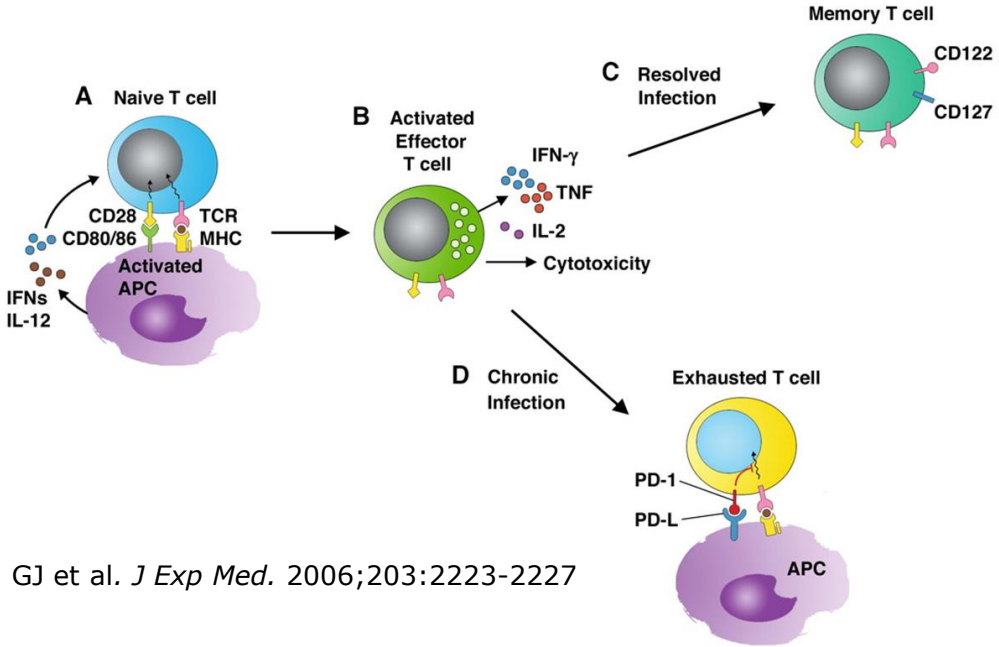
Single cell multiomics analysis for deep characterization of exhausted T cells

T-cell exhaustion

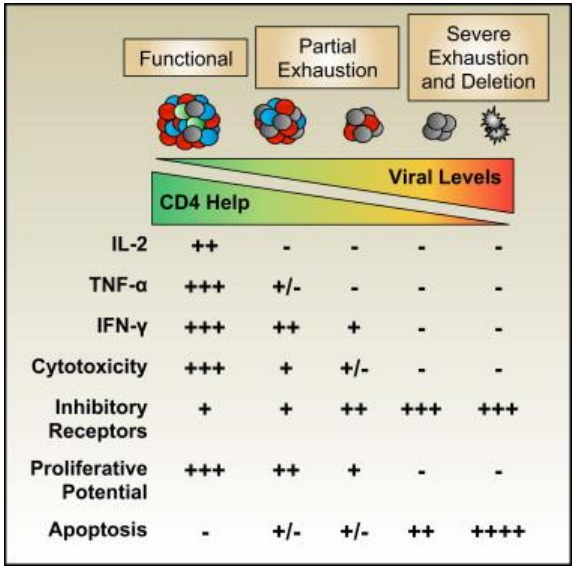
Chronic viral infections or cancer can lead to persistent stimulation of T cells, which results in T-cell dysfunction.



Adapted from Freeman GJ et al. *J Exp Med.* 2006;203:2223-2227

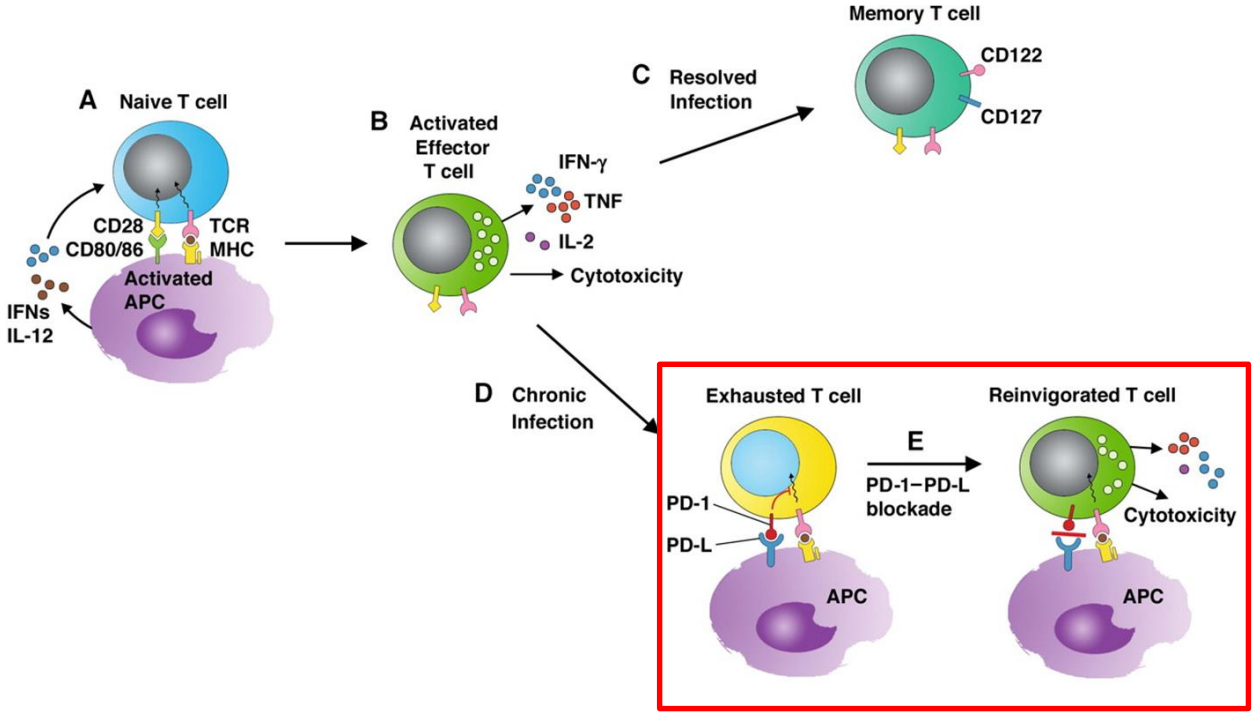
Hallmarks of T-cell exhaustion

T-cell exhaustion is characterized by the progressive loss of function and sustained expression of inhibitory receptors.



Kahan SM et al. *Virology.* 2015 May;479-480:180-93

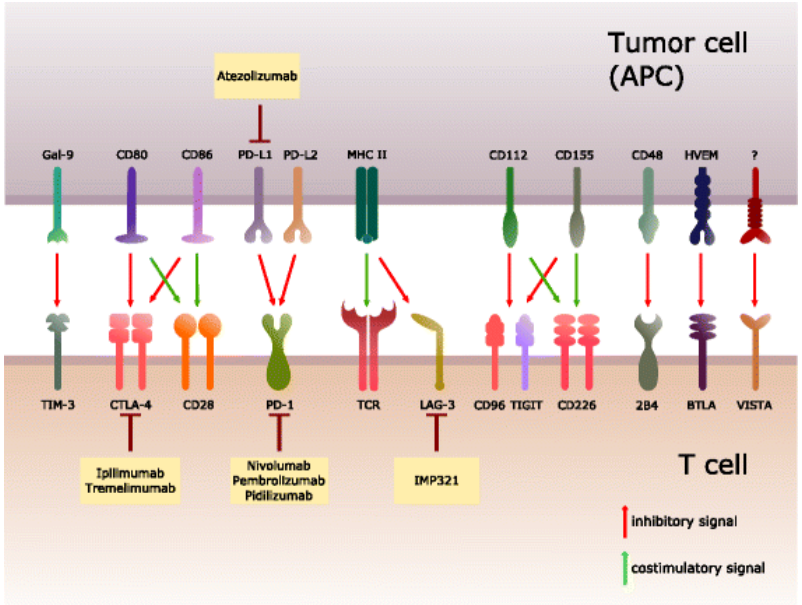
T-cell exhaustion is reversible



Adapted from Freeman GJ et al. *J Exp Med.* 2006;203:2223-2227

Immune checkpoint blockade therapy

Different inhibitory receptors can be simultaneously expressed on exhausted T cells.



