October 27th, 2022

Monocyte SFC panel #5

	Specimen	Status	~~Location~~ Lym+Mon	Conc	Date	Tasks	Volume	Ly	~~Ly~~+Mon	Total	3E+6	2E+6
44C,	ND006 (1)	adult	4.29	15E6	Sep-1-2020	lying lymp	3	3.14	1.500	9.42	.955	
4950/μl	ND006 (2)	adult	4.81	15E6	Sept-1 2020		3	3.41	1.750	10.23	.879	
2900/μl	ND050 (1)	adult	2.86	15E6	Aug-24-2021		3	2,11	0.838	6.33	1.42	
255/μl	ND050 (2)	adult	2.79	15E6	Aug-24-2021		3	1.90	0.880	5.70	1.57	
2500/μl	~~NY062~~	~~adult~~	~~5.30~~	~~20E6~~	~~Aug12-21~~		~~3~~	~~3.79~~	~~1.600~~	~~11.37~~	~~.791~~	
1900/μl	NY062	adult	5.30	20E6	Aug12-12	mon? viable	3	3.88	1.200	11.64	.773	

Count stain @ 12:49 pm → 12:56 pm

Everything was treated w/ 40μl DNASE-I

ND006 substantive "dying" lymphocyte sign

ND050 clean, monocytes ?

NY062 >, alive monocytes

~~Oct-27-2020~~ PPD: 1 μg/1μl ~ 5ug/ml ?

Dec 18, 2020 DTx: 10mg/ml → 10μg/μl ~5μg/ml

5.0μl ~~500μl~~ → ~~50 μg/ml~~ 10^7

0.5μl 500 ~~ml~~ 1ml → ~~5~~ μg/ml

R10 alone

	beads	R10	CRM197	PPD	
1)	O	O 3M / O 1M	O 3M / O 1M	O 3M / O 1M	} Δ conc/cell ~ AF?
2)	O	O 3M / O 1M	O 3M / O 1M	O 3M / O 1M	~ 170μl
3)	O	O 3M / O 1M	O 3M / O 1M	O 3M / O 1M	Monocyte Panel O + CD64
		12 Million	12 million	12 million	~125μl for SCs

//Calculations done @ 1:32pm //

PTA inc 2:07 (3μl in ~~3?~~ Rx/5μl aliq)

cells in @ 2:09 → 8:09 pm

3M cells/ml in 1ml

w/ 5μg/ml of PPD, & DTx (CRM197)

O — O — O ND006 Series w/ CD64 spillover

/O ND050 Medium/alt-b/

3M/21 → 0.150M Lym (+.075 Mon)

~ 200,000 //

UltraComp eBeads plus Lot 2291583 + 64 sc's + unstaineds

×.2

12.8 → 13.2 from a single donor

→ 25 million ~ 23 million NY062

→ 12 million ~ 19.65 ND006

→ 12 million ~ 12.03 ND050

(+ 3)

Starting prep @ 4:00 pm

4:42 ab PBS aliquots

Calcs done @ 5:30 pm

All reagents prepped @ 6:49 pm

Beads @ 7:55

In @ 4°C @ 20:09 pm

Cells spin down @ 8:38pm

Beads rinsed @ 8:43pm spin @ 8:49

cells dumped @ 8:56pm

Beads

22 sc's • 2ml 2% FBS-PBS

resuspend in 50μl 0.4% PFA-PBS

66 sc's spin incubate temp

rbc lyse rinse 2ml 2% FBS-PBS

resuspend in 50μl 0.4% PFA-PBS

9 unstained 500K in ~~50~~ μl 0.4% PFA-PBS

resus

* 6 ml 2% FBS-PBS

13 samples in 100μl 0.4% ~~PFA-PBS~~

* 6ml 2% FBS-PBS

3 dregs → 50μl 0.4% PFA-PBS

* 6 ml 2% FBS-PBS

22 × 3 = 66

~~18~~ 9

9

~~72~~ 84

+3 ND006 (Accord) → diff + CD64

+ 1 ND005 (1 med)

+ 1 NY062 (unstained 3M vs 500K)

