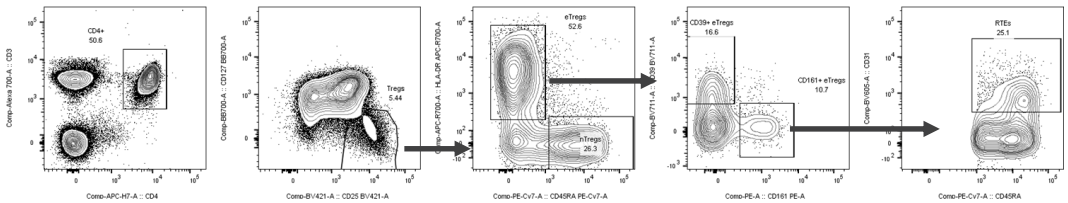
One solution for comprehensive Treg characterization via single cell multiomic analysis

Overcoming challenges of single cell sequencing on rare cell populations

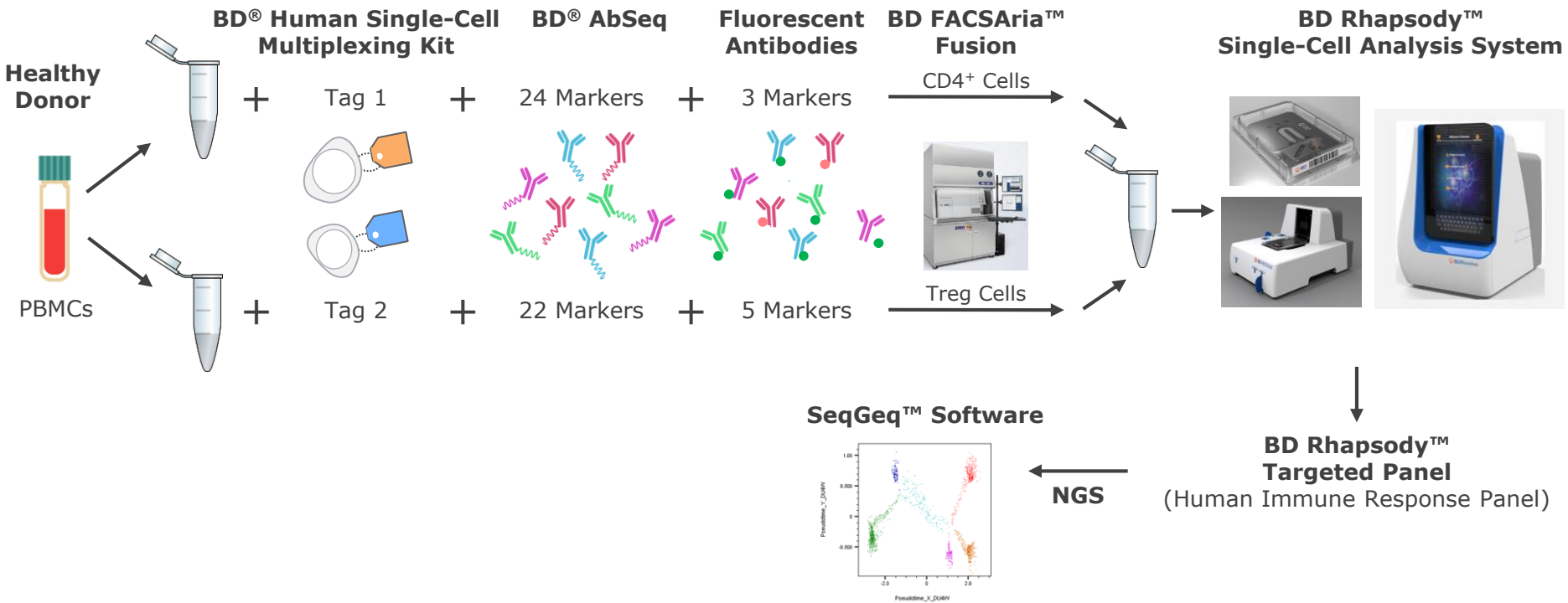


Approximate values. High donor-to-donor variability is observed

Pop.	% total cells	% CD4+ cells	% Tregs
CD4+	40%	___	___
Tregs	2%	7%	
eTregs	1%	3%	52%
nTregs	0.5%	1.5%	26.3%
CD39+ eTregs	0.2%	0.5%	8.5%
CD161+ eTregs	0.1%	0.25%	4.4%
RTEs	0.1%	0.4%	6.8%

- Regulatory T cells (Tregs) represent only ~2% of the total PBMCs and 7-10% $CD4^+$ T cells.
- Using PBMCs for characterization of Tregs requires:
  - Analysis of a large number of PBMCs.
  - Loading a large number of cells in multiple cartridges.
  - Analysis of a large number of non-relevant cells that result in high sequencing costs.
- Benefits of cell sorting:
  - Targeted and exclusive analysis of Tregs devoid of contaminants.
  - Lower number of cells to be processed for downstream analysis.
  - Ability to sequence at high depth, at a reduced cost due to the lower number of analyzed cells.

Experimental design and workflow



Panel design

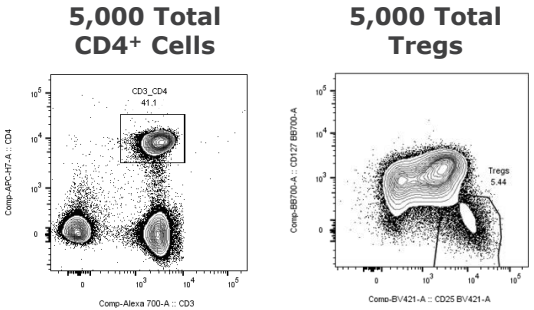
24-plex AbSeq Panel		
HLA-DR	CD278 (ICOS)	CD62L
CD45RA	CD49D	CD95
CD31	CD183 (CXCR3)	CD27
CD39	CD185 (CXCR5)	CD28
CD161	CD194 (CCR4)	CD7
CD279 (PD-1)	CD196 (CCR6)	CD103
CD152 (CTLA-4)	CD294	CD127*
CD357 (GITR)	CD38	CD25*

* Not included for sorted Treg sample because they were included in the FACS panel.

FACS Panel: Treg	
Marker	**Fluorochrome**
CD3	BUV395
CD4	APC-H7
CD8	Alexa-700
CD25	BB515
CD127	BUV786

FACS Panel: CD4^{+}	
Marker	**Fluorochrome**
CD3	BUV 395
CD4	APC-H7
CD8	Alexa-700

Cell sorting for CD4+ and Treg cells

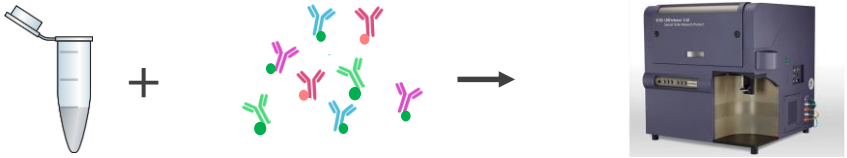


PBMCs from a healthy donor were sorted to obtain 5,000 CD4+ and 5,000 Tregs. The two samples were pooled and loaded on the same cartridge for downstream library preparation using the BD Rhapsody™ Targeted Immune Response Panel.

Flow cytometry analysis

Fluorescent Antibodies

BD LSRFortessa™ X-20 Flow Cytometer



Flow Cytometry Panel Backbone	
Marker	**Fluorochrome**
CD3	BUV395
CD4	BUV805
CD8	AF700
CD127	BV786
CD25	BB515
HLA-DR	BV480
CD45RA	APC-H7

Flow Cytometry Panel 1	
Marker	**Fluorochrome**
CD31	BV605
CD39	APC
CD161	BV421
CD7	PE

Flow Cytometry Panel 2	
Marker	**Fluorochrome**
PD-1	BB515
CTLA-4	PE
GITR	BV421
ICOS	BUV737
CD49d	PerCP-Cy™5.5

Flow Cytometry Panel 3	
Marker	**Fluorochrome**
CD183	PE-Cy™7
CD185	BV711
CD194	BV421
CD196	BUV737
CD294	PE

Flow Cytometry Panel 4	
Marker	**Fluorochrome**
CD38	BUV737
CD62L	APC
CD95	PE
CD27	BV421
CD28	BV605

Cells from the same donor were stained with four multicolor panels and acquired on the BD LSRFortessa™ X-20 flow cytometer for side-by-side comparison between flow cytometry and AbSeq data.