Catakovic et al. *Cell Commun Signal.* 2017 Jan 5;15(1):1

The importance of studying T-cell exhaustion

- Understand the diversity and complexity of exhausted tumor-infiltrating T cells.
- Discover new mechanisms regulating T-cell exhaustion and promoting cancer progression.
- Optimize protocols for CAR T-cell generation.
- Screen new molecules able to prevent or revert T-cell exhaustion.

Limitation of current single cell technologies

- High-parameter flow cytometry (conventional flow cytometry, CyTOF®, spectral flow cytometry): Allows simultaneous analysis of 28-40 proteins on a single cell level.
- Single cell RNA sequencing: Allows comprehensive assessment of mRNA expression on a single cell level.
- These technologies are complementary and can be used in parallel for proteomic and transcriptomic analysis of cells of interest. However, these assays are not performed on the same sample/cell, therefore the two datasets cannot be correlated.

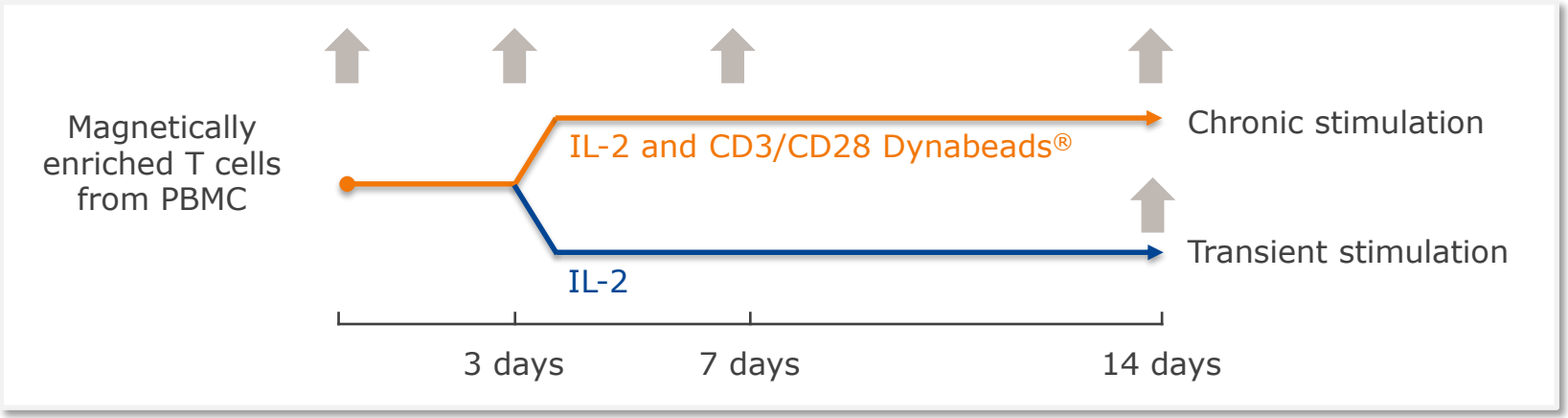
Power of single-cell multiomics

- BD® AbSeq reagents, in conjunction with the BD Rhapody™ Targeted Panel, allows for **simultaneous proteomic and transcriptomic analysis at the single cell level** and enables deeper characterization of cells, understanding of mechanisms governing cell processes, identification of novel populations or patterns of differentiation/activation/transformation based on the correlation of immunophenotype and gene expression profile.
- BD Rhapsody provides useful insight into the markers of T-cell exhaustion following chronic or transient stimulation of cells.

Key findings

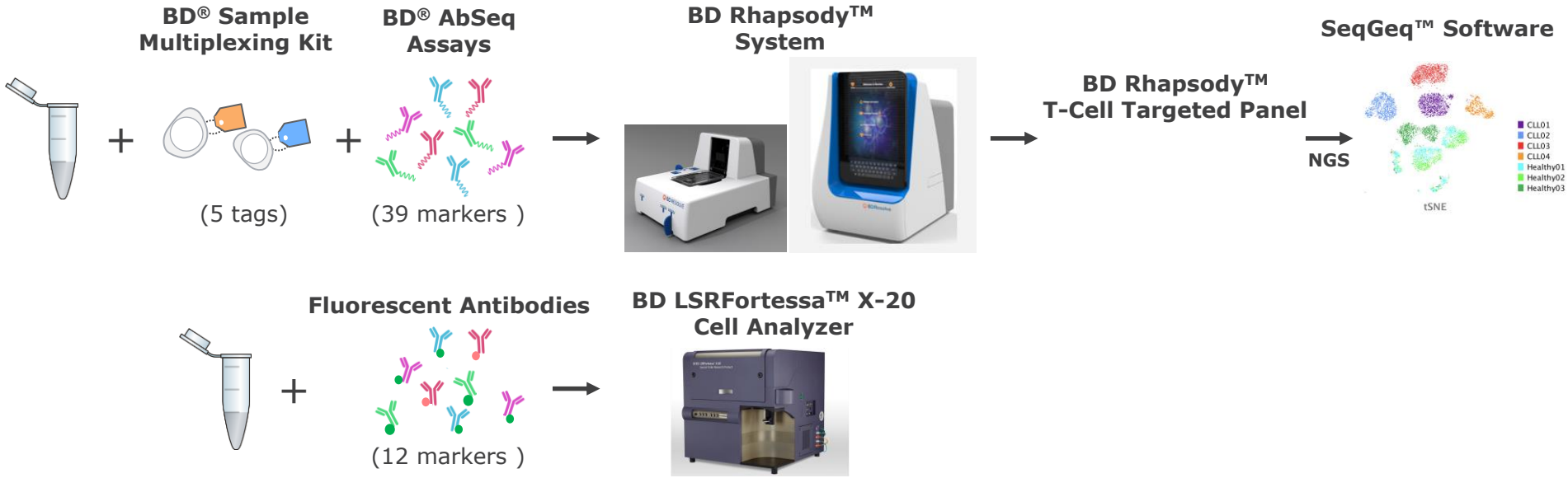
1. Demonstrated the concordance between flow cytometry and BD AbSeq technology.
2. Assessed kinetics and differential expression of 39 proteins and 400 T-cell specific genes over time.
3. Identified discrete subsets of cells within a single sample and across different samples, allowing correlation of protein and mRNA expression to reveal the mechanism by which protein level is regulated.
4. Resolved heterogeneity of exhausted T cells using supervised analysis.
5. Identified additional populations of interest using unsupervised analysis.

Experimental design and workflow



- Magnetically enriched T cells from PBMC were stimulated for 3 days in the presence of IL-2 and CD3/CD28 Dynabeads®.
- Chronic stimulation: Stimulation was carried over for an additional 11 days.
- Transient stimulation: Dynabeads® were removed and cells were cultured in the presence of IL-2 only for an additional 11 days.
- Total of 5 samples were collected and frozen: Fresh T cells, cells stimulated for 3 days, 7days, 14 days (chronically stimulated), and cells stimulated for 3 days and rested for 11 days (transiently stimulated).

