February 8th, 2023

JMC: SEB/Cord

~~PMA: Kitchen Sink~~

Specimen	Status	Location	Conc	Date	Tasks	Volume	Ly	Ly+Mon	Total	~~3E+6~~	~~3E+6~~
ND006	Adult		1SEB 17.4µl	1/20/2023		2	5.63	6.87	11.26 / 13.74	9.26ml	
Inf-000374-A-5	HEU-10 SV4 W		14.0µl		5 vials	2	4.34	5.98	8.68 / 11.96	6.68ml	
ND006			20.38µL			2.5	4.31	5.08	10.775 / 12.7		
Inf000374			11.72			2.5	3.69	4.84	9.225 / 12.1		

1.100

1 million/ml

9:04 am Thaw

9:34 ~~stain~~ stain

9:49 am count start

10:13 am cells into incubator,
(6 well plate 1m/mL 5-6mls)

5:18 pm spining down (16:20 Med. a morning)

5:43 pm stain

5:57pm Cells SEB (CD107A for CMs)
→ 8:57 pm golgi block

95% Monocytes
5% DCs
Antigen-pres
10% B cells

Cord = 9.225 million

2Million cells ○ ○ ○

1.3 ○ ○ ○

Adult 10.775 million

6 million cells

Media 1.5 % 3.69 = 407 µl + 593µl R10 = 1.5E6/mL

SEB 2.0 % 3.69 = 542µL + 458µl R10 = 2.0 E6/mL

10 µg/mL SEB (1µg/µL) = 10 µL/mL

CD107a sc

TBref + 1 Monensin + 19 µL R10 → golgi Mix
→ 10µl of the mix into each tube.

5 wells for SEB
6 wells for media

2.225 Lym cord → 2 wells for unstained 1M/mL
1 will be SEB → sc 2 centre sc
1 will be for Media → CD4SR sc CD8

Adult 2 % 4.31 = 464 µL + 536µl R10 = 2.0E6/mL
+ 10µL SEB
+ 10µl golgi mix

○ ○ ○ wells media

○ ○ wells SEB

cord unstained 500µl

cord 1ml

Adult 1m

9 doses

10 doses
×10µl
100

Brd Monen 120
1:1:19
×6 ×6 ×6

February 10th, 2023

JMC: Cord - SEB Cytokines and AIM

9:44 pm pulled from incubator

9:54 samples spin

~~Time~~ 10:06

CD107A – 100

Zombie – 100

45/CD8 cord → 20 each

Sc unstaineds → 50 each

10:32 4°C with nothing Single colors

4°C out single colors

37°C samples @ 10:45 → 11:15 am

11:18 37°C Sample spin 1400 rpm 6 min

11:29 4°C Sample stain 30 min (→ 11:59)

1:3

11:36 am scs start

1:10

11:42 Zombie single color cord 15 min (→ 11:57)

11:49 single colors 37°C (hot) stains 30 min (→ 12:19)

1ml

40 × 8 = 320

11:59 Zombie single color cord centrifuge 1500 rpm 8 min (→ 12:07)

30 − 12.5 → 37.5

4 | 50 (20)

Scs for 4°C @ 12:00 pm → 12:30 pm

12:01 RBC lyse samples (→ 12:04)

4°C Samples spin (RBC lysed)

12:08 centrifuge samples 1400 rpm 6 m (→ 12:14)

12:17 Samples Nuclear Perm → 27 → 37 → 47 pm

12:21 RBC lyse Single color hot 37°C → (→ 12:24)

12:27 RBC lyse single color hot 37°C centrifuge 1400 rpm 6 m → (12:33)

12:30 1/2 Single colors cold 4°C RBC lyse → (12:33)

12:31 2/2 " " (12:34)

