

Chapter 10

Analysis of Microglial Proliferation in Alzheimer's Disease

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Abstract

The expansion and activation of the microglial population is a hallmark of many neurodegenerative diseases. Despite this fact, little quantitative information is available for specific neurodegenerative disorders, particularly for Alzheimer's disease (AD). Determining the degree of local proliferation will not only open avenues into understanding the dynamics of microglial proliferation, but also provide an effective target to design strategies with therapeutic potential. Here we describe immunohistochemical methods to analyse microglial proliferation in both transgenic murine models of AD and in human post-mortem samples, to provide a broad picture of the microglial response at the different experimental levels. The application of a common and universal method to analyse the microglial dynamics across different laboratories will help to understand the contribution of these cells to the pathology of AD and other neurodegenerative diseases.

Key words Alzheimer's disease, Microglia, Proliferation, Bromodeoxyuridine (BrdU), Ki67, Phospho Histone H3, CSF1R, PU.1, Immunohistochemistry

1 Introduction

Alzheimer's disease is a chronic neurodegenerative disease and the most common form of dementia in the Western countries. Despite much interest in the inflammatory response in AD, and the extensive research focused on understanding the role of microglia in this disease, the scientific community has failed to shed clear and uniform light into their contribution to the disease [1–3]. The neuropathology of AD shows a robust innate immune response characterized by the presence of activated microglia, with increased or de novo expression of diverse macrophage antigens [3, 4], and at least in some cases production of inflammatory cytokines [5, 6]. Microglial activation in neurodegeneration is accompanied by an increase in their density. In addition, other brain macrophages, perivascular macrophages (PVMs) and meningeal macrophages (MMs), play a critical role in signaling from the periphery to the brain. Recent studies report a minor or even absent contribution of circulating progenitors to the microglial population in a mouse

model of AD [7], pointing to in situ microglial proliferation as the mechanism regulating microglial turnover, with little or no contribution of circulating progenitors [8, 9]. Microglia are maintained and function largely independently of circulating progenitors in health [10] and disease [7, 11, 12]. Therefore, the analysis of PVMs, MMs and microglial proliferation under pathological conditions with widespread chronic neurodegeneration, as is the case of Alzheimer's disease, is critical for understanding how innate inflammation contributes to disease onset and progression.

Although proliferation was assumed to be responsible for the increased number of microglial cells observed in AD samples, direct evidence of proliferating microglial cells (Ki67 expression in Iba1+ cells) was reported only recently, together with the upregulation of the transcription factor PU.1 and the mitogen IL-34, key components of the pathway regulating microglial proliferation [13]. An important signaling pathway for microglial proliferation, the CSF1-receptor (CSF1R) pathway, has also been shown to be upregulated in microglial cells during AD, indicating prominent activity of this pathway [14]. The expansion of the microglial population has been consistently documented in transgenic mouse models of AD, mainly accumulating around plaques [15, 16]. However, direct evidence of microglial proliferation (incorporation of bromodeoxyuridine (BrdU) in Iba1+ cells) was only recently reported, suggesting a direct effect of the plaque microenvironment over the regulation of microglial mitogenesis [17].

These studies pinpoint the importance of the control of microglial proliferation during AD, offering new avenues for the regulation of the innate immune response in the brain. Establishing reproducible and universal methods to monitor microglial proliferation in models mimicking aspects of AD and in post-mortem AD brains will provide the scientific community with valuable tools to better compare results across experimental models or cohorts of patients, contributing to a better understanding of the pathophysiology of AD.

2 Materials

The immunohistochemical identification of proliferating microglial cells can be performed using the following materials.

2.1 Tissue Samples

2.1.1 Mouse/Rat Tissue Samples

To provide a reliable correlate of cell proliferation we recommend the use of thymidine analogues such as bromodeoxyuridine (BrdU), which gets incorporated into the nuclear DNA in dividing cells (*see Note 1*). The use of fixed tissue obtained from intracardiac perfusion (4 % paraformaldehyde; *see Note 2*) is highly encouraged, although the methods are also applicable to the use of fresh-frozen brain tissue. We also encourage the use of reporter mice with