Experimental design and workflow



- An aliquot of each samples was thawed and stained with 39 BD® AbSeq panels and BD® Single-Cell Multiplexing antibodies. The samples were pooled and loaded into 2 BD™ Rhapsody cartridges (~3000 cells/sample). BD Rhapsody™ T-cell targeted panel was used to assess mRNA expression level.
- An aliquot of frozen cells was stained with a 12-color flow cytometry panel and analyzed on the BD LSRFortessa™ X-20 cell analyzer.

Panel design

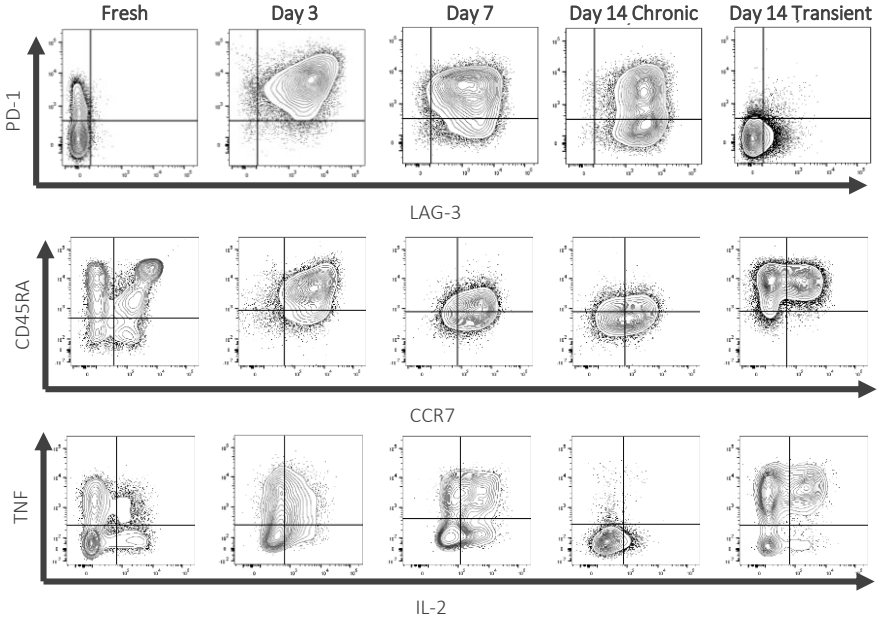
39-Plex AbSeq Panel			
CD4	CD278 (ICOS)	CD103	CD94
CD8	CD178 (FAS-L)	CD30	CD95
CD279 (PD-1)	CD183 (CXCR3)	CD38	CD98
CD223 (LAG-3)	CD185 (CXCR5)	CD39	CD2
CD366 (TIM-3)	CD194 (CCR4)	CD44	TCRab
CD272 (BTLA)	CD196 (CCR6)	CD45RA	CD154
CD357 (GITR)	CD137 (4-1BB)	CD49a	CD7
CD226 (DNAM-1)	CD134 (OX40)	CD54	CD2
CD152 (CTLA-4)	CD27	CD62L	CD25
CD270 (HVEM)	CD28	CD69	

12-color Flow Cytometry Panel	
Marker	**Fluorochrome**
CD4	BUV805
CD8	BUV395
CD279 (PD-1)	PE-Cy7
CD152 (CTLA-4)	PE
CD223 (LAG-3)	BV480
CD366 (TIM-3)	BV711
CD39	BUV737
CD103	APC
CD45RA	APC-H7
CD62L	FITC
CD95	BV786
CD357 (GITR)	BV421

- The AbSeq panel identifies 39 proteins that play roles in T-cell activation, inhibition/exhaustion, differentiation or chemotaxis.
- A companion 12-color flow cytometry panel with selected, overlapping specificities was also designed to assess concordance between flow cytometry and AbSeq technology.
- scRNA-Seq panel includes 400 genes involved in immune responses.
- All data shown here are from $CD8^+$ T cells. Equivalent data analysis can be performed on $CD4^+$ T cells

Validation of the in vitro model system

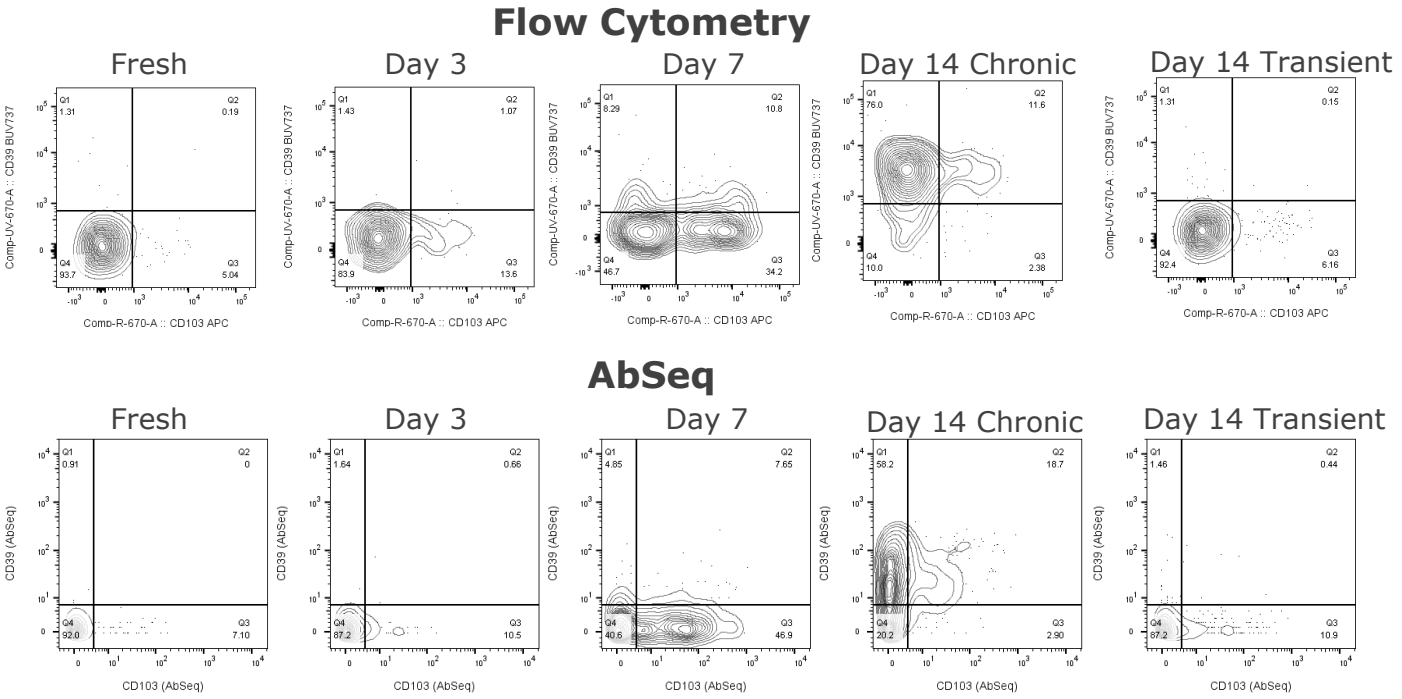
- The optimized panel confirmed that the in vitro model system for chronic stimulation induced immunophenotypic and functional changes associated with T-cell exhaustion:
  - Progressive expression of inhibitory receptor and marker for T-cell differentiation
  - Progressive loss of the ability to produce pro-inflammatory cytokines
- Removal of stimulus after 3 days led to a reversal of the exhausted phenotype.



