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OMIP-037: 16-Color Panel to Measure Inhibitory Receptor Signatures from Multiple Human Immune Cell Subsets

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• **Key terms**
immune cell; checkpoint inhibition; T cell; NK cell; $\gamma\delta$ T cell; iNKT cell; inhibitory receptors; PD-1; TIGIT; CD160; LAG-3; TIM-3; cancer; tumor

Purpose and Appropriate Samples

The panel was developed to determine the combinational inhibitory receptor expression ("IR signatures") of CD4+ T cells, CD8+ T cells, Natural Killer (NK) cells, invariant Natural Killer T (iNKT) cells, and gamma delta ($\gamma\delta$) T cells from individual human samples. The inhibitory receptors measured are PD-1, TIM-3, CD160, LAG-3, and TIGIT. The activation marker CD137 (4-1BB) is also included in the panel, as is IL-7Rα (CD127). This panel works well with cryopreserved PBMC from healthy and HIV-infected individuals well as fresh tumor specimens. For optimum performance of this panel with digested tumor specimens, the pan-lymphocyte marker CD45 is included (swapped for LAG-3). Other tissues/sample types have not yet been evaluated. Also, a modification of this panel has been optimized that includes CD45RO and CD25 in place of CD160 and CD137.

Background

During many chronic diseases, populations of antigen-specific CD4+ and CD8+ T cells exhibit low functional activity, little/no proliferative capacity, and distinct molecular profiles; this state is defined as "exhaustion" or "checkpoint inhibition" (1–3). In addition to conventional T cells, there is evidence that "innate-like" immune subsets, such as Natural Killer (NK) cells, invariant Natural Killer T (iNKT) cells, and $\gamma\delta$ T cells undergo immune exhaustion as well (4–6). Exhausted immune cells are often characterized via the expression of inhibitory receptors such as PD-1, TIM-3, CD160, LAG-3, and TIGIT (7–10) Programmed Death 1, or PD-1, is upregulated on the surface of immune cells upon activation; after PD-1 binding with ligands PDL-1 and PDL-2 on neighboring cells, cell proliferation, cytokine production, and survival is inhibited (11–14). T cell immunoglobulin and mucin-containing protein 3, or TIM-3, is expressed on fully differentiated Th1/Tc1 lineage T cells, T regulatory cells (Tregs), and NK cells, as well as monocytes, macrophages, and dendritic cells (15). TIM-3 binding to Galectin-9 results in an inhibitory signal to T cells (16) and can induce T cell apoptosis in vitro (17). Also, NK cytotoxicity is abrogated upon TIM-3 cross-linking in vitro (18), and TIM-3 signaling is integral for tolerance induction and EAE amelioration in mouse models (17,19,20). CD160 has been defined as an activating receptor on NK cells (21); however, CD160+ human T cells receive an inhibitory signal upon binding with the ligand HVEM (her-

