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OMIP-016: Characterization of Antigen-Responsive Macaque and Human T-cells

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• **Key terms**
intracellular cytokines; T cells; HIV; SIV; vaccines

Purpose and Appropriate Sample Types

The present panel was optimized to assess the quality and phenotype of antigen-specific CD4 and CD8 T cells in both cynomolgus macaques and humans. The use of an identical protocol for specimens collected in the two species allows for an immediate translation of research from the macaque model to humans. Our protocol works well with cryopreserved and freshly collected PBMC. Following the fixing and permeabilization procedure, we introduced a freezing step that breaks the experimental procedure and allows the shipment of freshly stimulated and permeabilized samples to facilities equipped with instruments able to measure ten distinct fluorescences. Our procedure is thus adapted to multicenter studies where stimulation is performed on fresh PBMC and flow cytometry acquisition is done in a centralized facility.

Background

Preclinical data generated in macaque models can be used to validate research hypotheses in humans. In the field of viral immunology, macaque models are used to study new vaccine formulations and therapies (1). To facilitate the translational process from the macaque model to humans, we developed an intracellular cytokine staining protocol in which all the procedures have been optimized in parallel using specimens collected from the two species. The optimization procedure started from a panel previously used for human samples (2). Antibodies were titrated simultaneously on macaque and human PBMC (Supporting Information Figs. 1–5).

Unlike similar OMIPs, anti MIP-1β and anti-CD154 antibodies were included in the panel. MIP-1β increases detection of CD8 T cell responses (2,3) and CD154 expands the detection of antigen specific CD4 T lymphocytes to cells that do not necessarily express the cytokines included in the panel (Tables 1 and 2) (4). To our knowledge, the present OMIP validates for the first time the use of CD154 to characterize antigen specific CD4 T-cell responses in macaques (Supporting Information Fig. 4).

CD45RA was chosen to discriminate the antigen experienced cells with a terminally effector phenotype (CD45RA+ CCR7−) from memory and effector memory cells (CD45RA− CCR7+/−). The number of naïve T cells (CD45RA+ CCR7+) carrying a T cell receptor specific for a given antigen will be too low to be detected within the 5 h incubation time of the present protocol assuring that antigen activated CD45RA+ T cells are truly terminally effector cells.

Various antibody-conjugates were tested to select the brightest. As an example, the optimization of the TNF-α staining is shown in Supporting Information Fig. 6.

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A blue-fluorescent reactive dye excited by the UV laser was used as live/dead cells discrimination marker leaving other channels free for the detection of functional, lineage and differentiation markers. The live/dead discrimination marker has not been included in a dump channel to precisely evaluate the quality of the sample in terms of living cells. This is of paramount importance when frozen samples are analyzed.

All antibodies were added in a unique staining step immediately after the post-fixation/permeabilization thawing procedure (Supporting Information Table 2). This experimental sequence allows for multicenter studies and storage of samples that can be simultaneously stained according to instrument accessibility.

Figure 1 shows the adopted gating strategy in a macaque sample and in Supporting Information Figure 9 a representative staining on human PBMC is shown. To avoid exclusion of any relevant cell that, following activation, might have downregulated the CD3 molecule, we adopted a special gating strat-