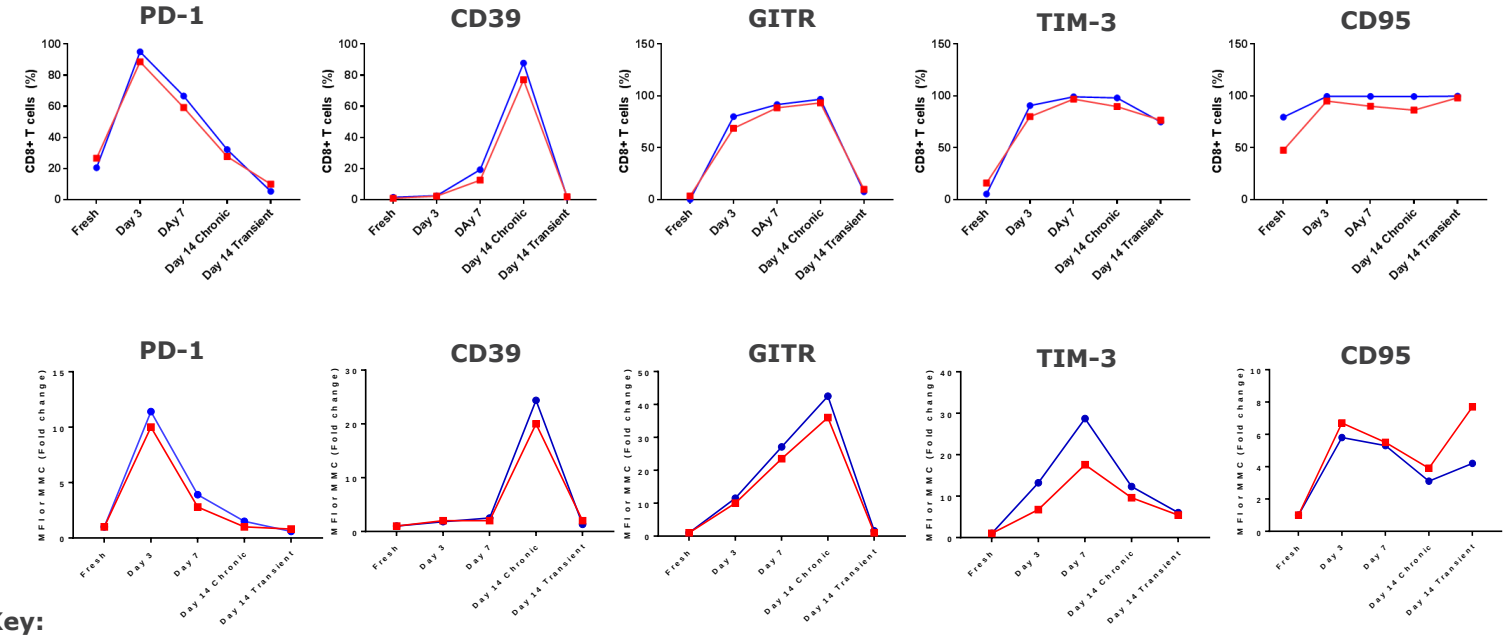
Sequencing metrics

Filtered Reads (M)	Putative Cell #	AbSeq Mean Reads/Cell	RNA Mean Reads/Cell	AbSeq Mean Raw Seq Depth	RNA Mean Raw Seq Depth	AbSeq Saturation (%)	RNA Saturation (%)
325	18800	5746	1650	1.15	2.13	24	99

Correlation between flow cytometry and AbSeq technology

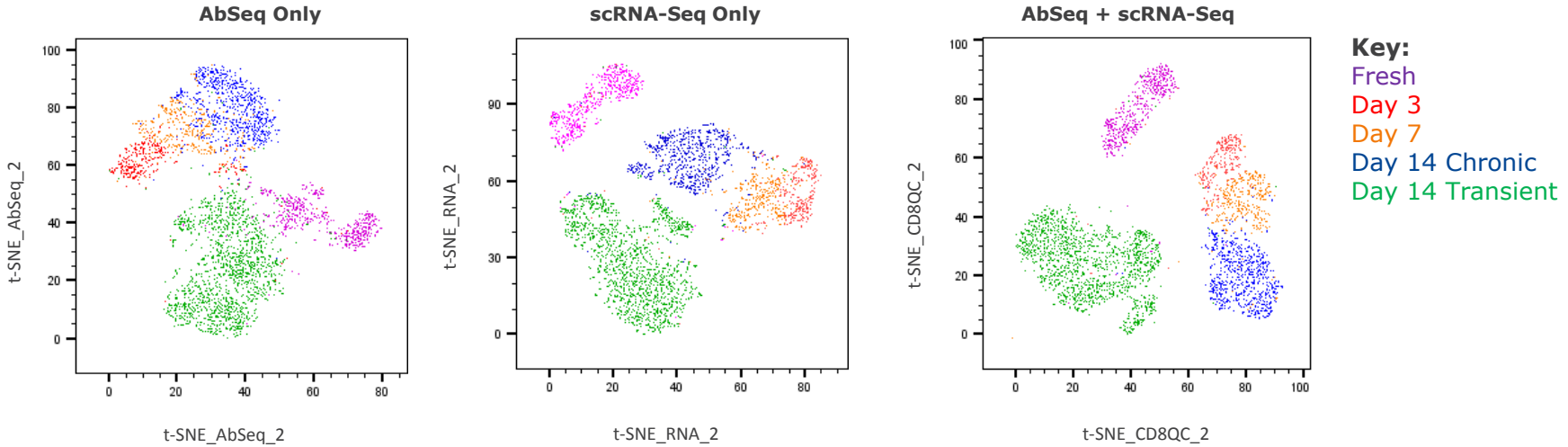


Concordance between flow cytometry and AbSeq data



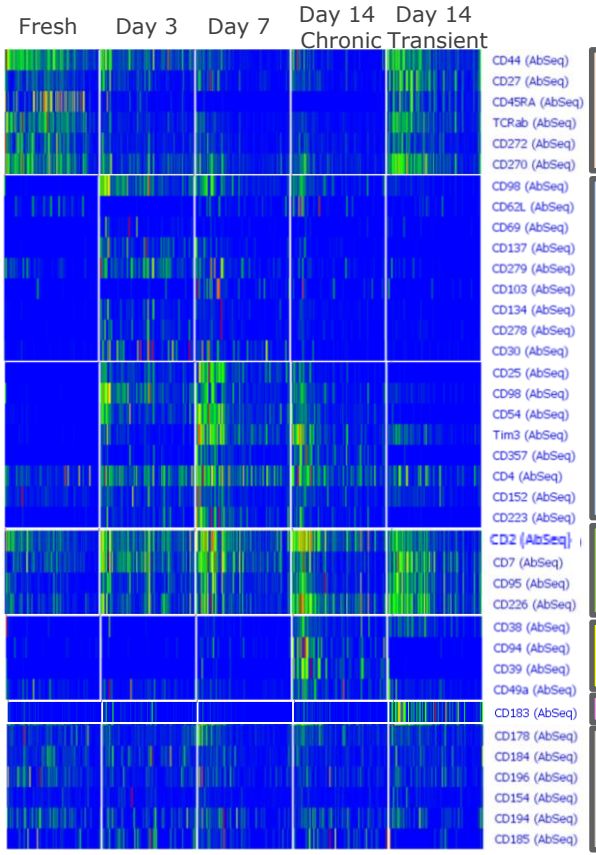
Key:
Flow Cytometry
AbSeq

Clustering of T cells



- Distinct clusters are resolved for each samples and the resolution is equivalent for clustering based on protein only, mRNA only or a combination of both.
- Transiently stimulated cells (Day 14) cluster separately from both fresh T cells and chronically stimulated cells, suggesting a distinct signature, in contrast to previous observation suggesting they are phenotypically and functionally similar to fresh cells.
- Chronically stimulated cells (Day 3, 7 and 14) form clusters that are separate from fresh cells. The three samples form distinct clusters, suggesting they each have unique signatures.

