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

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OMIP-033: A Comprehensive Single Step Staining Protocol for Human T- and B-Cell Subsets

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Received 21 January 2016; Revised 20 April 2016; Accepted 11 May 2015

Grant sponsor: Swiss Multiple Sclerosis Society

Grant sponsor: The Progressive Multifocal Leukoencephalopathy (PML) Consortium (Switzerland).

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

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Conflict of interest: Authors declare no conflict of interest.

Published online 8 June 2016 in Wiley Online Library (wileyonlinelibrary.com)

DOI: 10.1002/cyto.a.22889

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• **Key terms**
T cell memory subsets; B cell memory subsets; plasma cells; chemokine receptors; cerebrospinal fluid; whole blood; multiple sclerosis

Purpose and Appropriate Sample Types

Limited sample availability is a common problem for research with patient material, and this factor has hampered phenotypic studies. This work addresses the clear need for a thorough immunophenotyping panel tailored for biological samples with few cells. This panel is optimized for staining of both cerebrospinal fluid (CSF) lymphocytes (containing 10–100,000 cells), as well as whole blood. The usually very low CSF cell numbers make a one-step staining protocol necessary to minimize cell loss in washing steps. CSF cells must be stained immediately due to the CSF's low protein content (1), which renders cells vulnerable. Ideal CSF samples are no older than 1 h and have no fewer than a total of 10,000 cells. This panel identifies human CD4 and CD8 memory subsets as well as T helper subsets, CD4+ CD28− costimulation-independent T cells, B cell memory subsets, and plasma cells. Optimal whole blood samples should be no older than 5 h after sample acquisition (due to plasma cell loss). In these conditions, samples contain extremely few dead cells, and due to the necessity of a one-step staining, we did not include a live-dead cell discriminator. This panel can be successfully performed on frozen PBMCs, but authors then recommend inclusion of a live/dead marker, and also it needs to be noted that plasma cells are sensitive to freeze/thawing.

Background

OMIP-033 was designed to phenotypically characterize T and B cell subsets in the CSF and whole blood from multiple sclerosis (MS) patients with different forms/stages of the disease and under different treatments. CSF is in direct contact with brain tissue, and hence it can be expected that CSF lymphocytes are biologically relevant and enriched in MS patients (2,3). Comparison of T and B cell subsets in whole blood and CSF can lead to insight about the cellular exchange between these two niches.

Myelin-specific T cells play a major role in both the induction and maintenance of neuroinflammation in MS (4,5). The T cell memory markers CCR7 and CD45RA were selected for identification of naïve (TN), central memory (TCM), effector memory (TEM), and effector memory RA+ (TEMRA) subsets (6,7). Within these memory populations chemokine receptor expression identifies T helper 1 (Th1) (CCR4− CCR6−), T helper 2 (Th2) (CCR4+ CRTh2+), and T helper 17 (Th17) (CCR4+ CCR6+) populations (7,8). Since there are multiple ways to define a T cell subset, this panel allows for the identification of diverse Th2 and Th1 subsets. Th2