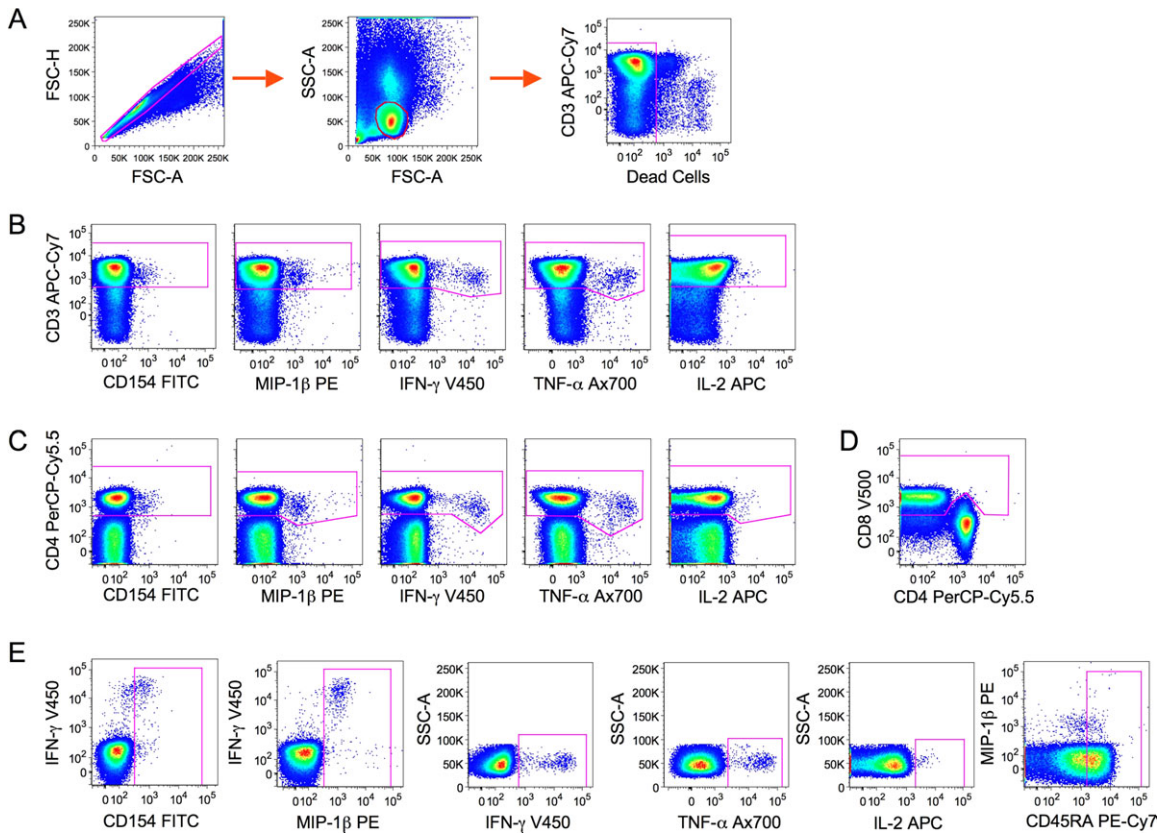

Figure 1. Example of gating strategy and staining results on cynomolgus macaque PBMC stimulated with peptides spanning the SIV Gag protein p15. PBMC were obtained from a macaque infected with SIV mac251. **A**: Singlets are selected using a FSC-A versus FSC-H plot. Lymphocytes are then selected using a FSC-A versus SSC-A plot and subsequently live cells are selected using a live/dead cell discriminator. **B**: CD3+ cells are selected plotting the CD3-axis versus all the functional markers used in the protocol in order to detect potential CD3 downregulation. CD3+ cell gates are adapted to include functional positive cells that downregulate CD3. The CD3+ population is calculated combining all the gates shown in (B) using the Boolean operator ''OR.'' **C**: Similar to CD3 T cells, CD4+ cells are selected taking in account the CD4 downregulation. The CD8 gate in (**D**) was used to discriminate functional positive CD4 T cells with a downregulated CD4 from functional positive CD8. Total CD4+ cells are then obtained by gates in (C) combined by the operator ''OR'' and the exclusion of the CD8 cells identified in (D). **E**: Live lymphocytes expressing CD3 and CD4 are then analyzed according to the expression of five functional markers (CD154, MIP-1β, IFN-γ, TNF-α, and IL-2) and the differentiation marker CD45RA. Combination of axis was chosen to allow easy positioning of gates and optimal discrimination between positive and negative events. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Table 1. Summary table for application of OMIP-016

Purpose	Quality and phenotype of antigen-specific CD4 and CD8 T cells
Species	Cynomolgus macaque or Human
Cell types	Fresh or cryopreserved PBMCs
Cross reference	OMIP-005 and OMIP-009

egy where the CD3 marker is gated against all the possible activation markers. CD3+ cell gates are then combined using a Boolean operator "OR." The same gating procedure is adopted to identify CD4+ and CD8+ cells.

Similarity to Published OMIPs

This OMIP shares some similarity to OMIP-005 and OMIP-009. Our OMIP-016 can be used to simultaneously analyze macaque and human samples. It is more oriented to CD4 activation with the inclusion of CD154 and it includes a breaking point in the experimental procedure allowing multicenter setting.

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Table 2. Reagents used for OMIP-016

SPECIFICITY	FLUOROCHROME	CLONE	PURPOSE
Dead Cells	Blue fluorescent dye	na	Viability
CD3	APC-Cy7	SP34-2	Lineage
CD4	PerCP-Cy5.5	L200	Lineage
CD8	V500	RPA-T8	Lineage
CD45RA	PE-Cy7	L48	Memory/ differentiation
CD154	FITC	TRAP1	CD4 T cell activation
MIP-1β	PE	D21-1351	Function
IFN-γ	V450	B27	Function
TNF-α	Ax700	MAb11	Function
IL-2	APC	MQ1-17H12	Function

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