Common Methods in immunology

And their difficulties and pitfalls

Dr. Lea Taylor

Topics

1. Flow Cytometry
2. RNA Sequencing
3. TCR and BCR sequencing

1. Flow Cytometry

1. Acquire events

2. Normalize Data

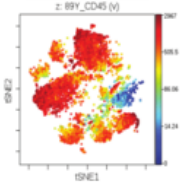
3. Debarcode files (if applicable)

4. Gate for live singlet events

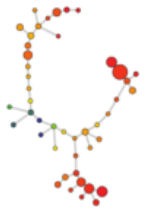
Analyze by traditional boolean gating

Analyze with algorithm(s)

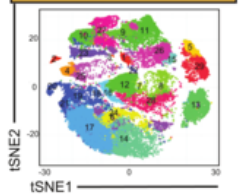
viSNE



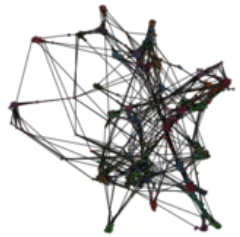
SPADE



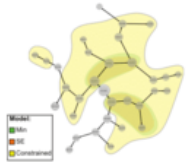
PhenoGraph



X-shift



Citrus



When to use FlowJo

FlowJo is the work horse of Flow Cytometry analysis

You have Flow Cytometry Data or Mass Cytometry

Anything between 2 and 14 colors is feasible

COMPENSATION!!!!

What is a .fsc file

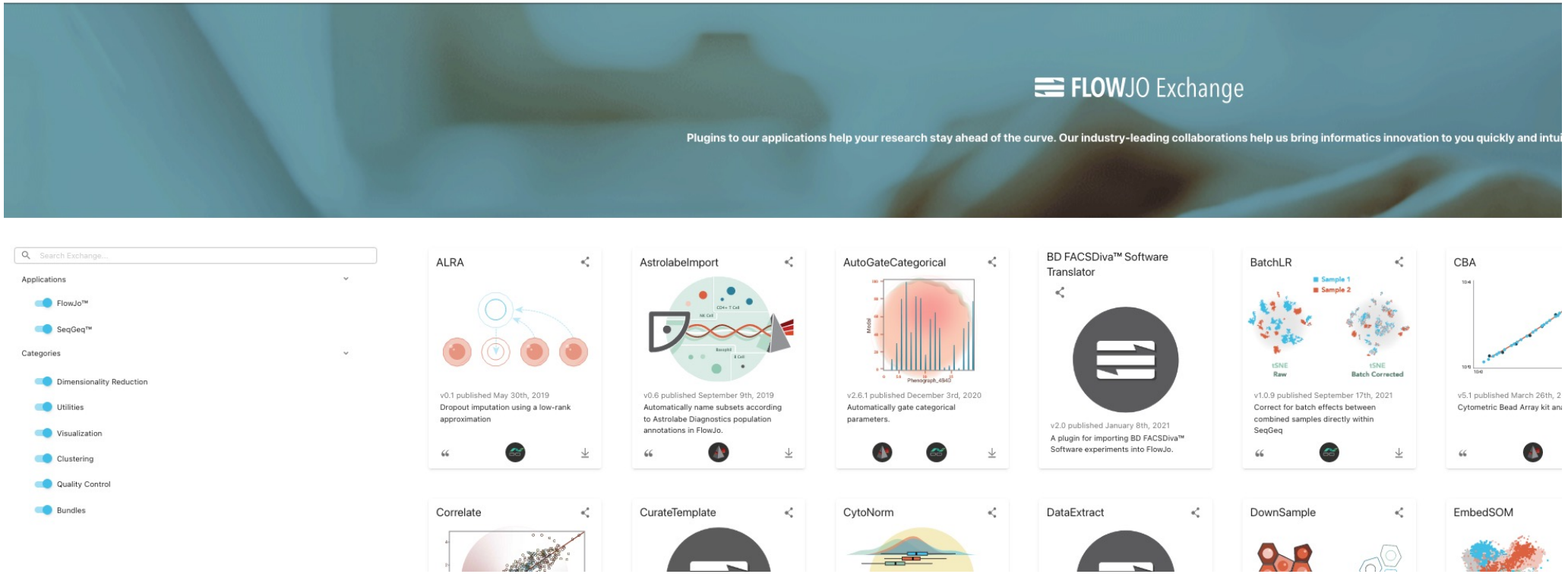
1. Cytometry data
2. Metadata

FSC-A	FSC-H	SSC-A	FJComp-AARD-A	FJComp-APC-H7-A	FJComp-Ax488-A	FJComp-Ax700-A	FJComp-PE-A	FJComp-PE-Cy5-A	FJComp-PE-Cy7-A	FJComp-PE-TxRed-A	FJComp-PacBlue-A
120579.12	104987	44034.00	220.80475	81.8041763	80.103394	-34.6044350	7.469240e+03	3518.786377	-3.6115351	47.048943	92.091324
111824.64	96847	37833.75	217.28133	2803.1440430	50.608536	42.9815979	2.677187e+01	510.473633	87.8014755	34.959339	18.914070
118530.73	105923	29655.75	140.39104	1.5925758	47.308800	605.2854614	1.315144e+01	97.072586	88.4622421	2874.945801	25.834587
130061.52	114730	26773.50	153.88135	7.2521353	69.706207	1338.0178223	4.201168e+01	143.403503	99.0259781	7.943945	2821.871094
131541.84	113050	37986.00	226.82951	30.5443878	84.445320	686.7761230	2.826719e+01	-70.994576	96.8211746	3493.785889	61.496696
94222.80	81149	30656.25	166.84659	16.7258968	85.709511	1149.7573242	7.893163e+01	2891.302002	224.1487122	2588.175537	59.880505
84591.37	69472	38713.50	209.24405	66.0515900	78.988998	1406.6749268	5.802124e+01	-45.092579	230.4299164	3589.281250	91.244644
126300.24	111266	43332.75	221.62988	23.7860966	89.275154	914.4905396	7.237708e+01	285.010925	125.6522293	11.781715	2730.924316
118645.92	105392	35396.25	162.32948	-32.8473549	79.566658	1455.0655518	-1.067613e+01	876.413391	82.7316055	3242.814941	115.997368
127788.48	112747	31811.25	182.83990	84.6169434	97.154762	985.9942017	3.686142e+01	-15.333862	89.6051254	4028.061523	92.006165
103549.69	91614	31973.25	140.58505	55.5311890	77.519653	1465.4819336	6.771473e+00	1020.770203	86.9379807	3051.562012	80.758972
106204.33	93730	31484.25	176.10905	-94.7903900	38.823074	2241.2373047	-6.503075e+01	589.882080	81.9102554	3655.586670	66.216530
80729.28	72802	47832.75	203.79915	9.2464190	41.240608	50.6559715	4.358342e+03	-22.028620	163.3323822	186.100464	1038.423096
105510.24	90569	48492.00	294.49133	-1.5806031	79.825333	-9.7515669	6.435017e+03	4485.864746	237.9988861	298.391937	1070.301392
93592.80	78883	36726.75	213.31126	2002.1612549	76.494049	57.7512512	8.127861e+01	979.643311	149.6526794	6.016162	109.540733
125699.05	108850	41855.25	238.19644	226.4483643	90.828094	1328.6246338	4.018067e+01	221.950775	136.8989716	5058.541992	163.276764
77925.60	51885	51897.75	234.30276	114.1668243	66.071167	553.6314087	1.795088e+02	46.148647	150.5481110	31.300348	88.303879
119127.60	105138	35553.00	204.95068	31.3594837	48.278599	1271.8177490	4.003592e+02	154.268906	29.9994526	4065.328857	61.479176
113652.73	101108	20745.75	146.40933	-7.4413428	75.987579	918.4487915	1.178633e+01	1055.306152	112.2102661	2864.214600	24.751148
122265.37	107919	28829.25	166.98257	-32.5024948	60.992855	1353.9030762	4.140195e+01	-12.530173	163.6040039	3156.037842	125.227768
107834.41	95547	22797.75	56.34419	48.6063805	29.924494	1226.3300781	1.012048e+01	-14.087471	40.6047401	2050.024902	129.801193

FlowJo Plugins

FlowJo exchange

- https://www.flowjo.com/exchange/#/



Further tutorials

Material for a 5 day course on using R for Flow Cytometry analysis

- https://taniawyss.github.io/flow-cytometry-analysis-with-R/
- Much more detailed than this one hour introduction and purely R based.

