Comprehensive 18 color pan leukocyte flow cytometry analysis for immune surveillance

The human immune system is highly complex and immune status is associated with disease state, treatment efficiency, and response to external stimuli such as vaccines. Simultaneous quantification of multiple leukocytes allows for better surveillance of the immune response to infectious disease and the immune status of patients. This multi-color flow cytometry staining panel is based on OMIP-024, which was originally designed to measure leukocyte subsets in the peripheral blood mononuclear cells (PBMCs) of children to monitor vaccine efficacy. Therefore it was important to obtain the maximum information concerning the status of the immune system with a small amount of blood. Monitoring the frequency of numerous immune cell population as well as the differentiation/activation status of specific cell subsets such as monocytes, NK cells, T and B cells is essential as they may influence the immunogenicity of a vaccine and its efficiency. With the increasing capabilities of flow cytometers to perform complex multi-color analysis, the opportunity to monitor more immune subsets is made possible. Through comprehensive consideration and analysis of the expression of each marker, fluorescence intensity, spectral overlap, and NovoCyte Quanteon™ configuration, an 18-color immunophenotyping panel was designed for studying human PBMCs using the NovoCyte Quanteon **(Figure 1)**. In this 18-color immunophenotyping stain, we examined the frequency of monocytes, B cells, plasmablasts, CD4+ and CD8+ T cells, regulatory T cells, γδ T cells, NK T cells, NK cells, and dendritic cells. NK and T cell status are indicators of a proper immune response to a vaccine, therefore NK and T cell activation and differentiation states were identified to obtain a deeper understanding of immune status.

SPECIFICITY	CLONE	FLUOROCROME	PURPOSE
CD3 CD4 CD8	UCHT1 S3.6 SK1	PE-TR (ECD) PE-Alexa 700 PerCP-Cy5.5	Lineage T cells
CD19	J3-129	PerCP-eFluor 710	B cells
CD14	MφP9	BV711	Monocytes
CD56	HCD56	BV605	NK cells and NK T-like cells
CD16	3G8	APC-Cy7	NK cells and monocytes
γδ TCR Vδ2 TCR	11F2 B6	PE-Cy7 PE	γδ T cells
CD25	M-A251	BV421	Tregs
CD127	A019D5	APC	Tregs/memory/differentiation
CD45RA CCR7 CD57	HI100 G043H7 NK-1	BV650 BV785 FITC	Memory/differentiation
HLA-DR	B169414	BV570	Activation
CD38	HIT2	PE-Cy5	Activation/plasmablasts
NKG2C Dead Cells	134591	Alexa 700 AViD	NK receptor Dead cell exclusion

Figure 1: 18 color pan leukocyte panel antibody table

Antibody Titration for optimization of antibody concentration

Antibody titration is an important step in panel optimization allowing one to obtain optimal signal resolution, population identification, and expression level measurements. It is more time consuming but will improve your flow data resulting in saving time and money, as this becomes especially important in large multi-parameter stains. Too little antibody can result in dim signal and inadequate separation of the positive cells from the negative cells, however, the addition of too much antibody increases non-specific binding and increases the spread and background of the negative population. To titrate the antibodies, a single stain of each was performed at multiple concentrations to determine which concentration resulted in the best stain index. The stain index is the ratio of the separation between the positive population and negative population divided by two times the standard deviation of the negative population **(Figure 2A)**. The Stain Index can be automatically determined in the statistics table functions in NovoExpress® software. Antibody titrations were performed for all 17 antibodies used in this immunophenotyping panel that include 6, 2-fold dilutions of each antibody starting with the manufacturer's recommended concentration. Two example plots of antibody titration results can be seen in **Figure 2B**. After obtaining the optimized antibody concentration for all 17 colors determined by the highest stain index, the final stain was performed.

