CAR T cell characterization by flow cytometry

Andrea R Daniel[1]

[1]Duke University

Andrea R Daniel: This protocols was adapted form the M. Brown lab at Duke University

Andrea R Daniel
Duke University

NOV 17, 2023

OPEN ACCESS

Protocol Citation: Andrea R Daniel 2023. CAR T cell characterization by flow cytometry. **protocols.io** https://protocols.io/view/car-t-cell-characterization-by-flow-cytometry-c48qyzvw

MANUSCRIPT CITATION: Transcriptional and epigenetic regulators of human CD8$^+$ **T** cell function identified through orthogonal CRISPR screens.McCutcheon SR, Swartz AM, Brown MC, Barrera A, McRoberts Amador C, Siklenka K, Humayun L, Ter Weele MA, Isaacs JM, Reddy TE, Allen AS, Nair SK, Antonia SJ, Gersbach CA.Nat Genet. 2023 Nov 9. doi: 10.1038/s41588-023-01554-0. Online ahead of print.PMID: 37945901

ABSTRACT

Flow cytometry of tumor tissue and blood from mice with human tumors. Mice bearing HCC1954 tumors were euthanized at days 3 and 19 post CAR T cel delivery. This protocol describes methods for preparing input CAR T cells and tumor infiltrating CAR T cells for phenotypic characterization of by flow cytometry. Data collection can be performed using a Fortessa X 20 and analyzed using Flow Jo V10.8.1.

MATERIALS

Antibodies Table

Antibody Target	Fluorophore/Sequence	Clone	Isotype	Dilution	Application	Manufacturer	Catalog #	Notes
CD2	PE	RPA-2.10	Mouse / IgG1, kappa	1:50	Flow cytometry	Thermo	12-0029-42	-
B2M	PE	A17082A	Mouse IgG1, κ	1:50	Flow cytometry	Biolegend	395704	-
IL2RA	PE-Cy7	BC96	Mouse / IgG1, kappa	1:50	Flow cytometry	Thermo	25-0259-42	-
EGFR	bv-421	EGFR.1	Mouse IgG2b, κ	1:50	Flow cytometry	BD Biosciences	742602	-
CCR7	FITC	150503	Mouse IgG2a	1:100	Flow cytometry	BD Biosciences	561271	Stain at 37C
CD8	bv-421	HIT8a	Mouse IgG1, κ	1:50	Flow cytometry	BD Biosciences	740078	-
IL7RA	PE-Cy5	eBioRDR5	Mouse / IgG1, kappa	1:100	Flow cytometry	Thermo	15-1278-42	-
LAG3	PE	3DS223H	Mouse / IgG1, kappa	1:50	Flow cytometry	Thermo	12-2239-42	-

Protocol status: Working

We use this protocol and it's working

Created: Nov 17, 2023

Last Modified: Nov 17, 2023

PROTOCOL integer ID: 91120

Keywords: CAR T cells, flow cytometry

Funders Acknowledgement:

