

# Comprehensive Immunophenotyping of Peripheral Human Whole Blood

A 24-color panel design and optimization using the  
Agilent NovoCyte Penteon flow cytometer

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## Abstract

Multiparameter flow cytometry is an incredibly powerful tool to monitor and characterize immune cell subsets. High-dimensional analysis of immune cells provides in-depth information on the state of the immune system at homeostasis or in response to stimulation. In this application note, a 24-color immunophenotyping panel for the five-laser Agilent NovoCyte Penteon flow cytometer was designed for human peripheral blood to characterize the major immune cell subsets, providing a comprehensive overview of immune cell status.

## Introduction

With constant advancements, it is possible to design increasingly complex, multicolor flow cytometry experiments that evaluate more immune cells simultaneously. With the enlistment of the five-laser Agilent NovoCyte Penteon flow cytometer equipped with ultraviolet (349 nm), violet (405 nm), blue (488 nm), yellow-green (561 nm), and red (640 nm) lasers, researchers can easily generate highly complex, multicolor flow cytometry panels addressing their biological questions. Immune status had been shown to be a critical component of the immune response to various stimuli.

In this application note, a 24-color immunophenotyping panel for human blood samples was designed to identify the major immune cell populations. To design this panel, fluorophores were selected by analyzing the expression of each marker, fluorescence intensity, spectral overlap, and Agilent NovoCyte Penteon configuration. Resulting data was analyzed using both NovoExpress software and t-stochastic neighbor embedding (t-SNE) in FlowJo for high dimensional analysis. t-SNE analysis visualizes and characterizes cell populations by arranging high-dimensional data points into a two-dimensional space so that events which are highly related by many variables are located closer to one another. This permits a large number of markers to be assessed

simultaneously. The resulting data support a comprehensive assessment of all immune cells, including T cells, B cells, monocytes, dendritic cells (DC), natural killer (NK) cells, neutrophils, eosinophils, and basophils. In addition, this immunophenotyping panel can provide in-depth analysis of T cell and B cell differentiation, activation, and polarization. Thus, this panel characterizes not only the cells present in the sample, but also illuminates their cellular state.

## Experimental

### Materials

- Agilent NovoLyse lysing solution (894B605)
- Phosphate buffered saline, PBS (Genom Biomedical Technology, GNM-14190)
- 8% paraformaldehyde in water, PFA (Electron Microscopy Sciences, 157-8)
- Flow cytometry antibodies, listed in Table 1
- FlowJo software (Becton, Dickinson and Company)
- FlowSOM (CytoBank)
- Agilent NovoExpress software
- Agilent NovoCyte Penteon flow cytometer

### Flow cytometry staining protocol

1. Dilute antibody cocktail to specifications described in Table 1.
2. Spin antibody cocktail at 12,000 g for 5 minutes to remove any aggregates.
3. Add antibody cocktail to 100  $\mu$ L of a human blood sample.
4. Incubate sample at room temperature (RT) for 15 minutes in the dark.
5. Add 500  $\mu$ L of Agilent NovoLyse lysing solution for 10 minutes at RT to remove red blood cells.
6. Rinse cells with 500  $\mu$ L of PBS for 5 minutes.
7. Pellet cells by centrifugation and resuspend in 1 mL PBS.
8. Repeat wash in steps 6 and 7.
9. Pellet cells by centrifugation and resuspend in 150  $\mu$ L of 1% PFA.
10. Acquire samples on the Agilent NovoCyte Penteon flow cytometer.

## Results and discussion

Antibody titration is a key step for the optimization of a multicolor flow cytometry panel. This step determines the optimal amount of antibody needed to achieve the highest signal resolution, population identification, and expression level measurements. To titrate an antibody, a single stain was performed at multiple concentrations to determine which concentration resulted in the highest stain index while minimizing any potential nonspecific binding. Antibody titrations were performed on all 24 antibodies and the concentrations in the final stain were determined by the stain index (data not shown). Antibody clone, detection channel, vendor information, and the amount of each antibody added are described in Table 1.

**Table 1.** Antibody panel used for the 24-color immunophenotyping panel on the Agilent NovoCyte Penteon flow cytometer. The laser and filter used for detection for each antibody can be found in the table, as well as concentration and product details for each antibody.