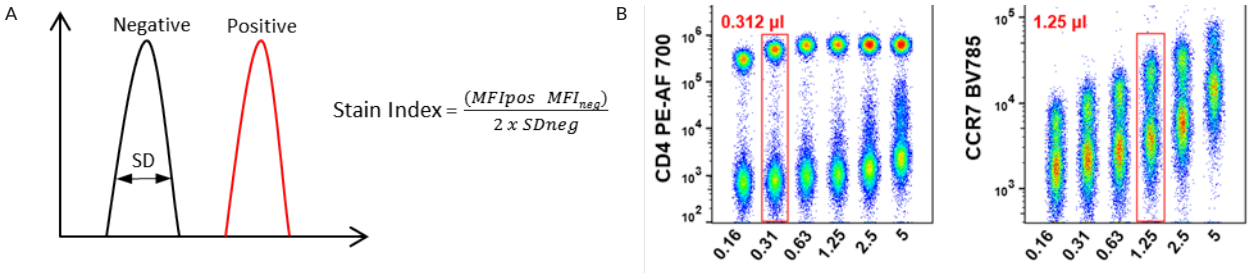

Figure 2: Antibody titration for 18 color panel
(A) graphic representation and equation for determination of stain index. **(B)** representative plots of antibody titrations at 6 dilutions of antibody for αCD4 PE-Alexafluor700 and αCCR7 BV785. PBMCs were stained with 6 serial dilutions of antibody starting with manufacturer recommended concentrations. All 6 dilutions are shown on the same plot with the X-axis demonstrated the µL of each antibody added to 50µL of PBMCs. 20µL of diluted antibody was added to 50µL of isolated PBMCs and incubated for 30 minutes on ice. The red box highlights the concentration that was determined to be the best concentration for staining.

