Application Note

Cell Analysis

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Comprehensive 18-Color Pan-Leukocyte Flow Cytometry Analysis for Immune Surveillance

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Abstract

This application note describes the use of an 18-color immunophenotyping stain to examine leukocyte subsets in human peripheral blood mononuclear cells (PBMCs), using the Agilent NovoCyte Quanteon flow cytometer. This method simultaneously identifies and quantifies 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 statuses were evaluated to obtain a deeper understanding of immune status.

Introduction

The human immune system is highly complex and research has shown that immune status is a critical component of the immune response to various stimuli. Simultaneous quantification of multiple leukocyte subsets allows for better surveillance of the immune response to infectious diseases and the immune status of subjects.

This multicolor flow cytometry staining panel is based on OMIP-024, which was originally designed to measure leukocyte subsets in the PBMCs of children to evaluate vaccine efficacy. It was, therefore, 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 populations, as well as the differentiation/activation status of specific cell subsets, such as monocytes, NK cells, T cells, 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 multicolor analysis, the opportunity to evaluate more immune subsets is made possible. By analyzing the expression of each marker, fluorescence intensity, spectral overlap, and Agilent NovoCyte Quanteon configuration, an 18-color immunophenotyping panel was designed for studying human PBMCs using the NovoCyte Quanteon (Table 1).

Table 1. 18-color pan leukocyte panel antibody table.

Specifcity	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 Vγ2 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

Antibody titration for optimization of antibody concentration

Antibody titration is an important step in panel optimization, allowing optimal signal resolution, population identification, and expression level measurements. Adding too little antibody can result in dim signal and inadequate separation of the positive cells from the negative cells; however, too much antibody increases nonspecific binding, and the spread and background of the negative population.

To titrate an antibody, a single stain 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 1A). The stain index can automatically be determined in the statistics table functions in NovoExpress software. Antibody titrations were performed for all 17 antibodies used in this immunophenotyping panel. This included six 2-fold dilutions of each antibody starting with the manufacturer's recommended concentration. Figure 1B shows two example plots of antibody titration results. After obtaining the optimized antibody concentration for all 17 colors determined by the highest stain index, the final stain was performed.

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

The panel in Figure 2 was originally designed to evaluate the immune response to a malaria vaccine where NK and γδ T cell responses seemed to be especially important. Therefore, it was important to focus on identifying these cell populations. 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 2). As the original OMIP 024 authors described, one to three different populations of γδ T cells can be detected based on the expression levels of Vδ2 TCR. This may indicate functional differences as they show different profiles of CD16 and CD57, which have been known 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 costaining 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 and granted a comprehensive overview of the immune status.

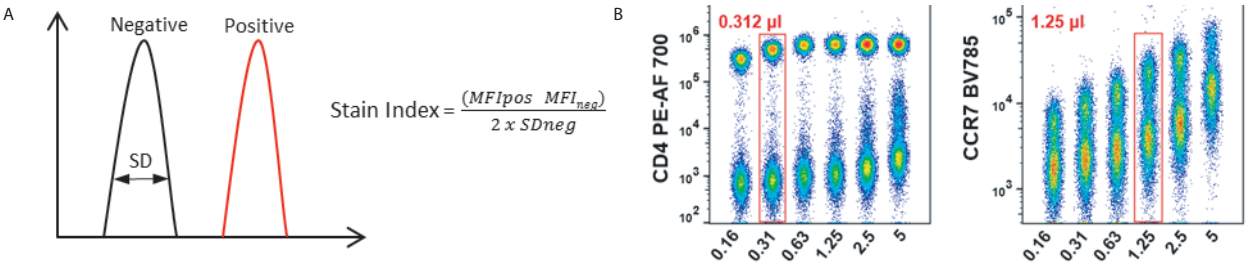

Figure 1. Antibody titration for an 18-color panel. (A) graphic representation and equation for determination of stain index. (B) representative plots of antibody titrations at six dilutions of antibody for anti-CD4 PE-Alexafluor700 and anti-CCR7 BV785. PBMCs were stained with six serial dilutions of antibody starting with the manufacturer's recommended concentration. All six dilutions are shown on the same plot with the X-axis as the volume (µL) of each antibody added to 50 µL of PBMCs. The red box highlights the optimal concentration for staining.

