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Analysis of the Microglial Sensome

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Abstract

Sensing changes in the brain milieu is a major microglial function that regulates the ability of these cells to perform other tasks including host defense and homeostasis. Microglia express a cluster of genes that allow them to perform their sensing functions termed the sensome. It is important to be able to assess expression of these genes and their corresponding proteins in isolated microglia. Here we describe a step by step procedure to isolate microglia from adult mouse brains and provide examples of analyzing sensome genes and proteins by quantitative PCR and flow cytometry ex vivo. We also describe an in situ hybridization method to detect sensome RNA in the mouse brain. These approaches can be applied to all known sensome genes and can be used in analyzing sensome expression in physiological and pathological conditions.

Keywords

Microglia; Isolation; Sensome; Adult mice; In situ hybridization

1 Introduction

1.1 The Microglial Sensome

A key microglial function is to sense changes in their cellular environment and respond to these changes. Morphologically, microglia have multiple processes that constantly move in the area surrounding the cell body allowing them to survey these areas and detect any disturbances. Such changes could be caused by microbial invasion, cell injury, or death associated with neurodegeneration, by deposition of neurotoxic substances such as amyloid β or by inflammatory molecules such as chemokines and cytokines. Additional milieu alterations could include changes in pH, in the composition or integrity of the extracellular matrix, or in extracellular metabolites such as nucleotides or amino acids. A large armamentarium of proteins and receptors may be used by a given cell for sensing such changes in the environment. Using RNAseq, we have defined the full spectrum of the cellular receptors and proteins used by microglia to sense changes in their environment and defined these proteins as the “sensome” of microglia [1] (see Table 1).

Because of the importance of sensome genes for the functioning of microglia in physiological and pathological conditions, it is important to be able to assess expression of these genes and their corresponding proteins in isolated microglia. A key step in this assessment is the isolation of pure and intact cells from adult healthy or diseased brains. This is followed by global gene expression profiling of these purified cells by RNAseq or

targeted expression profiling by qPCR. Subsequently, validation of gene expression data by flow cytometry allows correlation between mRNA and protein levels. In this chapter, we describe a detailed protocol for such isolation as well as use of these cells to measure expression of several sensome proteins including Cd11b, CD45, Cx3cr1, and P2ry12 by flow cytometry. We also describe an in situ hybridization method to detect sensome RNA transcripts in paraffin-embedded or fresh frozen sections of the mouse brain. These approaches can be universally used to measure expression of any sensome gene and/or protein.

1.2 Isolation of Microglia from Adult Mice

Multiple protocols have been developed for isolating microglia from adult mice [1, 2]. We will discuss the two major methods that we currently use in our lab. Generally, both protocols use (A) enzyme digestion, (B) mechanical dissociation, followed by (C) removal of myelin/debris, and then (D) selection of microglia by flow cytometry (Fig. 1). When we started isolating microglia 15 years ago, we tried various enzymes for dissociation and used hand trituration for mechanical dissociation of enzyme-digested tissue. A combination of neutral protease (aka dispase) and collagenase III worked well without affecting CD11b and CD45 antigens [2] but did reduce CD4 and CD25 receptors. Papain digestion also worked very well and did not reduce levels of CD11b or CD45. Trypsin reduced levels of CD11b and CD31 and resulted in higher cell death so we do not recommend using this enzyme. After enzyme digestion, addition of DNase I was required to digest DNA strands that were released by lysed cells resulting in stickiness and cell clumping. Once the brain was adequately dissociated, the removal of myelin and other debris was imperative. We tried various methods of debris and myelin removal. This was initially done using Percoll gradients that removed debris and enriched for cell types, including microglia [3]. The problem with using hand trituration is that good cell yield and high viability were user dependent. Rough trituration formed bubbles which killed cells and resulted in lower viability. Too gentle trituration gave inadequate dissociation and lower cell yield. With the advent of mechanical dissociation instruments and digestion kits from various companies, which give optimized enzyme conditions, yields and viabilities are more consistent between users in the same or different labs. However, with kits we often do not know the concentration of enzymes used or buffer compositions, but they are optimized for successful isolation. We will outline the protocols we currently use or have used successfully in the past. Two methods of myelin/debris removal are discussed: removal using anti-myelin beads and magnetic separation and Percoll gradient.