+ 2 dreg tubes unstaineds

3.75

4μl Edta

1.5 ml Edta

10 of 10% → 75 ~~80~~ mls

40 PBS 300 mls

2% FBS-PBS = 358.6 mls

PFA-PBS ≈ 11.385 mls

14mls w/o Zombie NIR

(6μl in 15 mls)

~~3.73 mls~~ 30 mls 1x RBC lyse

UltraComp Beads ~1 drop

~1 test × of (5μl +/- 3 drams) pulse vortex

~15-30min @ fridge temp

2-3 mls buffer 400-600g 3-5 min FCS Staining

/resuspend//

9:06 beads sc's fixed & away
everything vortexed, cell samples
went into fridge

condensing sc's → by color.

Ab addition @ 9:11 am

sb @ 9:26 am → :56 pm @ 9:36 pm hoh's were swapped incubator

9:33 L/D in
→ :48 pm

L/D spin @ 9:52 pm ✓

22
×4
88
× 2
176 ~ 3hrs

10:16 pm samples in @ 37°c.

SC's done @ 10:43 (no RBC lysis (not a sadist and I have a 5011 in 36 hours...)

37c spin @ 10:50 pm

23:04 $ cold samples in → 11:34 pm
spin @ 11:36 pm

Done @ 11:52 pm

October 27th, 2022 Monocyte SFC panel

5 μl aliquots for SCs
AB + PBS

#	Filter	Single color (ul)	Ref ctrl type	Unmixing ctrl name	Fluorochrome	Marker	Clone	21 - Vial Lot #	L/D + FC blocker 3ul/rxn	37oC (RT) for 30min	10	4oC for 30min	10
1)	UV2	2.4			BUV395	CD18	(6.7)	2.4	18.6	0.6	6		
	UV6				AF350								
	UV7				BUV496								
2)	UV9	2.4			BUV563	CCR2	(Is132.1D9)	2.4	18.6			0.6	6
	UV10												
3	UV11				BUV661	CX3CR1	(2A9-1)	4.0	17.0	1	10		
4	U14				BUV737	CD19		4.8	16.2			1.2	12
5	UV16				BUV805	CD14	(M5E2)	4.0	17.0			1.0	10
6	V1				BV421	FcER1a		4.0	17.0			1.0	10
	V3				Pacific Blue	CD5						<1>	####
	V5				BV480	CD11a	(HI111)					<0.4>	####
	V7				BV510 (dim)								
7	V8				BV570	CD16	(3G8)	6.0	15.0			1.5	15
8	V10				BV605	CD56	(5.1H11)	4.0	17.0			1.0	10
9	V11				BV650	CD11c	(B-ly6)	4.0	17.0			1.0	10
	V13				BV711								
	V14				BV750	CCR5	(2D7)			<1.5>	####		
10	V15				BV785	CD141		4.8	16.2			1.2	12
11	B2				FITC	CD11b		1.6	19.4			0.4	4
	B3				Spark blue 550								
12	B4				PE	CD88	(S5/1)	1.6	19.4			0.4	4
13	B6				PE-Dazzle594	CD3		2.4	18.6			0.6	6
14	B8				PE-Cy5	CD86	(IT2.2)	2.0	19.0	0.5	5		
15	B10				PerCP-Cy5.5	CD123	(7G3)	8.0	13.0	2	20		
16	B13				PE-Vio770	HLA-DR	(REA805)	1.2	19.8			0.3	3
	R1												
17	R2				Alexa Fluor 647	CD163	(GHI/61)	4.0	17.0			1.0	10
18	R4				APC-R700	CD40	(5C3)	4.0	17.0	1.0	10		
19	R6				Zombie NIR	L/D		<3200 μl?>	<1:2500>				
(21)	R7				APC/Fire 750	CD64		<3.2?>	17.8			<0.8>	
20	R8				APC/Fire 810	CD38	(HIT2)	4.0	17.0	1.0	10		
~~20~~					True Stain FcX					0.5	5	0.5	5
~~21~~					Antibody Total					6.6	66	11.7	117
					Brilliant Stain					50	500	50	500
	And UNSTAINED CONTROLS !!!				Pippette draw volume/sample					55.6		60.7	