Four part (youtube) course on using R for Flow cytometry analysis

- https://www.youtube.com/watch?v=2INqQNMNaV0

Workshop for CyTOF analysis using R, can also be used for Flow Cytometry

- http://bioconductor.org/help/course-materials/2017/BioC2017/Day2/Workshops/CyTOF/doc/cytofWorkflow_BioC2017workshop.html

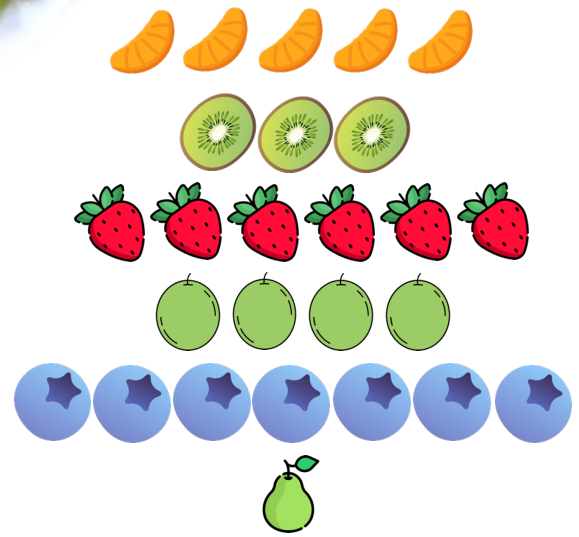
2. RNA Sequencing

Bulk vs single cell vs spatial

Spatial transcriptomics
Spatial resolved expression level

Bulk RNA-seq
Average expression level

scRNA-seq
Single cell expression level



www.flaticon.com

Many datasets are presorted:

- Quality of sorting varies a lot!
- Good questions:
  - Where did you sort? -> Core facility is much better than own sorter
  - Sorting quality –> did they do a quality check
- Which markers did they use for sorting?

Sample preparation

1. Digestion?
   - What digestion did they use?
     - To get tissue DCs you need much harder digestion that would kill most other cells (especially Eosinophils and Neutophils)
2. Cell dissotiation
   - Mechanical?
     - Scissors work great, sometimes even better than GentleMACS or similar
     - Do you feel like they know what they are talking about

Annotation challenges

a) How do we define a cell type?

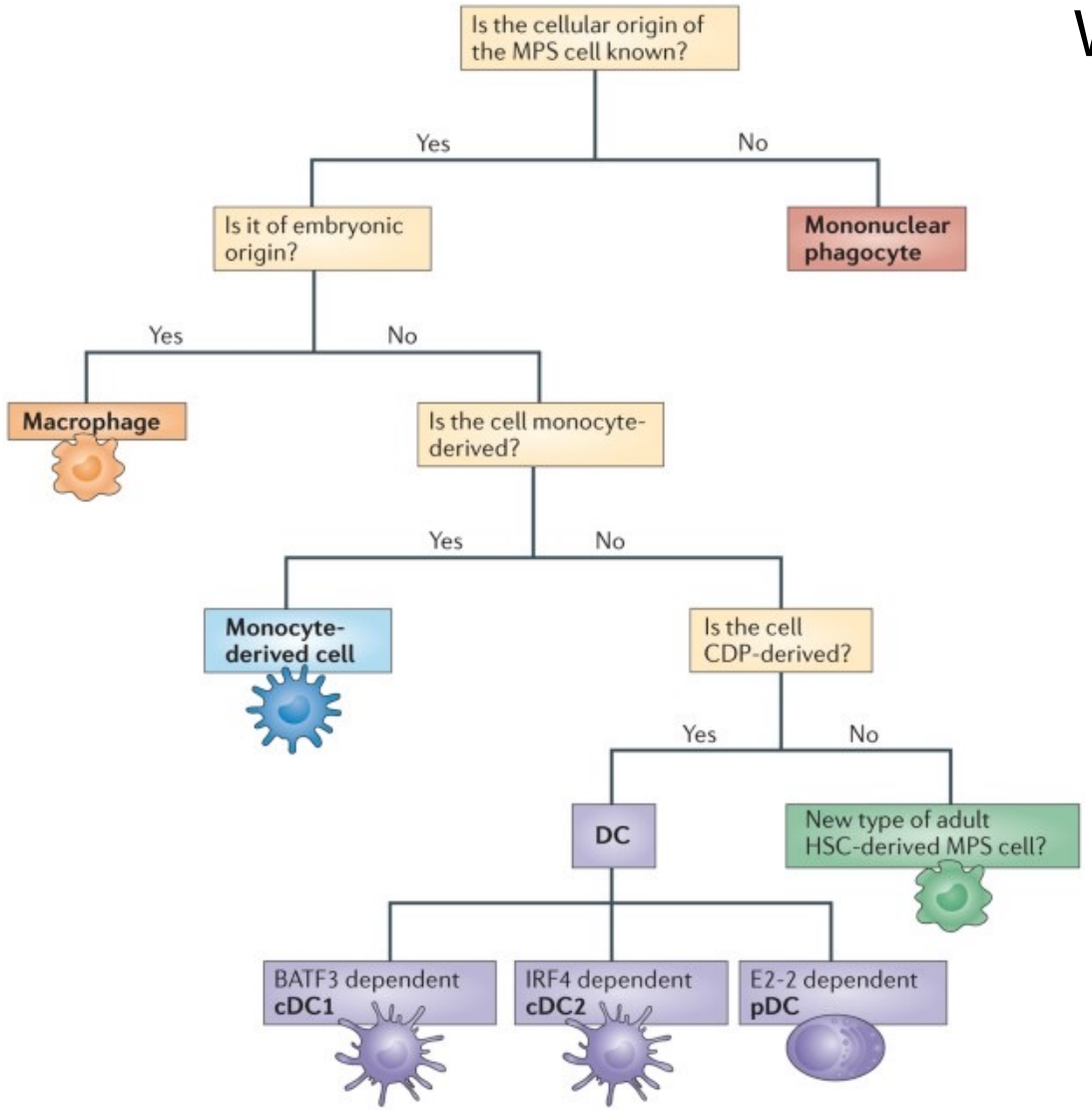
b) Cells have very different expression profiles
   - Dependent on tissue, age, status (resting, infection, …)

c) We don’t know how many cell types exist
   - Are the cell sub-types stable or intermediate
   - How “deep” should we annotate

d) Cell annotation is difficult in non model species

a) How do we define a cell type?

What is a macrophage anyway?

