Poster #81

Optimizing ex vivo CAR-T cell-mediated cytotoxicity assay through multimodality imaging

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Abstract

CAR-T cell–based therapies have demonstrated remarkable efficacy in treating malignant cancers, especially liquid tumors, and they are increasingly being evaluated in clinical trials for solid tumors. With FDA's initiative for advancing alternative methods for drug discovery and development, full human ex vivo assays are increasingly essential for precision CAR-T development. However, prevailing ex vivo CAR-T cell–mediated cytotoxicity assays are limited by their use of radioactive materials, lack of real-time measurement, low throughput, and automatability, among others. To address these limitations, we optimized the assay using multimodality imaging methods, including bioluminescence, impedance tracking, phase contrast, and fluorescence, to track CAR-T cells cocultured with CD19, CD20, and HER2 luciferase reporter cancer cells in real-time. Additionally, we varied the ratio of CAR-T cells to cancer cells to determine optimal cytotoxicity readouts. Our findings demonstrated that the CAR-T cell group effectively attacked cancer cells, and the optimized assay provided superior temporal and spatial precision measurements of ex vivo CAR-T killing of cancer cells, confirming the reliability, consistency, and high throughput of the optimized assay.

Results

	ATCC® Cell Line ID	CRL-8885-LUC2™	CCL-86-LUC2™	HTB-20-LUC2™	CRL-2630-LUC2™	CCL-213-LUC2™	CCL-121-LUC2™	CCL-185-LUC2™	CCL-225-LUC2™	CCL-228-LUC2™	CCL-240-LUC2™	CCL-247-LUC2™	CRL-1435-LUC2™	CRL-1469-LUC2™	CRL-1555-LUC2™	CRL-1619-LUC2™	CRL-1739-LUC2™	CRL-2003-LUC2™	HTB-14-LUC2™	HTB-22-LUC2™	HTB-43-LUC2™	HTB-96-LUC2™
Cell Line Info	Cell Line Name	WIL2-S	RAJI	BT474	FARAGE	DAUDI	HT1080	A549	HCT15	SW480	HL60	HCT116	PC3	PANC1	A431	A375	AGS	TF1	U87MG	MCF7	FADU	UZ0S
	Lineage Subtype	N/A	Non Hodgkin Lymphoma	Breast Ductal Carcinoma	Non Hodgkin Lymphoma	Non Hodgkin Lymphoma	Fibrosarcoma	NSCLC	Colorectal Adenocarcinoma	Colorectal Adenocarcinoma	AML	Colorectal Adenocarcinoma	Prostate Adenocarcinoma	Exocrine	Skin Squamous	Melanoma	Gastric Adenocarcinoma	AML	Glioma	Breast Carcinoma	Upper Aerodigestive Squamous	Osteosarcoma
Haematological Tumor CAR Targets	TNFRSF17 (BCMA)	N/A	3.8807	0.1635	1.3785	4.1094	0.0000	0.0000	0.0426	0.1243	0.2869	0.0704	0.1110	0.2987	0.0000	0.1375	0.0000	0.0000	0.2388	0.0000	0.0000	0.2016
	IL3RA (CD123)	N/A	0.0566	0.2141	0.0000	0.3103	0.1890	0.0841	0.0566	0.1890	0.4436	0.0000	0.0000	0.1635	0.0000	0.1635	0.1110	1.9928	0.0566	0.0566	0.0841	0.3785
	SDC1 (CD138)	N/A	0.3785	6.2052	0.0144	0.3103	7.1797	6.5703	5.7142	4.7719	0.6229	4.9303	7.6454	6.3425	7.7041	4.5027	5.0866	1.0000	5.7230	3.9653	6.3100	6.3583