Sequencing metrics

AbSeq Mean Reads/Cell	RNA Mean Reads/Cell	AbSeq Mean Raw Seq Depth	RNA Mean Raw Seq Depth	AbSeq Saturation (%)	RNA Seq Saturation (%)
9,117	2,886	4.2	7.2	92	95

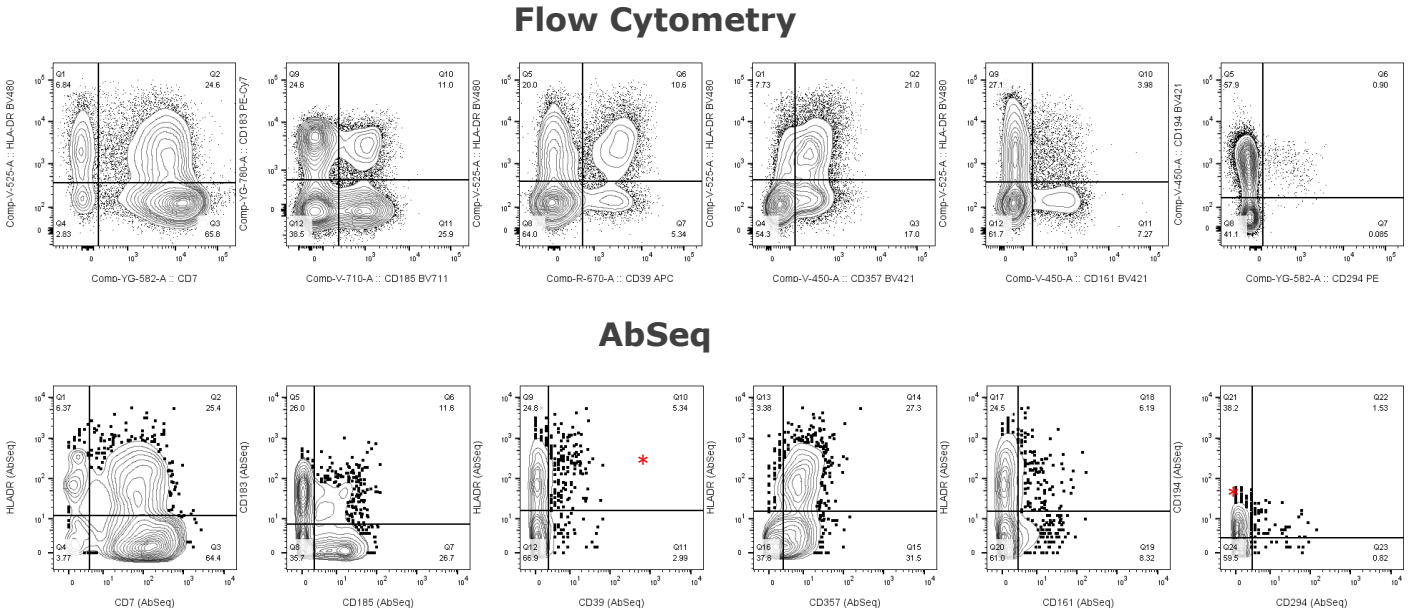
A total of 230 million mRNA and AbSeq reads were identified, reaching saturation.

Concordance between BD® AbSeq and flow cytometry results

Flow cytometry analysis of the samples

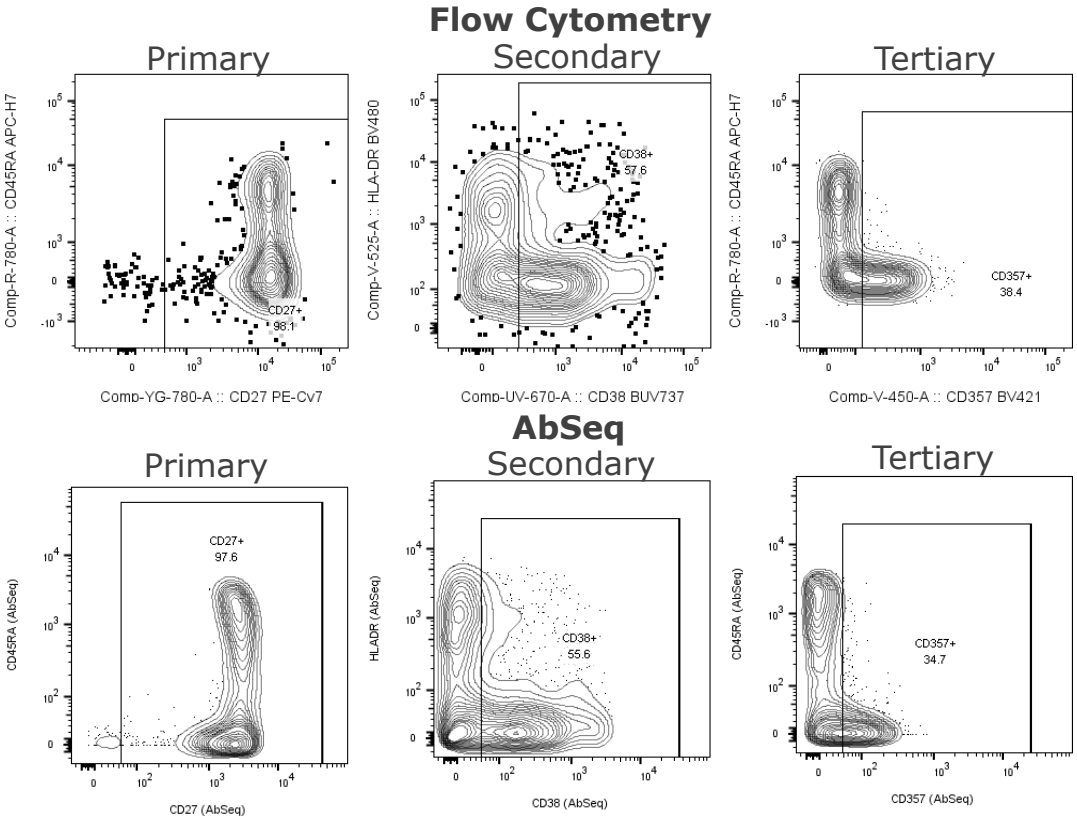
- Sorted Tregs were also analyzed by flow cytometry on the same day and the results were compared to the AbSeq data.
- High-dimensional analysis was performed.
- Performed qualitative and quantitative assessment of antigen expression on total Tregs.
- Analyzed antigen expression and distribution in three main Treg subsets (naïve, memory and effector/activated).

Qualitative analysis

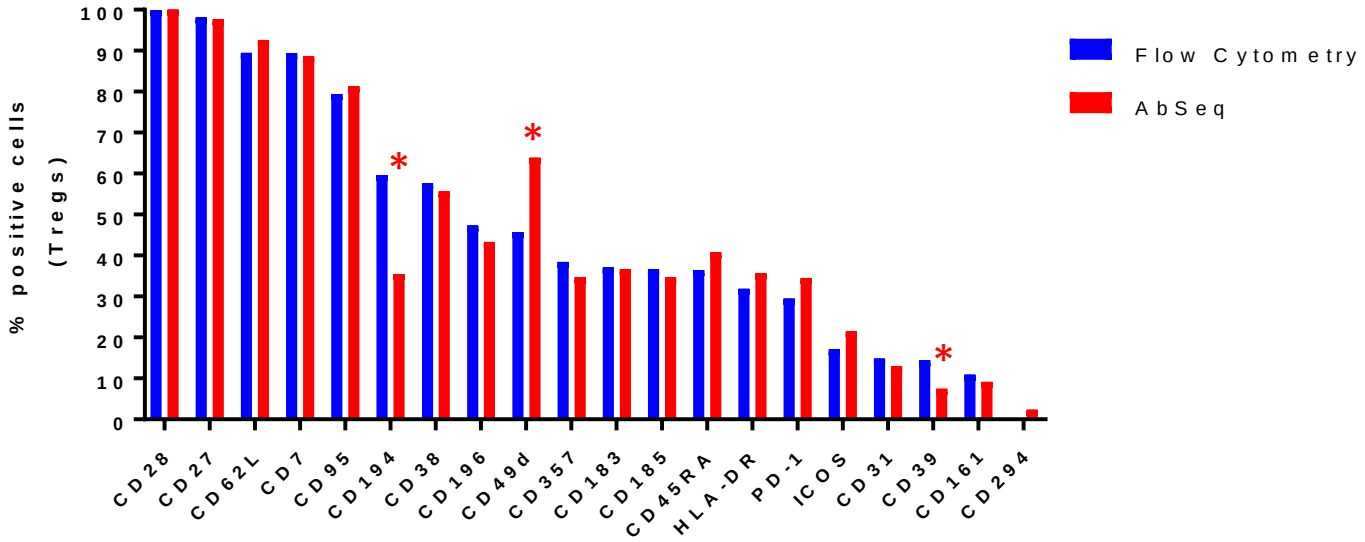


- Overall, good quantitation correlation between flow cytometry and AbSeq was seen, especially for clearly resolved markers.
- Gating is arbitrary and might not be accurate for AbSeq data because of the lack of negative controls/FMOs.