Differential protein expression analysis



Cluster 1: Proteins expressed on fresh T cells which are downregulated upon stimulation and then re-expressed after removal of the stimulatory agonist. This pattern is expected for CD45RA and CD27 because cells differentiate upon stimulation. TCRab downregulation is expected as well.

Cluster 2: Proteins expressed at variable levels upon stimulation throughout the 14-day stimulation period. Some show increased expression at day 3 and then decrease; others show increased expression at day 7. Expression of these proteins is downregulated upon stimulation removal, indicating their dependence on active TCR stimulation. All of the proteins fall in the expected categories of activation markers and inhibitory receptors. Interestingly, expression of most co-stimulatory and inhibitory receptors is downregulated at day 14.
Some expected patterns are observed: CD69, an early activation marker, is barely detected at day 3 only, whereas CD25, a late activation marker, shows an expected pattern of elevated expression at day 7. Expression of CD4 on activated $CD8^+$ cells has been largely documented.

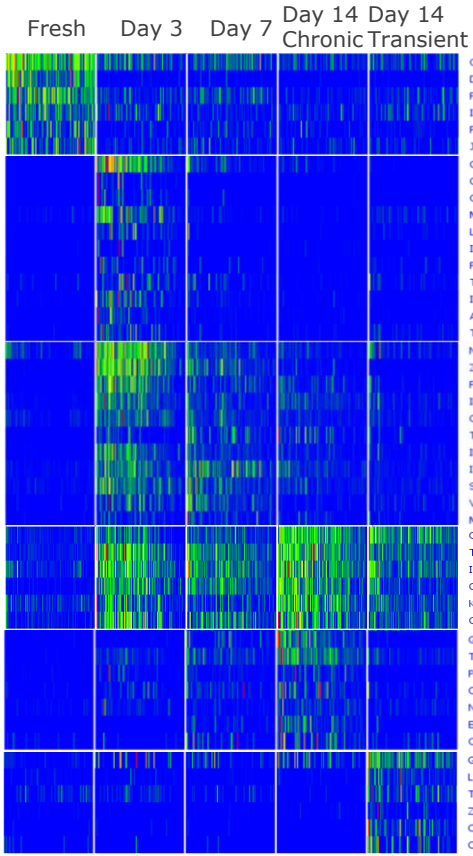
Cluster 3: Similar to cluster 2, but the expression of these markers appear to be TCR stimulation-independent as they are still expressed upon stimulus removal.

Cluster 4: Proteins whose expression is significantly upregulated after 14 days.

Cluster 5: Protein upregulated upon transient stimulation.

Cluster 6: Proteins with patterns of expression apparently not associated with a specific activation state.

Differential gene expression analysis



Cluster 1: Genes expressed on fresh T cells and downregulated upon activation. These genes are not significantly upregulated upon stimulus removal.

Cluster 2: Genes upregulated upon activation but transiently expressed and downregulated by day 7. Interestingly, a number of these genes code for proteins involved in cytotoxicity (GZMB, IFNG, TNF) and migration (CCL3, CCL4). This might suggest a progressive loss of function over chronic stimulation, consistent with the decrease of cytokine production observed using flow cytometry.

Cluster 3: Genes expressed throughout chronic stimulation, but lost upon stimulus removal. As observed for the protein expression, expression of these genes is significantly downregulated by day 14.

Cluster 4: Genes expressed throughout chronic and transient stimulation without significant downregulation over time. Some genes are progressively expressed and peak at day 14 (KLRK1, CD52).

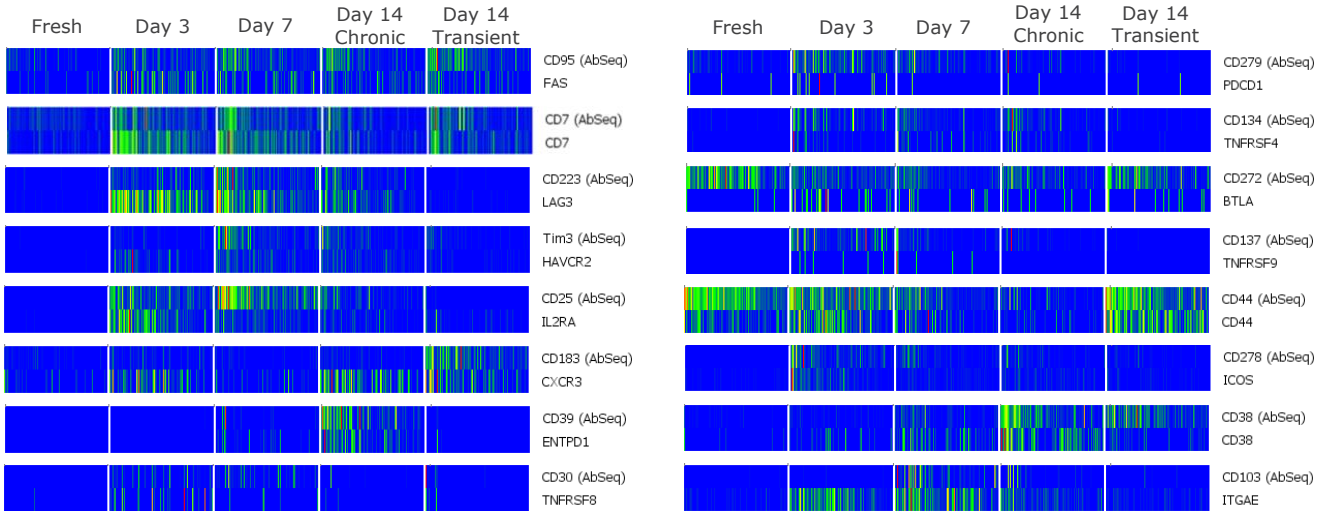
Cluster 5: Genes predominantly expressed at day 14 of chronic stimulation. Interestingly, GNLY, encoding the cytotoxic molecule Granulysin, is in this cluster. ENTPD1 encodes CD39, which belongs to a similar protein cluster.

Cluster 6: Genes almost exclusively expressed at day 14 of transient stimulation. Even though these cells resemble fresh T cells functionally and phenotypically, these genes might indicate the presence of unique cells that originate following transient activation.

Benefits of simultaneous analysis of protein and gene expression

- High-parameter flow cytometry and single cell RNA sequencing can independently produce data for differential protein expression and gene expression, respectively.
- However, using AbSeq reagents provides protein and gene expression data at the single cell level for a deeper, more precise cell characterization to allow:
  - Correlation of gene and protein expression of selected targets to understand mechanisms of regulation upon activation (transcriptional or post-transcriptional regulation)
  - Superior identification and separation of cell clusters
  - Further dissection of T-cell subsets based on combined immunophenotype and gene expression profiles

Correlating protein and gene expression on a single cell level

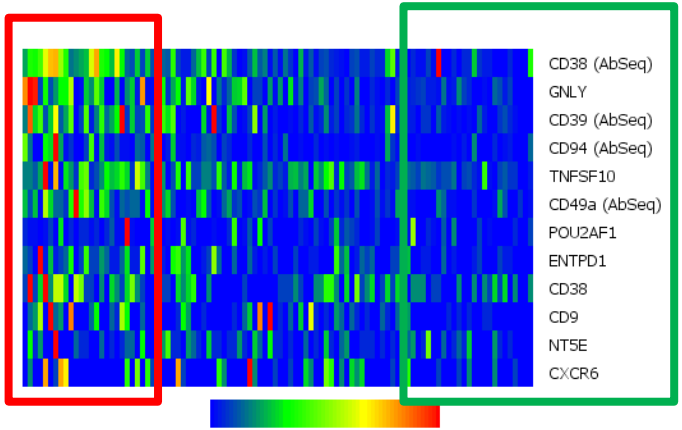


- CD7 protein and mRNA expression show strong correlation throughout the time course.
- While CD103 (ITGAE) mRNA is upregulated at day 3 of activation, protein expression increases later, at day 7.
- In chronically stimulated cells, CD38 mRNA is upregulated at day 3 and increases over time, whereas protein expression increases later, at day 14. In transiently stimulated cells, CD38 protein is expressed at day 14 even though mRNA expression is not maintained.

