Innovative, Intuitive, Flexible.

Luminex Flow Cytometry Solutions with **Guava®** and **Amnis®** **Systems**

Learn More >

Luminex®

complexity **simplified.**

Cytometry PART A
Journal of the International Society for Advancement of Cytometry

OMIP-025: Evaluation of Human T- and NK-Cell Responses Including Memory and Follicular Helper Phenotype by Intracellular Cytokine Staining

Gemma Moncunill,[1,2]* Carlota Dobaño,[2] M. Juliana McElrath,[1,3] Stephen C. De Rosa[1,4]

[1]Vaccine and Infectious Disease Division, Fred Hutchinson Cancer Research Center, Seattle, Washington 98109

[2]Barcelona Centre for International Health Research (CRESIB, Hospital Clínic-Universitat de Barcelona), Barcelona, Catalonia, Spain

[3]Department of Medicine, University of Washington, Seattle, Washington

[4]Department of Laboratory Medicine, University of Washington, Seattle, Washington 98195

Received 4 August 2014; Revised 29 September 2014; Accepted 28 October 2014

Grant sponsor: HIV Vaccine Trials Network Laboratory Center (HVTN, National Institute of Allergy and Infectious Diseases), Grant number: UM1 AI068618

Grant sponsor: Human Immunology Project Consortium (HIPC, National Institute of Allergy and Infectious Diseases), Grant number: P30 AI027757

Grant sponsor: Instituto de Salud Carlos III, Grant number: CD10/00156

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

Correspondence to: Gemma Moncunill, Barcelona Centre for International Health Research (CRESIB), Hospital Clínic-Universitat de Barcelona, Carrer Roselló

ISAC
International Society for Advancement of Cytometry

Purpose and Appropriate Sample Types

This panel was developed to assess antigen-specific T cells using peptide pools to various antigens of interest, although other types of antigens such as recombinant proteins or whole pathogens could be considered using different stimulation times. In addition to multiple functional markers, the panel includes differentiation markers and markers to assess follicular helper T cells and NK cells (Table 1). It was optimized using cryopreserved peripheral blood mononuclear cells (PBMC) from human immunodeficiency virus (HIV) uninfected and HIV infected adults with known cytomegalovirus (CMV) responses and it underwent assay qualification. The panel is being used to evaluate the responses to HIV and malaria vaccine candidates in adults and children from different geographic areas.

Background

Vaccines are one of the most cost-effective interventions, saving millions of lives every year. Yet, despite all the efforts invested, vaccines against complex diseases such as HIV, TB, and malaria remain resistant to development. One of the main impediments is the lack of correlates of protection, whose identification would improve and accelerate the design and development of vaccine candidates for these infectious diseases.

This panel was designed starting from the OMIP-014 panel (1) to include the maximum number possible of relevant markers for vaccine immunogenicity that could lead to the identification of correlates of protection. High-level polychromatic panels are a requirement when assessing vaccine responses with a limited amount of sample, particularly when the population under study is infants and children, as is often the case in vaccine development efforts for malaria and tuberculosis (2–4).

A prerequisite of the new panel was to retain at least the same sensitivity for interferon-gamma (IFN-γ), interleukin-2 (IL-2), and tumor necrosis factor alpha (TNF-α) as in OMIP-014, which is widely used by the HIV Vaccine Trials Network (HVTN). Therefore, our priority was the assessment of IFN-γ, IL-2, and TNF-α markers. Of importance, the OMIP-014 panel was validated for IFN-γ and IL-2 responses and the current panel underwent assay qualification comparing the responses with this panel (Supporting Information). After testing a few new reagents for these three cytokines that did not prove to be better than the bright and sensitive ones used in OMIP-014, we opted to utilize the same reagents for the new expanded panel. Ultimately, only these three cytokine reagents and the viability marker remained unchanged from OMIP-014; the reagents for the remainder of shared

153 (CEK building), E-08036 Barcelona, Catalonia, Spain. E-mail: gemma.moncunill@cresib.cat

Published online 18 November 2014 in Wiley Online Library (wileyonlinelibrary.com)

DOI: 10.1002/cyto.a.22590

markers (CD3, CD4, CD8, CD154, and IL-4) had to be changed through the panel development process (Supporting Information). For instance, CD4 conjugated to APC-Ax750 was replaced for several reasons (Supporting Information), including suspected breakdown of the tandem fluorochrome resulting in non-specific events detected in the APC channel that was reserved for the low-frequency functional marker IL-4 in OMIP-014, and which was eventually used here for IL-21.

