

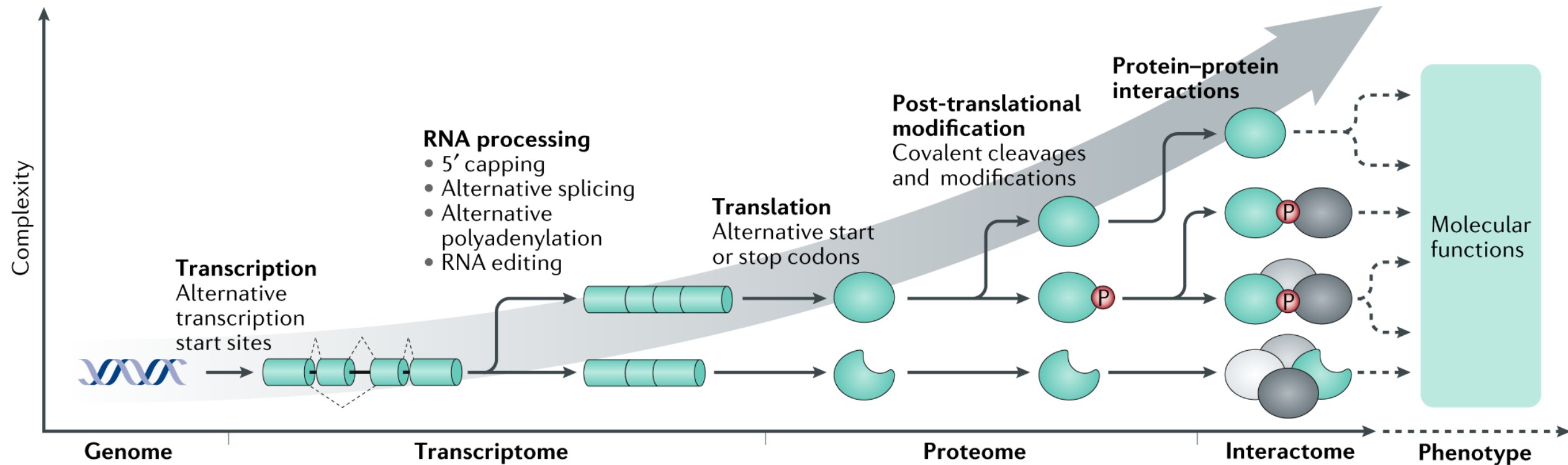
Proteomica funzionale ed Immunobiologia

sviluppo ed applicazione preliminare di una metodologia analitica

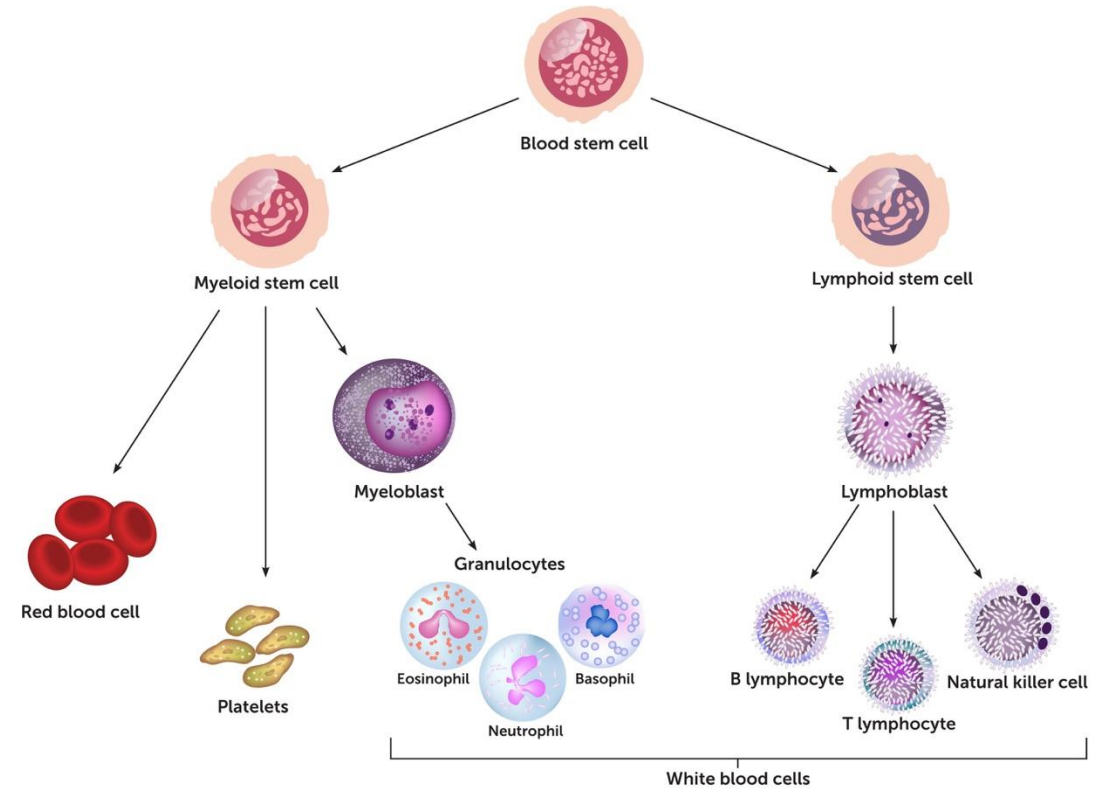
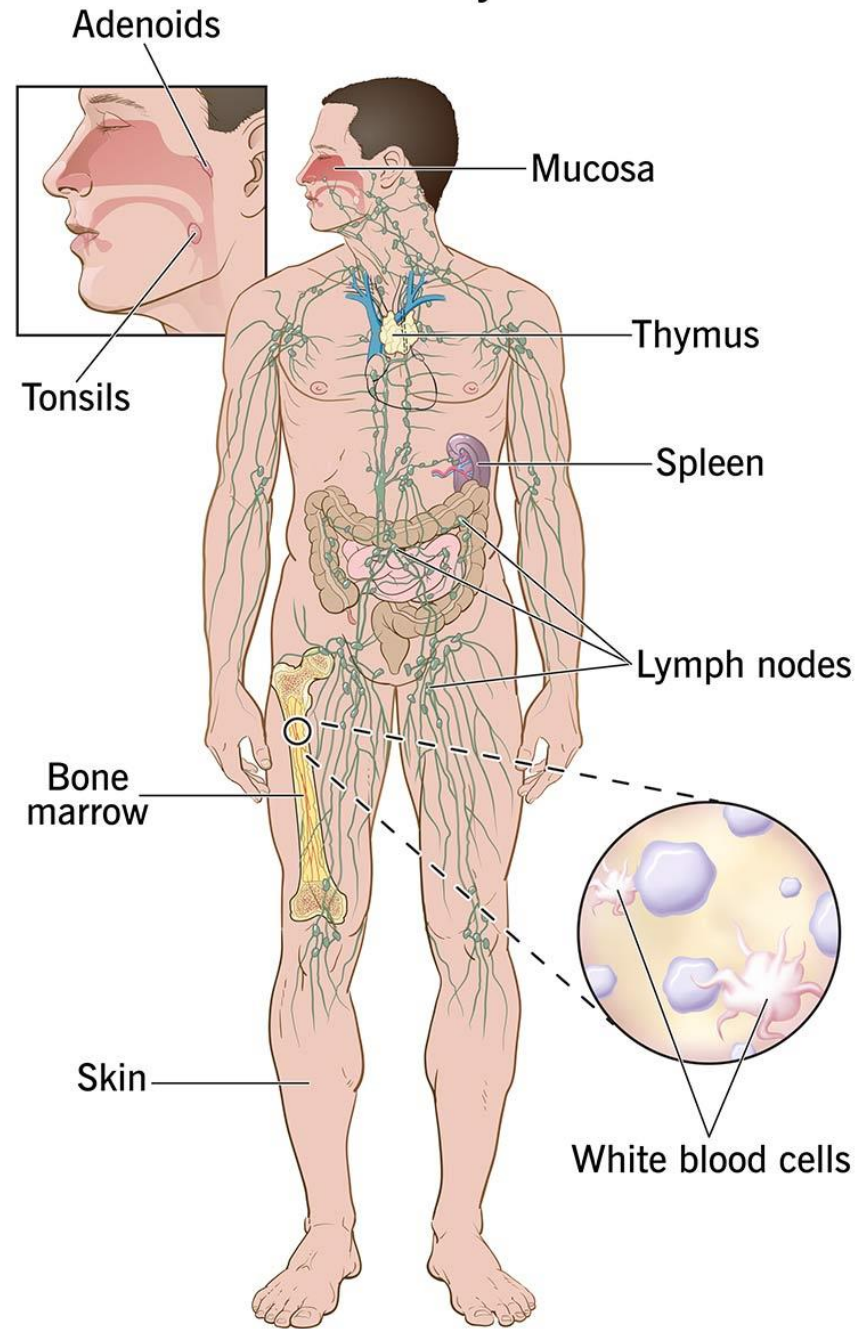
Gianluca Degliesposti

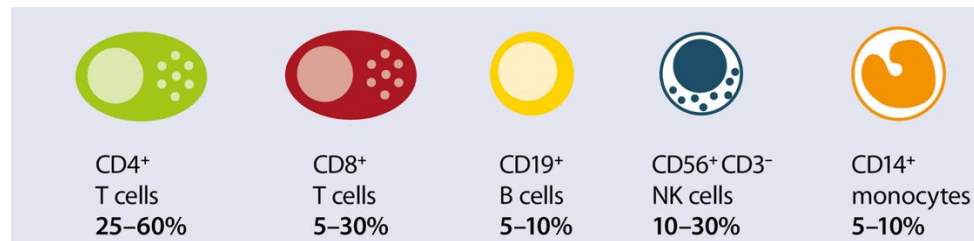
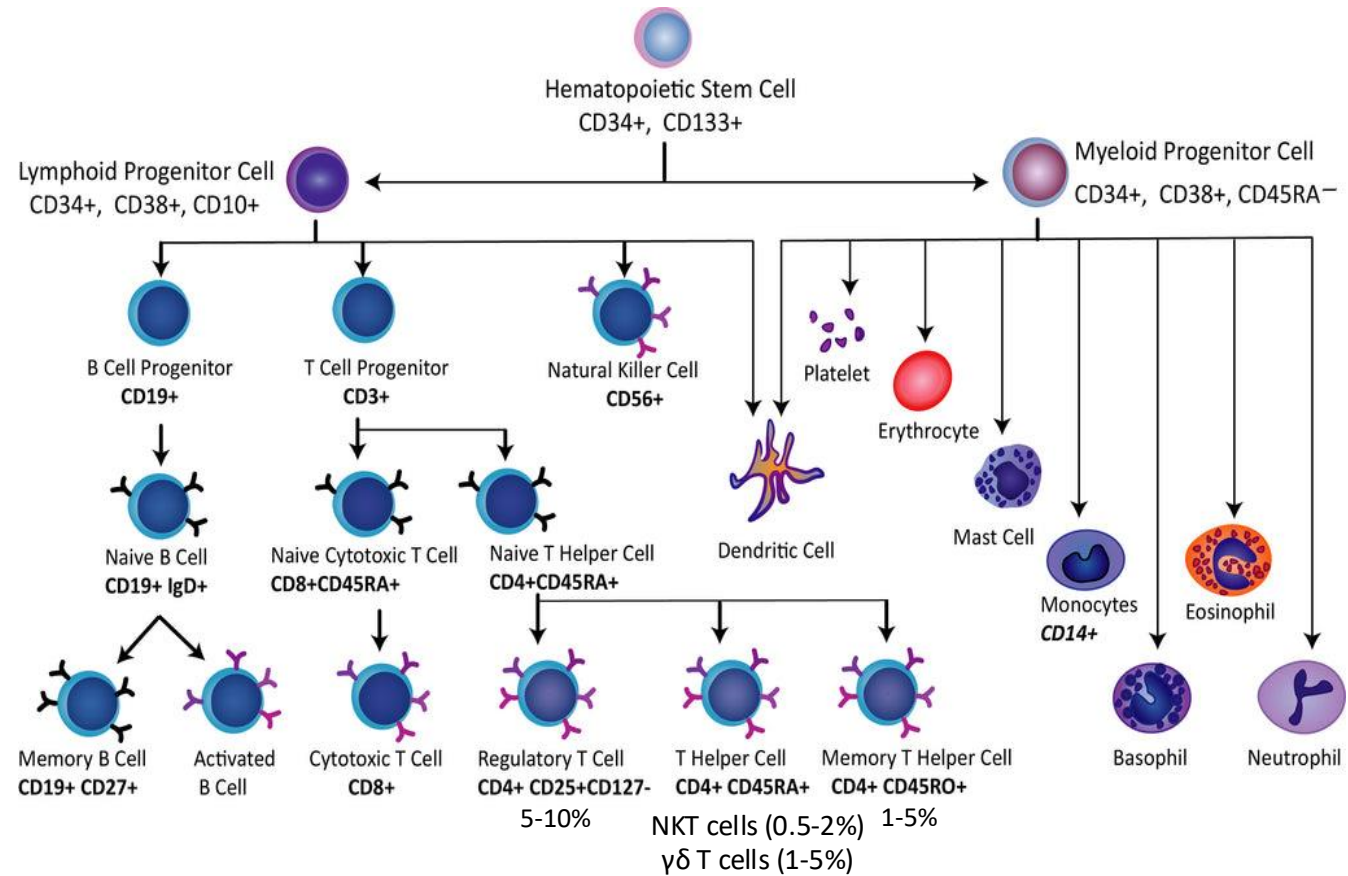
Dipartimento di Scienze Mediche e Chirurgiche Materno-Infantili e dell'Adulto