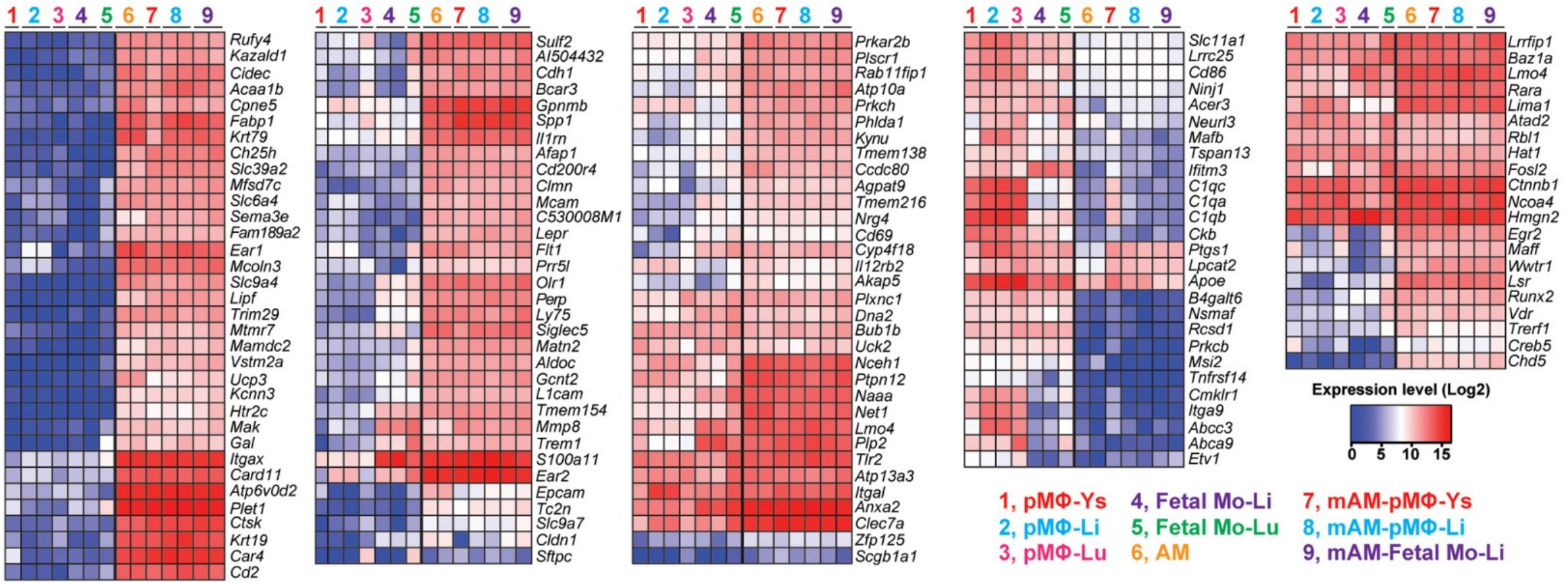


Dendritic cells, monocytes and macrophages: a unified nomenclature based on ontogeny

Martin Guilliams, Florent Ginhoux, Claudia Jakubzick, Shalin H. Naik, Nobuyuki Onai, Barbara U. Schraml, Elodie Segura, Roxane Tussiwand & Simon Yona

Nature Reviews Immunology **14**, 571–578 (2014) | Cite this article

a+ b) Origin and tissue effect



Fetal monocytes possess increased metabolic capacity and replace primitive macrophages in tissue macrophage development

Fengqi Li, Katarzyna Maria Okreglicka, Lea Maria Pohlmeier, Christoph Schneider, Manfred Kopf

Author Information

The EMBO Journal (2020) 39: e103205 | https://doi.org/10.15252/embj.2019103205

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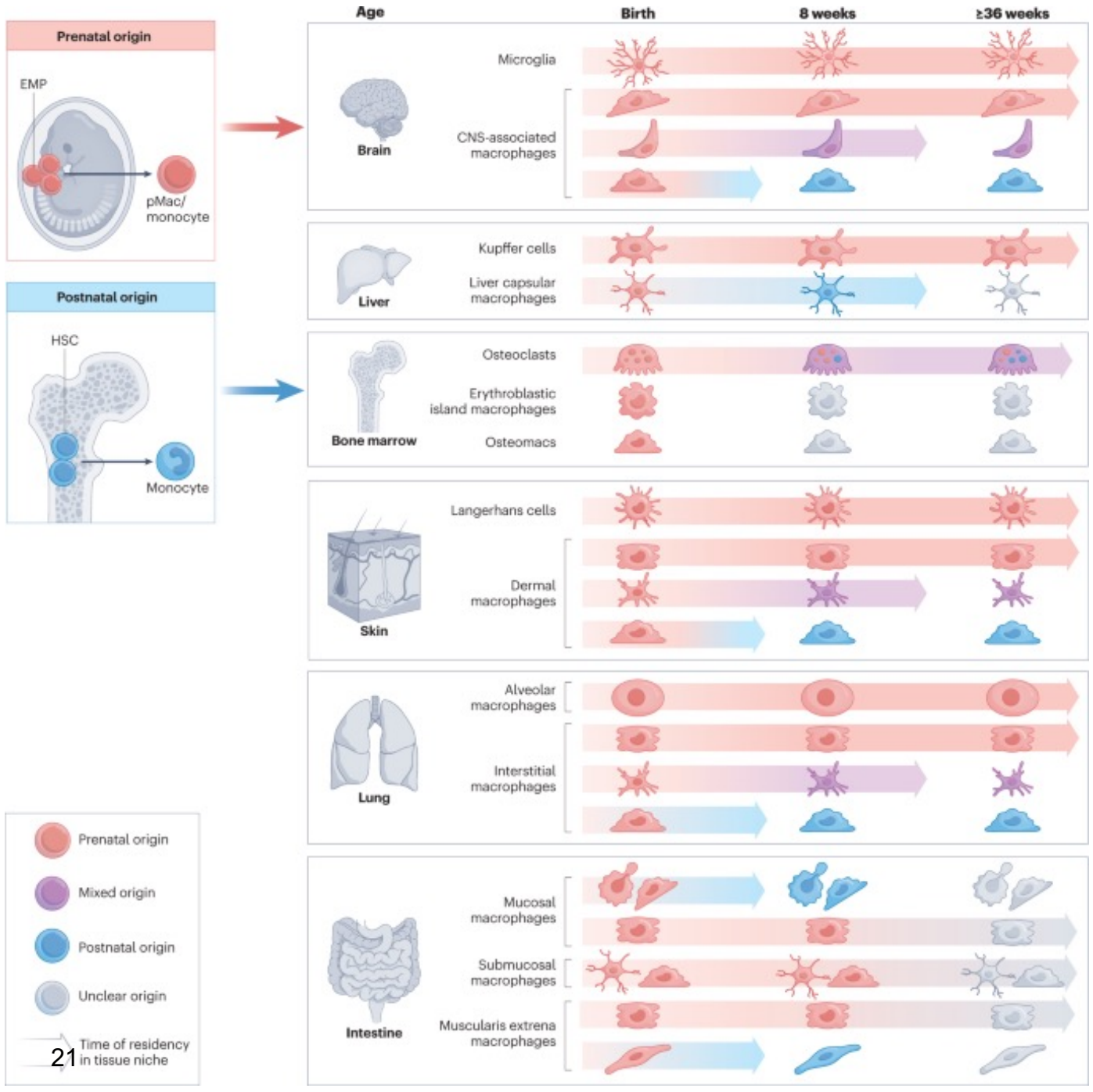