NIH

	TIM3	PE-Cy5	F38-2E2	Mouse IgG1, κ	1:50	Flow cytometry	Biolegend	345052	-
	TIGIT	PerCP-eFluor710	MBSA43	Mouse / IgG1, kappa	1:50	Flow cytometry	Thermo	46-9500-42	-
	PD1	PE-Cy7	EH12.1	Mouse IgG1, κ	1:100	Flow cytometry	BD Biosciences	561272	-
	Myc-tag	Alexa Fluor 647	9B11	Mouse IgG2a	1:50	Flow cytometry	Cell Signaling Technology	2233S	-
	Thy1.1	PE	OX-7	Mouse IgG1, κ	1:300	Flow cytometry	StemCell Technologies	60024PE	-
	Note: Antibodies below were used for in vivo TIL characterization experiment (more details in Supplemental Methods 4)								
	CD3	BUV737	UCHT1	Mouse IgG1 kappa	1:100	Flow cytometry	BD Biosciences	612750	
	CD8	BUV395	RPA-T8	Mouse IgG1 kappa	1:100	Flow cytometry	BD Biosciences	563795	
	TIGIT	BV605	A15153G	Mouse IgG2a, kappa	1:100	Flow cytometry	Biolegend	372712	
	LAG3	BV785	11C3C65	Mouse IgG1, Kappa	1:100	Flow cytometry	Biolegend	369322	
	CD127	PERCP-Cy5.5	A019D5	Mouse IgG1, Kappa	1:100	Flow cytometry	Biolegend	351322	
	PD1	BV711	EH12.2GH7	Mouse IgG1 kappa	1:100	Flow cytometry	Biolegend	329928	
	TIM3	PE-Cy5	F38-2E2	Mouse IgG1, Kappa	1:100	Flow cytometry	Biolegend	345052	
	Granzyme B	PE-Cy7	QA16A02	Mouse IgG1 kappa	1:100	Flow cytometry	Biolegend	372214	

	TCF1	BV421	S33-966	Mouse IgG1 kappa	1:100	Flow cytometry	BD Biosciences	566692	
	Ki-67	BV510	Ki-67	Mouse IgG1 kappa	1:100	Flow cytometry	Biolegend	350518	
	IFN-g	PE	B27	Mouse IgG1 kappa	1:100	Flow cytometry	Biolegend	506507	
	CD39	PE/Dazzle-594	A1	Mouse IgG1, Kappa	1:100	Flow cytometry	Biolegend	328224	
	CD56	BV605	5.1H11	Mouse IgG1 kappa	1:100	Flow cytometry	Biolegend	362538	
	CD45RO	BV786	UCHL1	Mouse IgG2a, kappa	1:100	Flow cytometry	Biolegend	304234	
	CD45RA	PE-Cy5	HI100	Mouse IgG2b, kappa	1:100	Flow cytometry	Biolegend	304110	
	CD28	PE-Cy7	S20013B	Mouse IgG1, Kappa	1:100	Flow cytometry	Biolegend	377812	
	CCR7	BV711	G043H7	Mouse IgG2a, kappa	1:100	Flow cytometry	Biolegend	353228	
	CD62L	BV510	DREG-56	Mouse IgG1, Kappa	1:100	Flow cytometry	Biolegend	304844	
	CTLA4	BV421	BNI3	Mouse IgG2a, kappa	1:100	Flow cytometry	Biolegend	369606	
	Tbet	PERCP-Cy5.5	4B10	Mouse IgG1, Kappa	1:100	Flow cytometry	Biolegend	644806	
	EOMEs	PE	X4-83	Mouse IgG1 kappa	1:100	Flow cytometry	BD Biosciences	566749	
	CD45	FITC	HI30	Mouse IgG1, Kappa	1:100	Flow cytometry	Biolegend	304054	
	CXCR3	BV711	G025H7	Mouse IgG1, Kappa	1:100	Flow cytometry	Biolegend	353732	
	TNF	BV605	MAb11	Mouse IgG1, Kappa	1:100	Flow cytometry	Biolegend	502936	
	ID2	PE-Cy7	ILCID2	Mouse IgG1, Kappa	1:100	Flow cytometry	Thermo-Fisher	25-9475-82	
	GATA3	BV421	16E10A23	Mouse IgG2b, kappa	1:100	Flow cytometry	Biolegend	653814	
	IRF4	PERCP-Cy5.5	IRF4.3E4	Rat IgG1, Kappa	1:100	Flow cytometry	Biolegend	646416	

	ID3	PE	S30-778	Mouse IgG1, Kappa	1:100	Flow cytometry	BD Biosciences	564564	
	CD4	BV510	OKT4	Mouse IgG2b, kappa	1:100	Flow cytometry	Biolegend	317444	

