Intracellular Cytokine (ICS) Staining Protocol

Materials

- Human Fc Block (BD/564220)
- 10% BSA
- Iono (1mg/ml) and PMA (100ug/ml)
- FACS buffer
- Golgi Plug & Golgi Stop
- Blocking Buffer
- Sodium azide at permeabilization step with saponin

Prepare Individual Peptides of Peptide Pools

Stimuli	Stock Concentration	Final Concentration
Peptide Pool	1mg/mL	Antigen Dependent
DMSO (Negative Control)		Same concentration as peptide
PMA + Ionomycin	100ug/mL (PMA)1mg/mL (Iono)	0.1ug/mL(PMA)0.5ug/mL(Iono)

Protocol:

Stimulus Solution

1. Label U-bottom plate with donor, stimulation solution, name and date.
2. Prepare PMA+Ionomycin and DMSO mix separately
3. Prepare and arrange the remaining stimulation solution. Mix thoroughly by pipetting up and down before adding to the experimental plate.
4. Add appropriate stimulus solution to each well in 96-well U-bottom plates.
5. Store stimuli-loaded plates in 37°C incubator while thaw cells in next step.

anti-CD40		
Antibody	Clone/vendor/catalog	
anti-CD40 1.5ug/mL per 10million cells	RF8B2/BD/740266	
HR5		
Total Volume		

PBMC Counting and Stimulus Preparation

1. Obtain indicated number of vial(s) of PBMCs.
2. For each donor, prepare sterile 50ml tubes with 10ml HR5 and 20µL Benzonase per vial to be thawed.
3. Thaw PBMC vials.
4. Centrifuge @ 1200 rpm for 7 min.
5. Resuspend cells in HR5 and determine cell number.
6. Centrifuge @ 1200rpm for 7 min.
7. While sample(s) spinning, prepare the CD40 antibody solution the stock for all donors.

8. Resuspend each donor at 1.5ug/mL per 10 million cells per ml in prepared 1.5µg/ml CD40 antibody solution
9. Add CD40 antibody solution to all wells already containing stimulus.
10. Keep plate in incubator at 37℃ while thawing/preparing cells in the following steps.
11. Obtain indicated number of vial(s) of PBMCs.
12. For each donor, prepare sterile 50ml tubes with 10ml HR5 and 20µL Benzonase per vial to be thawed.
13. Thaw PBMC vials.
14. Centrifuge @ 1200 rpm for 7 min.
15. Resuspend cells in HR5 and determine cell number.
16. Centrifuge @ 1200rpm for 7 min.
17. While sample(s) spinning, prepare the CD40 antibody solution the stock for all donors.
18. Resuspend each donor at 10 million cells per ml in prepared 1.5µg/ml CD40 antibody solution.
19. Incubate the tube for 15 minutes at 37°C/5% CO_2.
20. Add 100µL of CD40 antibody-treated PMBCs to each well already containing stimulus.
21. Incubate plate for a total of 20-24 hours at 37°C/5% CO_2.
22. After 20-24 HR, add 50µL Intracellular Transport Blocking solution to each well and incubate for an additional 4 hours at 37°C/5% CO_2.

Intracellular Transport Blocking Solution			
Reagent	*Vendor/catalog*	*Minimum (µl)*	
Golgi Plug	BD/#555029	4	
Golgi Stop	BD/#554724	4	
HR5	[redacted]	992	
Total Volume	[redacted]	1000	

23. After incubation, spin plate at 1400rpm/4°C/2min.
24. Wash plate by adding 200µL PBS and spinning at 1400rpm/4°C for 2 min.
25. Resuspend cells in 100µL of LIVE/DEAD and FC block mix. Prepare as follows:

	Reagent	Clone/Vendor/Catalog/Peak	Amount per well (uL)
1	Fixable Live/Dead Blue	Thermo/L23105/UV6	0.2
2	Human FC Block	BD/564220	5
3	PBS	[redacted]	94.8
	*Total Volume	[redacted]	100

26. Incubate at 4°C for 30 minutes, protected from light. Wrap plate in aluminum foil and place in fridge.
27. After incubation, add 100µL PBS buffer and spin plate at 1400rpm/4°C/2 min. Decant.

28. Resuspend cells in 100µL of surface antibody mix and incubate at 4°C for 30 minutes, protected from light.

Surface Stain (100µl per well)				
Membrane Antibody	Fluorochrome	Clone/vendor/catalog	Amount per well (uL)	
CD3	BUV395	UCHT1/BD/563546	1	
CD8	BUV661	RPA-T8/BD/750699	0.5	
CD16	BV510	3G8/Biolegend/302048	0.5	
CD14	BV510	63D3/Biolegend/367124	0.5	
CD20	BV510	2H7/Biolegend/302340	0.5	
CD45RA	BV570	HI100/Biolegend/304132	2	
CD4	BV711	RPA-T4/BD/740769	1	
CCR7	PE-Cy7	G043H7/Biolegend/353226	1	
FACS Buffer			83	
BSB plus		BD Horizon/566385	10	
*Total Volume			100	

29. After incubation, add 100µL FACS buffer and spin plate at 2000 rpm/4°C/1 min.
30. Wash plate 2X using 200µL FACs buffer at 2000 rpm/4°C/1 min.
31. Add 200µL 4% PFA to each well, pipette to mix, cover and incubate at 4°C for 10 minutes.
32. After incubation spin plate at 2000rpm/RT/5 min.
33. Wash 1X with 200µL PBS at 2000 rpm/RT/5 min. Meanwhile, prepare saponin buffer as follows:

	Saponin Buffer					Blocking Buffer
	Saponin powder	10% BSA	0.01% azide	Vol. PBS	Total Vol.	Blocking buffer (10% Human serum in SB)
Example	0.05 g	1mL	100µL of 1% azide	8.9mL	10mL	100 µL+ 900 µL SB

34. Wash 1X with 200µL of saponin buffer at 2000 rpm/RT/5 min. Meanwhile, prepare blocking buffer (use the rule 50µl blocking buffer per well + 3ml excess).
35. Add 50µL blocking buffer to each well and incubate protected from light at RT for 5 min.
36. Add 50µL of prepared intracellular stain to each well. Incubate protected from light at RT for 30min.

Intracellular Stain (50µl per well)					
	IC Antibody	Fluorochrome	Clone/vendor/catalog	Amount per Well (µL)	
1	IL-4	BUV737	MP4-25D2/BD/612835	0.5	

2	IL-17	BV785	BL168/Biolegend/512338	1	
3	TNFa	eFluor450	Mab11/eBioscience/48-7349-42	0.2	
4	IFNg	FITC	4S.B3/eBioscience/11-7319-82	0.2	
5	IL-2	BB700	MQ1-17H12 /BD/566405	0.5	
6	IL-10	PE-Dazzle594	JES3-19F1/BioLegend/506812	1	
7	CD40L	APC-ef780 Changed from percp-ef710	24-31/LifeTech/47-1548-42	2	
	PBS			34.6	
	BSB plus		BD Horizon/566385	10	
	*Total Volume			50	

37. Wash 1X with 100µL saponin buffer at 2000 rpm/RT/5 min
38. Wash 1X with 200µL PBS at 2000rpm/RT/5 min.
39. To store plate overnight, add 200µL FACS buffer. Wrap in foil and store at 4°C until analysis.