	CD19	N/A	7.1104	0.0000	6.5132	6.9925	0.0841	0.0704	0.0000	0.0144	0.1890	0.0286	0.0426	0.0704	0.0000	0.0144	0.0000	1.6182	0.0000	0.0000	0.0976	0.0426
	MS4A1 (CD20)	N/A	8.0865	0.0000	9.0930	8.8855	0.0704	0.0426	0.1763	0.0000	0.0000	0.1375	0.0000	0.0144	0.0000	0.1110	0.0841	0.1763	0.0000	0.0976	0.0566	0.1243
	CD22	N/A	7.5211	0.2141	8.6641	9.8023	3.4436	0.2388	0.2510	0.9336	0.0286	2.1210	0.2388	1.0000	2.0000	2.9030	0.5059	3.4141	2.5558	1.5607	0.2141	1.6781
	CD38	N/A	5.8276	0.0286	3.8032	7.4682	0.0841	2.5008	0.0000	0.0144	0.6041	0.0000	0.0426	0.0976	0.6323	0.0000	0.1110	3.1570	0.1890	0.2016	0.2987	0.0704
	CD5	N/A	0.0144	0.1506	0.0286	0.0976	0.0286	0.0000	0.0144	0.0144	0.0000	0.0144	0.1506	0.0286	0.0426	0.0144	0.0144	0.0144	0.0426	0.1763	0.0144	0.0976
	FUT3 (LeY)	N/A	0.0144	0.1506	0.0566	0.0841	0.0000	0.0286	1.7442	0.0426	0.0426	0.1506	2.4222	0.0566	2.8875	0.0841	0.1763	0.0286	0.1110	0.1763	3.7560	0.1375
	KLRK1 (NKG2D)	N/A	0.0286	0.0704	0.7049	1.9745	0.0000	0.0000	0.0566	0.1506	1.1440	1.1440	0.0841	0.0286	0.0704	0.0841	0.2510	0.2750	0.2869	0.0000	0.1890	1.2327
	ROR1	N/A	0.7312	0.0286	0.0000	0.3334	1.7570	3.8620	0.5460	1.6041	0.0704	1.2327	2.0496	4.0036	0.2630	1.4114	5.5097	0.0144	0.1506	0.0144	0.1506	3.6508
	WT1	N/A	0.0144	0.0000	0.1243	0.1763	2.3190	2.1699	0.0286	1.4594	3.4370	0.1243	1.9964	3.9495	0.0000	2.9165	0.0841	4.6118	0.1635	0.0704	1.7137	1.5705
Solid Tumour CAR Targets	MET (C-Met)	N/A	2.7506	3.4222	1.5607	1.4803	6.2300	6.4411	4.7613	1.4699	0.5558	6.7623	6.2038	5.9873	4.4456	4.5844	4.8324	0.7740	6.1445	3.0036	5.0794	3.6450
	CA9 (CAIX)	N/A	0.0144	0.2141	0.0000	0.0144	5.4877	0.8319	4.5336	0.5460	0.0000	0.0704	0.6229	0.0426	1.9782	2.1985	0.0426	0.0000	7.5568	0.1243	0.4222	2.0215
	PROM1 (CD133)	N/A	0.0976	0.8074	0.0000	0.9411	0.0841	0.0841	0.0566	0.0841	0.0426	3.9079	0.0144	0.1506	0.0144	0.4542	0.1506	0.0704	0.1375	0.0286	0.0704	0.1110
	L1CAM (CD171)	N/A	0.3896	0.8156	0.6229	0.1243	0.5753	2.3477	0.2141	5.6363	0.3334	2.7334	3.6769	8.2491	0.1375	4.1643	6.7570	0.5261	0.5361	4.3491	1.7004	6.8354
	CD70	N/A	6.9706	0.1635	7.4529	6.2919	6.8347	3.5361	0.0426	3.1619	0.8797	0.4114	1.4647	5.8202	0.0286	6.2223	0.1635	0.2750	5.2384	0.2016	0.5059	4.9621
	CEACAM5 (CEA)	N/A	0.1243	2.0670	0.0000	0.2265	0.0566	0.1890	0.2987	0.1635	0.1110	0.4222	1.4436	0.2388	1.4489	0.1110	2.1440	0.1110	0.0841	2.0704	0.4114	0.0704
	EGFR	N/A	0.1635	1.5410	0.0000	3.0790	4.2950	5.3459	3.9241	3.7159	0.2265	4.7634	4.8283	5.8497	7.6442	1.4906	3.8012	0.2141	5.1085	0.2630	6.5534	2.1635
	EPCAM (Ep-CAM)	N/A	0.5361	7.4039	0.0000	0.6599	0.4222	2.9523	9.6185	8.5089	0.6323	8.3334	7.5399	4.2342	7.1480	0.7740	9.1172	0.8718	0.9336	8.4341	7.0353	3.6859
