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Mitochondrial antigen presentation (MitAP) to primary 2C CD8 T (proliferation and suppression) assay

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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 primary 2C CD8 T (proliferation and suppression) assay. In order to assess MitAP in an assay that incorporated all signals for naïve T cells activation in vivo, we tested whether H.pylori-exposed BMDCs could trigger activation of mitochondria-reactive primary CD8 T cell.

Materials

Complete RPMI 1640:

	A	B
	RPMI 1640 with Glutamax (Gibco)	with 10% FBS
	sodium pyruvate	1%
	Hepes	1 mM
	penicillin/streptomycin	1%

MACS buffer: PBS, 2% FBS and 2mM EDTA

StemCell Isolation Buffer: PBS, 2% FBS and 1 mM EDTA

ACK (Ammonium-Chloride-Potassium) red blood cell lysis buffer: 8.29g NH4Cl, 1g KHCO3, 0.0367g EDTA, distilled water to 1L)

090-150- FBS, Premium Canadian Origin, 500mL **Wisent Bioproducts Catalog #**090-150

PBS 10x (Sigma; Cat# P5493-4L)

Phosphate buffered saline **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**P5493-4L without calcium/magnesium

Recombinant Mouse GM-CSF (carrier-free) **BioLegend Catalog #**576308

Purified anti-mouse CD3ε Antibody **BioLegend Catalog #**100302

EasySep™ Mouse CD8+ T Cell Isolation Kit For processing 1 x 10^9 cells **STEMCELL Technologies Inc. Catalog #**19853

EasySep™ Magnet For isolating 2.5 x 10^8 cells **STEMCELL Technologies Inc. Catalog #**18000

CD4+CD25+ Regulatory T Cell Isolation Kit, mouse **Miltenyi Biotec Catalog #**130-091-041

CFSE Cell Division Tracker Kit **BioLegend Catalog #**423801

eBioscience™ Fixable Viability Dye eFluor™ 780 **Thermo Fisher Catalog #**65-0865-18

Anti-mouse CD8a; -FoxP3; CD4; CD25 conjugated to selected fluorochromes (except PE).

e-Bioscience Foxp3 / Transcription Factor Staining Buffer Set **Invitrogen - Thermo Fisher Catalog #** 00-5523-00

MACS® LD and LS Columns Catalog #130-042-401, Catalog #130-042-901

QuadroMACS™ Separators

Tag-it Violet Biolegend Cat#455101

H. Pylori PMSS1 strain sonicate

70 µm cell strainers

Paraformaldehyde 1%

1M glycine in PBS

Mice

Pink1$^{+/+}$ (n=6) and Pink1$^{-/-}$ mice (n=6). 2C TCR transgenic mouse (n=1-2). OT-1xRAG1-/- (n=1-2).

Bone Marrow-derived Dendritic cells (BMDC, 0-9 days of experiment): 1m

1 Femurs from three Pink1$^{+/+}$ and three Pink1$^{-/-}$ mice are cleaned manually from soft tissues and flushed with ice cold PBS.

2 Cells are pelleted and 2 mL of ACK RBC lysis buffer are added for 00:01:00 and mixed gently with 1 ml pipette tip. 1m

3 Lysis buffer is blocked with 13 mL of complete RPMI and pelleted with centrifugation.

4 Cells are resuspended, counted with hemocytometer and brough to 200,000 cell/ml in complete RPMI.

5 10 ng/ml of mr-GMCSF is added to BMDC for differentiation.

6 Cells are plated in non-tissue culture treated plasticware (6 well plate, sarstedt) for 72:00:00 at 37 °C . 3d

7 On day 3 equal volume of the starting culture of complete RPMI with 10 ng/ml mr-GMCS is gently added on top of the well (avoid any additional shaking of the cells).

