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OMIP-008: Measurement of Th1 and Th2 Cytokine Polyfunctionality of Human T Cells

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Additional Supporting Information may be found in the online version of this article.

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• **Key terms**
immunophenotype; T cells; intracellular cytokine staining; melanoma

Purpose and Appropriate Sample Types

This panel was optimized to assess $CD4^+$ and $CD8^+$ T cell responses to various tumor antigens from melanoma patients. The panel was tested on single-cell derived T cell isolates (SCD-T) and T cell lines derived from peripheral blood mononuclear cells (PBMC) from melanoma patients, T cell lines from the tumor environment of melanoma patients, and fresh and cryopreserved PBMC (healthy donors). Staining can be performed in 96-well plates for high-throughput.

Background

The T cell response to human melanoma is broad, encompassing both effector and regulatory T cell populations with diverse functional profiles (1). Tumor specific T cells with cytokine profiles that do not follow the standard Th1/Th2 dichotomy have been demonstrated (2,3). These descriptions include tumor antigen-specific effector and regulatory T cells that co-produce Th1 and Th2 cytokines. Thus, immunological monitoring of T cells from melanoma patients needs to assess a wide spectrum of functions and often has access to only a limited number of cells. To provide a tool for this immunological monitoring, we developed a panel of Ab-conjugates for assessing cytokine profiles that cross "traditional" T cell subset boundaries. As such, this panel would be useful for those interested in human T cells with a mixed cytokine profile, such as induced regulatory T cells, $IL\text{-}10^+$ $CD8^+$ T cells, T cells involved in allergic responses, etc. Further, it should be useful for samples with limited cell numbers, such as tumor biopsies, fine-needle aspirations, or cerebral spinal fluid, as well as samples from which multiple separate samples for Th1 versus Th2 intracellular cytokine staining are not possible. This panel was developed following guidelines described previously (4). To maximize sensitivity, the brightest fluorochromes were reserved for IL-2, IL-4, IL-10, IFN-γ, and TNF-α. A dump channel was used to exclude dead cells, B cells, and monocytes/macrophages. Next, testing a collection of Ab-conjugates optimized the differentiation of T cell subsets. APC-Cy7 and Quantum dot (QD) 605 were selected for discrimination of CD4 and CD8, respectively, because of their stability in this panel (Fig. 1 and Table 1). CD3 was labeled after fixing and permeabilizing the cells to ensure that activated T cells having down-regulated their TCR/CD3 complex were included in the analysis (4). To permit resolution of CD4 following activation of T cells with PMA and ionomycin (used as a mitogenic control), CD4 was also labeled post-fixation/permeabilization for all samples. Specificity of the cytokine staining is demonstrated by reduced staining following blocking steps prior to intracellular staining with the fluorochrome-conjugated anticytokine reagents: (i) the surface-stained, fixed, and permeabilized cells are blocked with purified anticytokine antibodies of the identical clones and (ii) the fluorochrome-conjugated reagents are blocked with recombinant human cytokine (Table 2). These blocking steps typically reduce $cytokine^+$ staining to background levels, or to a level allowing unambiguous gate setting, and as such these fully stained and blocked samples are used to determine placement of $cytokine^+$ gates. This provides an alternative to individual FMO controls for each cytokine, which may not be feasible due to limited cell numbers from patient samples. The level of autofluorescence of the T cell lines has not been a substantial confounder in these experiments and we have not noted marked differences between the autofluorescence of cultured as compared with uncultured T cells from healthy donors.

