PITT ISL METHODS

1) Flow-based CTL assay

a. Purified $CD8^+$ T cells are co-cultured with fresh, autologous, infected $CD4^+$ T cells at various effector/target ratios for 18 hours at 37°C.
b. Baseline percentage of infection is determined by incubation of $CD4^+$ T cells without $CD8^+$ T cells.
c. Co-cultures are harvested and stained for surface expression CD8-PerCP-Cy5.5 (BD Pharmigen) and intracellular expression of HIV-1 core antigen using the KC57-FITC antibody (Beckman Coulter) per manufacturer's instructions.
d. Samples are then assessed on a BD LSRFortessa flow cytometer and analyzed using FlowJo.
e. The percentage of infected $CD4^+$ T cells is determined by gating on the CD8-negative population and then on the KC57-positive subset.
f. The percentage reduction in infected $CD4^+$ T cells is determined for each condition at each effector-target ratio and in relation to the baseline infection of the targets alone.
g. Percent reduction in infected targets at each E:T ratio is transformed to effector units 20% (EU20), i.e., lytic units calculated based on the relative number of effectors required to yield 20% killing of target cells.

2) Gut Mucosal Processing

a. Immediately after collection, gut mucosal biopsies are placed in a conical tube with transport media (tRPMI) containing RPMI, 1640 with L-glutamine and hydroxyethyl piperazineethanesulfonic acid (Invitrogen, #22400-105), 7.5% fetal bovine serum (FBS, Invitrogen, #10082-147), and 1% antibiotic/antimycotic (Invitrogen #15240-104).
b. Specimens are kept at room tempature or on ice for 1-2 hours until transport to the processing laboratory.
c. In the processing lab, biopsies are transferred to a 5mL Falcon snap-cap tube with media (cRPMI; similar to the tRPMI but with 10% instead of 7.5% FBS).
d. Biopsies are then minced with dissecting scissors to ~1mm-sized pieces, and then transferred to a 50ml conical tube with 20ml of freshly prepared Digest solution (contains 50mg of collagenase type II [Sigma #C-1764] in 100mL of cRPMI).
e. The 50ml conical with the minced tissue is then placed on a heated shaker-incubator at 37°C at 250 RPM, 30'.
f. Tissue is then pass digested through a 40 µM BD Falcon cell sieve and collected in a new 50mL conical tube and is centrifuged at 800xg, 10'.
g. The supernatant is decanted and the pellet resuspended in 2ml cRPMI and kept on ice.
h. Remaining tissue on the cell sieve is rinsed off on a petri dish with Digest solution and is returned to a new 50ml conical containing 20ml of Digest solution. (The step of rinsing remaining tissue with Digest solution can be repeated if necessary.)
i. The conical is also placed on a heated shaker-incubator at 37°C at 250 RPM, 30', and centrifuged at 800xg, 10'.
j. The supernatant is decanted and the pellet is resuspended in 1ml cRPMI. Cells are pooled with the cells of the first digested tissue (conical tubes should be placed on ice).
k. The pool is then centrifuged at 800xg, 10' and then resuspended in the desired volume of cRPMI for cell counting.
l. Cells can then be used for phenotyping or can be frozen in 90%FBS/10%DMSO.

3) Polyfunctional Flow

a. Cryopreserved PBMC are thawed, washed, and then rested in media overnight.
b. Aliquots of 10^6 cells are then co-cultured with a Gag peptide (10 µl at 0.1mg/ml), anti-CD28/CD49d monoclonal antibody (mAB, 2 µl at 1mg/ml; BD Biosciences), anti-CD107a antibody (PE-Cy5, 20 µl, BD Pharmingen), monensin (2 µl at 5 µg/ml, Sigma), brefeldin A (2 µl at 5 µg/ml), BD Bioscience), PBS (14 µl) in a 96-well plate.
c. Negative control consists of medium only and positive control includes stimulation with *Staphylococcus* enterotoxin B (4 µl at 50 µg/ml, Sigma).
d. Plates are incubated at 37°C for 6 hours and then a 4°C for 16 hours.
e. Cells are washed, stained with anti-CD3 mAB (PE-Cy7, 5 µl, BD Pharmingen), anti-CD8 mAb (APC-Cy7, µl, Biolegend), and anti-CD4 mAb (5 µl; BD Pharmingen), incubated in the dark for 30 minutes at room temperature (RT), and then washed.
f. BD FACS Lysing solution is added (100 µl/well) and incubated for 10 mins at RT and then washed with 200 µl/well cold buffer solution. The cells are treated with BD FACS Permeabilizing Solution 2 (200 µl/well), incubated for 10 mins at RT, and then washed with 200 µl/well of cold buffer.
g. The following mAb are added: anti-TNFα (PB, 20 µl, eBioscience), anti-MIP-1β (PE, 5 µl, BD Pharmingen), anti-IL2 (APC, 5 µl, Biolegend), and anti-IFNγ (FITC, 1 µl, BD Pharmingen). Plates are incubated in the dark for 30 minutes, washed with 150 µl/well of cold buffer, and fixed with 1% paraformaldehyde in PBS.
h. Cells are then assessed using a BD LSRII or BD LSRFortessa and analyzed using FlowJo. Polyfunctional responses are evaluated by creation of Boolean combinations of single functional gates and interpreted using the SPICE program (Roederer, Vaccine Research Center, NIAID, NIH).

