Characterization of Cytotoxic Immune Cells in Human Peripheral Whole Blood

Assessment of phenotypic and functional markers using a 16-color panel and the BD FACSymphony™ A1 Cell Analyzer

Features

- Attain premium flow cytometry performance with the BD FACSymphony™ A1 Cell Analyzer that leverages BD FACSymphony™ instrument technology in a compact size
- Analyze up to 16 colors and 19 parameters (including optional small particle side scatter channel) to detect various subsets of cytotoxic cells in blood using four high-power lasers
- Get clear resolution of dim signals when optimal panel design is combined with high instrument sensitivity

Cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells have become attractive therapeutic targets for cancer immunotherapy because of their efficient mechanisms of killing cancerous cells. Both effector CTLs and mature NK cells present a complex expression pattern of cytolytic proteins that dictates their ability to kill target cells. Using an optimized buffer system that allows simultaneous red blood cell lysis and cell fixation, we could examine the intracellular levels of cytolytic proteins directly in peripheral blood cells. The cells were analyzed in a BD FACSymphony™ A1 Cell Analyzer, which enabled the use of several bright or very bright fluorochrome-conjugated antibodies for optimal resolution of the cytolytic proteins and other markers that are rather low expressed in cytotoxic cells. Altogether, a coordinated analysis of a total of 16 intracellular or surface markers helped to detect various subsets of cytotoxic cells in blood.

BD

Protocol

Human peripheral blood was collected in sterile BD Vacutainer® Blood Collection Tubes containing sodium heparin. Predetermined optimal concentrations of fluorochrome-conjugated antibodies against surface markers (Table 1) were mixed in the presence of BD Horizon™ Brilliant Stain Buffer Plus and dispensed in 5 mL Corning™ Round-Bottom Polysterene Test Tubes. Next, 200 µL of heparinized blood and 2 µL of BD Horizon™ Fixable Viability Stain 575V at 0.6 µg/µL were sequentially added to the tubes and briefly vortexed. After 20 min incubation at room temperature in the dark, 4 mL of BD Phosflow™ Lyse/Fix Buffer (1X) was added to the tubes for red blood cells lysis and leucocytes fixation. Following 15 min incubation, the cells were spun down and washed twice with BD Pharmingen™ Stain Buffer (FBS). Then, the fixed cells were permeabilized in 100 µL of BD Phosflow™ Perm/Wash I Buffer for 20 min at 4 °C and incubated with antibodies against intracellular markers for an additional 60 min protected from light. After two washes, the cells were acquired in the BD FACSymphony™ A1 Cell Analyzer.

Table 1. Instrument configuration and reagents in the cytotoxic immune cells panel

Laser	Filter	Fluorochrome	Specificity	Clone	Catalog Number	Purpose	[§]Antibody Volume (mL) / 100 mL Blood or Perm Buffer
Violet 405 nm	450/50	BV421	Perforin	dG9	563393	Cytolytic abilities	5
	525/50	BV480	CD159a (NKG2A)	131411	747923	Inhibitory receptor	5
	610/20	BV605	CD19	HIB19	740394	Exclusion/B cells	1.25
			CD14	M5E2	564054	Exclusion/monocytes	5
			CD123	7G3	564197	Exclusion/plasmacytoid dendritic cells and eosinophils	5
			CD141	1A4	740421	Exclusion/myeloid cells and platelets	5
		FVS575V	-	-	565694	Viability	1
	670/30	BV650	CD3	UCHT-1	563851	T cells	5
	710/50	BV711	CD314 (NKG2D)	1D11	563688	Activating receptor	5
	780/60	BV786	HLA-DR	G46-6	564041	Activation marker	2.5
Blue 488 nm	530/30	FITC	CD57	NK-1	555619	Maturation and differentiation marker	10
	710/50	PerCP-Cy5.5	CD8	RPA-T8	560662	Cytotoxic T cells	5
Yellow-Green 561 nm	586/15	PE	CD158 (KIRs)	HP-MA4	567158	Maturation and differentiation marker	5
	610/20	PE-CF594	CD56	R19-760	564963	NK cells/activation marker	5
	670/30	PE-Cy5	CD95 (Fas)	DX2	559773	Differentiation marker	20
	710/50	PE-Cy5.5	CD127 (IL7R-a)	eBioRDR5	35-1278-42[#]	Differentiation marker/innate lymphoid cells	1.25
	780/60	PE-Cy7	CD38	HIT2	560677	Differentiation marker	2.5
Red 637 nm	670/30	AF647	Granzyme K	G3H69	566655	Cytolytic abilities	5
	710/50	R718	Granzyme B	GB-11	566964	Cytolytic abilities	1.25
	780/60	APC-H7	CD16 (FcgRIII)	3G8	560195	NK cells/cytolytic abilities	5