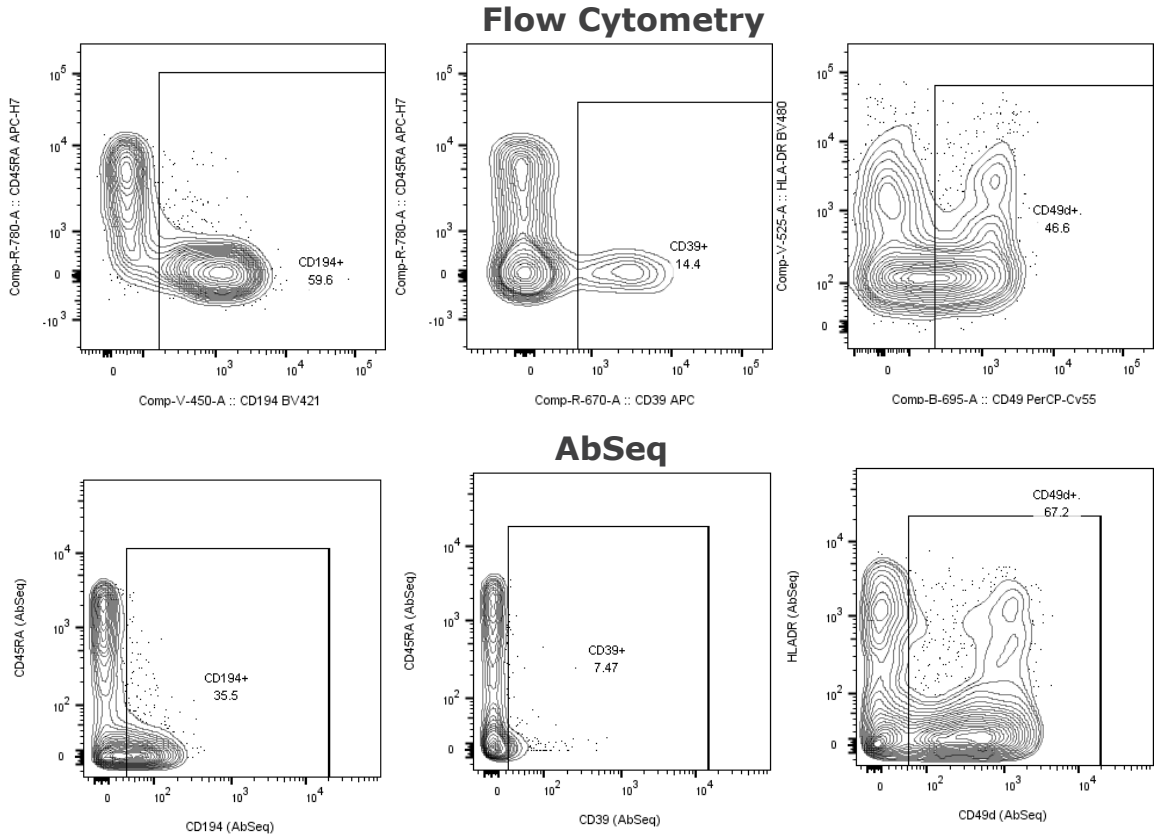
Resolution of different antigen classes



Quantitative analysis



Outliers



Defining Treg subsets

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doi:10.1182/blood-2013-02-482539

Multiparameter single-cell profiling of human CD4$^+$FOXP3$^+$ regulatory T-cell populations in homeostatic conditions and during graft-versus-host disease

Shen Dong, Sylvie Maiella, Aliénor Xhaard, Yuanyu Pang, Lynn Wenandy, Jérome Larghero, Christophe Becavin, Arndt Benecke, Elisabetta Bianchi, Gérard Socié and Lars Rogge

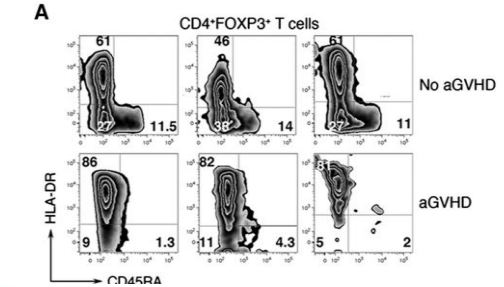
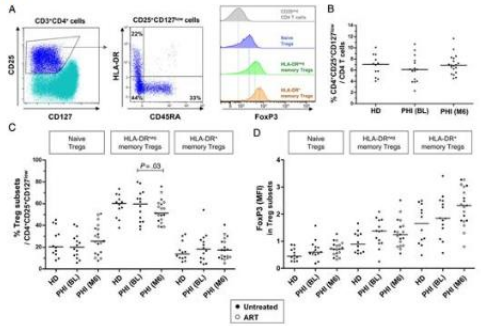


Figure 1.



Phenotype Alterations in Regulatory T-Cell Subsets in Primary HIV Infection and Identification of Tr1-like Cells as the Main Interleukin 10–Producing CD4$^+$ T Cells FREE

Mathieu F. Chevalier, Céline Didier, Gaël Petitjean, Marina Karmochkine, Pierre-Marie Girard, Françoise Barré-Sinoussi, Daniel Scott-Algara, Laurence Weiss ✉
Author Notes

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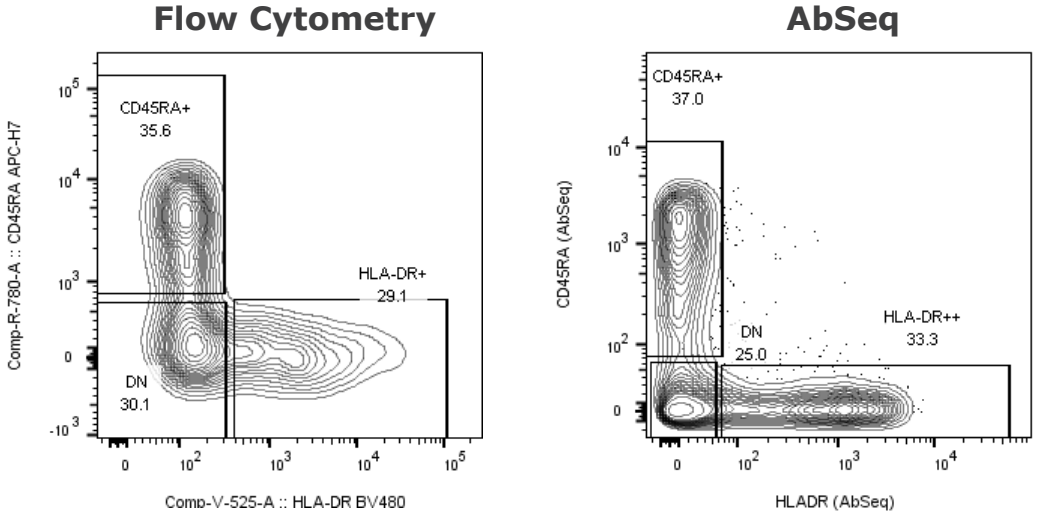
https://doi.org/10.1093/infdis/jiu549
Published: 03 October 2014 **Article history** ▾

- HLA-DR and CD45RA expression defines three main Treg subsets (naïve, memory, memory/activated).
- Distribution of these subsets can be altered in disease.
- These markers are expressed over a continuum (secondary antigens). Optimal resolution is required to fully resolve the heterogeneity of different subsets of cells expressing different levels of these markers.

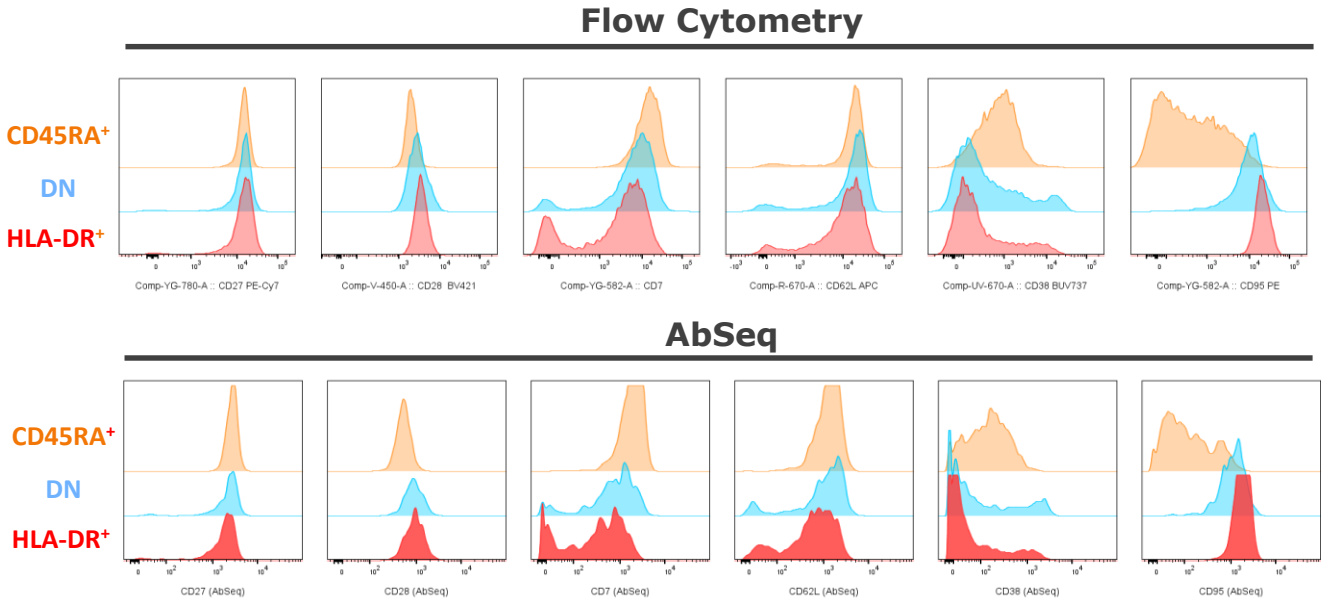
Resolving Treg subsets



Expression of the remaining 20 markers was analyzed within each main Treg subset: RA^+DR^- (naïve), RA^-DR^- (memory) and RA^-DR^+ (memory/activated).