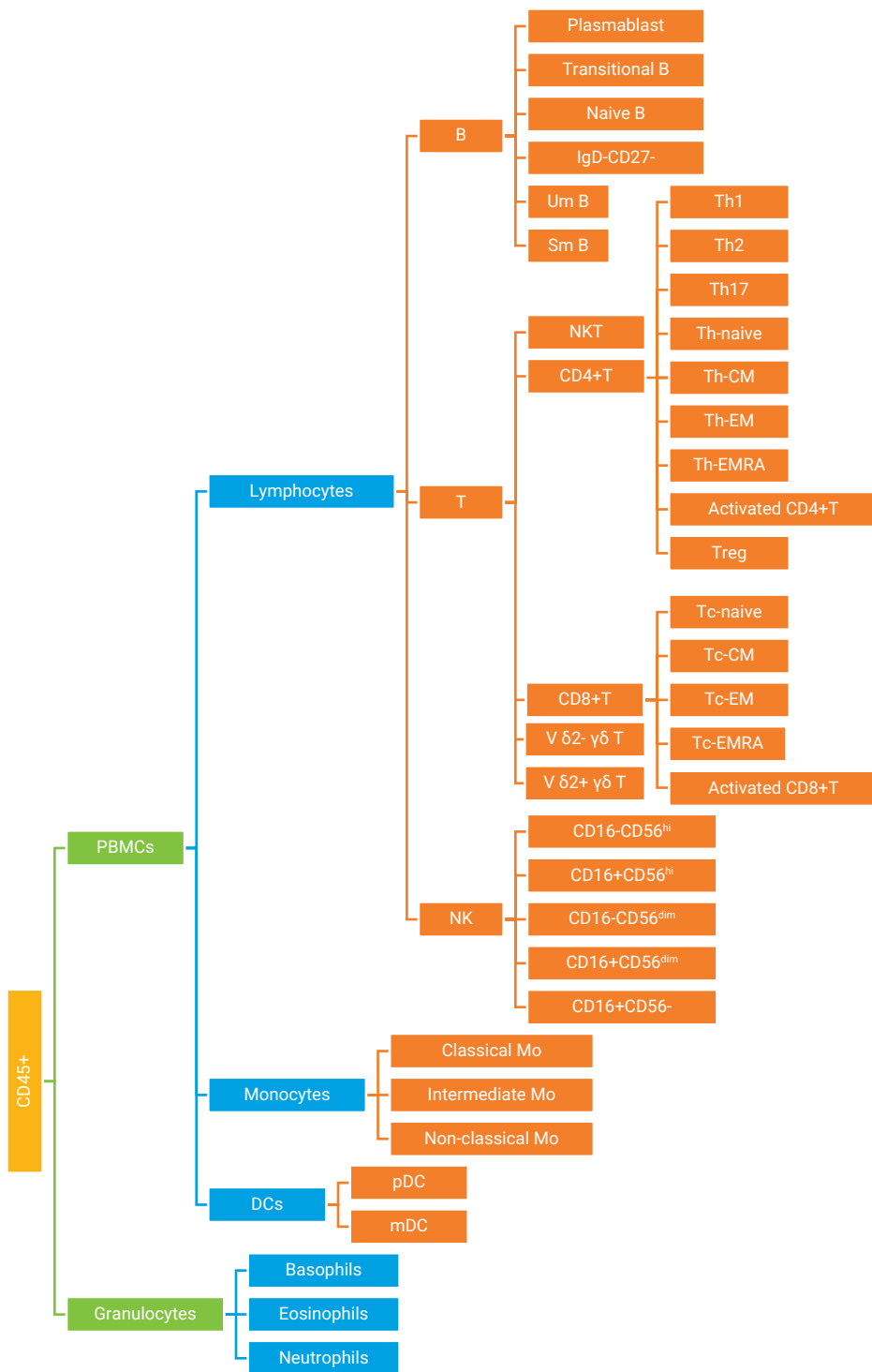
Laser	Filter	Specificity	Fluorochrome	Clone	Vendor	Part Number	Amount Added to 100 $\mu$ L of Blood ( $\mu$ L)
349	445/45	V $\delta$ 2 TCR	BUV395	B6	Becton, Dickinson and Company	743754	1
	525/45	CD27	BUV496	M-T271	Becton, Dickinson and Company	741145	0.50
	586/20	CD33	BUV563	WM53	Becton, Dickinson and Company	741369	0.25
	615/20	CD56	BUV615	NCAM16.2	Becton, Dickinson and Company	613001	2.50
	667/30	CD11c	BUV661	B-ly6	Becton, Dickinson and Company	612967	0.63
	725/40	IgD	BUV737	IA6-2	Becton, Dickinson and Company	612798	1.25
	780/60	CD8	BUV805	SK1	Becton, Dickinson and Company	612889	1.25
405	445/45	CD25	BV421	M-A251	BioLegend	356114	1.25
	525/45	CD45	BV510	HI30	BioLegend	304036	0.63
	586/20	CD3	BV570	UCHT1	BioLegend	300436	2.50
	615/20	CD16	BV605	3G8	BioLegend	302040	5
	667/30	HLA-DR	BV650	L243	BioLegend	307650	2.50
	725/40	CD14	BV711	M $\phi$ P9	Becton, Dickinson and Company	563373	1.25
	780/60	CCR7	BV785	G043H7	BioLegend	353229	2.50
488	525/45	CD123	FITC	6H6	BioLegend	306014	0.31
	725/40	CD19	PerCP-eF710	J3-129	Thermo Fisher Scientific	46-0197-42	2.50
561	586/20	CD24	PE	ML5	BioLegend	311106	1.25
	615/20	CXCR3	PE-Dazzle594	G025H7	BioLegend	353736	1.25
	667/30	CD38	PE-Cy5	HIT2	Becton, Dickinson and Company	555461	1.25
	725/40	CD4	PE-AF700	S3.6	Thermo Fisher Scientific	MHCD0424	0.63
	780/60	$\gamma$ $\delta$ TCR	PE-Cy7	11F2	Becton, Dickinson and Company	655434	1.25
640	667/30	CD127	APC	A019D5	BioLegend	351315	1.25
	725/40	CCR6	APC-R700	11A9	Becton, Dickinson and Company	565173	1.25
	780/60	CD45RA	APC-Cy7	HI100	BioLegend	304128	1.25

In the 24-color flow cytometry panel used in this application note, many immune cells are delineated by the expression of surface receptors, in addition to cell activation and functional status of T and B cell populations (Figure 2). To generate this immunophenotyping panel, antibodies were added to 100  $\mu$ L of blood from a healthy donor. After staining, the sample data was acquired and analyzed using the NovoCyte Penteon flow cytometer and NovoExpress software (Figure 3). First, the gating hierarchy used to identify immune cells populations and subsets will be described. After initial gating using forward scatter (FSC) and side scatter (SSC) to remove debris and doublets, granulocytes and peripheral blood mononuclear cells (PBMC) were

identified by SSC and CD45 expression (Figure 3A). Next, granulocyte subsets were distinguished by CD16 expression as neutrophils (CD16+) or eosinophils (CD16-) (Figure 3B). In the PBMC population, CD14 expression was used to identify lymphocytes (CD14-SSC<sup>lo</sup>) from monocytes (CD14+SSC<sup>hi</sup>). Monocytes were further characterized into classical (CD14<sup>lo</sup>CD16<sup>lo</sup>), intermediate (CD14<sup>hi</sup>CD16<sup>int</sup>), and nonclassical (CD14<sup>lo</sup>CD16<sup>hi</sup>) monocytes.

