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OMIP-XXX: An 18-color panel for measuring Th1, Th2, Th17, and Tfh responses in rhesus macaques

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Purpose and Appropriate Sample Types

This 18-color panel was designed to measure vaccine-induced T cell responses in rhesus macaques by intracellular cytokine staining (ICS). It detects seven cytokines, IFN- γ IL-2, IL-4, IL-5, IL-13, IL-17, and IL-21, two memory markers, CD45RA and CCR7, and four follicular helper T cell (Tfh) markers, CXCR3, CXCR5, ICOS, and PD-1. While this panel was optimized for use on cryopreserved rhesus macaque peripheral blood mononuclear cells (PBMC) (Table 1), it can also be used on rhesus macaque tissue samples and human PBMC and tissue samples.

Background

Experimental vaccines are often tested in nonhuman primates (NHP) for immunogenicity and protection in preclinical trials before proceeding to clinical trials in humans. Historically, vaccine-induced T cell responses have been measured by IFN- γ ELISpot or intracellular cytokine staining (ICS) for IFN- γ , IL-2, and TNF. However, antibody levels, rather than Th1 responses, correlate with protection for many vaccines including the HIV vaccine used in the RV144 clinical trial (1). As such, measuring only Th1 responses may not be adequate for determining T cell correlates of protection or risk in vaccine studies. Therefore, we expanded our 11-color intracellular cytokine staining (ICS) panel, described in OMIP-005, to 18 colors in order to assess a fuller spectrum of T cell responses.

To decrease the variability of the assay due to age-related differences in T cell differentiation, cytokine measurements can be reported as a frequency of memory T cells using the markers CD45RA and CCR7 (2). Similarly, IL-21 can be reported as a frequency of Tfh using a combination of the markers CXCR3, CXCR5, ICOS, and PD-1. This combination of markers allows for the delineation of Tfh cells by all currently accepted methods (3–5).

Similarity to Published OMIPs

This 18-color rhesus macaque panel is an extension of the 11-color panel described in OMIP-005. The cytokines IL-4, IL-5, IL-13, IL-17, and IL-21 were added as well as the Tfh

subset markers CXCR3, CXCR5, ICOS, and PD-1. To accommodate these additional markers, CD28 and TNF were removed. It is similar to the following human panels also used to measure antigen-specific responses by intracellular staining: OMIP-001, OMIP-008, OMIP-009, OMIP-14, OMIP-16, OMIP-22, OMIP-25. However, this panel is designed for staining rhesus macaque PBMC (using NHP cross-reactive antibody clones) and includes additional Tfh markers, as well as IL-5 and IL-13. It is also the only panel with this combination of seven cytokines.

Online Material

Developmental Strategy

Our previous panel and staining protocol, described in OMIP-005 (1), was designed to measure Th1 responses from macaques in preclinical HIV vaccine studies. However, the RV144 HIV vaccine clinical trial results suggest that antibody responses, not Th1 responses, correlate with protection from acquisition of HIV and that IL-13 may have a protective role (2). In addition, the antibodies in the RV144 trial waned rapidly, so the identification of a correlate of antibody durability would be useful. Therefore, since Tfh cells producing IL-21 drive the differentiation of B cells and affinity maturation of antibodies (3), we added Tfh markers and IL-21 to our panel. Furthermore, there is evidence that Th2 and Th17 responses may be deleterious in the generation of protective vaccine responses to some pathogens (4, 5). Combined, these data suggest that further characterization of T cells is necessary in vaccine development.

Recent advancements in cytometer instrumentation and the discovery of new dyes enable development of more complex flow cytometry panels which measure more parameters. As such, we developed an 18-color flow cytometry panel that identifies Th1, Th2, Th17, and Tfh responses. The strategy for developing this panel was based generally on our previous panel design, in which the brightest fluorochromes were reserved for cytokines in order to increase sensitivity to detect rare or low responses. Next, priority was given to the dimmer and less widely available memory and Tfh markers. Last, T cell lineage markers were given the lowest priority since the reagents used to identify these populations are more abundant and available on many different fluorochromes (6). All reagents were titrated and the dilutions that gave the brightest signal and the least amount of background staining were chosen (Online Fig. 1).