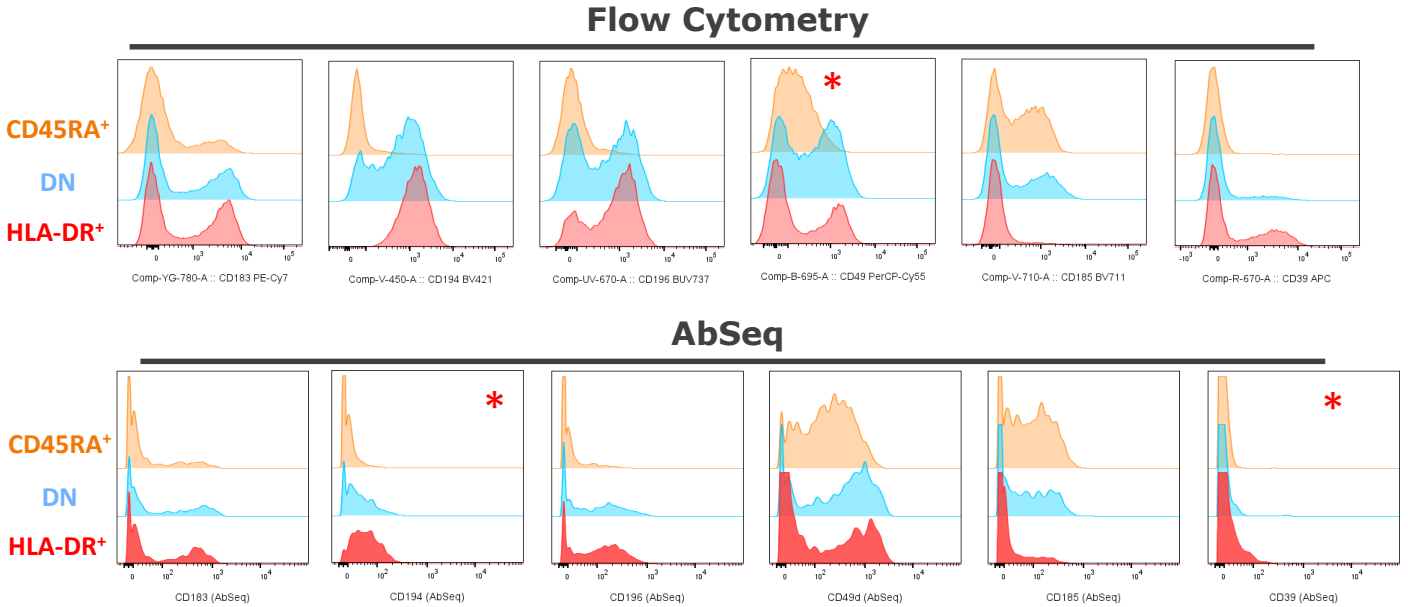
Note: Different scales and transformations were required for visualization of flow cytometry and AbSeq data. A true side-by-side comparison using the same scale and transformation cannot be performed.

Resolution of primary and secondary antigens



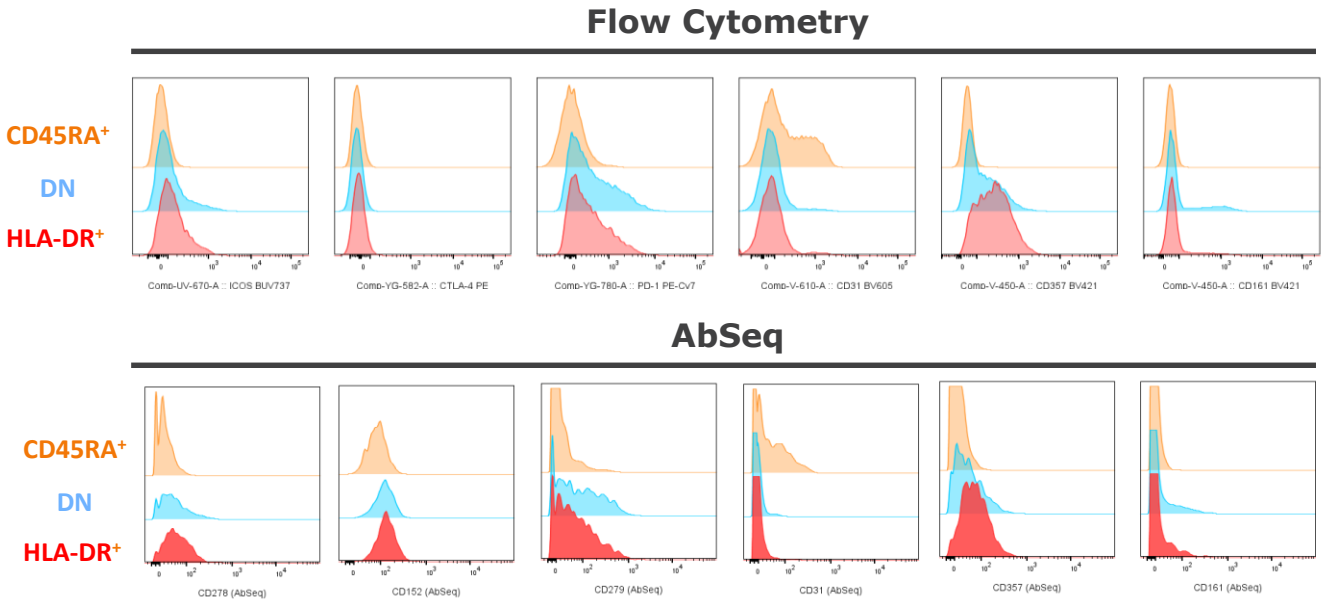
Note: Different scales and transformations need to be used to visualize flow cytometry and AbSeq data. Histograms are used here for a side-by-side comparison, but ultimately histograms are not recommended for AbSeq data visualization.

Resolution of secondary and tertiary antigens



- For CD194 and CD39, AbSeq resolution was poorer compared to that of flow cytometry.
- Check flow-proxy titration and/or perform new titration as needed.

