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

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Alexandra Kazanova¹

¹McGill Research Centre on Complex Traits and Department of Microbiology and Immunology, McGill University



Alexandra Kazanova

McGill University

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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)
- SIYRYGL 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 μL 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 ( 2 millimolar (mM) , penicillin (100 U ml^{-1}) and streptomycin ($100 \mu\text{g ml}^{-1}$).
- 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 $\mu\text{g/ml}$ of *Helicobacter pylori* and sonicate for  06:00:00 . Control - unstimulated APC. 
- 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 . 
 
- 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 . 
- 7 Quench PFA with at least 9 times exceeding volume (with  18 mL in case of  2 mL PFA) of  0.1 Mass Percent Glycine in complete RPMI (dilute  1 Mass Percent Glycine with complete RPMI 1:9).
- 8 Spin  600 x g, 4°C , 00:05:00 and decant. 

- 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  0.63 micromolar (μM) (Tag-it Violet Biolegend). 
- 13 Incubate  00:12:00 at  37°C . 

- 14 Add  1 mL of FBS and incubate another  00:05:00 at  Room temperature in the dark. 
 
- 15 Add  13 mL of complete RPMI mix and spin down at  $400 \times g$, 4°C , 00:05:00 . 
 
- 16 Wash two more times with complete RPMI, count and bring to a concentration 0.5×10^6 cell/ml. 
- 17 Co-culture at  37°C 5% CO_2  Overnight fixed APC with Tag-itViolet+2CZ in round bottom 96 well plate in technical triplicates. 50,000 2cz cells –  $100 \mu\text{L}$ and 100,000 APC in another  $100 \mu\text{L}$ per well. 
- Negative control  $100 \mu\text{L}$ of 2cz cells plus  $100 \mu\text{L}$ of complete RPMI; Positive control  $100 \mu\text{L}$ of 2cz cells plus  $100 \mu\text{L}$ of  $0.2 \mu\text{L}$ 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.
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