Cell Signaling Course, LACI Satellite Meeting at CIML

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Experiment Overview

Overall Goal: Stain, collect, and analyze samples of stimulated primary mouse lymph node cells and quantify signaling by measuring protein phosphorylation in cell subsets using flow cytometry.

Experiment Outline (see next page for reagents)

1. There are two different experiments: a signaling profile timecourse and an analysis of BCR and TCR signaling. Each group will stain and collect the files from one of these experiments.

2. **Obtain cells** – Mouse lymph node cells have been prepared for you by the course instructors. The cells were stimulated as described below, fixed in 2% para-formaldehyde for 15 minutes, and then permeabilized in -80 ºC methanol following the standard phospho-flow protocol (detailed protocol in Irish et al., *PNAS* 2010, supporting information). Take tubes with approximately 0.5 million cells in 100 uL PBS + 1% BSA.

Group 1
(Signaling Profile Timecourse)

	Stimulation	Staining Panel
1.	0 min IL-7 (Unstim.)	Panel 1
2.	2 min IL-7	Panel 1
3.	4 min IL-7	Panel 1
4.	8 min IL-7	Panel 1
5.	16 min IL-7	Panel 1
6.	45 min IL-7	Panel 1
7.	0 min IFNα (Unstim.)	Panel 1
8.	2 min IFNα	Panel 1
9.	4 min IFNα	Panel 1
10.	8 min IFNα	Panel 1
11.	16 min IFNα	Panel 1
12.	45 min IFNα	Panel 1
13.	Unstimulated	Unstained

Group 2
(BCR and TCR Signaling)

	Stimulation	Staining Panel
1.	Unstimulated	Panel A
2.	4' TCR stim	Panel A
3.	4' BCR stim	Panel A
4.	16' PMA + iono	Panel A
5.	45' TCR stim + H2O2	Panel A
6.	45' BCR stim + H2O2	Panel A
7.	Unstimulated	Panel B
8.	4' TCR stim	Panel B
9.	4' BCR stim	Panel B
10.	16' PMA + iono	Panel B
11.	45' TCR stim + H2O2	Panel B
12.	45' BCR stim + H2O2	Panel B
13.	Unstimulated	Panel C
14.	4' TCR stim	Panel C
15.	4' BCR stim	Panel C
16.	16' PMA + iono	Panel C
17.	45' TCR stim + H2O2	Panel C
18.	45' BCR stim + H2O2	Panel C
19.	Unstimulated	Unstained

3. **Stain cells for flow cytometry** – Prepare enough of each antibody staining panel for your samples. Below is the recipe for each group. Once the antibody staining panel mix is prepared, add 50 uL of mix to each tube of 100 uL of cells. For group 2, prepare the 'Lineage master mix' first to ensure even lineage staining in panels.

Group 1 (Signaling Timecourse)

Staining **Panel 1**, stain tubes 1 – 14:
- 800 µL PBS + 1% BSA
- 70 µL α-CD44-PE
- 70 µL α-CD4-Pacific Blue
- 70 µL α-TCRβ-PE-Cy7
- 70 µL α-B220-PerCP-Cy5.5
- 140 µL α-p-STAT5-Ax488
- 140 µL α-p-STAT1-Ax647

Both Groups: *Don't forget to set aside the last tube, your unstained control!*

Group 2 (BCR and TCR Signaling)

Lineage Master Mix
- 2600 µL PBS + 1% BSA
- 210 µL α-CD4-PacBlue
- 210 µL α-TCRβ-PE-Cy7
- 210 µL α-B220-PerCP-Cy5.5

Staining **Panel A**, stain tubes 1 - 6:
- 800 uL Lineage Master Mix
- 140 µL α-p-PLCγ-Ax488
- 140 µL α-p-SFK/LCK -PE
- 140 µL α-p-SYK/ZAP70-Ax647

Staining **Panel B**, stain tubes 7 - 12:
- 800 uL Lineage Master Mix
- 140 µL α-p-ERK-Ax488
- 140 µL α-p-NFκB -PE
- 140 µL α-p-p38-Ax647

Staining **Panel C**, stain tubes 13 - 18:
- 800 uL Lineage Master Mix
- 140 µL α-p-AKT-Ax488
- 140 µL α-p-STAT5-PE
- 140 µL α-p-S6-Ax647