Materials Table

	Name	Manufacturer
	RBC lysis buffer	Sigma
	RPMI-1640 medium	Gibco
	Liberase	Sigma-Aldrich
	DNaseI	Roche
	70mm cell strainer	Olympus Plastics
	PBS	Gibco
	Zombie NIR	Biolegend
	FBS	Sigma
	Tru-stain Fc block	Biolegend

CAR T Panels Table

	HUMAN Panel 1 main		HUMAN panel 2		Human Panel 3		Antigen	fluorophore			
	GFP	CD45		GFP	CD45		GFP	CD45		Zombie live/dead	APCy7
	MYC	APC		MYC	APC		MYC	APC		CD3	BUV737
	Live Dead	APCcy7		Live Dead	APCcy7		Live Dead	APCcy7		CD8	BUV395

	CD3	BUV737		CD3	BUV737		CD3	BUV737		CD4	BV510
	CD8	BUV395		CD8	BUV395		CD8	BUV395		TiGIT	BV605
	TIGIT	BV605		CD39	PECF594		TNF	BV605		LAG3	BV786
	LAG3	BV786		CD56	BV605		LAG3	BV786		PD1	BV711
	CD127	PERCPcy5.5		CD45RO	BV786		TIM3	PEcy5		TIM3	PEcy5
	Granzyme B	Pecy7		CD45RA	PEcy5		ID2	PeCy7		TCF1	BV421
	PD1	BV711		CD28	PeCy7		CXCR3	BV711		Ki67	BV510
	TIM3	PEcy5		CCR7	BV711		CD4	BV510		ID3	PE
	TCF1	BV421		CD62L	BV510		GATA3	BV421		ID2	PEcy7
	Ki67	BV510		CTLA4	BV421		IRF4	PERCP cy5.5			
	IFNg	PE		Tbet	PERCP cy5.5		ID3	PE			
	CD39	PECF594		EOMEs	PE						

Tissue collection

1 Place mice under deep isoflurane anesthesia (5% isoflurane, minimum of 15 minutes)

2 Spray the abdomen of the mouse with 70% ethanol, this is to avoid fur contamination of samples

3 Perform a bilateral thoracotomy, cut the right atrium of the heart.

4 Collect blood using a 1ml pipette and place in a labelled EDTA tube (2ml K2 coated EDTA)- mix and store at room temperature (RT) until processing.

5 Dissect the tumor from the skin, place in a labelled 5ml tube containing ice cold RPMI 1640 (no serum); place sample on ice until processing.

Tissue processing

6 Blood

6.1 Add 1ml RBC lysis buffer (Thermo) directly to the blood tube

6.2 Incubate 10min at RT

6.3 Centrifuge (500Gx3min) at RT

6.4 Remove supernatant and discard

6.5 Repeat steps 6-9

6.6 Resuspend cells in 1ml PBS;

6.7 Transfer to a labelled FACS tube and place at 4^{O}C until downstream processing

6.8 Immediately prior to staining with Zombie live/dead dye, centrifuge (500Gx3min), and dump supernatant, retaining the cell pellet

7 Tumor

7.1 Prepare dissociation media, enough for 5ml per tumor (e.g. if 15 tumors prepare 75ml), mix well.

7.2 a. HBSS (5ml per tumor)

7.3 b. Liberase (5mg/ml): 1:100 dilution (e.g. 750ul in 75ml)

7.4 c. DNAse (10mg/ml): 1:500 dilution (e.g. 150ul in 75ml).

7.5 Place 5ml of dissociation solution in a 50ml conical for each mouse tumor labelled with corresponding ID

7.6 Remove the tumor with forceps and place in a 60mm dish

7.7 Use a #10 scalpel to mince the tumor tissue

7.8 Transfer the minced tumor tissue into the 50ml conical containing 5ml dissociation media- using the scalpel to dump tumor tissue into the tube

7.9 Clean the forceps and scalpel in 70% ethanol.

7.10 Repeat steps 16-19 until for all tumors.

7.11 Once all samples are minced and in dissociation media, put tubes in a 37C shaker set to rotate at 200rpm for 45min.

7.12 After incubation in step 21, use a 5 ml serological pipet to pipet up and down tumor tissue, force through a 70micron cell strainer back into the same 50ml tube, sliding the pipet back and forth over the cell strainer to mechanically dissociate any remaining fragments.

