Supplementary Material

Supplementary Methods

Source of primary cells

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation from peripheral blood and either cryopreserved at < -80°C in cell culture medium containing 10% DMSO, or directly used for experiments. T cells were negatively isolated from fresh HD PBMCs with the EasySep™ Human T Cell Isolation Kit (Stemcell Technologies, Vancouver, Canada) and either cryopreserved as described above, or immediately used for CAR T cell generation.

Flow cytometry

All measurements were conducted on a CytoFLEX flow cytometer (Beckman Coulter, Brea, CA) and analyzed using FlowJo software (version 10, BD Biosciences, Franklin Lakes, NJ). All antibodies used in the following experiments are listed in Supplementary Table 1. Median fluorescence intensity (MFI) was determined, and the MFI ratios were calculated based on the corresponding isotype controls.

Cytometric bead array

Cell culture supernatants from cytotoxicity experiments were analyzed for levels of cytokine secretion using the Th1/Th2 cytometric bead array (BD Biosciences, Franklin Lakes, NJ) or the LEGENDplex™ human CD8/NK panel (Biolegend, San Diego, CA). The assays were performed according to the manufacturers' instructions using flow cytometry.

Statistical analysis

Prism 10.4.2 (534) (GraphPad Software, LLC) was used for data visualization and statistical analysis. The two-way ANOVA and Sidak's multiple comparison test, the Log-rank (Mantel Cox) test and Gehan-Breslow-Wilcoxon tests, and the Kruskal-Wallis and Dunn's multiple comparison tests were used for statistical testing, as specified in the respective figure legends. Not significant ns_P > .05; *P < .05; **P < .01; ***P < 0.001; ****P < 0.0001.

Supplementary Tables

Supplementary Table 1. Media and buffers.

Description	Ingredients
2x complete blast medium	2x blast medium with 40 ng/ml rh IL-3, rh TPO, rh G-CSF, 114.4 µM β-mercaptoethanol
Blast medium	Alpha MEM (PAN Biotech) with 12.5% FBS, 12.5% horse serum, 1% pen–strep–L-glutamine

CAR T-cell medium	Human TexMACS (Miltenyi Biotec) + 1% pen–strep–L-glutamine and 10 ng/ml IL-7/IL-15 (Peprotech)
Complete blast medium	Blast medium with 20 ng/ml rh IL-3, rh TPO, rh G-CSF, 57.2 µM β-mercaptoethanol
Cryo-conservation medium	Cell lines: RPMI 1640 + 10% DMSO + 10% FCS Primary AML: RPMI 1640 + 10% DMSO + 45% FCS
FACS buffer	PBS + 0.1% BSA and 2 mM EDTA
FACS Fix	47.3 ml FACS buffer + 2.7 ml formaldehyde
HD blast medium	Blast medium with 20 ng/ml rh IL-3, rh IL-6, rh TPO, rh GM-CSF, FLT3L, SCF, 57.2 µM β-mercaptoethanol
R10	RPMI 1640 (PAN Biotech) + 10% FCS, 1% pen–strep–L-glutamine, 1% HEPES

Supplementary Table 2. Cell lines.

Name	**Species**	**Cell type**
MS5	Mouse	Stromal cells
MV4-11	Human	AML
MOLM13	Human	AML
OCI-AML-3	Human	AML
HEL92.1.7	Human	AEL

Supplementary Table 3. Antibodies for flow cytometry.

Antibody	**Clone**	**Conjugate**	**Manufacturer**	**Cat. #**
AquaLiveDead	-	-	Thermo Fisher Scientific	L34957
CD2	RPA-2.10	PerCP/Cy5.5	BioLegend	300216
CD2	REA1130	APC	Miltenyi Biotec	130-119-509
CD2	TS1/8	BV421	BioLegend	309218
CD3	HIT3a	PerCP/Cy5.5	BioLegend	300328
CD4	RPA-T4	PC7	BioLegend	300512
CD8	SK1	APC-Cy7	BioLegend	344714
CD33	REA775	APC	Miltenyi Biotec	130-111-020
CD33	REA775	PE	Miltenyi Biotec	130-111-019
mCD45	30-F11	BV510	BioLegend	103138
CD45	HI30	APC-Cy7	BioLegend	304014
CD45RA	REA1047	VioBlue	Miltenyi Biotec	130-117-854
CD86	IT2.2	BV421	BioLegend	305418
CD107a	H4A3	FITC	BioLegend	328606
CD135	4G8 (RUO)	PE	BD Pharmingen	558996

CD197 (CCR7)	G043H7	PE	BioLegend	353204
CD223 (LAG-3)	REA351	APC	Miltenyi Biotec	130-119-567
CD279 (PD-1)	EH12.2H7	FITC	BioLegend	329904
CD366 (TIM-3)	F38-2E2	BV421	BioLegend	345008

Supplementary Table 4. Reagents.