Resolution of tertiary antigens

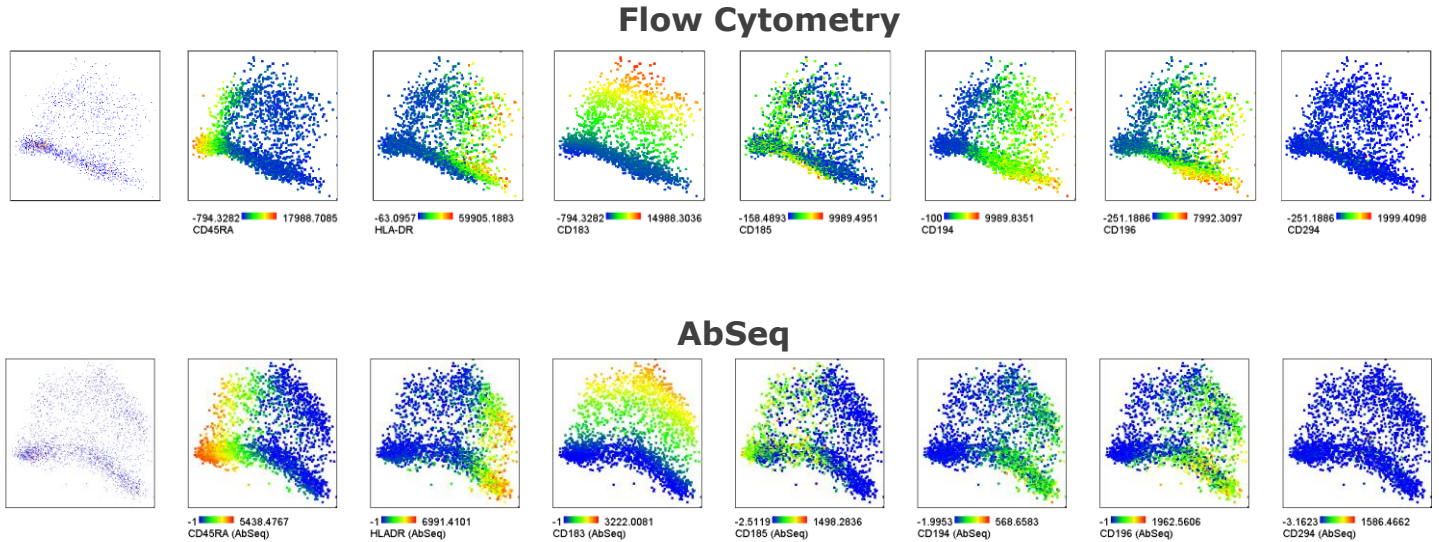


High-dimensional data analysis approach

- High-dimensional analysis was used to assess concordance between a 12-color flow cytometry panel and the corresponding AbSeq panel.
- The panel includes chemokine receptors known to identify discrete subsets of Tregs.
- For the flow cytometry analysis, Tregs were gated first as $CD3^{+}CD4^{+}CD8^{-}$ $CD127^{low/-}$ $CD25^{+}$ cells before downstream high-dimensional analysis.
- For AbSeq analysis, high-dimensional analysis was performed on purified total Tregs sorted as $CD3^{+}CD4^{+}CD8^{-}CD127^{low/-}CD25^{+}$ cells from the same sample.
- Only the overlapping markers, out of the 22 AbSeq Oligo plex, were used to trigger high-dimensional analysis.
- Different visualization and analysis tools were used:
  - Principal component analysis (PCA)
  - t-SNE
  - FlowSOM

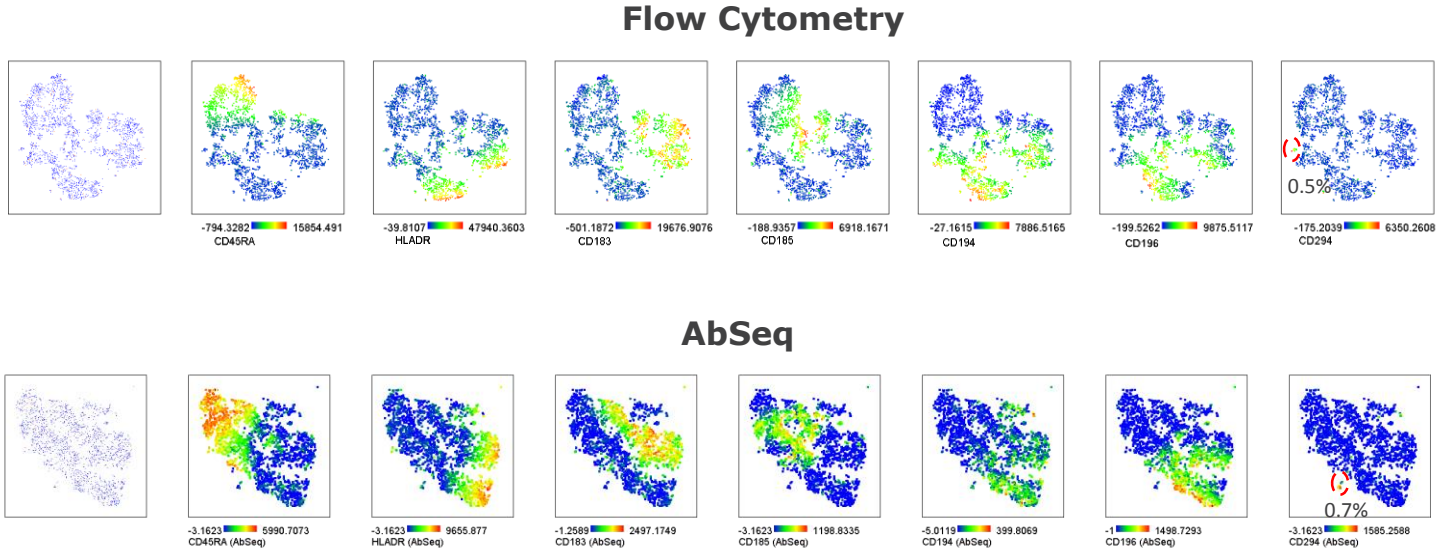
Marker	Fluorochrome
CD3	BUV395
CD4	BUV805
CD8	AF700
CD127	BV786
CD25	BB515
HLA-DR	BV480
CD45RA	APC-H7
CD183	PE-Cy7
CD185	BV711
CD194	BV421
CD196	BUV737
CD294	PE

Principal component analysis



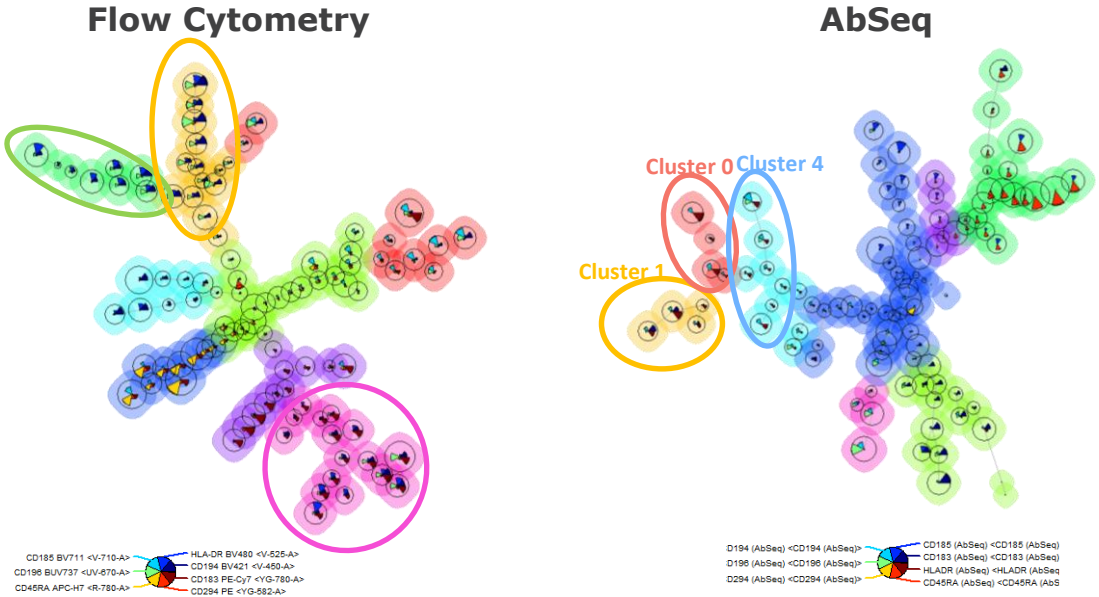
Equivalent relative spatial distribution was observed for distinct Treg subsets based on differential expression of the seven markers tested.

t-SNE analysis



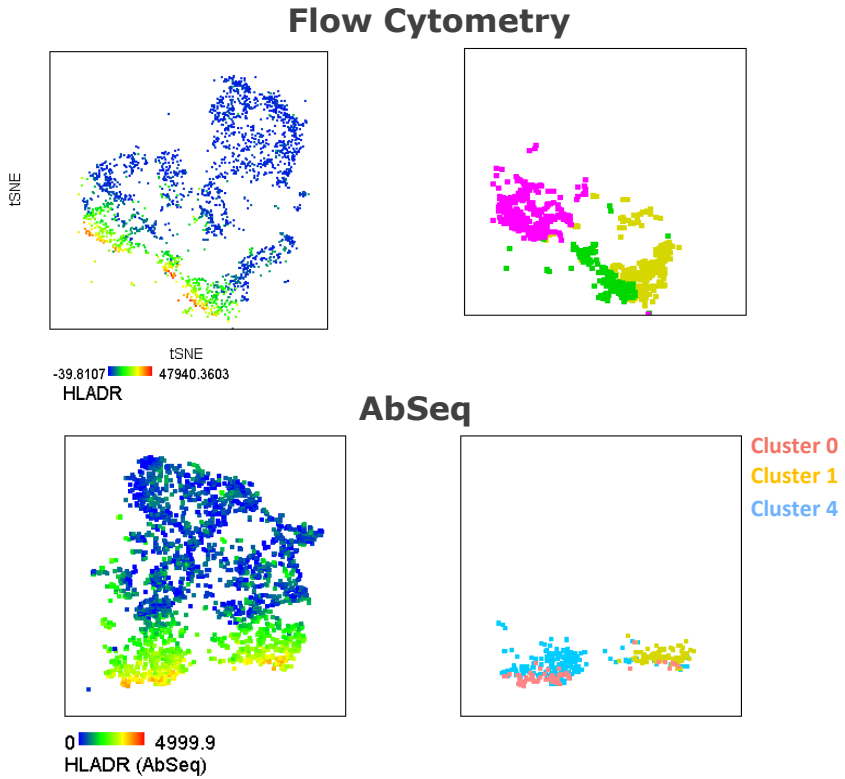
Equivalent relative spatial distribution was observed for distinct Treg subsets based on differential expression of the seven markers tested.