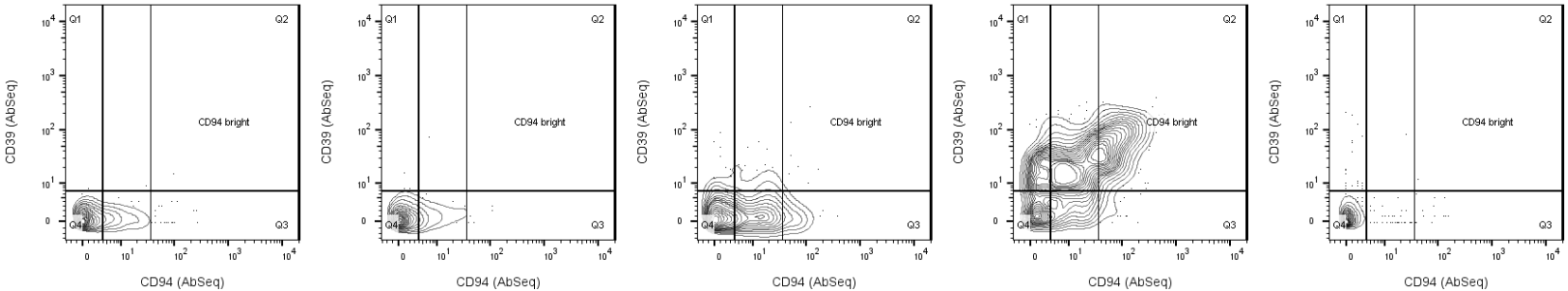
Subpopulation emerges within chronically stimulated CD8+ T cells

- Two populations (red and green boxes) emerge based on the expression level of these markers.
- Co-expression patterns are observed for CD38, CD39, CD94.
- A correlation between CD39 and CD73 expression, shown by CD39 (AbSeq)/ENTPD1 and NT5E, respectively; validates their known function of working together in ATP hydrolysis.

Expression of 12 proteins and genes that are significantly upregulated at day 14 of chronic stimulation

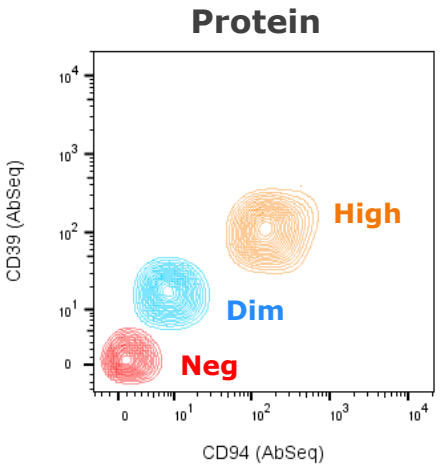


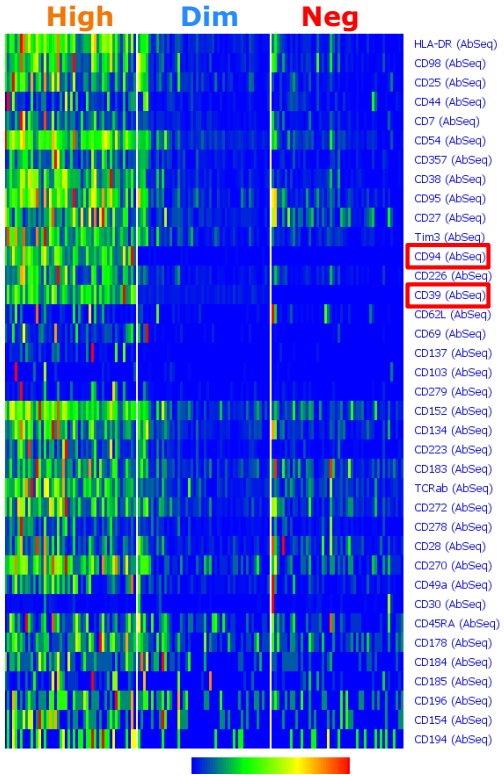
Resolution of subpopulations expressing CD39 and CD94 during chronic stimulation

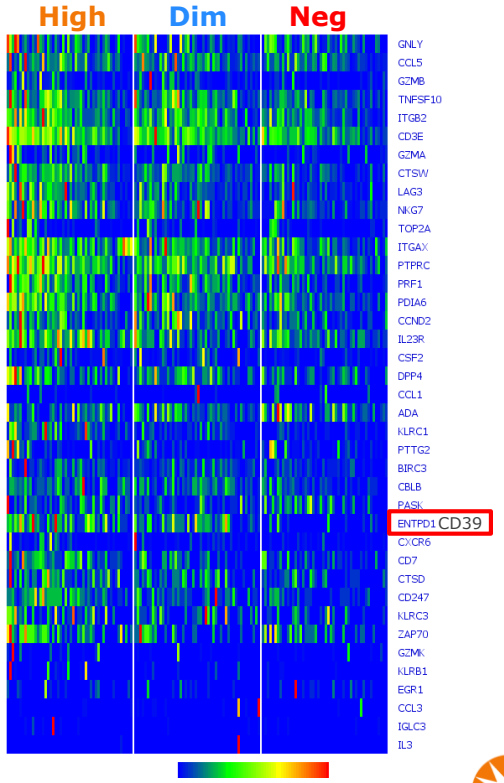


- Using AbSeq reagents enables you to resolve distinct populations of cells expressing intermediate or high levels of CD39 and CD94.
- We can now perform a comprehensive analysis in these subsets of cells by combining the protein and gene expression information, without the need to further sort these subsets for deeper analysis.

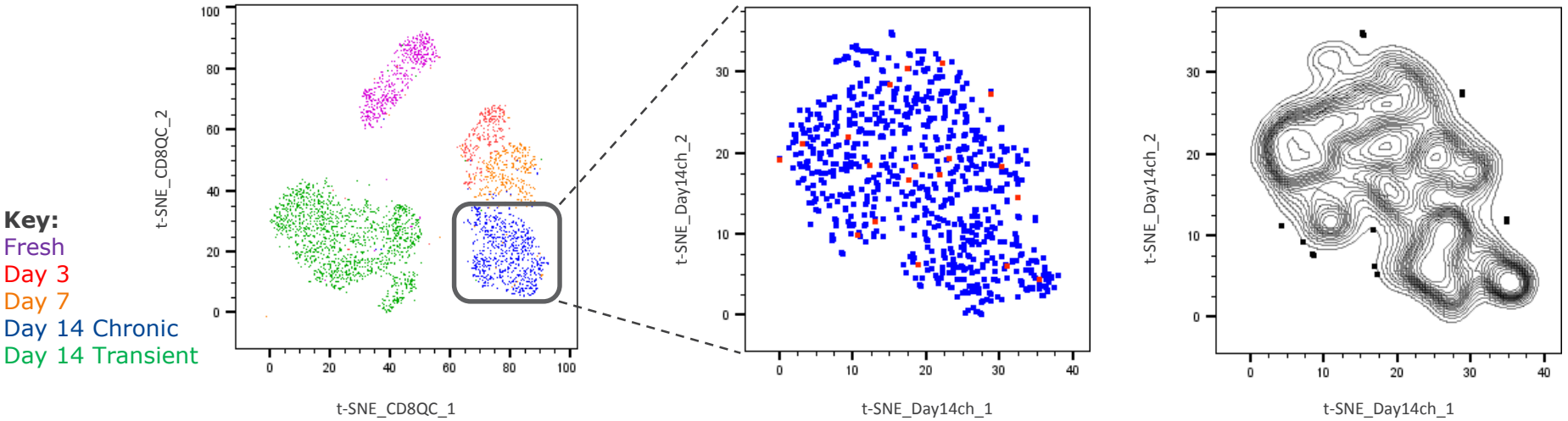
DPE and DGE between subsets expressing different levels of CD39 and CD94