b) Effect of age

Review Article | Published: 15 March 2023

Tissue-specific macrophages: how they develop and choreograph tissue biology

Elvira Mass ✉, Falk Nimmerjahn, Katrin Kierdorf & Andreas Schlitzer

Nature Reviews Immunology **23**, 563–579 (2023) | Cite this article

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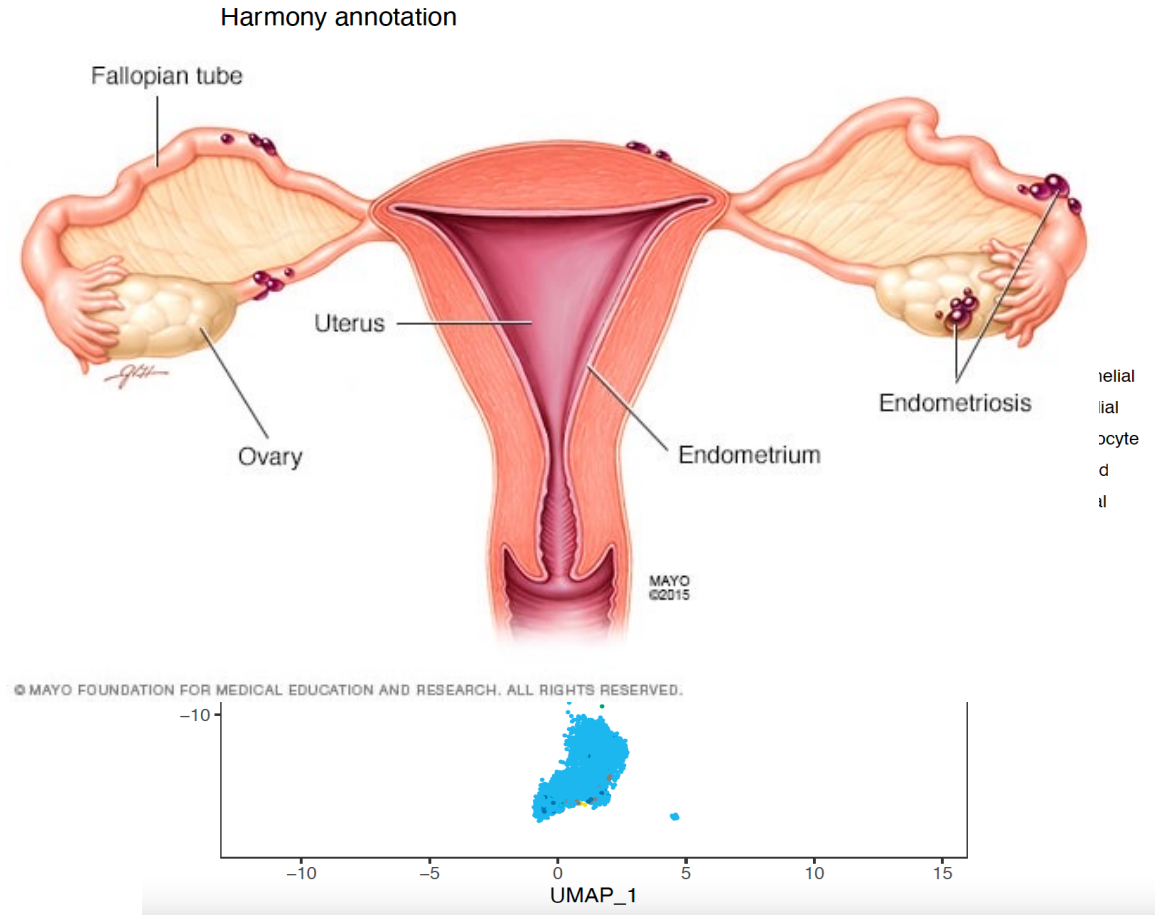
c) How many cell types exist?

1. Biopsies of endometriotic lesions
2. Single nucleus sequencing
3. Annotated major cell types
4. Annotation of “minor cell types”



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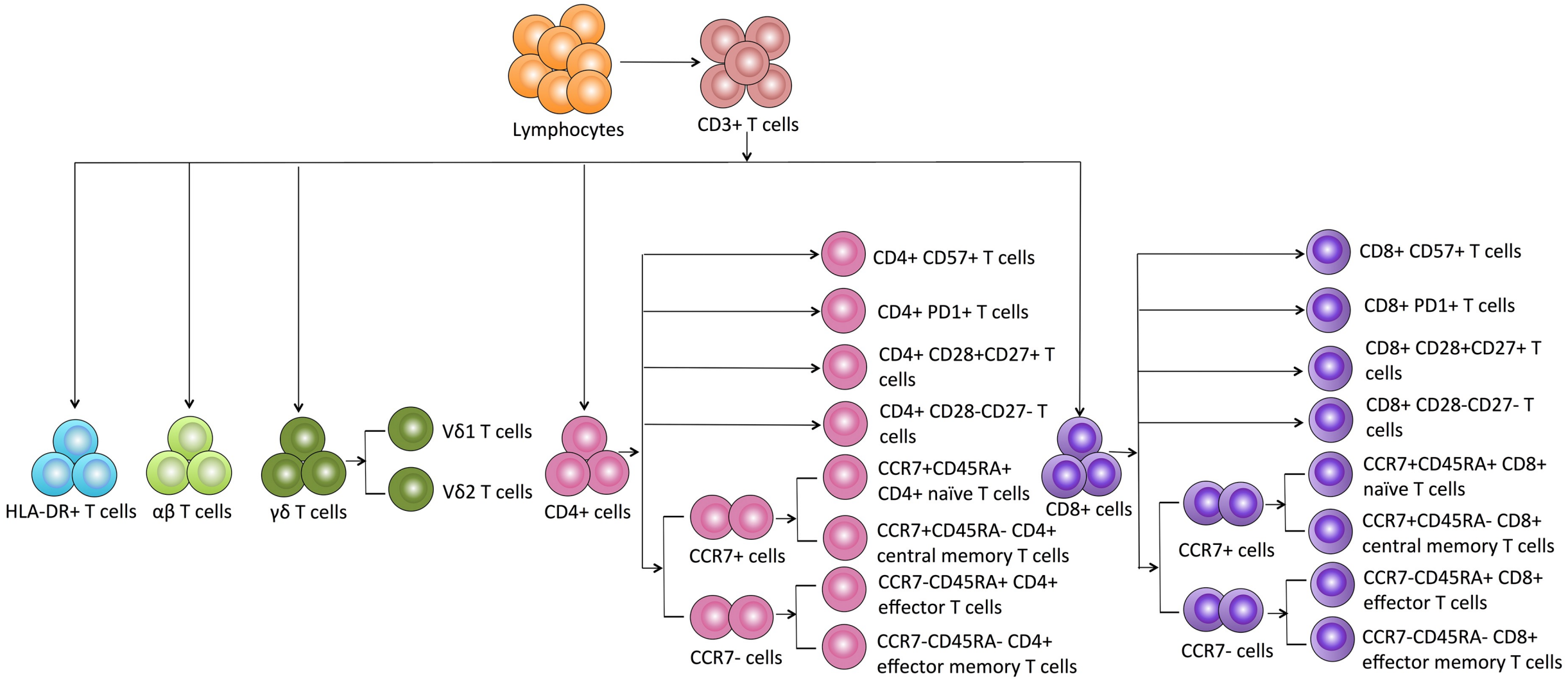
c) How many cell types exist?

	p_val	avg_log2FC	pct.1	pct.2	p_val_adj	cluster	gene	mean.in.cell_type	mean.out.of.cell_type
ITGAD	0	3.086294964	0.474	0.005	0	NK1	ITGAD	2.19396744	0.050885454
B4GALNT1	0	1.544004483	0.165	0.004	0	NK1	B4GALNT1	1.102642643	0.031177658
ILDR2	1.23E-216	1.10072698	0.102	0.005	3.44E-212	NK1	ILDR2	0.810789748	0.04839456
STYK1	1.34E-195	1.047112546	0.11	0.007	3.74E-191	NK1	STYK1	0.779595606	0.054146239
DPF3	8.99E-185	1.030089264	0.104	0.007	2.51E-180	NK1	DPF3	0.766504999	0.050245524
TRGV9	9.93E-85	0.894831036	0.1	0.001	2.77E-80	NK2	TRGV9	0.634978312	0.014787231
NMUR1	8.95E-78	1.072033915	0.15	0.003	2.50E-73	NK2	NMUR1	0.771180083	0.029102055
KIR2DL4	1.44E-77	0.828338172	0.117	0.002	4.01E-73	NK2	KIR2DL4	0.594807269	0.021516427
SCUBE1	4.84E-44	0.624765981	0.1	0.003	1.35E-39	NK2	SCUBE1	0.458147778	0.026288886
PLCH2	6.32E-34	0.785372663	0.133	0.006	1.77E-29	NK2	PLCH2	0.607080231	0.063949964
XCL1	1.24E-58	0.929970452	0.111	0.002	3.46E-54	NK3	XCL1	0.669050507	0.025217098
AC017104.1	8.99E-31	1.164906974	0.111	0.005	2.51E-26	NK3	AC017104.1	0.843514065	0.035668979
AC026979.2	2.25E-10	1.024808688	0.13	0.017	6.29E-06	NK3	AC026979.2	0.861052856	0.152251131
TRMO	4.74E-09	0.906697766	0.111	0.015	0.000132464	NK3	TRMO	0.743126441	0.115262444
SNHG22	2.68E-08	0.798941627	0.13	0.021	0.000749359	NK3	SNHG22	0.733162018	0.180653732