	EPHA2 (EphA2)	N/A	0.4854	2.2265	0.0000	0.3674	7.0613	5.7926	5.6488	5.5647	0.4114	6.3918	7.8590	7.9525	5.1094	5.9300	7.1738	0.4222	4.7698	3.3854	6.0027	6.0721
	FAP	N/A	0.0566	0.0704	0.0000	0.0000	0.1506	0.0566	0.0286	0.0144	0.2016	0.1506	0.0704	0.0144	0.4330	0.3561	0.0976	0.0704	5.2407	0.0566	0.0286	1.5059
	GPC3	N/A	0.0000	0.8400	0.0000	0.0000	0.1635	0.1375	0.4751	0.0841	0.0426	0.6135	0.1110	0.1110	0.0000	0.4222	0.6960	0.0000	0.0426	1.2388	0.6323	2.3646
	ERBB2 (HER2)	N/A	1.2510	10.5222	1.4647	1.3089	4.2510	4.2063	5.9669	3.6159	1.2449	4.7570	4.1102	4.7258	4.8435	4.5033	6.2246	1.5945	2.1890	5.5065	5.7578	5.4343
	IL13RA2 (IL13Rα2)	N/A	0.0566	0.0976	0.0000	0.0000	0.2265	0.0000	0.0000	0.0566	0.5361	0.0566	1.8400	0.0144	0.7312	9.0446	0.0000	0.1635	6.1183	0.0704	0.0566	0.0000
	FUT3 (LeY)	N/A	0.0144	0.1506	0.0566	0.0841	0.0000	0.0286	1.7442	0.0426	0.0426	0.1506	2.4222	0.0566	2.8875	0.0841	0.1763	0.0286	0.1110	0.1763	3.7560	0.1375
	MAGEA3	N/A	0.0704	0.0976	0.0000	0.1110	7.1033	8.8797	0.2750	0.0976	0.1110	7.0889	0.8953	0.1635	0.0286	7.3091	0.1243	4.3090	1.7949	0.2016	0.1110	4.4060
	MAGEA4	N/A	0.1243	0.0426	0.0000	0.1243	0.0000	0.1375	0.2265	0.2510	0.1375	0.2265	0.3561	0.2141	8.5461	9.6843	0.1506	0.2141	0.2750	0.1763	7.7173	6.0350
	MLANA (MART1)	N/A	0.3219	0.0286	0.1635	0.8797	0.3219	0.1506	0.3561	0.0000	0.0704	0.2016	0.1375	0.1243	0.1763	0.3896	0.2750	0.6135	0.4330	0.3103	0.5945	0.3896
	MSLN (Mesothlin)	N/A	0.1763	0.3103	0.0286	0.1506	0.1506	2.0807	0.4436	2.7929	0.2016	4.1043	1.7527	1.7991	0.0286	0.7049	5.7968	0.0841	0.2016	0.0976	1.6781	4.7286
	MUC1	N/A	0.1763	4.6616	0.0704	0.8953	1.2690	1.0000	1.3448	1.6554	0.5753	1.7698	3.2750	4.4436	1.7181	1.9928	7.0095	3.7991	1.8875	5.1922	3.7602	3.3882
	MUC16	N/A	0.0144	0.0286	0.0144	3.4854	0.0144	0.0704	0.0426	0.1110	0.0144	0.0566	0.3334	0.5656	2.6668	0.0144	0.0426	0.4647	0.0704	1.4699	0.6599	0.0566
	CTAG1B (NY-ESO-1)	N/A	0.0426	0.1110	0.0000	0.1110	6.4000	0.0426	0.0976	0.0841	0.4222	0.0000	0.0426	0.0000	5.9816	0.0000	0.0000	0.0000	0.0000	0.0426	0.0976	0.3103
	CD274 (PD-L1)	N/A	0.7485	0.1506	3.6005	0.5160	5.0605	1.7866	1.2869	1.2810	0.2388	1.5607	2.5261	0.0976	2.5435	1.7698	1.3505	0.5460	2.8094	0.2630	2.9982	1.4005
	PSCA	N/A	0.3103	5.0700	0.1243	0.1763	1.5410	0.6323	0.0426	0.2987	0.0976	0.3674	1.9523	1.0704	6.4091	1.1243	1.4276	0.9855	2.8053	0.4854	1.3448	0.9411
	FOLH1 (PSMA)	N/A	0.0000	1.0976	0.0000	0.0976	0.4957	0.4114	0.0000	0.0000	0.0000	0.2750	0.2265	0.0286	0.0144	2.0670	0.0286	0.0000	0.0000	0.0566	1.1043	1.6323
	ROR1	N/A	0.7312	0.0286	0.0000	0.3334	1.7570	3.8620	0.5460	1.6041	0.0704	1.2327	2.0496	4.0036	0.2630	1.4114	5.5097	0.0144	0.1506	0.0144	0.1506	3.6508
	KDR (VEGFR2)	N/A	0.0000	0.0000	0.0000	0.0286	0.0976	0.0000	0.0000	0.0144	0.0976	0.0841	0.8875	0.0426	0.0000	2.1603	0.0000	3.0514	1.7782	0.0286	0.0000	0.0566