2 Materials

2.1 Reagents

1. Neural Tissue Dissociation Kit (P) (Miltenyi Biotec).
2. MACS C Tubes (Miltenyi Biotec).
3. LS columns (Miltenyi Biotec).
4. Myelin removal beads II (Miltenyi Biotec).

5. β -Mercaptoethanol 70 μ m strainer for 50 ml Falcon tube.
6. Dulbecco's Phosphate Buffered Saline with Ca^{2+} and Mg^{2+} [PBS (w)].
7. Dulbecco's Phosphate Buffered Saline without Ca^{++} and Mg^{++} [PBS (w/o)].
8. 7.5% bovine serum albumin.
9. $10 \times$ phosphate buffered saline.
10. Percoll™ PLUS (only for alternative protocol for myelin removal) (GE Healthcare cat# 17-5445-01).
11. Anti-CD11b Alexa 488 labeled (CD11b-A488) (BioLegend Clone M1/70 cat#101217).
12. Anti-CD45 Pacific Blue (CD45-PacBlue) (BioLegend Clone 30-F11 cat# 103216).
13. Anti-CCR2 APC labeled (R&D Systems Cat#FAB5538A).
14. Fc block (BioLegend Cat#101320).
15. Anti-mouse P2ry12-PE (BioLegend Clone S16007D).
16. Anti-mouse Cx3cr1 (BioLegend Clone SA011F11).
17. Propidium iodide (PI) stock 250 g/ml in PBS diluted to 100 μ g/ml.
18. LS columns (Miltenyi Biotec).
19. RNeasy® Plus Micro Kit (Qiagen).
20. RNeasy® Lipid Tissue Mini Kit (Qiagen).
21. RNA 6000 Nano Kit (Agilent Technologies).
22. RNA 6000 Pico Kit (Agilent Technologies).
23. Multiscribe™ reverse transcriptase (Applied Biosystems) or any RT that works with your qPCR protocol.
24. RNAscope® 2.5 HD Duplex Reagent Kit (Advanced Cell Diagnostics, Cat# 322430).
25. RNAscope® Fluorescent Multiplex Assay (Advanced Cell Diagnostics, Cat# 320850).
26. RNAscope® user-specified target probes (Advanced Cell Diagnostics).
27. Optimal cutting temperature (OCT) solution.
28. Superfrost® Plus slides.

2.2 Equipment

1. gentleMACS™ Dissociator (Miltenyi Biotec Cat#130-045-401).
2. MACS Separator (magnet and holders) (Miltenyi Biotec).

3. Centrifuge with swinging bucket rotor.
4. MasterFlex L/S peristaltic pump. Agilent 2100 Bioanalyzer or Agilent 4200 TapeStation System.
5. Fixed angle microcentrifuge.
6. Roche LightCycler II or any qPCR machine available.
7. FACS Aria II (BD Biosciences) or another cell sorter to which you have access.
8. ACD HybEZ™ II Hybridization System (Advanced Cell Diagnostics).
9. Multiquick steamer (Braun) or HS3000 steamer (Black and Decker).
10. Cryostat for frozen sectioning.

3 Methods

3.1 Preparation of Brain for Enzyme Digestion

For brain dissociation we use the Miltenyi Neural Tissue Dissociation Kit P and adapted the protocol for optimum yields in our experiments.

1. Neural Tissue Dissociation Kit P uses papain for brain digestion. It is important to read through the protocol, aliquot enzyme P, and keep frozen at -20°C to avoid loss of proteolytic activity. The enzyme digestion buffer is Buffer X with addition of $13.5\ \mu\text{l}$ $50\ \text{mM}$ β -mercaptoethanol (made from $14.3\ \text{M}$ stock) per $10.0\ \text{ml}$ Buffer X. Enzyme P ($50\ \mu\text{l}$) is added to $1900\ \mu\text{l}$ Buffer X per brain or up to $400\ \text{mg}$ tissue and prewarmed to 37°C for $10\ \text{min}$ before use.
2. Euthanize a mouse according to your institution's guidelines. Perfuse with PBS (w) for $2\text{--}4\ \text{min}$. We use the MasterFlex L/S peristaltic pump from Cole-Parmer set to a flow rate of $30\ \text{ml}$ over $2\ \text{min}$ to avoid perfusion pressure being too high. If no pump is available, perfusion can be done manually using a $30\ \text{ml}$ syringe with a 19 gauge needle.
3. Remove the brain and transfer to tissue culture dish containing PBS (w). You can either use whole brain (approximate weight $400\ \text{mg}$) or cut out the sections of interest only.