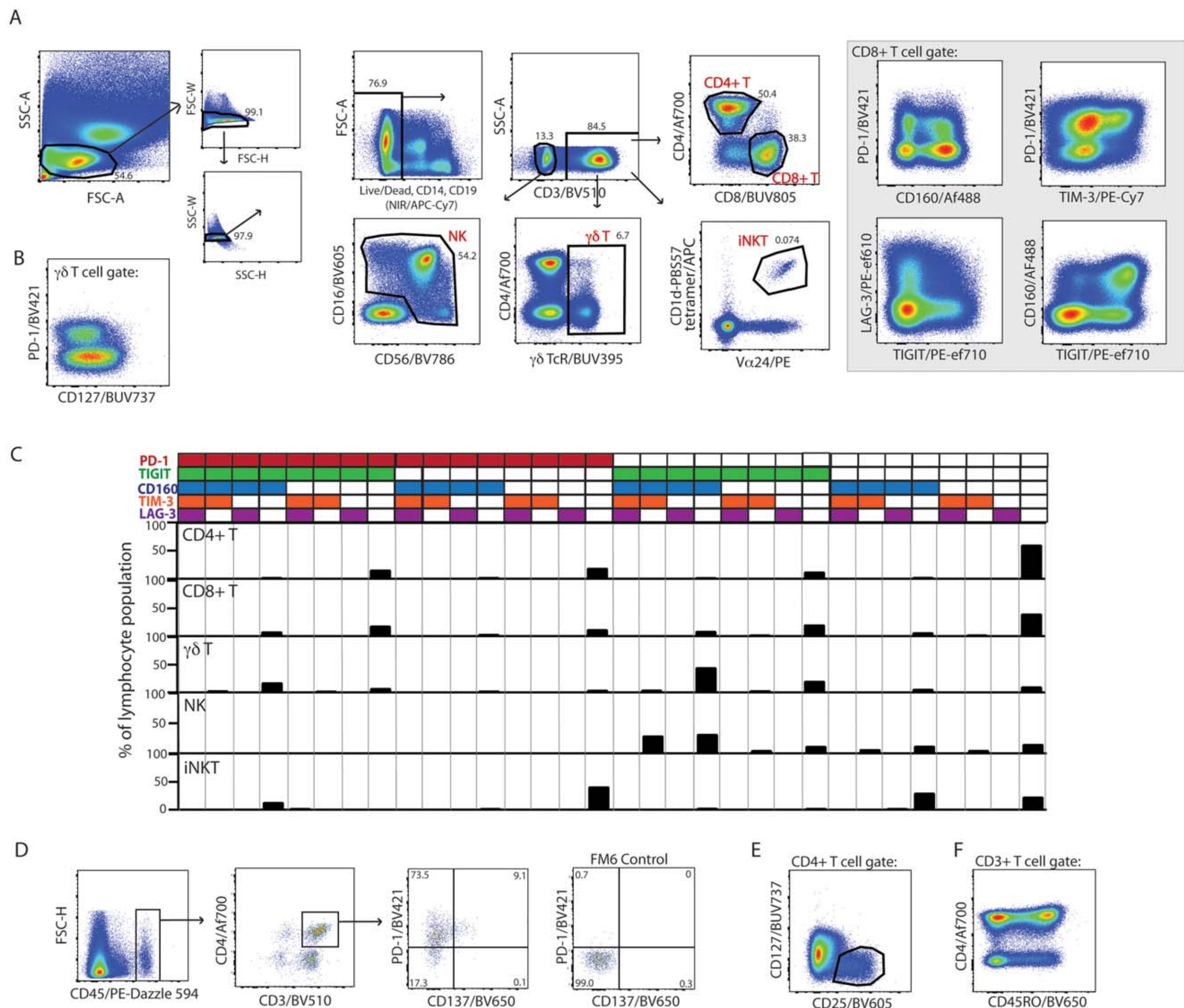

Figure 1. Gating Strategy for OMIP-037. (**A**) Bivariate plots from PBMC samples show lymphocyte gating via FSC:SSC, exclusion of doublets and non-viable, CD14+, and CD19+ cells. Next, immune cell subsets were defined as follows: (1) NK cells; CD3-; then variable expression of CD16 and CD56 (as shown), (2) $\gamma\delta$ T cells CD3+, $\gamma\delta$ TcR+; (3) iNKT cells; CD3+, Vα24+, CD1d-PBSS7 loaded tetramer+; (4) CD4+ T cells; CD3+, CD4+; and (5) CD8+ T cells; CD3+ CD8+. Examples of IR expression patterns from CD8+ T cells in ex vivo PBMC samples are shown the in gray box. It should be noted that the high percentage of LAG-3+ and TIM 3+ events show was found in only one healthy donor sample, much lower expression is typical. (**B**) CD127 staining is shown vs PD-1form $\gamma\delta$ T cells of a healthy PBMC sample. (**C**) Graphs of the IR expression signatures of five PBMC immune cell subsets from one healthy donor. Black bars indicate the percentage of cells positive for all possible combinations of five IRs listed. (**D**) TILs from a fresh oral cancer tissue specimen were stained ex vivo with a modified form of the 16–color IR panel (with CD45 in place of LAG-3). In this sample, CD137 expression is found on CD4+ T cells and is co-expressed with PD-1. The plot labeled "FM6 Control" shows staining of the same tumor sample stained without the CD137 and anti-IR antibodies. (**E**) "Treg" gating of PBMC via CD127 and CD25 and (**F**) CD45RO expression visualized with a modified version of the panel.