Next, lymphocytes populations were further segregated into NK cells, natural killer T (NKT) cells, T cells, and B cells. NKT cells were distinguished by expression of both CD3 and CD56 while remaining lymphocytes were separated into B cells (CD19+), T cells (CD3+), and a

CD3-CD19- cells (Figure 3C). Subsequent gating of T cells determined the frequencies of V $\delta$ 2+, V $\delta$ 2-  $\gamma$  $\delta$  T cells, as well as CD4 and CD8 T cells (Figure 3E). Next, in-depth characterization of T cell differentiation and activation was performed to illuminate T cell and immune status. Using the differential expression patterns of CD45RA and CCR7, naïve (CCR7+CD45RA+), central memory (CCR7+CD45RA-), effector (CCR7-CD45RA-), and effector memory (CCR7-CD45RA+) were identified for both CD4 and CD8 T cell populations (Figure 3F to 3G). Within the CD4+ T cells, Tregs were classified by high CD25 expression and low CD127 expression.

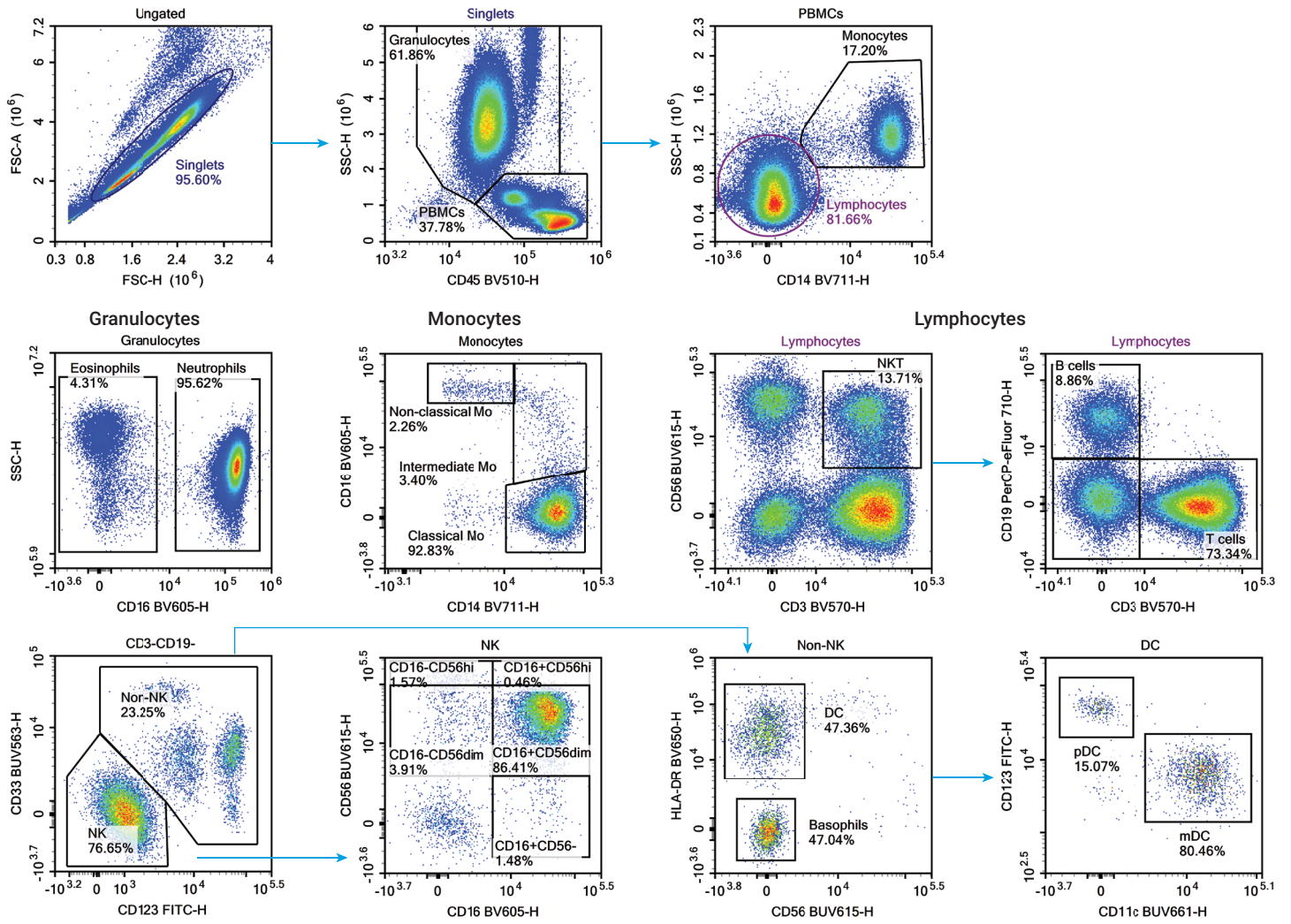


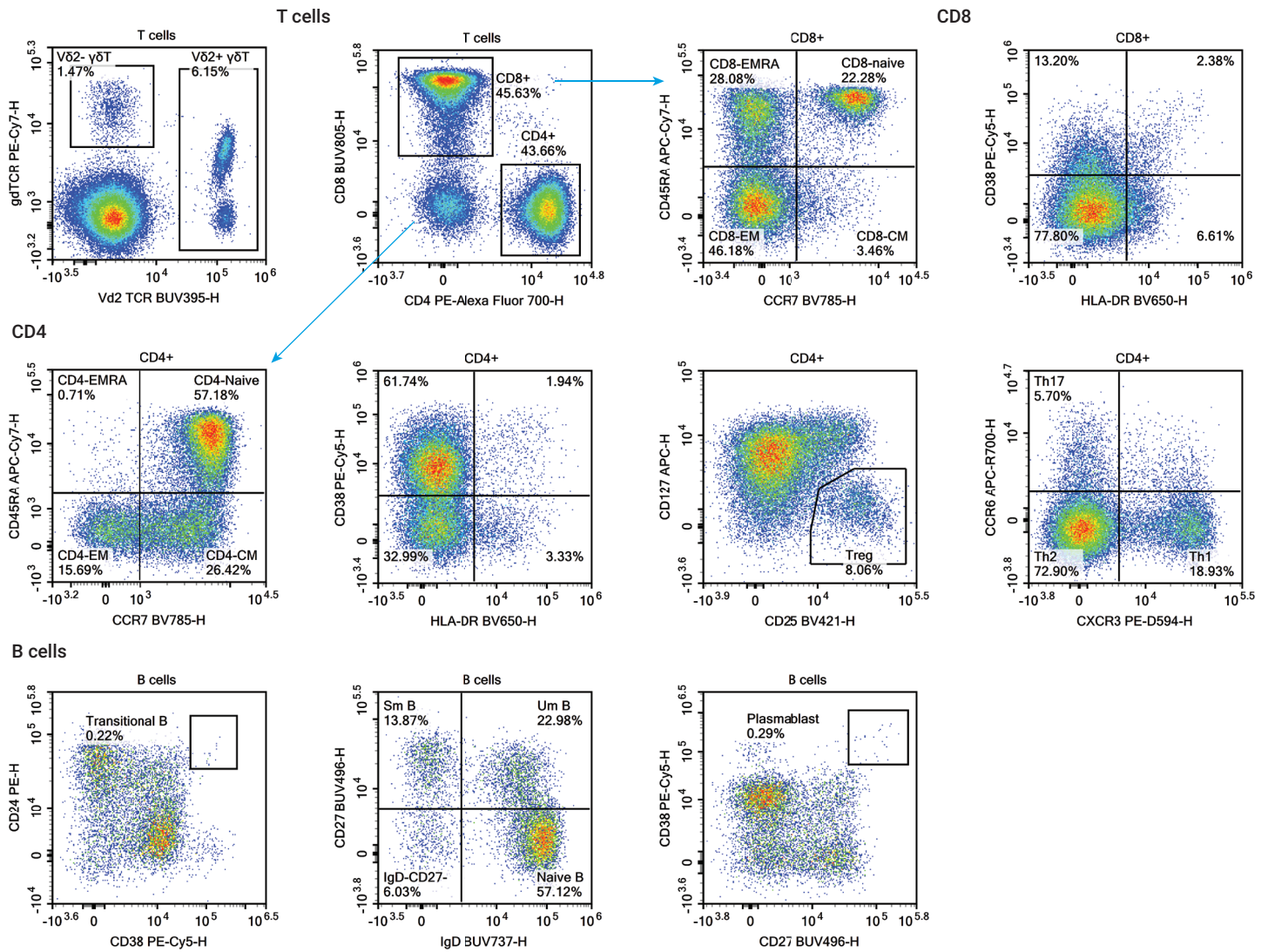
**Figure 1.** Flowchart of all cell populations identified in the 24-color immunophenotyping panel on the Agilent NovoCyte Penteon flow cytometer.

Polarization of CD4 T cells can be classified into TH1, TH2, TH17, as well as many other helper T cell subsets. These helper T cell subsets are associated with the production of specific cytokines and a unique functions *in vivo*. TH1, TH2, and TH17 CD4 T cells were identified by the expression of CCR6 and CXCR3, while activated CD4 and CD8 T cells were identified by both expression of CD38 and HLA-DR.