10 ✓ 1:00, 10 1:10, 10 1:20

12:36 37°C stain 4°C

12:50 hot & cold single colors Nuclear Fix Perm

12:49 Samples wash 1 1500 rpm 6 m, 12:59 second wash

Intracellular @ 1:09 → 1:49pm

Sc's first Wash @ 1:27pm

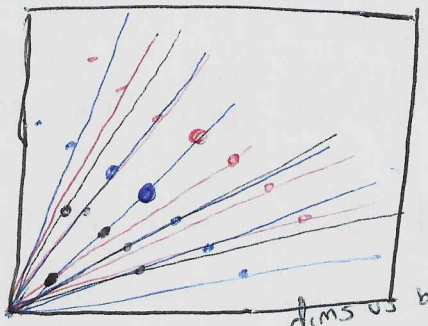
1:59 Sc's away

2:01 Samples finish Wash 1
Wash 2 not performed

2:02 PFA 50 µl added → 4°C

2:06 Spikes single colors RT 40min →(2:46)

Done @ 3:04pm



Aurora @ 9.40 am

QC @ 9:42 am Event rate 140

passed

// start @ 9:53am //

restore @ 11:23

Dropped Threshold to 150,000 ~~eds~~ FSC from 250,000
(other future option is ↑ FSC-A for nuclear stains)

Works ~ CD8 card

3/4 losers in an hour

40µl ~ 1:30 sec

Granulocytes contam in card?

CCR4 ~ 40 exact,

2 sporadic ssc drops real, one on CD45RA card

Icos (signal) FoxP3, eleagnea?

blip CD40L weak CXCR5

IL-17??

Done @ 11:16 pm

sc CD3 ≠ intra spike

Quarto/YouTube tutorial series

back to classroom/workshop

February 10th, 2023 MADI Cytokines

#	Filter	Single color (ul)	Ref ctrl type	Unmixing ctrl name	Fluorochrome	Marker	Clone	Vial Lot #	During stim!!!	2	L/D RT 15 min	RT for 10 min	HotStain 30min @37C	2	ColdStain 30min @4C	2	4*C for 15 min	RBC Lyse, then Fix/Perm	Intracellular Stain 40 min @RT	2
1	UV2				BUV395	CD62L	(DREG-56)								1.2	2.4				
2	UV7				AF	AF-UV6														
3	UV9				BUV496	CD8	(RPA-T8)								0.7	1.4				
4	UV10				BUV563	CD69	(FN50)								0.5	1			1	2
5	UV11				BUV615	CCR4	(1G1)						2.5	5						
6	U14														1.3	2.6				
7	UV16				BUV737	CXCR3	(1C6/CXCR3)								2.5	5				
8	V1				BUV805	CD4	(SK3).						1.5	3					0.5	1
9	V3				BV421	CD127	(A019D5)						1.5	3						
	V3				Pacific Blue	CD14	(M5E2)								2.0	4				
10	V5				Pacific Blue	CD19	(HIB19)								2.0	4				
11	V7				BV510	CD45RA	(Hi100)													
12	V10				BV570										0.7	1.4				
13	V11				BV605	CXCR5	(J252D4)						2.5	5						
14	V13				BV650	CCR7	(G043H7)						1	2					1	2
15	V14				BV711	ICOS									1.2	2.4				
16	V15				BV750	IFNg	(B27)						1.5	3					1.5	3
	B3				BV786	CCR6	(11A9)													
18					FITC	FoxP3									1.2	2.4			5	10
19	B4				Spark blue 550	CD3	(SK7)								1.2	2.4			0.5	1
20	B6				PE	PD-1	(EH12.2H7						1.2	2.4					0.5	1
21	B8				PE-CF594	CD26	(M-A261)						1.2	2.4						
22	B10				PE-Cy5	CD25	(M-A251)								1.2	2.4			0.5	1
23	B13				PerCP-eF710	CD40L														
24	R1				PE-vio770	TNFa									1.2	2.4			<1.2	####
25	R2				APC	CD39	(A1)													
26	R4				Alexa Fluor 647	IL2	(MQ1-						1.2	2.4					1.2	2.4
27	R6				APC-R700	CD107a	(H4A3)		6.0	12										
28	R7				Zombie NIR	L/D					<1:2500>									
29	R8				APC-Efluor780	IL-17A														
					APC/Fire 810	CD38	(HIT2)													
								Antibody Total	6.0		Antibody Total		1.6	3.2					1.5	3
And UNSTAINED CONTROLS !!!								R10 Media	14.5		Brilliant Stain		15.7	31.4	14.4	28.8			13.2	26.4
								Pippette draw volume /sample	19.5		Pippette draw volume /sample		50	100	50	100			50	100
													62.7		61.4				60.2	

