

Comprehensive Analysis of T Cell Status Following Activation Using a 16-Color Immunophenotyping Panel on the NovoCyte Advanteon

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Abstract

T cells are an essential part of the immune response, capable of recognizing and eliminating foreign pathogens and tumor cells. However, tight control of the T cell response is necessary to prevent autoimmunity and immunopathology. A 16-color T cell immunophenotyping panel was developed for the Agilent NovoCyte Advanteon to simultaneously analyze T cell differentiation, activation, exhaustion, and functional output through degranulation and cytokine expression. This flow cytometry panel provides comprehensive analysis of T cell function in an immune response.

Introduction

The role of the immune system is to fight off pathogens while limiting autoimmunity. T cells are an important part of the adaptive immune response, capable of recognizing specific antigens and resolving infections. In a normal immune response, T cells are activated to rapidly divide and destroy all infected or cancerous cells. Activation through the T cell receptor (TCR) and costimulatory receptors must be balanced with inhibitory receptors such as programmed cell death protein 1 (PD-1), lymphocyte-activation gene 3 (LAG-3), and T cell immunoglobulin and mucin-domain containing-3 (TIM-3). This is needed to prevent the development of autoimmunity and immunopathology.

When stimulation of T cells is sustained, there is a progressive increase in the expression of inhibitory receptors and a decrease in T cell effector function and proliferative capacity (T cell exhaustion). This can be seen during a chronic infection or cancer. Inhibitory receptors have become a focus of cancer immunotherapy research because reversing inhibitory receptor expression through checkpoint inhibitor blockade has restored T cell function and improved tumor immunity in the clinic. The severity of T cell exhaustion correlates heavily with the co-expression of multiple inhibitory receptors, making combination therapy promising because multiple inhibitory receptors are targeted, demonstrating an increase in tumor immunity. Analysis of T cell inhibitory receptor expression alone does not provide a comprehensive view of T cell state. Measuring additional markers for T cell differentiation, activation, previous antigen exposure, degranulation, and cytokine production provides a more comprehensive understanding.

Studies have identified inherent differences in inhibitory receptor expression among naive, effector, and memory T cell subsets. A 16-color immunophenotyping panel was designed to run on the NovoCyte Advanteon (Table 1) to examine CD4 and CD8 T cell differentiation and activation state, and measure functional T cell responses. Using the NovoCyte Advanteon's high capacity for multiparameter analysis, these data demonstrate a comprehensive look at the kinetic changes that occur in T cell subsets and function after stimulation.

In depth analysis of T cell state after TCR stimulation

To fully examine the T cell response to TCR activation, we looked at differentiation, activation, and inhibitor markers as well as degranulation and cytokine production in CD4 and CD8 T cells (Table 1). Isolated peripheral blood mononuclear cells (PBMCs) were stimulated with anti-CD3, anti-CD28 antibodies, and interleukin-2 (IL-2) to

induce T cell activation. Six hours before flow cytometric analysis, cytokine secretion was blocked with a protein transport inhibitor. Samples were measured initially after two and four days in culture, and again on day 5 after being restimulated.

Figure 1 demonstrates a representative gating strategy for the day 4 sample. First, dead cells were excluded using viability dye followed by examining the frequency of CD4 and CD8 T cells within the total CD3 T cell population. Next, T cell differentiation was assessed by determining the relative frequency of T cell subsets based on surface expression of CD45RA and CCR7. T cells are separated into Naive-like T_{naive-like} (CD45RA+/CCR7+), central memory T_{CM} (CD45RA-/CCR7+), effector memory T_{EM} (CD45RA-/CCR7-), and CD45RA+ effector memory T_{EMRA} (CD45RA+/CCR7-). Furthermore, T_{naive-like} are separated into true (T_{naive}) and T stem-cell memory T cells (T_{SCM}) by the expression of CD95 on T_{SCM}.