UniMORE

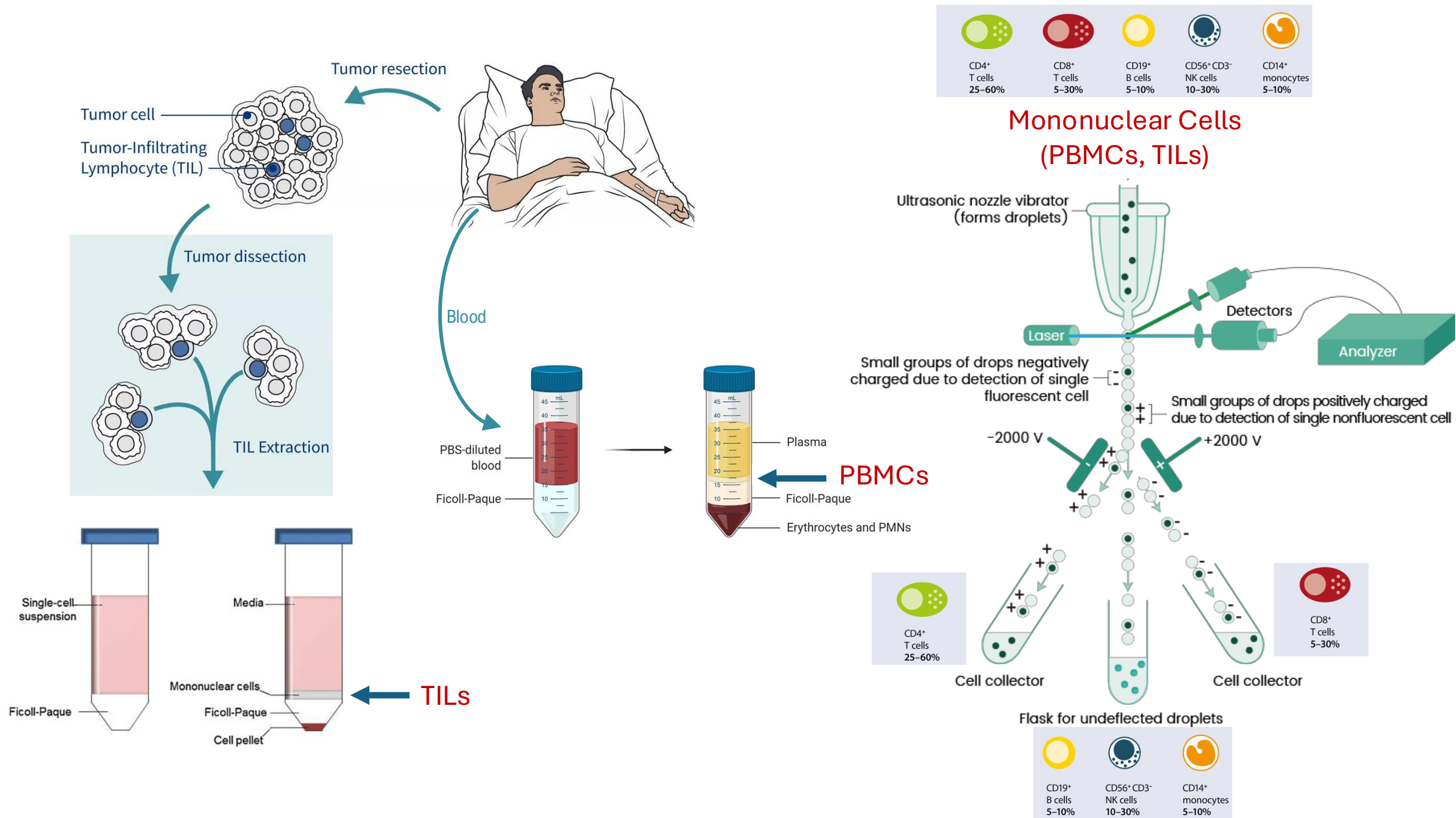


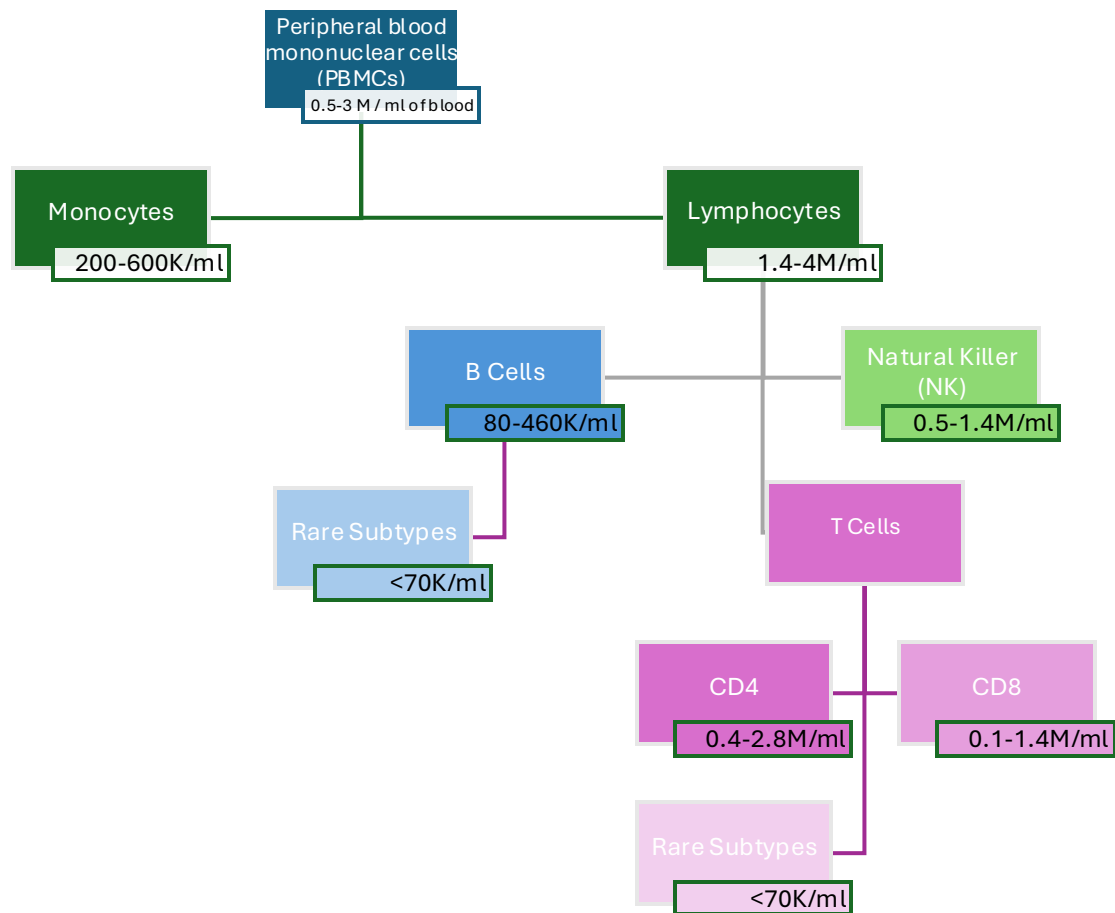
Immune System





% referred to PBMCs





0.5 and 3 million PBMCs per milliliter of blood

Proteomics on T-Cells

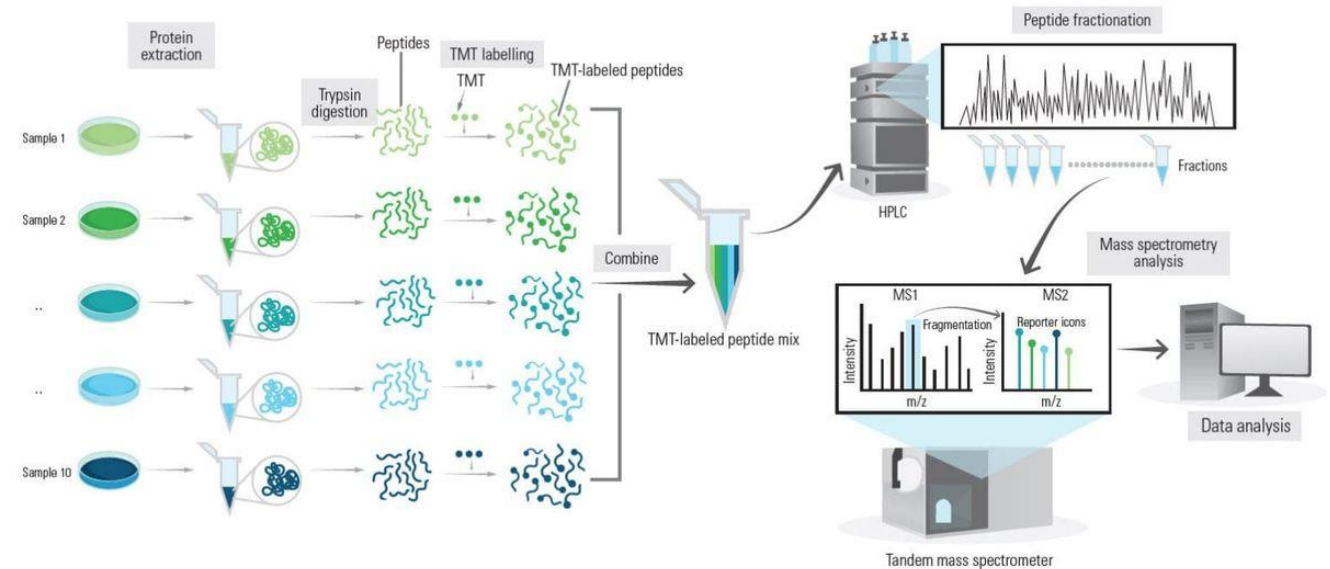
	Protein (pg/cell)	Cells/ml of blood	Protein (ug/ml)
T-lymphocyte (CD3)	~ 40	~3M	~ 120
T-lymphocyte (CD4)		~2M	~ 80
T-lymphocyte (CD8)		~1M	~ 40
Rare T-Subtype		<70K	< 0.280

TMT labelled proteomics

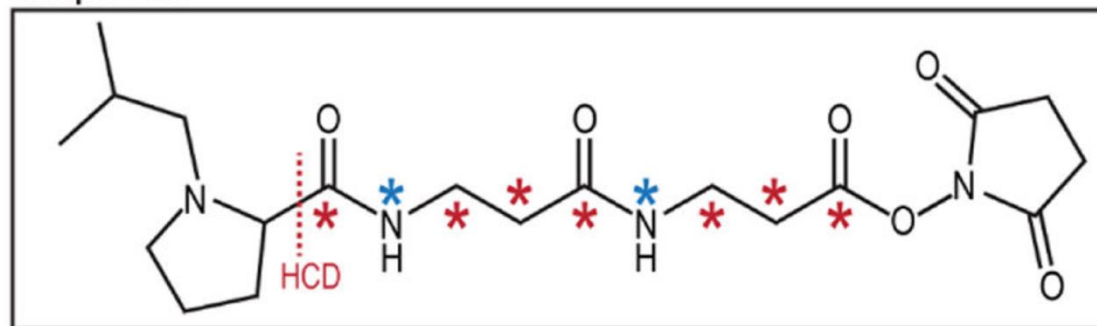
TMT proteomics simultaneously identify and quantify proteins across multiple samples. Different samples are tagged with chemically identical molecules that differ only in their reporter ion masses.

Sample Preparation:

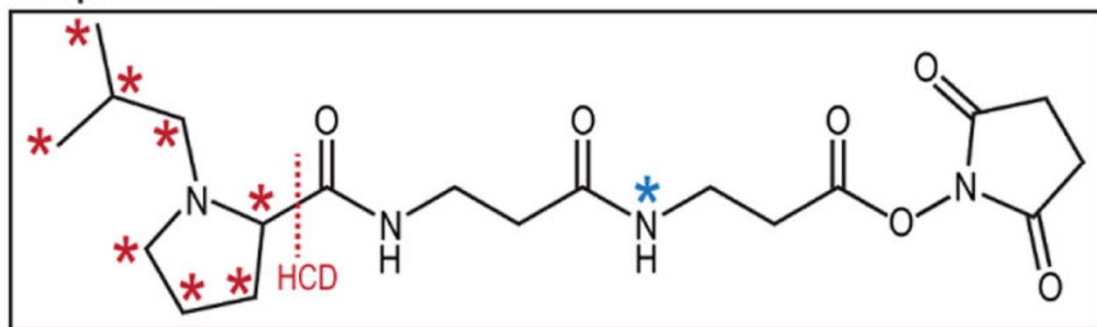
- Proteins from different samples are **digested into peptides**
- TMT Labeling:**
 - Each sample is labeled with a specific TMT reagent
 - The TMT reagent reacts with the primary amine groups of peptides
 - Each TMT reagent has a unique reporter ion mass, but the total mass of the labeled peptide is the same across all samples
- Sample Pooling:**
 - The labeled samples are pooled together
- Mass Spectrometry Analysis:**
 - The pooled samples are analyzed by mass spectrometry
 - During fragmentation, the TMT label is cleaved, releasing reporter ions with different masses
 - The intensity of each reporter ion reflects the relative abundance of the corresponding peptide in the original sample
- Data Analysis:**
 - The mass spectrometer data is analyzed to identify and quantify the peptides
 - The relative intensities of the reporter ions are used to calculate the relative abundance of proteins across different samples