Notes:

~60 ~62-5 58.2

Simplified Protocol

Thaw cells, DNAse, count.
<Resuspend at 1E6 Lym/ml of R10, rest cells for <6> hrs in a 6-well plate
Collect, count, aliquot cells 2.0E+6 Cells/ 5ml polystyrene tube
Add antigens, <stim antibodies>, and CD107a in 1 ml R10
Cap and incubate at 37*C for 2 two hours
Add golgi block, and continue to incubate for 12 hours

Wash with 2 ml PBS, spin down 1300rpm 8min
800 ul of LiveDead mix (1:2500) @RT for 15min
Wash 2 ml 5% PBS-FBS, spin 1300 rpm, 8min

Add to it HotStain mix, incubate @37C for 30 min
Wash 2 ml 5% PBS-FBS 1400 rpm, 6min

Add ColdStain mix, incubate @ 4C for 30min
Add 300-500 ul 1x RBC Lysis for 3 minutes
Wash 2 ml 5% PBS-FBS 1400 rpm, 6min

1 ml Nuclear FixPerm, incubate @ 4C for 30min
(vortex every 10 minutes)
First PermWash: 2 ml NuclearWash 1500 rpm 6 min
Second Perm Wash: 2 ml NuclearWash 1500 rpm 6 min

1.5 RG1

Add Intracellular Stain, incubate @ RT for 40min
First PermWash: 2 ml NuclearWash 1500 rpm 6 min
Second Perm Wash: 2 ml NuclearWash 1500 rpm 6 min

Resuspend in 100 ul 0.4% PFA-PBS
Cap tubes, wrap rack in foil, store at 4*C

FoxP3 can 4°C 1 day?

CD40L, ICOS in cells intracellular?

February 10th, 2023 MADI AIM

#	Filter	Single color (ul)	Ref ctrl type	Unmixing ctrl name	Fluorochrome	Marker	Clone	Vial Lot #	During stim!!!	2	L/D RT 15 min	RT for 10 min	HotStain 30min @37C	2	ColdStain 30min @4C	2	4°C for 15 min	RBC Lyse, then Fix/Perm	Intracellular Stain 40 min @RT	2
1	UV2				BUV395	CD62L	(DREG-56)								1.2	2.4				
2	UV7				AF	AF-UV6														
3	UV9				BUV496	CD8	(RPA-T8)								0.7	1.4				
4	UV10				BUV563	CD69	(FN50)								0.5	1			1	2
5	UV11				BUV615	CCR4	(1G1)						2.5	5						
6	U14				BUV661	~~Vd2~~	~~(B6)~~								~~0.7~~ 2.5	~~1.4~~ 3.5				
7	UV16				BUV737	CXCR3	(1C6/CXCR3)													
8	V1				BUV805	CD4	(SK3)						1.5	3					0.5	1
9	V3				BV421	CD127	(A019D5)						1.5	3						
	V3				Pacific Blue	CD14	(M5E2)								2.0	4				
10	V5				Pacific Blue	CD19	(HIB19)								2.0	4				
11	V7				BV480							1.5					Strep <0.8>			
12	V10				BV510	CD45RA	(Hi100)								0.7	1.4				
13	V11				BV570															
14	V13				BV605	CXCR5	(J252D4)						2.5	5						
15	V14				BV650	CCR7	(G043H7)						1	2					1	2
16	V15				BV711	ICOS									1.2	2.4				
	B3				BV750	IFNg	(B27)						1.5	3					1.5	3
18					BV786	CCR6	(11A9)													
19	B4				FITC	FOXP3													5	10
20	B6				Spark blue 550	CD3	(SK7)								1.2	2.4			0.5	1
21	B8				PE	PD-1	(EH12.2H7								1.2	2.4			0.5	1
22	B10				PE-CF594	CD26	(M-A261)						1.2	2.4						
23	B13				PE-Cy5	CD25	(M-A251)						1.2	2.4					0.5	1
24	R1				PerCP-eFlour710	CD40L									1.2	2.4				
25	R2				PE-Fire810	OX40									1.2	2.4				
26	R4				APC	CD39	(A1)						1.2	2.4						
27	R6				Alexa Fluor 647															
28	R7				APC-R700	CD107a	(H4A3)		6.0	12									1.2	2.4
29	R8				Zombie NIR	L/D					<1:2500>									
					APC/Fire 750	4-1BB														
					APC/Fire 810	CD38	(HIT2)								1.2	2.4				
								Antibody Total	6.0		Antibody Total		1.6	3.2						
And UNSTAINED CONTROLS !!!								R10 Media	14.5		Brilliant Stain		15.7	31.4	17.5	35.0			11.7	23.4
								Pippette draw volume /sample	19.5		Pippette draw volume /sample		50	100	50	100			50	100
													62.7		64.5				58.7	