3.2 Mechanical Dissociation

1. Mechanical dissociation with gentleMACS™: If using whole brain, cut into four pieces and, using tweezers, add pieces to gentleMACS™ C tube containing prewarmed enzyme solution ($1950\ \mu\text{l}$ per brain). If cutting out sections of interest, add them directly to C tube containing enzyme solution ($1950\ \mu\text{l}$). We use one brain per C tube or up to $400\ \text{mg}$ of tissue containing prewarmed Buffer X/papain solution.
2. Invert C tube onto gentleMACS™ Dissociator and run program *m_brain_01* once and then check for tissue chunks caught in top and dislodge before running the program one more time.

3. Incubate for 25 min in water bath or incubator at 37 °C or in cell culture incubator.
4. Remove from water bath or incubator, invert C tube into the Dissociator, and run program *m_brain_02* twice.
5. Add 30 µl enzyme A solution (20 µl Buffer Y + 10 µl enzyme A).
6. Return to water bath or incubator and incubate for 10 min.
7. Remove from water bath or incubator and run program *m_brain_03* twice.
8. Incubate for 5 min more at 37 °C.
9. Remove from water bath or incubator and add 5.0 ml cold PBS containing 0.5% BSA (PBS/BSA), pipette a few times to mix, and then pass cell digest over a 70 µm filter set into 50 ml conical tube.
10. Rinse C tube with cold 10.0 ml PBS (w) and pass over filter into conical tube.
11. Spin at $400 \times g$ for 10 min and aspirate supernatant.
12. Resuspend pellet in either 1800 µl cold PBS/0.5% BSA for myelin/debris removal using magnetic separation OR resuspend pellet in 1.0 ml cold PBS/0.5% BSA/0.5 mM EDTA for Percoll™ PLUS method.

3.3 Removal of Myelin/Debris

Two techniques that we have used are discussed: removal of myelin with magnetic bead separation or by Percoll™ gradient centrifugation.

3.3.1 Myelin Removal Using Magnetic Bead Separation

1. To the cell pellet that was resuspended in 1800 µl cold PBS/0.5% BSA, add 200 µl anti-myelin beads, mix well, and let it sit in the refrigerator for 15 min.
2. Add 20 ml cold PBS/0.5% BSA and mix well by gentle pipetting.
3. Centrifuge at $300 \times g$ for 10 min.
4. Aspirate supernatant and resuspend pellet in 2.0 ml PBS/0.5% BSA.
5. Meanwhile, set up two LS columns (Miltenyi Biotec) in the magnetic holders and rinse each column with 3.0 ml PBS/0.5% BSA, collecting flow-through in 15 ml conical tube.
6. After all rinse buffer has drained through, load 1.0 of the dissociated brain preparation onto each column.
7. Let it completely flow through before adding 1.0 ml PBS/0.5% BSA for the first rinse.
8. After each rinse has completely flowed through the LS column, add another 1.0 ml PBS/0.5% BSA and let it flow through.
9. Repeat rinse step one more time and centrifuge tube at $300 \times g$ for 10 min.

10. Aspirate supernatant and resuspend pellet in appropriate buffer for the intended use. For staining and sorting cells, resuspend in PBS/0.5 mM EDTA/0.5% BSA and Fc block (10 μ l/ml). Let cells sit on ice for 10 min.