Cell calling by both SingleR and Symphony from the Tan dataset (annotating cell when they both agree)

d) How ”deep” should we annotate



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e) Cell annotation of non model

1. SingleR
   - HumanPrimaryCellAtlasData(...) Human
   - BlueprintEncodeData(...) Human
   - ImmGenData(...) Mouse
   - MouseRNAseqData(...) Mouse
   - DatabaseImmuneCellExpressionData(...) Human
   - NovershternHematopoieticData(...) Human
   - MonacoImmuneData(...) Human
2. Symphony
   - Needs a annotated dataset

e) Cell annotation of non model

1. Can they provide you with a list of marker genes?
   - Not just surface if possible!
2. You can use orthologs but be careful since these cell may vary a lot between species!

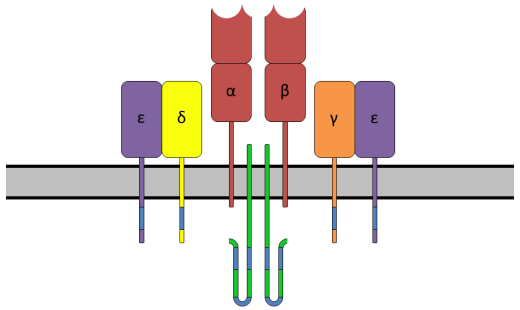
- Ask where these lists are from?
  - Do you feel like they know what they are talking about or

Resources

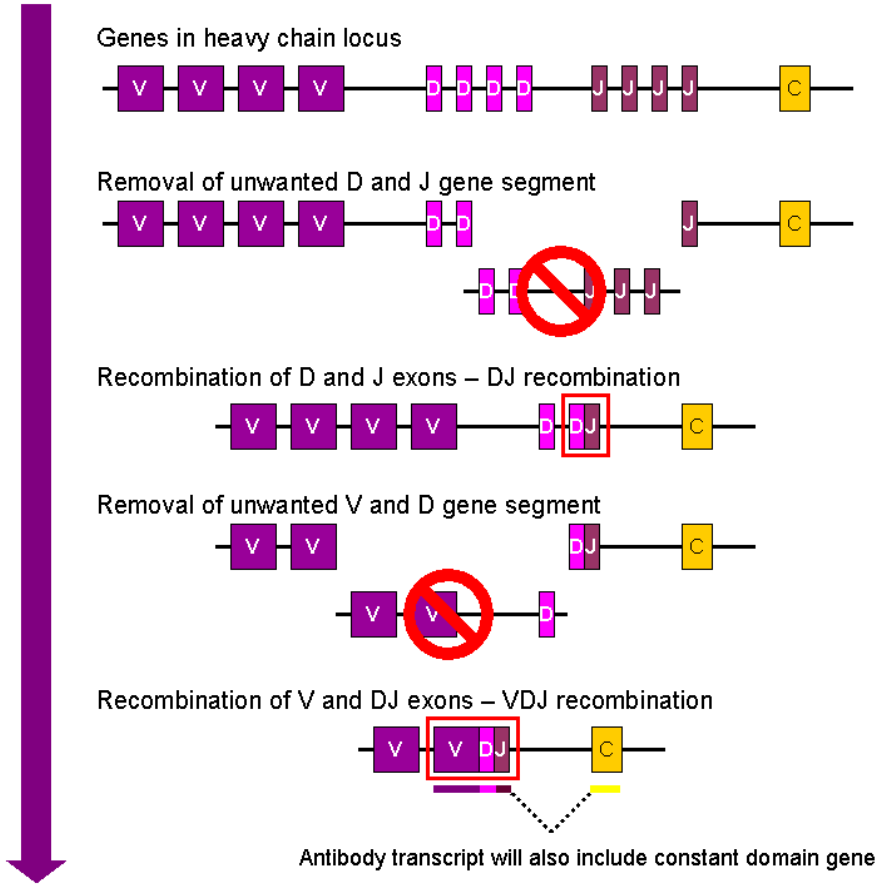
- SIB course material: https://sib-swiss.github.io/single-cell-training/
- 10X: https://www.10xgenomics.com/
- Seurat: https://satijalab.org/seurat/
- Scanpy: https://scanpy.readthedocs.io/en/stable/

3. TCR sequencing

T cell receptor (TCR)



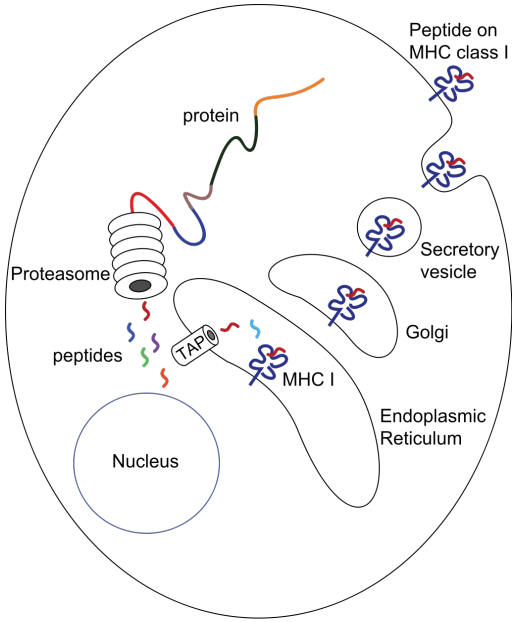
- The TCR alpha chain is generated by VJ recombination, whereas the beta chain is generated by VDJ recombination (both involving a random joining of gene segments to generate the complete TCR chain).
- Likewise, generation of the γδ TCR gamma chain occurs by VDJ recombination.
- For example, the human immunoglobulin heavy chain region contains 2 Constant (Cμ and Cδ) gene segments and 44 Variable (V) gene segments, plus 27 Diversity (D) gene segments and 6 Joining (J) gene segments



Major histocompatibility complex (MHC)

MHC class I

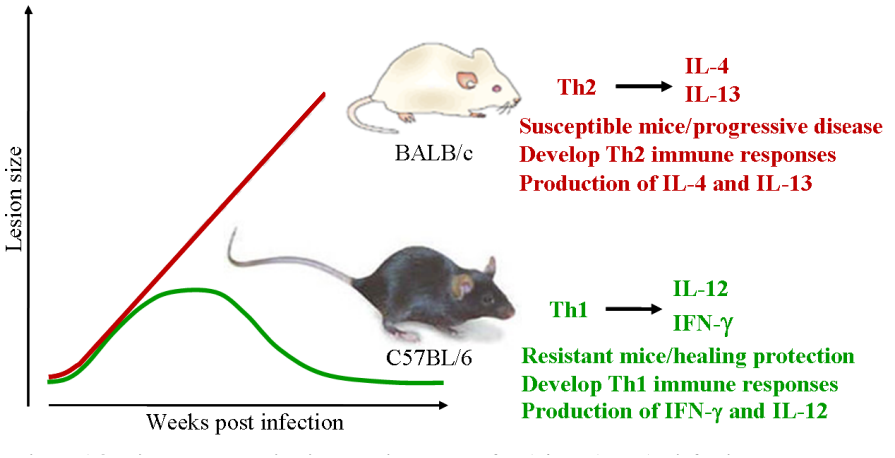
- Present on all nucleated cells
- Normally presents cytosolic peptides, mostly self peptides derived from protein turnover and defective ribosomal products
- During viral infection, intracellular microorganism infection, or cancerous transformation, such proteins degraded in the proteosome are as well loaded onto MHC class I molecules and displayed on the cell surface
- CD8⁺ T cells surveil MHC I and kill cells that bind ”their” TCR. Cell that downregulated MHC (virus infected or tumor cells) get killed my NK cells.