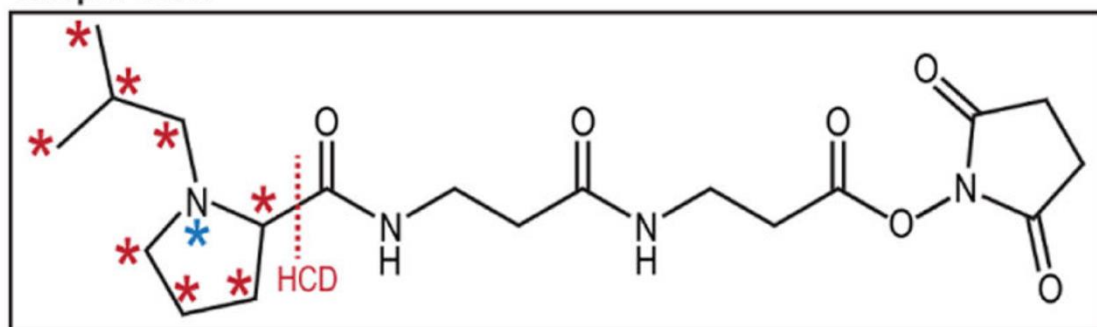
TMTpro-126



TMTpro-134C



TMTpro-135N



Reporter ion group

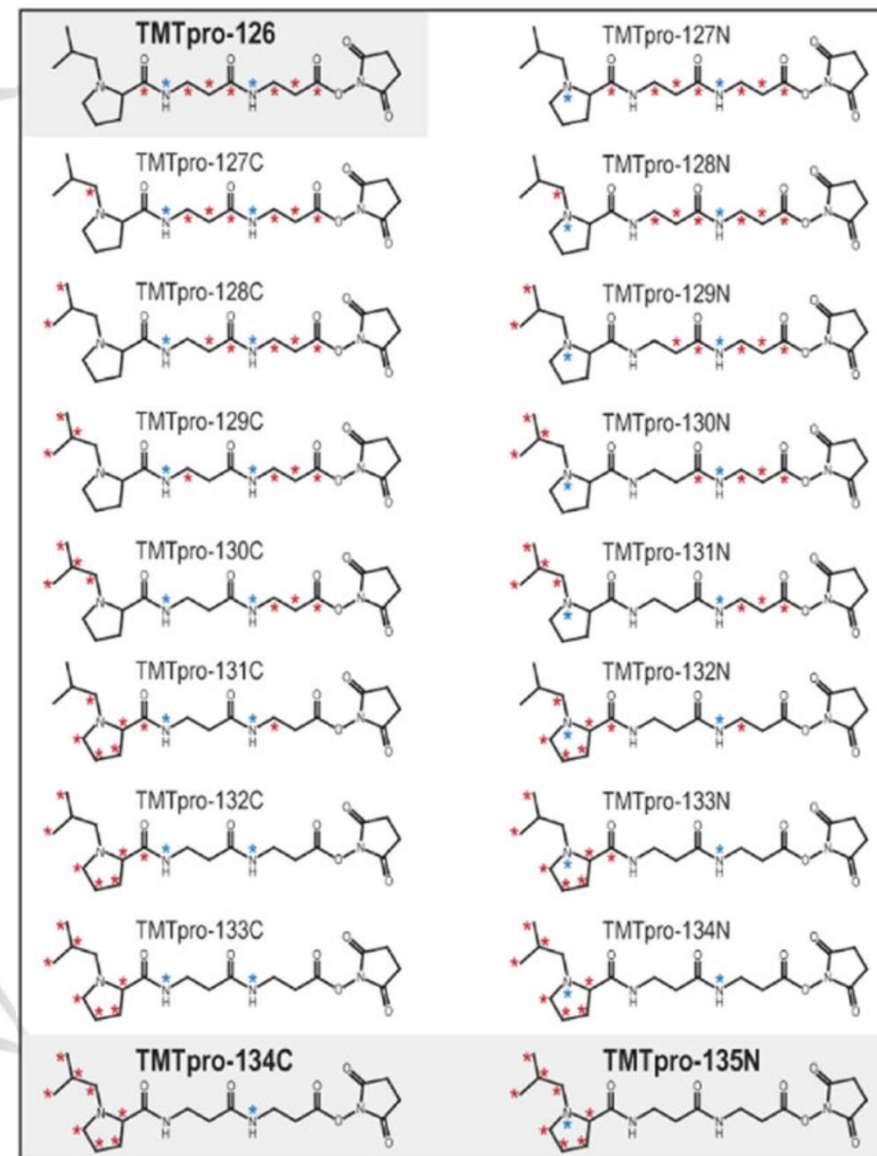
Mass normalization group

Amine reactive group

* ^{13}C

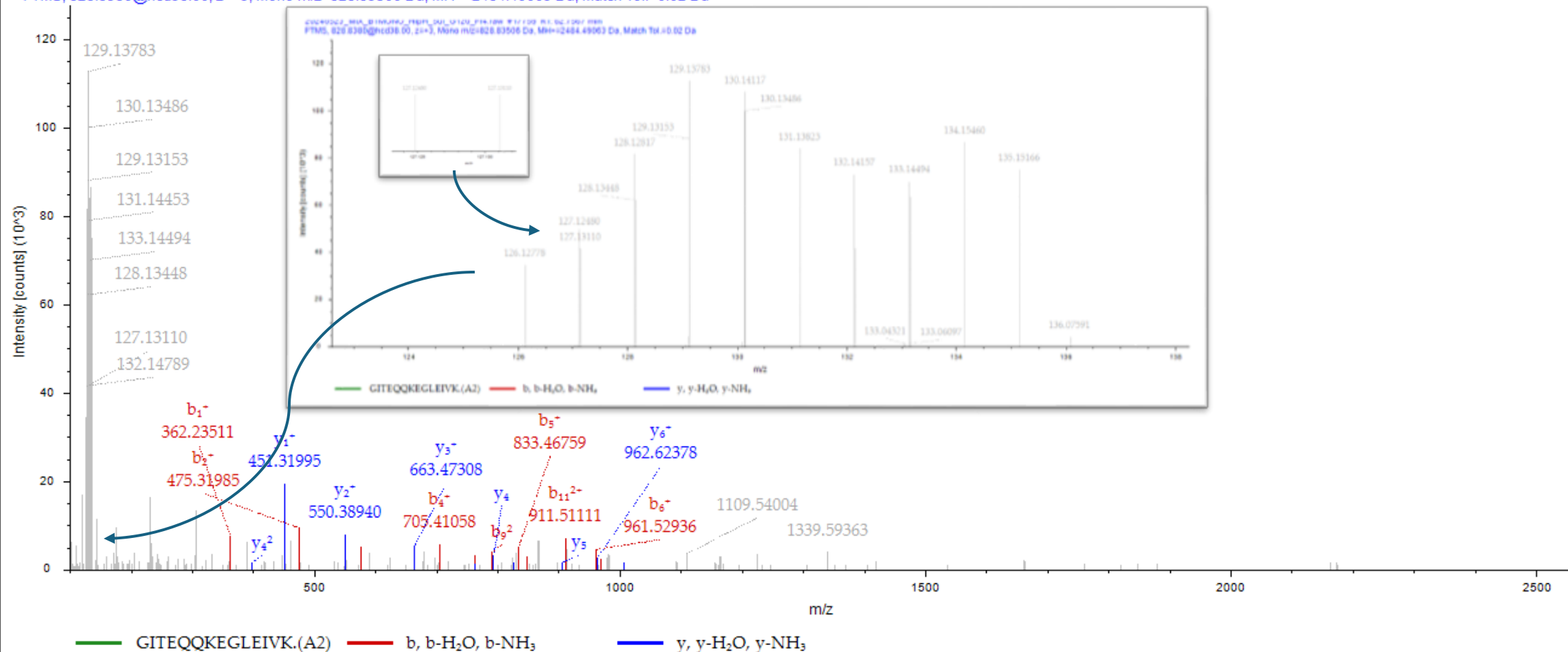
* ^{15}N

..... Fragmentation site by HCD



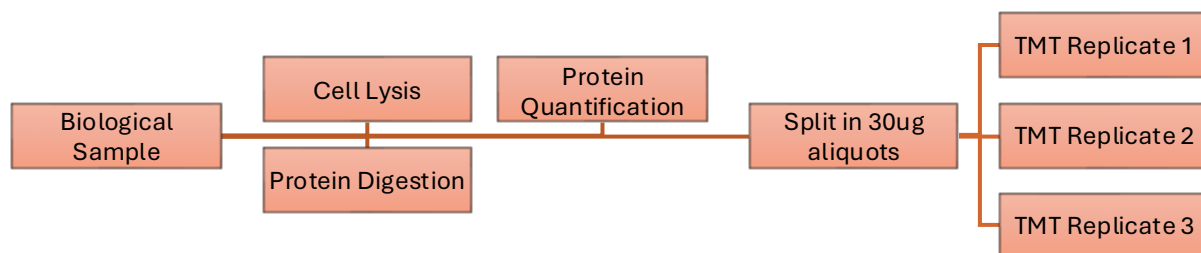
TMTpro-18plex

20240523_MIX_BTMONO_HipH_5ul_G120_Fr4.raw #17759 RT: 62.7567 min
 FTMS, 828.8380@hcd38.00, z=+3, Mono m/z=828.83506 Da, MH+=2484.49063 Da, Match Tol.=0.02 Da

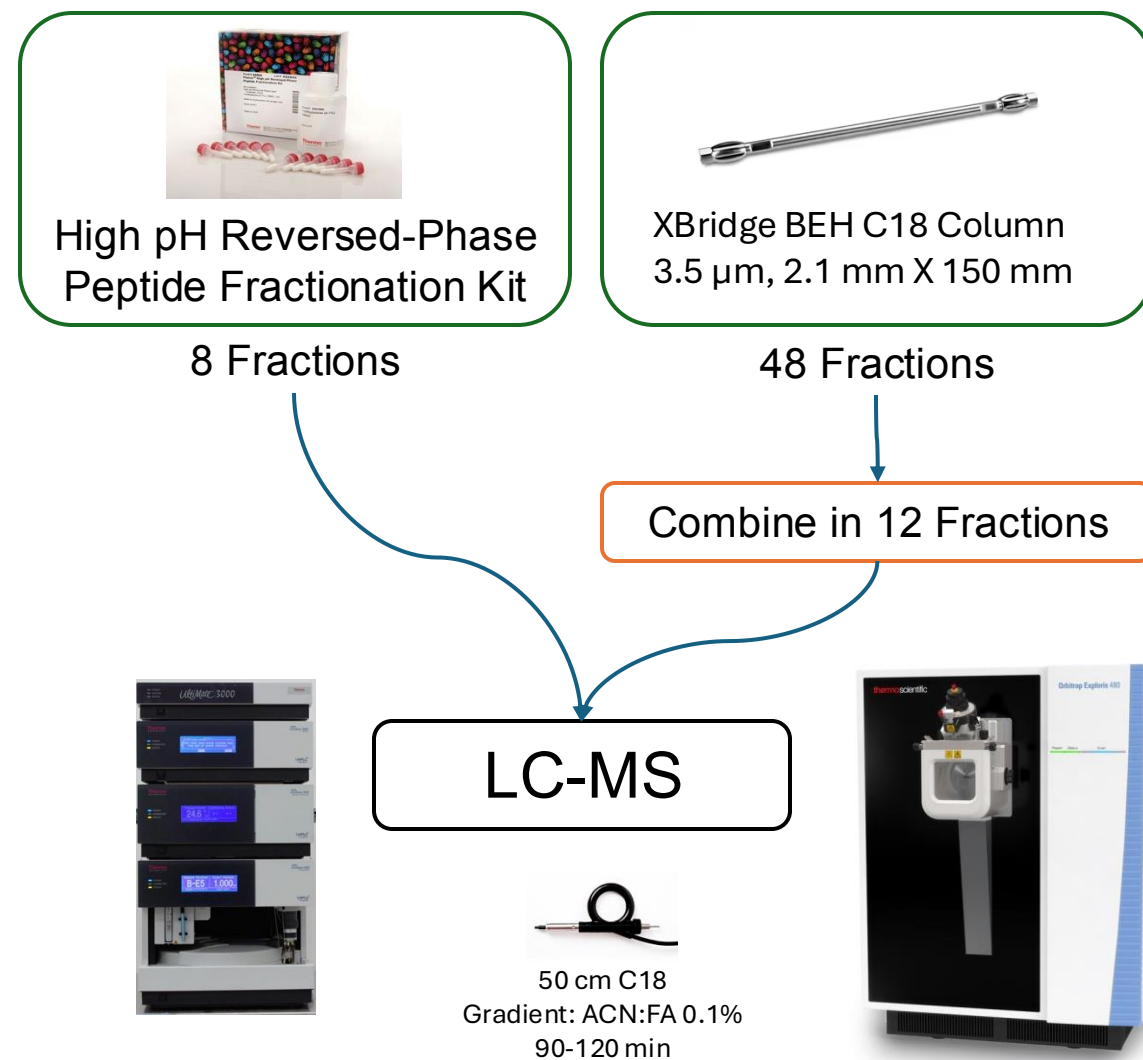


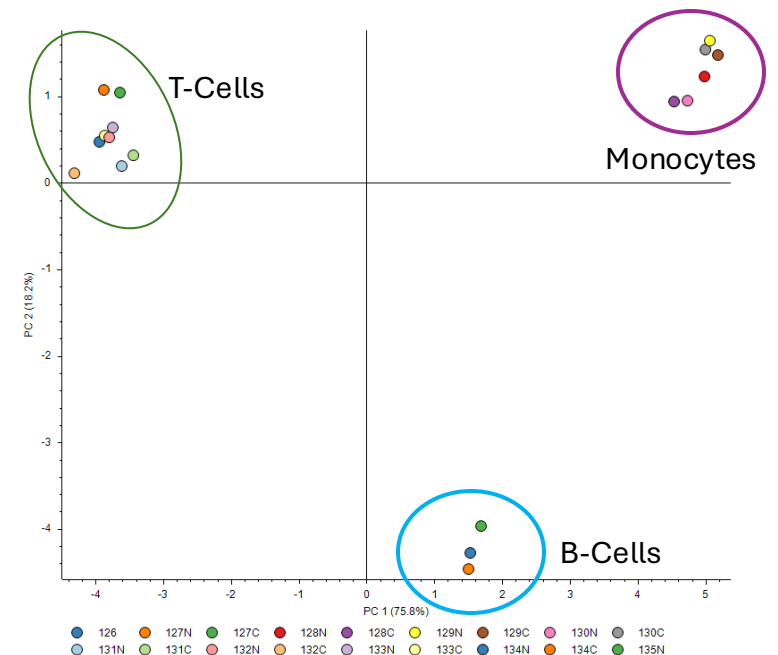
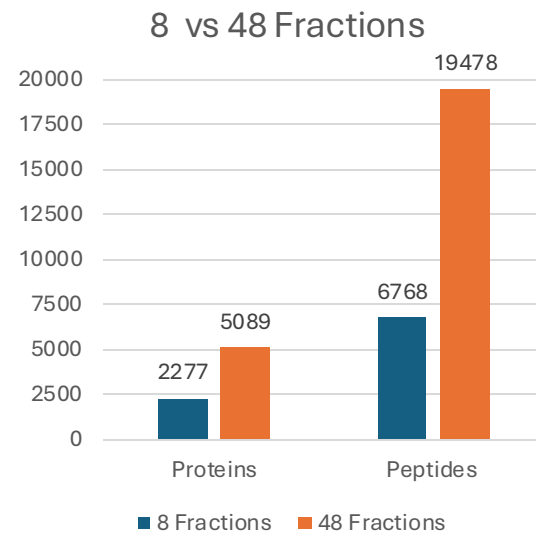
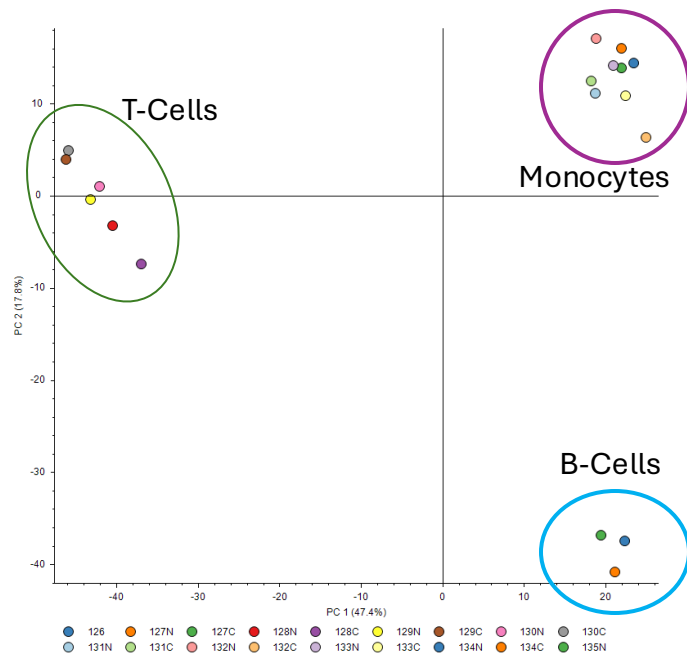
Method set up and pilot test

	Replicates		Number of cells per sample	ug of TMT labelled protein
	Biological	Technical (TMT)		
Monocytes	2	3	2 Millions	30
T Cells	3	3	2.5 Millions	30
B Cells	1	3	2.5 Millions	30