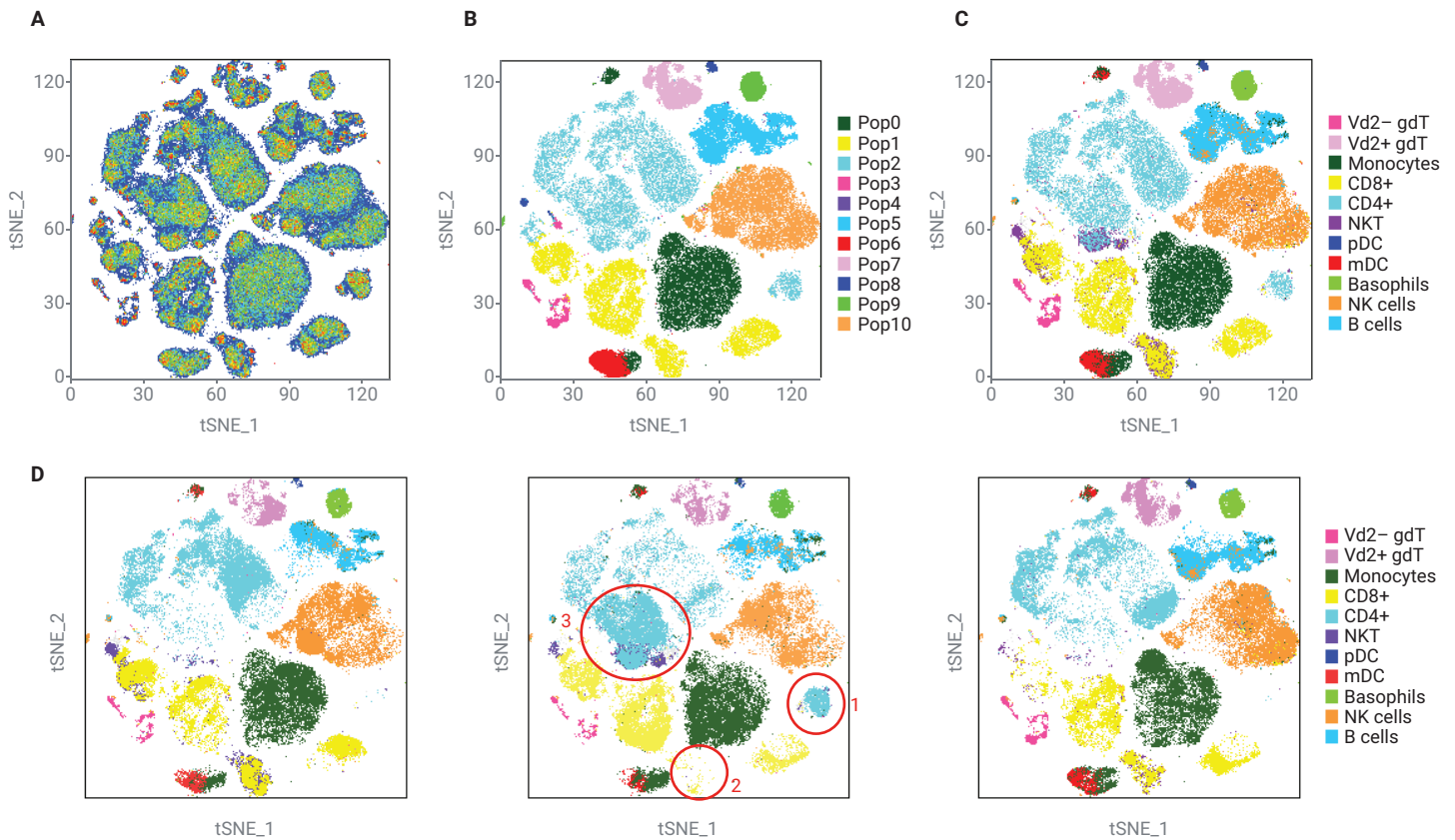
Additional characterization of B cells identified plasmablasts (CD27+CD38+) and transitional B cells (CD24+CD38+). Activation of B cells was described by the expression of CD27 and IgD, to classify naive (IgD+CD27-), CD27-switched memory B cells (IgD-CD27-), CD27+ switched memory B cells (IgD-CD27+), and unswitched memory B cells (IgD+CD27+). Within the CD3-CD19- lymphocyte population, NK cells were labeled by co-expression of CD16 and CD56 and HLA-DR was used to identify DCs (HLA-DR+) and basophils (HLA-DR-) from the remaining lymphocyte population. Finally, DCs were subdivided into two subtypes, plasmacytoid DC (CD11c-CD123+), and myeloid DC (CD11c+CD123-). Through the gating strategy described here, all major subsets of the immune cells can be identified with simultaneous in-depth analysis of T and B cell populations.

The complex gating strategy outlined in Figure 3 demonstrates that with increased flow cytometer capabilities, a vast number of markers can be analyzed in a single tube. With the ever-expanding number of markers, there is increasing interest in utilizing analysis algorithms. Using automated, nonbiased clustering of flow cytometry data with traditional gating can yield discoveries of yet-to-be described cell population/subpopulations and interactions between the expression of specific markers.





**Figure 2.** Identification of immune cells in peripheral human blood with a 24-color immunophenotyping panel on the Agilent NovoCyte Penteon flow cytometer. Gating strategy for the identification of immune cells in 24-color immunophenotyping panel: whole blood was stained with Vδ2 TCR-BUV395, CD27-BUV496, CD33-BUV563, CD56-BUV615, CD11c-BUV661, IgD-BUV737, CD8-BUV805, CD25-BV421, CD45-BV510, CD3-BV570, CD16-BV605, HLA-DR-BV650, CD14-BV711, CCR7-BV785, CD123-FITC, CD19-PerCP-eF710, CD24-PE, CXCR3-PE-Dazzle 594, CD38-PE-Cy5, CD4-PE-AF700, γδ TCR-PE-CY7, CD127-APC, CCR6-APC-R700, and CD45RA-APC-Cy7 antibodies. After staining, samples were acquired on the NovoCyte Penteon and analyzed with Agilent NovoExpress software. Hierarchical gating was used to identify all major cell subsets in human blood. (Continued from previous page).