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4. **Wash samples and prepare compensation tubes** – Allow staining to proceed for 15 minutes at room temperature in the dark. Then wash the samples by adding 2 mL flow cytometry staining media (PBS + 1% BSA) to each tube, spin at 800 rcf (2000 RPM) for 5 minutes, check for a pellet, and discard the supernatant
5. **Collect samples and upload to the Public Cytobank** – Add 250 uL PBS to the cell pellet and collect. When done, export the FCS files and then upload them to Cytobank (http://www.cytobank.org/). When at the cytometer, use the tube names from Step 2 to label each sample. This will speed up analysis later.

Reagent List

Items Needed			*Amount Needed*	*Cat # / Source*
Cells		Murine Lymph Nodes	#stims x [0.5 x 10^6 cells / 100 µL PBS+1%BSA]	LN prep
Stimuli		IL-7	50 ng/mL per stim of 1 µg/mL (20X)	MyltenyiBiotech (130-094-066)
		IFN Universal Type I (IFN-α)	1 ng/mL per stim of 0.02 µg/mL (20X)	MyltenyiBiotech (130-093-131)
		phorbol 12-myristate 13-acetate (PMA)	5 µg/mL per stim of 100 µg/mL (20X)	P1585-1MG / Sigma
		Ionomycin	5 µg/mL per stim of 100 µg/mL (20X)	I0634-1MG / Sigma
		TCR stim: α-CD3-biotin	0.5 mg/mL per stim of 10 µg/µL (50X)	
		TCR stim: streptavidin		
		TCR stim: α-CD28	0.5 mg/mL per stim of 10 µg/µL (50X)	
		BCR stim: α-IgM F(ab')$_2$	0.5 mg/mL per stim of 10 µg/µL (50X)	
		H_2O_2	165 mM per stim of 3.3 mM (50X)	
Fix, perm & buffers		para-formaldehyde (PFA)	16 x [10 µL of 16% (10X) PFA], room temp.	15710 / Electron Microscopy Sciences
		cold methanol (-80 °C)	500 mL, kept at -80 °C [-20 °C is OK]	
		phosphate buffered saline (PBS)	500 mL, kept at 4 °C	Lab stock
		PBS + 1% BSA for staining	500 mL, kept at 4 °C [other media OK]	Lab stock
Lineage antibodies		α-TCRβ-PE-Cy7 H57-597	#tubes x [10 µL/stain]	560729 / BD
		α-CD4-Pacific Blue RM4-5	#tubes x [10 µL/stain]	558107 / BD
		α-CD44-PE IM7	#tubes x [10 µL/stain]	553134 / BD
		α-B220-PerCPCy5.5 RA3-6B2	#tubes x [10 µL/stain]	561101 / BD
Phospho-antibodies	P1	α-p- STAT5(Y641)-Ax488 47	10 µL per stain	612600 / BD
		α-p-STAT1(Y701)-Ax647	10 µL per stain	612597 / BD
	PA	α-p- PLCγ(Y759)-Ax488 K86-689.37	10 µL per stain	558507 / BD
		α-p-SFK/LCK(Y505)-PE 4/LCK-Y505	10 µL per stain	558552 / BD
		α-p-SYK/ZAP70(Y352/Y319) 17a	10 µL per stain	557817 / BD
	PB	α-p-ERK(T202/Y204)-Ax488 D13.14.4E	10 µL per stain	4344 / CST
		α-p-NFκB p65 (S529)-PE K10-895.12.50	10 µL per stain	558423 / BD
		α-p-p38(T180/Y182)-Ax647 36/p38	10 µL per stain	612595 / BD
	PC	α-p-AKT(S473)-Ax488 193H12	10 µL per stain	2336 / CST
		α-p-STAT5(Y641)-PE 47	10 µL per stain	612567 / BD
		α-p-S6(S235/S236)-Ax647 D57.2.2E	10 µL per stain	4851 / CST
Comp Beads		(+) α-mouse-κ	5 x [10 µL beads per comp tube]	51-BP80212-01 / BD
		(-) FBS	5 x [10 µL beads per comp tube]	51-BP80212-02 / BD