IL-4, a representative T_{H2} cytokine, is difficult to detect and the responses have been low in HIV vaccine studies conducted through the HVTN. Despite this, when combined with other functional markers in polyfunctionality analyses, polarized T_{H1} and T_{H2} CD4+ T cells have been detected for different vaccine regimens (5) and T cells co-expressing IL-4 with CD154, IFN-γ, IL-2, and TNF-α inversely correlate with infection risk in the RV144 HIV vaccine trial (unpublished data). Therefore, we decided to retain IL-4, changing the conjugated fluorochrome from APC to PerCP-Cy5.5. In the APC channel we added IL-21, a T_{FH} immunoregulatory cytokine, which is also difficult to detect due to its low expression in circulating T cells. CD154 (CD40L) is expressed mainly on activated CD4+ T cells, is key to providing T-cell help to B cells, and is a relevant sensitive and specific marker for the detection of antigen-specific T cells upon ex vivo stimulations (6,7). Therefore, we also kept it in the new panel, although the reagent was changed to an antibody conjugated to BV605 instead of PE-Cy5 because the latter showed extensive fluorescence spreading into other channels, including those detecting functional markers. As in OMIP-014, CD154 staining was performed intracellularly (1). Although CD154 can be detected by surface staining by including the CD154 reagent during the ex vivo antigen stimulation, the usage of brefeldin A is incompatible with this method because it completely blocks CD154 surface expression (8) and brefeldin A rather than monensin alone is critical for optimal detection of some cytokines, especially TNF-α.

To discern the memory subset of the antigen-specific T cells, the differentiation markers CCR7 and CD45RA were included. The combination of both markers allows the identification of naïve (CD45RA+CCR7+), central memory (CD45RA−CCR7+), effector memory (CD45RA−CCR7−), and terminal effector (CD45RA+CCR7−) subsets (9–11). After several tests, CCR7 conjugated to BV785 and CD45RA to APC-H7 were selected.

One of our main interests was the inclusion of markers to identify peripheral follicular helper T cells (T_{FH} cells), a T_H subset involved in germinal center reactions necessary for the development of high affinity and long-term antibody responses (12). Circulating T_{FH}-like cells have been mostly defined as CXCR5+ CD4+ memory T cells (13,14), although different subsets of CXCR5+ CD4+ T cells have been studied including the $CCR7^{lo}$ $PD\text{-}1^{hi}$ cells, which have been described as T_{FH} precursors that correlate with active T_{FH} differentiation in secondary lymphoid organs and antibody responses (15). Therefore, the chemokine receptor CXCR5 and the inhibitory marker PD-1 were added in the panel conjugated to PE-eFluor610 and PE-Cy7, respectively. Additionally, T_{FH} and T_{FH}-like cells express inducible costimulator (ICOS) at high density in the lymphoid organs and in the periphery,

Table 1. Summary table for application of OMIP-025

Purpose	Characterization of antigen-specific T cells, T_{FH}-like cells and NK cells
Species	Human
Cell types	Cryopreserved PBMC
Cross-references	OMIP-014

T_{FH}, follicular helper T cells; NK, natural killer; PBMC, peripheral blood mononuclear cells.

Table 2. Reagents used for OMIP-025

SPECIFICITY	CLONE	FLUOROCHROME	PURPOSE
CD3	UCHT1	BV570	T lineages
CD8	RPA-T8	BV711	
CD4	SK3	BUV395	
CXCR5	MU5UBEE	PE-eFluor 610	T_{FH}
PD-1	eBioJ105	PE-Cy7	
CD45RA	HI100	APC H7	Memory/ Differentiation
CCR7	G043H7	BV785	
CD56	HCD56	BV650	NK cells, NKT-like cells
IFN-γ	B27	V450	Function
IL-2	MQ1–17H12	PE	
TNF-α	MAb11	FITC	
IL-4	MP4–25D2	PerCP-Cy5.5	
IL-21	3A3-N2	APC	
CD154	24–31	BV605	
CD14	M5E2	BV510	Monocytes (Dump)
Live/Dead	NA	AViD	Dead cells (Dump)

APC, allophycocyanin; Ax, Alexa; AViD, LIVE/DEAD fixable aqua dead cell stain; BUV, brilliant ultraviolet; BV, brilliant violet; Cy, cyanine; FITC, fluorescein isothiocyanate; NK, natural killer; PE, R-phycoerythrin; PerCP, peridinin chlorophyll protein; T_{FH}, follicular helper T cells.

