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OMIP-015: Human Regulatory and Activated T-Cells Without Intracellular Staining

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Additional and updated information including technical details may be found in the online version of this article.

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Purpose and Appropriate Sample Types

The present panel was optimized to investigate the frequency and phenotype of regulatory T-cells (T_{reg}), as well as the activation status of $CD4^+$ and $CD8^+$ T-cells in peripheral blood mononuclear cells (PBMC) from healthy individuals, without the use of intracellular staining (i.e., excluding the use of the canonical T_{reg} marker, FoxP3). The panel has been developed using cryopreserved PBMC and we have observed similar results with fresh specimens. Other tissue types have not been tested.

Background

Two OMIPs designed for the investigation of T_{reg} have recently been published (1,2). These use the expression of surface CD25 and intracellular FoxP3 to identify the T_{reg} subset of $CD4^+$ T-cells. This panel is designed to avoid experimental complexity induced by fixation, permeabilization, and intracellular staining. Notably this panel is potentially compatible with live cell sorting needed for functional and genomic interrogation of T_{reg}. A combined measurement of the IL-2Rα chain (CD25) and the IL-7R α-chain (CD127) was used, with T_{reg} being defined as $CD25^{hi}$ $CD127^-$ (3). Only about 13% of this population is $FoxP3^-$ (unpublished data) (3,4). T_{reg} were further characterized functionally and phenotypically by the expression of CD39, CD73, CD45RA, CD45RO, and PD-1. The nucleoside triphosphate dephosphorylase CD39 has been shown to identify cells with *in vitro* immunosuppressive capacity (5,6). CD73 is an ecto-5′-nucleotidase that, together with CD39, contributes to the inhibitory function of T_{reg} and other suppressive immune cells, as well as the tumor microenvironment, by generating adenosine (7–9). CD45RA (10) and CD45RO (11) were included in order to define naïve and activated T_{reg}, respectively, while PD-1 was selected as it has been demonstrated to negatively regulate T_{reg} function (12).

While the primary objective of the present panel was to identify and characterize T_{reg}, some of the markers included also provide information on the differentiation and activation status of other $CD4^+$ and $CD8^+$ T-cells. For instance, though CD39 is mainly expressed on immunosuppressive T_{reg}, it is also found on pathogenic $CD4^+$ $CD25^-$ effector memory T-cells (13). CD45RA, CD45RO, and CD127 are differentially expressed during T-cell differentiation, while CD25 and PD-1 are expressed in an activation-dependent manner.

Finally, CD38 and HLA-DR were included; differential expression of these two T-cell activation markers identifies subsets disparately correlating with disease control, particularly in HIV-1 infection (14,15).

To explore the expression of activation markers on T_{reg}, gates were set on total $CD4^+$ T-cells (Fig. 1B) and then applied to T_{reg} (Fig. 1C). For dim markers such as HLA-DR and PD-1, careful choice of the second marker visualized in the dot plot allowed identifying the best threshold for separating positive and