FlowSOM analysis



- Three distinct subsets of HLA-DR^{+} Tregs were identified using either flow cytometry or AbSeq.
- The subsets are defined based on differential co-expression of HLA-DR, CD183, CD194 and CD196.
- CD185 appears to be expressed in naïve Treg and in a transitional CD45RA^{-}HLA-DR^{-} population.

FlowSOM analysis: HLA-DR subsets



Concordance between BD® AbSeq and flow cytometry results

Assessing concordance between flow cytometry and AbSeq:

- Good concordance is observed overall, in terms of resolving different classes of antigens that are distributed over a broad range of expression levels.
- Both approaches produced similar quantitation of Treg subsets, with the exception of few outliers.
- Overall, similar expression patterns within different Treg subsets were observed via either conventional or high-dimensional data analysis.

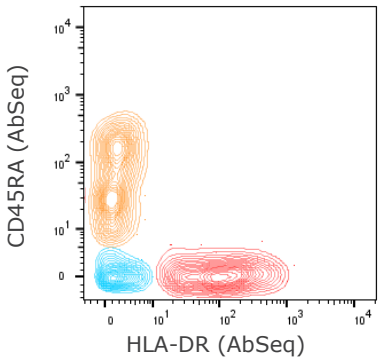
Resolution of Treg heterogeneity via single cell multiomics analysis

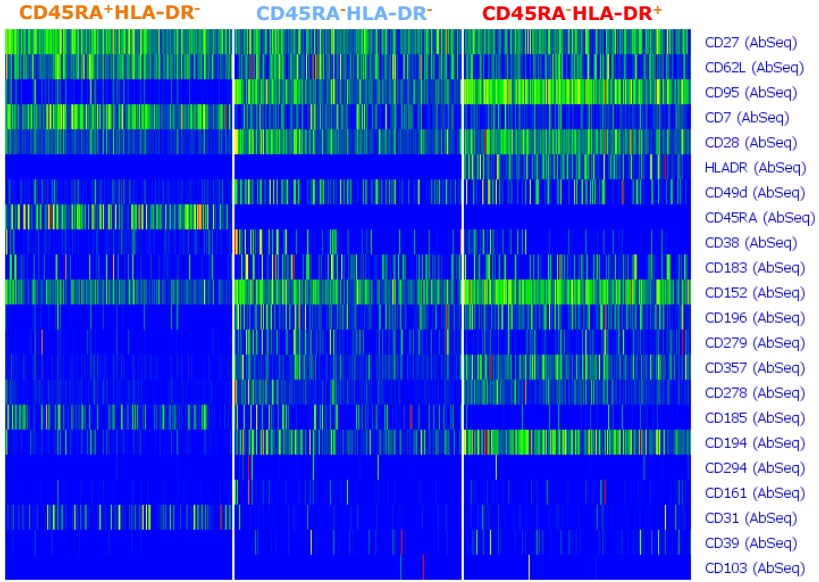
Unsupervised multidimensional analysis provides deeper cell characterization

Using a sorted Treg sample, we:

- Performed differential gene expression (DGE) and differential protein expression (DPE) analysis on three main Treg subsets based on manual gating and bi-variate analysis of CD45RA and HLA-DR.
- Used a high-dimensional analysis tool to identify Treg subsets based on unsupervised, simultaneous analysis of 22 proteins and 399 gene expressions.

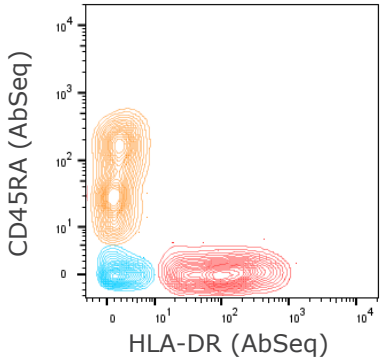
DPE analysis on manually defined Treg subsets

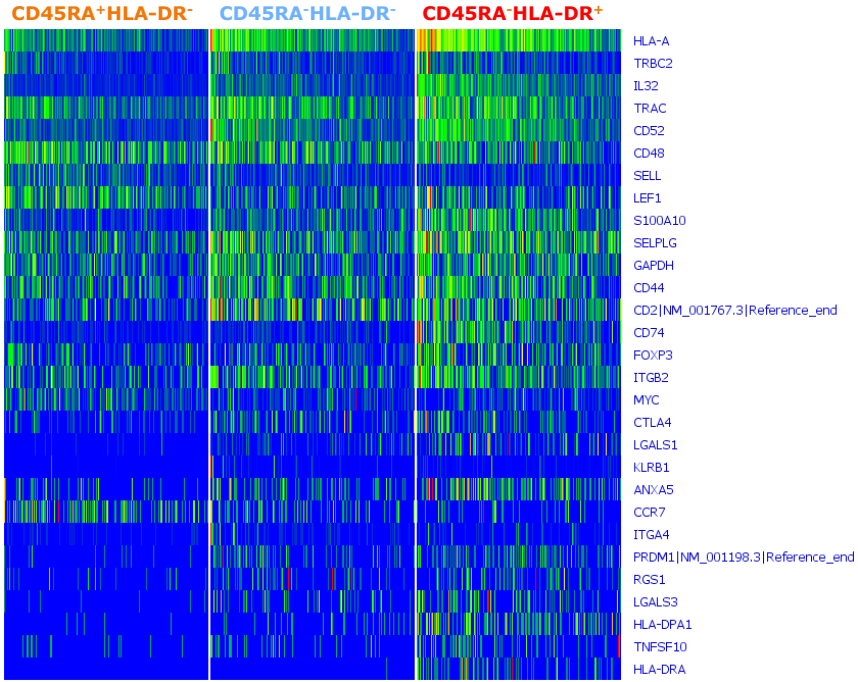




Note: 200 cells per population are represented.

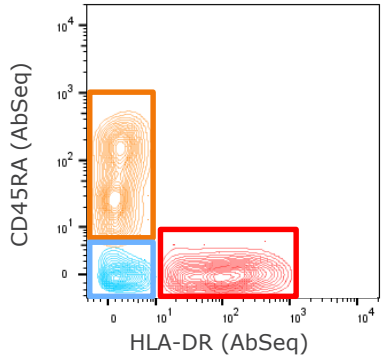
Differential gene expression analysis





- A similar analysis can be performed to visualize DGE.
- DGE analysis was performed by selecting genes upregulated (>1.25X) in each subset.

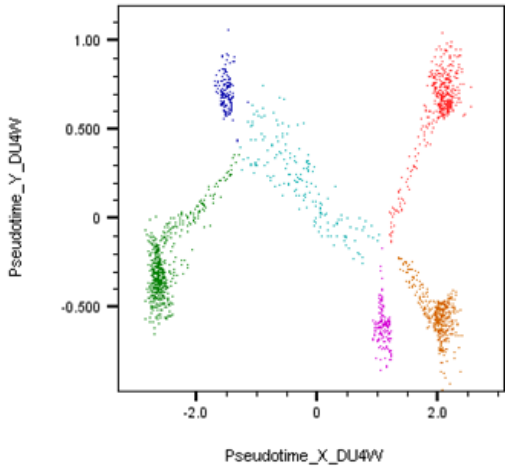
Deeper dive into Treg subsets



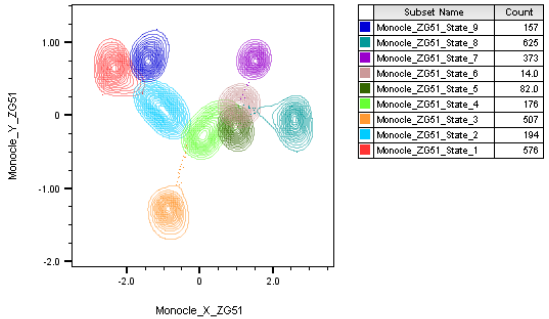
- The DPE and DGE analyses were performed on three main Treg subsets identified by supervised manual gating.
- However, DPE and DGE might be present within the cells included in the broad gates drawn. For example, two distinct populations of CD45RA^{dim} and CD45RA^{bright} can be detected.
- We could further dissect these populations by manually drawing more gates, or taking an unsupervised approach where no manual gating strategy is used and an algorithm can identify different clusters based on the differential gene and expression patterns.
- For this study we used Monocle, as this algorithm is well suited to cluster cells based on changes in expression patterns consistent with cell activation/differentiation.