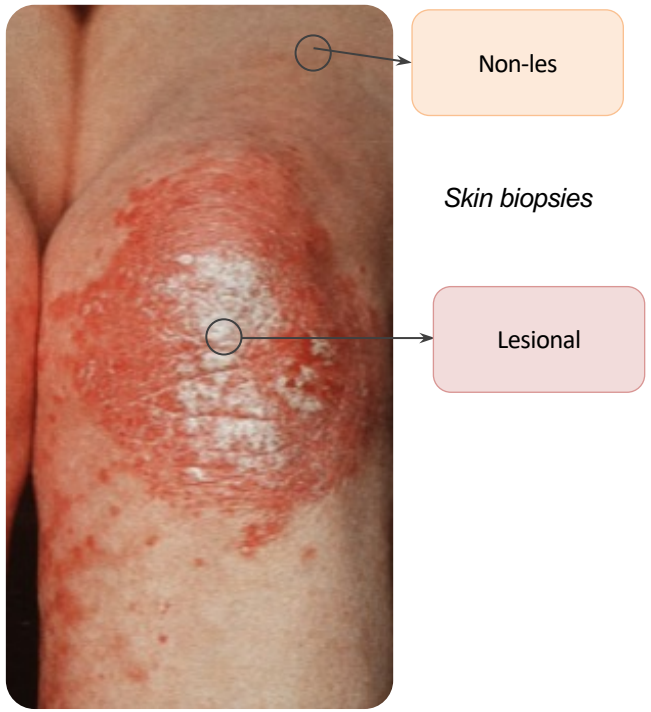
cells (Bretscher *et al.*, 1992; Menon and Bretscher, 1998; Uzonna *et al.*, 2004).

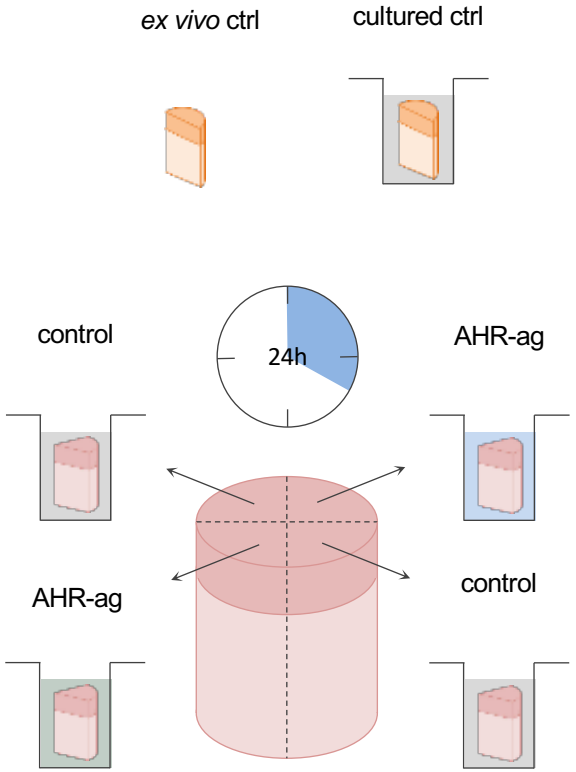
MHC class II

- Present only on antigen presenting cells (APCs) such as macrophages and dendritic cells
- Entities taken up by phagocytosis into phagosomes fuse with lysosomes. MHC class II expressed in the lysosome gets loaded with a peptide.
- These are trafficked to and externalized on the cell surface.
- Recognized by CD4⁺ T cells, during activation the T cells “decide” which Th response is needed. (It is not completely understood how)



The project:





Staining (TotalSeq-Ab, FACS-Ab)

FACS sort (CD45+/CD3+)

scRNAseq / V(D)J-seq

Bioinformatics

QC

– Correlation TotalSeq vs RNAseq?

Transcriptome

Change in T cell population frequency?

- High risk of sample bias

Normalized to T cell population → change in transcriptome?

T cell clones (VDJ-Seq)

Distribution of T cell clones between conditions?

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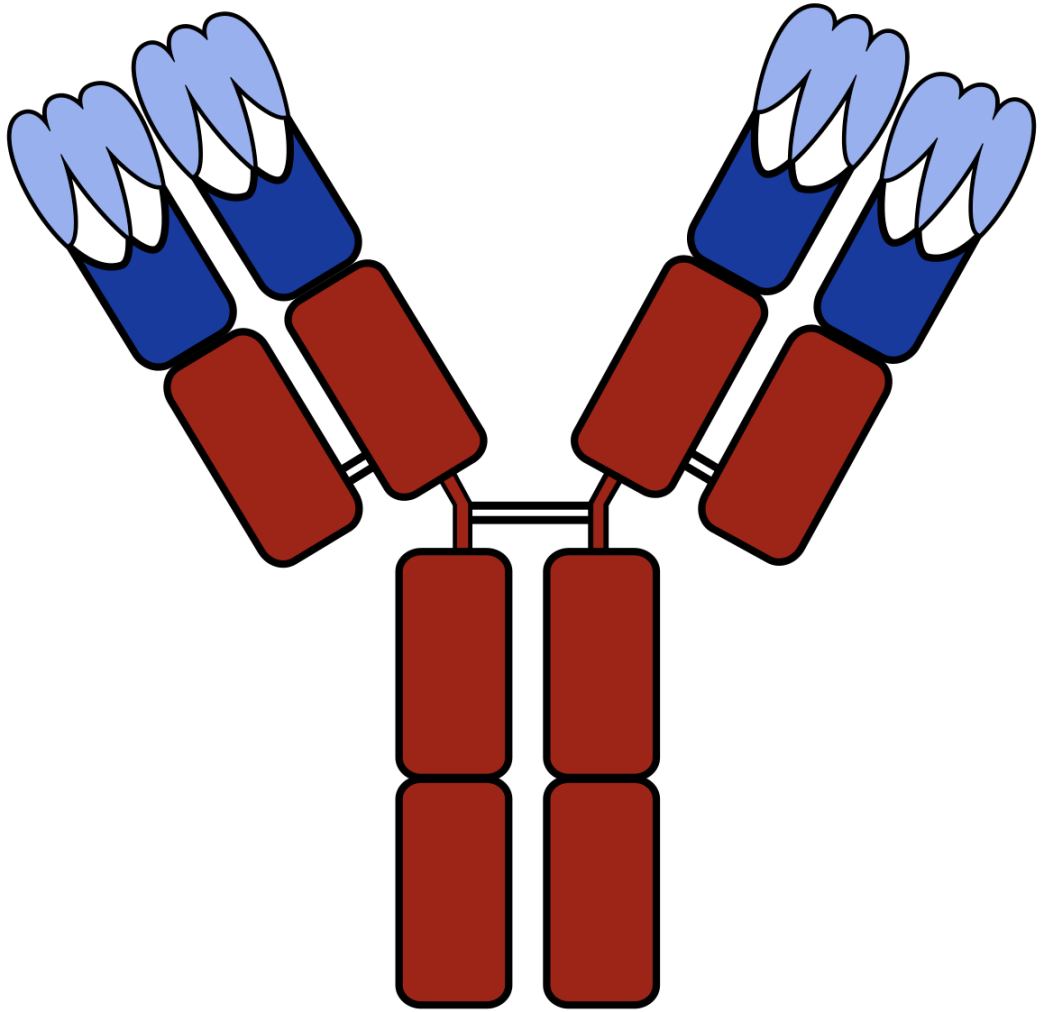
Complementarity-determining regions

Sketch of an antibody with the variable domains shown in blue, and the CDRs (which are part of the variable domains) in light blue.