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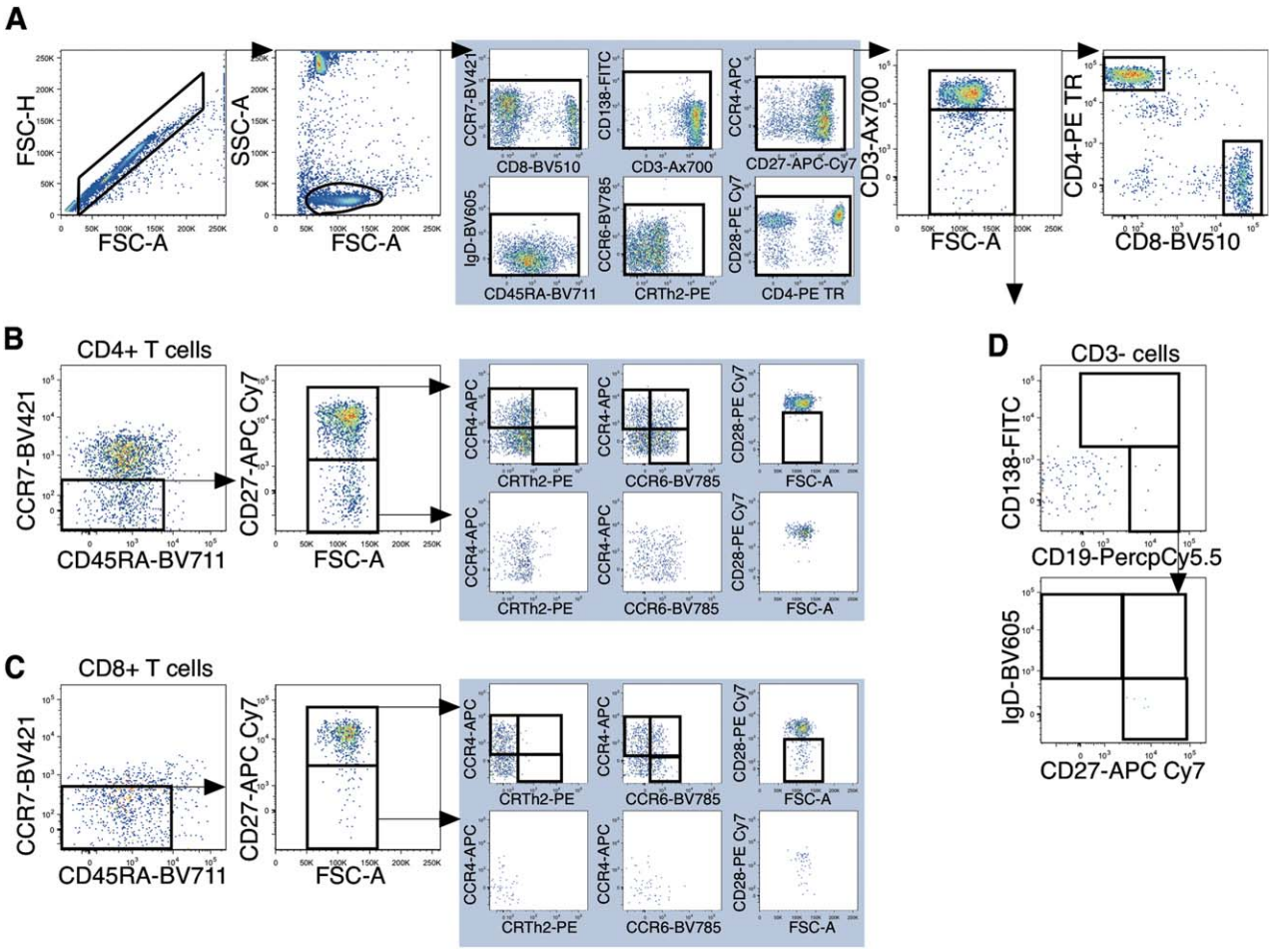

Figure 1. Gating strategy for human CSF staining. **A**: Shown are cleaning gates followed by distinguishing between CD3+ T cells and CD3− cells. Briefly, first doublets were excluded, followed by identification of lymphocytes and exclusion of aggregates (bright events outside the main populations). Once each detector is cleaned for aggregates, CD3−, CD3+ CD4+, and CD3+ CD8+ T cell populations are identified. **B**: CD3+ CD4+ T cells and (**C**) CD3+ CD8+ T cells are identified. For example, purposes, CCR7− CD45RA− effector memory cells are shown and further characterized using CD27 expression. Within CD27+ and CD27− cells, chemokine receptor expression shows diverse Th2 subsets with CRTh2 and CCR4 combinations and Th1 (CCR4− CCR6−), Th2 (CCR4+ CCR6−), Th17 (CCR4+ CCR6+), and Th1 CCR6+ cells. Possible autoreactive T cells are identified as CD28−. **D**: Among CD3− cells CD138 + plasma cells and CD19+ B cell subsets are shown. B cell subsets include naïve (IgD+ CD27−), marginal zone like (IgD+ CD27+), and memory (IgD− CD27+) B cells. CSF was obtained within 1 h of acquisition, stained immediately and 12,000 CSF cell events were collected. [Color figure can be viewed in the online issue which is available at wileyonlinelibrary.com]

cells can be described as CCR4 + CCR6−, or CCR4+ CRTh2+, or just CRTh2+ (7). For Th1, an IL-17 expressing Th1 subset has been described that is characterized by the expression of CCR6 (9). This panel is capable of revealing these subsets within any memory subset. CD27 was included as an additional memory discriminator since CD8+ CD45RA− CCR7− cells expressing CD27 lack immediate killing activity, while CD27 negative cells are cytotoxic (7,10). CD28 was selected due to findings that CD28- CD4 T cells are enriched in autoantigen-specific cells in MS, and these cells are considered capable of initiating autoimmune responses in the central nervous system, where expression of costimulatory molecules is low ((11–13); Figs. 1B and 1C).