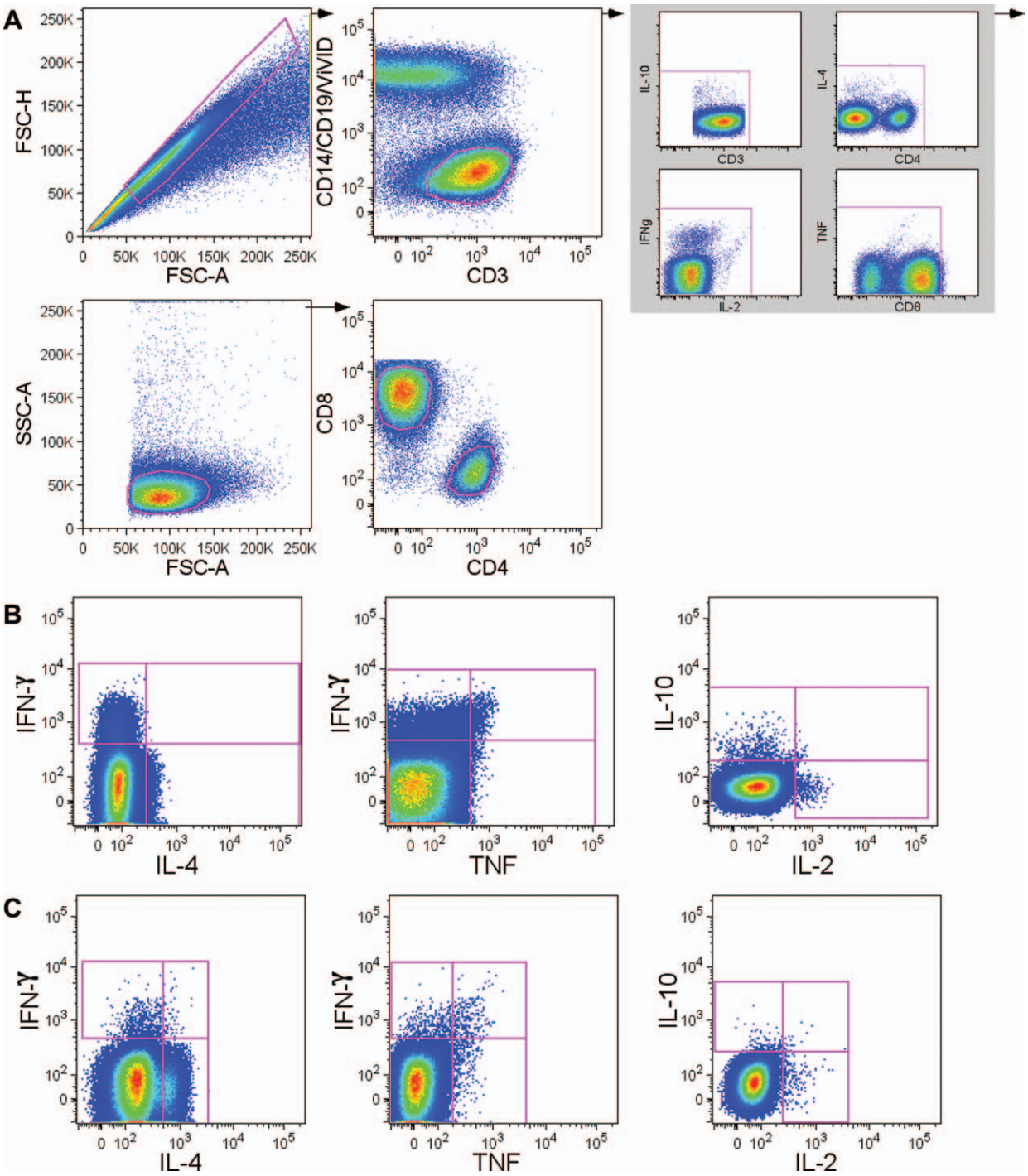

Figure 1. Gating strategy and example staining. **A**: Cells in the singlet gate are selected, live $CD3^+$ $CD14^-$ $CD19^-$ cells identified, followed by exclusion of dye aggregates (gray box) and after gating on small lymphocytes, $CD4^+$ and $CD8^+$ T cells are selected for further analysis. $CD4^+$ and $CD8^+$ T cells positive for individual cytokines are separately gated and subsequently, a Boolean gate encompassing all combinations (2^5) of $cytokine^{+/-}$ T cells is created. The median fluorescence intensity (MFI) of $cytokine^+$ T cells is evaluated, providing a measure of the magnitude of the response. **B** and **C**: Determination of $cytokine^+$ T cells following gating described in (A). **B**: Shown is a $CD4^+$ T cell line derived from the tumor of a melanoma patient stimulated with an allogeneic melanoma tumor cell line. The frequency of T cells positive for IFN-γ, TNF-α, IL-2, or IL-10 following antigenic stimulation was significantly greater than unstimulated T cells. **C:** Shown is a $CD4^+$ T cell line derived from the tumor of a melanoma patient stimulated with an autologous melanoma tumor cell line. The frequency of T cells positive for TNF-α, IL-10, or IL-4 following antigenic stimulation was significantly greater than unstimulated T cells. The T cell lines in B and C are from two different patients stimulated with the same melanoma cell line. Data in B and C are presented as low-resolution dot plots. Robust cytokine responses from mitogen-stimulated PBMC are presented in Supporting Information Figure S7.

Table 1. Summary table for application of OMIP-008

Purpose	T cell cytokine production following in vitro stimulation
Species	Human
Cell types	PBMC, T cell clones/lines, tumor-resident T cell lines
Cross references	OMIP-001

Table 2. Reagents used for OMIP-008

SPECIFICITY	CLONE	FLUOROCHROME	PURPOSE
IL-2	MQ1-17H12	PerCP-Cy5.5	Function
IL-4	8D4-8	Ax488	
IL-10	JES3-9D7	PE	
IFN-γ	B27	APC	
TNF-α	MAb11	PE-Cy7	
CD3	UCHT1	Ax700	Lineage
CD4	RPA-T4	APC-Cy7	
CD8	3B5	QD605	
CD14	M5E2	PacBlu	Dump
CD19	HIB19	eFluor450	
Dead cells	–	ViViD	

APC, allophycocyanin; Ax, Alexa; Bi, biotin; Cy, cyanine; PacBlu, pacific blue; PE, R-phycoerythrin; PerCP, peridinin chlorophyll protein; QD, quantum dot; ViViD, LIVE/DEAD fixable violet dead cell stain.

Human Subjects

The Health Sciences Institutional Review Board that serves both the William S. Middleton Memorial Veterans Hospital and the University of Wisconsin-Madison approved this study (Human Subjects Protocol # 1992-031). Written informed consent was obtained from the participants in this study.

Similarity to Published OMIPs

OMIP-001 was designed to detect cytokine-producing T cells, including IL-2, IFN-γ, and TNF-α (5). Our panel extends OMIP-001 with the addition of IL-4 and IL-10, thus broadening its potential application for the discrimination of T cells exhibiting traditional Th1 and Th2 responses, as well as those with unconventional cytokine profiles.

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