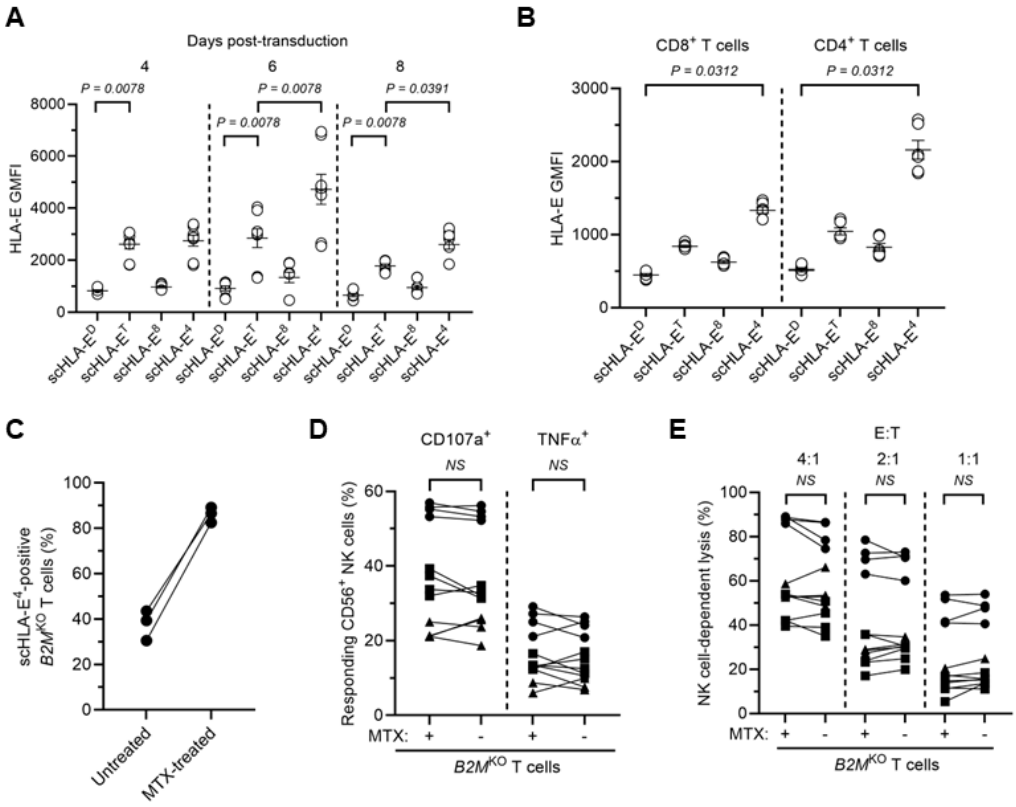
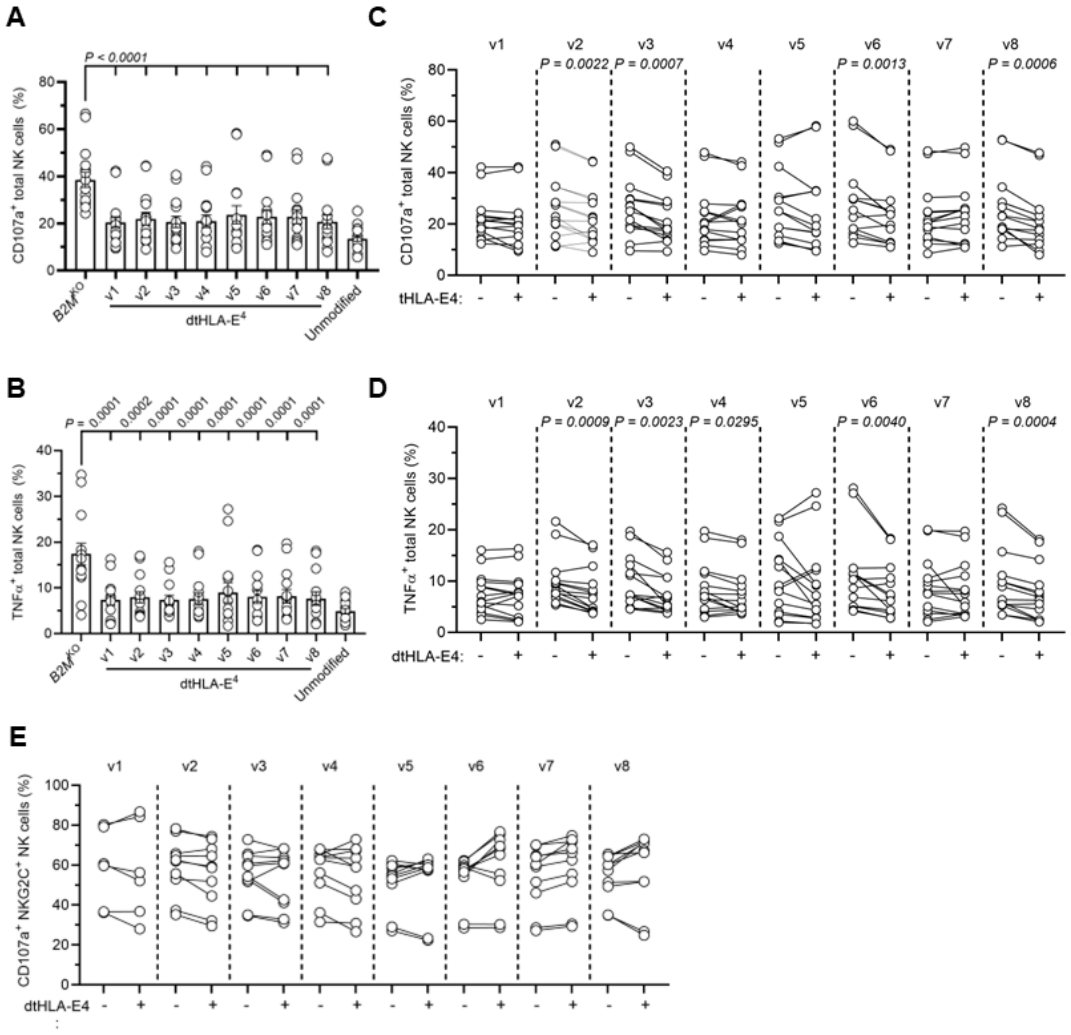
Supplemental Fig. 1



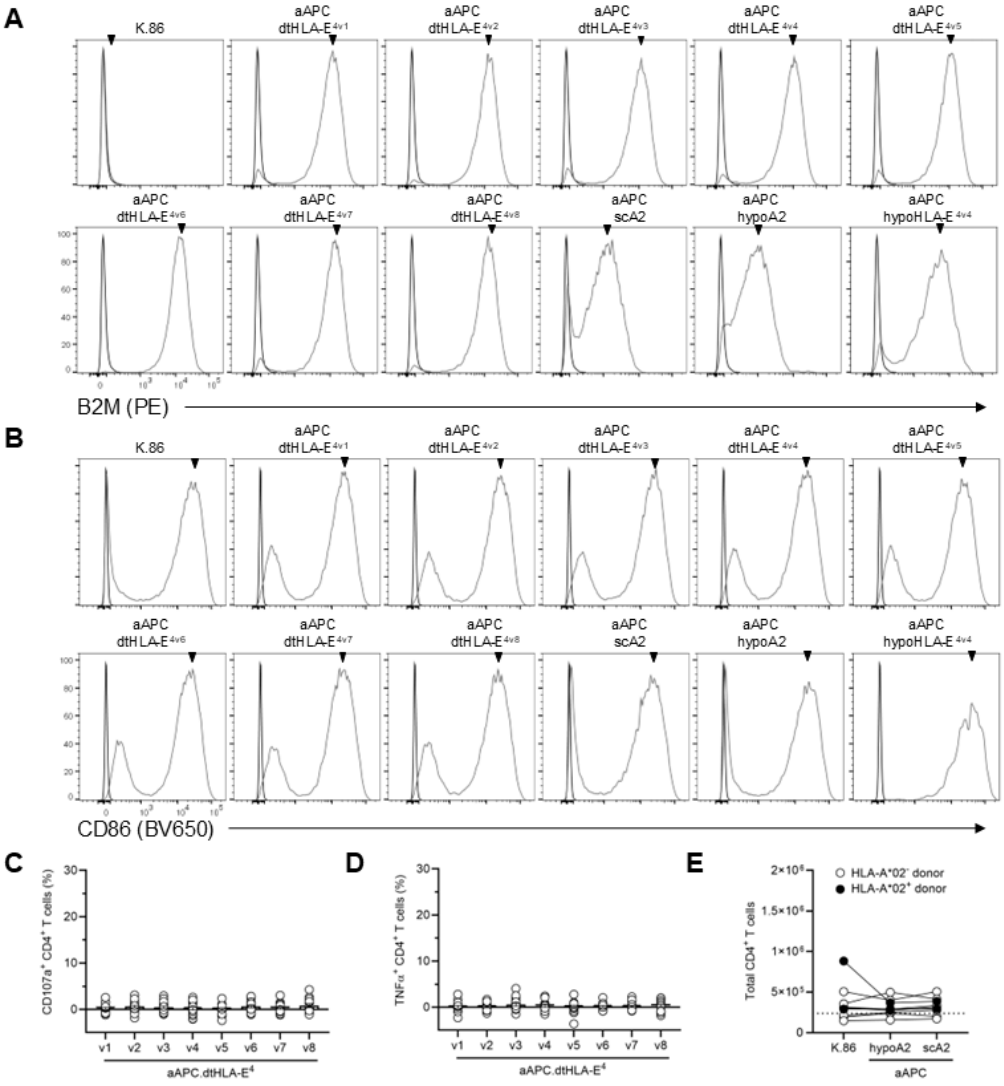
CD4 transmembrane domain and cytoplasmic regions enhance surface expression of scHLA-E^4. *B2M*^{KO} T cells were transduced with an equivalent titer of lentiviral vector encoding a single-chain (sc) HLA-E dimer (D), scHLA-E trimer (T), or scHLA-E constructs comprising the transmembrane domain and truncated cytoplasmic regions of CD8α (8) or CD4 (4) coreceptors. **A** Geometric median fluorescence intensity (GMFI) of HLA-E on HLA-E positive *B2M*^{KO} T cells expressing a scHLA-E variant at 4-, 6-, and 8-days post-transduction. Symbols represent 4 independent T cell donors in duplicate. **B** GMFI of HLA-E on HLA-E positive *B2M*^{KO} CD8$^+$ and CD4$^+$ T cells expressing a scHLA-E variant at 11-days post-transduction. Symbols represent 3 independent donors in duplicate. For **A** and **B**, bars and lines represent mean, and error bars show ±SEM. Non-transduced *B2M*^{KO} T cells served as a gating control