8 On day 6 replace 50% of the culture media with new complete RPMI with 10 ng/ml mrGMCSF.

9 On day 9 collect all non-adherent and loosely adherent cells and count. Bring to 500,000 cell/ml in complete RPMI.

10 3 mL of BMDC cells/ per well of a 6-well plate. Add sonicate of H. pylori 100 µg/ml (300 µg/well) and incubate for 06:00:00 at 37 °C . Leave at least two wells without bacterial sonicate for a control. 6h

11 After 6 hours collect BMDC gently using cell scrapper and wash.

12 Pellet cells by spinning down at 800 x g, 00:05:00 (Eppendorf 5810 – 2000 RPM). 5m

13 Remove supernatant completely and add 2 mL of Room temperature (RT) 1% PFA and mix gently with a pipette. Leave at RT for 9 minutes.

14 Quench with 13 ml of 0.1M Glycine in complete RPMI (1 1M Glycine : 9 Complete RPMI).

15 Pellet cells by spinning down at 800 x g, 00:05:00 and dumping the supernatant. 5m

16 Repeat go to step #13 - go to step #15 two more times.

17 Resuspend in complete RMPI count and bring to 1x10^6 cells/ml .

2C CD8 T cells (day 9)

18 Collect spleens from 2C TCR transgenic mice of 6-8 weeks old, verify no presence of thymoma in the mouse.

19 Mash spleen through a PBS pre-primed 70 µm cell strainer using a 3 ml syringe plunger. Wash strainer with additional 10 mL of PBS.

20 Add 3 mL of ACK RBC lysis buffer to the splenocyte pellet and leave for 00:01:00 , mix gently. 1m

21 Block lysis buffer with 12 mL of complete RPMI, count cells and take 100 µL aliquot for purification control (a) into a separate Eppendorf tube.

22 Proceed with EasySep™ Mouse CD8+ T Cell Isolation Kit (Stemcell; Cat#19853) according to the manufacturer protocol. After spinning resuspend cell pellet in Stemcell Isolation Buffer 10^8/ml.

23 Transfer cell to a 5 ml round bottom conical tube and add 20 µL of Fc Blocking reagent per 1 ml of cell suspension.

24 Add 50 µl/ml of Isolation cocktail to cell suspension and incubate 00:20:00 On ice . 20m

25 Add 125 µl/ ml of pre-vortexed for 00:00:30 Rapid spheres and incubate 00:05:00 at Room temperature 5m 30s

26 Top to 2.5 mL with Stemcell isolation buffer, transfer to EasySep™ Magnet and let stand for 00:03:00 in the hood. 3m

27 Dump negatively selected CD8 T cells from the falcon tube in the magnet to a new 15 ml conical tube. Take a 50 µL aliquot of cell suspension for purification control (b).

28 Top the 15 ml tube with PBS and spin down at 450 x g, 00:05:00 (Eppendorf 5810 – 1500 rpm). 5m

29 Stain pelleted 2C CD8 T cells with 2 mL 2.5 µM cell tracker Tag-it Violet in PBS 00:12:00 at 37 °C in the dark. 12m

30 Add 2 mL of the FBS and incubate an additional 5 min in the dark at Room temperature to efflux and quench excessive Tag-it violet dye.

31 Wash cells three times with 10 ml of complete RPMI.

32 Resuspend in complete RMPI count and bring to $1x10^6$ cells/ml.

33 Take an aliquot of 500 µL Tag-it Violet-2C CD8 T cells and store in the fridge (a undiluted Tag-it violet signal, one of undivided cells control for flow cytometry).

34 Stain purification controls (a) and (b) with antiCD8 and viability to access CD8 T cell purification quality.

OT-1 CD8 T cells (TCR Tg CD8 T cell control)

35 collect inguinal, popliteal, axillar, and neck subcutaneous lymph nodes from OT-1 x Rag1 -/- mice.

> **Note**
>
> Make sure not to take any fat tissue.

