

Accurately Differentiate Microglia from Macrophages to Understand Their Role in the Central Nervous System (CNS)

INTRODUCTION

Microglia are the primary resident immune cells in the brain parenchyma and play a variety of roles, such as releasing of cytokines and phagocytosis of foreign and dead cells. Numerous studies have reported that microglia are involved in various brain diseases. On the other hand, macrophages are found in the meninges and perivascular spaces in the central nervous system (CNS), and their properties are very similar to those of microglia. To understand the functional significance of microglia, especially in CNS-associated disease, it is essential to distinguish microglia from macrophages.

P2RY purinoceptor 12 (P2RY12) and trans-membrane protein 119 (TMEM119) are currently used as markers to discriminate between microglia and macrophages. However, commercially-available antibodies for microglial specific markers are confined to a limited number of animal species and lack versatility in multiple immunostaining.

In this study, we developed a guinea pig antibody against mouse P2RY12 with highly reproducible immunohistochemistry.

MATERIALS AND METHODS

Antibody preparation

We prepared the Anti-P2RY12 guinea pig polyclonal antibody by first determining the target sequence of mouse P2RY12 from protein conformation prediction by using AlphaFold2 and the synthesized peptides corresponding to the C-terminus of P2RY12, which were immunized to guinea pigs. After immunization, antisera were collected from the animals and the polyclonal antibody was purified using protein-A affinity chromatography.

Immunohistochemistry

Adult male mice (ICR) were perfused transcardially with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA). After perfusion of the fixative, brains were dissected out, postfixed in 4% PFA at 4°C for 24 hours, cryoprotected by 30% sucrose in PBS, and frozen quickly in Tissue-Tek OCT Compound. Sections were obtained by sagittal or coronal cutting on a cryostat at a thickness of 30 µm.

For immunohistochemistry, the section was incubated with a blocking solution consisting of 0.3% Triton X-100/PBS and 5% normal goat serum. The primary and secondary antibodies used in the experiment are listed in **Table 1**.

Table 1. Antibodies

Primary Antibodies		Conjugated Secondary Antibodies
<ul style="list-style-type: none"> • P2RY12 - Guinea Pig IgG* (Fujifilm, Catalog # 011-28873; dilution 1:1,000) • Iba1 - Rabbit IgG* (Fujifilm, Catalog # 019-19741; dilution 1:1,000) • Iba1 - Goat IgG* (Fujifilm, Catalog # 011-27991; dilution 1:1,000) 	<ul style="list-style-type: none"> • TMEM119 - Rabbit IgG (Commercially-available antibody; dilution 1:100) • P2RY12 - Rabbit IgG (Commercially-available antibody; dilution 1:200; [0.5 g/mL]) • Laminin - Rat IgG (Kyoto Institute of Technology Miyata Lab; dilution 1:200) 	<ul style="list-style-type: none"> • Dylight 405 (Jackson ImmunoResearch; dilution 1:500) • Alexa Fluor 488 (Jackson ImmunoResearch; dilution 1:500) • Alexa Fluor 594 (Jackson ImmunoResearch; dilution 1:500) • Alexa Fluor 647 (Jackson ImmunoResearch; dilution 1:500)

*Manufactured by FUJIFILM Wako Pure Chemical Corporation. Available from FUJIFILM Biosciences.

RESULTS

Immunohistochemistry (P2RY12 vs Iba1 vs DAPI)

The sections of the mouse hippocampus were immunolabeled with the polyclonal antibodies against P2RY12 and Iba1 with nuclear staining of DAPI. As shown in **Figure 1**, P2RY12 signals were detected in the hippocampus, all of which were localized in Iba1-positive microglia.

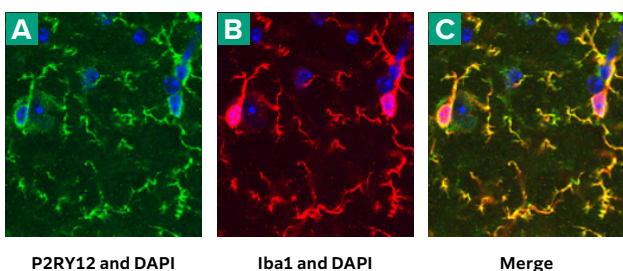


Figure 1. Visualization of P2RY12- and Iba1-immunopositive microglia. Double labeling immunohistochemistry showed the presence of P2RY12⁺ and Iba1⁺ microglia (**A-C**).

Specific detection of microglia with P2RY12 antibody

Triple immunostaining was performed using antibodies against P2RY12, Iba1, and Laminin. P2RY12⁺ (positive) and Iba1⁺ microglia (arrowheads) were observed in the brain parenchyma of the mouse area postrema. On the other hand, P2RY12⁻ (negative) and Iba1⁺ macrophage-like cells (arrow) were observed in the perivascular space within laminin⁺ vasculature (**Figure 2**).