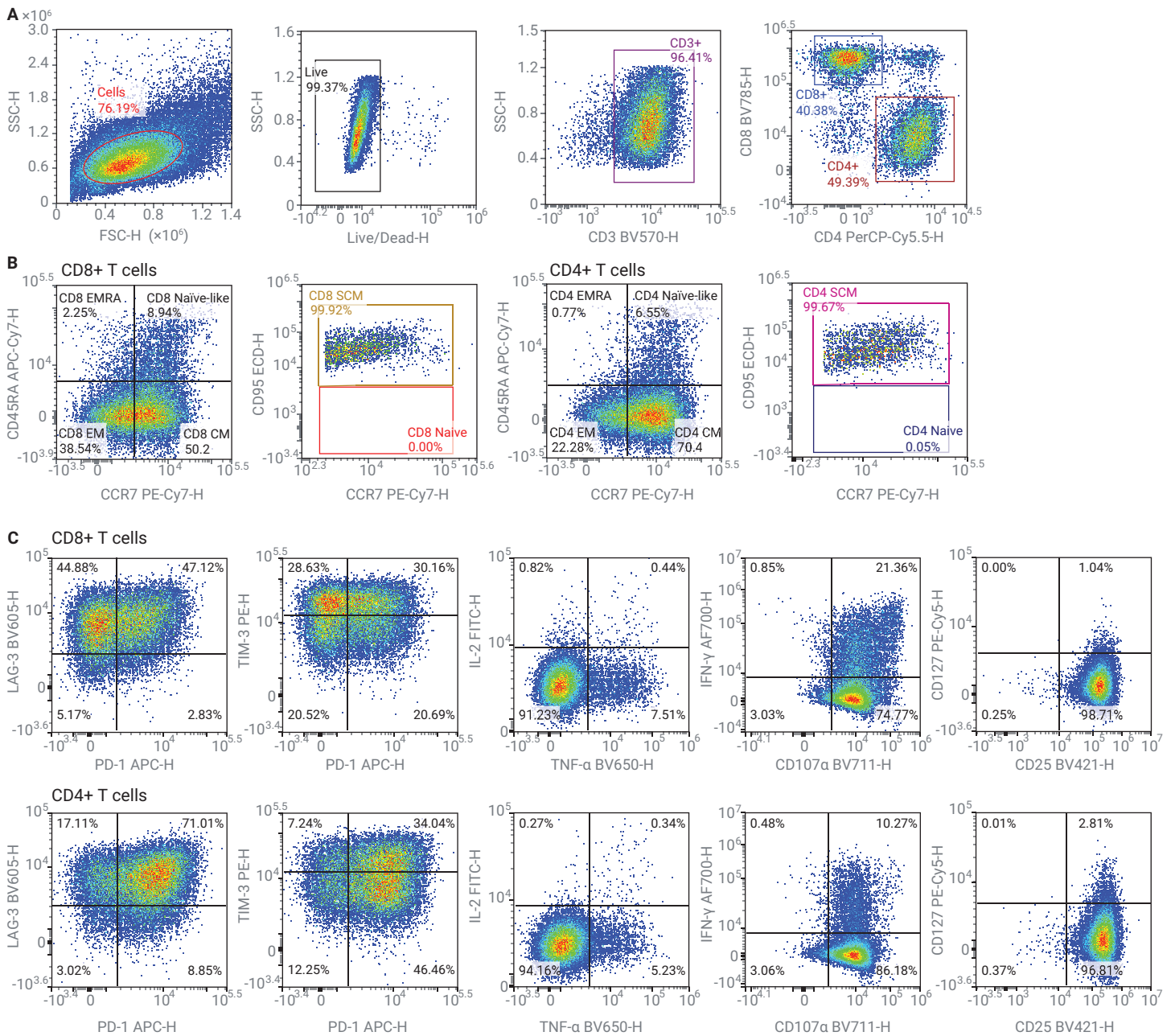
Table 1. Fluorochromes used in the 16-color immunophenotyping panel.

Marker	Fluorochrome	Clone	Purpose
Live/Dead	AVID		Viability
CD3	BV570	UCHT1	Lineage
CD4	PerCP-Cy5.5	RPA-T4	
CD8	BV785	SK1	
CD45RA	APC-Cy7	HI100	Activation/Differentiation
CCR7	PE-Cy7	G043H7	
CD25	BV421	M-A251	
CD127	PE-Cy5	A019D5	
CD95	PE-Dazzle594	DX2	Inhibitory receptors
PD-1	APC	EH12.2H7	
TIM-3	PE	F38-2E2	
LAG-3	BV605	11C3C65	Cytokine
IL-2	FITC	MQ1-17H12	
IFN-γ	AF700	B27	
TNF-α	BV650	MAB11	Degranulation
CD107α	BV711	H4A3	