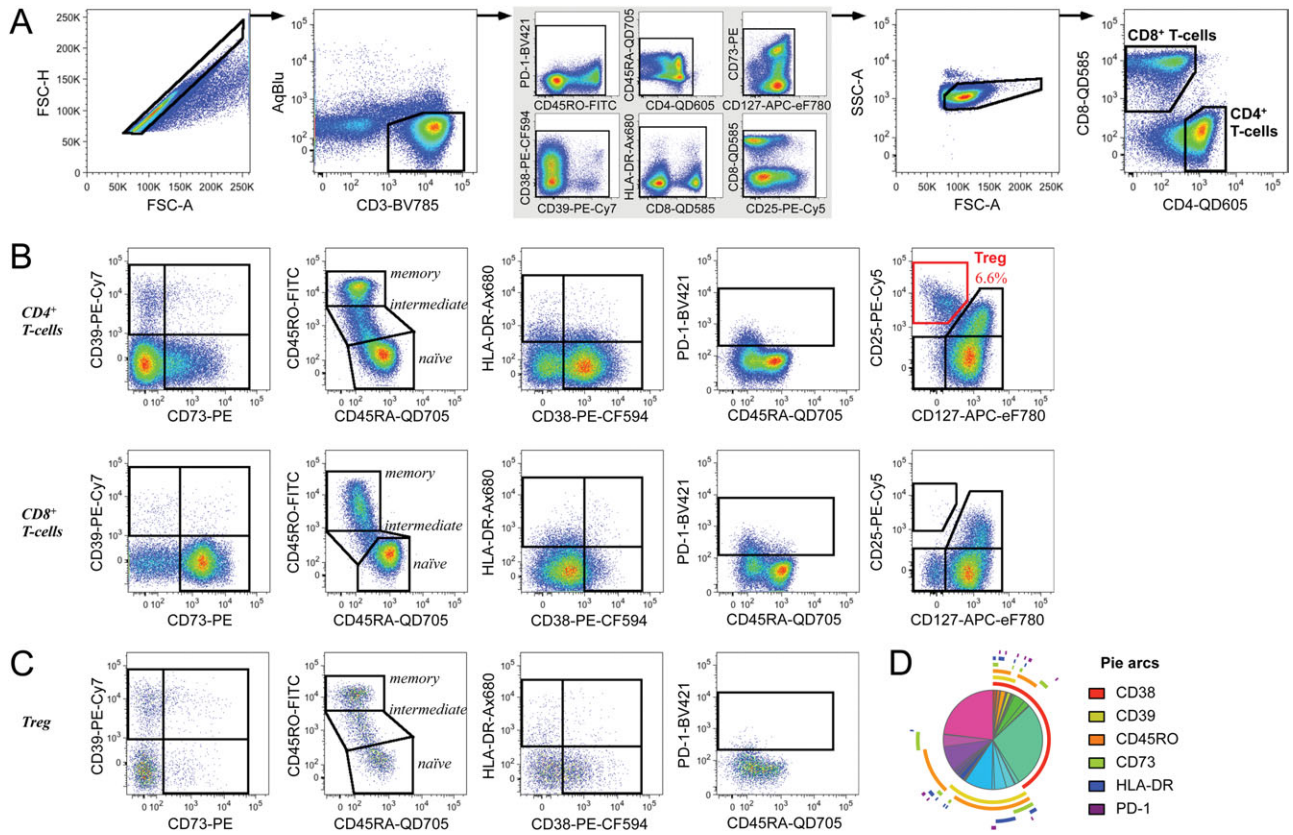

Figure 1. Example staining and gating. **A:** Identification of $CD4^+$ and $CD8^+$ T-cells in healthy donor PBMC. After selecting live $CD3^+$ single cells, any cells nonspecifically labeled with dye aggregates are excluded (gray box) and a lymphocyte gate set. $CD4^+$ and $CD8^+$ T-cells are then selected for further analysis. **B:** Evaluation of $CD4^+$ and $CD8^+$ T-cell activation, and identification of T_{reg}. The expression level of CD25, CD38, CD39, CD45RA, CD45RO, CD73, CD127, HLA-DR, and PD-1 are investigated within $CD4^+$ and $CD8^+$ T-cell subset as gated in (A). Within $CD4^+$ T-cells, T_{reg} are $CD127^-$ while expressing high levels of CD25. **C:** T_{reg} phenotype. The analysis of CD39 and CD73, CD45RA, and CD45RO, and CD38, HLA-DR, and PD-1 on their surface provides insight into maturation states, regulatory potential, and activation of the cells, respectively. Gates were set on total $CD4^+$ T-cells (see B) and were then applied to T_{reg}. **D:** Exploration of T_{reg} activation. The pie chart illustrates the proportion of total T_{reg} expressing any given combination of the activation markers measured.

negative cells. Neither isotype nor fluorescence minus one (FMO) controls were used as many years of working with these markers have taught us what the staining patterns typically look like and where to set gate boundaries.

Similarity to Published OMIPs

OMIP-004 and -006 were also designed for the investigation of human T_{reg}.

Table 1. Summary Table for application of OMIP-015

Purpose	T-reg characterization and T-cell activation
Species	Human
Cell types	PBMC
Cross-references	n.a.

Table 2. Reagents used for OMIP-015

SPECIFICITY	CLONE	FLUOROCHROME	PURPOSE
CD3	OKT3	BV785	Lineage
CD4	OKT4	QD605	
CD8	RPA-T8	QD585	
CD25	M-A251	PE-Cy5	T-reg
CD127	eBioRDR5	APC-eF780	
CD39	eBIOA1	PE-Cy7	T-reg functionality
CD73	AD2	PE	
CD38	HIT2	PE-CF594	Other activation/ differentiation
CD45RA	5H9	QD705	
CD45RO	UCHL1	FITC	
HLA-DR	G46-4	Ax680	
PD-1	EH12.2H7	BV421	
Dead cells	-	AqBlu	Dump

BV, brilliant violet; QD, quantum dot; PE, R-phycoerythrin; Cy, cyanine; APC, allophycocyanin; eF, eFluor; CF, cyanine-based fluorescent dye; FITC, fluorescein; Ax, Alexa; AqBlu, LIVE/DEAD Fixable Aqua Dead Cell Stain.