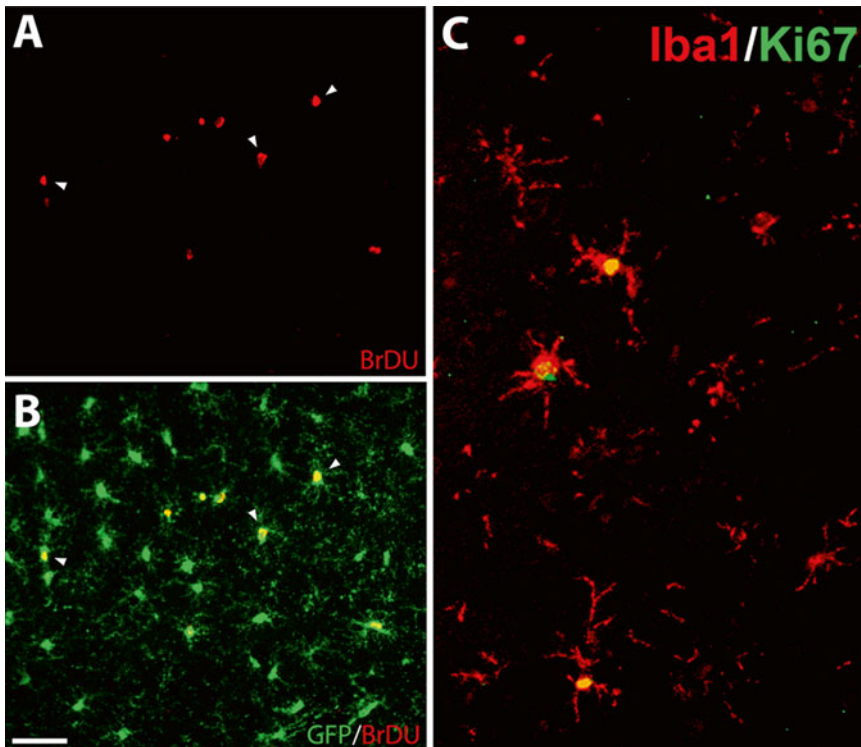


Fig. 1 Microglial proliferation in murine and human chronic neurodegeneration. (**a** and **b**) Representative image of the immunohistochemical detection of bromodeoxyuridine (BrdU) (**a**, **b**; red) in microglial cells (**b**; c-fms-EGFP+, green) in the hippocampus of a mouse having prion disease (ME7 model). (**c**) Representative image of the immunohistochemical detection of Ki67 (green) in microglial cells (Iba1+, red) from the temporal cortex of an AD patient. Scale bar in (**a** and **b**) 20 μm . In (**c**) 100 μm . Reproduced from Gomez-Nicola et al. [13], with permission from Journal of Neuroscience. Society for Neuroscience (www.jneurosci.org; reuse of own material)

fluorescent microglia/macrophages, such as c-fms EGFP mice (macgreen) [18] or CX3CR1 EGFP mice [19], to facilitate the detection of microglial cells in the brain (Fig. 1) (*see Note 1*).

2.1.2 Human Tissue Samples

Samples from post-mortem human tissue are usually obtained from brain banks as paraffin-embedded tissue. Tissue obtained from any brain bank should have appropriate consent and ethical permission to use the tissue. It is the responsibility of the experimenter to ensure that this is in place when tissue is obtained from a source.

For human tissues: The method can be used with wax-embedded or fresh-frozen tissue. Brain sections can be sectioned at a range of thickness from 5 to 30 μm , depending on the experimental needs, although the use of 30 μm sections combined with free-floating immunohistochemistry (*see Subheading 3*) is highly encouraged.

2.2 Buffers and Solutions

1. Citrate buffer: Mix 2.1 g of citric acid in 1 L of distilled water (dH₂O). Adjust pH to 6.0 with NaOH. Store at 4 °C.
2. Phosphate buffered saline with Tween 20 (PBST 0.1/0.2): Prepare a stock solution of PBS (10×) by dissolving 80 g of NaCl, 2 g of KCl, 26.8 g of Na₂HPO₄·7H₂O and 2.4 g of KH₂PO₄ in 800 mL of dH₂O. Adjust volume to 1 L with dH₂O. Adjust pH to 7.4 with HCl or NaOH when diluted to (1×) (PBS 1×). Add 0.1 or 0.2 % (v/v) of Tween 20 to the PBS solution and mix gently to get the final 'PSBT0.1' and 'PBST0.2' solutions. Store at room temperature (RT).
3. Mowiol/DABCO mounting medium for immunofluorescence: Combine 2.4 g of Mowiol 4-88 (e.g. Sigma-Aldrich), with 6 g of glycerol and 6 mL of H₂O. Mix for approx. 3 h. Add 12 mL of 0.2 M Tris-HCl (pH 8.5). Incubate with mixing at 50 °C until it dissolves. Centrifuge at 5,000×*g* for 15 min to pellet insoluble material. Add 1,4-diazabicyclo-[2,2,2]-octane (DABCO) as antibleaching agent (to reduce fading of fluorophores) to a final concentration of 2.5 % (w/v). Store in 500 µL aliquots at -20 °C.
4. Fluorescence quenching solution: 0.1 % (w/v) Mix Sudan Black in 70 % ethanol. Mix and filter. Store solution at RT, protected from light.