Next, the expression of inhibitory receptors PD-1, TIM-3, and LAG-3 was examined as well as activation markers CD25 and CD127. These markers provide additional information on the activation state of the T cells. Finally, production of cytokines IL-2, tumor necrosis factor alpha (TNF α), and interferon gamma (IFN γ) were measured as well as degranulation by expression of CD107 α , which demarcates cytolytic T cells. All gates were determined with the use of

fluorescence minus one (FMO) controls (Figure 1D). Four days after stimulation, most cells had undergone T cell activation, and there were no longer any T_{naive} cells in both the CD4 and CD8 T cell populations. A large upregulation in CD25 expression and downregulation of CD127 was seen in T cells, also consistent with highly activated T cells. Both CD4 and CD8 T cells had upregulated inhibitory receptor expression, although not all cells had positive expression.

Cytokine production of TNF α and IFN γ was also present while only minimal IL-2 production was observed. Most T cells were also CD107 α positive, showing that they had cytolytic potential. This high-parameter staining panel with the NovoCyte Advanteon allows extensive analysis of T cells after stimulation. Next, kinetic changes in the parameters measured were examined in T cells after TCR activation.



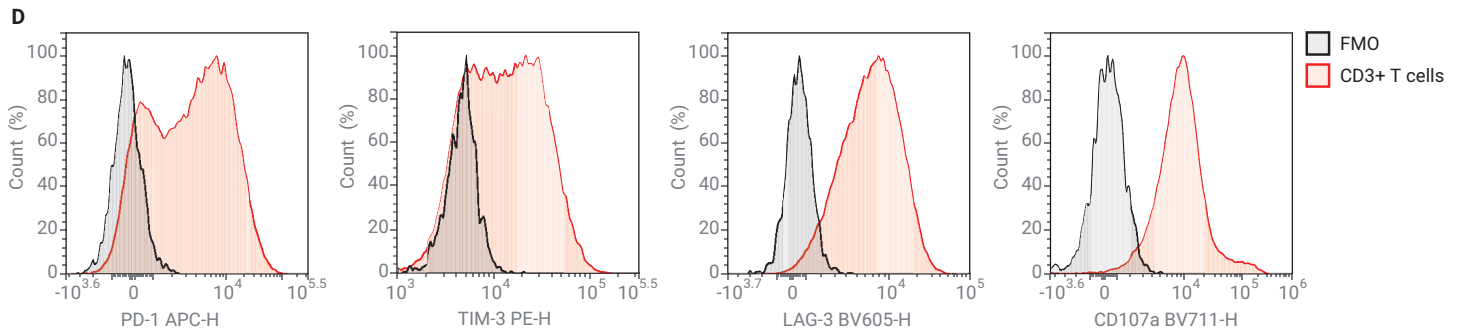


Figure 1. Immunophenotyping of stimulated PBMCs by a 16-color flow cytometry panel. PBMCs isolated from donor blood were stimulated with 5 $\mu\text{g}/\text{mL}$ anti-CD3 antibody, 2 $\mu\text{g}/\text{mL}$ anti-CD28 antibody, and 100 ng/mL IL-2. Stimulated PBMCs were stained with the 16-color immunophenotyping panel (Table 1) on day 4. Protein transport inhibitor cocktail (2 $\mu\text{L}/\text{well}$) and anti-CD107a BV711 antibody (5 $\mu\text{L}/\text{mL}$) were added directly to the wells for six hours before analysis. (A) Gate strategy. FSC-H and SSC-H were used to identify target cells from debris; dead cells were excluded by Live/Dead dye. CD3 was used to identify T cells. Subsequent gating of CD3+ cells identified CD4+ and CD8+ T cells. Upon gating on CD4+ or CD8+ cells, T_{CM} , T_{EM} , and T_{EMRA} cells were identified based on the differential expression of CD45RA and CCR7. From the CD45RA+CCR7+ population, CD95+ TSCM and CD95- naive T cells were further detected (B). The expression level of CD25 and CD127, exhaustion level of PD-1, LAG-3, and TIM-3, cytokine secretion level of IL-2, TNF- α , and IFN- γ and degranulation level of CD107a were also examined within CD4+ and CD8+ T cell subsets. The data shown here were from the sample harvested on day 4. (D) FMO controls. The gates were defined using FMO controls. FMO controls of PD-1, TIM-3, LAG-3, and CD107a shown above. Samples were acquired on the Agilent NovoCyte Advanteon, and analyzed using NovoExpress software.