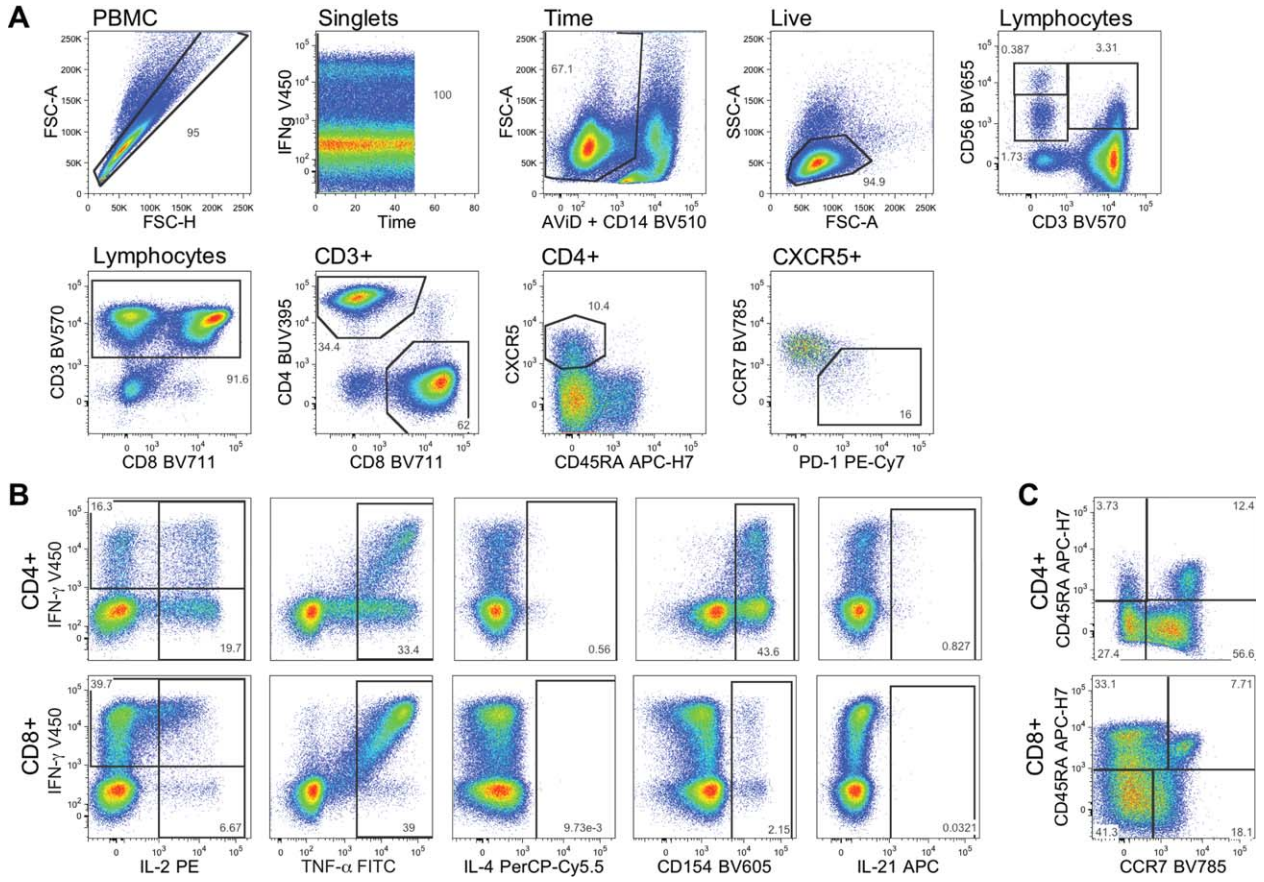

Figure 1. Example of the staining and gating strategy for PBMC stimulated with Staphylococcal enterotoxin B (SEB). All gates for non-functional markers were defined using fluorescence minus one (FMO) controls whereas gates for functional markers were defined using the unstimulated samples. **A**: Gating hierarchy to identify NK cells, NKT-like cells, CD4+ and CD8+ T cells, and T_{FH}-like cells. Initial gating is done on FSC-H and FSC-A to discriminate singlets, followed by the exclusion of events collected during a period of time early in collection when fluctuations may occur. In this example, there were no problems of fluctuations and the time gate was minimized to avoid exclusion of any events. Dead cells and monocytes are excluded by an amine reactive dye and the CD14 marker in the same dump channel. Lymphocytes are gated using FSC-A and SSC-A. Subsequent gating discriminates two subsets of NK cells by CD56 and CD3 expression (CD56dimCD3− and CD56hiCD3−) and NKT-like cells (CD56+CD3+). Within the gate of lymphocytes, CD3+ cells are identified, followed by identification of CD4+ and CD8+ T cells. Of note, NKT-like cells are not excluded from classical T cells and therefore are overlapping populations. Finally, T_{FH}-like cells are identified as CXCR5+ CD45RA− CD4+ T cells that have a low expression of CCR7 and are PD-1+. **B**: Functional markers for CD4+ and CD8+ T cells. A gate is applied for each cytokine, not taking into account the coexpression of other markers. Boolean gates are then created based on these gates to identify cells expressing different combinations of markers. **C**: The expression level of CCR7 and CD45RA is examined within CD4+ and CD8+ T-cell subsets to later provide insight into the memory phenotype of the antigen specific cells.

