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T cell differentiation from mice spleen tissue

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ABSTRACT

This protocol is for T cell differentiation from mice spleen tissue to investigate T cell function (including differentiation and proliferation) in vitro.

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We use this protocol and it's working

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MATERIALS

1. Pre coating of 96 well plate with antibodies using following reagents

A	B	C
Reagent	Final concentration	Amount
PBS	n/a	100mL/well
Anti-CD3e (0.5mg/mL)	2.5mg/mL	0.5mL/well
Anti-CD28 (1mg/mL)	1mg/mL	0.1mL/well

2. **Prepare FACS buffer and T cell culture media (See Appendix 1 below)**

A	B	C
FACS buffer		
Reagent	Final concentration	Amount
PBS	n/a	490mL
FBS	2%	10mL
Total	n/a	500mL

Note: Can be prepared in advance and stored at 4℃ for at least 4 months. Filtration through a
0.2 µm vacuum filter is recommended.

A	B	C
T cell culture media		
Reagent	Final concentration	Final concentration
MEMα	n/a	429mL
FBS	10%	50mL
GlutaMAX 100×	2×	10mL
HEPES, 1M	0.02M	10mL

	A	B	C
	2-mercaptoethanol (55 mM)	55μM	0.5mL
	Normocin, 1g (50mg/ml)	50μg /ml	0.5mL
	Total	n/a	500mL

Note: Can be prepared in advance and stored at 4℃ for up to 1 month.

2. Prepare T-cell differentiation cocktails [1-4] (Appendixes 2 below)

	A	B	C
	Th0 differentiation cocktail (2.5×)		
	Reagent	Final concentration	Amount
	T cell culture media	n/a	1mL
	IL-2 (5×105U/mL)	100U/mL	0.5mL

	A	B	C
	Th1 differentiation cocktail (2.5×)		
	Reagent	Final concentration	Amount
	T cell culture media	n/a	1mL
	IL-2 (5×105U/mL)	100U/mL	0.5mL
	IL-12 (10μg/mL)	4ng/mL	1mL
	Anti-IL-4 (10mg/mL)	10μg/mL	2.5mL

	A	B	C
	Th2 differentiation cocktail (2.5×)		
	Reagent	Final concentration	Amount
	T cell culture media	n/a	1mL

	A	B	C
	IL-2 (5×105U/mL)	100U/mL	0.5mL
	IL-4 (10μg/mL)	10ng/mL	2.5mL
	Anti-IFNg (10mg/mL)	10μg/mL	2.5mL

	A	B	C
	Th9 differentiation cocktail (2.5×)		
	Reagent	Final concentration	Amount
	T cell culture media	n/a	1mL
	IL-2 (5×105U/mL)	100U/mL	0.5mL
	IL-4 (10μg/mL)	10ng/mL	2.5mL
	Anti-IFNg (10mg/mL)	10μg/mL	2.5mL
	TGF-b (5μg/mL)	1ng/mL	0.5mL
	IL-1b (10μg/mL)	10ng/mL	2.5mL

	A	B	C
	Treg differentiation cocktail (2.5×)		
	Reagent	Final concentration	Amount
	T cell culture media	n/a	1mL
	IL-2 (5×105U/mL)	300U/mL	1.5mL
	Anti-IFNg (10mg/mL)	10μg/mL	2.5mL
	TGF-b (5μg/mL)	5ng/mL	2.5mL
	Anti-IL-4 (10mg/mL)	10μg/mL	2.5mL

Note: Cocktails can be prepared in advance and stored at 4℃ for several days, seed 100mL in each well.

3. **Reagents or Resources**

A	B	C
MEMα	Fisher Scientific	Cat#32-571-101
FBS	R&D Systems	Cat#S11150
GlutaMAX 100×	Fisher Scientific	Cat#35050061
HEPES	Fisher Scientific	Cat#MT25060CI
2-mercaptoethanol	Fisher Scientific	Cat#21985023
Anti-CD3e	Cytek Biosciences	Cat#145-2C11
Anti-CD28	BD Biosciences	Cat#553294
IL-2	Prometheus Laboratories	Cat#NDC65483-116-07
IL-12	PeproTech	Cat#210-12
TGF-b1	BioLegend	Cat#763104
IL-1b	R&D Systems	Cat#401-ML/CF
IL-4	PeproTech	Cat#214-14
Anti-IL-4	BioXCell	Cat#BE0045
Anti-IFNg	BioXCell	Cat#BE0055
Mouse CD4+ T-cell Isolation Kit	STEMCELL Technologies	Cat#19852
Mouse IFN-gamma DuoSet ELISA	R&D Systems	Cat#DY485
Mouse IL-4 DuoSet ELISA	R&D Systems	Cat#DY404

