November 10th, 2022

ILT Panel Final Checks #1

180µl 40µl
250µl 40µl

Specimen	Status	Location	Conc	Date	Tasks	Volume	Ly (1:100)	Ly+Mon	Total	2E+6	.2E+6
ND006	adult wk2 after 2nd vax		15E+6	mar11-2021		3	2.32	3.23	6.92	862µl	86.2µl
ND006	adult wk2 2nd vax		15E+6			2	3.72	4.88	7.44	537µl	53.7µl
									14.36		

stain count @ 12:38 pm
Count on Guava @ 12:51 pm

2µl PMA + 500µl + 500µl

O O O O (~~@2M~~) @2M

Spiked L/D → TBD → from actual sample

→ 8M (6M/30) → 200K cells

act CD107A + / IFNg + TNFa + CD69 + unstained ×2 / → 1µl PMA + 500µl (69) (PBS)

ctrl CD62L unstained

3
463
×1.5
2315
4630
694.5
−463.0
261.5
238.5

4 million act + .5 tube (act controls)
4 million act + 1.5 tubes ctl controls

PMA/ctrl in @ 1:37, → 7:37pm, CD107a @ 6µl w/ samples

no direct rbc lyse for TNFa/IFNγ?

06:02 pm decided adjusted ab stain amounts to test
06:03pm ab spin down
Abs prepped by 6:54 pm

Spindown @ 7:54 pm → 36 (20 min)
L/D @ 20:09 → 20:24 pm

2µl 1:10 hCD1d
5µl 1:10 hMR1] Tets @ 20:43 → 21:23

SC's into done @ 23:20 pm
Cold Rbc lyse
10:42/48 → 50.
Spin @ 22:52pm

5
×.8
4.0 → 2.0µl L/D NIR in 5ml

6 * 4+2 cnst = 36 ml 5% FBS
30 * 2 = 60ml SC's FBS
96 ml → 100 ml ← 5ml

RBC lysis
300µl * 6 = 1.8
* 30 = 9.0

11000
1100 − 1:10
9900

6 * 6 = 36ml Permwash
30 * 2 = 60ml
96 ml Permwash

20ml in 200ml 10%

6 * 100µl = 600µl
30 * 50µl 1500µl
2100 ml 0.4% PFA-PBS

7:25pm reagents prepped

Sample Fix Perm @ 11:01 → 11→21

((SC's only got 1 2ml Permwash. Δ size correctly? and sufficient for intracellular SC's?))

1st sample permwash 1 ml @ 23:21pm
2nd wash @ 23:32pm
Intra @ 23:42 → 00:22
Done @ 12:30pm

Cold SC's @ 9:00 pm → 9:30
Hot SC's 9:06pm → 9:36

CD69 .5 @ 4°c @ .5 @ intra
Zombie NIR ✓ CD107A ✓
Rbc lysed & fixed

21:29 − 30 Rbc lyse → 33 → 36
Hot Rbc lyse @ 30

Hot Samples @ 9:41 → 10:11pm
SC's FixPerm @ 9:57 → :07 → :17
Cold Samples @ 10:20 → 10:50
Permwash spin 10:26pm for SC's.
Intra single colors @ 22:46 pm → 23:26 pm

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November 10th, 2022 NK and Innate-like T cell SFC panel - Final Tests