Name	**Manufacturer**	**Cat. #**
Alpha-MEM	PanBiotech	P04-21500
Anti-mouse IgG,k CompBeads	BD	51.90.9001229
Anti-REA CompBeads	Miltenyi Biotec	130-104-693
Brefeldin A	Sigma–Aldrich	B7651-5MG
Buffer RLT Plus	Qiagen	1053393
DMSO	Serva	20385.01
DPBS	PanBiotech	P04-36500
FcR Blocking Reagent	Miltenyi Biotec	130-059-901
Fetal bovine serum	Thermo Fisher Scientific	10270106
Ficoll Histopaque-1077 Hybri-Max	Sigma–Aldrich	H8889-500ML
Formaldehyde solution	Roth	7398.1
HEPES buffer solution (1 M)	Gibco Life Technologies	15630-056
Horse serum	Sigma–Aldrich	H1270
Human TexMACS	Miltenyi Biotec	130-097-196
MACS BSA stock solution	Miltenyi Biotec	130-091-376
Monensin, sodium salt	Sigma–Aldrich	M5273-1G
Penicillin–streptomycin–L-glutamine, 100x	Thermo Fisher Scientific	10378016
rh FLT3L	Peprotech	300-19
rh G-CSF	Peprotech	300-23
rh GM-CSF	Peprotech	300-03
rh IL-15	Peprotech	200-15
rh IL-3	Peprotech	200-3
rh IL-7	Peprotech	200-7
rh SCF	Peprotech	300-007
rh TPO	Peprotech	300-18
RPMI 1640	PanBiotech	P04-16500
Sodium heparin	Ratiopharm	N68542.05-Z01
Trypan blue	Thermo Fisher Scientific	T10282
T Cell TransACT human	Miltenyi Biotec	130-111-160
UltraPure 0.5 M EDTA	Invitrogen	15575-038
β-Mercaptoethanol	Sigma–Aldrich	M6250

Supplementary Table 5. Kits.