	A	B	C
	Mouse IL-9 DuoSet ELISA	R&D Systems	Cat#DY409
	Foxp3/transcription factor staining buffer set	Fisher Scientific	Cat#00-5523-00
	Anti-CD4-eFluor 450	eBioscience	Cat#48–0042–82
	Anti-CD25-PerCP	TONBO Biosciences	Cat#65-0251
	Anti-Foxp3-PE	Fisher Scientific	Cat#12-5773-82
	PMA	Sigma-Aldrich	Cat#P1585

Day 0

1 Prepare precoated 96-well plate using antibodies described in materials section.

Day 1

2 Prepare single-cell suspensions of mice spleen tissues as per steps below

3 Euthanize mice by CO_2 inhalation or other means of euthanasia.

4 Collect the spleen from the mice.

5 Put a 40μm vacuum filter on a 50mL tube. Mesh the spleen on the strainer using the back of a syringe.

6 Add 5mL of RPMI medium to the mashed spleen to collect splenocytes into a 50mL tube.

7 Spin down cells at 1500rpm for 3min. Discard supernatant.

8 Add 0.5mL ACK lysis buffer to the red cell pellet, resuspend, and wait 30 seconds to lyse RBC.

9 Directly add 5mL T cell medium.

10 Spin down cells at 1500rpm for 3min. Discard supernatant.

11 Prepare a new 50mL tube with a new 40μm vacuum filter.

12 Resuspend cells in 10mL T cell medium and filter through a new 40μm vacuum filter.

13 Count the cells.

Isolation of naïve CD4+ T cells from single-cell suspensions from spleen ti…

14 Naïve $CD4^+$ T cells were isolated from single-cell suspensions from spleen tissue by negative selection using the EasySep Mouse $CD4^+$ T-cell Isolation Kit (#19852, STEMCELL Technologies, Vancouver, Canada).

T cell differentiation on Day 1

15 Seed the cells into plate and culture with different conditions

16 Regulate the cell concentration at $5×10^5$/mL with T cell medium

	A	B	C
	Naïve CD4+ T cells	5×104/well	100μL/well
	T cell medium		50μL/well
	Total	Total	150μL/well

17 Take out the pre-coated plate from 4°C.

18 Remove the supernatant and add 200μL PBS to the pre-coated plate and then remove PBS to wash the coated plate. Repeat 2 times.

19 Seed wells of a 96-well plate with 150µL cells cocktail (100µL cells and 50µL T cell media.

20 Seed 100µL differentiation cocktail.

21 Incubate for 5 days at 37°C.

Detect T cell differentiation by ELISA on Day 5 and Day 6 (Note: The chose…

22 Collect cells and count.

23 Regulate cell concentration at 1×10^6/mL.

24 Seed 100µL cells into a new 96-well plate (U bottle)

25 Add 100µL PMA cocktail and culture overnight. For non-stimulate cells, add 100µL T cell medium.

	A	B	C
		Final concentration	Amount
	T cells	1×106/mL	100µL
	T cell Medium	n.a	100µL

	A	B	C
	PMA (50μg/mL)	50ng/mL	0.2μL

26 Collect supernatant for ELISA. (Note: The supernatant can be frozen at -20°C.)

Detect Treg cell differentiation by Flow cytometry on Day 5

27 Spin down cells at 1500rpm for 3min at RT.

28 Remove the supernatant over the sink in one motion.

29 Add 150μL FACS buffer and spin down cells at 1500rpm for 3min at RT. Remove the supernatant.

30 Surface staining, add 50μL FACS Buffer mAbs cocktail:

	A	B
	FACS buffer	50μL
	Anti-CD4-eFluor 450	0.3μL
	Anti-CD25-PerCP	0.3μL

31 Incubate at 4°C for 30min in the dark.

32 Directly add 150uL FACS buffer and spin down cells at 1500rpm for 3min at RT.

33 Remove the staining buffer and thoroughly resuspend cells.

34 Add 200µL of Foxp3 Fixation/Permeabilization working solution to each well for 1 hour at RT in the dark. (4×stock solution must be diluted prior to use with the Fixation/Permeabilization Diluent dilute 1 part concentrate with 3 parts diluents, make fresh).

35 Centrifuge samples at 400-600g for 5min at RT.

36 Add 200µL 1×Permeabilization Buffer (make fresh) to each well and centrifuge samples at 400-600g for 5min at RT. Discard the supernatant.

37 Add 50uL 1×Permeabilization Buffer mAb cocktail (Foxp3):

	A	B
	1×Permeabilization Buffer	50µL
	Anti-Foxp3-PE	0.3µL

38 Incubate at 4°C for 30min in the dark.

39 Directly add 200μL 1×Permeabilization Buffer to each well and centrifuge samples at 400-600g for 5min at RT. Discard the supernatant.

40 Add 200μL 1×Permeabilization Buffer for Flow Cytometry.