2.3 Reagents and Other Components

1. Liquid chemicals: Ethanol; xylene; 2 N HCl.
2. Solid chemicals: Bovine serum albumin (BSA); DAPI (4',6-diamidino-2-phenylindole dihydrochloride).
3. Serum from the host animal of the secondary antibody to be used (*see* Subheading 3).
4. Blocking solution: 5 % serum, 5 % BSA in PBST0.2.
5. Incubation chamber or tray.
6. Free-floating incubation plate: Starting from a plastic cell culture plate, divide each well into two chambers with a stainless metallic mesh adhered to the bottom and the sides of the well. The tissue sections are incubated in free-floating in one chamber. Washes and incubations are done through the communicating chamber (*see* Note 3).
7. ImmEdge Hydrophobic Barrier Pen (Vector Labs), to provide a heat-stable, water-repellent barrier that keeps reagents localized on tissue specimens (*see* Note 3).
8. Glass slides coated with gelatin or APES (3-aminopropyltriethoxysilane). Alternatively, use ionized slides (*see* Note 3).

2.4 Primary and Secondary Antibodies

2.4.1 Primary Antibodies (Recommended)

1. Microglial markers: Rabbit anti-Iba1 (Wako); goat anti-Iba1 (Abcam); rat anti-CD11b (ABD Serotec); rabbit anti-PU.1 (Cell Signaling).
2. Proliferation markers: Mouse anti-BrdU (Developmental studies Hybridoma Bank); rat anti-BrdU (Santa Cruz Biotechnologies); rabbit anti-PCNA (Abcam); rabbit anti-phospho Histone H3 (Cell Signaling); rabbit anti-Ki67 (Abcam).
3. Other: Chicken anti-GFP (Abcam).

2.4.2 Secondary Antibodies

Biotinylated, affinity purified, secondary antibodies (Vector Labs), and fluorescence-conjugated (Alexa 405, 488 or 594 recommended) secondary antibodies, or streptavidin (Life technologies).

3 Methods

Unless otherwise specified, carry out all procedures at room temperature.

3.1 Immuno-histochemical Detection of Microglial Proliferation in AD Mouse Models

1. Wash sections three times with PBST0.1 buffer, 5 min each (*see Note 3*).
2. [*Only if detecting BrdU*]. *DNA denaturation step for BrdU detection*: Incubate with 2 N HCl for 30 min at 37 °C. This step will provide access of the anti-BrdU antibodies to its epitope in the DNA (*see Note 4*).
3. [*Only if detecting BrdU*]. Wash with PBST0.1 buffer three times, 5 min each.
4. *Blocking*: Incubate with blocking solution (5 % serum, 5 % BSA in PBST0.2) for 1 h. This incubation will prevent unspecific binding of the primary or secondary antibodies to the tissue. Note it is not necessary to wash after the incubation.
5. *Primary antibodies*: Incubate with primary antibodies (choose one microglial marker (i.e. Iba1) and one marker of proliferation (i.e. BrdU), from different hosts) at manufacturer's recommended dilution in blocking solution, at 4 °C overnight (*see Note 5*).
6. Wash with PBST0.1 buffer three times, 5 min each.
7. *Secondary antibodies*: Incubate with appropriate fluorescent secondary antibodies at manufacturer's recommended dilution in blocking solution for 1 h. From this step, sections will need to be protected from light (*see Note 6*).
8. Wash with PBST0.1 buffer three times, 5 min each.
9. *Counterstain* with DAPI: Incubate with DAPI (1:2,000) in PBST0.1, 10 min if the blue channel is available (*see step 7 and Note 6*). Nuclear staining will provide anatomical reference

and will also define the nuclear compartment to better identify proliferation-related markers.

10. Wash with PBST0.1 buffer three times, 5 min each.
11. *Mounting and coverslipping*: Use mowiol/DABCO mounting medium (*see Note 7*). Store slides at 4 °C, protected from light until imaging.

3.2 Immuno-histochemical Detection of Microglial Proliferation in Post-mortem Tissue from AD Patients

Samples from post-mortem human tissue are usually obtained from brain banks as paraffin-embedded tissue. In case human sections are obtained by alternative preservation methods please omit **steps 1 and 2**. Tissue samples obtained from any brain bank should have appropriate consent and ethical permission to be used.