~60 ~62.5 56.7

Notes:

Simplified Protocol

Thaw cells, DNAse, count.
<Resuspend at 1E6 Lym/ml of R10, rest cells for <6> hrs in a 6-well plate
Collect, count, aliquot cells 2.0E+6 Cells/ 5ml polystyrene tube
Add antigens, <stim antibodies>, and CD107a in 1 ml R10
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Add golgi block, and continue to incubate for 12 hours

Wash with 2 ml PBS, spin down 1300rpm 8min
800 ul of LiveDead mix (1:2500) @RT for 15min
Wash 2 ml 5% PBS-FBS, spin 1300 rpm, 8min

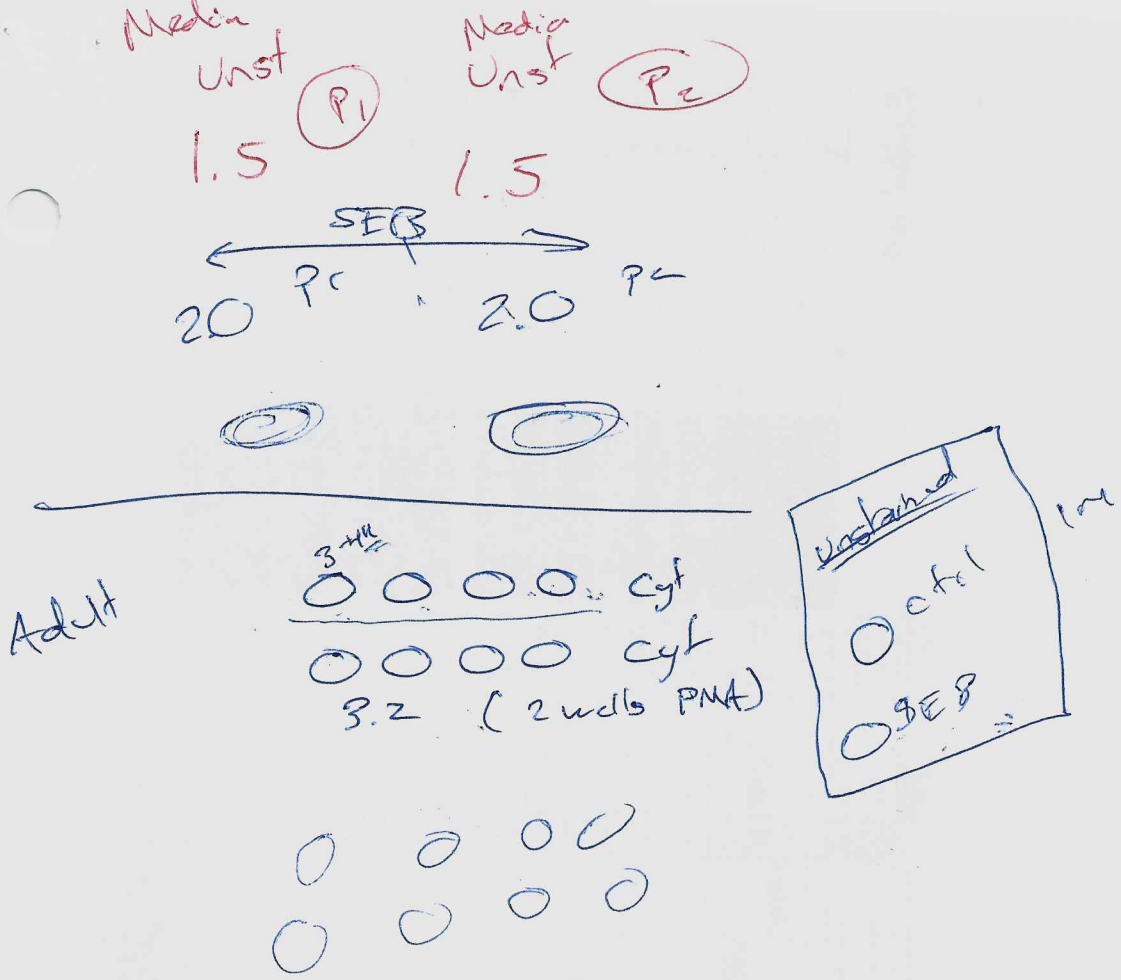
Add to it HotStain mix, incubate @37C for 30 min
Wash 2 ml 5% PBS-FBS 1400 rpm, 6min

