Polyfunctional T Cell Panel

An optimized multicolor flow cytometry panel for simultaneous assessment of inhibitory receptor expression and cytokine production

Polyfunctionality is defined as the ability of individual T cells to produce multiple pro-inflammatory cytokines (e.g., IFN-γ, IL-2 and TNF) in response to activation[1]. Progressive loss of polyfunctionality and concomitant upregulation of inhibitory receptor(s) expression are hallmarks of T cell exhaustion, a dysfunctional state induced by chronic viral infections or cancer. Consequently, polyfunctionality is routinely assessed to measure the T cell response or determine the quality of that response upon stimulation by foreign pathogens, tumor cell antigens or vaccine candidates. The polyfunctional T cell panel is an 11-color flow cytometry panel designed for examining surface expression of well-characterized inhibitory receptors as well as cytokine production by $CD4^+$ and $CD8^+$ T cell subsets. The role of the five inhibitory surface receptors (PD-1, LAG-3, TIM-3, TIGIT and BTLA) included in the panel are well-documented in negative regulation of T cell function[2].

Table 1. Instrument configuration and reagent selection

Laser Line	Marker	Fluorochrome	Clone	Volume Per Test	Catalog Number
Violet 405 nm	TIGIT	BV421	741182	2.5 µL	747844
	CD223 (LAG-3)	BV480	T47-530	5 µL	746609
	CD272 (BTLA)	BV711	J168-540	5 µL	743987
	CD366 (TIM-3)	BV786	7D3	2.5 µL	742857
Blue 488 nm	IFN-γ	FITC	B27	0.6 µL	554700
	IL-2	PE	MQ1-17H12	5 µL	559334
	Live/Dead	FVS620	N/A	1 µL	564996
	CD297 (PD-1)	PE-Cy™7	EH12.1	5 µL	561272
Red 640 nm	TNF	APC	MAb11	20 µL	551384
	CD4	APC-R700	RPA-T4	5 µL	564975
	CD8	APC-H7	SK1	5 µL	560179

BD

Protocol

T cells were enriched from fresh human peripheral blood mononuclear cells from healthy donors (N=2) using BD IMag™ Human T Lymphocyte Enrichment Set. Freshly isolated T cells were stimulated for 14 days with Dynabeads® Human T-Activator CD3/CD28 Beads (Thermo Fisher Scientific) (25 µL/well; bead-to-cell ratio of 1:1) and recombinant human interleukin-2 (rhIL-2; 25 U/mL; Sigma-Aldrich). Either fresh T cells (Day 0) or stimulated T cells (Day 14) were treated for 5 hours with phorbol 12-myristate 13-acetate (PMA; 50 ng/mL; Sigma-Aldrich) and ionomycin (500 ng/mL; Sigma-Aldrich) in the presence of the BD GolgiPlug™ and BD GolgiStop™ Transport Inhibitors. Cells were then stained with the antibodies from the panel recognizing surface markers for 30 minutes at room temperature (RT), in the dark and in the presence of BD Horizon™ Brilliant Stain Buffer. Cells were then washed twice and stained with FVS620 in 1 mL PBS for 10 minutes at RT in the dark. Cells were washed twice in PBS with 5% FBS, prior to fixation and permeabilization with BD Cytofix/Cytoperm™ Fixation/Permeabilization Solution. Cells were then stained with the antibodies from the panel recognizing intracellular cytokines for 30 minutes at RT in the dark. Samples were acquired on a 3-laser (Violet/Blue/Red), 12-fluorescent parameter BD FACSLyric™ Flow Cytometer. The panel can also be run on any 3-, 4- or 5-laser flow cytometer with equivalent filter configuration, although panel performance may vary between different instruments.