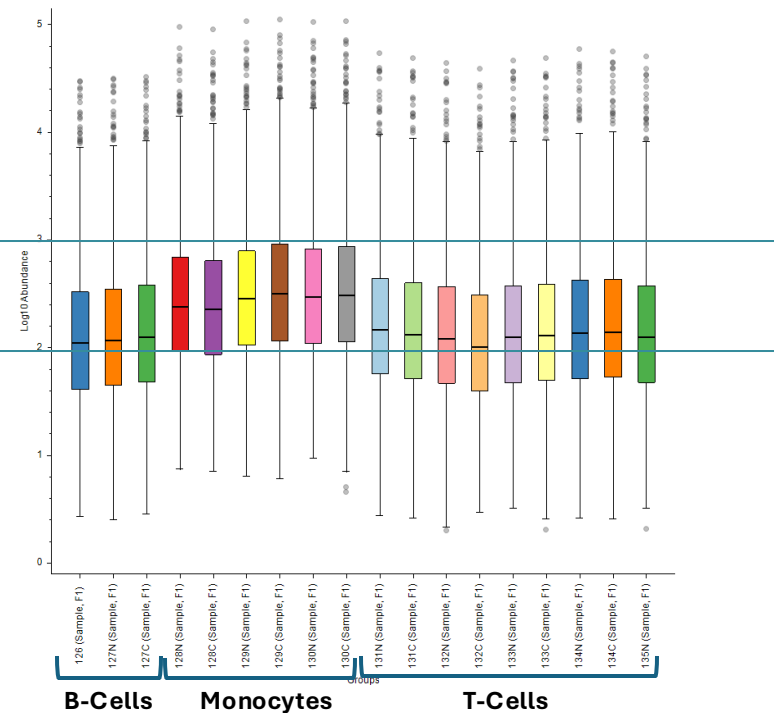
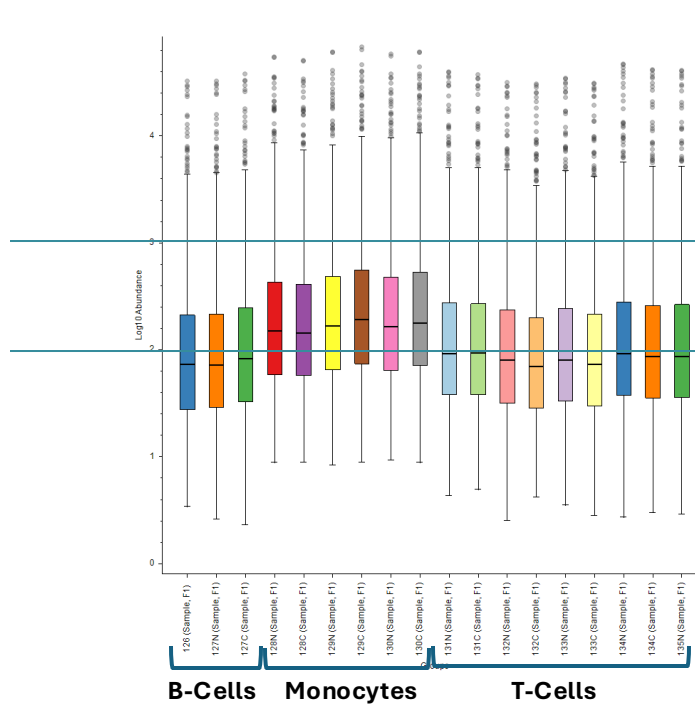


Hi-pH Peptide Fractionation



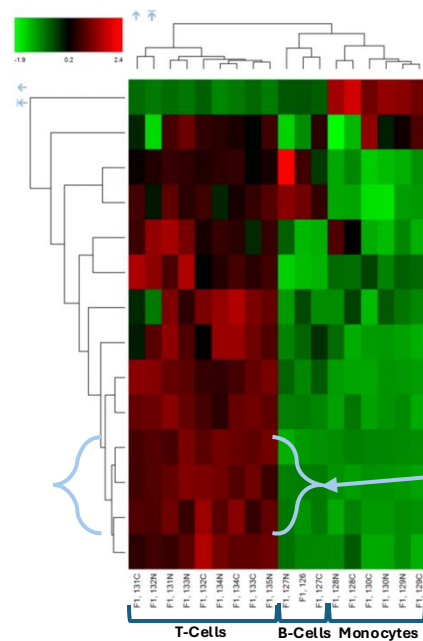


8 Fractions



48 Fractions

8 Fractions - 90 min gradient

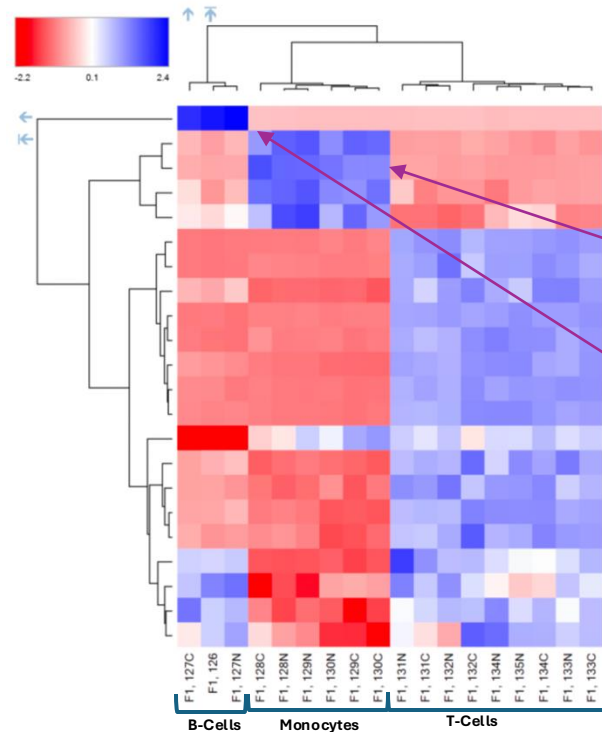


Master	Accession	Description	Exp. q-value	Sum PEP Score	Coverage [%]	Sequence Coverage
✓	Q9Y5K6	CD2-associated protein [OS=Homo sapiens]	0.030	1.307	1%	
✓	P16070	CD44 antigen [OS=Homo sapiens]	0.000	10.446	5%	
✓	P09326	CD48 antigen [OS=Homo sapiens]	0.000	8.023	10%	
✓	P13987	CD59 glycoprotein [OS=Homo sapiens]	0.028	1.355	6%	
✓	P14209	CD99 antigen [OS=Homo sapiens]	0.001	3.639	5%	
✓	Q08722	Leukocyte surface antigen CD47 [OS=Homo sapiens]	0.000	7.095	9%	
✓	P08571	Monocyte differentiation antigen CD14 [OS=Homo sapiens]	0.000	17.515	22%	
✓	P09564	T-cell antigen CD7 [OS=Homo sapiens]	0.006	2.203	5%	
✓	P30203	T-cell differentiation antigen CD6 [OS=Homo sapiens]	0.001	6.649	6%	
✓	P06729	T-cell surface antigen CD2 [OS=Homo sapiens]	0.001	6.803	11%	
✓	P04234	T-cell surface glycoprotein CD3 delta chain [OS=Homo sapiens]	0.003	2.854	5%	
✓	P07766	T-cell surface glycoprotein CD3 epsilon chain [OS=Homo sapiens]	0.001	5.688	7%	
✓	P20963	T-cell surface glycoprotein CD3 zeta chain [OS=Homo sapiens]	0.001	6.766	13%	
✓	P06127	T-cell surface glycoprotein CD5 [OS=Homo sapiens]	0.001	3.740	5%	

Monocytes

T lymphocytes

48 Fractions in 12 - 120 mins gradient

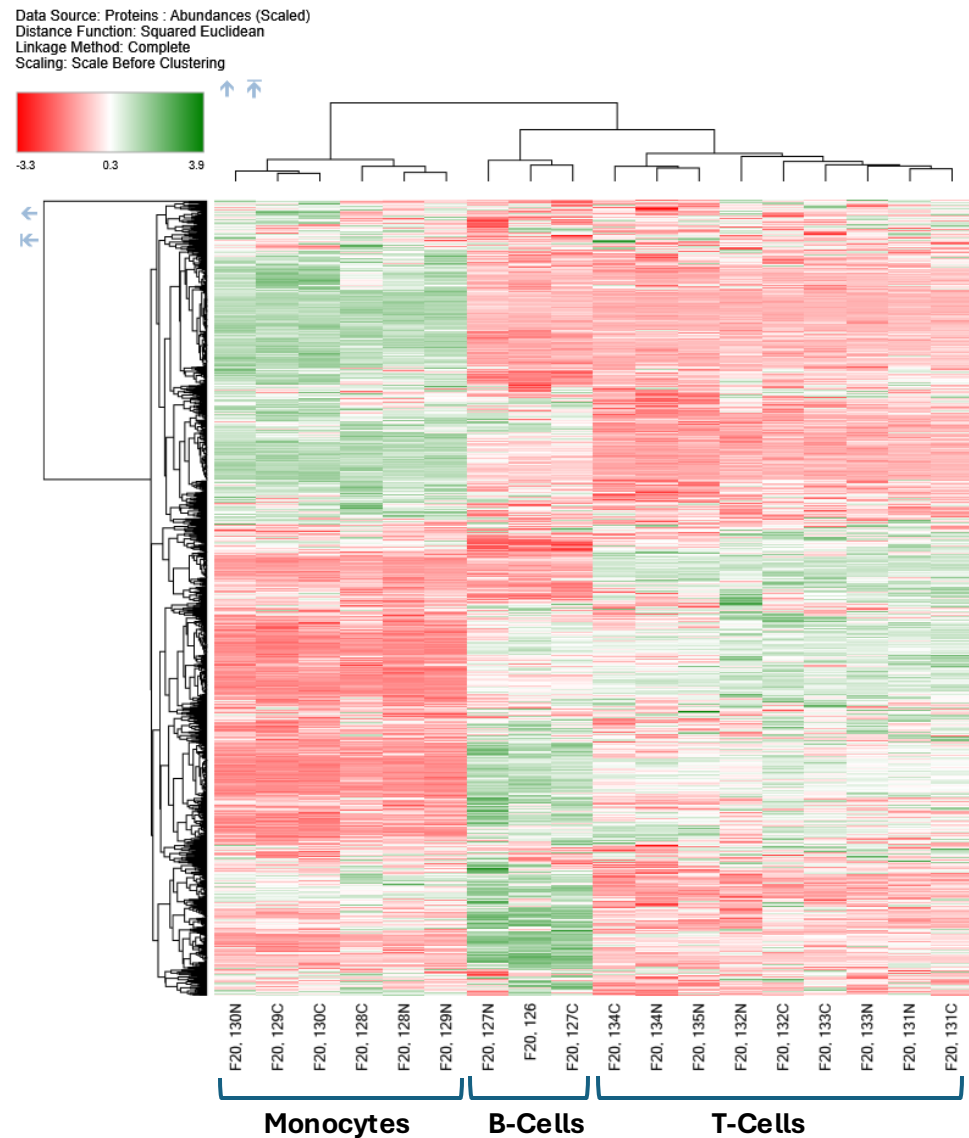
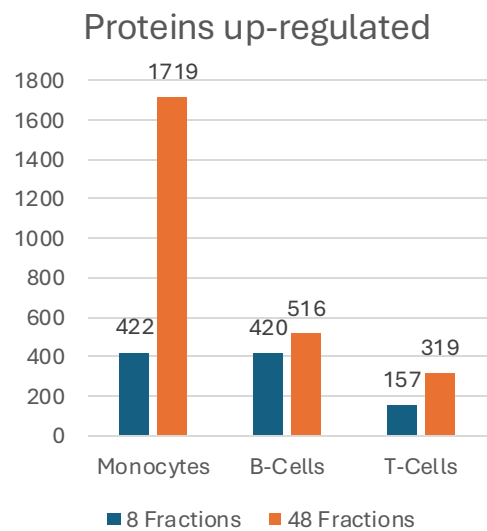
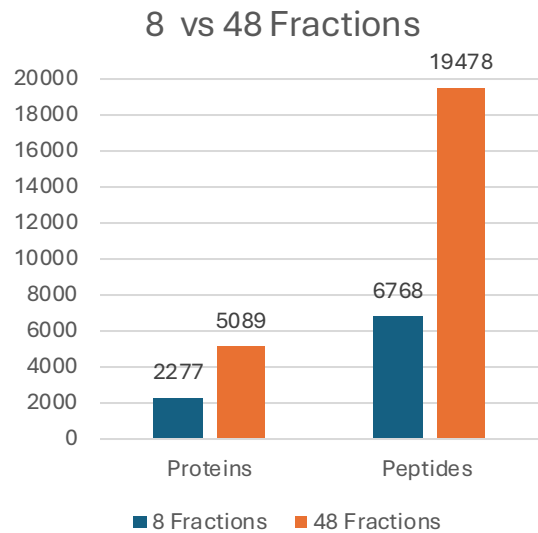


Master	Accession	Description	Exp. q-value	Sum PEP Score	Coverage [%]	Sequence Coverage
✓	Q95400	CD2 antigen cytoplasmic tail-binding protein 2 [OS=Homo sapiens]	0.000	9.937	11%	
✓	P01732	T-cell surface glycoprotein CD8 alpha chain [OS=Homo sapiens]	0.001	2.695	4%	
✓	P04234	T-cell surface glycoprotein CD3 delta chain [OS=Homo sapiens]	0.000	6.059	7%	
✓	P06127	T-cell surface glycoprotein CD5 [OS=Homo sapiens]	0.000	13.588	8%	
✓	P06729	T-cell surface antigen CD2 [OS=Homo sapiens]	0.000	18.454	19%	
✓	P07766	T-cell surface glycoprotein CD3 epsilon chain [OS=Homo sapiens]	0.000	19.217	26%	
✓	P08571	Monocyte differentiation antigen CD14 [OS=Homo sapiens]	0.000	32.748	21%	
✓	P09326	CD48 antigen [OS=Homo sapiens]	0.000	18.289	21%	
✓	P09564	T-cell antigen CD7 [OS=Homo sapiens]	0.001	2.867	5%	
✓	P13987	CD59 glycoprotein [OS=Homo sapiens]	0.000	4.646	9%	
✓	P14209	CD99 antigen [OS=Homo sapiens]	0.000	6.668	11%	
✓	P15391	B-lymphocyte antigen CD19 [OS=Homo sapiens]	0.000	7.696	4%	
✓	P16070	CD44 antigen [OS=Homo sapiens]	0.000	45.324	13%	
✓	P19397	Leukocyte surface antigen CD53 [OS=Homo sapiens]	0.000	8.568	11%	
✓	P20138	Myeloid cell surface antigen CD33 [OS=Homo sapiens]	0.000	12.574	8%	
✓	P20963	T-cell surface glycoprotein CD3 zeta chain [OS=Homo sapiens]	0.000	12.225	19%	
✓	P26842	CD27 antigen [OS=Homo sapiens]	0.004	2.095	8%	
✓	P30203	T-cell differentiation antigen CD6 [OS=Homo sapiens]	0.000	22.430	12%	
✓	P42081	T-lymphocyte activation antigen CD86 [OS=Homo sapiens]	0.013	1.496	3%	
✓	Q08722	Leukocyte surface antigen CD47 [OS=Homo sapiens]	0.000	8.988	8%	
✓	Q9UKY7	Protein CDV3 homolog [OS=Homo sapiens]	0.000	30.662	40%	
✓	Q9Y5K6	CD2-associated protein [OS=Homo sapiens]	0.000	15.160	10%	