Add ColdStain mix, incubate @ 4C for 30min
Add 300-500 ul 1x RBC Lysis for 3 minutes
Wash 2 ml 5% PBS-FBS 1400 rpm, 6min

1 ml Nuclear FixPerm, incubate @ 4C for 30min
(vortex every 10 minutes)
First PermWash: 2 ml NuclearWash 1500 rpm 6 min
Second Perm Wash: 2 ml NuclearWash 1500 rpm 6 min

Add Intracellular Stain, incubate @ RT for 40min
First PermWash: 2 ml NuclearWash 1500 rpm 6 min
Second Perm Wash: 2 ml NuclearWash 1500 rpm 6 min

Resuspend in 100 ul 0.4% PFA-PBS
Cap tubes, wrap rack in foil, store at 4°C


Media Unst
P1
Media Unst
P2
1.5
1.5
SEG
20
Pc
2.0
Pc
Adult
3rd
cyl
cyl
3.2 (2 wdls PMA)
Unbanded
ctrl
SEG

February 8th, 2023 PMA Kitchen Sink

#	Filter	Single color (ul)	Ref ctrl	Unmixing ctrl name	Fluorochrome	Marker	Clone	Vial Lot #	During stim!!!	8	L/D 15 min (RT)	Tetramer 40 min @ RT	HotStain 30min @37C	8	ColdStain 30min @4C	8	RBC Lyse, then Fix/Perm	Spiked 30 min @RT	8
1	UV2				BUV395	CD62L	(DREG-56)								1.2	9.6			
					AF	AF-UV6													
2	UV7				BUV496	CD8	(RPA-T8)								0.7	5.6			
3	UV9				BUV563	CD69	(FN50)						2.0	16	0.5	4.0		1	8.0
4	UV10				BUV615	CCR4	(1G1)								0.7	5.6		0.5	4.0
5	UV11				BUV661	Vδ2	(B6)								1.3	10.4			
6	U14				BUV737	CXCR3	(1C6/ CXCR3)						1.5	12				0.5	4.0
7	UV16				BUV805	CD4	(SK3)						1.5	12					
8	V1				BV421	CD127	(A019D5)								2.0	16.0			
9	V3				Pacific Blue	CD14	(M5E2)								2.0	16.0			
	V3				Pacific Blue	CD19	(HIB19)						2	16					
10	V5				BV480	CD161	(REA631)								0.7	5.6			
11	V7				BV510	CD45RA	(Hi100)												
12	V10				BV605	CD56							1.0	8				1	8.0
13	V11				BV650	CCR7							1	8					
28	V13				BV711	CD7							1	8				1.5	12.0
14	V14				BV750	IFNγ	(B27)						1.5	12					
15	V15				BV786	CCR6	(11A9)					< : >( )			< : >(1.2)				
16	B2				AlexaFluor 488	hCD1d									1.2	9.6		0.5	4.0
17	B3				Spark blue 550	CD3	(SK7)						1.5	12				0.5	4.0
18	B4				PE	NKG2D							1.2	9.6					
19	B6				PE-CF594	CD26	(M-A261)						1.2	9.6				0.5	4.0
20	B8				PE-Cy5	CD25	(M-A251)											2.5	20.0
21	B10				PerCP-Cy5.5	TNFα	(MAB11)								1.5	12.0		0.5	4.0
22	B13				PE-vio770	PD1									0.7	5.6			
29	R1				APC	CD16						< : >( )			1.2	9.6		0.5	4.0
23	R2				AlexaFluor647	Va7.2													
24	R4				APC-R700	CD107a	(H4A3)		6.0	48	<1:2500>								
25	R6				Zomble NIR	L/D							2	16					
26	R7				APC/Fire 750	CD27	(O323)						1.6	12.8					
27	R8				APC/Fire 810	CD38	(HIT2)												
								Antibody Total	6.0	48	Antibody Total	19.0	152	13.7	109.6		9.5	76
								R10 Media	14.5	116	Brilliant Stain	50	400	50.0	400		50	400
And UNSTAINED CONTROLS !!!								Pippette draw volume /sample	19.5		Pippette draw volume /sample	66		60.7			56.5	

