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OMIP-022: Comprehensive Assessment of Antigen-Specific Human T-Cell Functionality and Memory

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• **Key terms**
OMIP; cytometry; FACS; tuberculosis; T cell; ICS; cytokine staining; T-cell memory

Purpose and Appropriate Sample Types

This flow cytometry antibody panel was developed and optimized for the characterization of CD4+ and CD8+ T-cell memory and functional responses in adult and infant cryopreserved peripheral blood mononuclear cells (PBMC) stimulated with peptide pools to various antigens of interest (Table 1). The panel has been used to evaluate *Mycobacterium tuberculosis* (TB) antigen-specific responses in clinical trial specimens, and is currently undergoing assay qualification.

Background

The lack of a correlate of immunity is a source of frustration in TB research (1). As such, it is crucial for TB vaccine researchers to "cast a wide net" when assessing immune responses to clinical trial candidates while minimizing the amount of specimen required to generate these data. This panel (Table 2) began as an incremental enhancement to OMIP-014 (2), and included markers for dump (fixable viability dye, CD14, and CD19), T-cell phenotype (CD3, CD4, and CD8), Th1 cytokines (IFN-γ and TNF), a Th2 cytokine (IL-4), T-cell proliferation cytokine (IL-2), degranulation (CD107a), and activation (CD154). We have also expanded the panel to include memory markers (CCR7 and CD45RO), a Th17 cytokine (IL-17A), and an IL-10 superfamily cytokine (IL-22, used in place of IL-4).

We chose to combine viability dye and dump markers on the same channel using AViD and V500 (for CD14 and CD19), thus freeing additional channels on the violet laser. For phenotyping, CD3 remains on ECD and CD4 on APC-eFluor 780.

Th1 cytokines have been shown to be critical for controlling TB (3–9). To assess Th1 responses, we utilize antibodies against tumor necrosis factor (TNF) and IFN-γ. In designing this panel, we moved TNF to PE-Cy7 to improve detection of this cytokine, as fluorescein isothiocyanate (FITC) is comparatively dimmer and more prone to photobleaching. The recently introduced Brilliant Violet series of fluorochromes represents a bright and economical set of tools for detecting markers via the violet laser. Brilliant Violet 421 (BV421) was previously shown to be of similar brightness to PE (10), and was initially chosen as a replacement fluorochrome for IFN-γ. However, further investigation showed that any increase in antigen-specific response detected using the BV421 conjugate was matched with a significant increase in IFN-γ background. This increased background was observed using both the B27 (BD Bio-

Table 1. Summary table for application of OMIP-022

PURPOSE	T-CELL PHENOTYPING, MEMORY CATEGORIZATION, CYTOKINE PRODUCTION, AND FUNCTION FOLLOWING IN VITRO STIMULATION
Species	Human
Cell types	Cryopreserved PBMC (adult and infant)
Cross references	OMIP-001, OMIP-008, OMIP-009, and OMIP-014

sciences, San Jose, CA) and the 4S.B3 (Biolegend, San Diego, CA) clones. When this background population was excluded from the IFN-γ gate of the BV421 conjugates, the responses detected using either the BV421 conjugates or the V450 conjugate were similar. This issue, combined with the increased cost of the reagent, led us to choose to continue to use the V450 conjugate for IFN-γ detection in this panel.

Interleukin-2 (IL-2) is a cytokine responsible for T-cell proliferation and differentiation (11,12) that also may play a role in TB immunity (13). In our panel, we use PE for bright and efficient detection of this cytokine.

As *M. tuberculosis* is a facultative intracellular bacteria, cytolytic activity may be necessary to help control the disease. CD107a (LAMP-1) is a marker for degranulation and an indirect marker for cytolytic function (14,15). Ideally, lytic proteins such as Perforin would be costained with the CD107a to confirm the cytotoxic potential of the CD107a+ cells. To this point, we are examining future iterations of this panel that may include a lytic marker. However, the current constraints of this panel limit our ability to add an additional marker without harming sensitivity in detecting the other markers of interest. To accommodate the change in fluorochrome for the detection of TNF, we moved CD107a to FITC initially, and then replaced FITC with the enhanced stability of Alexa Fluor 488 (Ax488).