Figure 1A

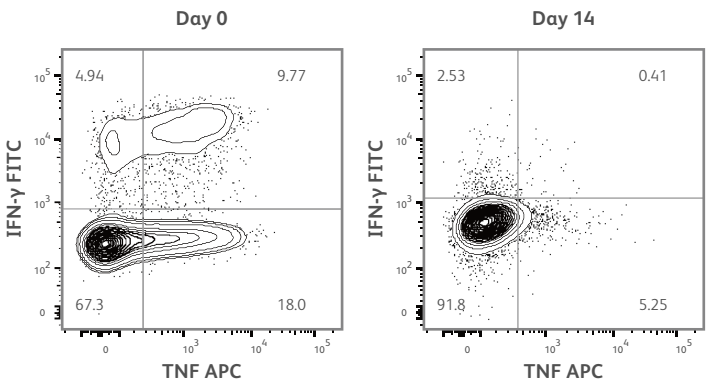


Figure 1B

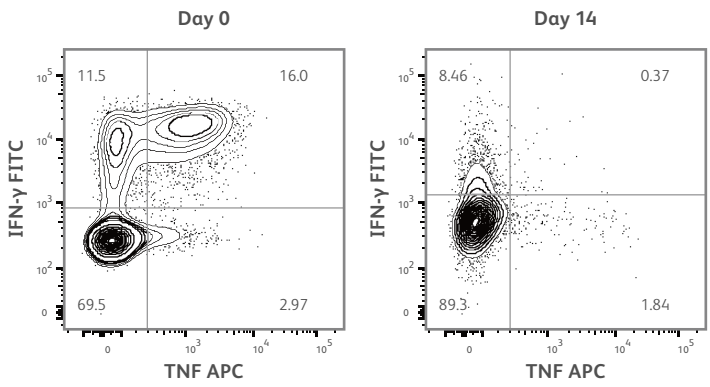
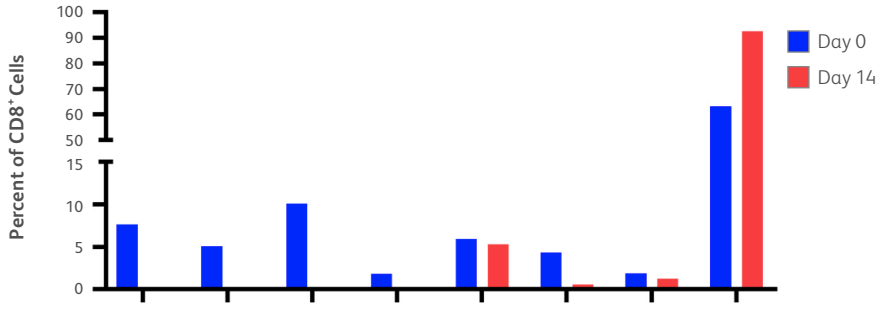


Figure 1C



IFN-γ	+	+	+	-	+	-	-	-
IL-2	+	+	-	+	-	+	-	-
TNF	+	-	+	+	-	-	+	-
Number of Cytokines	3	2			1			0

Figure 1. Representative analysis of intracellular cytokine expression

T cells were first gated based on scatter properties typical of lymphocytes (not shown). **A-B.** Production of IFN-γ and TNF is dramatically reduced in both CD4+ and CD8+ T cells upon chronic stimulation (Day 14), as compared to fresh cells (Day 0). **C.** Boolean gates were used to determine the frequency of CD8+ T cells producing either 3, 2, 1 or no cytokines. Polyfunctionality was lost after 14 days of chronic stimulation, as shown by the lack of cells producing more than one cytokine. Data were analyzed using FlowJo™ Software v10.6.2. The bar graph was created using GraphPad Prism 8.