respectively, when they are activated (12–15). We tested several ICOS reagents but this marker was ultimately excluded, as it did not provide satisfactory results in combination with the other markers in the panel.

Additionally, CD56 BV650 was incorporated to evaluate effector NK cell responses, since this leukocyte population has been shown to be a key producer of IFN-γ in acquired immune responses, contributing to the vaccine-induced response (16,17). The marker CD56 allows the discrimination of the two main subsets of NK cells that have different receptors and functions (18): CD56high (CD56hiCD3−) and CD56dim (CD56dimCD3−). The inclusion of CD56+ permitted also the identification of NKT-like cells defined as CD56+CD3+ cells, which are different than the CD1d-restricted invariant NK T cells (19).

Finally, to improve the specificity of the assay, a viability marker was included for the exclusion of dead cells that may non-specifically bind antibodies and contribute to background (20). CD14 was similarly included to exclude monocytes, and in contrast to OMIP-014, it is detected in the same channel as the viability marker, thus creating a dump channel.

The reagents used for this panel are listed in Table 2. Figure 1 shows an example staining profile for PBMC stimulated with Staphylococcal enterotoxin B. Further developmental strategies and details for the panel may be found in the Supporting Information.

Similarity to Published OMIPs

This panel is an expansion and modification of the OMIP-014 panel (1), which now includes differentiation and

T_{FH} markers, whereas MIP-1β and CD107a were removed. In addition, it presents similarities to other OMIP panels with intracellular cytokine staining to identify antigen specific T cells: OMIP-001 (21), OMIP-008 (22), OMIP-009 (23), and OMIP-022 (24). However, our panel includes T_{FH} markers, the NK marker CD56, and cytokine IL-21, which are not addressed in any of the other ICS OMIP panels.

Acknowledgments

The authors wish to thank all the individuals enrolled in the Seattle Assay Control cohort, from which PBMC were used for optimization and testing of the panel. The authors are grateful to HVTN technicians Hannah H. Han for her help in the titrations and panel tests, and also Nathaniel Chartrand, Paul Newling, Bryce Manso, Kevin Hawkins and Terry Stewart for their help in the lab. Authors acknowledge Stephen Voght for his help with editing. They also thank the Vaccine Immunology Workgroup (VIW) of the RTS,S immune correlates study (Mal067) for their support in developing the panel and particularly the VIW Cellular Working Group for their inputs and discussion in the panel design. Finally, authors thank the James B. Pendleton Charitable Trust for their generous equipment donation.