BV, BD Horizon Brilliant Violet™; FVS, BD Horizon™ Fixable Viability Stain; AF, Alexa Fluor™; [#]Thermo Fisher Scientific; [§]See Certificate of Analysis for antibody concentration; Perm: Permeabilization.

Cell Analysis Results

The BD FACSymphony™ A1 Cell Analyzer is equipped with four lasers (Violet, Blue, Yellow-Green and Red) and allowed the detection of cells stained with 13 bright or very bright fluorochrome-conjugated antibodies. The high sensitivity of the instrument combined with the bright fluorochromes resulted in highly efficient resolution of markers that are expressed at variable or low levels among cytotoxic cell populations, such as CD3, CD56, perforin, CD314 (NKG2D) and CD159 (NKG2A) (Figures 1 and 2).

Detection of rare populations was achieved by recording a minimum of 150,000 lymphocytes and then excluding doublets, dead cells and non-relevant cell lineages during cell analysis using FlowJo™ v10.7.2 Software. A total of eight healthy donors were pre-evaluated and the results shown in this panel sheet are from one donor whose peripheral blood presented high frequencies of five major cell subsets of interest: (pink) $CD56^{bright}CD3^{-}$ cytokine-producing NK cells, (blue) $CD56^{dim}CD3^{-}$ cytotoxic NK cells, (brown) $CD56^{dim}CD3^{+}$ including NKT cells, (green) $CD56^{dim}CD3^{bright}$ including γδ T cells and (purple) conventional CD8 cytotoxic T (Figure 1).

