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Mitochondrial Antigen Presentation (MitAP) to 2CZ CD8+ T cell hybridoma

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ASAP Collaborative Rese…

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We use this protocol and it's working

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Abstract

This protocol details the Mitochondrial Antigen Presentation (MitAP) to 2CZ CD8+ T cell hybridoma. Previously, quantification of the number of IL-2 producing 2cz hybridoma cells in an ELISPOT assay was used as a readout for MitAP (Matheoud et al., 2016). Here, we adapted the assay for the detection of activation-induced markers (AIM) on 2cz cells with flow cytometry.

Materials

Reagents:

- Recombinant Mouse GM-CSF (carrier-free) **BioLegend Catalog #**576308
- The OGDH/Ld and OGDH/Kb-restricted 2CZ CD8T+ cell hybridoma.
- Complete RPMI media: RPMI 1640 with GLUTAMAX

	A	B
	Heat inactivated FBS (Wisent)	10%
	NEAA	1%
	Sodium pyruvate	1%
	Hepes	1mM
	penicillin/streptomycin	1%

- PBS without Ca2+/Mg2+
- 1M Glycine in PBS
- 4% paraformaldehyde (PFA)
- SIYRYYGL peptide (Genscript) 0.5 mg/ml
- Tag-it Violet (Biolegend, Cat#455101)
- LPS-EB (LPS from E. coli O111:B4) **InvivoGen Catalog #**tlrl-3pelps

9 days prior to experiment

1 Collect bone marrow from femur of mice and start Bone Marrow Dendritic Cell (BMDC) culture with 20 undetermined mrGM-CSF (according to BMDC protocol).

Three days prior to experiment (minimum)

2 Start 2CZ hybridoma from a frozen stock. Maintain the hybridomas in RPMI-1640 medium supplemented with 5% (v/v) FCS, glutamine ([M] 2 millimolar (mM) , penicillin (100 U ml^{-1}) and streptomycin (100 µg ml^{-1}).

3 Culture 2CZ cells at no point exceeding 10^6 cell/ml in complete RPMI in flasks for suspension cell culture (2CZ on average undergo 2 divisions per day).

On the day of experiment:

14h 40m

4 Stimulate your antigen presenting cells (APC) with 80-100 ug/ml of Helicobacter pylori and sonicate for 06:00:00 . Control - unstimulated APC.

6h

5 Collect APC gently with cell scraper into 50 ml tubes, wash with ice cold PBS and spin down at 600 x g, 4°C, 00:05:00 .

5m

6 Discard supernatant and resuspend APC pellet in 2 mL of 1% PFA (dilute stock 4% PFA in PBS), leave for fixation 00:09:00 at Room temperature .

9m

7 Quench PFA with at least 9 times exceeding volume (with 18 mL in case of 2 mL PFA) of [M] 0.1 Molarity (M) Glycine in complete RPMI (dilute [M] 1 Molarity (M) Glycine with complete RPMI 1:9).

8 Spin 600 x g, 4°C, 00:05:00 and decant.

5m

9 Repeat step 7-8 two more times.

10 Resuspend in complete RPMI, count cells and bring the concentration of fixed APC to 10^6 cell/ml.

11 Collect 2CZ cells from a culture flask and wash it in PBS.

12 Gently mix a pellet of 2cz cells with 1 mL of cell tracker diluted in PBS to [M] 0.63 micromolar (µM) (Tag-it Violet Biolegend).

13 Incubate 00:12:00 at 37 °C . 12m

14 Add 1 mL of FBS and incubate another 00:05:00 at Room temperature in the dark. 5m

15 Add 13 mL of complete RPMI mix and spin down at 400 x g, 4°C, 00:05:00 . 5m

16 Wash two more times with complete RPMI, count and bring to a concentration 0.5x10^6 cell/ml.

17 Co-culture at 37 °C 5% CO2 Overnight fixed APC with Tag-itViolet+2CZ in round bottom 96 well plate in technical triplicates. 50,000 2cz cells – 100 µL and 100,000 APC in another 100 µL per well. 8h

- Negative control 100 µL of 2cz cells plus 100 µL of complete RPMI; Positive control 100 µL of 2cz cells plus 100 µL of 0.2 undetermined SIYRYYGL peptide in complete RPMI.

Next morning

18 Collect cells and proceed to staining for flow cytometry (Viability and activation induced markers (AIM): CD69, CD137; PD1)

19 For analysis in FlowJo gate on live (Viability stain negative), Tag-it violet+ single cells.

20 Make a positive gate on CD69/PD1/CD137.

21 Use Tools → Boolean to create OR gates to acquire total activated 2CZ cells.

22 Deduct from % AIM+ in samples % of AIM+ in of 2CZ cells cultured without any APC.

23 Discard results if AIM% of 2CZ with SIYRYYGL well doesn't exceed 2CZ alone.

Protocol references

Matheoud, D., Sugiura, A., Bellemare-Pelletier, A., Laplante, A., Rondeau, C., Chemali, M., Fazel, A., Bergeron, J. J., Trudeau, L.-E., Burelle, Y., Gagnon, E., McBride, H. M., & Desjardins, M. (2016). Parkinson's Disease-Related Proteins PINK1 and Parkin Repress Mitochondrial Antigen Presentation. Cell, 166(2), 314–327. doi:10.1016/j.cell.2016.05.039