to discriminate positive and negative HLA-E expression. Wilcoxon matched pairs-signed rank test was used to calculate significance. *NS*, not significant ($P > 0.05$). **C** Lentivirus encoding scHLA-E^4 also contained methotrexate-resistant dihydrofolate reductase (mDHFR) separated by a T2A sequence. At 4-days post-transduction, the T cells were divided, and the culture medium was supplemented with methotrexate (MTX) or untreated. Data indicates the frequency of scHLA-E^4-positive *B2M*^{KO} T cells 4-days after MTX addition. Symbols indicate 3 independent T cell donors. **D,E** NK cells were stimulated with *B2M*^{KO} T cells that were treated with MTX or untreated. Symbols represent 3 independent NK donors that were stimulated in duplicate with T cells generated from 4 independent donors transduced with a lentivirus encoding mDHFR.T2A.EGFR. **D** Frequency of total NK cells that upregulated expression of CD107a and TNFα. **E** NK cell cytotoxicity assay as described in Methods. NK cell-dependent lysis of MTX-treated and untreated *B2M*^{KO} T cells 48-hours post-culture with NK cells at the indicated E:T ratios.

Supplemental Fig. 2



VL9 epitope stabilization enhances scHLA-E^4 NK cell inhibitory function without increasing NKG2C$^+$ NK cell stimulation. Frequency of total NK cells that upregulated expression of CD107a (**A,C**) and TNFα (**B,D**) post-stimulation with unmodified T cells, or *B2M*^{KO} T cells that were non-transduced or expressed unique disulfide trap (dt) HLA-E^4 VL9 variants (**A,B**) or the corresponding scHLA-E4 VL9 variant (**C,D**) from 3 independent donors. Symbols represent 8 independent NK cell donors in duplicate. **E** Frequency of CD107a$^+$ NKG2C$^+$ NK cells post-stimulation with *B2M*^{KO} T cells that expressed a unique dtHLA-E^4 VL9 variant or the corresponding scHLA-E^4 VL9 variant. Symbols represent 3 independent NK cell donors in duplicate for dt- and sc-HLA-E^{4v1}, and 4 independent NK cell donors in duplicate for all other molecules. Bars indicate mean and error bars show ±SEM. Wilcoxon matched pairs-signed rank test was used to calculate significance.