Continuous changes present in T cell differentiation state after stimulation

TCR activation induces rapid changes in T cell differentiation. Differentiation status was examined by identifying each subset: T_{naive} , T_{SCM} , T_{CM} , T_{EM} , and T_{EMRA} , as described in the previous section. Previous studies have identified functional differences between T cell subsets; T_{naive} and T_{CM} have high proliferative capacity but lack rapid effector function while T_{EM} and T_{EMRA} have less proliferative capacity but more extensive effector capabilities.

T_{SCM} have experienced antigen stimulation but maintain the same stem-like properties of self-renewal as T_{naive} cells. To understand the changes in T cell differentiation after stimulation, the relative frequencies of all T cell subsets were examined in unstimulated PBMCs and stimulated PBMCs after two and four days, and restimulated PBMCs at day 5 (Figure 2). As expected, the frequency of T_{naive} cells decreases dramatically after two days of TCR stimulation; most CD45RA+ CCR7+ cells are T_{SCM} as they express CD95 as well

(Figure 2B). This is typical with *ex vivo* activation with anti-CD3/anti-CD28 antibody since it should activate all T cells present. The proportion of T_{CM} increases steadily over time following stimulation, however, the proportion of T_{CM} decreases after restimulation (day 5 sample). This indicates that restimulation induces a shift in T cells to an effector phenotype. Overall, *ex vivo* stimulation of PBMCs induces rapid T cell differentiation into multiple effector and memory cell subsets.