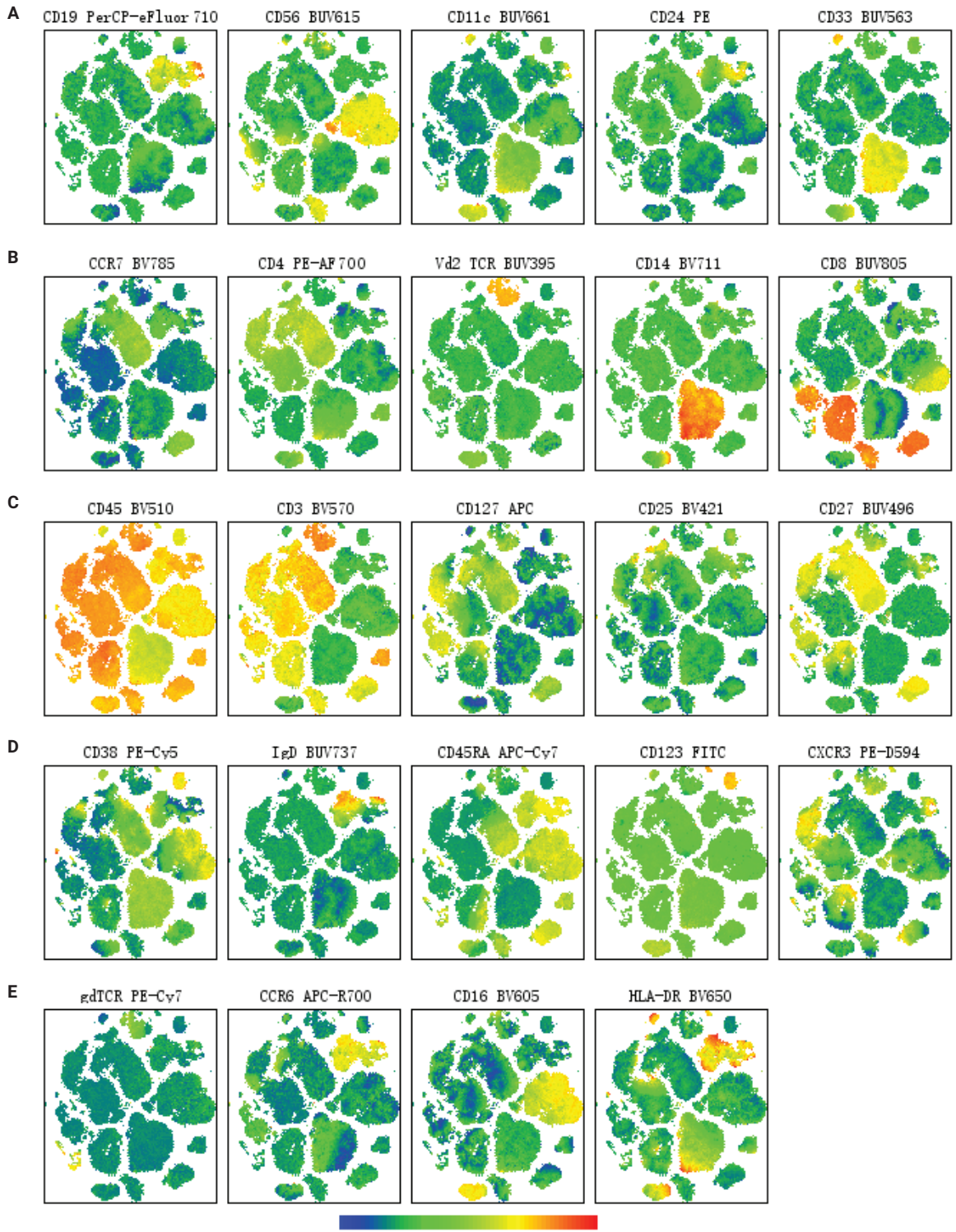


**Figure 3.** t-SNE and FlowSOM (CytoBank) analysis in FlowJo software (Becton, Dickinson and Company). Automated computational analyses showing the distribution of PBMC populations across three donors. (A) During cell analysis in FlowJo software, doublets were excluded and t-SNE analysis was performed on 110,000 PBMCs from each blood sample; all 24 markers were used to provide insight into the complexity of the high-dimensional data obtained with this 24-color panel. (B) The colored cell clusters corresponding to clusters generated with FlowSOM software were applied to the t-SNE map. (C) Various cell populations that were manually gated as described in Figure 3 were overlaid onto the t-SNE plot. (D) Individual blood samples were separated and visualized in the t-SNE map generated from the merged dataset.

A commonly used algorithm for analysis and visualization of high-dimensional flow cytometry data is t-distributed stochastic neighbor embedding (t-SNE), which groups cells based on their phenotypic similarities across all markers. Also, to visualize and easily analyze flow cytometry data, FlowSOM, a widely used clustering algorithm, can be used to provide a detailed overview of the phenotypic heterogeneity of cells. In this application note, blood from three donors was stained with the 24-color immunophenotyping panel, as previously described. After initial individual analysis within NovoExpress, the data was further

analyzed in FlowJo software. In the FlowJo software, cells within the PBMC gate from different donors were reduced to 110,000 events each and merged into one file, then t-SNE was applied to the merged file (Figure 4A). The FlowSOM clustering algorithm was applied to the same dataset and generated the colored clusters shown on the t-SNE map (Figure 4B). To determine the location of each manually gated population (as shown in Figure 3), the cell groups were overlaid onto the t-SNE map where each of the gated cell populations occupied distinct regions in the t-SNE map (Figure 4C). Using t-SNE and FlowSOM

analysis of the flow cytometry data allows the visualization of highly complex data into a two-dimensional space. Each cluster represents a distinct population of cells that shares similarities in the expression of all 24 markers. Using t-SNE heat maps can also be used to visually represent the expression levels of all 24 markers in each of the cell islands. The individual marker heat maps can be used to evaluate key differences between marker expression within each immune cell population rapidly without the need to look at individual plots (Figure 4E).



