/Δ) T cells were repeatedly stimulated with LM7 tumor cells at a 0.5:1 T-cell to tumor cell ratio, and cytolysis measured by an impedance based assay (xCelligence). Assays performed in triplicate. The dashed line represents 50% killing of target cells. The final stimulation is indicated for each donor.

Supplementary Figure 7



Supplementary Figure 8: CD8α/CD28- and 41BBL-CAR T cells have robust anti-glioma activity *in vivo*. NSG mice received intracranial (i.c.) injection of 5x10^4 U373.ffLuc cells on day 0, followed by 2x10^6 CAR or control (CD8α/Δ) T cells i.c. on day 7. (**A**) Bioluminescent signal (flux = photons/second). (**B**) Kaplan-Meier survival curve for injected mice. Data, log-rank (Mantel-Cox) test; N=10 mice per group; **** p<0.0001.