4) Treg and Th17 Staining

<u>Treg</u>

a. Thaw cryopreserved PBMC and wash. Divide $5x10^6$ cells into 2 wells (Treg isotype and Treg)
b. Add 1 µl Live/Dead stain, mix, and incubate for 30min in the dark at RT.
c. Add 200 µl D-PBS, centrifuge at 300g, 5', decant, and vortex.
d. Add surface stain as per table below and mix.

WELL 1: TREG ISOTYPE		**WELL 2: TREG**	
STAIN	**AMOUNT (in µl)**	**STAIN**	**AMOUNT (in µl)**
CD3 PE-Cy7	10	CD3 PE-Cy7	10
CD4 AF700	10	CD4 AF700	10
CD8 BV711	10	CD8 BV711	10
CD127 FITC	40	CD127 FITC	40
CD25 BV421	10	CD25 BV421	10
IgG1 PE	40	CD39 PE	20
IgG1 PerCP-Cy5.5	40	CD73 PerCP-Cy5.5	10

e. Incubate for 30 mins in the dark at RT.
f. Add 100µl/well of cold D-PBS and mix. Centrifuge (300G, 5 min), decant and vortex.
g. Add 200µl/well **FOXP3 Fix/Perm Buffer A** Solution mix. Incubate 10 min, RT, dark.
h. Centrifuge (500G, 5 min), decant and vortex.

i. Add 200µl/well cold D-PBS, centrifuge (500G, 5 min), decant and vortex.
j. Add 200µl/well **FOXP3 Fix/Perm Buffer C** Solution. Incubate for 30 min, RT, dark.
k. Add 200µl/well **BD Staining Buffer**. Mix and centrifuge (500G, 5 min), decant.
l. Add 100 ul **BD Staining Buffer** to each well.
m. Add intracellular stains and mix.

WELL 1: TREG ISOTYPE

STAIN	**AMOUNT (in µl)**
FOXP3 **ISO** PC-CF594	10

WELL 2: TREG

STAIN	**AMOUNT (in µl)**
FOXP3 PC-CF594	10

n. Vortex and incubate for 30 mins, RT, dark.
o. Add 200µl/well of PBS. Centrifuge (500G, 5 min), decant and vortex.
p. (Repeat) Add 200µl/well of **BD Staining Buffer**. Centrifuge (500G, 5 min), decant and vortex.
q. Add 250µl Formaldehyde solution to each well. Keep in 4°C for at least 15 min. Read within 24 hours on BDLSR II or BDLSRFortessa.

Th17

DAY 1

1. Prepare Brefeldin A, PMA, and Ionomycin working solutions.
   a. **BFA** (stock: 10mg/ml, final conc: 5µg/mL); *BD Cat # 347688*
      1. 10µl frozen aliquots; **dilute 1:10 in PBS**
         1mL of 10x stock + 9mL DiH2O
   b. **PMA** (stock: 1mg/ml, final conc: 50ng/ml) *Sigma Cat # (P 1585)*
      1. **Dilute stock 1:400 in PBS**, making a 2.5µg/ml solution. ?
         1 uL stock + 399uL PBS
   c. **Ionomycin** (1 mM ; final conc: 1µM) *Sigma Cat # (I 0634)*
      1. **Dilute stock 1:100 in PBS**
         1uL stock + 99uL PBS
   d. **EDTA** *BD Cat # 347689*
      1. Dilute 1:20

   Additional reagents needed:
   e. **D-PBS** *Fisher Cat # SH30028.02*
   f. **FACS staining buffer with FBS** *BD Cat # 554656*

2. Prepare the stimulation panel in a 96 well plate as below (note: BFA is added later)

Well #	Condition	PMA (50ng/ml)	Ionomycin (1µM)	aCD28/49 (1µg/ml)	BFA (5µg/ml)	PBS	Total volume
1	Th17 Control	1µl	5µl	2µl	2µl	40µl	50µl
2	Th17 Test	1µl	5µl	2µl	2µl	40µl	50µl

3. Add 0.5 x10^6 PBMC to the control well and 1.0 x10^6 PBMC to the TH17 #1 well (total volume of cells should be approx. 150 µl).
4. Incubate the covered plate for 2 hours at 37°C/5% CO2.
5. Add 2µl/well BFA. Incubate for 2 hours at 37°C/5% CO2.
6. Add 20µl Diluted EDTA (1:20 DiH2O) to each well, mix and incubate 15 min, RT, dark.
7. Centrifuge (300G, 5 min), decant and vortex.
8. Add 1µl prepared Live/Dead stain, mix, incubate 30 min, dark.
9. Add 200µl PBS and centrifuge (300G, 5 min), decant and vortex.
10. Add surface stains, mix, and incubate 30 min, RT, dark.