Literature Cited

1. Biancotto A, Dagur PK, Fuchs JC, Langweiler M, McCoy JP Jr. OMIP-004: In-depth characterization of human T regulatory cells. Cytometry Part A 2012;81A:15–16.
2. Murdoch DM, Staats JS, Weinhold KJ. OMIP-006: Phenotypic subset analysis of human T regulatory cells via polychromatic flow cytometry. CytometryPart A 2012;81A:281–283.
3. Seddiki N, Santner-Nanan B, Martinson J, Zaunders J, Sasson S, Landay A, Solomon M, Selby W, Alexander SI, Nanan R, et al. Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. J Exp Med 2006;203:1693–1700.
4. Liu W, Putnam AL, Xu-Yu Z, Szot GL, Lee MR, Zhu S, Gottlieb PA, Kapranov P, Gingeras TR, Fazekas de St Groth B, et al. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. J Exp Med 2006;203:1701–1711.
5. Dwyer KM, Hanidziar D, Putheti P, Hill PA, Pommey S, McRae JL, Winterhalter A, Doherty G, Deaglio S, Koulmanda M, et al. Expression of CD39 by human peripheral blood CD4+ CD25+ T cells denotes a regulatory memory phenotype. Am J Transplant 2010;10:2410–2420.
6. Schulze Zur Wiesch J, Thomssen A, Hartjen P, Toth I, Lehmann C, Meyer-Olson D, Colberg K, Frerk S, Babikir D, Schmiedel S, et al. Comprehensive analysis of frequency and phenotype of T regulatory cells in HIV infection: CD39 expression of FoxP3+ T regulatory cells correlates with progressive disease. J Virol 2011;85:1287–1297.
7. Alam MS, Kurtz CC, Rowlett RM, Reuter BK, Wiznerowicz E, Das S, Linden J, Crowe SE, Ernst PB. CD73 is expressed by human regulatory T helper cells and suppresses proinflammatory cytokine production and Helicobacter felis-induced gastritis in mice. J Infect Dis 2009;199:494–504.
8. Beavis PA, Stagg J, Darcy PK, Smyth MJ. CD73: A potent suppressor of antitumor immune responses. Trends Immunol 2012;33:231–237.
9. Deaglio S, Dwyer KM, Gao W, Friedman D, Usheva A, Erat A, Chen JF, Enjyoji K, Linden J, Oukka M, et al. Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. J Exp Med 2007;204:1257–1265.
10. Hoffmann P, Eder R, Boeld TJ, Doser K, Piseshka B, Andreesen R, Edinger M. Only the CD45RA+ subpopulation of CD4+CD25high T cells gives rise to homogeneous regulatory T-cell lines upon in vitro expansion. Blood 2006;108:4260–4267.
11. Haas J, Fritzsching B, Trubswetter P, Korporal M, Milkova L, Fritz B, Vobis D, Krammer PH, Suri-Payer E, Wildemann B. Prevalence of newly generated naive regulatory T cells (Treg) is critical for Treg suppressive function and determines Treg dysfunction in multiple sclerosis. J Immunol 2007;179:1322–1330.
12. Franceschini D, Paroli M, Francavilla V, Videtta M, Morrone S, Labbadia G, Cerino A, Mondelli MU, Barnaba V. PD-L1 negatively regulates CD4+CD25+Foxp3+ Tregs by limiting STAT-5 phosphorylation in patients chronically infected with HCV. J Clin Investig 2009;119:551–564.
13. Moncrieffe H, Nistala K, Kamhieh Y, Evans J, Eddaoudi A, Eaton S, Wedderburn LR. High expression of the ectonucleotidase CD39 on T cells from the inflamed site identifies two distinct populations, one regulatory and one memory T cell population. J Immunol 2010;185:134–143.
14. Giorgi JV, Ho HN, Hirji K, Chou CC, Hultin LE, O'Rourke S, Park L, Margolick JB, Ferbas J, Phair JP. CD8+ lymphocyte activation at human immunodeficiency virus type 1 seroconversion: development of HLA-DR+ CD38− CD8+ cells is associated with subsequent stable CD4+ cell levels.The Multicenter AIDS Cohort Study Group. J Infect Dis 1994;170:775–781.
15. Meditz AL, Haas MK, Folkvord JM, Melander K, Young R, McCarter M, Mawhinney S, Campbell TB, Lie Y, Coakley E, et al. HLA-DR+ CD38+ CD4+ T lymphocytes have elevated CCR5 expression and produce the majority of R5-tropic HIV-1 RNA in vivo. J Virol 2011;85:10189–10200.