The presence of oligoclonal bands in the CSF and antibody deposits in MS lesions supports a role for antibodies and B cells in disease pathogenesis (14,15). The importance of B cells in MS was clearly demonstrated by the success of anti-CD20 treatment (i.e., Rituximab and more recently Ocrelizumab) in relapsing-remitting MS patients (16,17). B cells may also be important for neuroinflammation as evidenced by the 4 patterns of demyelination described by Lucchinetti et al. (15). Pattern 2 shows evidence of antibody- and complement-mediated tissue damage, and CSF cells from these patients may provide clues to disease pathogenesis (Table 1). Further evidence of B cell involvement in MS is the presence and association with disease exacerbation, of ectopic B-cell follicle-like

Table 1. Summary table

PURPOSE	PHENOTYPE T- AND B-CELL SUBSETS; AND PLASMA CELLS
Species	Human
Cell types	Cells from fresh CSF Fresh whole blood
Cross references	OMIPS-003, 009, 013, 018

Table 2. Reagents used in OMIP-033

SPECIFICITY	CLONE	FLUOROCHROME	PURPOSE
CD3	HIT3a	AF700	T-cell lineage
CD4	S3.5	PE TR	
CD8	SK1	BV510	
CD45RA	HI100	BV711	Naïve and memory subsets
CCR7	G043H7	BV421	
CD27	O323	APC Cy7	
CD28	CD28.2	PECy7	Putative autoreactive cells
CCR4	L281H4	APC	T helper subsets
CCR6	G034E3	BV785	
CRTh2	BM16	PE	
CD19	HIB19	PerCPCy5.5	B cells
IgD	IA6-2	BV605	
CD138	MI15	FITC	Plasma cells

AF700, Alexa Fluor 700; PE, R-phycoerythrin; TR, texas red; BV, brilliant violet; APC, allophycocyanin; Cy, cyanin; PerCP, peridinin chlorophyll protein.

structures in the meninges of secondary progressive MS (18,19). In the present panel, the B cell markers CD27 and IgD were chosen for identification of naïve (NB), marginal zone-like (MZLB), and memory B (MB) cells as previously published (20), and plasma cells based on expression of CD138 ((21); Fig. 1D).

The strategy used to develop this panel was to start with a wish list containing the most important antibodies for our study and then assigning fluorochromes to these markers based on their known expression levels (22). We placed brighter fluorochromes for dimmer markers showing a continuous staining range (22), and dimmer fluorochromes for proteins expressed as on or off (Table 2). Over time we added an increasing number of T cell markers and removed B cell markers due to results with the previous panels showing redundancy or lack of cells in our compartment of interest (CSF). An important aspect of designing the present panel was switching from the BD Cytometer Setup and Tracking (CST) beads (the recommended BD LSR Fortessa settings) to settings outlined by Perfetto et al. (23). This set-up allowed for better resolution of T- and B cell populations (Supporting Information Fig. S4), which is particularly important in this single-step protocol.

A benefit of this panel is that it can be performed quickly, keeping cell loss during staining to a minimum. Its simplicity as a one-step staining may contribute to its successful use across clinics and research centers. A possible drawback is that the use of CSF cells requires close proximity to a hospital and that fresh samples must arrive in the lab in a timely manner (within one hour).

Human Subjects

These studies were conducted as part of two research projects: EC-ZH-No.2013–0001 and EC-ZH-No.2015–0477, which were approved by the Cantonal Ethics Committee of Zurich on June 5, 2013 and September 9, 2015, respectively.

Similarity to Published OMIPs

This panel includes a few T cell memory markers used in OMIPs 009 and 0013; T helper subsets used in OMIP 018 and B cell markers used in OMIP 003, but no panel yet incorporates these markers together.

Statement of Importance

This work will aid researchers interested in tracking both B and T cell subsets as well as plasma cells for both basic research and clinical trials. The one-step staining protocol makes it practical and possible to retain as many cells as possible when cell numbers are limited.

Acknowledgments

The authors would like to thank Juliane Hill and Steve Perfetto for their help with questions regarding the setup of the Nature Protocols cytometer quality control and Paula Thomas Ojer for technical support. They would also like to thank Roland Martin for critical reading of the manuscript. The Neuroimmunology and Multiple Sclerosis Research (nims) programs are supported by the Clinical Research Priority Program MS (CRPP-MS) at the University of Zurich.