Figure 2A

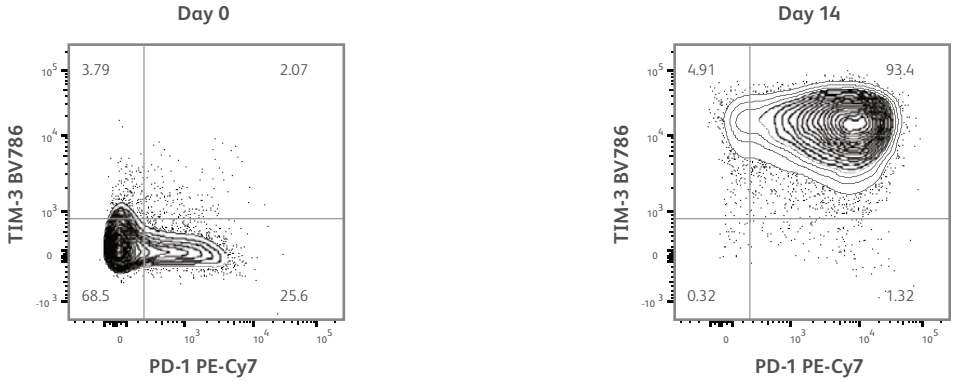


Figure 2B

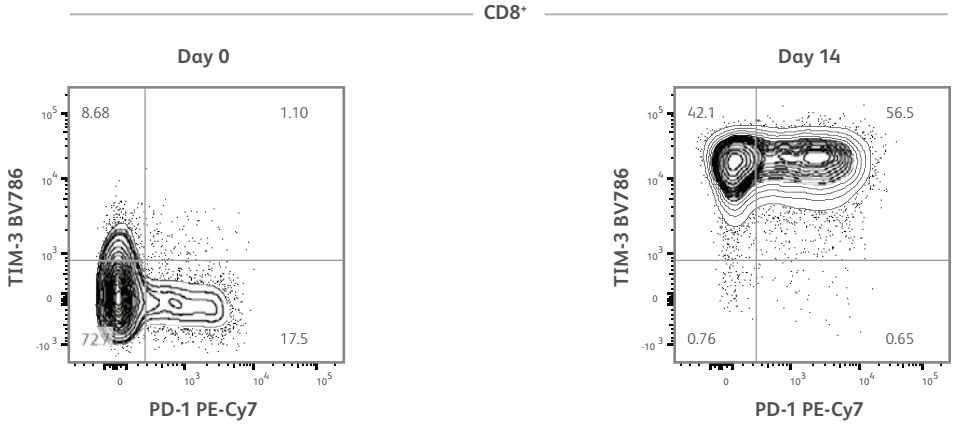
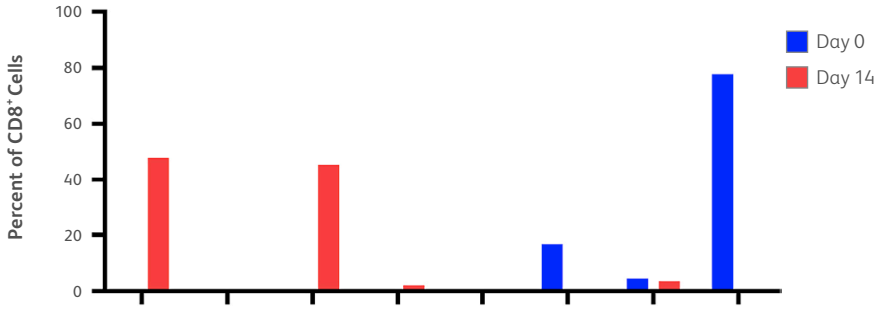


Figure 2C



LAG-3	+	+	+	-	+	-	-	-
PD-1	+	+	-	+	-	+	-	-
TIM-3	+	-	+	+	-	-	+	-
Number of Receptors	3	2			1			0

Figure 2. Representative analysis of inhibitory surface receptor combinatorial expression

T cells were first gated based on scatter properties typical of lymphocytes (not shown). **A-B.** Expression of TIM-3 and PD-1 was dramatically increased in both CD4+ and CD8+ T cells upon chronic stimulation (Day 14), as compared to fresh cells (Day 0). **C.** Boolean gates were used to determine the frequency of CD8+ T cells expressing either 3, 2, 1 or no inhibitory receptors. Expression of 2 or 3 inhibitory surface receptors on individual CD8+ T cells was observed only after 14 days of chronic stimulation. Data were analyzed using FlowJo™ Software 10.6.2. The bar graph was created using GraphPad Prism 8.

Conclusion

The data show that the polyfunctional T cell panel is a useful tool for simultaneous measurement of cytokine production and expression of inhibitory receptor(s) on T cell subsets.

References:

1. Boyd A, Almeida JR, Darrah PA, et al. Pathogen-specific T cell polyfunctionality is a correlate of T cell efficacy and immune protection. *PLoS One.* 2015;10(6):e0128714. doi: 10.1371/journal.pone.0128714.
2. Odorizzi PM, Wherry EJ. Inhibitory receptors on lymphocytes: insights from infections. *J Immunol.* 2012;188(7):2957-2965. doi: 10.4049/jimmunol.1100038.

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