Figure 1. Cytotoxic cells sub-setting

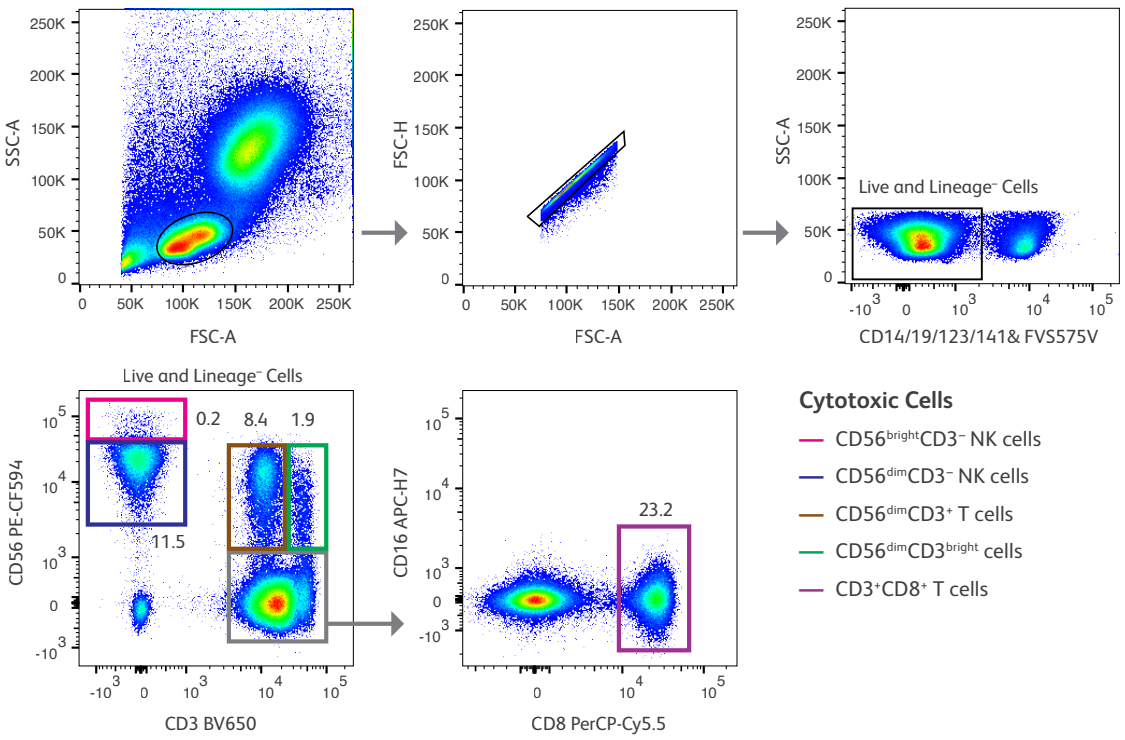

Figure 1. Clear separation of cytotoxic immune cell populations in healthy human peripheral blood
Based on forward and side scatter cell features, an initial gate around lymphocytes excluded debris and most monocytes/granulocytes. Subsequent gates eliminated cell doublets. Then, FVS575V-labeled dead cells and other lineage cells (CD19⁺/CD14⁺/CD123⁺/CD141⁺) were also excluded enriching the samples with T cells and NK cells. Within live and lineage-negative cells, analysis of CD56 versus CD3 revealed various cell populations that were color coded as cytokine-producing NK cells (pink), cytotoxic NK cells (blue), CD56⁺ T cells containing NKT cells (brown), CD56⁺ T cells containing γδ T cells (green) and cytotoxic CD8⁺ T cells (purple).

NK and cytotoxic T cell subsets utilize common mechanisms to kill infected or transformed cells via the granule exocytosis pathway. The key components of the cytolytic granules are perforin and several serine esterases, termed granzymes. Herein, we simultaneously assessed the intracellular levels of granzyme K (GrzmK), granzyme B (GrzmB) and a series of differentiation markers for a comprehensive analysis of the functional status of these cells (Figures 2 and 3).

Figure 2A. Cytolytic proteins

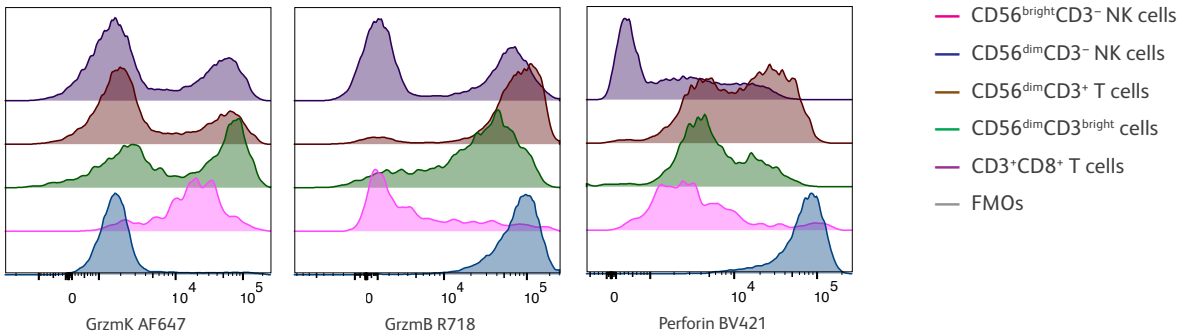

Figure 2B. Cell differentiation markers

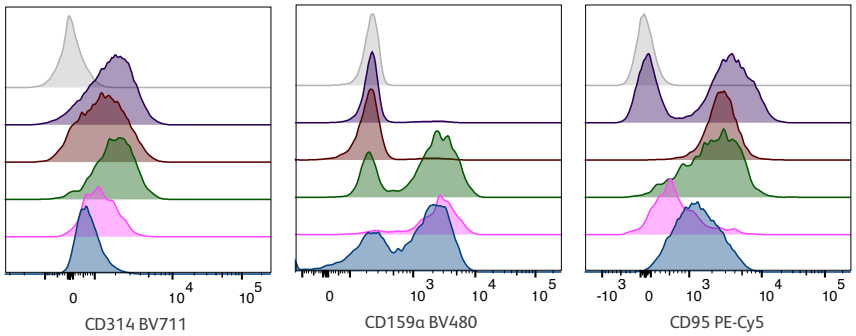

Figure 2. Comparative analysis of circulating cytotoxic cells
Each colored histogram represents a cell population gated as depicted in Figure 1. **A.** The top row reveals the expression of key granzymes (GrzmK and GrzmB) and perforin. **B.** The bottom row shows the expression of two major activating (CD314, NKG2D) and inhibitory (CD159a, NKG2A) receptors and the death receptor CD95 (Fas). The fluorescence minus one (FMO) histograms were generated after gating on the total live and lineage negative cells.