Figure 1: Analysis of common hematological and solid tumor CAR targets from selected cancer cell lines. Using the RNA seq data from the CCLE database, a heat map was generated by screening 21 cancer cell lines that have a luciferase-derived daughter cell line available from ATCC® for 12 hematological tumor CAR targets and 26 solid tumor CAR targets. Note: WIL2-S did not have RNA seq data within the CCLE database.

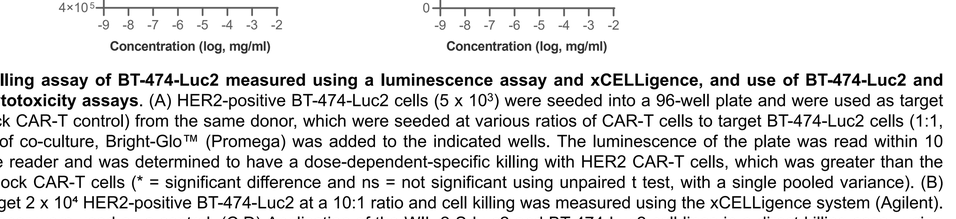

Figure 4: HER2 CAR-T in vitro killing assay of BT-474-Luc2 measured using a luminescence assay and xCELLigence, and use of BT-474-Luc2 and WIL2-S-Luc2 in CAR-T and NK cytotoxicity assays. (A) HER2-positive BT-474-Luc2 cells (5 x 10^3) were seeded into a 96-well plate and were used as target cells for either HER2 CAR-T or Mock CAR-T control) from the same donor, which were seeded at various ratios of CAR-T cells to target BT-474-Luc2 cells (1:1, 2:1, 5:1, and 10:1). After 24 hours of co-culture, Bright-Glo™ (Promega) was added to the indicated wells. The luminescence of the plate was read within 10 minutes using a luminescence plate reader and was determined to have a dose-dependent-specific killing with HER2 CAR-T cells, which was greater than the non-specific killing observed with mock CAR-T cells (* = significant difference and ns = not significant using unpaired t test, with a single pooled variance). (B) HER2 CAR-T cells were used to target 2 x 10^4 HER2-positive BT-474-Luc2 at a 10:1 ratio and cell killing was measured using the xCELLigence system (Agilent). Mock CAR-T cells from the same donor were used as a control. (C,D) Application of the WIL-2-S-Luc2 and BT-474-Luc2 cell lines in a direct killing assay using primary natural killer cells. (C) WIL2-S-Luc2 cells or (D) BT-474-Luc2 cells were co-cultured with NK cells for 16 hours at various NK cell to target ratios (1:4, 1:2, 1:1, 2:1, 4:1, 8:1, and 16:1) after which the luciferase activity was measured. (E,F) Application of an antibody-dependent cell-mediated cytotoxicity (ADCC). (E) Rituximab (anti-CD20) or (F) Trastuzumab (anti-HER2) monoclonal antibodies were added at concentrations from 10 pg to 1 µg/mL to wells containing co-cultures of WIL2-S-Luc2 or BT474-Luc2, respectively, with primary NK cells at a 2:1 ratio of target-to-effector ratio for 4 hours after which a luciferase activity assay was measured. Human IgG1 was used an isotype control. N=3 in all experiments. *, P<0.05.

	ATCC® Cell Line ID	CRL-8885™	CRL-8885™	CRL-8885-LUC2™	CRL-8885-LUC2™	CCL-86™	CCL-86™	CCL-86-LUC2™	CCL-86-LUC2™
Cell Line Info	Experiment ID	E1	E2	E1	E2	E1	E2	E1	E2
	Cell Line Name	WIL2-S	WIL2-S	WIL2-S-LUC2	WIL2-S-LUC2	RAJI	RAJI	RAJI-LUC2	RAJI-LUC2