Supplemental Fig. 3



CD4⁺ T cells do not react against aAPCs. A,B K-562 cells were transduced with lentiviral constructs encoding CD86 and the indicated scHLA molecule. Histograms show cell-surface expression of B2M (**A**), which denotes the scHLA molecule, and CD86 (**B**) relative to non-transduced K-562 cells (black line). **C,D** Bulk T cells were first stimulated with aAPC.dtHLA-E⁴ variants and then restimulated with their respective aAPC or K.86 control cells. Frequency of CD107a⁺ (**C**) and TNFα⁺ (**D**) CD4⁺ T cells after restimulation. Data represent 9 independent T cell donors in duplicate and values are background subtracted from restimulation with K.86 cells. Bars indicate mean and error bars show ±SEM. **E** Bulk T cells were stimulated for 1-week in culture with aAPC.scA2, aAPC.hypoA2 or K.86 cell lines. Total CD4⁺ T cells were quantified following the activation period by flow cytometry. Symbols represent 9 independent T cell donors that were either HLA-A*02⁺ (black) or HLA-A*02⁻ (white).

Supplemental Table 1

Amino acid sequences for scHLA-E, scHLA-A*02 and CD86 molecules

Component	Amino Acid Sequence
B2M signal peptide	MSRSVALAVLALLSLSGLEA
v1 VL9 epitope	VMAPRTLFL
v2 VL9 epitope	VMAPRTLLL
v3 VL9 epitope	VMAPRTLVL
v4 VL9 epitope	VMAPRTLIL
v5 VL9 epitope	VMAPRALLL
v6 VL9 epitope	VTAPRTVLL
v7 VL9 epitope	VMAPRTVLL
v8 VL9 epitope	VTAPRTLLL
$(G_4S)_3$ linker	GGGGSGGGGSGGGGS
GCGGS$(G_4S)_2$ linker	GCGGSGGGGSGGGGS
B2M chain	IQRTPKIQVYSRHPAENGKSNFLNCYVSGFHPSDIEVDLLKNGERIEKVEHSDLSFSKDWSFYLLYYTEFTPTEKDEYACRVNHVTLSQPKIVKWDRDM
$(G_4S)_4$ linker	GGGGSGGGGSGGGGSGGGGSG
HLA-E*01:03 extracellular domain	SHSLKYFHTSVSRPGRGEPRFISVGYVDDTQFVRFDNDAASPRMVPRAPWMEQEGSEYWDRETRSARDTAQIFRVNLRTLRGYYNQSEAGSHTLQWMHGCELGPDGRFLRGYEQFAYDGKDYLTLNEDLRSWTAVDTAAQISEQKSNDASEAEHQRAYLEDTCVEWLHKYLEKGKETLLHLEPPKTHVTHHPISDHEATLRCWALGFYPAEITLTWQQDGEGHTQDTELVETRPAGDGTFQKWAAVVVPSGEEQRYTCHVQHEGLPEPVTLRWKPASQPTIPI