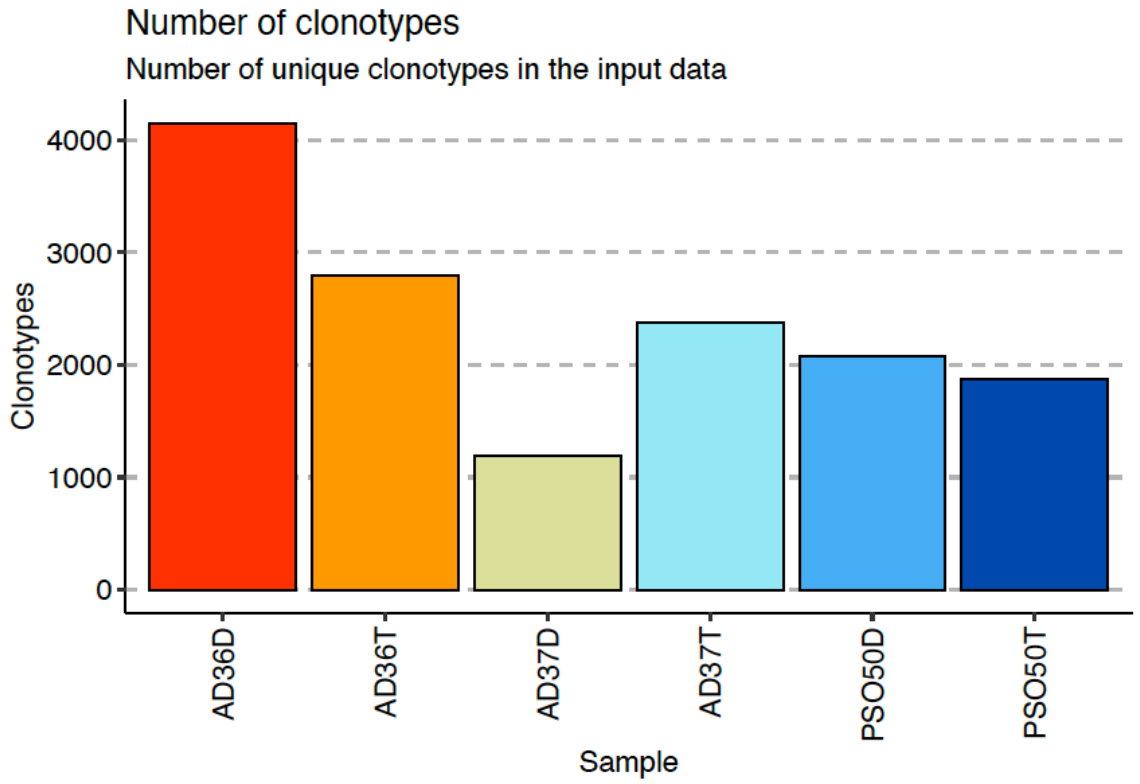


Output of Cellranger-VDJ

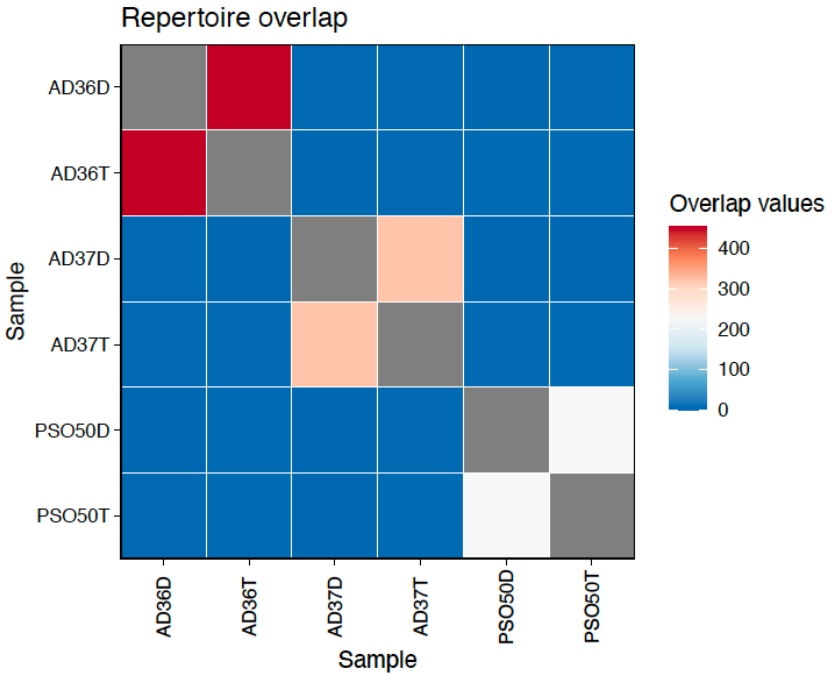
Clones	Proportion	CDR3.nt	CDR3.aa	V.name	D.name	J.name	Sequence	chain	Barcode	raw_clonotype_id	ContigID
37	0.00768592	TGTGCAGAG	CAETNDYKLS	TRAV13-2;TRBV5-1	NA;NA	TRAJ20;TRBJ1-2	TGTGCAGAGACTAACGAC	TRA;TRB	AACCGCGAGCACGCCT-1;AAGAC	1	AACCGCGAGCACG
30	0.00623182	TGTGTGGTG	CVVSVSGGY	TRAV12-1;TRBV5-6	NA;NA	TRAJ4;TRBJ1-4	TGTGTGGTGAGCGTTTCT	TRA;TRB	AACACGTCAAGAGTCG-1;AAGGA	2	AACACGTCAAGAG
12	0.00249273	TGTGCCAGC	CASSLELGIEK	TRBV5-6	NA	TRBJ1-4	TGTGCCAGCAGCTTGGAG	TRB	ACTGTCCAGAAAGTGG-1;CAACT	2	ACTGTCCAGAAAG
12	0.00249273	TGTGCTCTG	CALSAGYNTI	TRAV19;TRBV4-1	NA;NA	TRAJ34;TRBJ1-2	TGTGCTCTGAGTGCCGGG	TRA;TRB	AACTCAGCATACGCTA-1;ATGGG	4	AACTCAGCATACG
11	0.002285	TGCGCCAGC	CASSSTGDTN	TRBV5-1	NA	TRBJ1-2	TGCGCCAGCAGCTCCACA	TRB	AAACCTGAGTGTCCAT-1;ACATAC	1	AAACCTGAGTGTC
11	0.002285	TGTGCCGCC	CAAYNFNKF	TRAV8-1;TRBV4-3	NA;NA	TRAJ21;TRBJ1-2	TGTGCCGCCTACAACTTCA	TRA;TRB	AAACGGGTCGGGAGTA-1;AATCC	5	AAACGGGTCGGG
9	0.00186955	TGTGCTACG	CATDGETDN	TRAV17;TRBV2	NA;NA	TRAJ26;TRBJ2-3	TGTGCTACGGACGGGGA	TRA;TRB	ACTGAGTGTTCATGGT-1;CCATTC	6	ACTGAGTGTTCAT
8	0.00166182	TGCCTCGTG	CLVGALFNTN	TRAV4;TRBV20-1	NA;TRBD1	TRAJ27;TRBJ2-5	TGCCTCGTGGGCGCCCTC	TRA;TRB	AAACGGGTCTAGAGTC-1;AGCGT	10	AAACGGGTCTAGA
8	0.00166182	TGTGCAATG	CAMSPPHILT	TRAV14/DV4;TRBV7-9	NA;NA	TRAJ10;TRBJ2-2	TGTGCAATGAGTCCTCCT	TRA;TRB	AACACGTGTAATCGTC-1;ATTATC	8	AACACGTGTAATC
8	0.00166182	TGTGCAGCA	CAASETSYDK	TRAV13-1;TRBV7-9	NA;NA	TRAJ50;TRBJ1-5	TGTGCAGCAAGTGAAACC	TRA;TRB	ACTTGTTCAATCTGCA-1;AGATTG	13	ACTTGTTCAATCTC
8	0.00166182	TGTGCAGCT	CAASKGDSG	TRAV13-1;TRBV3-1	NA;NA	TRAJ11;TRBJ2-7	TGTGCAGCTTCGAAGGG	TRA;TRB	AGTCTTTAGCTTTGGT-1;CATCAA	11	AGTCTTTAGCTTTC
8	0.00166182	TGTGCTTTCA	CAFMKRGGC	TRAV38-1;TRBV2	NA;NA	TRAJ42;TRBJ1-2	TGTGCTTTCATGAAGCGT	TRA;TRB	AAACGGGCATCCCATC-1;AACACG	3	AAACGGGCATCCC
7	0.00145409	TGCATCTCCG	CISEYNFNKF	TRAV26-1;TRBV2	NA;NA	TRAJ21;TRBJ2-6	TGCATCTCCGAATACAACT	TRA;TRB	CAGATCATCCAAGCCG-1;CGGACA	12	CAGATCATCCAAG
7	0.00145409	TGTGCAATG	CAMSPYNFN	TRAV12-3;TRBV20-1	NA;NA	TRAJ21;TRBJ1-2	TGTGCAATGAGCCCTTAC	TRA;TRB	ATGAGGGCAGACTCGC-1;ATTGG	16	ATGAGGGCAGAC