1. *Dewaxing and rehydration*. Transfer the slides with the samples to a rack and incubate 40 min at 60 °C in an oven. After heating, directly transfer slides to xylene (15 min), followed by sequential incubation in the rehydrating solutions (100, 95, 80 and 75 % ethanol, ending with dH₂O; 5 min each). Wash three times in PBS, 5 min each.
2. *Antigen retrieval*: Transfer slides to a plastic rack and cover with excess citrate buffer (to prevent drying due to evaporation). Heat at full power in a microwave for 25 min. Then, transfer quickly to cold running tap water.
3. Wash with PBST0.1 buffer three times, 5 min each (*see Note 3*).
4. *Blocking*: Incubate with blocking solution for 1 h. This incubation will prevent unspecific binding of the primary or secondary antibodies to the tissue. Note it is not necessary to wash after the incubation.
5. *Primary antibody*: Incubate with primary antibodies (choose one microglial marker (i.e. Iba1) and one marker of proliferation (i.e. Ki67), from different hosts) at manufacturer's recommended dilution in blocking solution. Incubate overnight at 4 °C.
6. Wash with PBST0.1 buffer three times, 5 min each.
7. *Quenching autofluorescence step*: Incubate with fluorescence quenching solution (Sudan Black) for 10 min. This step is particularly important in the case of AD human tissue, as the occurrence of autofluorescent artefacts (e.g. lipofuscin granules) is very frequent and can confound the interpretation of results.
8. Wash with PBST0.1 buffer three times, 5 min each.
9. *Secondary antibodies*: Incubate with appropriate fluorescent secondary antibodies at manufacturer's recommended dilution in blocking solution for 1 h. From this step, sections will need to be protected from light (*see Note 6*).
10. Wash with PBST0.1 buffer three times, 5 min each.

11. *Counterstain* with DAPI: Incubate with DAPI (1:2,000) in PBST0.1, for 10 min if blue channel is available (*see step 9* and **Note 6**). Nuclear staining will provide anatomical reference and will also define the nuclear compartment to better identify proliferation-related markers.
12. Wash with PBST0.1 buffer three times, 5 min each.
13. *Mounting and coverslipping*: Use mowiol/DABCO mounting medium (*see Note 7*). Store slides at 4 °C, protected from light until imaging.

4 Notes

1. If allowed by the experimental conditions, the use of birthdating studies with thymidine analogues (e.g. tritiated-thymidine birthdating) is highly recommended. To date, we recommend one single window of proliferation using BrdU (50 mg/kg body weight, in 0.9 % (w/v) sterile solution of NaCl (sterile saline)), administered by intraperitoneal injection. Each dose of BrdU will label approximately 2–3 h of proliferation, so we recommend using cumulative dosage paradigms (i.e. three to four consecutive injections at 3 h intervals), to maximize the readout of proliferating microglia and facilitate the analysis and quantification. Multiple windows of proliferation can be differentiated by sequentially administering complementary analogues, such as CldU, IdU or EdU, with detection methods similar to that of BrdU [20, 21].
2. In case of using BrdU for the detection of proliferation, avoid long post-fixation times (no longer than 2 h at 4 °C) which might interfere with the accessibility to the BrdU epitope in the DNA. If long post-fixation is necessary due to experimental needs, add a step of antigen retrieval (*see Subheading 3.2*) to the method, before DNA denaturation.
3. Immunohistochemical detection of microglial proliferation can be performed on sections mounted on glass slides or on free-floating sections in incubation plates (encouraged). In the first case, start by tracing an area around the section with ImmEdge pen, to limit diffusion of buffers (*see Subheading 2*).
4. When using tissue from transgenic reporter mice (i.e. macrogreen or CX3CR1-EGFP), the DNA denaturation step required for BrdU detection might eliminate the native fluorescence from the enhanced green fluorescent protein (EGFP). We suggest using anti-GFP primary antibodies combined with secondary antibodies coupled to green fluorescence to retrieve the EGFP signal (Fig. 1).
5. As a complementary study, we strongly recommend analysing the expression of the different components of the main pathway

regulating microglial proliferation: the activation of CSF1R. The expression of the transcription factor PU.1 is specific for microglia, and correlates with the proliferative status. Also, analysing the expression levels of CSF1R (c-fms), CSF1 or IL34 by immunohistochemistry will inform about the proliferative activity of microglia [13].

6. The immunohistochemical method can be adapted to the specific experimental aims allowing, for example, the simultaneous detection of up to four epitopes using conventional imaging methods. In these cases, matching each primary antibody with a specific color of the fluorescent-coupled secondary antibody will depend on the expected intensity for each epitope. Thus, green fluorescence is usually better registered by conventional microscopes, so it will be used for the antigen expected to have the worse signal. Signals expected to be optimal and intense will be assigned to the red or blue channels. If required, biotin-conjugated secondary antibodies could be used, bridging to fluorescence with the use of fluorescent-coupled streptavidin conjugates (streptavidin-biotin binding enables detection of biotinylated antibodies).
7. If using free-floating immunohistochemistry, sections will need to be previously transferred to gelatine-coated or ionized glass slides with the help of a paintbrush.

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