#	Filter	Single color (ul)	Ref ctrl	Unmixing ctrl name	Fluorochrome	Marker	Clone	Vial Lot #	During stim!!!	4	L/D 15 min (RT)	Tetramer (RT) for 40 min	37oC (RT) for 30min	4	Surface staining 4oC for 30min	4	Lysing, then CytoFix/ Perm	Intra mixRT for 40min Intra mix	3
1	UV2				BUV395	CD62L	(DREG-56)								1.2	4.8			
2	UV7				AF	AF-UV6													
3	UV9				BUV496	CD8	(RPA-T8)								0.7	2.8		0.25	0.75
4	UV10				BUV563	CD69	(FN50)												
5	UV11				BUV615	CCR4	(1G1)												
6	U14				BUV661	Vδ2	(B6)								0.7	2.8			
7	UV16				BUV737	CXCR3	(1C6/ CXCR3)												
8	V1				BUV805	CD4	(SK3)								1.5	6.0		0.25	0.75
9	V3				BV421	CD127	(A019D5)												
	V3				Pacific Blue	CD14	(M5E2)								2.0	8.0			
10	V5				Pacific Blue	CD19	(HIB19)								2.0	8.0			
11	V7				BV480	CD161	(REA631)												
					BV510	CD45RA	(Hi100)												
12	V10				BV605	CD56									0.7	2.8			
13	V11				BV650	CCR7													
28	V13				BV711	CD7?													
14	V14				BV750	IFNγ	(B27)												
15	V15				BV786	CCR6	(11A9)												
16	B2				AlexaFluor 488	hCD1d													
17	B3				Spark blue 550	CD3	(SK7)					<1:xx> (1.2)							
18	B4				PE	NKG2D													
19	B6				PE-CF594	CD26	(M-A261)								1.2	4.8		0.25	0.75
20	B8				PE-Cy5	CD25	(M-A251)												
21	B10				PerCP-Cy5.5	TNFα	(MAB11)						1.2	4.8	1.2	4.8		0.25	0.75
22	B13				PE-vio770	PD1												2.5	7.50
29	R1				APC	CD16									1.5	6.0			
23	R2				AlexaFluor647	hMR1													
24	R4				APC-R700	CD107a	(H4A3)					<1:500> (5)							
25	R6				Zombie NIR	L/D			6.0	24									
26	R7				APC/Fire 750	CD27	(O323)				<1:2500>								
27	R8				APC/Fire 810	CD38	(HIT2)						2	8					
													1.6	6.4					
And UNSTAINED CONTROLS !!!						Antibody Total			6.0	24	Antibody Total		12.1	48.4		69.6		5.75	17.25
						R10 Media			14.5	58	Brilliant Stain		50	200	50.0	200		50	150
						Pippette draw volume /sample			19.5		Pippette draw volume /sample		61		66.4			54.75	

Simplified Protocol

- Aliquot cells 3.0E+6 Cells/tube
- Raise R10 + cell volume to 500 ul
- Add 500 ul of PMA-ionomycin/Ctrl
- Incubate 6 hours @37C

Wash with 2 ml PBS, 1400rpm, 6min

800 ul Live/Dead @RT for 15min
Wash 2 ml 5% PBS-FBS 1400 rpm, 6min
Add Tetramers, incubate @RT for 40min
Wash 2 ml 5% PBS-FBS 1400 rpm, 6min

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

Add cold Surface mix, incubate @ 4C for 30min
Add 300-500ul 1x RBC Lysis for 3 minutes
Wash 2 ml 5% PBS-FBS 1500 rpm, 6min

300 ul Cytofix/perm, incubate @ 4C for 20min
Wash twice 1 ml PermWash 1500rpm, 6min

Add Intra Staining, incubate @ RT for 40min
Wash once w/ 2ml Perm Wash
Resuspend 100 ul 0.4% PFA-PBS, store 4C

... alternate sets for CD1c and AF488/647 using Biotin

Ctrl

PMA

107A
CD62L
TNF-
+IFNg
Zombie NIR

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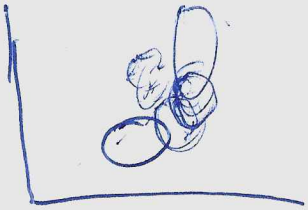
12:32pm start

ctrl unstained ~1400 events.
PMA unstained ~900 events (no monocytes for all effective purposes)

SCs @ 400-470 events/sec

1:07pm → 2:10pm

~200K ~50µl



4 UV7 V7 B3 1 4
(3) ↓UV7 ↑V7 =B3 2
(2) ↓UV7 =V7 =B3 3
(1) ↓UV7 ↓V7 ↓B3

PMA vs ctrl monocytes (resided) similar UV7, V7-A, B3-A (little lower in PMA)

"not dying" vs ctrl monocytes "not dying" slightly higher, ghosts unique to PMA well

deads share ctrl monocyte AF signature, tail out right to ghost location?

These ghost represent the most fully AF (edge/extreme pseudop)

PMA Lymphocytes very similar in UV as controls, but from V2 → V10 have an increase

PMA edge (lesser extent ctrl) ↑UV7 ↑V6/V8 ↑B3 (20)

Ctrl unique ↓UV7 ↑V5/6

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PD1 is squished in SC control, needs more!!

CD62L merge // ↑ CCR4

S6 bright vs average

PMA+ CD69 are not in the dying CD3 pop

Might be better to do a spiked in zombie NIR control given gating