36 Mesh lymph nodes through a pre-primed with PBS 70 µm 1 ml syringe plunger.

37 Wash the strainer with 10 mL of PBS. Take 500 µL aliquot for CD8 frequency assessment using flow cytometry.

38 Spin cells down 450 x g, 00:05:00 . 5m

39 Stain pelleted OT-1 CD8 T cells with 2 ml 2.5 µM cell tracker Tag-it Violet in PBS 00:12:00 at 37 °C in the dark. 12m

40 Add 2 mL of the FBS and incubate an additional 00:05:00 in the dark at Room temperature to efflux and quench excessive Tag-it violet dye. 5m

41 Wash cells three times with complete RPMI.

42 Resuspend in complete RMPI count and bring to 1x10^6 cells/ml.

CD4CD25- (CD4 conv) and CD4CD25 T (Tregs) (day 9) 3h 40m

43 Collect spleens of three Pink1$^{+/+}$ and three Pink1$^{-/-}$ mice.

44 Mash spleen individually through the 70 µm cell strainers and lyse RBC (as in step 20-21).

45 Pull three spleens of each genotype together and count cells with hemocytometer and take a 100 µL aliquot for purification control (c). (Expected spleen count for B6.129 mixed genetic background mouse spleen is 5x10^{7}).

46 Spin cells down at 450 x g, 00:05:00, resuspend pellets in 600 µL of MACS buffer per 1.5x10^{8} (adjust the volume for more cells) and transfer to a new labeled 15 ml conical tube. 5m

47 Add 150 µL of the CD4+CD25 regulatory T cell Biotin-Antibody Cocktail (100 µl/ 400µl cell suspension) and incubate On ice 00:15:00. 15m

48 Wash cells by adding 10 mL of MACS buffer and spinning it down 450 x g, 00:05:00. 20m
Resuspend pellets in 1200 mL of MACS buffer (800/ 10^{8}) and add 300 µL of anti-Biotin MicroBeads (200/10^{8} cells) incubate 00:15:00 On ice.

49 Immediately transfer the QuadroMACS separator with two LD columns into the hood and prime columns with 2 mL of MACS buffer.

50 Transfer cells to the columns and collect negatively selected unlabeled total CD4 T cells to a new 15 ml labeled conical tubes.

51 Wash columns three times with 1 mL MACS buffer (passing through LD columns take a long time, make sure collection tubes under the columns is placed On ice).

52 Take a 50 µL aliquot of total CD4 T cells in a separate Eppendorf cup – control (d). Spin cells down and add to the pellet 600 µL of MACS buffer with 15µl of anti CD25-PE.

53 Incubate On ice 00:20:00 in the dark. 20m

54 Top with 10 of MACS buffer, mix and take 100 µL aliquote (control e).

55 After spinning cells down 450 x g, 00:05:00 aspirate supernatant and resuspend cells in 1350 of buffer (900/10^8). 5m

56 Add 150 µL of anti-PE microbeads (100/108) and place On ice for another 00:15:00 . 15m

57 Place 2 LS columns on the magnet and prime them with 3 ml buffer and discard the pass through.

58 Transfer samples to the columns and collect the pass through into a new 15 ml conical tube on ice labeled CD4+CD25-.

59 Wash column with 3 ml buffer three times and collect flow through. Take a 500 µL aliquot from the flow through for a control (f).

60 Spin CD4 CD25- cells down 450 x g, 00:05:00 and resuspend in 2 ml of complete RPMI with 1 µg/ml anti-CD3 (145-2c11) and place in the CO_2 incubator to culture for 01:00:00 at 37 °C . 1h 5m

61 Simultaneously with the go to step #60 transfer LS columns from the magnet to the new conical tube labeled CD4+CD25+.

62 Add 3 mL of buffer to the LS column and use column plunger to isolate CD25+ cells.

63 Replunge dry columns 5 times to completely recover the cells.

64 Add 10 mL MACS buffer and spin down the cell.

65 Completely remove the supernatant containing unbound microbeads and resuspend in 1 mL of complete media (take 30 µL aliquot for a control (g).