HLA-E*01:03 extracellular domain (Y84C)	SHSLKYFHTSVSRPGRGEPRFISVGYVDDTQFVRFDNDAASPRMVPRAPWMEQEGSEYWDRETRSARDTAQIFRVNLRTLRGCYNQSEAGSHTLQWMHGCELGPDGRFLRGYEQFAYDGKDYLTLNEDLRSWTAVDTAAQISEQKSNDASEAEHQRAYLEDTCVEWLHKYLEKGKETLLHLEPPKTHVTHHPISDHEATLRCWALGFYPAEITLTWQQDGEGHTQDTELVETRPAGDGTFQKWAAVVVPSGEEQRYTCHVQHEGLPEPVTLRWKPASQPTIPI
HLA-E*01:03 extracellular domain (Y84C/D227K/T22A/A245V)	SHSLKYFHTSVSRPGRGEPRFISVGYVDDTQFVRFDNDAASPRMVPRAPWMEQEGSEYWDRETRSARDTAQIFRVNLRTLRGCYNQSEAGSHTLQWMHGCELGPDGRFLRGYEQFAYDGKDYLTLNEDLRSWTAVDTAAQISEQKSNDASEAEHQRAYLEDTCVEWLHKYLEKGKETLLHLEPPKTHVTHHPISDHEATLRCWALGFYPAEITLTWQQDGEGHTQKAELVETRPAGDGTFQKWVAVVVPSGEEQRYTCHVQHEGLPEPVTLRWKPASQPTIPI
HLA-E*01:03 transmembrane and cytoplasmic domains	VGIIAGLVLLGSVVSGAVVAAVIWRKKSSGGKGGSYSKAEWSDSAQGSESHSL
CD8α transmembrane and cytoplasmic domains	IYIWAPLAGTCGVLLLSLVITLYC
CD4 transmembrane and cytoplasmic domains	MALIVLGGVAGLLLFIGLGIFFCVRC
scA2	MSRSVALAVLALLSLSGLEAIQRTPKIQVYSRHPAENGKSNFLNCYVSGFHPSDIEVDLLKNGERIEKVEHSDLSFSKDWSFYLLYYTEFTPTEKDEYACRVNHVTLSQPKIVKWDRDMGGGGSGGGGSGGGGSGGGGSGGGGSGSHSMRYFFTSVSRPGRGEPRFIAVGYVDDTQFVRFDSDAASQRMEPRAPWIEQEGPEYWDGETRKVKAHSQTHRVDLGTLRGYYNQSEAGSHTVQRMYGCDVGSDWRFLRGYHQYAYDGKDYIALKEDLRSWTAADMAAQTTKHKWEAAHVAEQLRAYLEGTCVEWLRRYLENGKETLQRTDAPKTHMTHHAVSDHEATLRCWALSFYPAEITLTWQRDGEDQTQDTELVETRPAGDGTFQKWAAVVVPSGQEQRYTCHVQHEGLPKPLTLRWEPSSQPTIPIVGIIAGLVLFGAVITGAVVAAVMWRRKSSDRKGGSYSQAASSDSAQGSDVSLTACKV
hypoA2	MSRSVALAVLALLSLSGLEAIQRTPKIQVYSRHPAENGKSNFLNCYVSGFHPSDIEVDLLKNGERIEKVEHSDLSFSKDWSFYLLYYTEFTPTEKDEYACRVNHVTLSQPKIVKWDRDMGGGGSGGGGSGGGGSGGGGSGGGGSGSHSMRYFFTSVSRPGRGEPRFIAVGYVDDTQFVRFDSDAASQRMEPRAPWIEQEGPEYWDGETRKVKAHSQTHRVDLGTLRGYYNQSEAGSHTVQRMYGCDVGSDWRFLRGYHQYAYDGKDYIALKEDLRSWTAADMAAQTTKHKWEAAHVAEQLRAYLEGTCVEWLRRYLENGKETLQRTDAPKTHMTHHAVSDHEATLRCWALSFYPAEITLTWQRDGEDQTQKAELVETRPAGDGTFQKWVAVVVPSGQEQRYTCHVQHEGLPKPLTLRWEPSSQPTIPIVGIIAGLVLFGAVITGAVVAAVMWRRKSSDRKGGSYSQAASSDSAQGSDVSLTACKV
CD86	MDPQCTMGLSNILFVMAFLLSGAAPLKIQAYFNETADLPCQFANSQNQSLSELVVFWQDQENLVLNEVYLGKEKFDSVHSKYMGRTSFDSDSWTLRLHNLQIKDKGLYQCIIHHKKPTGMIRIHQMNSELSVLANFSQPEIVPISNITENVYINLTCSSIHGYPEPKKMSVLLRTKNSTIEYDGVMQKSQDNVTELYDVSISLSVSFPDVTSNMTIFCILETDKTRLLSSPFSIELEDPQPPPDHIPWITAVLPTVIICVMVFCLILWKWKKKRPRNSYKCGTNTMEREESEQTKKREKIHIPERSDEAQRVFKSSKTSSCDKSDTCF