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

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OMIP-041: Optimized Multicolor Immunofluorescence Panel Rat Microglial Staining Protocol

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Received 17 January 2017; Revised 14 September 2017; Accepted 26 September 2017

Grant sponsor: Clare A. Glassell Family Pediatric Stem Cell Research Fund

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

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Published online 14 October 2017 in Wiley Online Library (wileyonlinelibrary.com)

DOI: 10.1002/cyto.a.23267

• **Key terms**
rat microglia; M1 polarized microglia; M2 polarized microglia; traumatic brain injury; spinal cord injury; neuro-immuno-phenotyping

Purpose and Appropriate Sample Type

The common usage of animal models in a variety of preclinical studies is supported by appropriate species-specific antibodies to be utilized in immunohistochemistry (IHC), western blotting, and flow cytometry (FC) assays. Other than the technical advantages (sophisticated surgical manipulations due to their size), modest cost (relative to larger animals), and the standardized results, the similarities in metabolic activity and physiology of neurological disorders to humans make rats appropriate for neurological disease or disorders models (1,2). However, rat-based assays are not as comprehensive or standardized as mouse or human based assays are, partially because there is a shortage in rat-specific antibodies. Rat-specific antibodies are now becoming commercially available, which allows us to set standardize criteria for rat origin cells of interest. As our research focus is in neurotherapy, we are interested specifically in microglial cells, which are the innate immune cells in the brain (3) and spinal cord (4). Microglial cells play a critical role in traumatic brain injuries (TBI) and spinal cord injuries (SCI), and their presence, activation, and effect are highly investigated in those models (4–6). Microglial characterization via FC would save many hours of work as a substitute for IHC analysis, yield unbiased statistics, and overall help research move at a faster pace (7). Here we present multicolor phenotyping panels for assessing microglia derived from rat brain or spinal cord for their activation states, polarization, and number (see Table 1). The microglial cells used are immediately isolated from fresh brain or spinal cord tissues, using a Neural Tissue Dissociation kit, followed by myelin removal and purification using anti-rat CD11b/c microbeads.

Background

The Optimized Multicolor Immunofluorescence Panel (OMIP) was designed to phenotypically characterize rat-derived microglial cells isolated from brain or spinal cord, in TBI or SCI rat models. Central nervous system (CNS) injury immediately results in a robust inflammatory cell-mediated response. The key players in the response to the injury are microglial cells, which are a major cellular component of the CNS innate immune system. In normal situations, microglia maintains a dynamic and ramified phenotype, while an injury results in a dramatic amoeboid phenotype change. CNS injuries also lead to infiltration of macrophages to the injury site and both microglia and macrophages share the expression of surface markers such as CD45, CD11b/c, and CX_3CR_1. The common way of identifying microglia

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Table 1. Summary table for OMIP-041

PURPOSE	MICROGLIA IDENTIFICATION AND CHARACTERIZATION OF ACTIVATION
Species	Rat
Cells type	Primary Microglia Cells
Cross reference	None to date.

using FC is as CD11b/c^{+} CD45^{low} (8,9). In this study, we developed a new multiparametric flow cytometric analysis that not only enables differentiation between macrophages and resident microglia but also defines phenotypic changes in microglia in rat model.

Recently, microglial specific markers such as transmembrane protein 119 (Tmem119) and the p2y12 receptor were found to distinguish between resident microglia and infiltrating macrophages which originate from the same myeloid lineage (10). The p2y12 receptor is an APD-responsive G protein-coupled receptor. In the CNS, its expression is restricted to microglia. It is now known that the p2y12 receptor is selectively expressed in nonactivated microglia and mediates process motility during early injury responses (11). Both anti-p2y12 and anti-Tmem119 antibodies were produced and studied well in mouse and human (8), but less so in rat models. As far as we know, only one anti-rat p2y12 antibody (Alomone Labs, Jerusalem, Israel) is currently available for FC purposes.

Microglial activation is not one defined path of phenotypic changes, but a heterogeneous phenotype expression profile that is unique to the cells, particular conditions/diseases,