Notes:

Simplified Protocol

Thaw cells, DNAse, count.
Collect, count, aliquot cells 3.0E+6 Cells R10 / 5ml polystyrene tube
Bring volume upto 1 ml R10, add 2 ul PMA/Ctrl and CD107a
Cap and incubate at 37°C for 6 hours

Wash with 2 ml PBS, spin down 1300rpm 8min
800 ul of LiveDead mix (1:2500) @RT for 15min
Wash 2 ml 5% PBS-FBS, spin 1300 rpm, 8min

Add HotStain mix, incubate @37C for 30 min
Wash 2 ml 5% PBS-FBS 1400 rpm, 6 min

Add Tetramers, incubate @RT for 10 min
Add ColdStain mix, incubate @ 4C for 30min
Add 300-500 ul 1x RBC Lysis for 3 minutes
Wash 2 ml 5% PBS-FBS 1400 rpm, 6min

300 ul BD FixPerm, incubate @ 4C for 20min
(vortex every 10 minutes)

First PermWash: 1 ml PermWash 1500 rpm 6 min
Second Perm Wash: 1 ml PermWash 1500 rpm 6 min

Add Intracellular Stain, incubate @ RT for 40min
First PermWash: 2 ml PermWash 1500 rpm 6 min

Resuspend in 70 ul 0.4% PFA-PBS
Cap tubes, wrap rack in foil, store at 4°C

5 11 +1.6 / 13.6 2 in 5 ml 6 µl in 15 ml

February 8th, 2023

PMA: Kitchen Sink

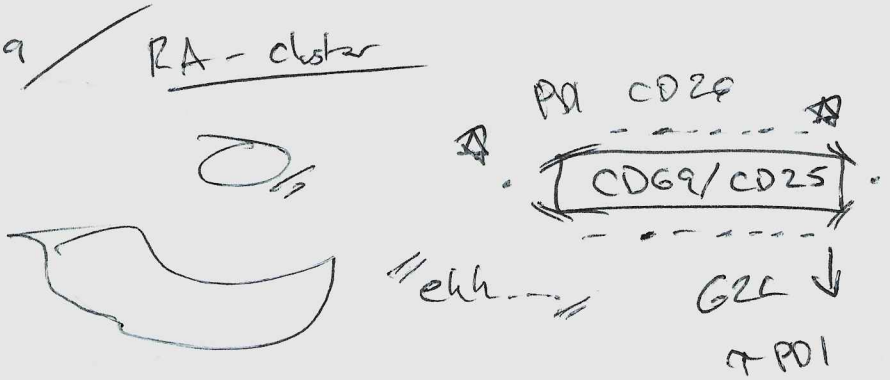
Initial Unmix:

(splitting acquired into 2 separate panels for unmixing)

TNFa on Pe-Cy7 nearly off scale 11 (for adult)

No clear signal for IL-17a (wide uncertainty bands)

Aim: CD69 / RA - cluster



CCR7 ± ↔ Δ icos

ΔCD40L

Wait... intracellular spike remember?