3.3.2 Myelin Removal Over Percoll™ PLUS Gradient

1. This protocol is a less expensive alternative to myelin removal using magnetic separation (*see Note 1*).
2. Prepare physiologic Percoll™ PLUS (GE) by adding 5.0 ml 10 $\times$ PBS to 45 ml Percoll™ PLUS and mix gently by inversion.
3. Resuspend cell pellet in 1.0 ml of cold PBS/0.5% BSA/0.5 mM EDTA using a P1000 pipette tip and mix gently being careful not to create bubbles (*see Note 2*). Next, add 9.0 ml PBS/0.5% BSA/0.5 mM EDTA and triturate gently several times with a 10.0 ml pipette.
4. Add 4.5 ml of the physiologic Percoll™ PLUS solution to cell suspension and mix gently by inversion several times. A gradient will be formed upon centrifugation.
5. Spin in a swinging bucket rotor at 800 $\times g$ for 30 min, with brake on lowest setting.
6. At the end of the spin there will be two phases: the top fluffy/sticky beige-colored layer is the debris/myelin and should be carefully aspirated off first (Fig. 1). Then, continue to aspirate the supernatant to the 5.0 ml mark on the 50 ml tube. Cells are in both the pellet and in the few milliliters above the pellet.
7. Add 40 ml cold PBS/0.5 mM EDTA and spin at 400 $\times g$ for 10 min.
8. Remove supernatant down to the pellet careful not to disturb the pellet.
9. Resuspend pellet in 10 ml PBS/0.5 mM EDTA and spin at 400 $\times g$ for 8 min to rinse.
10. Aspirate supernatant and resuspend pellet in appropriate buffer for the intended use.
11. For staining and sorting cells, resuspend in PBS/0.5 mM EDTA/0.5% BSA and Fc block (10 μ l/ml). Let cells sit on ice for 10 min.

3.4 Selection of Microglia by FACS

Microglia can be isolated using magnetic beads coupled to anti-CD11b to isolate CD11b-expressing cells which would be mostly microglia with some monocytes, neutrophils, or macrophages. Depending on the disease studied, the percentage of non-microglia CD11b⁺ cells in the brain can increase due to influx from the blood stream, such as in traumatic brain injuries, stroke, advanced Alzheimer's disease, and glioblastoma growth. Several companies, such as Miltenyi Biotec and Stemcell Technologies, have products and protocols for isolation of immune cells from the brain using magnetic bead separation. However, we prefer using fluorescence-activated cell sorting (FACS) to isolate highly pure microglia

leaving an option of separating other CD11b⁺ cells that are present in response to a disease state. Therefore, we will only discuss the FACS protocol and gating strategies that we used to sort microglia for performing transcriptomic analysis.

1. After myelin removal and centrifugation, aspirate supernatant and resuspend pellet in 210 µl PBS/0.5 mM EDTA/0.5% BSA containing Fc block (10 µl/ml). Let cells sit on ice for 10 min.
2. For “no stain” control, remove 10 µl of cells and put in the tube with 400 µl PBS/EDTA and pass over blue cap filter tube. Put on ice until ready to sort (*see* Note 3).
3. Stain for CD11b, CD45, and CCR2 and use a viability dye to gate out dead and dying cells.
4. To the remaining 200 µl of cells, add 4 µl CD11b-A488, 2 µl CD45-PacBlue, and 20 µl CCR2-APC, and stain for 40 min on ice, with gentle mixing every 15 min.
5. Add 2.0 ml cold PBS (w/o) and spin at $300 \times g$ for 10 min.
6. Resuspend pellet in 400 µl PBS/0.5 mM EDTA and add 4 µl stock PI (100 µg/ml) and let it sit for 5–10 min before sorting.

3.5 Gating Strategies

After appropriately compensating for the fluorophores used, cells should be plotted as follows: FSC-H vs FSC-A to gate on singlets and gate out clumps of cells (P1). The cells within the singlet gate (P1) are further distinguished by plotting FSC vs SSC and drawing a gate (P2) to eliminate small cells and debris. Cells from gate P2 are plotted as PI intensity vs SSC and a gate drawn comprising live cells (*live*) and excluding dead/dying cells that are permeable to PI. This is a standard gating strategy for obtaining single live cells with FACS, before further differentiating cells with target antibodies (Fig. 2a).

1. Plot *live* cells (PI negative) as intensity of CD11b-A488 vs intensity of CD45-PacBlue. This gating reveals the dominant “microglia” population (about 94–97%) as well as mixed populations of CD11b⁺/CD45⁺ cells that do not fall within the microglia population (3–5% but can be higher in certain disease states).
2. Draw the “microglia” gate around the dominant population and then assess cells in that gate for CCR2 expression by plotting SSC vs CCR2-APC (Fig. 2b). CCR2-negative cells are approximately 98% of the cells in the “microglia gate” and are sorted directly into tubes containing 400 µl RLT lysis buffer (*see* Notes 4 and 5).