7.13 10. Using the same strainer, pipette the solution through the strainer into corresponding FACs tube

7.14 11. After all samples are complete, spin at 500gx3min at RT

7.15 12. Discard supernatant.

Staining

8 Tumor and Blood

8.1 Ensure no more than ~50ul volume of cell pellet (using reference FACs tube with 50ul water as guide) is stained, remove cell volume as needed to maintain under this amount; excessive cell density can impair staining and introduce artifacts.

8.2 Pulse vortex to disperse pellets

8.3 Add 100ul of PBS containing 1:500 Zombie NIR (Biolegend, diluted in 100ul per manufacturer instructions) and 1:500 DNAse I (to prevent clumping).

8.4 Pulse vortex

8.5 Incubate for 15min at RT

8.6 Pulse vortex

8.7 Add 1ml of FACs buffer (2% FBS in PBS) to each sample to neutralize the Zombie staining

8.8 Spin 500G x 3min, dump and dab

8.9 Pulse vortex

8.10 10. Add mouse and human FC block at 1ul per sample in FACS buffer at 100ul per tube (make a master mix in FACs buffer for ease, e.g. 100x # samples- total volume, 1ul x #samples for FCX amount- add a few to the # samples to account for dead volume).

8.11 11. Pulse vortex after adding the 200ul FCX solution for each tube.

8.12 12. Transfer 50ul of each sample to second correspondingly labelled tube (4 panels, thus, for sets of tubes). Incubating at RT for at least 10min after FCX solution addition.

8.13 13. Within each tube panel series, generate pooled isotype control florescence minus one (FMO) controls by taking 5-10ul of each sample and transferring to isotype controls.

8.14 14. Resuspend antibody master mixes (see panels spreadsheet, all at 1:100 dilution, note include separate isotype control antibody master mixes) in enough FACs buffer to transfer 50ul per tube (need 500ul total volume per 10 tubes- but add a few samples to account for dead volume). Add 1:500 DNAse I.

8.15 15. Add antibodies to each tube (minimum incubation of FCX block is 10min)

8.16 16. Vortex

8.17 17. Incubate for 20 minutes at RT

8.18 18. Vortex again

8.19 19. Incubate another 20 minutes at RT

8.20 20. During the incubation prepare 1x Fix/Perm buffer, enough for 500ul per tube (Invitrogen FOXP3 Staining kit II, per manufacturer's instructions)

8.21 21. Add 500ul of FACs buffer per tube

8.22 22. Vortex

8.23 23. Centrifuge at 500G x 3min at RT

8.24 24. Resuspend cells in 500ul 1x Fix/perm buffer (step 43)

8.25 25. Incubate for 45min- 1 hour

8.26 26. Add 1ml 1x Perm buffer (Invitrogen FOXP3 Staining kit II, per manufacturer's instructions)

8.27 27. Centrifuge at 500G x 3min at RT

8.28 28. Resuspend cells in 100ul 1x Perm buffer containing 1:100 mouse and human FC block, in addition to panel specific antibodies (at 1:100); along with panel specific isotype controls.

8.29 29. Incubate overnight at 4C.

8.30 30. Pulse vortex to resuspend cells

8.31 31. Wash cells in 500ul 1x Perm buffer

8.32 32. Centrifuge at 500G x 3min at RT

8.33 33. Dump supernatant, dab tube

8.34 34. Pulse vortex to disperse pellet

8.35 35. Resuspend cells in 115ul of FACs buffer containing 1:500 DNAse I

8.36 36. Vortex

8.37 37. Cells are ready for analysis on the Flow Cytometer