In-depth analysis of T and NK lymphocyte activation and differentiation of PBMCs

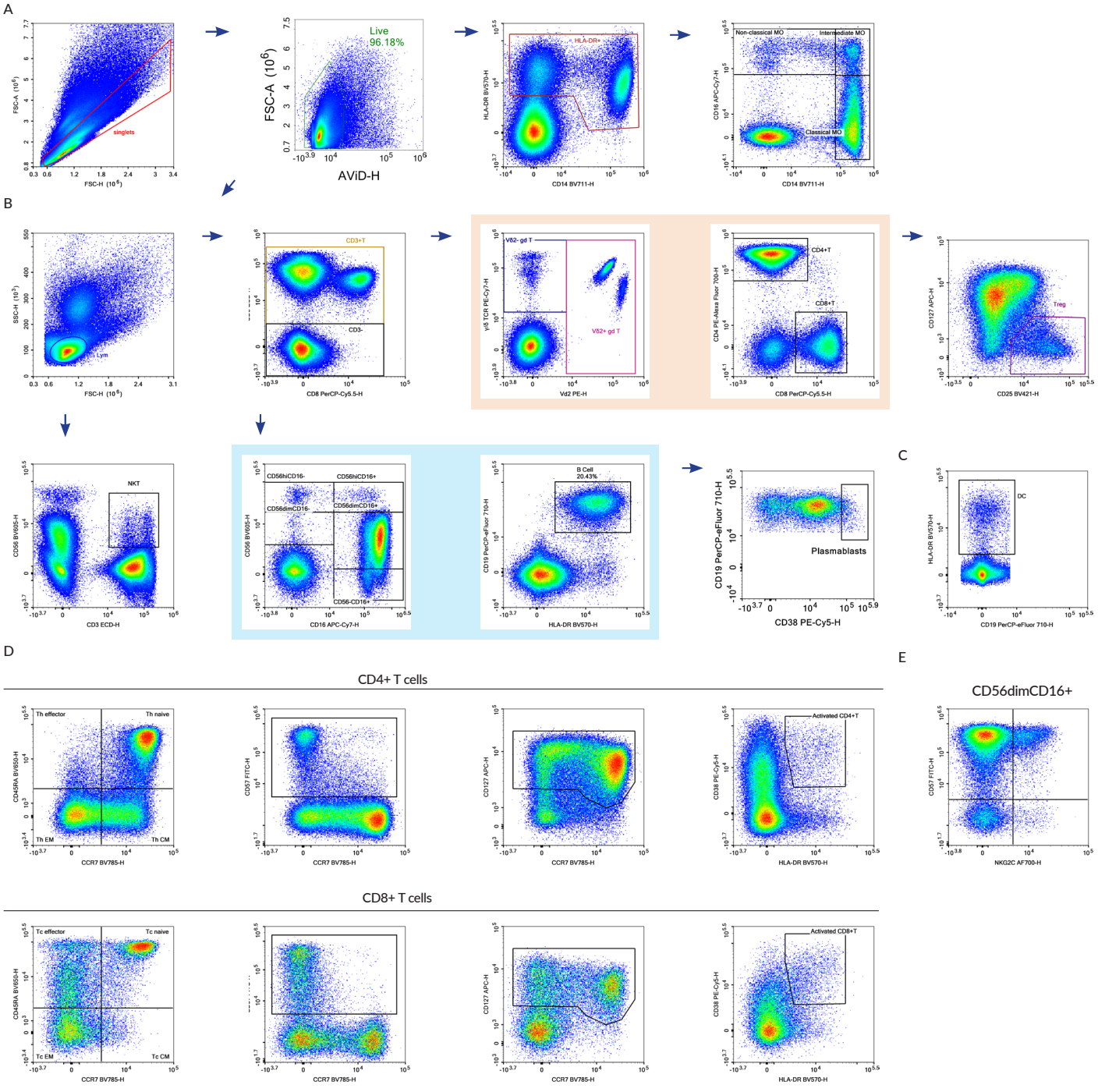

Figure 3: 18-color Pan-Leukocyte flow cytometry panel

PBMCs from a normal donor were stained with the 18 color panel stain. 50µl of isolated PBMC were first stained with AViD for 30 minutes at room temperature and then treated with Fc Block solution (PBS containing 10% heat-inactivated human serum, 0.5% BSA, and 0.5% heat inactivated FBS) for 30 minutes on ice. After, cells were stained with 20µL diluted antibodies for 30 minutes on ice, then cells were washed and analyzed on the NovoCyte Quanteon **(A)** Initial gating was done on FSC-H and FSC-A to discriminate single cells. Monocytes were identified as HLA-DR+;Three monocyte subsets were identified: classical (CD14+CD16-), intermediate (CD14+CD16+), and non-classical (CD14dimCD16+). **(B)** FSC-H and SSC-H were used to identify lymphocytes. CD3 was used to identify T cells. Subsequent gating of CD3+ cells identified Vδ2+ and Vδ2- γδ T cells as well as CD4+ and CD8+ T cells. Regulatory T cells (Tregs) were identified as CD25hi CD127lo CD4+. NK T cells were identified by the co-expression of the NK marker, CD56, and CD3. Among the CD3- cells, five NK cell subsets were identified by expression of CD56 and CD16 (CD56hiCD16-. CD56dimCD16-, CD56hiCD16+, CD56dimCD16+, and CD56-CD56+). B cells were identified as CD19+HLA-DR+. Plasmablasts were identified within the CD19+ cells as CD38hi. **(C)** Dendritic cells were identified as being negative in all lineage markers but positive for HLA-DR **(D)** Activation status of CD4+ and CD8+ T cells were examined by the expression of CCR7, CD45RA, CD57, CD127, HLA-DR and CD38. **(E)** Activation of NK cells were examined by CD57 and NKG2C expression. Plots show expression of NKG2C and CD57 in CD56dimCD16+ NK Cells.

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This panel was originally designed to monitor the response to a malaria vaccine where NK and γδ T cell responses have been indicated as having particular importance. Therefore, focus on identifying these cell populations was the utmost importance. Expression levels of CD56 (neural cell adhesion molecule NCAM) and CD16 (Fcγ IIIa) were used to define five NK subsets; Vδ2 and γδ TCR were included to identify γδ T cells **(Figure 3)**. As described by the original OMIP 024 authors, one to three different populations of γδ T cells can be detected based on the expression levels of Vδ2 TCR may indicate functional differences as they show different profiles of CD16 and CD57 have been to correlate with expression of cytotoxic and differentiation markers. NK T cells were identified as CD3+CD56+ cells; NK activation was determined by NKG2C staining. Regulatory T cells were identified by the expression of CD25 (IL-2Rα-chain) and CD127 (IL-7Rα-chain) on CD4+ T cells to avoid intracellular staining for Foxp3. In depth analysis of T cell subsets were achieved by CD45RA and CCR7 co-staining to identify the following T cell populations: naïve (CD45RA+CCR7+), central memory (CD45RA-CCR7+), effector memory (CD45RA-CCR7-), and terminal effector memory (CD45RA+CCR7-). Further T cell subset analysis was performed by examining the expression of CD127 (homeostatic proliferation) and CD57 (cell senescence). CD38 and HLA-DR were included to evaluate T cell activation as well as identify plasmablasts (CD19+CD38hi). Dendritic Cells were identified as negative for all lineage markers but positive for HLA-DR. This panel allows simultaneous analysis of various leukocyte subsets granted a comprehensive overview of the immune status.

Conclusions

Here we demonstrated an 18-color multi-parameter panel on the NovoCyte Quanteon which identifies numerous leukocyte subsets. The cell types identified include monocytes, B cells, plasmablasts, T cells, γδ T cells, NK T cells, NK cells, and dendritic cells in PBMCs; allowing a broad overview of all leukocytes in only one stain. Complex multi-parameter flow cytometer experiments have been made possible with newer, high performance, multi-laser flow cytometers such as the NovoCyte Quanteon. The addition of more parameters to current flow cytometry experiments will expand the capability to understand complex interactions of the numerous cell subsets of the immune system as well as their activation/differentiation status.

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