Literature Cited

1. Felgenhauer K. Protein size and cerebrospinal fluid composition. Klin Wochenschr 1974;52:1158–1164.
2. Scolozzi R, Boccafogli A, Tola MR, Vicentini L Camerani A, Degani D, Granieri E, Caniatti L, Paolino E. T-cell phenotypic profiles in the cerebrospinal fluid and peripheral blood of multiple sclerosis patients. J Neurol Sci 1992;108:93–98.
3. Cepok S, Jacobsen M, Schock S, Omer B, Jaekel S, Beoddeker I, Oertel WH, Sommer N, Hemmer B. Patterns of cerebrospinal fluid pathology correlate with disease progression in multiple sclerosis. Brain 2001;124:2169–2176.
4. Sospedra M, Martin R. Immunology of multiple sclerosis. Annu Rev Immunol 2005; 23:683–747.
5. Dendrou CA, Fugger L, Friese MA. Immunopathology of multiple sclerosis. Nat Rev Immunol 2015;15:545–558.
6. Sallusto F, Geginat J, Lanzavecchia A. Central memory and effector memory T cell subsets: function, generation, and maintenance. Annu Rev Immunol 2004;22:745–763.
7. Mahnke YD, Brodie TM, Sallusto F, Roederer M, Lugli E. The who's who of T-cell differentiation: human memory T-cell subsets. Eur J Immunol 2013;43:2797–2809.
8. Sallusto F, Mackay CR. Chemoattractants and their receptors in homeostasis and inflammation. Curr Opin Immunol 2004;16:724–731.
9. Lindestam Arlehamn CS, Paul S, Mele F, Huang C, Greenbaum JA, Vita R, Sidney J, Peters B, Sallusto F, Sette A. Immunological consequences of intragenus conservation of Mycobacterium tuberculosis T-cell epitopes. Proc Natl Acad Sci U S A 2015;112: E147–155.
10. Hamann D, Baars PA, Rep MH, Hooibrink B, Kerkhof-Garde SR, Klein MR, van Lier RA. Phenotypic and functional separation of memory and effector human CD8+ T cells. J Exp Med 1997;186:1407–1408.

11. Scholz C, Patton KT, Anderson DE, Freeman GJ, Hafler DA. Expansion of autoreactive T cells in multiple sclerosis is independent of exogenous B7 costimulation. J Immunol 1998;160:1532–1538.
12. Lovett-Racke AE, Trotter JL, Lauber J, Perrin PJ, June CH, Racke MK. Decreased dependence of myelin basic protein-reactive T cells on CD28-mediated costimulation in multiple sclerosis patients. A marker of activated/memory T cells. J Clin Invest 1998;101:725–730.
13. Markovic-Plese S, Cortese I, Wandinger KP, McFarland HF, Martin R. CD4+CD28-costimulation-independent T cells in multiple sclerosis. J Clin Invest 2001;108:1185–1194.
14. Weber MS, Hemmer B, Cepok S., The role of antibodies in multiple sclerosis. Biochim Biophys Acta 2011;1812:239–245.
15. Lucchinetti C, Breuck W, Parisi J, Scheithauer B, Rodriguez M, Lassmann H. Heterogeneity of multiple sclerosis lesions: Implications for the pathogenesis of demyelination. Ann Neurol 2000;47:707–717.
16. Hauser SL, Waubant E, Arnold DL, Vollmer T, Antel J, Fox RJ, Bar-Or A, Panzara M, Sarkar N, Agarwal S, et al. B-cell depletion with rituximab in relapsing-remitting multiple sclerosis. N Engl J Med 2008;358:676–688.
17. Kappos L, Li D, Calabresi PA, O'Conner P, Bar-Or A, Barkhof F, Yin M, Leppert D, Glanzman R, Tinbergen J, et al. Ocrelizumab in relapsing-remitting multiple sclerosis: A phase 2, randomised, placebo-controlled, multicentre trial. Lancet 2011;378:1779–1787.
18. Magliozzi R, Howell OW, Reeves C, Roncaroli F, Nicholas R, Serafini B, Aloisi F, Reynolds R. A Gradient of neuronal loss and meningeal inflammation in multiple sclerosis. Ann Neurol 2010;68:477–493.
19. Serafini B, Rosicarelli B, Franciotta D, Magliozzi R, Reynolds R, Cinque P, Andreoni L, Trivedi P, Salvetti M, Faggioni A, et al. Dysregulated Epstein-Barr virus infection in the multiple sclerosis brain. J Exp Med 2007;204:2899–2912.
20. Klein U, Rajewsky K, Kuppers R. Human immunoglobulin (Ig)M+IgD+ peripheral blood B cells expressing the CD27 cell surface antigen carry somatically mutated variable region genes: CD27 as a general marker for somatically mutated (memory) B cells. J Exp Med 1998;188:1679–1689.
21. O'Connell FP, Pinkus JL, Pinkus GS. CD138 (syndecan-1), a plasma cell marker immunohistochemical profile in hematopoietic and nonhematopoietic neoplasms. Am J Clin Pathol 2004;121:254–263.
22. Mahnke YD, Roederer M. Optimizing a multicolor immunophenotyping assay. Clin Lab Med 2007;27:469–485.
23. Perfetto SP, Ambrozak D, Nguyen R, Chattopadhyay PK, Roederer M. Quality assurance for polychromatic flow cytometry using a suite of calibration beads. Nat Protoc 2012;7:2067–2079.