Figure 3A.

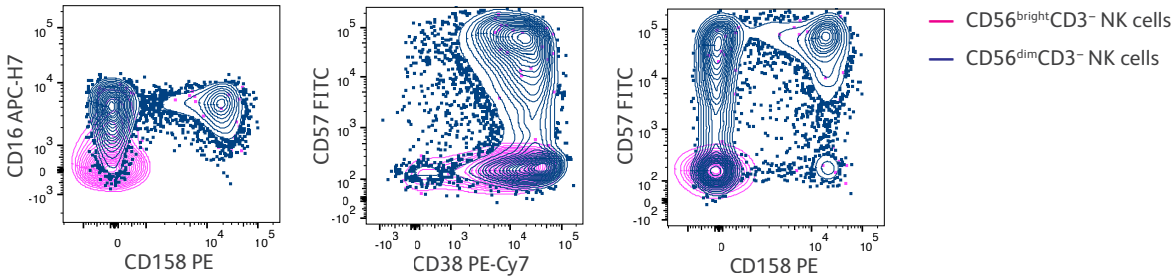

Figure 3B. CD56^{dim}CD3^{+} T cells

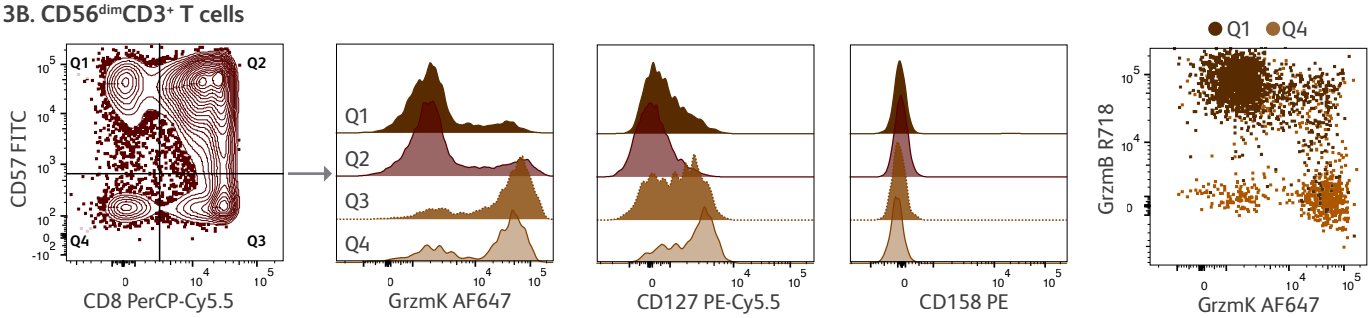

Figure 3C. CD56^{dim}CD3^{bright} T cells

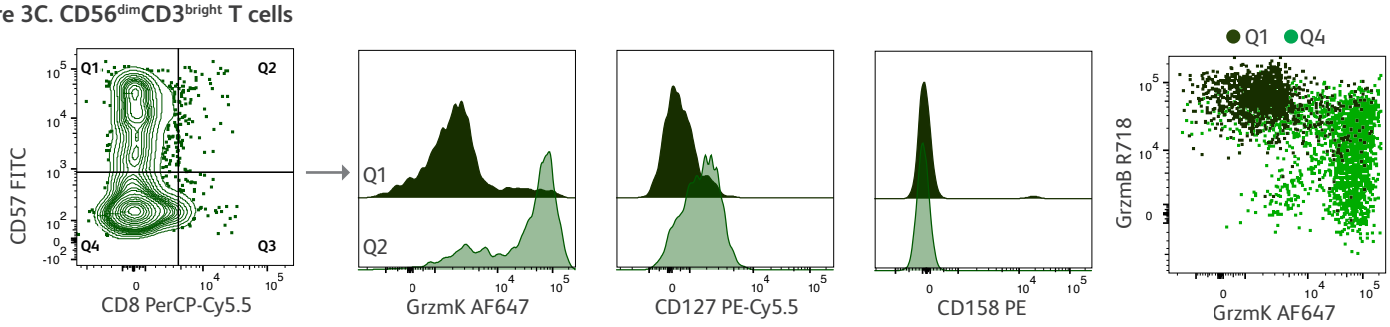

Figure 3D. CD3^{+}CD8^{+} T cells

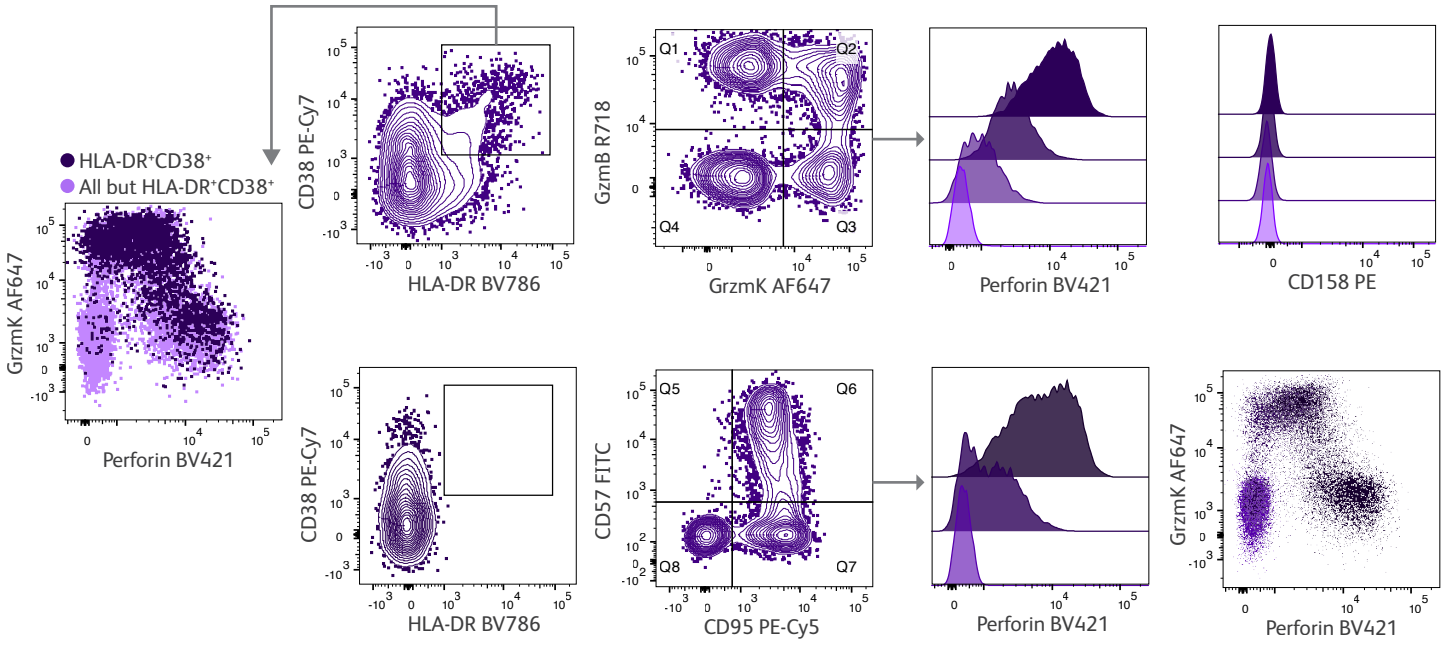

Figure 3. Phenotyping of circulating cytotoxic cells using a 16-color panel

The plots represent the analysis of cytolytic proteins in combination with various cell differentiation markers, enabling a deeper characterization of the cell populations gated in Figure 1. **A.** Overlay of NK cell subsets. **B.** Analysis of CD56^{dim}CD3^{+} T cell subsets, highlighting cell subsets that express either GrzmB or GrzmK. **C.** Analysis of CD56^{dim}CD3^{bright} T cell subsets also showing GrzmB and GrzmK in the different subsets. **D.** Identification of activated CD8 T cells based on the expression of CD38 and HLA-DR. The HLA-DR FMO staining helped to determine the gating boundaries for proper detection of the double positive cells. The figure also depicts different CD8 T cell subsets based on the expression of GrzmB versus GrzmK or CD95 versus CD57.

Conclusion

In this study, we described an assay that provided a means to compare different types of circulating cytotoxic cells based on the expression of effector cytolytic proteins. These proteins as well as other differentiation markers were expressed in a range from low to high levels across the different cell populations. Thus, the use of high-performance reagents and instrumentation were critical to examine even the lowest expressed proteins for a thorough assessment of circulating cytotoxic cell populations.

BD Life Sciences, San Jose, CA, 95131, USA

bdbiosciences.com