Monocle plug-in

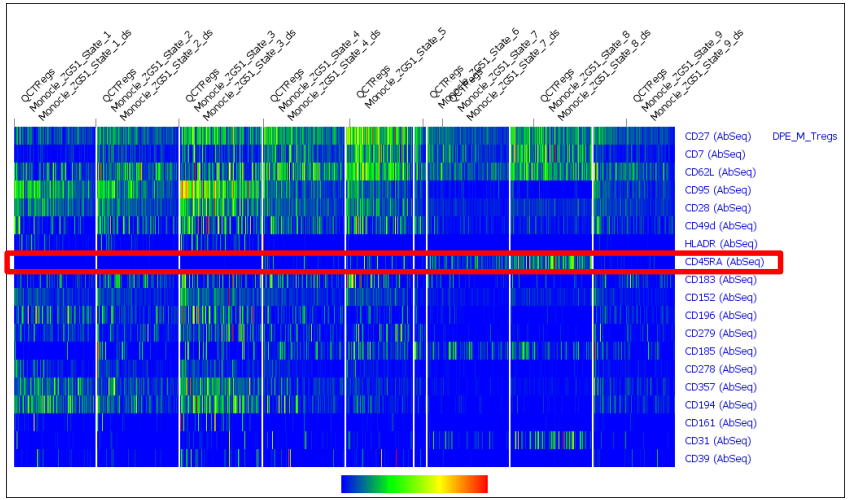
- Unsupervised algorithm able to define transcriptional dynamics and trajectories that might occur over time, for example during cell differentiation, activation, etc.
- Algorithm considers the whole set of data as a time series, or “pseudo-time”, where each cell represents a distinct time point over the continuum.
- Cells are then clustered based on the temporal regulation of genes that might be up or downregulated throughout different states.
- Major applications involve identification of differentiation, activation and tumor progression states.



Unsupervised data analysis: Monocle

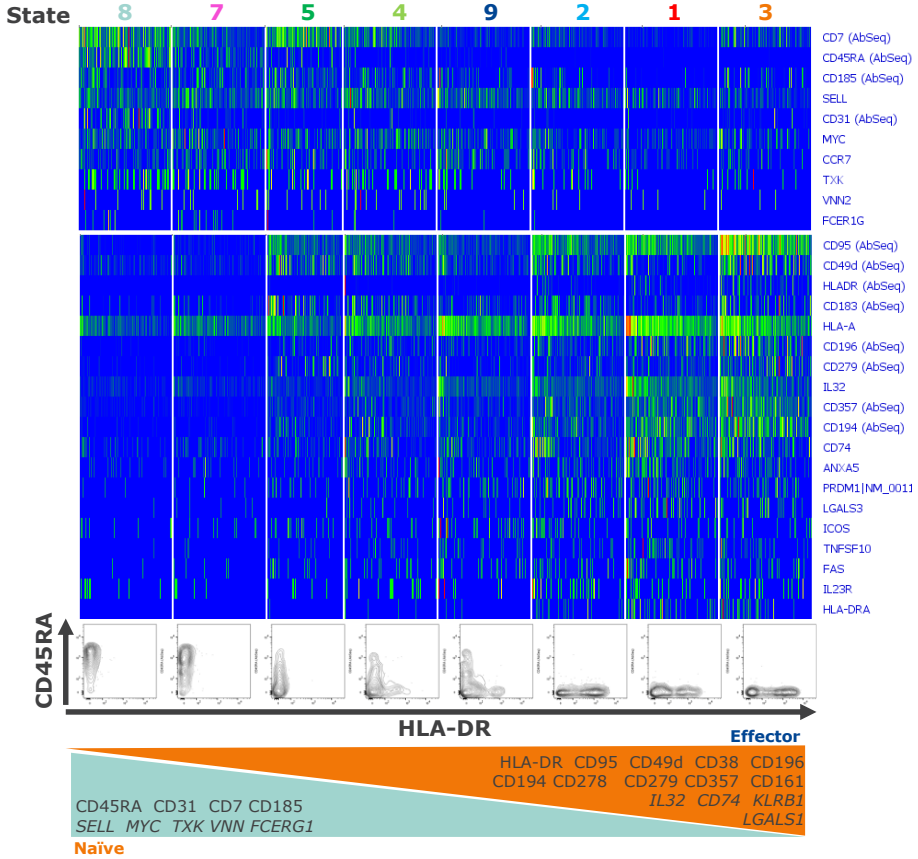


Subset Name	Count
Monocle_ZG51_State_9	157
Monocle_ZG51_State_8	625
Monocle_ZG51_State_7	373
Monocle_ZG51_State_6	14.0
Monocle_ZG51_State_5	82.0
Monocle_ZG51_State_4	176
Monocle_ZG51_State_3	507
Monocle_ZG51_State_2	194
Monocle_ZG51_State_1	576



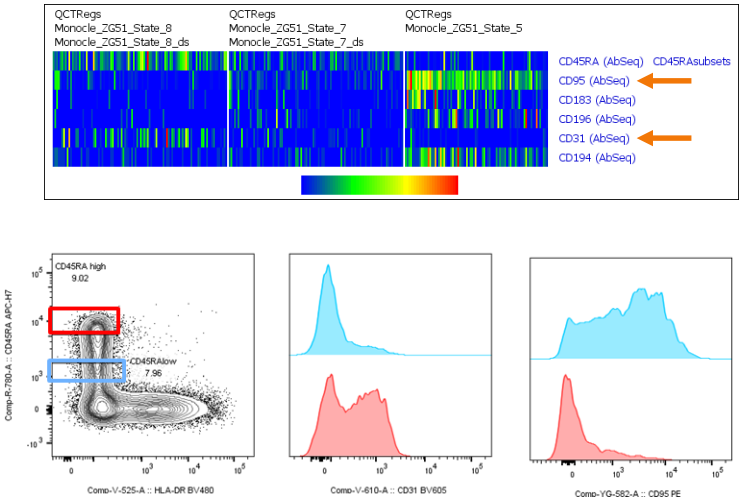
- A SeqGeq plug-in, Monocle, makes it possible to perform a highly refined characterization of Treg subsets compared to manual gating, evidenced by the identification of the three distinct subsets of CD45RA^{+} Tregs.
- Use of single cell heat maps facilitates the identification of unique expression patterns, as shown by the correlation between CD31 and high CD45RA expression in cluster 8, defining TREs.

Differentiation model



Combination of DGE and DPE allows for unprecedented resolution and identification of Treg subsets, defining different states of differentiation.

Flow cytometry validation



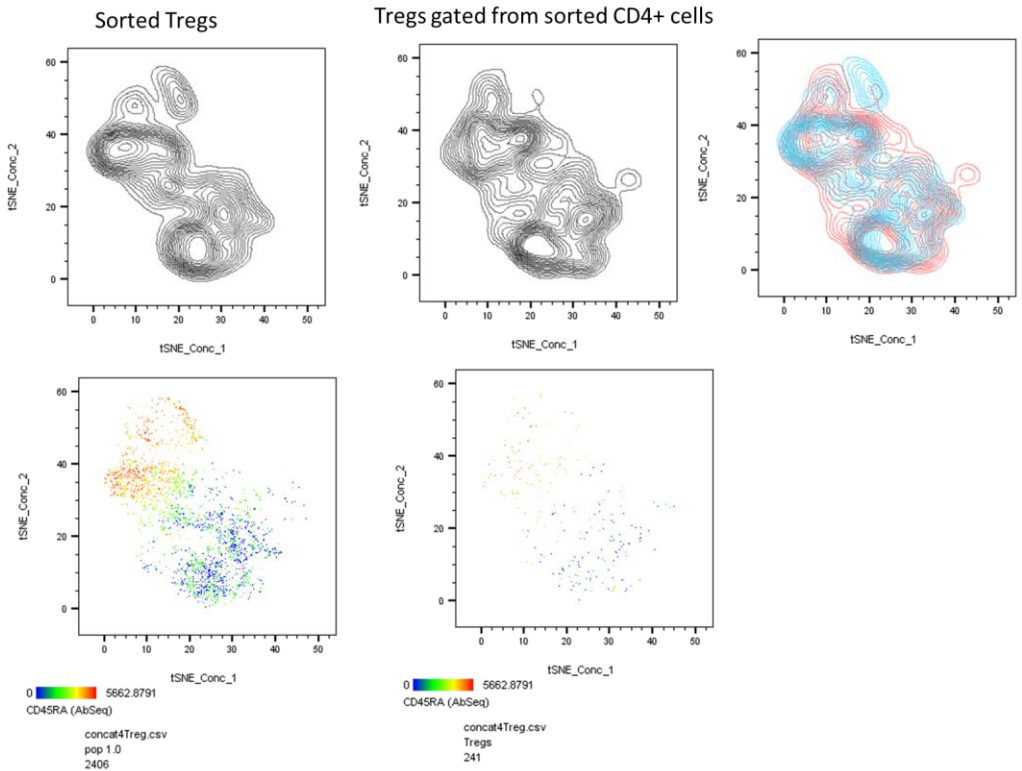
- The data shows the sensitivity of AbSeq and ability to cluster distinct subsets based on varying levels of CD45RA expression.
- Flow cytometry was used to validate the distinct phenotype of CD45RA^{high} and CD45RA^{dim} cells based on differential expression of CD31 and CD95.