Clonotypes

Distribution of CDR3 length



Heatmap of public clonotypes



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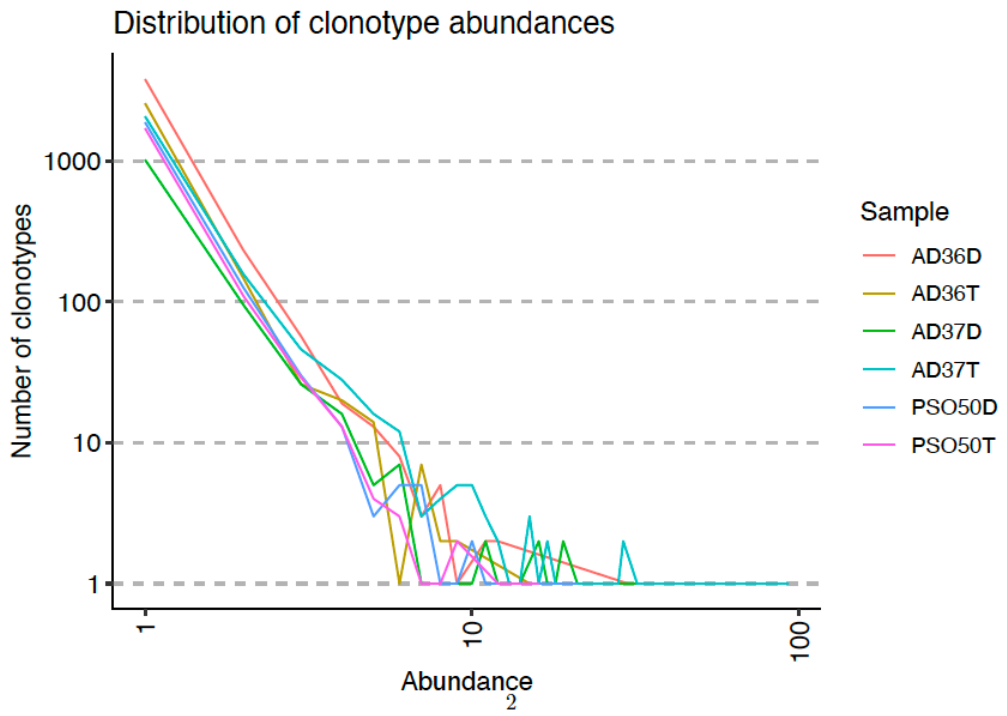
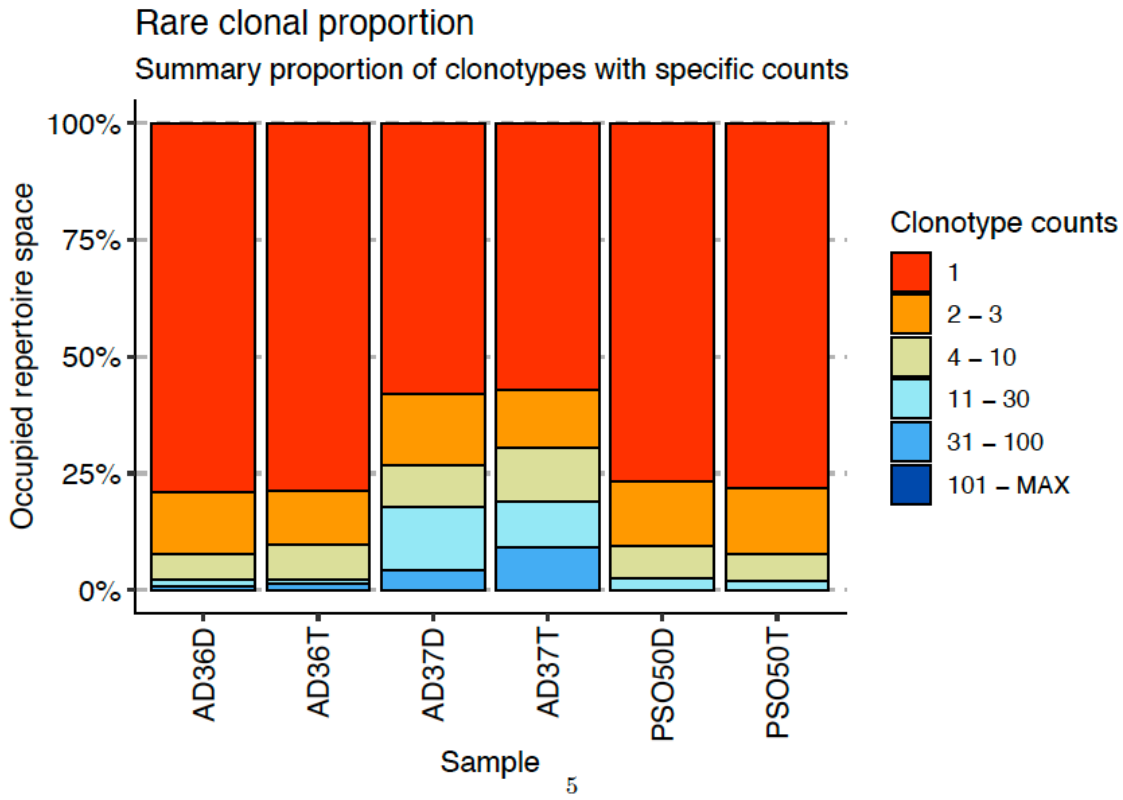

Distribution of clonotype abundances
Number of clonotypes
1000
100
10
1
1
10
100
Abundance
2
Sample
AD36D
AD36T
AD37D
AD37T
PSO50D
PSO50T


Rare clonal proportion
Summary proportion of clonotypes with specific counts
Occupied repertoire space
100%
75%
50%
25%
0%
AD36D
AD36T
AD37D
AD37T
PSO50D
PSO50T
Sample
5
Clonotype counts
1
2 – 3
4 – 10
11 – 30
31 – 100
101 – MAX

Resources

- Immunarch: https://immunarch.com//