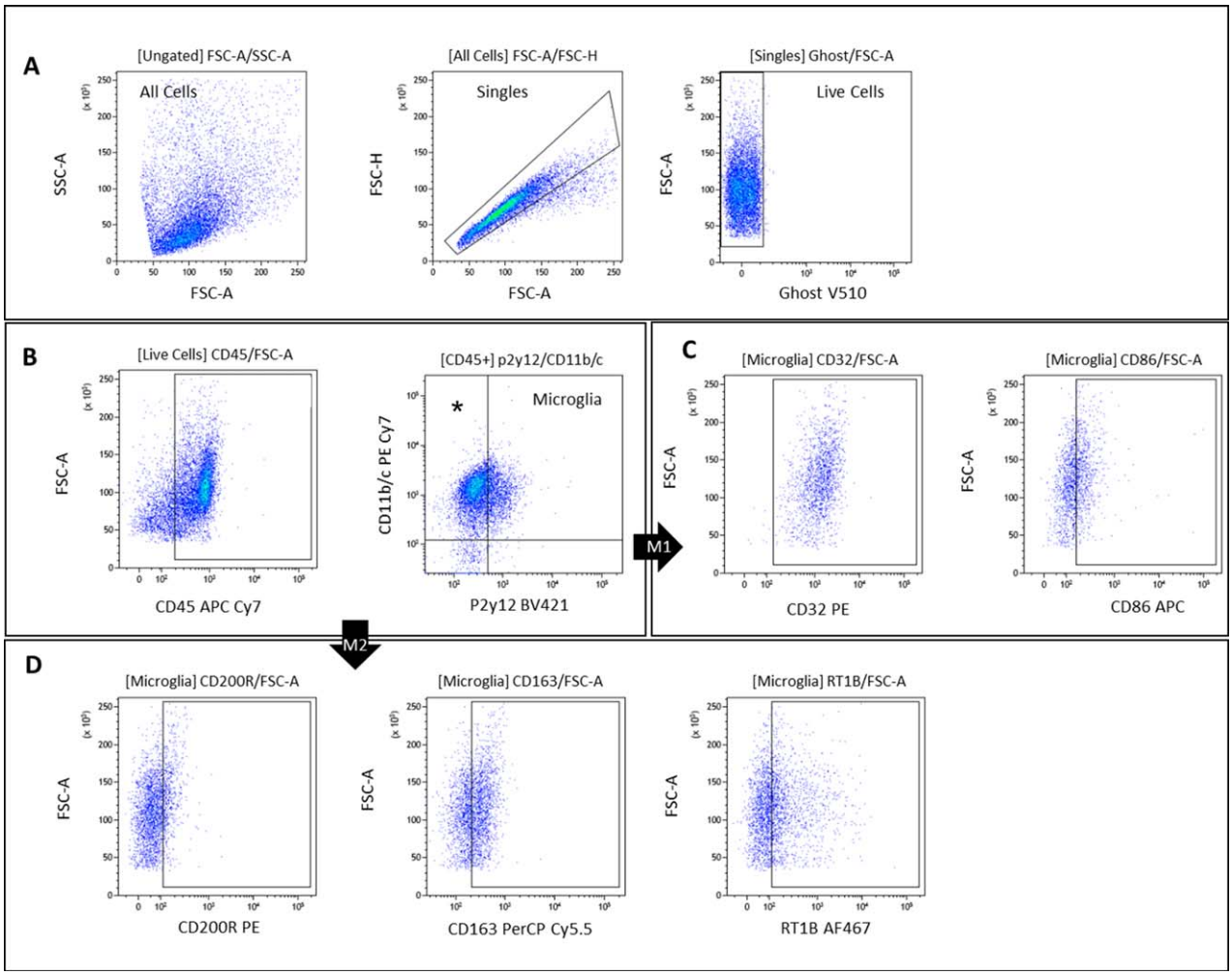

Figure 1. Gating strategy for rat microglia. After isolation, each cell sample was divided into 2 tubes. The pro- and anti- inflammatory paths were evaluated separately in tube 1 (M1) and tube 2 (M2), respectively. (**A**) The main cellular population was gated to exclude doublets, and viable cells were gated under the single cells gate. (**B**) Identification of microglia using the triplet anchor CD45, CD11b/c and p2y12. Microglia are positive for all 3 markers. Cells that are positive for CD45+ and CD11b/c but negative for p2y12 (noted with *) are considered all other myeloid cells (such as monocytes and macrophages). (**C**) The M1 markers acquired were CD32 and CD86, under the microglia gating. (**D**) M2 polarized microglia were detected by the M2 markers CD200R, RT1B, and CD163. The level of expression of all the markers in the panels may vary with the activation levels of the cells. Therefore, during experiments biological controls as sham or naïve rat derived cells are recommended for the comparison and precise analysis of cells derived from injured tissues.

Table 2. Reagents used in OMIP-041 for microglia phenotyping

SPECIFICITY	CLONE	FLUOROCHROME	PURPOSE	ASSOCIATION TO DIFFERENTIATION PATH	REFS.	PANEL
CD45	OX-1	APC-Cy7	Leucocyte common antigen. General microglia marker.	M1 and M2	(19)	M1 and M2
CD11b/c	OX-42	PE-Cy7	CD11b and CD11c are integrin αM and αX chains, respectively. General microglia marker.	M1 and M2	(20)	M1 and M2
P2Y12	Polyclonal	BV421	Selectively expressed on microglia, and mediates process motility during early injury responses.	M1and M2	(10,11)	M1 and M2
CD32	D34–485	PE	Immunoglobulin Fc receptor, associated with phagocytosis. CD32a (Fc_{γ}IIA) and CD32b (Fc_{γ}RIIB) activate inhibitory signaling.	M1and M2b	(17,21–23)	M1
CD86	24-F	APC	T cell receptor ligand. Allow for antigen- presenting activity.	M1 and M2b	(12,13)	M1
CD200R	OX-102	PE	A membrane glycoprotein. The interaction between CD200R and its ligand limits the inflammatory damage in the tissue.	M2a	(21,24)	M2
RT1B	OX-6	Alexa Fluor 647	Allow for antigen- presenting activity	M1 and M2a	(12,13,25,26)	M2
CD163	ED2	PerCP Cy5.5	Involves in clearing hemoglobin and in regulation of cytokine production.	M2c	(22,27)	M2
Live/Dead Reagent	–	V510	–	–	–	M1 and M2