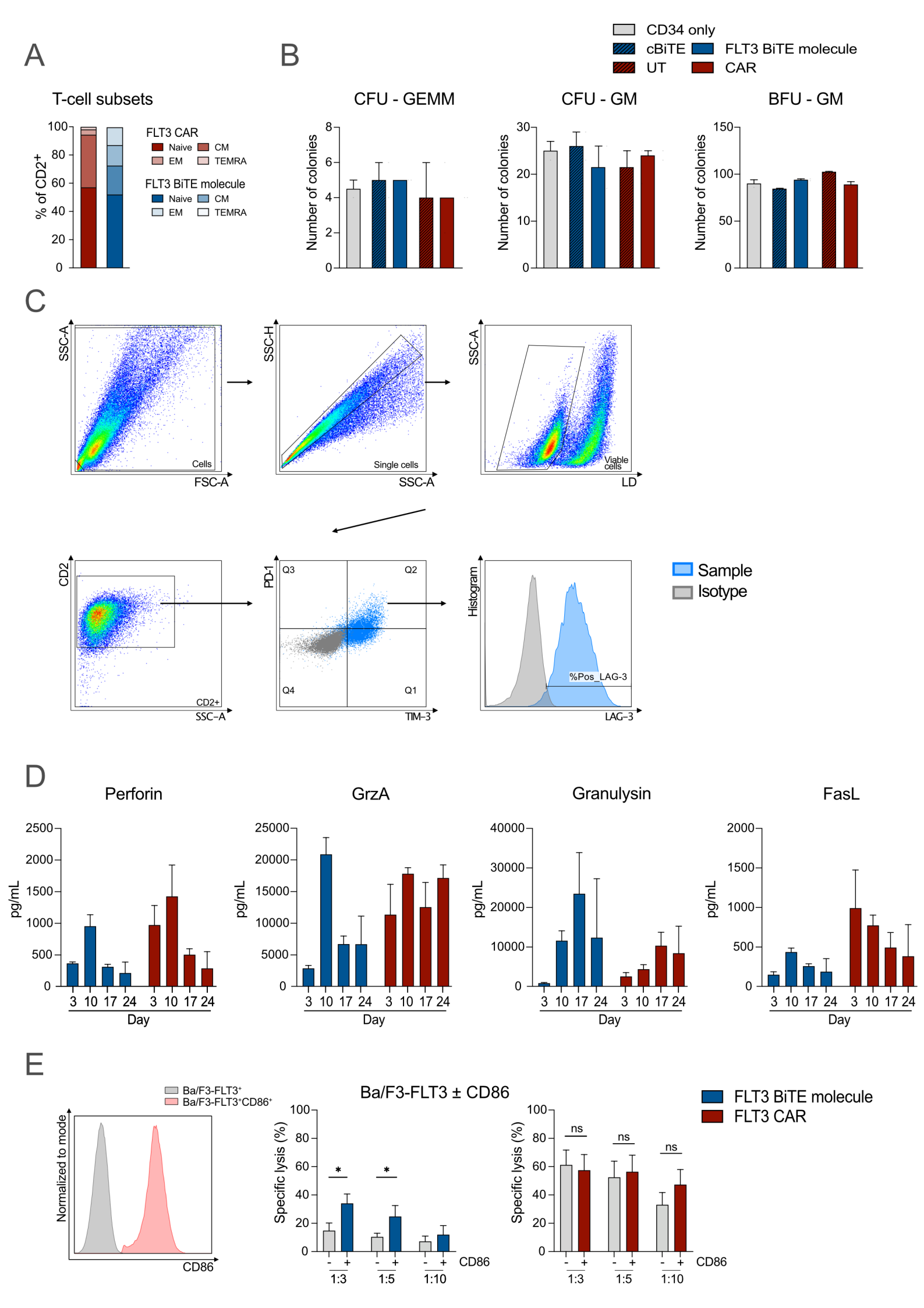
Name	Manufacturer	Cat. #
Cytofix/Cytoperm Fixation/Permeablization Kit	BD Biosciences	554714
CellTrace Far Red Cell Proliferation Kit	ThermoFisher Scientific	C34564
CellTrace CFSE Cell Proliferation Kit	ThermoFisher Scientific	C34570
EasySep Human CD3 Positive Selection Kit II	Stemcell Technologies	17851
EasySep Human T Cell Isolation Kit (Stemcell)	Stemcell Technologies	17951
Human Th1/Th2 Cytokine Kit II	BD Biosciences	551809
LIVE/DEAD Fixable Aqua Dead Cell Stain Kit	Invitrogen	L34957
LEGENDplex human CD8/NK panel	Biolegend	741186

Supplementary Figure Legends

Supplemental Figure 1. Comparison of BiTE molecule- and CAR T cell-mediated efficacy.

(A) T-cell subsets from HD unmanipulated and HD CAR T cells prior to the start of experiments. (B) Colony-forming units (CFU) following co-culture of BiTE molecule- and CAR-redirected T cells with $CD34^+$ cells isolated from healthy BMMCs for 6 h at an E:T ratio of 10:1. The total number of colonies was determined 2 weeks after plating and analyzed for the following progenitor subsets: CFU-granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM), CFU-ganulocyte/macrophage (CFU-GM), and burst-forming unit–erythroid (BFU-E). (C) Exemplary gating strategy for $PD\text{-}1^+/TIM\text{-}3^+/LAG\text{-}3^+$ triple-positive T cells. (D) Secretion levels of perforin, granzyme A (GrzA), granulysin, and FasL in the supernatants of long-term co-culture. (E) Representative histogram of CD86 expression on $Ba/F3\text{-}FLT3^+$ and $Ba/F3\text{-}FLT3^+CD86^+$ cell lines and *in vitro* cell line model demonstrating the effect of positive co-stimulation by CD86 on BiTE molecule- and CAR-mediated cytotoxicity.

Supplementary Figure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FLT3 BiTE molecule
FLT3 CAR