66 Immediately acquire control f and g (10 µl only) on a cytometer, using PE channel if purity of control (g)- PE+ cells are lower than 85% repeat steps [go to step #57 - go to step #59 and go to step #61 - go to step #63] one more time. If control f has CD25+ cells more than 1% repeat steps [- go to step #60] one more time.

67 Transfer controls d-g to a plate for staining with FoxP3 antibody.

68 Spin plate down and stain viability with FVS780 in PBS dilution 1:2000 50 µl per well, 00:15:00 On ice in the dark. 15m

69 Wash viability stain with 300 µL of MACS buffer and leave cells for fixation in FoxP3 fixing buffer in the fridge 120 µl/well.

70 After an hour take CD4+CD25- cells from the incubator and top the tube with PBS.

71 Spin CD4+CD25- cells down and resuspend in 2 ml of CFSE 1.25 µM stain in the same manner as B12-B14.

72 While staining CD4+CD25- count CD4CD25+ and bring them to the concentration of 10^6 cells/ml in complete RPMI.

73 Resuspend CFSE-CD4+CD25 in 3 ml complete RPMI, take 100 µL aliquote for undilute CFSE staining control and place it in the fridge.

74 using CD4+CD25+ Regulatory T Cell Isolation Kit (Miltenyi Biotech; Cat #130-091-041) according to manufacturer's protocol. CD4 conv T cells were resuspended in complete RPMI 10% FBS and incubated for 01:00:00 at 37 °C with 1 µg/ml of anti-mouse CD3 (145-2c11 clone; Biolegend; Cat#100363) or left untreated. Cells were washed in PBS and stained with CFSE 1.25 µM in the same manner as Tag-it violet staining. 1h

75 Purity of magnetic selection of 2C CD8, CD4+CD25+ and CD4+CD25- cells was determined with flow cytometry prior to co-culture. Regulatory CD25+CD4 T cells were stained with anti-FoxP3 and acquired with flow cytometry.

76 Co-culture was performed in 10% FBS complete RPMI in 96-well round bottom plates for suspension cells (Sarstedt; Cat#83.3925.500) at a following ratio: 50,000 2C to 50,000 CD4 conv cells to 100,000 fixed BMDC. For suppression assay Tregs were added to the co-culture at (1-0.25):1 2C CD8 T ratio. Each condition was performed in technical replicates. At 24- and 72-hours cells were collected and assessed by flow cytometry for Activation Induced Markers expression and proliferation.

77 Co-culture conditions for proliferation assay (excluding single cell type controls, and cell tracker controls from the fridge):

	A	B	C	D	E	F	G	H	I
	BMDC	*H. pylori* for BMDC	CD4 conv	aCD3 for CD4	OT-1 CD8T	2C CD8 T	SIINFEKL	SYIRYYGL	Function
	+	-	-	-	+	-	+	-	OT-1 proliferation – positive control
	+	-	-	-	+	-	-	-	OT-1 proliferation – negative control
	+	-	-	-	-	+	-	+	2C proliferation – positive control
	+	-	-	-	-	+	-	-	2C proliferation – negative control
	+	+	+	+	-	+	-	-	2C proliferation - experimental condition
	+	+	+	+	+	-	-	-	TCR 2C specificity control
	+	-	+	+	-	+	-	-	2C No proliferation – infection effect on MitAP control

	A	B	C	D	E	F	G	H	I
	+	+	+	-	-	+	-	-	CD4 help effect control
	+	+	-	-	-	+	-	-	CD4 help effect control
	-	-	+	+	+	-	-	-	Bystander activation control OT1
	-	-	+	+	-	+	-	-	Bystander activation control 2C
	+	+	+	+	-	-	-	-	CD4 proliferation
	+	-	+	+	-	-	-	-	CD4 proliferation
	-	-	+	+	-	-	-	-	CD4 proliferation
	-	-	+	-	-	-	-	-	CD4 proliferation negative control

78 Suppression of proliferation was calculated using the following formula:

% suppression = 100x(1-% of divided 2C in the presence of Treg/ -% of divided 2C at No Treg condition)