Adequate detection of cytokine responses poses a challenge due to background responses from non-specific cells. Therefore, the use of fluorochromes that will maximize resolution between negative and positive populations is critical. Brilliant Violet (BV) and Brilliant Blue (BB) fluorochromes are amongst the brightest and were used for detecting five of the seven cytokines. IL-2, IL-4, IL-5, IL-13, and IL-17 were paired with BV750, BB700, BB515, BV421, and BV605, respectively. We also wanted to measure the Th2 cytokines IL-4 and IL-5, however, the commercial conjugates available for both reagents were limited. Thus, options for obtaining custom conjugates for both reagents were pursued. Since IL-4 and IL-5 are expressed at low levels, both were conjugated to the brightest fluorophores available BB700 and BB515, respectively. To ensure that the custom conjugates performed as expected, we compared the frequencies of IL-4 and IL-5 positive cells obtained with

commercial PE reagents to those obtained with the custom conjugates and observed similar results (Online Fig. 2). While the separation between positive and negative events wasn't as high with the IL-5 BB515 custom conjugate as with the commercial PE reagent, we chose to use BB515 because frequencies were similar between the two antibody conjugates and we needed PE for CXCR5. Last, we chose IL-21 Ax647 and IFN- γ Ax700 because expression levels of these cytokines are more robust, and we were able to distinguish negative and positive populations with moderately bright fluorochromes. As with OMIP-005, the activation marker CD69 was included in the panel to exclude background responses from CD69⁻ cells (7).

Next, we prioritized memory subset (CD45RA and CCR7) and Tfh markers (PD-1, CXCR5, CXCR3, and ICOS). Relatively bright fluorochromes were also reserved for these markers. CD45RA was placed in the open PE-Cy5 channel and CCR7 BV650 was selected since BV650 is relatively bright. Using a bright fluorochrome for CCR7 is necessary since this receptor is particularly dim in nonhuman primates. In a similar manner, the dim markers CXCR3, CXCR5, ICOS, and PD-1 were put on the moderately bright fluorochromes BV711, PE, PE-Cy7, and BV785, respectively. We initially had CXCR5 in the surface staining portion of the panel; however, we noticed that the frequency of CXCR5⁺ cells decreased upon stimulation in some animals and that all of the IL-21⁺ cells were CXCR5⁻ (Online Fig. 3A). Therefore, we tested staining for CXCR5 in the intracellular portion of the assay and discovered that the frequency of CXCR5⁺ cells increased upon stimulation and approximately 50% of the IL-21⁺ cells were CXCR5⁺ (Online Fig. 3B). Thus, although optimal separation was observed during surface staining, we decided to stain for CXCR5 during the intracellular step of the assay since we are interested in measuring IL-21 from Tfh (CXCR5⁺) cells.

Last, we assigned fluorochromes to the lineage marker antibodies. CD3, CD4, and CD8 were assigned to the open channels APC-Cy7, PE-Cy5.5, and BV570, respectively. As with OMIP-0005, CD3 was stained for during the intracellular step of the assay since the CD3 molecule can be internalized along with the TCR complex upon stimulation (8).

Staining Protocol

Commercial Materials:

- PBS (GIBCO, Carlsbad, CA)
- RPMI 1640 with phenol red (GIBCO)
- RPMI 1640 without phenol red (GIBCO)
- Heat-inactivated FBS (GIBCO)
- Penicillin/Streptomycin/Glutamine (GIBCO)
- BD Cytotfix/Cytoperm Fixation/Permeabilization kit (BD Biosciences, San Diego, CA)
- Monensin (GolgiStop, BD Biosciences)
- 96 well tissue-culture treated V-bottom plates (Corning Costar, Corning, NY)

Anti-CD28 BD Biosciences

Anti-CD49d BD Biosciences

BD Horizon Brilliant Stain Buffer BD Biosciences

In-house media:

R10 culture medium:

RPMI 1640 with phenol red

10% FBS

100 IU/ml penicillin

100 µg/ml streptomycin

2mM L-glutamine

Wash buffer:

RPMI 1640 without phenol red

4% FCS

0.02% sodium azide

Note: cells are pelleted in a desktop centrifuge with a 25 cm rotor while reagent master mixes are centrifuged in a microfuge with a 7 cm rotor.