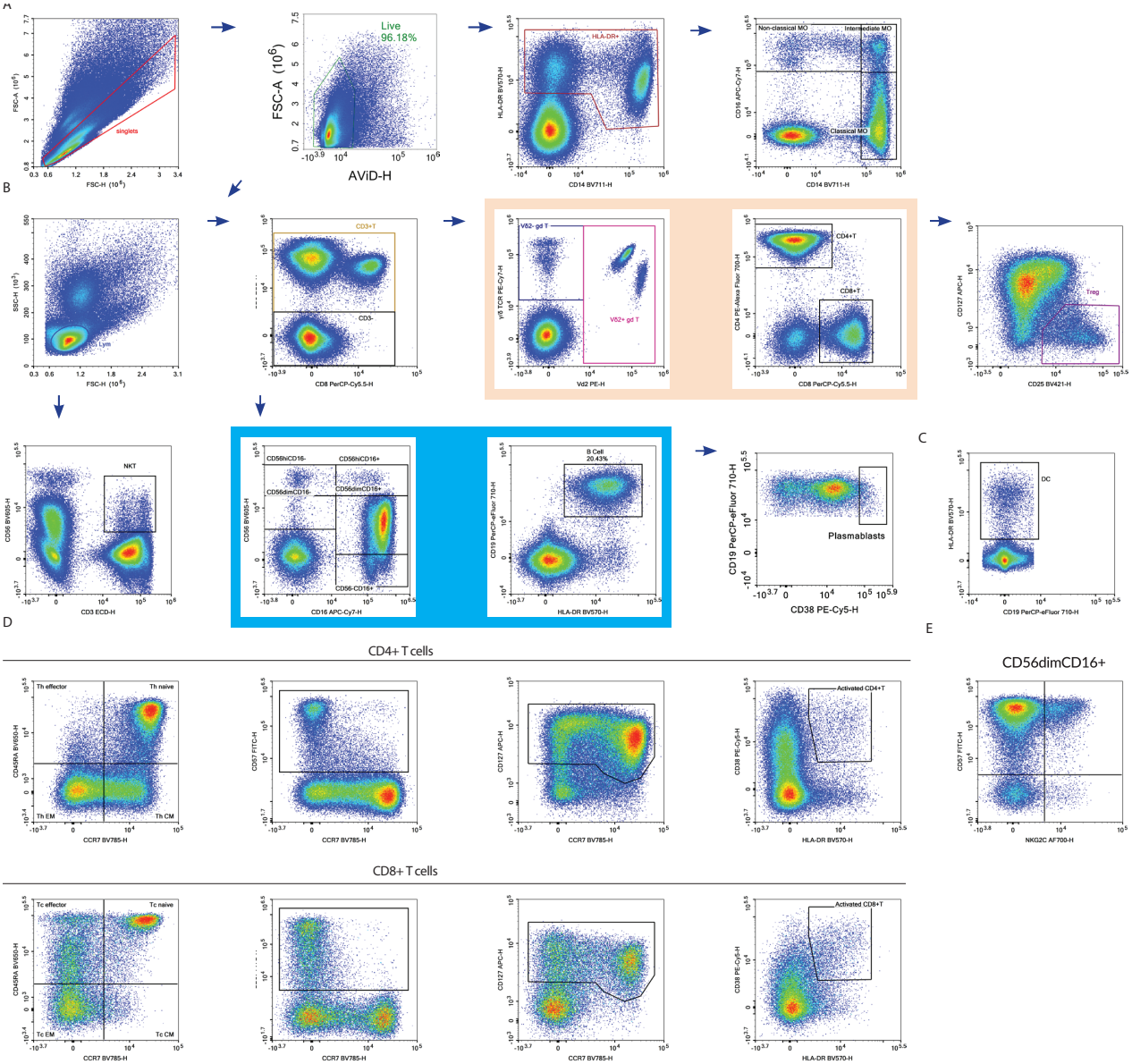

Figure 2. 18-color Pan-Leukocyte flow cytometry panel. PBMCs from a normal donor were stained with the 18-color panel stain. Isolated PBMC (50 µL) was first stained wih AViD for 30 minutes at room temperature, then treated with Fc Block solution (PBS containing 10% heat-inactivated human serum, 0.5% BSA, and 0.5% heat-activated FBS) for 30 minutes on ice. Afterwards, cells were stained with 20 µL of diluted antibodies for 30 minutes on ice. Afterwards, cells were stained with 20 µL of diluted antibodies for 30 minutes on ice, 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 nonclassical (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 was 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.

Conclusion

This application note demonstrates an 18-color multiparameter panel on the NovoCyte Quanteon that 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 multiparameter flow cytometry experiments have been made possible with newer, high performance, multilaser 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 immune system cell subsets, as well as their activation/differentiation status.

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