The Brilliant Violet series of fluorochromes has also allowed us to include the memory markers CCR7 and CD45RO. CCR7 is responsible for homing T-cells to lymph nodes (16) and is expressed on naïve and central memory T-cells (T_{CM}) (17). CD45RO is the smallest isoform of CD45, and has been shown to be expressed on effector memory (T_{EM}) and T_{CM} (18). The inclusion of both markers allows for T-cells to be categorized as naïve, T_{CM}, T_{EM}, or effector T-cells. We chose Brilliant Violet 605 for CCR7 as it was the next brightest available Brilliant Violet dye and CCR7 is expressed as a continuum (not distinct populations) (19). The binding kinetics of the CCR7 antibody has shown optimal staining when incubated at 37°C (see staining protocol in Supporting Information). Brilliant Violet 785 was chosen for CD45RO based on antibody availability and minimal spectral overlap to other markers.

IL-17A is secreted by Th17 T helper cells and acts to recruit innate immune effector cells to the site of inflammation (20). Recent works (21,22) have suggested a strong role for IL-17A in tuberculosis immunity. As such, we initially replaced the MIP-1β from OMIP-014 with and anti-IL-17A antibody. In our hands, the PerCP-Cy5.5 on CD8 causes high spectral overlap into the Ax700 channel used for IL-17A. As IL-17A is lowly expressed compared to CD8 (and as PerCP-Cy5.5 is fluorescently brighter), we exchanged the fluorochromes for these two markers to minimize the impact of the high compensation.

As an effect of changing CD8 to Ax700, a reduction in CD8+ events [perhaps an escapee phenomenon (23)] was noted using Ax700 to detect CD8, but was mitigated by staining for CD8 intracellularly.

IL-4, previously included with OMIP-014, is a representative Th2 cytokine. This interleukin is extremely difficult to detect and, in our hands, is inconsistent. We continue to include this marker as an option when assessing clinical trial specimens in which Th2 is considered a desired response. Recently, however, we have substituted IL-22 on APC instead

Table 2. Reagents used for OMIP-022

SPECIFICITY	CLONE	FLUOROCHROME	PURPOSE
Viability Dye	–	AViD	Dump
CD14	M5E2	V500	
CD19	HIB19	V500	
CD3	UCHT1	ECD	Phenotype
CD4	RPA-T4	APC-eFluor 780	
CD8	HIT8a	Ax700	
CCR7	G043H7	BV605	Memory
CD45RO	UCHL1	BV785	
IFN-γ	B27	V450	Th1
IL-2	MQ1–17H12	PE	
TNF	MAb11	PE-Cy7	
IL-17A	BL168	PerCP-Cy5.5	Th17
IL-22	IL22JOP	APC	Th22
CD107a	H4A3	Ax488	Degranulation
CD154	TRAP1	PE-Cy5	Activation/B-Cell Help