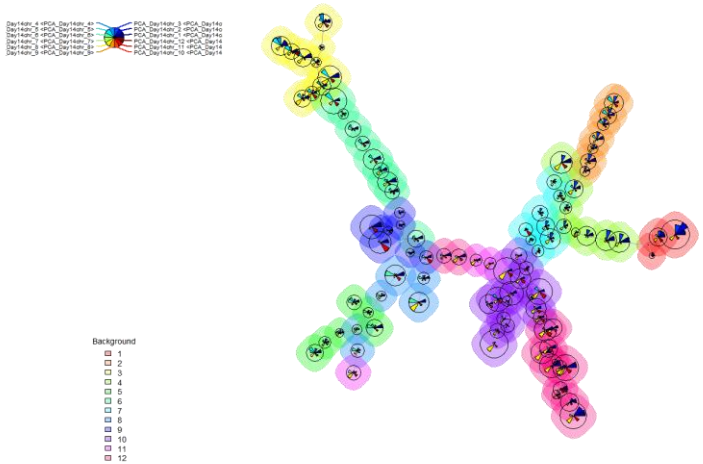


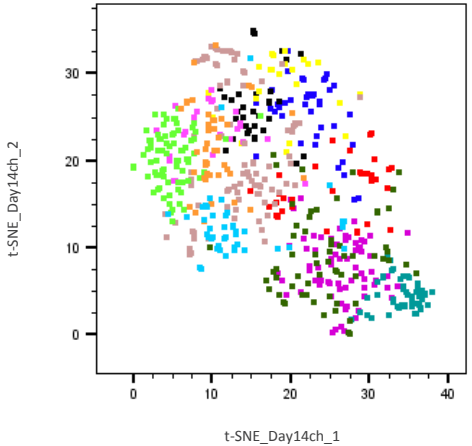
Unsupervised data analysis



- Unsupervised analysis of chronically stimulated cells (day 14) resolved the population (blue) into multiple clusters of cells, showing the heterogeneity of the population.
- Next, FlowSOM, a SeqGeq™ plug-in, was used to identify and separate the clusters in the third panel.

Unsupervised data analysis for a deeper characterization

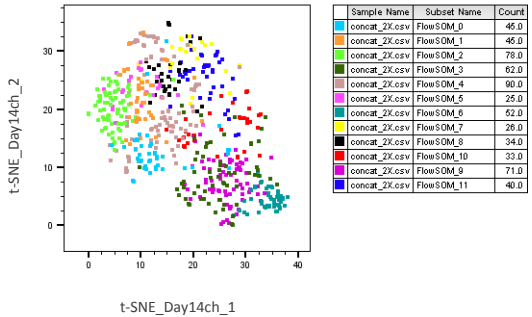




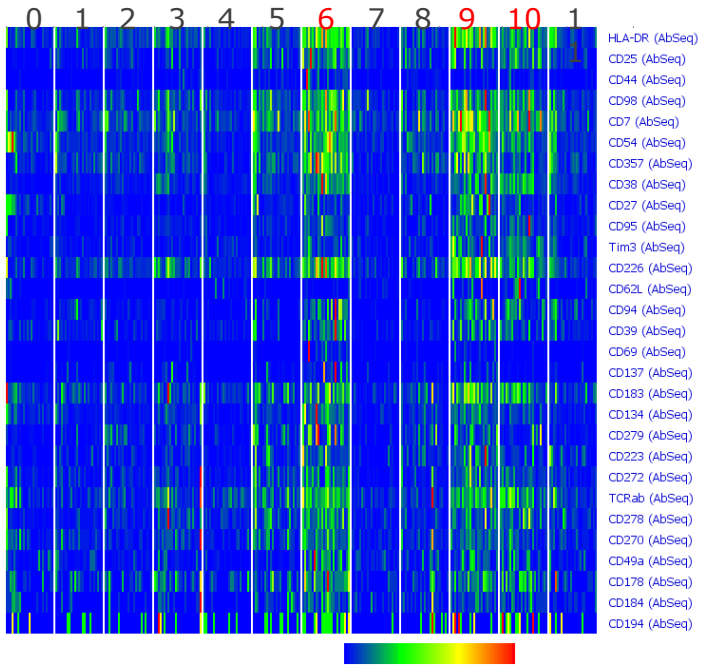
	Sample Name	Subset Name	Count
	concat_2X.csv	FlowSOM_0	45.0
	concat_2X.csv	FlowSOM_1	45.0
	concat_2X.csv	FlowSOM_2	78.0
	concat_2X.csv	FlowSOM_3	62.0
	concat_2X.csv	FlowSOM_4	90.0
	concat_2X.csv	FlowSOM_5	25.0
	concat_2X.csv	FlowSOM_6	52.0
	concat_2X.csv	FlowSOM_7	26.0
	concat_2X.csv	FlowSOM_8	34.0
	concat_2X.csv	FlowSOM_10	33.0
	concat_2X.csv	FlowSOM_9	71.0
	concat_2X.csv	FlowSOM_11	40.0

- At least 12 sub-clusters are identified. This allows us to perform differential protein and gene expression analysis to identify these subsets.
- A minimal spanning tree is generated to visualize different clusters of cells whose proximity is a function of phenotypical and transcriptional similarity.
- FlowSOM clusters can also be overlaid onto t-SNE plots for additional data visualization.
- Each cluster can be subjected to differential protein and gene expression analysis.