pesvirus entry mediator) (22) and NK cell function can be positively or negatively regulation via CD160 signaling, depending on the stimulus (23). Also, the functional impairment associated with CD160 on human CD8+ T cells was found to be distinct from PD-1 (24), underscoring the importance of combinational IR analysis to elucidate the breadth and mechanisms of checkpoint inhibition in chronic diseases. The Lymphocyte Activated Gene-3, or LAG-3, is expressed on several immune cell populations, including activated T cells and NK cells (25). After TcR stimulation of T cells, LAG-3 associates with TcR:CD3 complexes (26) and negatively impacts signal transduction (27). The T cell immunoreceptor with Ig and ITIM domains, TIGIT, is a member of the immunoglobulin superfamily that binds CD155 and CD112; soluble TIGIT suppresses T cell responses in vivo and in vitro (28). TIGIT is expressed on exhausted T cells in both cancer and chronic viral infections and anti-TIGIT antibody blockade can enhance T cell effector functions (29). CTLA-4, another well-characterized inhibitory receptor, is not included in this panel because it was not found on immune cells in the vast majority

Table 1. Summary table for OMIP-037

PURPOSE	INHIBITORY RECEPTOR PROFILING
Species	Human
Cell types	Immune cells
Cross-References	OMIP-031

of PBMC samples tested. CTLA-4 is primarily stored intracellularly and may be more readily detected if intracellular cytokine staining is performed; however, this panel is designed for use on a FACSARIA cell sorter or similar instrument to allow simultaneous phenotyping and sorting of viable cell populations of interest for further cell culture/study. The activation marker CD137 (4-1BB) is also included in the panel because of its established role as a co-stimulatory molecule that helps to maintain T cell activation (30) and data from mouse models show that when anti-CD137 and anti-PD-1 antibodies are used in combination, synergistic effects in tumor reduction ensue (31). CD127 (IL-7Rα) is a key regulator of T cell homeostasis and loss of CD127 expression is associated with exhaustion; analysis of virus-specific CD8+ T cells found that CD127 expression correlated positively with viral antigen-specific CD8+ T cell proliferation and negatively with PD-1 (32).

There is a more recent and rapidly expanding interest in measuring IR profiles on human immune cells due to the promising clinical trial results of cancer patients treated with therapeutics that target the PD-1: PD-1 L axis (33–37). A combination therapeutic regimen targeting multiple IRs (CTLA-4 and PD-1) resulted in a synergistic beneficial response in a clinical trial of melanoma patients (33); also, LAG-3 and PD-1 synergistically regulated T cell functions to promote cancer progression in a mouse tumor model (38). Taken together, these results indicate that combinational IR targeted therapies may lead to improved clinical trial results for individuals with cancer as well as other chronic diseases.

Given the ambiguities in how distinct patterns of IR expression reflect stages of the immune exhaustion process and the potential clinical benefit of combinational IR-based therapeutics, the ability to measure IR signatures from individual human immune cells is of high interest. Therefore, we developed an 18-parameter, 16-color panel that allows combinational analysis of five inhibitory receptors and one activation marker on five distinct immune subsets (Fig. 1, Tables 1 and 2, Supporting Information Online Methods). After gating on lymphocytes via FSC and SSC, and excluding doublets and dead cells, CD19+ cells, and CD14+ cells via a dump channel (CD19+ and CD14+ cells are removed for accurate NK cell gating), the following gating strategies are used to visualize immune subsets: 1) conventional CD4+ and CD8+ T cells (CD3+, CD4+ and CD3+, CD8+ gates, respectively), 2) iNKT cells (CD3+, vα24+, and CD1d-PBS57 loaded tetramer positive cells), 3) $\gamma\delta$ T cells (CD3+, $\gamma\delta$ TcR+), and 4) NK cells (CD3-, then differential expression of CD16 and CD56, as shown in Fig. 1A). Using this panel, IR signatures, comprised of all 32 possible combinations of PD-1, TIGIT, CD160, TIM-3, and LAG-3, are simultaneously measured from five immune cell subsets in PBMC directly ex vivo (Fig. 1B).