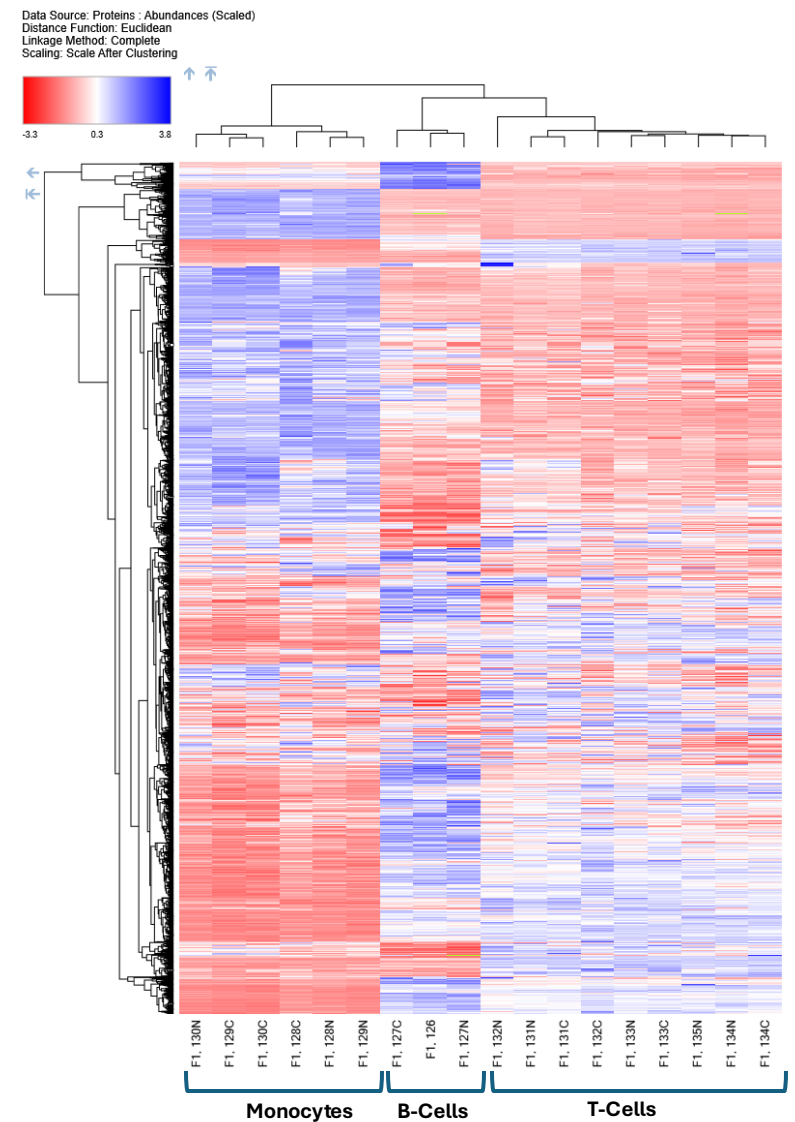
T lymphocytes

Monocytes

B lymphocytes

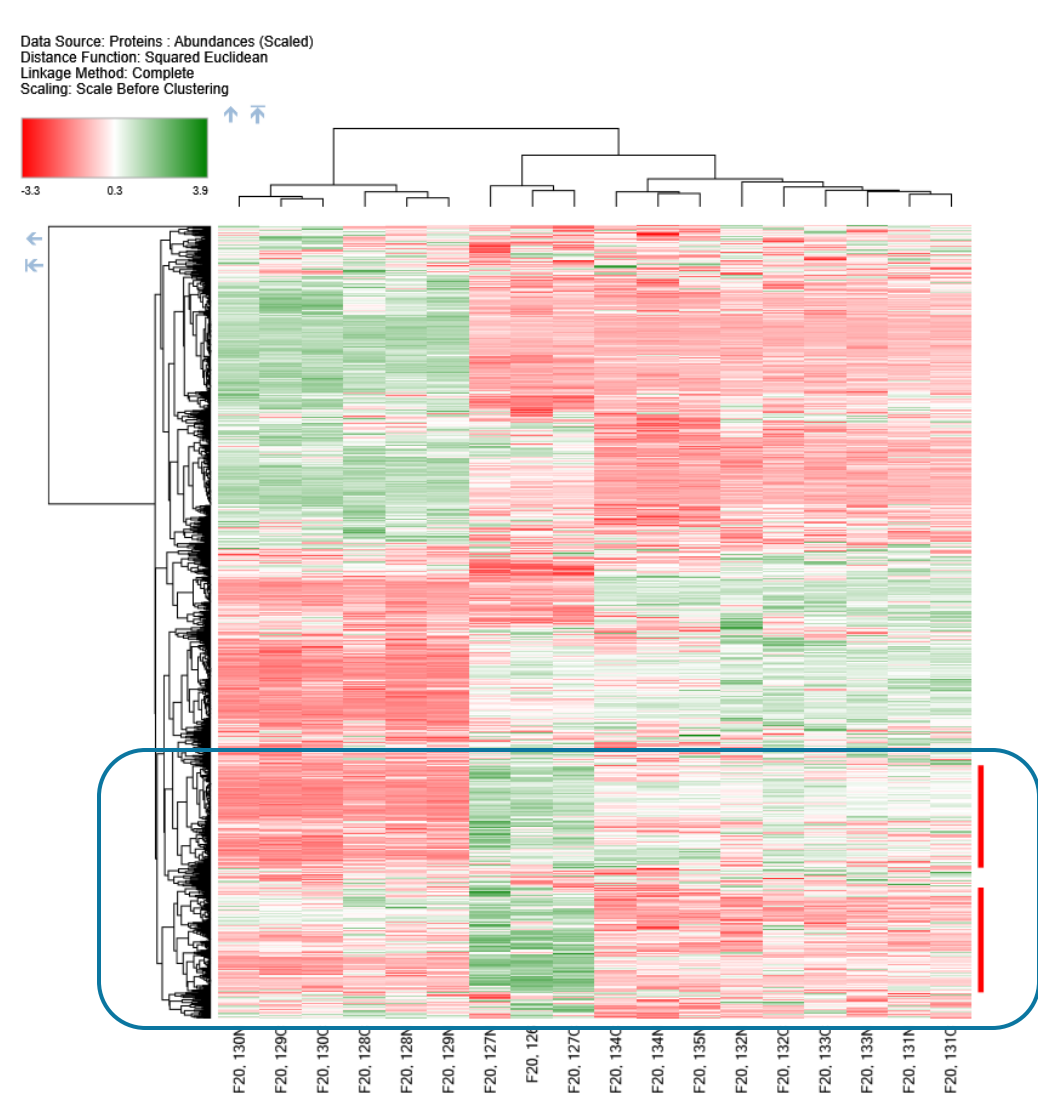
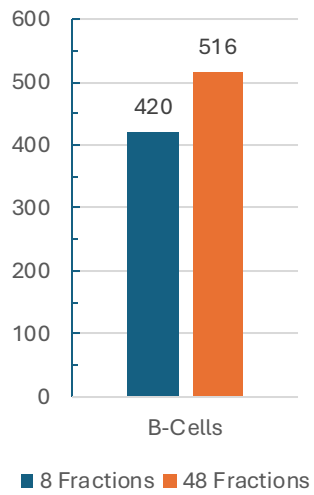


8 Fractions - 90 min gradient
2277 Proteins - 6768 Peptides - 15727 PSMs

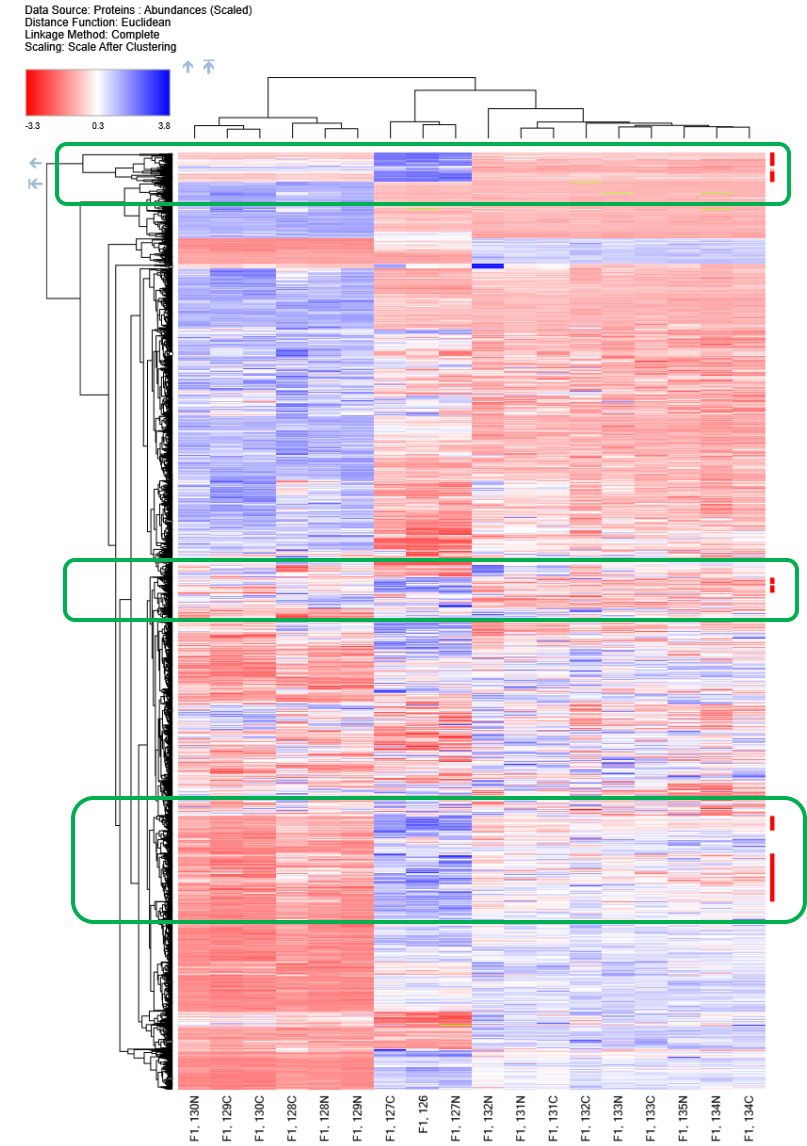


48 Fractions in 12 - 120 mins gradient
5089 Proteins - 19478 Peptides - 38341 PSMs

B Cells up-regulated cluster

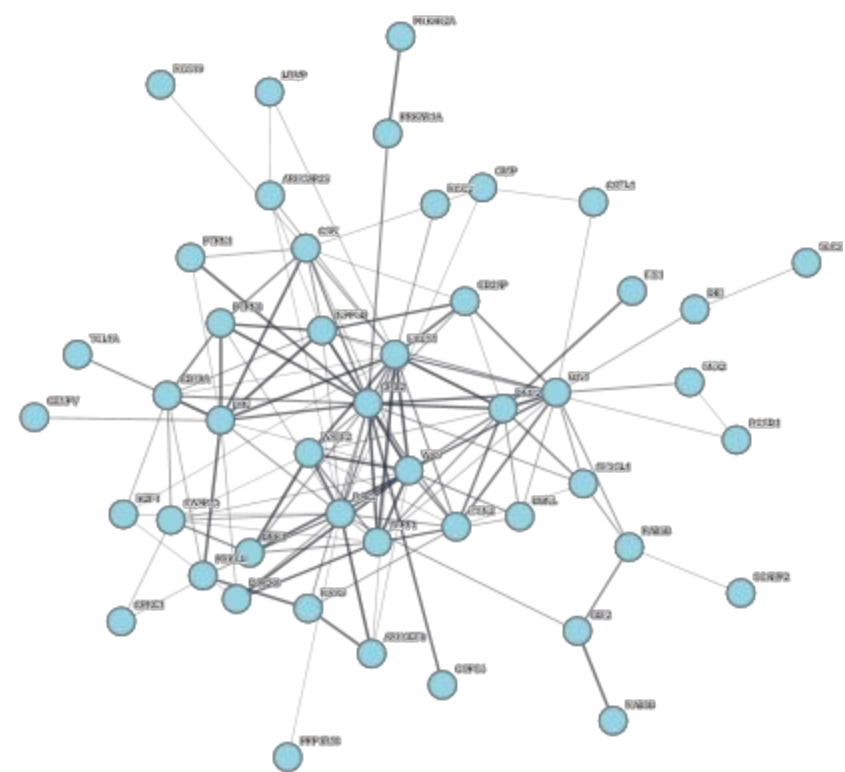
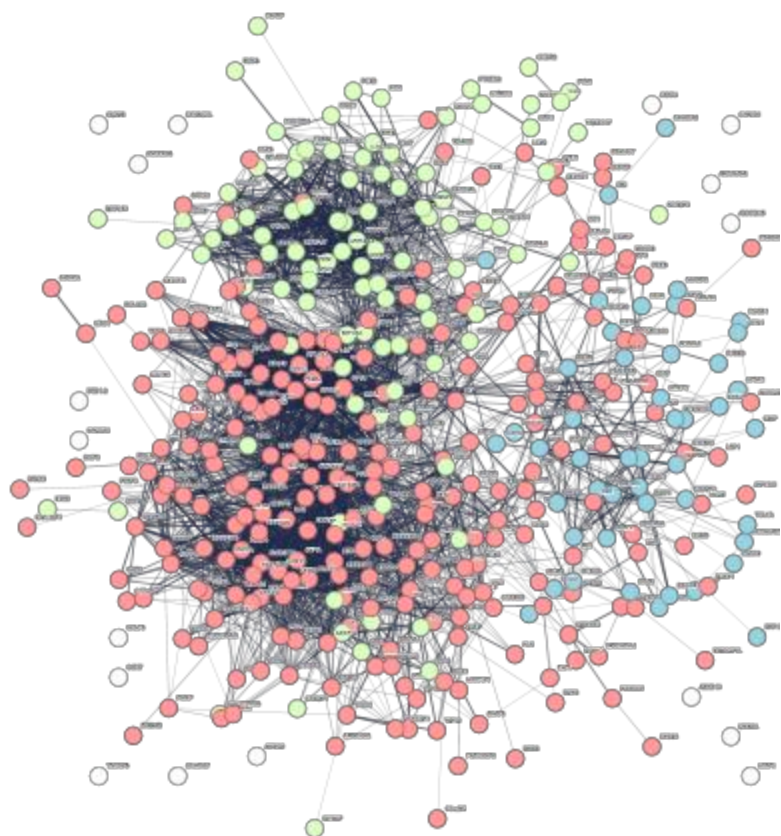