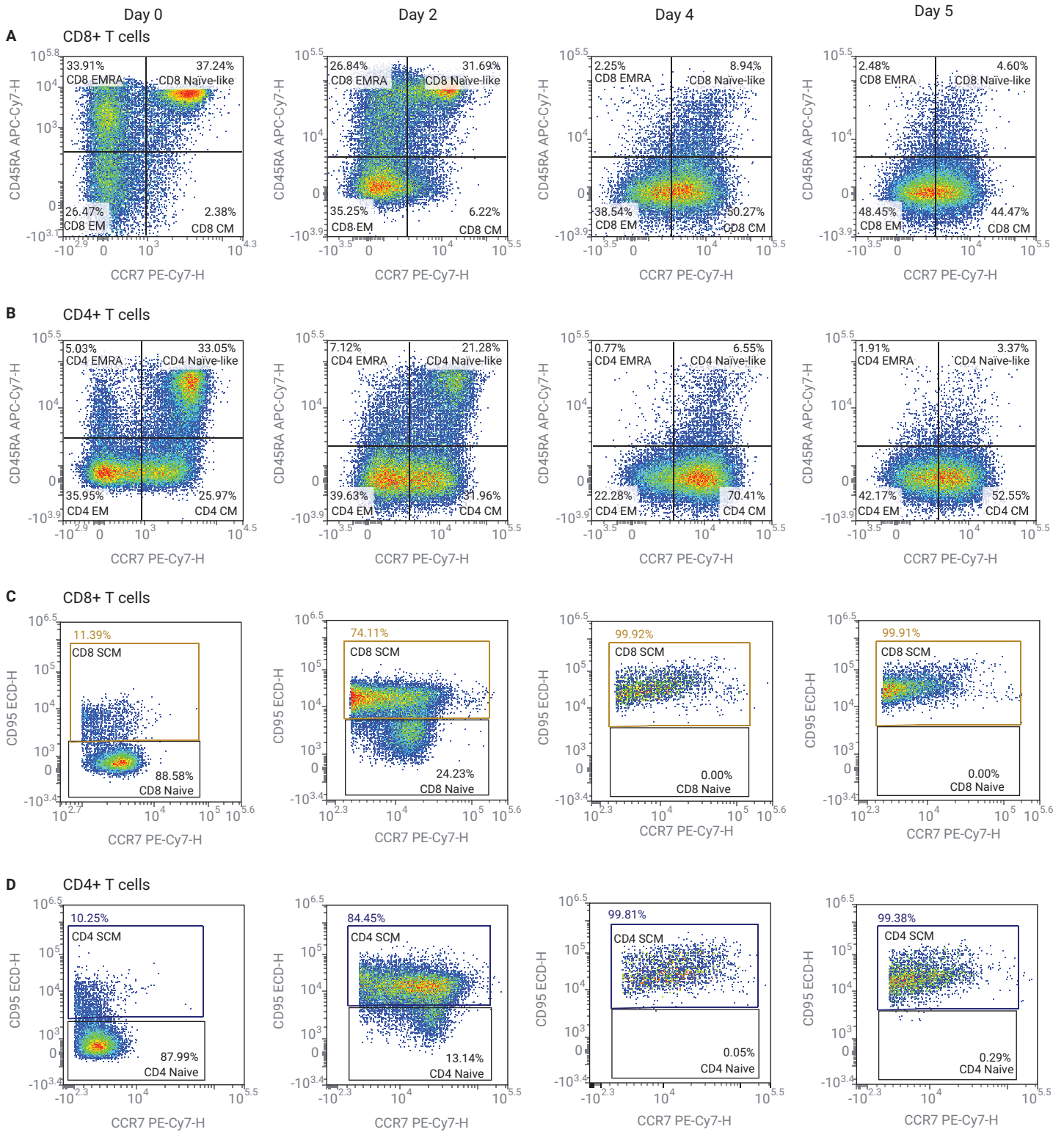


Figure 2. Kinetic changes of T-cell subsets is continuous. PBMCs isolated from donor blood were stimulated with 5 $\mu\text{g}/\text{mL}$ anti-CD3 antibody, 2 $\mu\text{g}/\text{mL}$ anti-CD28 antibody, and 100 ng/mL IL-2. Stimulated PBMCs were stained with the 16-color immunophenotyping panel (Table 1) on days 0, 2, and 4. Cells were restimulated on day 4, and analyzed 24 hours later (day 5). The expression CCR7 and CD45RA was analyzed at each time point for CD8 T cells (A) and CD4 T cells (B) to determine the relative change of T cell subsets; Naïve-like, central memory (CM), effector memory (EM), and effector memory RA (EMRA). CD8 and CD4 Naïve-like T cells were further analyzed for CD95 expression to distinguish stem cell memory cells (SCMs) from truly naïve T cells. The proportion of CD4+T cells, CD8+T cells and T_{SCM} , T_{CM} , T_{EM} , T_{EMRA} , and $T_{naïve}$ cells changed over time.

Progressive upregulation of inhibitor receptor upon TCR stimulation

With persistent stimulation, inhibitory receptor expression increases, and is a key indicator of T cell exhaustion. Efficient negative regulation by inhibitory receptors helps to restrain overexuberant immune responses. However, inhibitory receptors can also hinder effective immune responses to clear pathogens and tumors. Defining how inhibitory receptors are upregulated during acute and persistent T cell stimulation is essential for the development of safer and more effective therapeutic manipulations of these pathways.

Activation markers (CD25 and CD127) and inhibitory receptors (PD-1, LAG-3, and TIM-3) were examined on days zero, two, and four and after restimulation on day five (Figure 3). CD25 was rapidly upregulated while CD127 was downregulated in both CD8 and CD4 T cells, demonstrating rapid activation of T cells. No immediate change was observed in the expression of the inhibitory receptors after two days of stimulation. However, after four days of stimulation, PD-1, LAG-3, and TIM-3 were upregulated in both CD4 and CD8 T cell populations. After restimulation on day 5 with anti-CD3/CD28, all inhibitory receptors

were downregulated. This coincided with a decrease in CCR7 expression and decrease in the percentage of T_{CM} cells after restimulation. As T cells transition toward an effector phenotype, it is beneficial to decrease inhibitory receptor expression. This correlates with previous studies that suggest that T cell exhaustion occurs as cells move to a memory phenotype, despite the persistence of antigen. Examining inhibitory receptor expression in the context of various T cell phenotypes and functions provides an understanding of the how T cells are altered in the context of an immune response.

