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T cell differentiation from mice spleen tissue

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Protocol status: Working We use this protocol and it's working

Created: Feb 29, 2024

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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MATERIALS

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

A	В	С
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	В	С
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 $4\,^{\circ}\text{C}$ for at least 4 months. Filtration through a

0.2 µm vacuum filter is recommended.

A	В	С
T cell culture medi	a	
Reagent	Final con	centration Final concentration
ΜΕΜα	n/a	429mL
FBS	10%	50mL
GlutaMAX 100×	2×	10mL
HEPES, 1M	0.02M	10mL

A	В	С
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°C for up to 1 month.

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

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

A	В	С
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	В	С
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	В	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 $4\,^{\circ}\text{C}$ for several days, seed 100mL in each well.

3. Reagents or Resources

A	В	С
ΜΕΜα	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

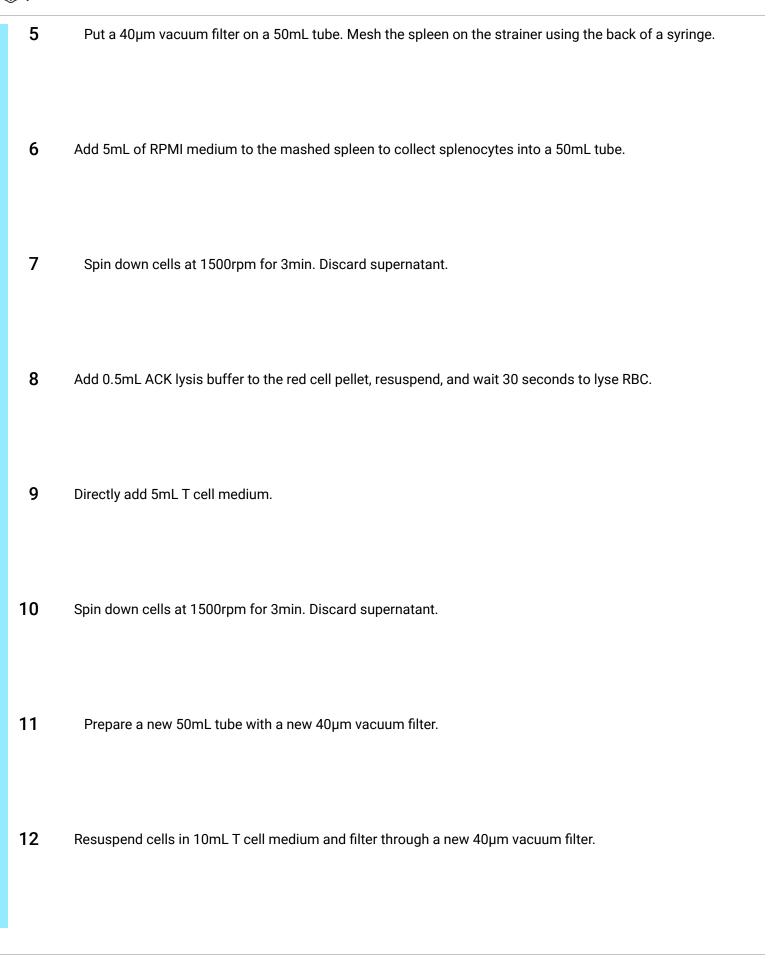
A	В	С
Mouse IL-9 DuoSet ELISA	R&D Systems	Cat#DY409
Foxp3/transcription factor staining buffer set	Fisher Scientific	Cat#00-5523-00
Anti-CD4-eFluor 450	eBioscience	Cat#48-0042-82
Anti-CD25-PerCP	TONBO Biosciences	Cat#65-0251
Anti-Foxp3-PE	Fisher Scientific	Cat#12-5773-82
РМА	Sigma-Aldrich	Cat#P1585

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 CO₂ inhalation or other means of euthanasia.
- 4 Collect the spleen from the mice.



13 Count the cells.

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

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

Regulate the cell concentration at 5×10⁵/mL with T cell medium

A	В	С
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.

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

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4.0			
19	Seed wells of a 96-well	plate with 150µL cells cocktail	(100µL cells and 50µL T cell media.

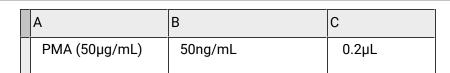
20 Seed 100µL differentiation cocktail.

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.
- Regulate cell concentration at 1×10⁶/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	В	С
	Final concentration	Amount
T cells	1×106/mL	100µL
T cell Medium	n.a	100μL



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.
- Remove the supernatant over the sink in one motion.
- 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	В
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.

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

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

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

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Directly add $200\mu L$ 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.