WELL 1: TH17 Control

STAIN	AMOUNT (in µl)
CD3 PE-Cy7	5
CD4 AF700	5
CD8 BV711	5

WELL 2: TH17

STAIN	AMOUNT (in µl)
CD3 PE-Cy7	5
CD4 AF700	5
CD8 BV 711	5

11. Add 100µl/well BD FACS Lyse Solution (1X) and mix well. Refrigerate overnight.

DAY 2

You will need: BD Perm 2 solution (Dilute 1:10 in DI H20), Formaldehyde solution (dilute 1:2 in PBS), TH17 #1 ICS stains, PBS

1. Remove **Th17** plate from the fridge. Add 100µl/well of FACS staining buffer and mix. Centrifuge (300G, 5 min), decant and vortex.

2. Add 200µl/well **BD Perm 2 Solution**, mix. Incubate 10 min, RT, dark.

3. Centrifuge (500G, 5 min), decant and vortex.

4. Add 200µl/well FACS staining buffer, centrifuge (500G, 5 min), decant and vortex. **REPEAT.**

5. Add intracellular stains, mix, and incubate in the dark, 30 min, RT.

WELL 1: TH17 #1 Control

STAIN	AMOUNT (in µl)
IgG1 PE	5
IgG1 APC	5

WELL 2: TH17 #1

STAIN	AMOUNT (in µl)
IL-17a PE	20
IFN-G APC	5

6. *Create compensation controls.* PE-Cy7, AF700, BV711, PE, APC, LIVE/DEAD (use ArC comp beads and 3µl L/D stain).

7. Add 200µl/well of FACS staining buffer. Centrifuge (300G, 5 min), decant and vortex.

8. Add 250µl Formaldehyde solution to each well. Keep in 4°C for at least 15 min. Read using BD LSR II or BD LSRFortessa within 24 hours.

STAINS	stain	Cat.#	Clone	manu.
CD3	PE-Cy7	557851	SK7	BD
CD4	AF700	564975	RPA-T4	BD
CD8	BV711	563677	RPA-T8	BD
LIVE/DEAD	AQUA	L34957	NA	Invitrogen
IgG1	PE	551436	MOPC-21	BD
IgG1	APC	554681	MOPC-21	BD
IL17a	PE	560436	SCLP1362	BD
IFNγ	APC	554702	B27	BD

5) Extended Culture ELISPOT

a) Day 1: Thaw cells according to the lab SOP. Each time point and control are plated into 2 wells /sample (0.5 x 10^6 cells/well) of a 48 well plate.
b) Add the peptides and controls at pre-determined optimal concentrations to each well. Incubate for 3 days at 37^0C at 5% CO_2.
c) Day 3: Add IL-2 to a final concentration of 500IU/mL and return to the incubator.
d) Day 7: Split the cells and place half the cells into another well. Add media to each well to return to starting volume. Return plate to the incubator.
e) Day 8: Label and coat the Elispot plate with the coating antibody and keep overnight at 4^oC.
f) Day 9: Block non-specific binding by washing 4 times and holding the plate with 250 mL media/well. To the test wells, add the appropriate peptides at predetermined concentrations. To the positive test well add the appropriate stimulant (e.g. CEF).
g) Harvest and count the cultured cells. Plate each sample and control into the ELISPOT plate. All samples are run in triplicate with the exception of the positive control which is run in a single well. The negative control wells contain cells and media only.
h) Incubate for 18hours at 37^0C at 5% CO_2.
i) Day 10: Wash the ELISPOT plate 6 times with PBS and add the biotinylated antibody to each well. Incubate 2 more hours. When there is one half hour left in the 2 hr incubation , make up the ABC peroxidase solution.
j) After the 2 hr incubation, wash the plate 6 times with PBS containing Tween 20. After the final wash, add the ABC-HPP and incubate for 45 minutes at room temperature.
k) Following this incubation, wash 3 times with PBS with Tween 20 followed by 3 washing with plain PBS.
l) Add of 3, 3'-diaminobenzidine (DAB) + H_2O_2 to each well and let the color develop for 5 minutes. (Note: DO NOT develop longer than 5 minutes. Timing is critical).
m) Wash with water, decant the plate, and dry overnight upside down in the hood.
n) Day 11: Read the plate on an ELISPOT reader.