1.0 Preliminary Operations

1.1 Determine desired 96-well plate layout. One column per animal is recommended. Each sample will likely have at least 2 stimulations each: peptide and negative (DMSO). If more than 3 million cells per sample are to be run, cells should be plated in duplicate or triplicate. Include an extra well of cells for the unstained control for gating on the flow cytometer.

1.2 Prepare the peptide pool(s) by mixing the peptides in DMSO to achieve a final concentration of 400 µg/ml. Peptides are used at a final concentration of 2 µg/ml when added to cells.

1.3 In separate tubes, prepare the appropriate Stimulation Mixes as listed below. The table below is for 100 µl of solution per well for 4 wells with 10% extra (total volume for 5 wells). Adjust the volumes proportionately as needed for individual experiments.

<i>Component</i>	<i>uL</i>	<i>uL</i>	<i>Final conc.</i>
<i>Stimulant</i>	Peptide-5	DMSO-5	2 µg/ml
<i>Anti-CD49d (1 mg/ml)</i>	1	1	1 µg/ml
<i>Anti-CD28 (1 mg/ml)</i>	1	1	1 µg/ml
<i>Monensin (GolgiStop 3 mM)</i>	1	1	3 µM

<i>Component</i>	<i>uL</i>	<i>uL</i>	<i>Final conc.</i>
<i>R10 Medium</i>	492	492	
<i>Total Volume</i>	500	500	

1.4 Determine the amount of Perm/Wash Buffer needed for the experiment and dilute the 10× Perm/Wash Buffer 1:10 with deionized water. Shake well and filter through a 0.22 micron vacuum flip filter. Store at 4°C for up to 1 week.

1.5 Prepare 25 ml of PBS + Benzonase per plate by diluting 5 µl of Benzonase nuclease stock in 25 ml of PBS.

1.6 Prepare 1% PFA by diluting the 4% stock PFA solution to 1% in 1× PBS. Make enough for 125 µl per sample (plus 10% extra).

1.7 Prepare the live/dead Amine Staining Mix no more than 1 – 2 hours prior to staining cells. Dilute the appropriate amount of amine reactive dye 1:40 in dH₂O, then 1:20 in 1× PBS at a total volume of 100 µl per sample (plus 10% extra). Store at 4°C in the dark until ready to use.

1.8 Prepare the Surface Staining Mix no more than 1 – 2 hours prior to staining cells. Add the appropriate antibodies to BD Horizon Brilliant Stain Buffer in a microcentrifuge tube, 15 ml conical tube, or a 50 ml conical tube depending on the final volume (**be sure to add the Brilliant Staining Buffer to the tube first!**). Spin the antibodies in a microcentrifuge at 13,000 rpm (15,700g) for 5 minutes to remove aggregates. For volumes larger than 1.5 ml, split antibody mix evenly between microcentrifuge tubes and spin. Remove the supernatant and adjust the total volume to 100 µl per sample (depending on panel) with Brilliant Staining Buffer.

1.9 Prepare the Intracellular Cytokine Staining Mix no more than 1–2 hours prior to staining cells. Add the appropriate antibodies to BD Horizon Brilliant Staining Buffer in a microcentrifuge tube, 15 ml conical tube, or a 50 ml conical tube depending on the final volume (**be sure to add the Brilliant Staining Buffer to the tube first!**). Spin the antibodies in a microcentrifuge at 13,000 rpm (15,700g) for 5 minutes to remove aggregates. For volumes larger than 1.5 ml, split antibody mix evenly between microcentrifuge tubes and spin. Remove the supernatant and add the appropriate volume of 10× Perm/Wash Buffer.

2.0 Procedure

2.1 Prepare cells according to the appropriate SOP (cell preparation and/or thawing) using R10 as the final buffer. Cells should be at a final concentration of 10 – 30 million cells/ml.

2.2 Pipette 100 µl of cells into the appropriate wells on the plate (1 to 3 million cells/well) according to plate layout.

2.3 Pipette 100 µl of the peptide and negative control Stimulation Mixes from the table above into the appropriate wells and mix thoroughly with a multi-channel pipette.