Improved workflow and cost efficiency by cell sorting

Assessing the benefits of cell sorting

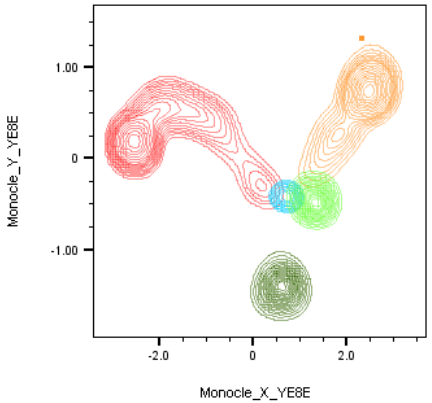
- We performed a side-by-side comparison of sorted total $CD4^+$ T cells and Tregs.
- 5,000 cells from each sorted sample were pooled and loaded in the same cartridge.
- After cell capture, 250 Tregs could be gated and analyzed from total $CD4^+$ cells, whereas 2,570 Tregs could be analyzed from the sorted Treg sample.
- Benefits of Treg purifications were investigated based on:
  - Comparison of unsupervised data analysis between total $CD4^+$ cells and Treg sorted samples.
  - Comparison of clusters, differentially expressed genes and proteins identified using unsupervised data analysis.

Dimensionality reduction



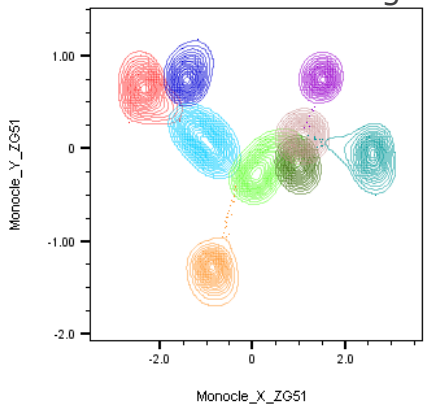
Monocle analysis

250 Tregs Gated
From Sorted CD4+



Subset Name	Count
Monocle_YE8E_State_5	43.0
Monocle_YE8E_State_4	23.0
Monocle_YE8E_State_3	74.0
Monocle_YE8E_State_2	2.00
Monocle_YE8E_State_1	121

2500 Purified Tregs



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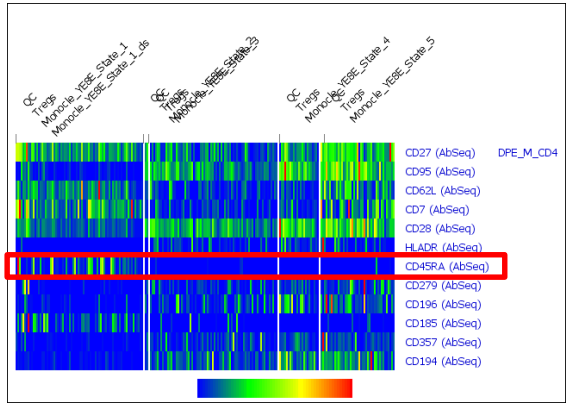
Monocle_ZG51_State_4	176
Monocle_ZG51_State_3	507
Monocle_ZG51_State_2	194
Monocle_ZG51_State_1	576

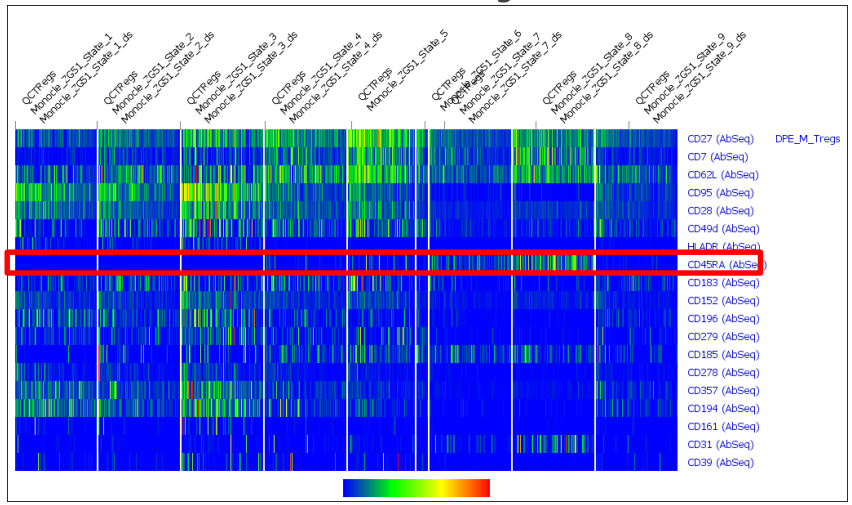
- A higher number of states were defined when a higher number of Tregs were analyzed.
- This is likely due to limit of detection of very rare cells defining intermediate states.

Immunophenotype of Treg subsets

Tregs Gated From Sorted CD4+

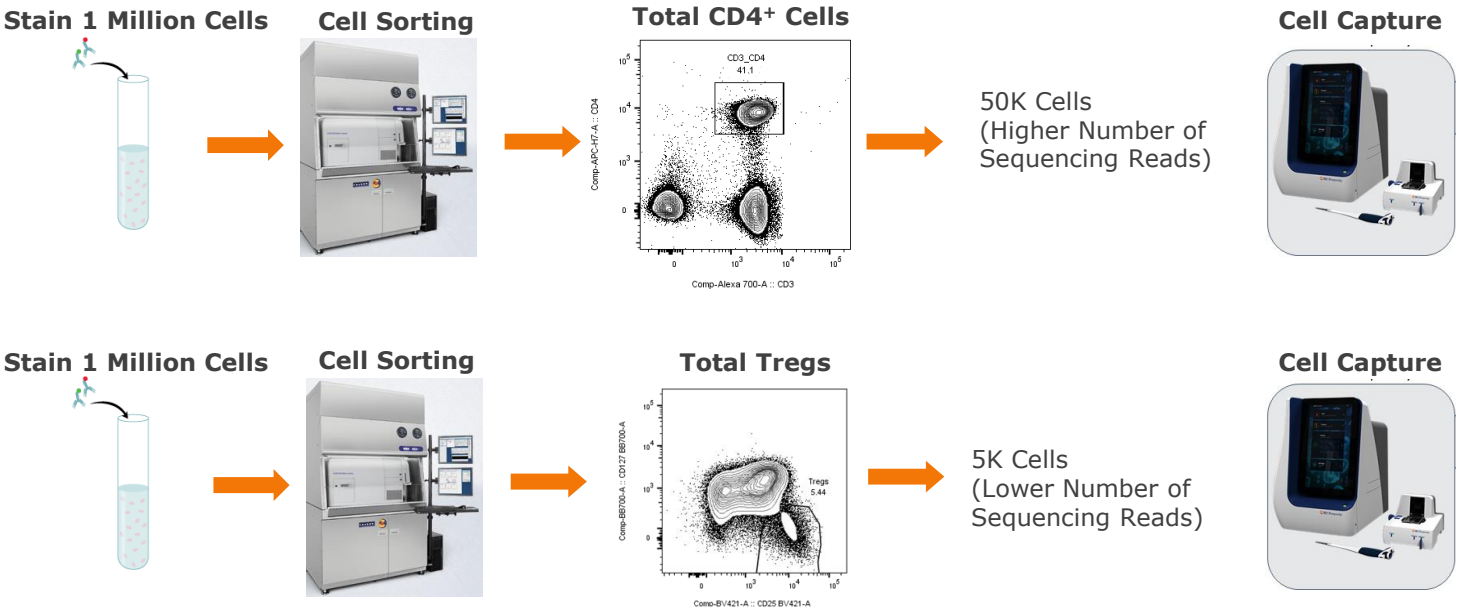


Purified Tregs



In addition to the ability to identify a higher number of states, analyzing purified Tregs revealed higher number of differentially expressed genes (61 vs. 29) and proteins (19 vs. 12) compared to analyzing Tregs gated from sorted total CD4+ cells. Furthermore, we also achieved more refined resolution of the three subsets of CD45RA+ naïve Treg cells with purified Tregs.

Workflow and cost efficiency



Note: Loading of 1/3 of a cartridge may not be cost-effective, because it would cost as much as fully loading a cartridge. You can fully load a cartridge, sub-sample 1/3 of the beads or cells, and keep the rest for a follow-up study (more reads or a different library prep). Alternatively, you could fill a cartridge with three donors.

Summary

Validation of the combined use of BD® AbSeq and BD Rhapsody™ mRNA sequencing technologies using a well-defined model system.

Workflow efficiency

- Sorting rare cells prior to the single cell multiomics analysis reduces sequencing costs and enhances the ability to perform in-depth characterization.

Validation of BD AbSeq

- High degree of concordance between the BD AbSeq assays and flow cytometry data.
- Equivalent resolution was seen for the BD Rhapsody regardless of antigen density or classification.

Power of single cell multiomic analysis

- Simultaneous analysis of 24 proteins and 399 genes at the single cell level enables unprecedented resolution of heterogeneous cells, as compared to conventional single cell multiomic approaches.
- High-dimensional data analysis tools available on SeqGeq™ allows for identification of cell subsets based on unique protein and gene expression profiles.

Thank you!

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