8 Fractions – 90 min gradient



48 Fractions – 120 min gradient

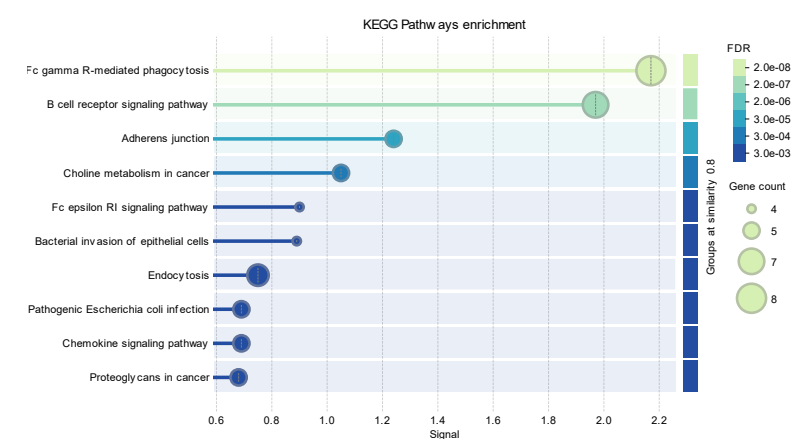
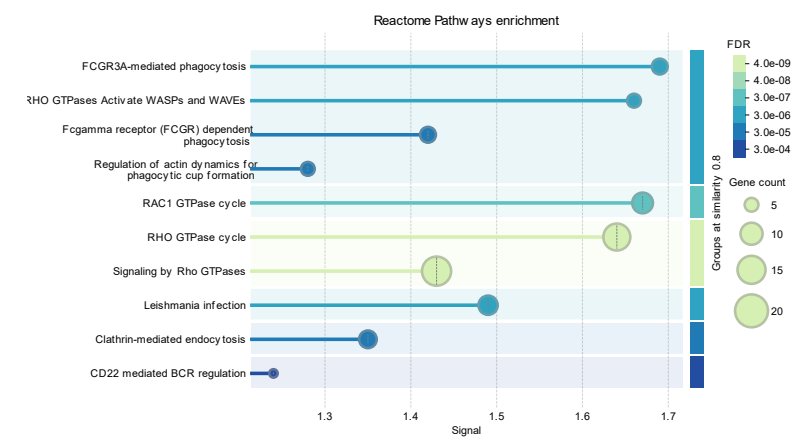
B Cells cluster - 8 Fractions



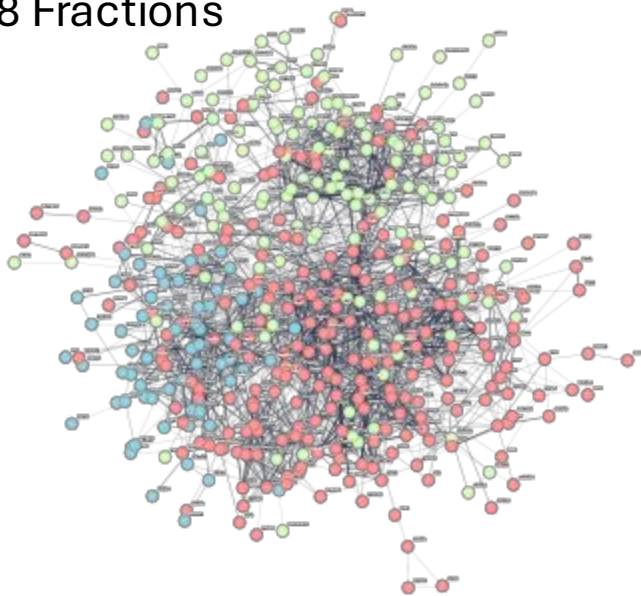
color	cluster Id	gene count	description
red	Cluster 1	249	Metabolism of RNA
green	Cluster 2	99	+ Aerobic respiration
blue	Cluster 3	47	ARHGAP25, ARHGEF6, BIN1, BRK1, CD2AP, CD79A, CENPV, COPS4, COTL1, CPNE1, CSK, DBI, DBNL, DNM2, DOCK8, FIS1, GDI2, GMIP, GRB2, HCLS1, IKZF1, INPP5D, ITSN2, LRMP, LYN, PPP1R18, PRKAR1A, PRKAR2A, PRKCB, PTPN1, PTPN6, RAB5B, RAB8B, RAC2, RCC2, RCSD1, RGS19, RRAS, SCAMP2, SH3GL1, SLC27A4, SNX2, SWAP70, TCL1A, WAS, WASF2, WIPF1

Network Stats

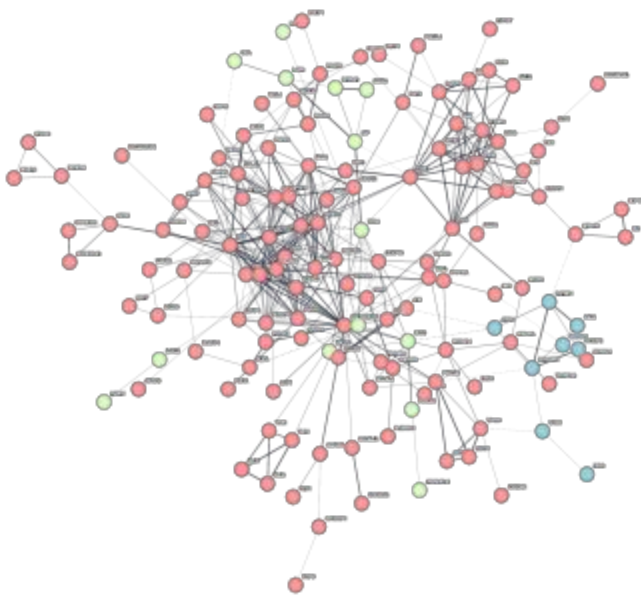
number of nodes: 47	expected number of edges: 16
number of edges: 135	PPI enrichment p-value: < 1.0e-16
average node degree: 5.74	your network has significantly more interactions than expected (what does that mean?)
avg. local clustering coefficient: 0.581	



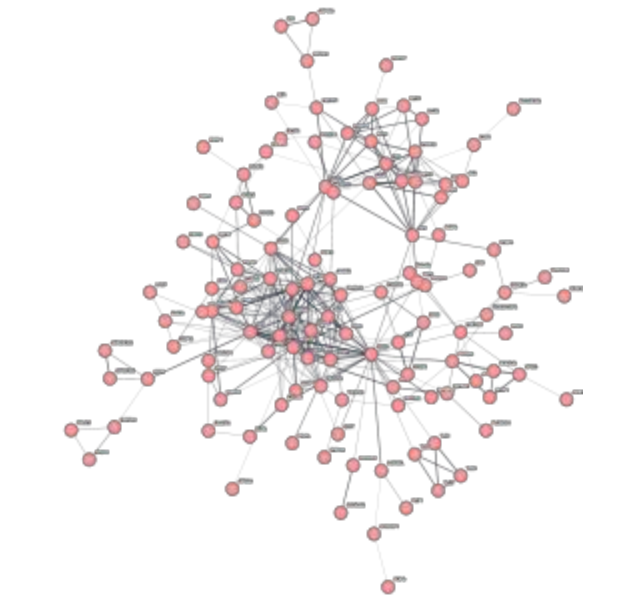
B Cells cluster - 48 Fractions



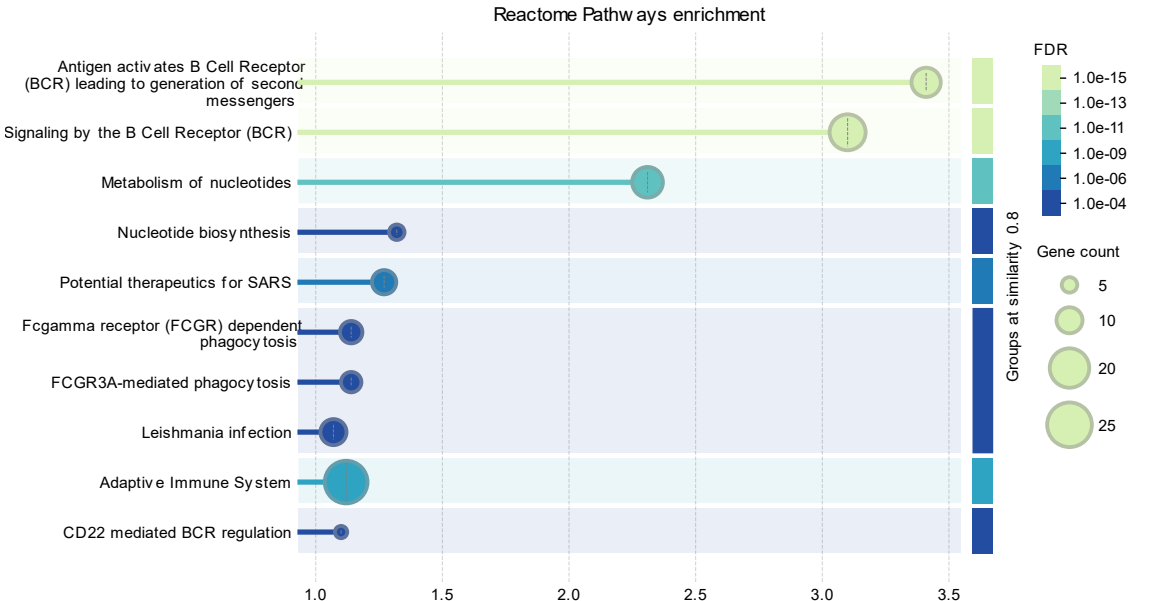
color	cluster Id	gene count	description
red	Cluster 1	245	Mitochondrion
green	Cluster 2	140	ABI1, ADK, AFTPH, AHCY, AMPD3, APPL1, ARHGAP44, ARRDC1, ATIC, ATP2A3, BCL...
blue	Cluster 3	65	+ RNA splicing



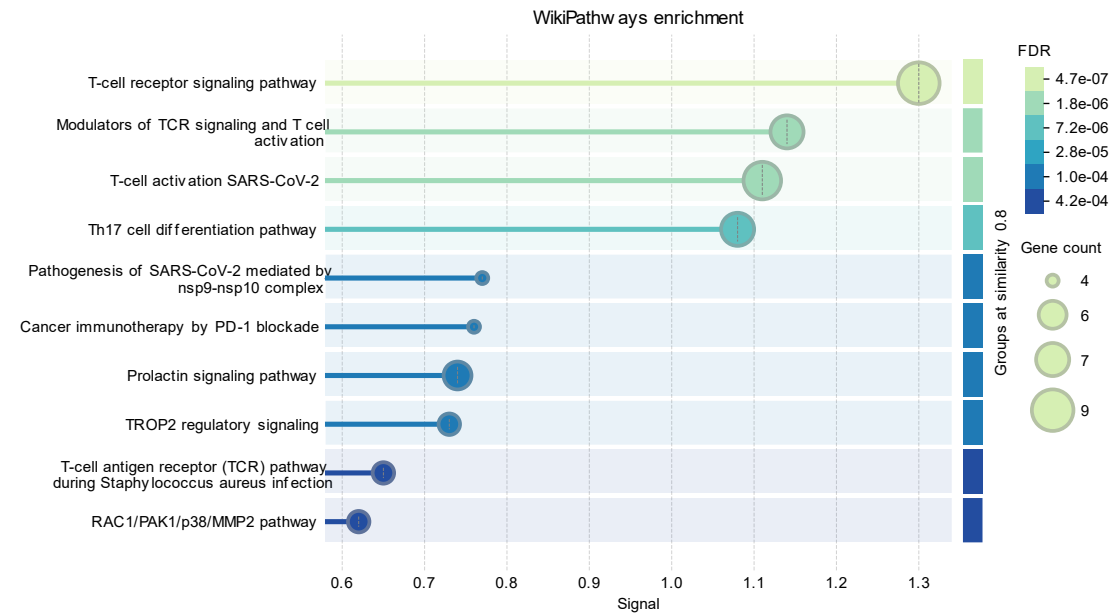
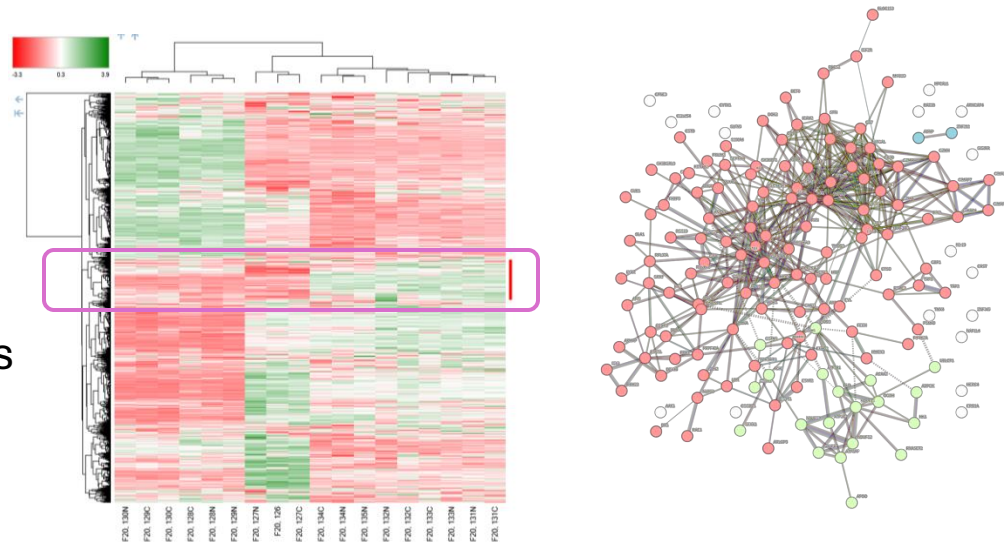
color	cluster Id	gene count	description
red	Cluster 1	118	ABI1, ADK, AFTPH, AHCY, AMPD3, ARRDC1, ATIC, ATP2A3, BCL10, BLNK, BRK1, BT...
green	Cluster 2	14	Golgi Associated Vesicle Biogenesis
blue	Cluster 3	8	1. TBC/RABGAPs 2. Regulation of Fc receptor mediated stimulatory signaling pathway 3. Mixed, incl. Endosomal vesicle fusion, and Mon1-Ccz1 complex