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Simplified Protocol

Aliquot cells 3E+6 cells/tube

Wash with 2ml PBS, 1300rpm, 8min

2500x diluted Zombie NIR, 1E+6/1ml + 3ul Fc blocker at RT for 15min

Wash with 2ml 2%FBS-PBS-2mM EDTA

Spin at 1300rpm for 8min

x ul of 37oC Ab mix, at 37oC for 30min

Remove RBC with 1ml lysing solution for 3min

Wash with 2ml 2%FBS-PBS-2mM EDTA

Spin at 1400rpm for 6min

x ul of 4oC Ab mix, at 4oC for 30min

Treat with 600ul of 1x Lysing solution at RT for 3min

Wash with 2ml 2%FBS-PBS-2mM EDTA

Spin at 1400rpm for 6min

Resuspend in 0.4%PFA-PBS

Error for master mix, thus ↑ so brightness

Monocytes SFC Panel

10/27/2022

Spectrum	UV				Violet				Blue				Red			
373	UV1															
388	UV2	**BUV395**	[2]	**CD18**												
428	UV3				V1	**BV421**	[4]	**FcER1a**								
443	UV4				V2											
458	UV5				V3	**PacBlue**	[1]	**CD5**								
473	UV6	AF350		<AF>	V4											
508					V5	**BV480**	[3]	**CD11a**	B1							
514	UV7	BUV496	[2]	<AF>												
525					V6				B2	**FITC**	[1.5]	**CD11b**				
542	UV8				V7	BV510	[1.5]	<AF>	B3	SparkBlue 550	[1]					
582	UV9	**BUV563**	[3]	**CCR2**	V8	**BV570**		**CD16**	B4	**PE**	[4]	**CD88**				
598					V9				B5							
613	UV10	BUV615	[3]		V10	**BV605**	[3]	**CD56**	B6	**PE-Dazzle**	[4]	**CD3**				
664	UV11	**BUV661**	[3]	**CX3CR1**	V11	**BV650**	[3.5]	**CD11c**	B7				R1	APC	[3.5]	
679									B8	**PE-Cy5**	[4.5]	**CD86**	R2	**AF647**	[3.5]	**CD163**
697	UV12				V12				B9	**PerCP-Cy5.5**	[2]	**CD123**	R3			
717	UV13				V13	BV711	[4]		B10				R4	**APC-R700**	[3]	**CD40**
738									B11				R5			
750	UV14	**BUV737**	[3]	**CD19**	V14	**BV750**	[2.5]	**CCR5**								
760									B12				R6	**Zombie NIR**	[ ]	**L/D**
783	UV15				V15	**BV785**	[3]	**CD141**	B13	**Pe-Vio770**	[3]	**HLA-DR**	R7	**APC-Fire 750**	[2]	**CD64**
812	UV16	**BUV805**	[1]	**CD14**	V16				B14				R8	**APC-Fire 810**	[3]	**CD38**

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Monocyte SFC panel #5

PPD CD14 (ran empty tube CD14, 2 sitflushes, overwrote actual CD14)
PPD elicited a wider scatter to the monocyte pop.
DTR appears active also.

	NDO50	NDO06	NY062
ctl	0	0	0
DTx	0	0	0
PPD	0	0	0

12 / 45 (1:45)
90 → 1:30

Media	22
DT	22
PPD	22
Bead	

Dr beads	100	250	300
Dr Monocyte	61	327	307

In spectroflo

Not enough CD14 beads ~ 5000 event.

☆ Look @ AF of unstained/ see where off for hints ☆

1 NDO06
2 62a
3 62b

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Monocyte SFC panel #5

Monocytes #5:
- cleaned up Desktop (56 GB)
- exported previous spectroFlo experiments to H: drive
- import the 4 sets of reference controls
  - append the samples to each
- into a common Monocyte #5 folder w/ subfolders,
  - raw files kept here for samples, not scs?

For starters, all just have NY062 media + unstained as samples for initial unmixing (to not overload memory) (44 GB free c/ memory)

W, H, A A

100	WHA	61
250	WHA	327
300	WHA	307

// Bead file not cooperating unfortunately //

A

CD86 looks constitutive

How to gate consistently?