	Lineage Subtype	B lymphoblast (ATCC®)				Non Hodgkin Lymphoma (CCLE), B lymphocyte (ATCC®)			
Haematological Tumor CAR Targets	TNFRSF17 (BCMA)	2.6034	2.5863	2.8300	2.5259	1.3341	0.9085	0.9724	1.5416
	IL3RA (CD123)	0.3835	0.4604	0.5875	0.6493	0.0000	0.0197	0.0000	0.0000
	SDC1 (CD138)	2.6672	2.6350	2.2521	2.1521	0.0000	0.0000	0.0000	0.0000
	CD19	4.7165	4.7405	4.5641	4.4393	6.7619	6.3809	6.6525	6.6966
	MS4A1 (CD20)	8.0526	8.0698	7.8880	7.8873	5.4484	6.1726	6.6860	6.6093
	CD22	7.4285	7.4467	6.9762	6.9798	5.1644	5.3359	6.2923	6.2771
	CD38	1.7122	1.5742	1.8423	1.9469	4.8609	5.7897	5.8123	5.7744
	CD5	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	FUT3 (LeY)	0.0000	0.0000	0.0512	0.0000	0.0000	0.0000	0.0000	0.0000
	KLRK1 (NKG2D)	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	ROR1	0.0000	0.0000	0.0000	0.0120	0.2722	0.9052	0.8378	0.8008
	WT1	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Solid Tumour CAR Targets	MET (C-Met)	0.0000	0.0000	0.0000	0.0000	1.0296	1.8161	2.7878	2.7289
	CA9 (CAIX)	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	PROM1 (CD133)	0.0282	0.0000	0.0000	0.0000	0.0000	0.0000	0.0367	0.0000
	L1CAM (CD171)	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	CD70	7.1310	7.1892	7.1458	7.2590	7.6370	7.0802	6.4572	6.4472
	CEACAM5 (CEA)	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	EGFR	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	EPCAM (Ep-CAM)	0.0000	0.1028	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	EPHA2 (EphA2)	0.0275	0.0000	0.0000	0.1871	0.0000	0.0000	0.0000	0.0000
	FAP	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	GPC3	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	ERBB2 (HER2)	1.6341	1.5860	1.4579	1.4976	0.8825	0.7710	0.6043	0.6806
	IL13RA2 (IL13Rα2)	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	FUT3 (LeY)	0.0000	0.0000	0.0512	0.0000	0.0000	0.0000	0.0000	0.0000
	MAGEA3	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	MAGEA4	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	MLANA (MART1)	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	MSLN (Mesothlin)	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	MUC1	0.0000	0.2049	0.0000	0.0599	0.0000	0.0000	0.0763	0.0518
	MUC16	0.0019	0.0000	0.0000	0.0000	0.0000	0.0209	0.0000	0.0000
	CTAG1B (NY-ESO-1)	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	CD274 (PD-L1)	3.5061	3.5668	3.6920	3.7440	0.0774	0.0000	0.2622	0.1348
	PSCA	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	FOLH1 (PSMA)	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	ROR1	0.0000	0.0000	0.0000	0.0120	0.2722	0.9052	0.8378	0.8008
	KDR (VEGFR2)	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000