and variable over time. Similar to the M1-M2 polarization process of macrophages in non-neuronal tissues, microglia present multiple activation phenotypes. However, the markers for each polarization state were defined using in vitro models, and as such, this paradigm are oversimplified. Nonetheless, given the limited data on the role microglia after TBI or SCI, this classification establishes a solid base to investigate the role of microglia and evaluate new treatment strategies. The classical M1 activation of microglia, in response to TNF-α and IFN-γ, involves phagocytosis, ability to kill pathogens and ROS release (12). As microglia are antigen presenting cells and communicate with T cells, activated microglia will upregulate their cell surface markers such as MHC-II and CD86 (13). M2 classification branch out to 3 major alternative pathways. While the phenotypic profile of the M2 cells may diverse, they are united in their purpose to downregulate, repair or protect the body from inflammation (14). Microglia can be stimulated using IL4 or IL13 in an alternative pathway that includes immunity against parasites, Th2 cell recruitment, and tissue repair. Under these conditions, cells are defined as M2a-polarized. The M2c phenotype, also called "acquired deactivation," is in response to IL10, glucocorticoids, or uptake of apoptotic cells where microglia are involved in tissue remodeling processes. In M2c, cells will overexpress transforming growth factor (TGF) beta, sphingosine kinase (SPHK1), and CD163, the membrane-bound scavenger receptor for haptoglobin/hemoglobin complexes (15–17). The M2b phenotype has characteristics of both M1 and M2 and is associated with memory immune response (18).

Microglia are being evaluated via FC, however, most data published are presenting human or mouse microglia with only few publications showing microglia of rat origin. Moreover, no uniform, validated, or standardized panel to evaluate rat microglia is currently available. Our method allows the evaluation of rat microglia originating from CNS-related tissues following an injury, as well as be useful in variety of applications other than one suggested here.

The procedure is optimized to sample a single brain hemisphere or a 1 inch long section of spinal cord tissue (see Supporting Information Fig. S4 for microglia isolation process illustration). We developed this panel (Figure 1) based on an existing mouse microglia panel that was previously developed and utilized in our lab (7). The rationale was to first identify the main microglia population using CD45, CD11b/c, and p2y12 antibodies. Next, we chose markers that are available for rat that could indicate the polarization state a cell is in, see Table 2. Antibodies for intracellular markers were omitted from this protocol (such as Iba1 and CD68), as the permeabilization conditions have been shown to critically affect the cells (28). Additional benefits of limiting the protocol to surface staining are the retained ability to sort live cells for functional experiments and the simplicity of the procedure.

The panel was designed with consideration of the brightness index of the fluorophores that were matched to each

marker. Finally, optimization was performed to validate the phenotypic changes of differentiated cells using antibodies titrations and fluorescence minus one controls.

Source of Cells

Male Sprague Dawley Rats (225–250 g, Harlan Labs) were the source of CNS tissue. The usage of the animals was approved by the Animal welfare committee at University of Texas Health Science Center at Houston, Texas, protocol: AWC16–0046 and AWC14–0023. Animals were handled in accordance with the standards of the American Association for the Accreditation of Laboratory Animal Care (AAALAC).

Statement of Importance

This work will provide researchers with a practical tool to evaluate the polarization state of the microglial cells following brain or spinal cord injuries in rat models. This surface staining is a simple and relatively quick procedure to evaluate microglia, which are freshly harvested from brain or spinal cord.

Acknowledgments

We would like to acknowledge the contributions of Deepa Bhattarai, Dr. Amit Srivastava, and Dr. Katherine Ruppert for their support and assistance in providing primary tissue for this study and final editing of the manuscript.

Author Contributions

Dr. Naama Toledano-Furman performed the final isolation of primary cells, staining, flow cytometry, analysis, and was the primary author of the manuscript.

Karthik S. Prabhakara was responsible for the majority of the surgery and primary isolation of tissue and cells used in this manuscript.

Dr. Supinder Bedi assisted in the design and marker selection for the panels and performed some surgical manipulations.

Drs. Olson and Cox provided guidance, assistance with writing and editing the manuscript, and financial and logistical support of the work.

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