**PROTOCOL:** **T cell proliferation/Th differentiation**

Day 0:

* Coat plates at RT for 2-3 hrs in 37°C incubator w/ NeutrAvidin [5 µg/mL, 1:2000 from 10 mg/ml stock] in PBS w/o Ca/Mg. OK to coat at 4°C overnight.
	+ 96well: 100µL/well
	+ 6well: 2-3mL/well
	+ 10cm dish: 10mL/dish
* ~~Coat plates at RT for 2-3 hrs in 37°C incubator w/ αCD3/28 [1/0.5µg/mL] in PBS w/o Ca/Mg (direct coating)~~
	+ ~~96well: 100µL/well~~
	+ ~~6well: 3mL/well~~
	+ ~~10cm dish: 10mL/dish~~
* Note: okay to coat plates longer during cell isolation and media preparation.
* Prep cells
	+ Harvest spleen +/- lymph nodes from mice.
	+ Perform CD4 negative selection with Stem Cell beads (EasySep Mouse CD4+ T Cell Isolation Kit).
* Wash plates 1x with PBS right before adding cytokine-laden media.
* Load plates with ½ volume of 2x stimulation media (made with complete Kool-aid media)
	+ See accompanying numbers sheet to calculate
		- Th0: biotin - αCD3, biotin - αCD28 (Non-polarizing)
		- ThN: αIL-4, αIFNg, biotin - αCD3, biotin - αCD28 (Neutralizing)
		- Th1: IL-12, anti-IL4, biotin - αCD3, biotin - αCD28
		- Th2: IL-4 sup, anti-IFNg, biotin - αCD3, biotin - αCD28
		- Th17: αIL-4, αIFNg, IL-6, TGFβ, biotin - αCD3, biotin - αCD28
		- Th17+αIL-2 αIL-2, αIL-4, αIFNg, IL-6, TGFβ, biotin - αCD3, biotin - αCD28
		- Treg αIL-4, αIFNg, TGFβ, biotin - αCD3, biotin - αCD28
* Count cells and resuspend at 2x concentration in complete Kool-Aid media w/o antibodies or cytokines.
	+ 96well: 1x105 cells/75µL media
	+ 6well: 2x106 cells/1.5 mL media
	+ 10cm dish: 10x106 cells/5mL media
* Plate resuspended cells into well/dish with cytokine-laden media, such that the final concentration of each is 1x (1:1 cells:stim media).
* FINAL CONCENTRATIONS:
	+ 96well: 1x105 cells/well, 150µL media/well
	+ 6well: 2x106 cells/well, 3 mL media/well
	+ 10cm dish: 10x106 cells/dish, 10ml media/dish
* Optional: spin down cells on plate at 500 x G 3 minutes so that all cells begin stimulation at the same time
* Incubate at 37°C in 10% CO2

Day 3:

* Withdraw cells from TCR stimulation
	+ Wash cells out of culture well
		- Cells will be somewhat adherent, Th2>Th1, and will need to be blown off the bottom of the plate
	+ Move cells to fresh plate. I typically upscale 2 plate sizes (ie. if I start with cells in a 24 well plate then I would move them to a 6 well plate) and add at least 2x fresh 1x maintenance media (which includes IL-2 + polarizing cytokines) (typically add 3x fresh media)
		- see chart below for appropriate amounts.
	+ Alternatively, split cells by taking 1/4 total volume and adding 3/4 x original volume (i.e., if you start with 200µL, take 50µL and add 150µL fresh media)

Day 4 (all but Th17):

* If you plan to stimulate for cytokines, then the cells need fresh media <24hrs before restimulation to get optimal results. I typically remove 50% of volume then add 1V of 1x maintenance media. Can also simply double media volume with 1x maintenance media.

Day 5 (Day 4 for Th17):

* Cells should expand 20-40x since the start of culture. Cells will remain viable for 1-3 days from this point.
* Stimulate with P + I
	+ Stocks: PMA 1 mM, ionomycin 10 mM (stored in -20 TC room)
	+ Goal dilution: PMA 1:50K, ionomycin 1:10K
	+ Logistics to stimulate T cells in 3 mls conditioning media in 6-well plates:
		- 1 ml Kool-aid Complete media
		- 1 ul PMA
		- 5 ul Ionomycin
	+ Mix, then add 60 ul of this to each well (an additional 50x dilution of both P and I)
	+ Incubate 4-6h at 37\*C 10% CO2
	+ Proceed to Golgi block at 2 hours if planning to do flow cytometry.
* Block Golgi w/Brefeldin A (trap cytokines intracellularly for flow detection)
	+ Stock 2 mg/ml
	+ Goal dilution 1:400
	+ Add 7.5 ul of stock solution to each well of a 6-well plate, containing 3 ml total volume.
	+ Incubate 2h additional.
	+ Harvest cells for flow cytometry.

APPENDIX:

Media:

“Kool-Aid”: DMEM high glucose with additives

 +4.5g/L Glucose

 +0.584g/L L-glutamine

 +3.7g/L NaHCO3

 +NEAA

 +MEM Essential Vitamins

 +0.116g/L L-arginine HCL

 +0.036g/ L-asparagine

 +0.006g/L folic acid

+10% FBS

+100mcg/ml Strep; 100u/ml PenG

+10mM HEPES

+1mM Na Pyruvate

+100uM 2ME

+ 2mM L glutamine

(\*we have 5ml aliquots of 2ME + L gutamine frozen at -20oC TC

Cytokines and antibodies:

|  |  |  |  |
| --- | --- | --- | --- |
| **Reagent** | **Stock Concentration** | **Dilution Factor** | **Final Concentration** |
| NeutrAvidin | 10mg/mL | 2,000 | 5µg/mL |
| Biotinylated Anti-CD3 | 2.8mg.ml | 5,600 | 0.5ug/ml |
| Biotinylated Anti-CD28 | 2mg/ml | 2,000 | 1ug/ml |
| Anti-IFNg | 5mg/ml | 500 | 10ug/ml |
|  | 3.5mg/ml | 350 | 10ug/ml |
| Anti-IL4 | 6mg/ml | 600 | 10ug/ml |
| Anti-IL2 | 1mg/ml | 100 | 10ug/ml |
| IL-2 | 1,000,000U/ml | 50,000 | 20U/ml |
| IL-4 | 10,000U/ml | 20 | 500U/ml (Th2) |
|  | 10,000U/ml | 1,000 | 10U/ml (Th2 low) |
| IL-12 | 20ug/ml | 2,000 | 10ng/ml (Th1) |
|  | 20ug/ml | 200,000 | 0.1ng/ml (Th1 low) |
| TGFβ | 10ug/ml | 2,000 | 5ng/ml |
| IL-6 | 100ug/ml | 5,000 | 20ng/ml |
| PMA | 1mM | 50,000 | 20nM |
| Ionomycin | 10mM | 10,000 | 1uM |
| Brefeldin A | 2mg/ml | 400 | 5ug/ml |

Cell numbers, media volume and TC pate size for starting cultures:

|  |  |  |
| --- | --- | --- |
| **Cell number** | **Media Volume** | **TC plate size** |
| 100,000  | 150ul | 96 well |
| 300,000 | 450ul | 48 well |
| 500,000 | 750ul | 24 well |
| 1,000,000 | 1.5ml | 12 well |
| 2,000,000 | 3ml | 6 well |
| 10,000,000 | 12ml | 10cm TC plate (\*\*use CellStar) |