2.4 Incubate the plate for 6 hrs (18 hrs if overnight stimulation) in a humidified 5% CO₂ incubator at 37°C. At the end of the incubation, the plate may be held at 4°C overnight if desired.

2.5 Centrifuge the plate at 2000 rpm for 3 minutes at RT (18–26°C). All centrifugation steps from this point will be done the same way.

2.6 Flick supernatant into a biohazardous waste container under a laminar flow hood and immediately and quickly blot the plate on a paper towel before returning to an upright position. All flicks and blots from this point will be done the same way.

2.7 Add 200 µl of PBS + Benzonase to each of the wells and mix thoroughly by pipetting up and down with a multi-channel pipette. Centrifuge, flick, and blot.

2.8 Add 100 µl of the prepared Amine Staining mix to each of the wells and mix thoroughly. Incubate for 20 min at RT protected from light.

2.9 Wash the cells by adding 120 µl of wash buffer directly to the wells containing the cells in the Amine Staining Mix. Centrifuge, flick, and blot.

2.10 Add 200 µl of wash buffer to the wells and mix thoroughly. Centrifuge, flick, and blot.

2.11 Add 100 µl of Surface Staining Mix to each of the wells, mixing well. Incubate for 20 min at RT protected from light.

2.12 Wash the cells by adding 120 µl of wash buffer directly to the wells containing the cells in Surface Staining Mix. Centrifuge, flick, and blot.

2.13 Add 200 µl of wash buffer to the wells and mix thoroughly. Centrifuge, flick, and blot.

2.14 Add 100 µl of Cytofix/Cytoperm reagent to each of the wells, mixing well. Incubate for 20 min at 4°C protected from light.

2.15 Wash the cells by adding 120 µl of 1× Perm/Wash buffer directly to the wells containing cells in Cytofix/Cytoperm. Centrifuge, flick, and blot.

2.16 Add 200 µl of 1× Perm/Wash buffer to the wells and mix thoroughly. Centrifuge, flick, and blot.

2.17 Add 100 µl of Intracellular Staining Mix to the wells, mixing well. Incubate for 20 min at RT in the dark.

2.18 Wash the cells by adding 120 µl of 1× Perm/Wash buffer directly to the wells containing cells in the Intracellular Staining Mix. Centrifuge, flick, and blot.

2.19 Add 200 µl of 1× Perm/Wash buffer to the cells and mix thoroughly. Centrifuge, flick, and blot.

2.20 Repeat step 2.19 for a third wash.

2.21 Add 125 µl of 1× PBS to the cells and mix.

2.22 Fix by adding 125 μ l of 1% formaldehyde to the cells in 1 \times PBS for a total volume of 250 μ l (0.5% formaldehyde) per well and mix. Store covered in foil at 4°C until ready to run on the flow cytometer (within 48 hours).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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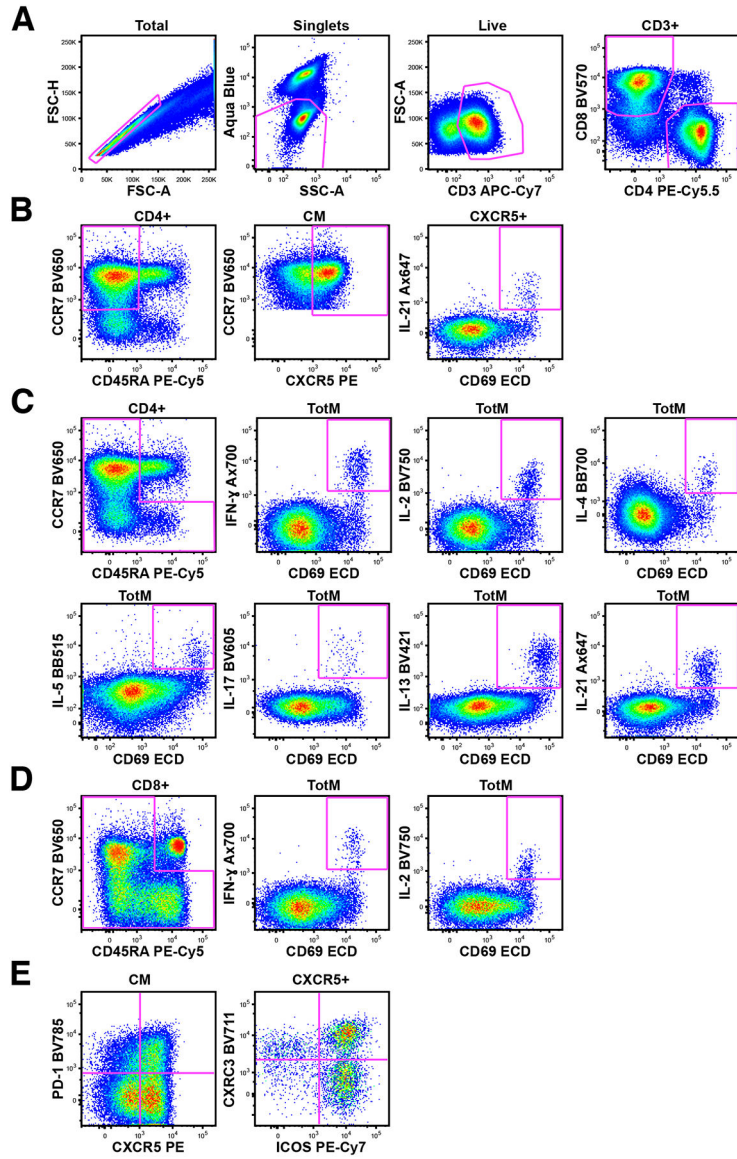