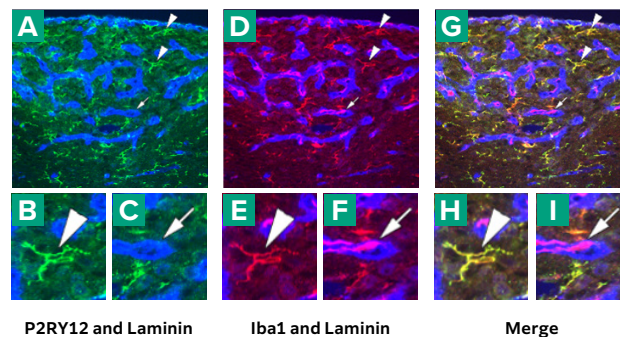


Figure 2. Triple immunostaining with anti-P2RY12, Iba1, and Laminin polyclonal antibodies. The immunoreactivity of P2RY12 was seen at Iba1⁺ microglia (arrowheads) in brain parenchyma. However, it was not observed at Iba1⁺ macrophages (arrows) in Laminin⁺ perivascular space (**A-I**).

Subcellular localization of P2RY12

The cortical and cerebellar sections were immunostained with antibodies against P2RY12, Iba1, or TMEM119. The P2RY12⁺ immunofluorescence was observed at the cellular surface of microglia and therefore fine cellular processes were clearly visualized compared to the Iba1 and TMEM119 antibodies (**Figure 3**).

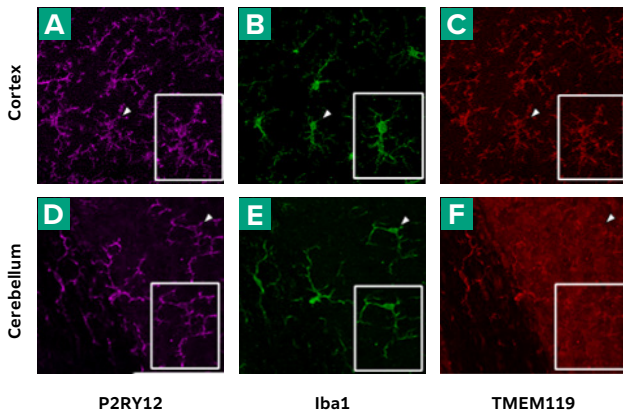


Figure 3. Comparison of other microglial markers. Triple labeling immunohistochemistry showed that Iba1⁺ microglia expressed P2RY12 and TMEM119 in the cortex and cerebellum (**A-F**).

DISCUSSION

P2RY12 is a G protein-coupled receptor with a primary ligand of ADP. In CNS, P2RY12 is specifically expressed in microglia and therefore, widely used as a microglia-specific marker in neuroscience research. Moreover, P2RY12 serves as a valuable marker for distinguishing microglia from macrophages found in vascular system in the CNS, since its expression in macrophages is scarcely detected. As shown in this report, the newly developed anti-P2RY12 polyclonal antibody allows us to perform highly specific visualization of microglia

Comparison with commercially-available polyclonal antibodies

The specificity of our anti-P2RY12 polyclonal antibodies was compared with a commercially-available Rabbit IgG antibody. When the rabbit anti-P2RY12 antibody was used, some neuron-like cells were immunostained as indicated by the arrowheads, while P2RY12 - Guinea Pig IgG did not show such non-specific reaction, as shown in **Figure 4**.

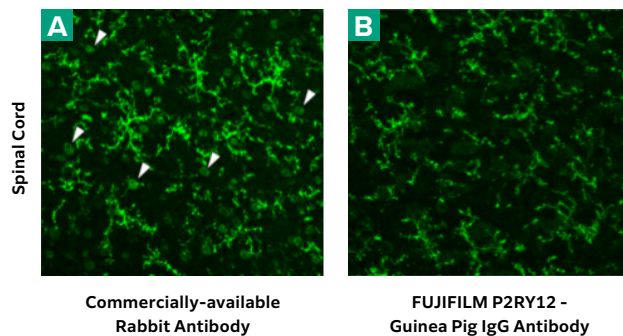


Figure 4. Specificity comparison with the commercially-available rabbit anti-P2RY12 polyclonal antibody detected some neuron-like cells (arrowheads) in the spinal cord, but P2RY12 - Guinea Pig IgG did not (**A, B**).

in mouse CNS. Because of its guinea pig origin, it also allows multiple immunostaining with antibodies from other animals, such as our highly reputed rabbit anti-Iba1 polyclonal antibody (FUJIFILM Wako Pure Chemical Corporation, Catalog # 019-19741). Thus, our P2RY12-specific polyclonal antibody is a useful tool to study how microglia maintain brain homeostasis and how they are involved in disease dynamics by clearly distinguishing them from CNS-resident macrophages.

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