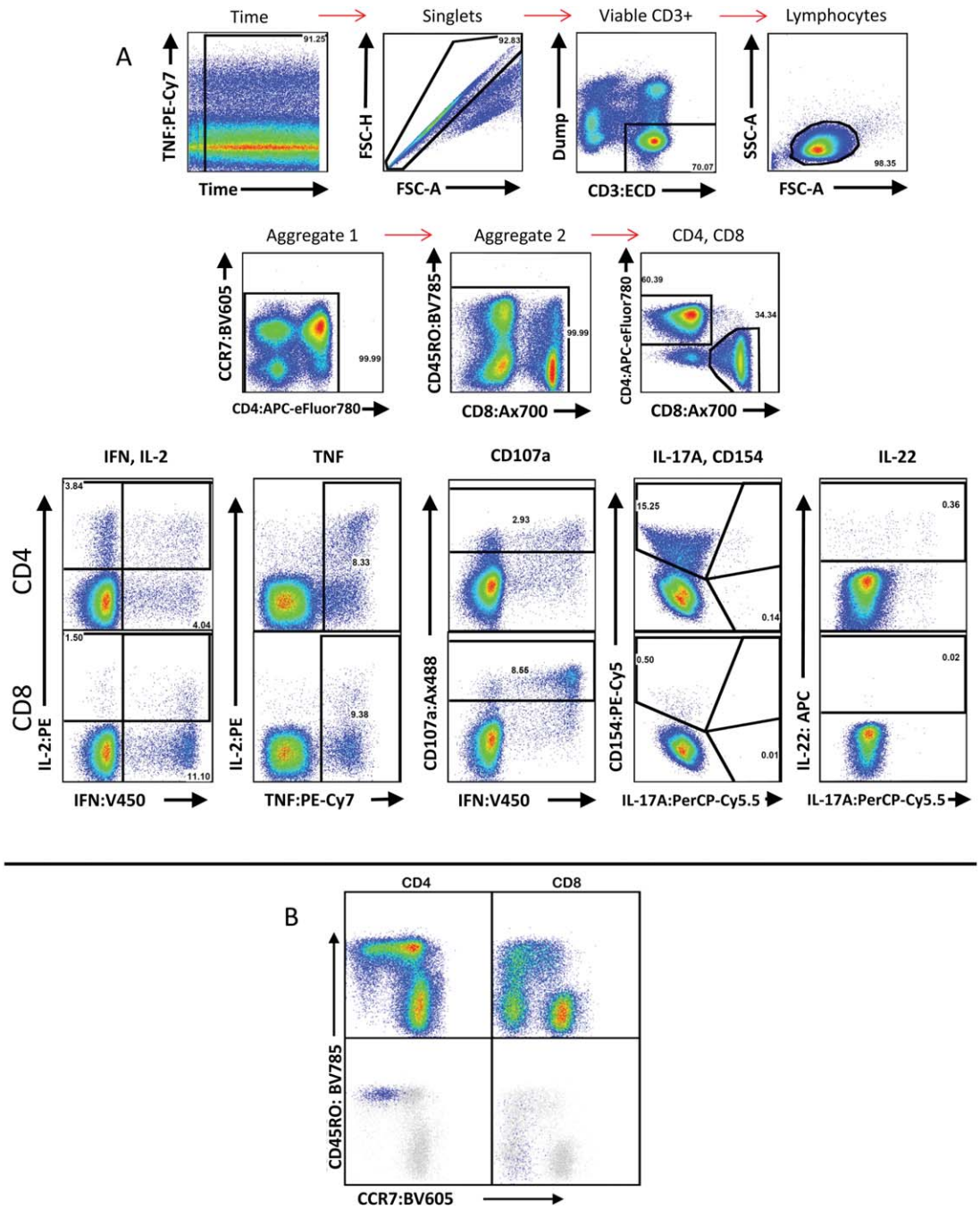

Figure 1. Example staining of adult human PBMC following stimulation. (**A**) The first two rows demonstrate the gating hierarchy from total sample to CD4/CD8 identification. A time gate is used to exclude pressure aberrations from the cytometer that may have occurred during sample acquisition. Aggregate gates are used to exclude brightly positive events that may result from antibody or cell aggregation. The bottom row demonstrates gating for cytokines and functions from CD4+ (top half) and CD8+ (bottom half) events resulting from stimulation with *Staphylococcal enterotoxin B*. Note that IL-17A and CD154 were gated on the same plot to avoid mischaracterization resulting from the increased spectral overlap observed from their fluorochromes. (**B**) Example plots showing memory profile of CCR7 versus CD45RO in CD4+ and CD8+ populations. Using CMV pp65 peptide pool-stimulated PBMC from a CMV-reactive donor, IFN-γ+ events (blue) were overlaid onto these plots (gray), confirming localization of these events to the effector memory and effector compartments.

of IL-4. IL-22 is part of the IL-10 superfamily (24), and when coexpressed with IL-17 enhances production antimicrobial peptides in the mucosa (25). Although poorly characterized to this point, recent research indicates that IL-22 plays a role in TB infection (21,26).

CD154 was maintained on this panel as emerging evidence indicates its role as a specific and sensitive marker in detecting CD4 response (27), as well as its roles in upregulating antimicrobial peptides (28) and its necessity for T-cell activation of B cells (29). As indicated in OMIP-014,

the inclusion of Brefeldin A in our stimulation protocol requires intracellular staining of CD154. Figure 1 shows an example staining and analysis for adult PBMC stimulated with *Staphylococcal enterotoxin B* (Fig. 1A) or CMV pp65 (Fig. 1B).

Similarity to Published OMIPs

As this panel assesses antigen-specific T-cell responses, it is similar to OMIP-001 (30), OMIP-008 (31), OMIP-009 (32), and OMIP-014 (2). Furthermore, this panel evolved from an initial desire to implement OMIP-014. Unlike these panels, however, our panel includes T-cell memory markers as well as an extensive combination of cytokines and functions associated with tuberculosis vaccine research. Our panel was developed for use in multicenter studies under good clinical laboratory practices conditions, and is currently undergoing assay qualification.

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