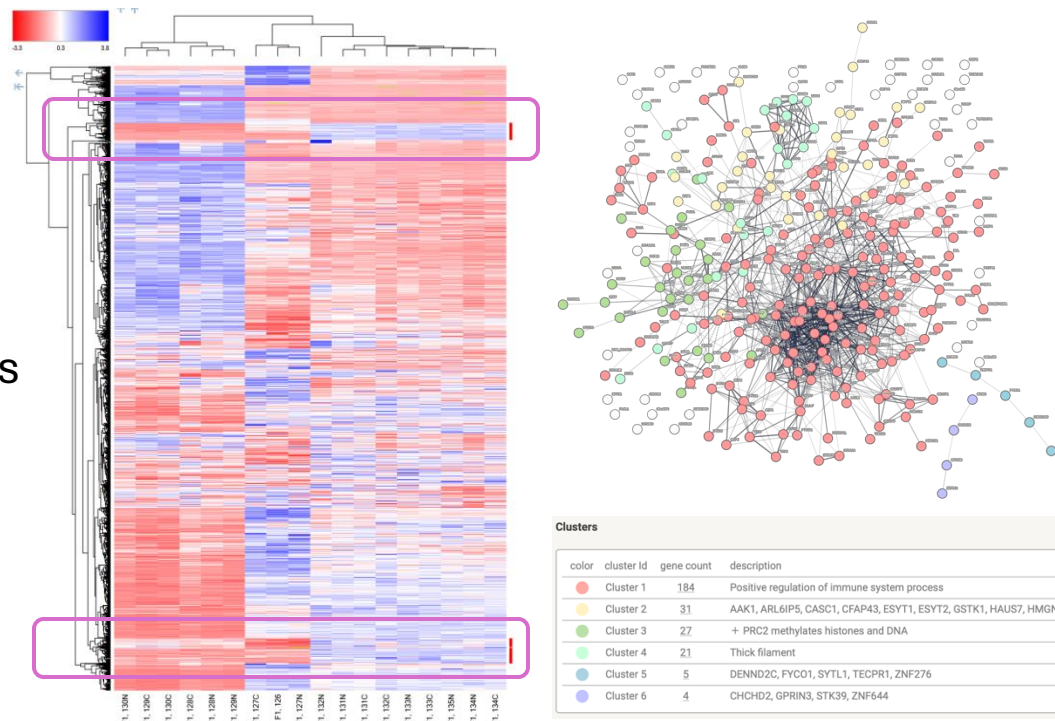
number of nodes: 118	expected number of edges: 95
number of edges: 366	PPI enrichment p-value: < 1.0e-16
average node degree: 6.2	your network has significantly more interactions than expected (what does that mean?)
avg. local clustering coefficient: 0.568	



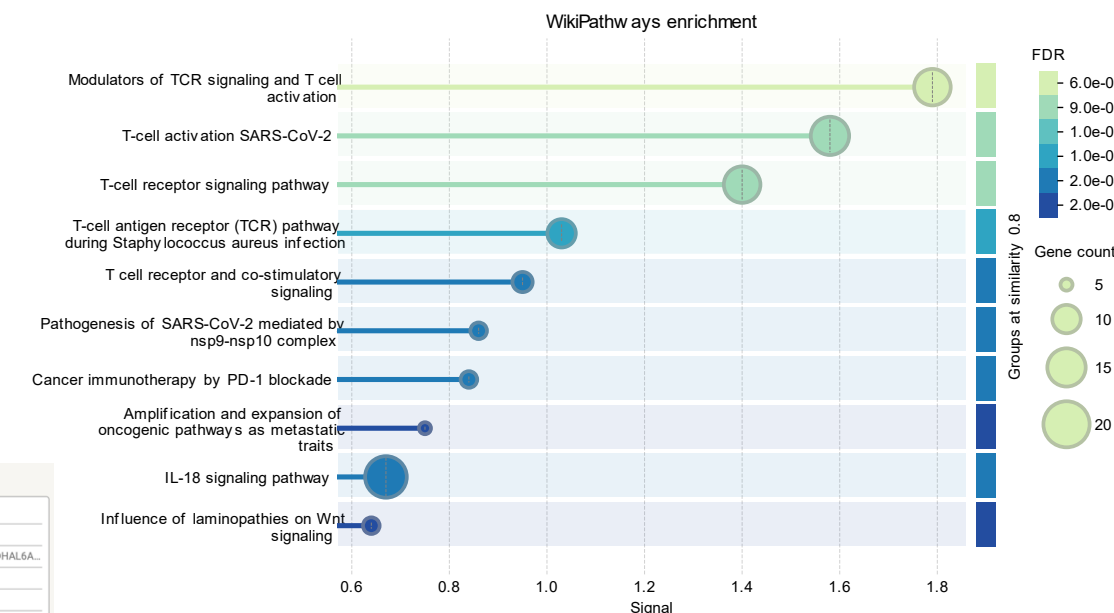
T Cells
8 Fractions



T Cells
48 Fractions

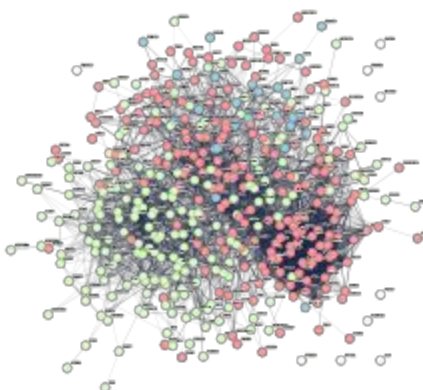
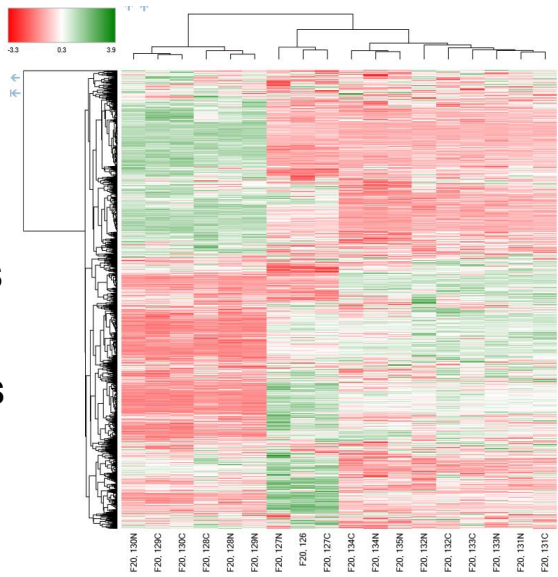


Clusters			
color	cluster Id	gene count	description
●	Cluster 1	184	Positive regulation of immune system process
●	Cluster 2	31	AAK1, ARL6IP5, CASC1, CFAP43, ESYT1, ESYT2, GSTK1, HAUS7, HMGN4, LDHAL6A...
●	Cluster 3	27	+ PRC2 methylates histones and DNA
●	Cluster 4	21	Thick filament
●	Cluster 5	5	DENND2C, FYCO1, SYTL1, TECPR1, ZNF276
●	Cluster 6	4	CHCHD2, GPRIN3, STK39, ZNF644

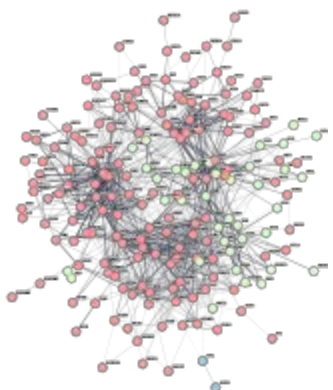


Monocytes

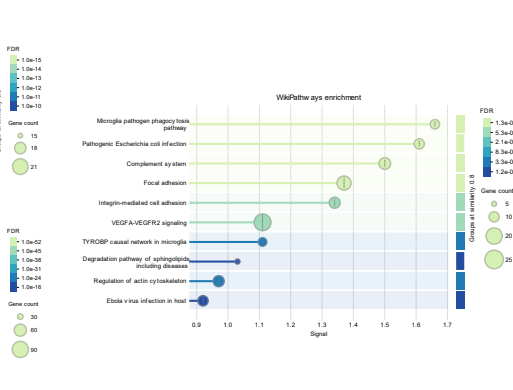
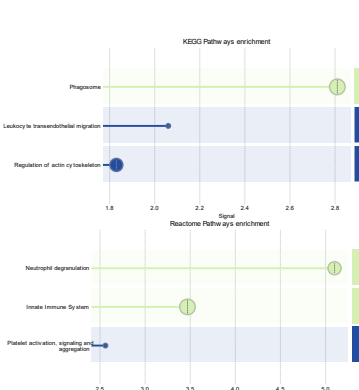
8 Fractions



color	cluster id	gene count	description
red	Cluster 1	199	Translation
green	Cluster 2	188	1. Neutrophil degranulation 2. Secretory granule
blue	Cluster 3	25	1. Endoplasmic reticulum to Golgi vesicle-mediated transport 2. Rab granety/granulation

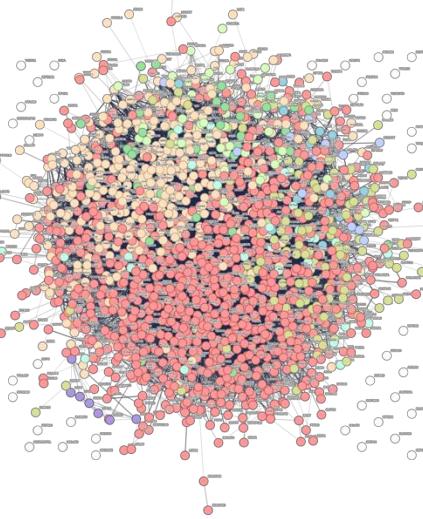
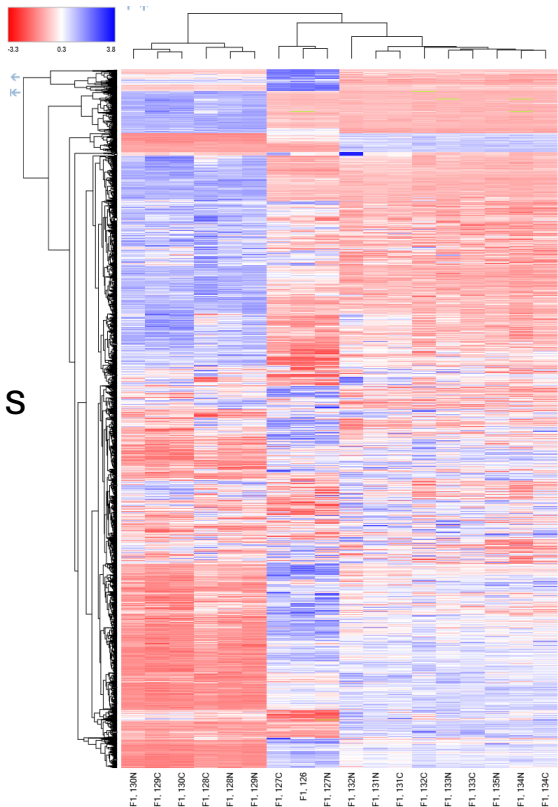


color	cluster id	gene count	description
red	Cluster 1	148	1. Neutrophil degranulation 2. Cell-cell junction
green	Cluster 2	38	1. Vacuolar lumen 2. Mixed, incl. S100/CaBP-9-k type, calcium binding, subdomain, and Cystatin superfamily
blue	Cluster 3	2	ACB03, CPV1

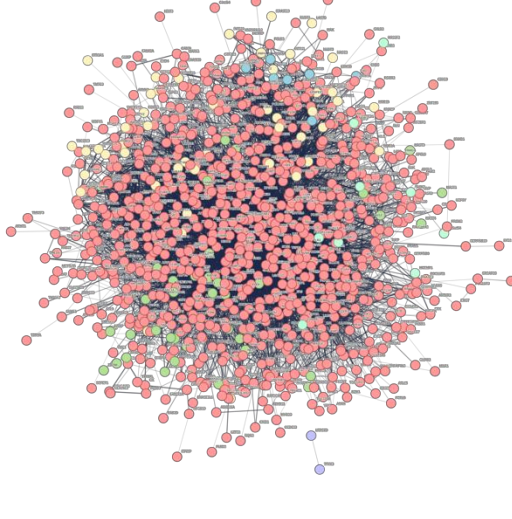


Monocytes

48 Fractions



color	cluster id	gene count	description
red	Cluster 1	976	+ Immune System
orange	Cluster 2	385	+ Cellular macromolecule biosynthesis
yellow	Cluster 3	127	Rab subfamily of small GTPases
light green	Cluster 4	48	Fatty acid beta-oxidation
green	Cluster 5	39	+ Pyrimidine metabolism
light blue	Cluster 6	32	COMM domain, and Ritscher-Schinzel syndrome 1
blue	Cluster 7	20	Lysosome
dark blue	Cluster 8	14	+ Glycerophospholipid metabolism
purple	Cluster 9	7	+ Intermediate filament organization



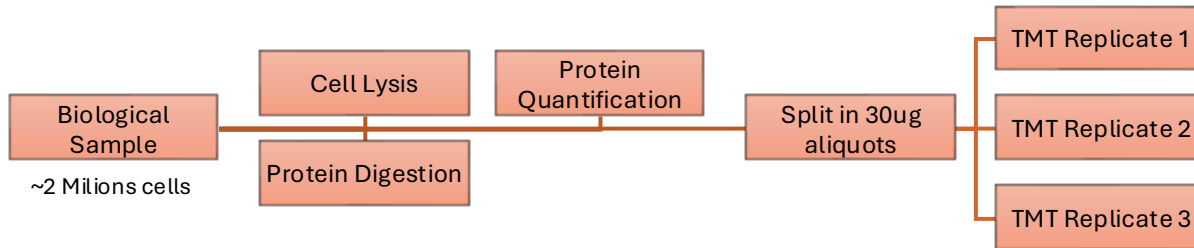
color	cluster id	gene count	description
red	Cluster 1	872	1. Immune System 2. Regulation of catalytic activity 3. Cytoplasmic vesicle
yellow	Cluster 2	49	BICD2, BTN1A1, CAPZA2, CAPZB, CEP128, CNOT10, CNOT11, DCTN1, DCTN3, DYN...
green	Cluster 3	37	Actin-binding
light green	Cluster 4	9	+ Mitotic G2 DNA damage checkpoint signaling
blue	Cluster 5	7	Cohesin Loading onto Chromatin
purple	Cluster 6	2	Volume-sensitive anion channel activity

KEGG Pathways				
pathway	description	count in network	strength	false discovery rate
hsa04145	Phagosome	45 of 141	0.86	2.52
hsa04562	Chemokine signaling pathway	49 of 186	0.77	2.23
hsa04540	Gap junction	20 of 87	0.89	2.18
hsa05170	Human immunodeficiency virus 1 infection	48 of 203	0.73	2.0
hsa04511	Platelet activation	34 of 122	0.8	1.97
(more...)				
Reactome Pathways				
pathway	description	count in network	strength	false discovery rate
HSA-437239	Recycling pathway of L1	29 of 48	1.14	3.16
HSA-6798695	Neutrophil degranulation	115 of 476	0.74	2.88
HSA-76502	Platelet activation, signaling and aggregation	68 of 260	0.77	2.57
HSA-6811434	COP1-dependent Golgi-to-ER retrograde traffic	35 of 99	0.9	2.4
HSA-168249	Innate Immune System	188 of 1041	0.61	2.39
(more...)				
WikiPathways				
pathway	description	count in network	strength	false discovery rate
WP5124	Alzheimers disease	59 of 260	0.71	2.08
WP2059	Alzheimers disease and miRNA effects	59 of 260	0.71	2.08
WP9129	Chemokine signaling pathway	41 of 165	0.75	1.91
WP3888	VEGFA-VEGFR2 signaling	75 of 428	0.6	1.78
WP2806	Complement system	28 of 96	0.82	1.76
(more...)				