**Figure 4.** Marker expression on the t-SNE cluster map. Merged data file t-SNE analysis was used to generate heat maps that represent the expression of each marker on the t-SNE cell clusters.

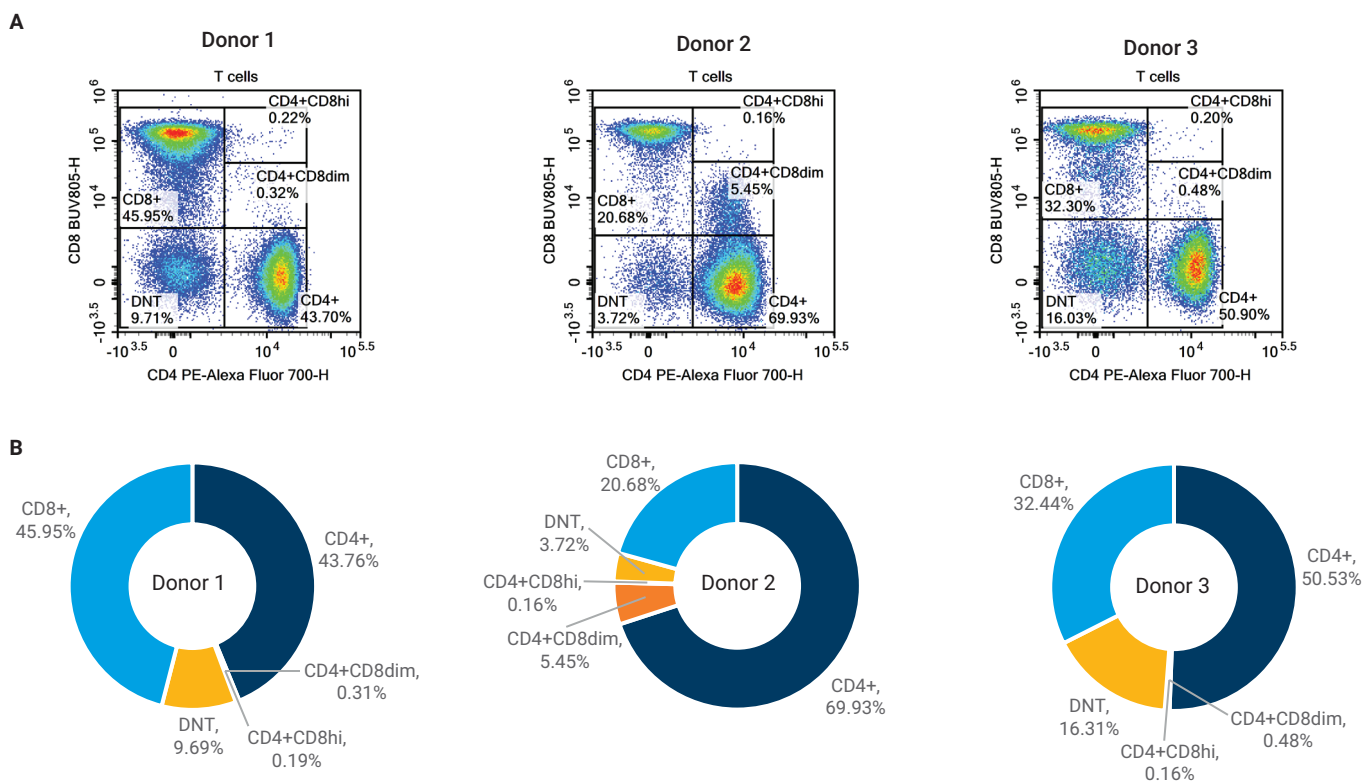


To study the distribution of cell populations across the donors, the individual samples were analyzed within the t-SNE map generated from the merged dataset (Figure 4D). While comparing the three different donors, the clustering pattern of Donor 2 displayed key differences, shown with red circles, compared to the other donors. These cluster differences were located within the CD4 (Figure 3, red circles 1 and 3) and CD8 (Figure 3, red circle 2) populations, which indicated that a deeper analysis of T cell population frequencies and marker expression was needed to understand the differences between individual donors in these samples. The differential clusters were compared to the individual marker heat maps showing that these clusters had high levels of CD45RA and low levels of CCR7, which are associated with