While the ex vivo phenotypic profiles of immune cells in the circulation have been linked to the health status of individuals with a variety of chronic diseases, analysis of cellular sources other than peripheral blood, such as tumors from cancer patients, has revealed that site-specific immunological characteristics track with disease prognosis (39–41). Therefore, we optimized a reagent swap option for this panel (CD45 for LAG-3; Table 3) to allow its use with human tissue

Table 2. Reagents used for OMIP-037

	SPECIFICITY	FLUOROCHROME	AB CLONE	PURPOSE
1.	$\gamma\delta$TCR	BUV 395	B1	$\gamma\delta$ T cells
2.	CD127	BUV 737	HIL-7R-M21	Exhaustion; Treg gating
3.	CD8	BUV 805	SK1	Lineage
4.	PD-1	BV 421	EH12.2H7	Exhaustion
5.	CD3	BV 510	OKT3	T cell lineage
6.	CD16	BV 605	3G8	NK cell lineage/subsets
7.	CD137	BV 650	4B4-1	Activation
8.	CD56	BV 786	NCAM16.2	NK cell lineage/subsets
9.	CD160	Af488	BY55	Exhaustion
10.	Vα24 TCR	PE	C-15	iNKT lineage
11.	LAG-3	PE-ef610	3DS223H	Exhaustion
12.	TIGIT	PE-ef710	MBSA43	Exhaustion
13.	TIM-3	PE-Cy7	F38-2E2	Exhaustion
14.	CD1d-PBS57 tetramer	APC	n/a	iNKT lineage
15.	CD4	Af700	RPA-T4	T_H cell lineage
16.	CD14	APC-Cy7	HCD14	Monocyte exclusion
17.	CD19	APC-Cy7	HIB19	B cell exclusion
18.	NIR Zombie	n/a	n/a	Viability
19.	CD16/32	n/a	n/a	FCγRIII blocking

Table 3. Additional reagents used in OMIP modifications

	SPECIFICITY	FLUOROCHROME	AB CLONE	PURPOSE
1.	CD45	PE-Dazzle 594	HI30	Hematopoietic cell lineage
2.	CD25	BV 605	BC96	Treg gating/T cell activation
3.	CD45RO	BV 650	UCHL1	Naïve/memory
4.	CD16	FITC	3G8	NK cell lineage/subsets

samples. We chose to remove LAG-3 due to the color availability of each and the low LAG-3 expression found in the tumors analyzed by our group to date. Bivariate plots generated from an oral cancer tumor specimen stained directly *ex vivo* with this panel show the high resolution of staining acquired and the clear presence of CD4+ T cells that express both PD-1+ CD137+ (Fig. 1C).

In addition, a modification of this panel has been developed where CD137 and CD160 were omitted and replaced with CD25 and CD45RO (Table 3) to allow enumeration of regulatory T cells ($CD3 + CD4 + CD25 + CD127^{low}$) and memory T cells ($CD3 + CD4+/CD8 + CD25^{var}CD45RO+$) (not shown, see Supporting Information Online Material).

All reagents were initially titrated as single color stains, then tested in Fluorescence Minus One (FMO) combinations (42) to evaluate spillover and staining artifacts in the channel of interest emerging from secondary channels. Different reagent combinations were tested and the optimal clones and/or conjugates that were selected for the final panel as listed in Table 2.

Further analysis of single cell IR signatures using flow cytometry panels including the one in this report could reveal previously unknown states of immune exhaustion, novel biomarkers for diagnostics, and new combinational targets for more effective therapeutic interventions for cancer and other diseases.

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Human Samples

All human blood samples were obtained via informed consent through multiple Boston hospitals, including the Boston University Medical Center. Approval of study protocols was obtained by the institutional review boards at each hospital. Tissue specimens, representing scalpel-generated incisional biopsies from primary tumors, were obtained at the Boston University Medical Center and approved for studies by the institutional review board.

Similarity to Published OMIPs

This OMIP is similar to OMIP-031 (43) in its objective to evaluate inhibitory receptors (i.e. checkpoint inhibitors); however, since OMIP-031 is applicable exclusively for mouse cells, no practical overlaps are possible between this panel and OMIP-031.

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