Differential protein expression analysis on FlowSOM clusters

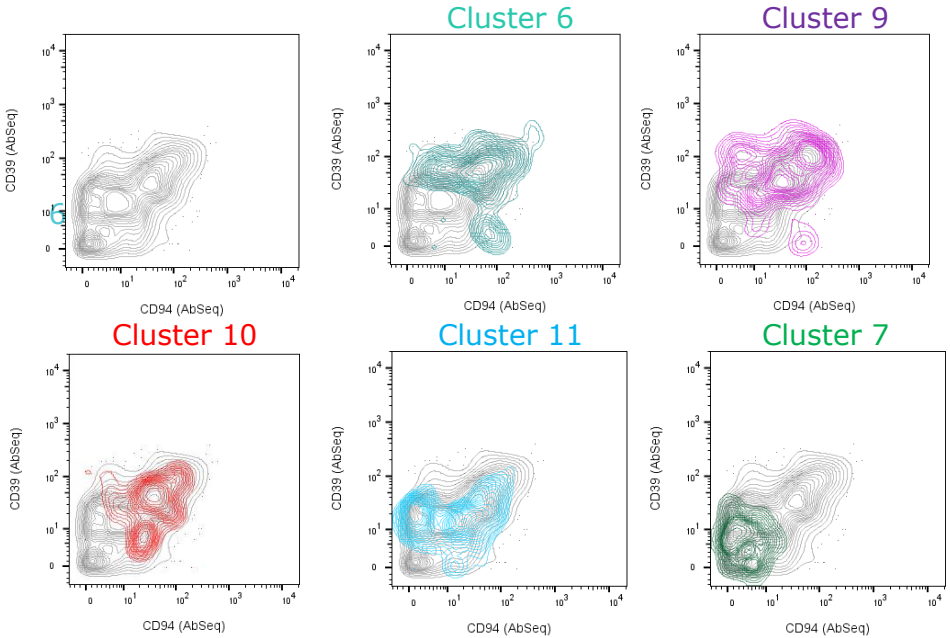


	Sample Name	Subset Name	Count
	concat_2X.csv	FlowSOM_0	45.0
	concat_2X.csv	FlowSOM_1	45.0
	concat_2X.csv	FlowSOM_2	78.0
	concat_2X.csv	FlowSOM_3	62.0
	concat_2X.csv	FlowSOM_4	90.0
	concat_2X.csv	FlowSOM_5	25.0
	concat_2X.csv	FlowSOM_6	52.0
	concat_2X.csv	FlowSOM_7	26.0
	concat_2X.csv	FlowSOM_8	34.0
	concat_2X.csv	FlowSOM_10	33.0
	concat_2X.csv	FlowSOM_9	71.0
	concat_2X.csv	FlowSOM_11	40.0



- DPE analysis revealed the presence of 3 distinct subsets characterized by high expression of activation markers and inhibitory receptors, including CD39, CD38, CD94 and CD49 resembling the single cluster identified via supervised analysis.

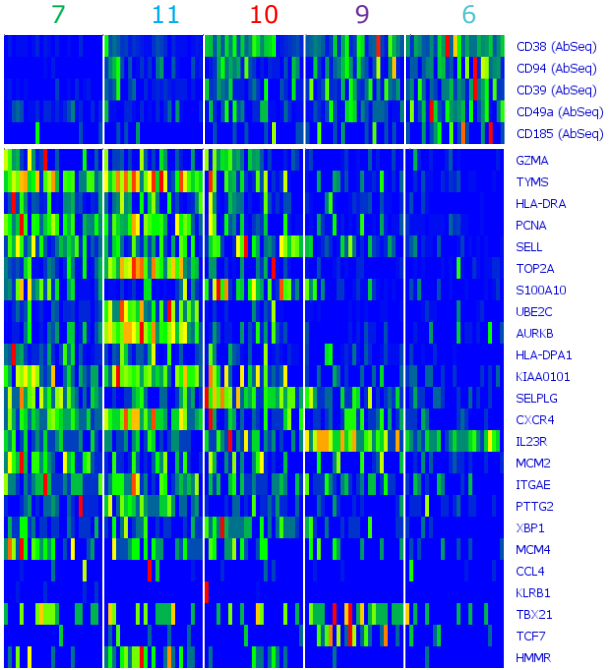
Differential protein expression analysis



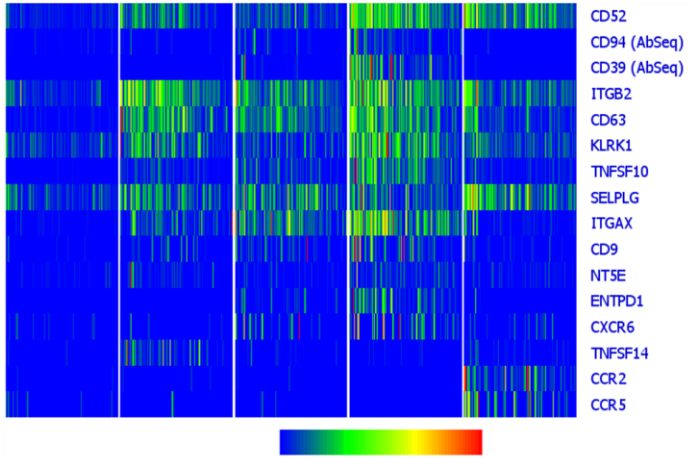
- Visual confirmation of the presence of different clusters expressing different levels of CD39 and CD94, spanning from negative to dim and high.
- Next, we performed DGE analysis to further dissect and define these clusters.

Differential protein and gene expression analysis on selected clusters

- Supervised analysis identified CD39, CD38, CD49a and CD94 as markers defining a distinct subset of cells. Unsupervised analysis further dissected these cells based on differential gene expression.
- Analysis of protein expression alone would have not been able to dissect the 3 clusters expressing CD38, CD39, CD49a and CD94.
- These genes are ideal candidates for further investigation to gain deeper insight into T-cell activation and exhaustion pathways.



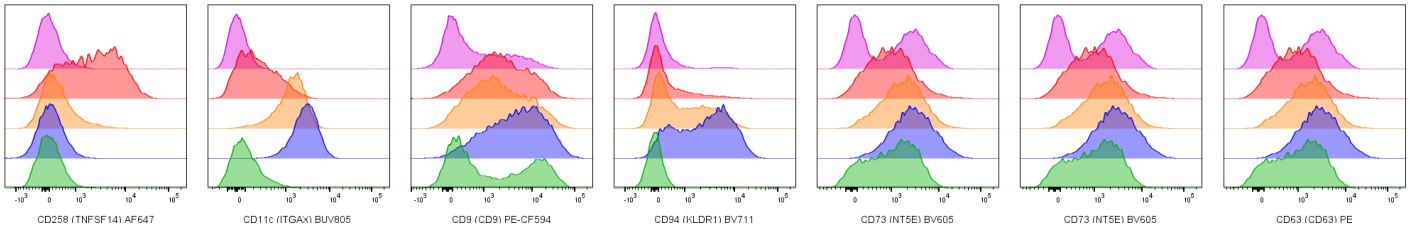
Validation of identified genes and proteins using flow cytometry



Marker	Target Genes	Format
Live/dead		7-AAD
CD4		APC-R700
CD8		APC-H7
CD39	*ENTDP1*	BB515
CD11c	*ITGAX*	BUV805
CD314	*KLRK1*	PE-Cy7
CD52	*CD52*	BV510
CD94	*KLRD1*	BV711
CD73	*NT5E*	BV605

Marker	Target Genes	Format
CD18	*ITGB2*	BUV395
CD163	*SELPLG*	BUV737
CD186	*CXCR6*	BV650
CD258	*TNFSF14*	AF647
CD253	*TNFSF10*	BV421
CD9	*CD9*	PE-CF594
CD63	*CD63*	PE
CD192	*CCR2*	BV786

Key:
Fresh
Day 3
Day 7
Day 14 Chronic
Day 14 Transient



Summary

- A single cell multiomic approach was used to resolve cell heterogeneity and identify potential novel cell populations in two biologically relevant models.
- Using AbSeq reagents allows an extensive analysis of a high number of proteins (39 in this study), in a simplified workflow.
- AbSeq technology enables you to resolve different classes of antigens (primary, secondary, tertiary) that are expressed at various densities (from low to high) on subsets of cells present at different frequencies (from rare to abundant).
- AbSeq reagents can sensitively detect changes in expression over time upon different stimulation conditions.
- Simultaneous analysis of protein and mRNA expression allows:
  - Assessment of correlation of mRNA and protein expression to determine potential mechanisms of regulation
  - Refined and deeper cell characterization
- Unsupervised data analysis approach using Monocle and FlowSOM enables further dissection of subsets of interest identified using biased, manual gating strategies.

Thank you!

BD LSRFortessa X-20 cell analyzer is a Class 1 Laser Product.
For Research Use Only. Not for use in diagnostic or therapeutic procedures.