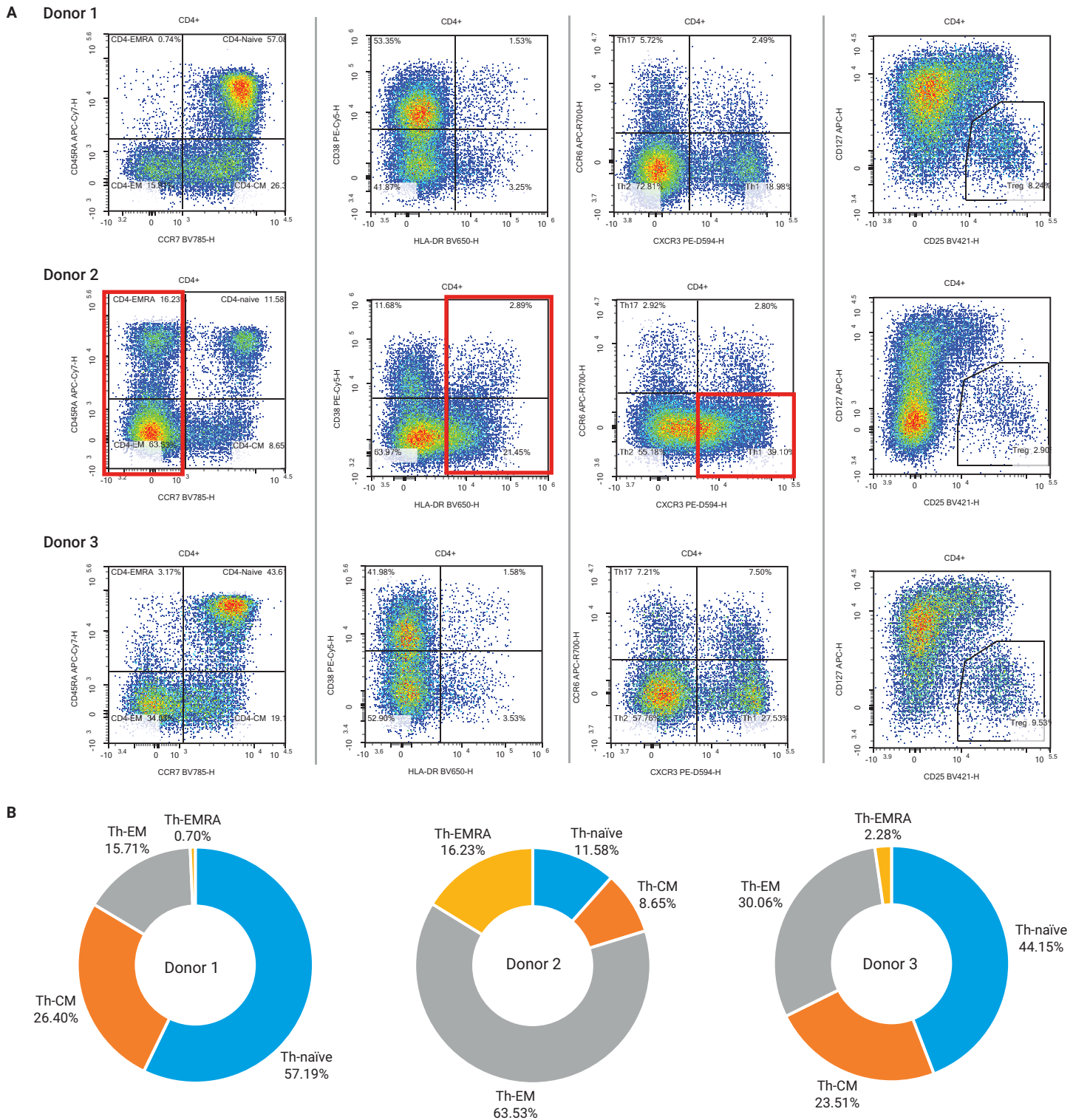
differentiated T cells (Figure 4E). Thus, it was indicated that there were differences in the frequencies of differentiated T cells between donors and to follow up, T cell plots for individual donor samples need evaluation. Using t-SNE and FlowSOM to visualize high-dimensional flow cytometry data can provide a rapid and simple way to compare biological samples.

To analyze the donor variation in T cells, T cell-specific flow cytometry plots were analyzed and compared across donors. First, the frequencies of CD4 and CD8 T cells were compared, demonstrating a higher frequency of CD4+ T cells and CD4+ CD8<sup>lo</sup> double positive T cells in Donor 2 (Figure 5). Interestingly, there is evidence that an increase in peripheral CD4+CD8+ T cells occurs during viral infections. In human immunodeficiency virus (HIV) and Epstein-Barr virus (EBV),

it has been reported that the percentage of CD4+CD8+ T cells can increase to 20% of all circulating lymphocytes. Further analysis of T cell populations examined the differences in effector, effector memory RA, central memory, and naïve CD4 T cell numbers identified by expression of CD45RA and CCR7 (Figure 6, first column). Donor 2 had a high frequency of effector CD4 T cells, 63.53%, and effector memory RA CD4 T cells, 16.23%, and fewer naïve T cells compared to other samples. The expression of CD38 and HLA-DR on T cells is an indicator of cellular activation. Higher numbers of activated CD4 T cells (Figure 6, second column) and TH1 polarized cells (Figure 6, third column) were also observed.



**Figure 5.** Comparison of T cell population across donors. The frequency of CD4 and CD8 T cells was compared between donors, as shown with (A) flow cytometry plots and (B) chart graphing frequency of each cell population.



In addition, the frequency of Treg cells was decreased within the CD4 T cell population of Donor 2. Together, these data suggest that Donor 2 may have recently encountered an antigen stimulating event, which resulted in more activated and primed T cells. The data shown in this application note, demonstrate the power in high-dimensional flow cytometry analysis to rapidly and efficiently uncover differences between biological samples.

## Conclusion

Polychromatic flow cytometry has become an essential tool for studying immunology. Here we have demonstrated that with a 24-color immunotyping antibody panel run on the five-laser Agilent NovoCyte Penton flow cytometer, all major immune cell subsets can be identified, including T cells, B cells, monocytes, DCs, NK cells, neutrophils, eosinophils, and basophils. Activation and differentiation of these cells can also be determined. Therefore, flow cytometry immunotyping with an increased number of markers allows a more comprehensive analysis of the immune system, in steady state conditions and in the context of immunological challenges, including infections and autoimmunity.

## Reference

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