- which gates differentially expressed? CD19,

Center the octoid gate from brightest ↑ SSC monocytes ↓ to lymphocyte gate edge

gate ↑ $10^{5\wedge1} \rightarrow 10^6$ (note exceptions)

for lymph @ edge add spur to top ↗ boundary →

edge for Dead

Quantifying this systematically is tricky

NY062
ND050
ND006

DTx missing CD88 pe

→ unmixing matrix failed to show comparable overlaps due to this ←

Media CCR2 $10^5 - 10^6$ (edge Δ)

Media CX3CR1 (lot high flying events)

Media CD19 $10^{4\wedge3} \rightarrow 10^5$

Media FcERIa $10^{4\wedge3} \rightarrow 10^{5\wedge1}$

Media CD16 brightest most cohesive signal $10^{5\wedge-2}$ tails $\rightarrow 10^{5\wedge6}$ tails

exclusion events

56 $10^{4\wedge3} \rightarrow 10^{5\wedge1}$

CD11c $\rightarrow 10^5 - 10^6$, CD141

CD3 $10^5 - 10^6$

CD86 $10^{4\wedge6} \rightarrow 10^{5\wedge1}$

CD123 $10^{4\wedge1} \rightarrow 10^{5\wedge1}$

CD163 $10^{4\wedge3} \rightarrow 10^{5\wedge1}$

L/D zombie $10^5 - 10^{\wedge6}$ (not cohesive)

CD38 $10^{4\wedge1} - 10^5$

debris → CD64 $10^{5\wedge-2} \rightarrow 10^6$

AFs - Keywords

* Treatment
  0 = Media 1 = DTx 2 = PPD
  ~ 6 hrs stimulation
* Donor
  0 = NY062 1 = ND050 2 = ND005
  ~ same processing

running solely on AFs, no FSC or SSC properties, etc.

Each unstained = 242,000 cells
~ 2,178,000 total cells

running PaCMAP @ 1:24 pm

CD141 650 ct
CD163 ~ 550
CD40 ~ 400

Media		DTx	PPD
CD8	$10^{5 \wedge 3} \rightarrow 10^{6}$		

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Monocyte SFC panel #5

Positive gate

(Fitc CD11b + AF) − (AF) = Pure Fitc

UV LV67 UV LV67

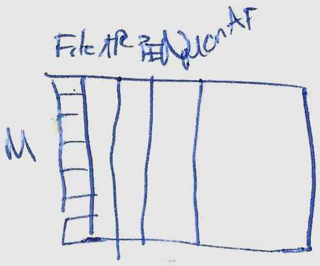
<UV V67

− UV high "UV high" Blue "+/-"

4% 96%

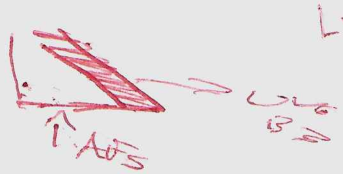
No Δ across donors
small Δ's across treatments
Δ across cell types
(DCs ~ Δ AF than mon & act Mon)

CD16 BV570 + reg hyMAF
+ small contam



positiv + AF − AF = positive
M
mon − new tags n mon

SC ref



Lympho

positive + AF − ? AF = positive
mon positi
− mon

SC ref

actv
AF 1
AF 2

What's the cutoff line?

* Avoidance → set your gate to avoid contaminant AFs in your reference controls
¿impact of brightness? ie, if your DCs are brighter and you just focused on monocytes
In pops w/ multiple AFs, set on Δ pop w/ unique AFs? Mon vs Mon act, Lym? ← this issue
→ under activation conditions, set on non-activated 1 AF
(~ w/ brightness caveats)

SC's from beads + AF tags instead ay caramba!!

Where do activated AF atypicals end up? (at peaks)

AF sinks

- FcER1a * DC AFs ← ¿as a sink? *
- CD11c ~ low expression on monocytes, bright expression on DCs.
- CD141 ~ low expression on monocytes, bright cDC1's
- CD123

>CD40 ↑ brightness on monocytes

errors multiple

~ cDC gates to monocytes

~ Lym gate ↑ size

- DC's markers
- Lym markers

} low abundance

Fuck you!

"This is too many samples to scroll down and see"

1115 299 947

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Monocyte SFC panel #5