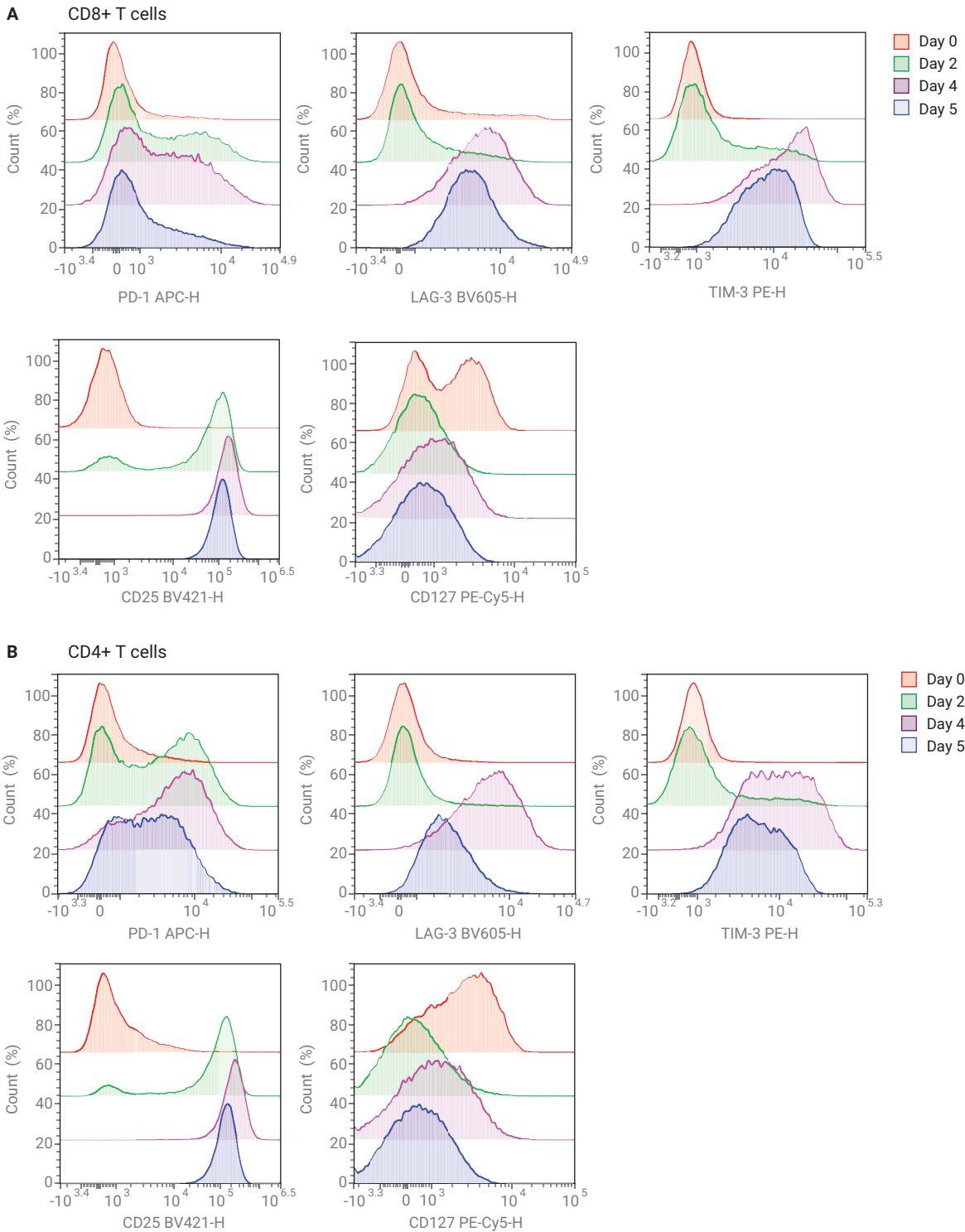


Figure 3. Change of expression level of inhibitory and activation receptors upon stimulation. PBMCs isolated from donor blood were stimulated with 5 $\mu\text{g}/\text{mL}$ anti-CD3 antibody, 2 $\mu\text{g}/\text{mL}$ anti-CD28 antibody, and 100 ng/mL IL-2. Stimulated PBMCs were stained with the 16-color immunophenotyping panel (Table 1) on days 0, 2, and 4. Cells were restimulated on day 4 and analyzed 24 hours later (day 5). Inhibitory receptors PD-1, LAG-3, and TIM-3 and activation markers CD25 and CD127 were analyzed over time.

Conclusion

Thorough analysis of T cells allows a closer look into the T cell response by examining differentiation, activation, cytokine production, and degranulation simultaneously so that a more comprehensive understanding of T cells can be achieved. Using the NovoCyte Advanteon to perform this 16-color immunophenotyping panel can help further elucidate the T cell response in the context of persistent infection and tumor immunity.

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