Literature Cited

1. De Rosa SC, Carter DK, McElrath MJ. OMIP-014: Validated multifunctional characterization of antigen-specific human T cells by intracellular cytokine staining. Cytometry A 2012;81A:1019–1021.
2. Agnandji ST, Lell B, Soulanoudjingar SS, Fernandes JF, Abossolo BP, Conzelmann C, Methogo BGNO, Doucka Y, Flamen A, Mordmüller B, et al. First results of phase 3 trial of RTS,S/AS01 malaria vaccine in African children. N Engl J Med 2011;365: 1863–1875.
3. Agnandji ST, Lell B, Fernandes JF, Abossolo BP, Methogo BGNO, Kabwende AL, Adegnika AA, Mordmüller B, Issifou S, Kremsner PG, et al. A phase 3 trial of RTS,S/AS01 malaria vaccine in African infants. N Engl J Med 2012;367:2284–2295.
4. Tameris MD, Hatherill M, Landry BS, Scriba TJ, Snowden MA, Lockhart S, Shea JE, McClain JB, Hussey GD, Hanekom WA, et al. Safety and efficacy of MVA85A, a new tuberculosis vaccine, in infants previously vaccinated with BCG: A randomised, placebo-controlled phase 2b trial. Lancet 2013;381:1021–1028.
5. Spearman P, Lally MA, Elizaga M, Montefiori D, Tomaras GD, McElrath MJ, Hural J, De Rosa SC, Sato A, Huang Y, et al. A trimeric, V2-deleted HIV-1 envelope glycoprotein vaccine elicits potent neutralizing antibodies but limited breadth of neutralization in human volunteers. J Infect Dis 2011;203:1165–1173.
6. Frentsch M, Arbach O, Kirchhoff D, Moewes B, Worm M, Rothe M, Scheffold A, Thiel A. Direct access to CD4+ T cells specific for defined antigens according to CD154 expression. Nat Med 2005;11:1118–1124.
7. Chattopadhyay PK, Yu J, Roederer M. A live-cell assay to detect antigen-specific CD4+ T cells with diverse cytokine profiles. Nat Med 2005;11:1113–1117.
8. Chattopadhyay PK, Yu J, Roederer M. Live-cell assay to detect antigen-specific CD4+ T-cell responses by CD154 expression. Nat Protoc 2006;1:1–6.
9. Sallusto F, Lenig D, Förster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature 1999; 401:708–712.
10. Larbi A, Fulop T. From "truly naïve" to "exhausted senescent" T cells: when markers predict functionality. Cytometry A 2014;85A:25–35.
11. Maecker HT, McCoy JP, Nussenblatt R. Standardizing immunophenotyping for the Human Immunology Project. Nat Rev Immunol 2012;12:191–200.
12. Crotty S. Follicular helper CD4 T cells (TFH). Annu Rev Immunol 2011;29:621–663.
13. Morita R, Schmitt N, Bentebibel S-E, Ranganathan R, Bourdery L, Zurawski G, Foucat E, Dullaers M, Oh S, Sabzghabaei N, et al. Human blood CXCR5(+)CD4(+) T cells are counterparts of T follicular cells and contain specific subsets that differentially support antibody secretion. Immunity 2011;34:108–121.
14. Pallikkuth S, Parmigiani A, Silva SY, George VK, Fischl M, Pahwa R, Pahwa S. Impaired peripheral blood T-follicular helper cell function in HIV-infected nonresponders to the 2009 H1N1/09 vaccine. Blood 2012;120:985–993.
15. He J, Tsai LM, Leong YA, Hu X, Ma CS, Chevalier N, Sun X, Vandenberg K, Rockman S, Ding Y, et al. Circulating precursor CCR7(lo)PD-1(hi) CXCR5(+) CD4(+) T cells indicate Tfh cell activity and promote antibody responses upon antigen reexposure. Immunity 2013;39:770–781.
16. Horowitz A, Hafalla JCR, King E, Lusingu J, Dekker D, Leach A, Moris P, Cohen J, Vekemans J, Villafana T, et al. Antigen-specific IL-2 secretion correlates with NK cell responses after immunization of tanzanian children with the RTS,S/AS01 malaria vaccine. J Immunol 2012;188:5054–5062.
17. Horowitz A, Behrens RH, Okell L, Fooks AR, Riley EM. NK cells as effectors of acquired immune responses: Effector CD4(+) T cell-dependent activation of NK cells following vaccination. J Immunol 2010;185:2808–2818.
18. Montaldo E, Del Zotto G, Della Chiesa M, Mingari MC, Moretta A, De Maria A, Moretta L. Human NK cell receptors/markers: A tool to analyze NK cell development, subsets and function. Cytometry A 2013;83A:702–713.
19. Godfrey DI, MacDonald HR, Kronenberg M, Smyth MJ, Van Kaer L. NKT cells: What's in a name? Nat Rev Immunol 2004;4:231–237.
20. Horton H, Thomas EP, Stucky JA, Frank I, Moodie Z, Huang Y, Chiu Y, McElrath MJ, De Rosa SC. Optimization and validation of an 8-color intracellular cytokine staining (ICS) assay to quantify antigen-specific T cells induced by vaccination. J Immunol Methods 2007;323:39–54.
21. Mahnke YD, Roederer M. OMIP-001: Quality and phenotype of Ag-responsive human T-cells. Cytometry A 2010;77A:819–820.
22. Zuleger CL, Albertini MR. OMIP-008: Measurement of Th1 and Th2 cytokine polyfunctionality of human T cells. Cytometry A 2012;81A:450–452.
23. Lamoreaux L, Koup R a, Roederer M. OMIP-009: Characterization of antigen-specific human T-cells. Cytometry A 2012;81A:362–363.
24. Graves AJ, Padilla MG, Hokey DA. OMIP-022: Comprehensive assessment of antigen-specific human t-cell functionality and memory. Cytometry A 2014;85A: 576–579.