Figure 2: mRNA seq analysis of common hematological and solid tumor CAR targets in B lymphoblast and B lymphocyte cancer cell lines. (A) A heat map was generated by screening the RNA seq data for 2 cancer cell lines and 2 single clone luciferase-expressing daughter cell lines for 12 hematological tumor CAR targets and 26 solid tumor CAR targets. RNA sequencing was performed with 2 duplicate samples by Psomagen. CD19 expression remained consistent pre- and post-luciferase transduction. (B) Schematic of CAR-T target luciferase reporter cells showing CAR-T target cells with expression of CD19-positive WIL2-S-Luc2 and Raji-Luc2, CD20-positive Daudi-Luc2 and Farage-Luc2, and HER2-positive BT-474-Luc2 being surrounded and attacked by CD19-, CD20-, and HER2-targeting CAR-T cells, respectively. Created with BioRender.com

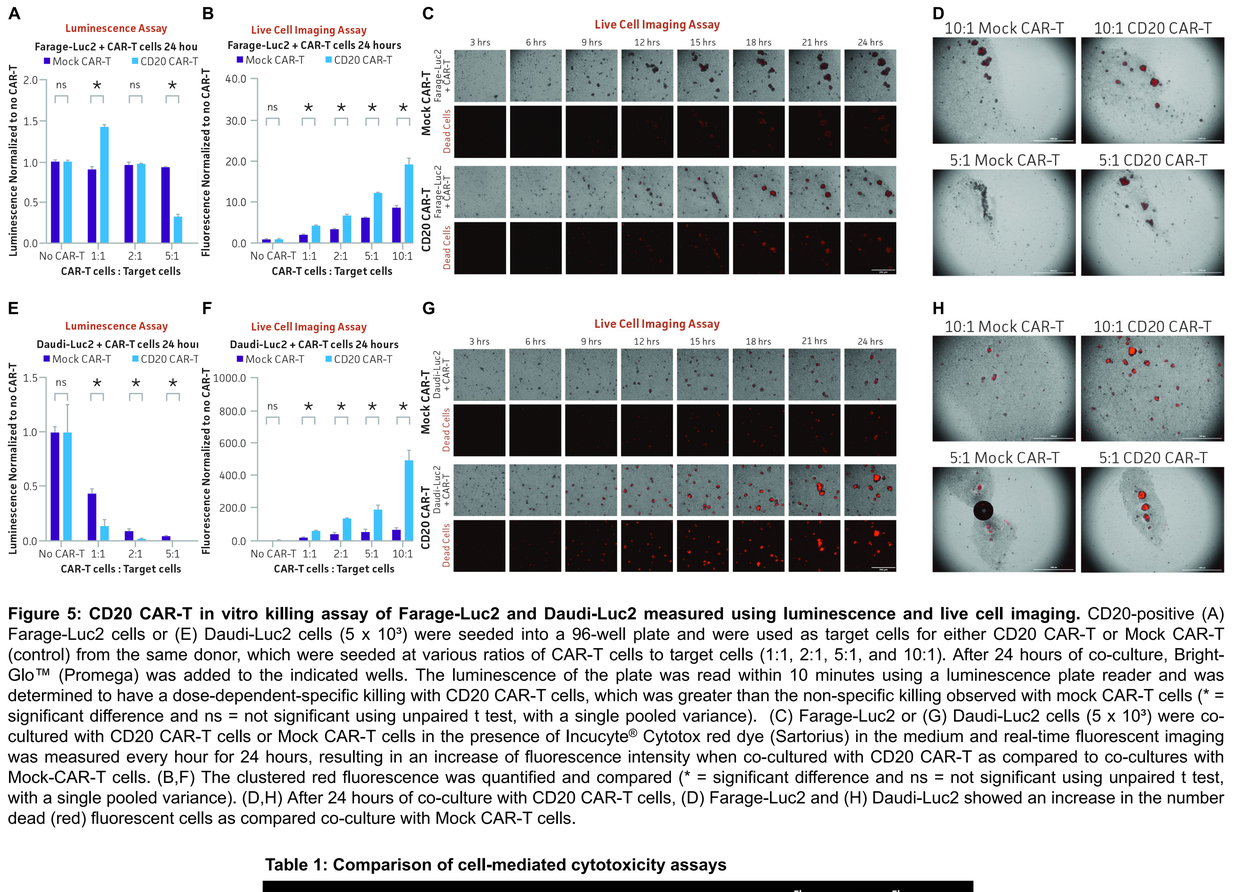

Figure 5: CD20 CAR-T in vitro killing assay of Farage-Luc2 and Daudi-Luc2 measured using luminescence and live cell imaging. CD20-positive (A) Farage-Luc2 cells or (E) Daudi-Luc2 cells (5 x 10^3) were seeded into a 96-well plate and were used as target cells for either CD20 CAR-T or Mock CAR-T (control) from the same donor, which were seeded at various ratios of CAR-T cells to target cells (1:1, 2:1, 5:1, and 10:1). After 24 hours of co-culture, Bright-Glo™ (Promega) was added to the indicated wells. The luminescence of the plate was read within 10 minutes using a luminescence plate reader and was determined to have a dose-dependent-specific killing with CD20 CAR-T cells, which was greater than the non-specific killing observed with mock CAR-T cells (* = significant difference and ns = not significant using unpaired t test, with a single pooled variance). (C) Farage-Luc2 or (G) Daudi-Luc2 cells (5 x 10^3) were co-cultured with CD20 CAR-T cells or Mock CAR-T cells in the presence of Incucyte® Cytotox red dye (Sartorius) in the medium and real-time fluorescent imaging was measured every hour for 24 hours, resulting in an increase of fluorescence intensity when co-cultured with CD20 CAR-T as compared to co-cultures with Mock-CAR-T cells. (B,F) The clustered red fluorescence was quantified and compared (* = significant difference and ns = not significant using unpaired t test, with a single pooled variance). (D,H) After 24 hours of co-culture with CD20 CAR-T cells, (D) Farage-Luc2 and (H) Daudi-Luc2 showed an increase in the number dead (red) fluorescent cells as compared co-culture with Mock CAR-T cells.