color	cluster id	gene count	description
red	Cluster 1	47	+ Rap1 signaling pathway
orange	Cluster 2	33	Mixed, incl. Immunoregulatory interactions between a Lymphoid and a non-Lymphoi...
yellow	Cluster 3	29	+ Clathrin coat
light green	Cluster 4	28	Protein folding
green	Cluster 5	28	CD42 GTPase cycle
light blue	Cluster 6	23	Nucleosome organization
blue	Cluster 7	20	RHO GTPase cycle
dark blue	Cluster 8	20	+ Collecting duct acid secretion
purple	Cluster 9	15	+ Microtubule-dependent trafficking of connexons from Golgi to the plasma memb...
light purple	Cluster 10	14	1. Retrograde vesicle-mediated transport, Golgi to endoplasmic reticulum 2. COP1-dependent Golgi-to-ER retrograde traffic
yellow-green	Cluster 11	13	+ Attenuation phase
green	Cluster 12	12	Mixed, incl. Positive regulation of plasma membrane repair, and Annexin
light green	Cluster 13	11	+ Respiratory burst
green	Cluster 14	11	+ Cell adhesion mediated by integrin
light green	Cluster 15	11	Nuclear events stimulated by ALK signaling in cancer
green	Cluster 16	11	+ Chromosome condensation
light green	Cluster 17	11	+ Amyloidosis
green	Cluster 18	11	Histone deacetylase complex
light green	Cluster 19	11	+ DARPP-32 events
green	Cluster 20	9	Formation of Fibrin Clot (Clotting Cascade)
light green	Cluster 21	9	Antiviral defense

Conclusions

	Replicates		Number of cells per sample	ug of TMT labelled protein
	Biological	Technical (TMT)		
Monocytes	2	3	2 Millions	30
T Cells	3	3	2.5 Millions	30
B Cells	1	3	2.5 Millions	30



- Both methods discriminate among cell types
- HPLC Hi-pH fractionation outperform the spin columns and provides a deeper insight of proteome

Hi-pH Peptide Fractionation



High pH Reversed-Phase Peptide Fractionation Kit

8 Fractions



XBridge BEH C18 Column
3.5 μ m, 2.1 mm X 150 mm

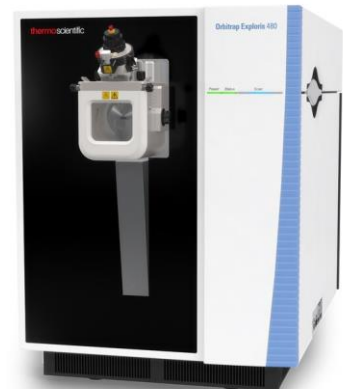
48 Fractions

Combine in 12 Fractions

LC-MS

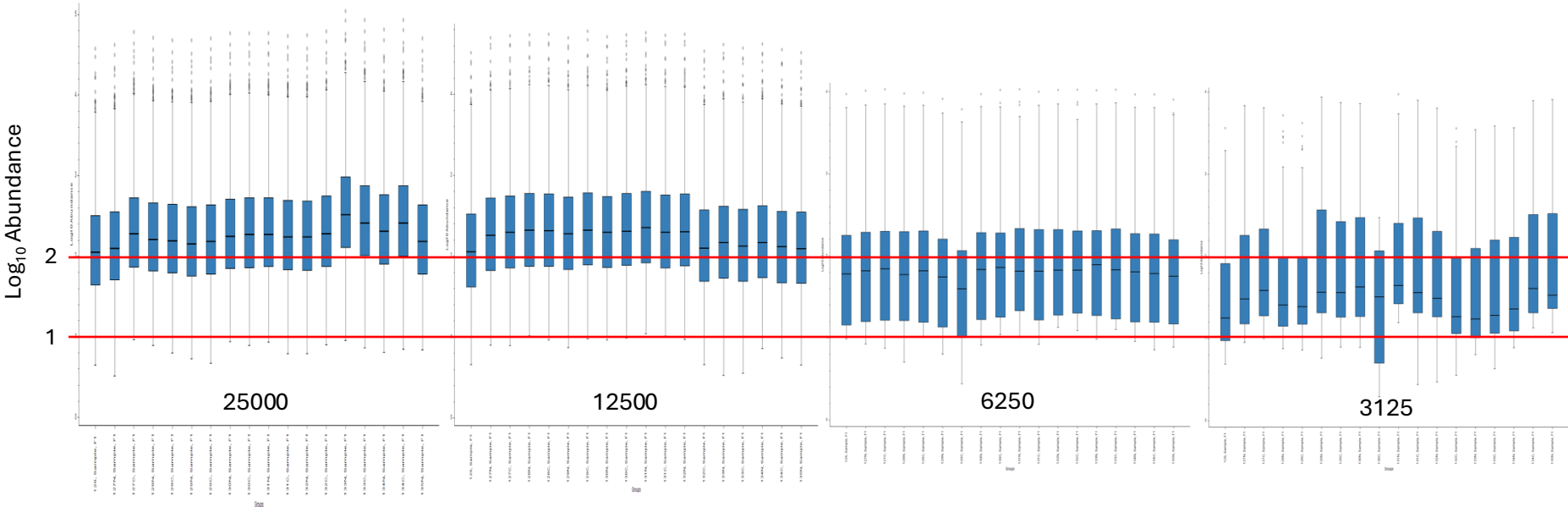
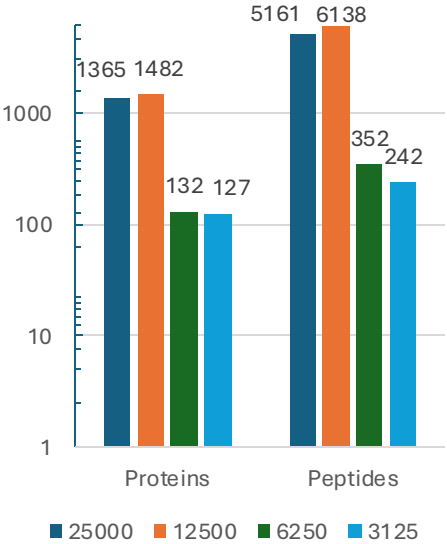


50 cm C18
Gradient: ACN:FA 0.1%
90-120 min
Inject ~2ug



Determining the Detection Limit

Num. of Cells	Protein (ug)	Cells Vol. (ul)	Digestion Vol. (ul)	Technical Replicates
25k	1.200	10	20	18
12.5k	0.600	5	10	18
6.25k	0.300	2.5	5	18
3.125k	0.150	1.25	2.5	18



Conclusions

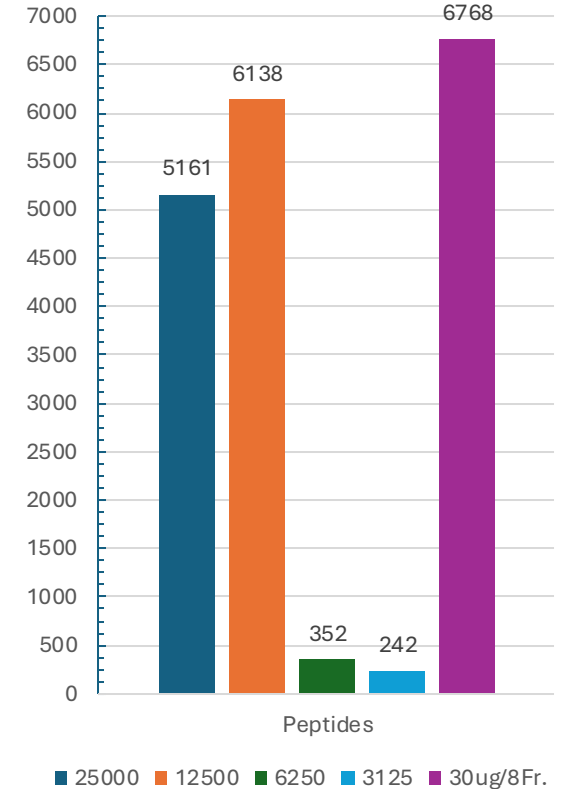
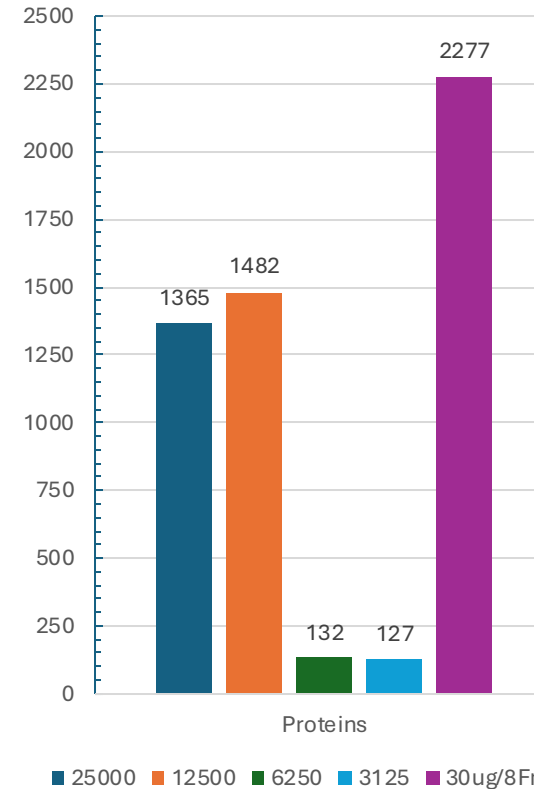
T-Cells (CD3+)

Num. of Cells	Protein (ug)	Cells Vol. (ul)	Digestion Vol. (ul)	Technical Replicates
25k	1.200	10	20	18
12.5k	0.600	5	10	18
6.25k	0.300	2.5	5	18
3.125k	0.150	1.25	2.5	18

0.6-1.2 ug of protein per sample (T-Cells)

8 Hi-pH fractions on spin columns

90 minutes gradient for LC-MS analysis



	Replicates		Number of cells per sample	ug of TMT labelled protein
	Biological	Technical (TMT)		
Monocytes	2	3	2 Millions	30
T Cells	3	3	2.5 Millions	30
B Cells	1	3	2.5 Millions	30

Labeling 0.6-1.2 ug of peptides per sample yields similar results to labelling 30 ug

Summary

- A pilot proteomics experiment successfully investigated 30 µg samples from 3 different immune cell types
- A minimum aliquot of 12,500 cells per cell type was required for the 18-plex proteomics analysis

Further Investigations

- Can we confirm these results by sorting and processing straightaway 12,500 cells only?
- Can HPLC fractionation improve the results dealing with 0.6-1.2 ug of protein per sample?
- Are we ready for a real proteomics campaign?

Acknowledgments

- **CIGS Team:**

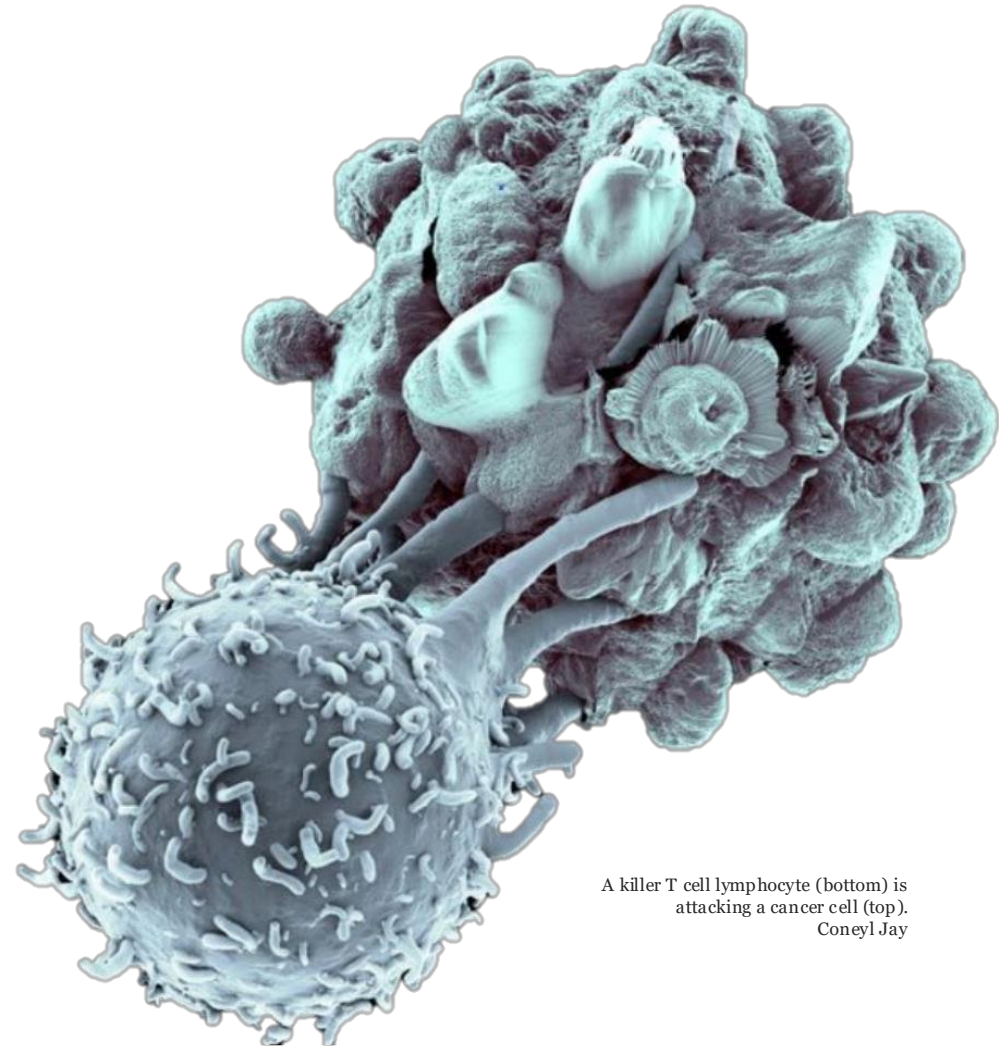
- Dr. Diego Pinetti
- Dr. Filippo Genovese

- **Cossarizza Lab:**

- Dr. Maristella Leccese
- Dr. Miriam D'Angerio
- Dr. Elena Santacroce
- Dr. Domenico Lo Tartaro
- Dr. Alin Liviu Ciobanu
- Prof. Andrea Cossarizza
- Dr. Sara De Biasi

- **Additional Support:**

- Dr. Francesco Demetrio Lofaro



A killer T cell lymphocyte (bottom) is attacking a cancer cell (top).
Coneyl Jay