Figure 1.

Representative staining and gating. Since this panel covers diverse populations and T cell polarization states, plots from multiple rhesus macaques in the same experiment were chosen in order to best display staining of all populations and production of all cytokines. (A) T cell populations were selected by subsequent gating: first single cells, followed by live/SSC low cells, then CD3⁺/FSC low cells, and finally CD4⁺ and CD8⁺ T cells. (B) To measure IL-21 production from Tfh, CD4 T cells were gated first on central memory (CM) T cells (CCR7⁺CD45RA⁻) then CXCR5⁺ CM cells to identify Tfh. Subsequently, from CXCR5⁺ Tfh, CD69⁺IL-21⁺ were gated. (C and D) Cytokine production from total memory (TotM) CD4 (C) and CD8 (D) T cells was measured by first gating on total memory cells (central memory CCR7⁺CD45RA⁻ plus effector memory CCR7⁻CD45RA⁻ plus terminal effector memory CCR7⁻CD45RA⁺ T cells) while excluding naïve CCR7⁺CD45RA⁺ T cells,

then CD69⁺cytokine⁺ gates were drawn on total memory T cells. (E) Tfh can be further characterized using CXCR3 and ICOS after gating on CXCR5⁺ CM CD4 T cells.

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Table 1.

Summary Table for Application of OMIP-XXX

Purpose	To measure Th1, Th2, Th17, and Tfh responses
Species	Rhesus macaque
Cell types	PBMC
Cross references	OMIP-005: Quality and phenotype of antigen-responsive rhesus macaque T cells

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Table 2.

Reagents Used for OMIP-XXX

Specificity	Clone	Fluorochrome	Purpose
Dead cells		Aqua Blue	Exclusion
CD3	SP34.2	APC-Cy7	T cells
CD4	S3.5	PE-Cy5.5	
CD8	RPA-T8	BV570	
CD45RA	5H9	PE-Cy5	Memory markers
CCR7 (CD197)	G043H7	BV650	
CXCR3 (CD183)	1C6/CXCR3	BV711	Tfh markers
CXCR5 (CD185)	MU5UBEE	PE	
PD-1 (CD279)	EH12.2H7	BV785	
ICOS (CD278)	C398.4A	PE-Cy7	
CD69	TP1.55.3	ECD	Background reduction
IFN- γ	B27	Ax700	Cytokines
IL-2	MQ1-17H12	BV750	
IL-4	MP4-25D2	BB700	
IL-5	JES1-39D10	BB515	
IL-13	JES10-5A2	BV421	
IL-17	BL168	BV605	
IL-21	3A3-N2.1	Ax647	