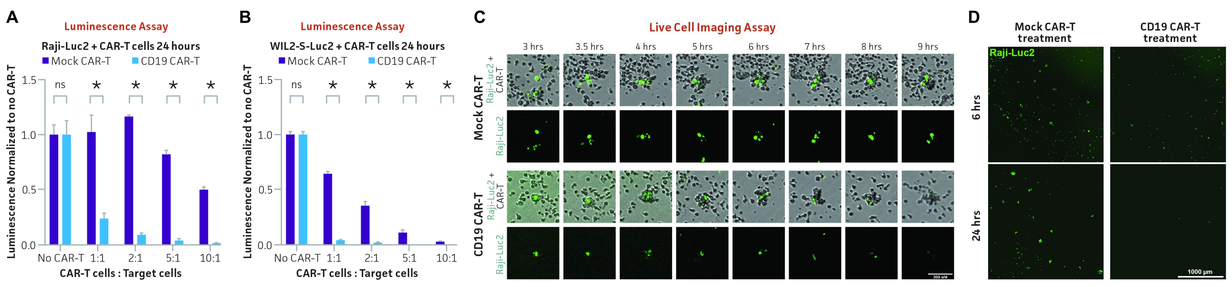

Figure 3: CD19 CAR-T in vitro killing assay of Raji-Luc2 and WIL2-S-Luc2 measured using luminescence and live cell imaging. (A) CD19-positive Raji-Luc2 cells (5 x 10^3) or (B) WIL2-S-Luc2 cells (5 x 10^3) were seeded into a 96-well plate and were used as target cells for either CD19 CAR-T or Mock CAR-T (control) from the same donor, which were seeded at various ratios of CAR-T cells to target Raji-Luc2 or WIL2-S-Luc2 cells (1:1, 2:1, 5:1, and 10:1). After 24 hours of co-culture, Bright-Glo™ (Promega) was added to the indicated wells. The luminescence of the plate was read within 10 minutes using a luminescence plate reader and was determined to have a dose-dependent-specific killing with CD19 CAR-T cells that was greater than the non-specific killing observed with mock CAR-T cells (* = significant difference and ns = not significant using unpaired t test, with a single pooled variance). (C) Raji-Luc2 cells were stained with Vybrant™ (Thermo Fisher Scientific) DiO dye and real-time fluorescent imaging was measured every 30 minutes for 24 hours during the co-culture of Raji-LUC2 cells with CAR-T cells. Two stained Raji-Luc2 cells (Green) from the co-culture experiment were tracked for 6 hours and became surrounded by CAR-T cells, resulting in a decrease of fluorescence when treated with CD19 CAR-T as compared to co-cultures with Mock-CAR-T cells. (D) After 24 hours of co-culture, CD19 CAR-T cells showed a decrease in fluorescent cells as compared to 6 hours; in a co-culture with Mock CAR-T cells numerous Raji-LUC2 cells were present.

Table 1: Comparison of cell-mediated cytotoxicity assays

	Chromium release	Bioluminescence imaging	Impedance	Flow cytometry	Fluorescence Imaging - cell labeling	Fluorescence imaging - cytotox dye in media
Principal measure of cytotoxicity	^{51}Cr release	Luciferase activity	Cell detachment	Live/dead staining phenotype	Decrease in fluorescent signal	Dye infiltration into dead cell
Radioactive materials needed	Yes	No	No	No	No	No
Target cell labeling required	Yes	No	No	Yes	Yes	No
Genetic modification of target cells	No	Yes (reporter gene)	No	Yes	No	No
Endpoint/kinetic	Endpoint	Endpoint	Temporal	Endpoint	Temporal & Spatial	Temporal & Spatial
Real-time measurement	No	No	Yes	No	Yes	Yes
Maximum time point measured	18 – 24 hours	Days	Days	Days	Days	Days
Ability to measure different cytotoxicity heterogenous targets	No	No	No	Yes	Yes	Yes
Throughput and automatability	Low	High	High	High	High	High

Conclusion

Overall, every single ex vivo CAR-T cell–mediated cytotoxicity assay has various limitations due to the assay technology itself. We generated a panel of luciferase reporter tumor cell lines that can be used to examine the function of CAR-T cells. The reporter cells in this panel naturally express high levels of clinically relevant CAR-T target antigens on cell surface, such as CD19, CD20, or HER2. By utilizing stable luciferase expression in these CAR-T target tumor cell lines, we can enhance the ex vivo CAR-T cell–mediated cytotoxicity assay by employing multimodality imaging techniques. This approach allows us to overcome certain limitations associated with individual single assay methods, such as radioactivity and the absence of spatial information, while optimizing the overall assay performance.

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