3.6 RNA Isolation and RNA Sequencing

1. Extract total RNA from sorted microglia using Qiagen RNeasy® Plus Micro Kit or from whole brains using RNeasy® Lipid Tissue Mini Kit according to manufacturer’s directions.

2. Briefly, pass RNA samples over mini-spin columns to bind the RNA, and rinse several times to remove impurities.
3. Elute RNA from the column with nuclease-free water supplied in the kit: 14 μ l elution volume for sorted cells and 25–40 μ l elution volume for brain tissue.
4. Assess RNA quality, by running samples on an Agilent Bioanalyzer using a RNA 6000 Nano or Pico kit chip or 4200 TapeStation System. Using this technique, most of the RNA integrity numbers (RIN) are usually above 9.0 and we do not use any RNA from samples that are below RIN of 7.0.

3.7 Quantification of the Microglial Sensome

Once microglia are isolated with high purity, analysis of their sensome can be achieved via several ways. Clearly, RNAseq is the approach that will allow differential analysis of sensome genes between microglia from different mice and disease conditions, as well as unbiased analysis of global gene expression differences [1]. Another approach is to design NanoString [4] probe sets for all sensome genes that have been identified. For RNAseq, we routinely create 3' libraries using the QuantSeq Forward library kit from Lexogen and sequence our libraries on the Illumina MiniSeq to generate FASTQ files, which are further analyzed through bioinformatics pipelines. There are some limitations to this approach. Creating 3' libraries allows gene counting but does not address alternative splicing or mutations. Further, making libraries or creating customized NanoString templates may be costly and may not be available in every lab. In addition to RNAseq, we use three methods to assess expression of individual sensome genes or their proteins: quantitative PCR to measure and detect differences in mRNA levels, flow cytometry to assess protein expression, and duplex in situ hybridization using sensome probes to examine location of cells expressing sensome genes in the mouse brains. In the following sections we provide examples of sensome genes that we have analyzed by these methods. These techniques can be scaled up to include all sensome genes and their proteins.

3.7.1 Quantitative RealTime PCR—Most labs have their favorite standard protocol for qPCR, so we will briefly outline our protocol with the understanding that any protocol can be used. Table 2 lists six sensome gene primer pairs that we validated for use with qPCR of microglia.

1. Isolate microglia using the protocols discussed in the previous sections, extract total RNA from each sample, quantify, and reverse-transcribe to cDNA.
2. Use dilutions of each cDNA prep to assess β 2-microglobulin RNA levels by qPCR and then adjust sample cDNAs to give equivalent levels of β 2-microglobulin per well in subsequent qPCR reactions for other genes.
3. Perform qPCR for sensome genes in duplicate or triplicate using SYBR Green to detect the amplification products. We use the Roche LightCycler II, but this can be adapted to any qPCR instrument.
4. Perform the following cycles for primers whose T_m are above 60 °C:
 - a. Initial denaturation cycle at 95 °C for 10 min.

- b. Then 40 amplification cycles at 95 °C for 15 s and 60 °C for 1 min.
- c. Final cycle at 25 °C for 15 s.
- d. Relative quantification of mRNA expression is calculated by the comparative cycle method to obtain the ratio of gene expression compared with β 2-microglobulin.

3.7.2 Flow Cytometry of P2ry12 and Cx3cr1—As antibodies that are suitable for use in flow cytometry become available, expression of sensome proteins can be assessed in microglia in normal and aging brains and during development of diseases, such as Alzheimer's disease or tumor progression or in models of traumatic brain injury. One group developed antibodies to Tmem119, a microglia-specific marker [5], to track Tmem119 expression during development. We stained microglia for two major sensome proteins Cx3cr1 [6] and P2ry12 [7] since there are now good antibodies to use in flow cytometry (see Subheading 2).

1. Isolate microglia using the method described in this chapter, as it allows preservation of most cell surface sensome proteins.
2. Costain for CD11b, CD45, and CCR2 and for Cx3cr1 or P2ry12 on ice for 40 min. Rinse samples and perform FACS.
3. Gate on singlet and live cells as previously described.
4. Plot live cells as intensities of CD11b vs CD45. Create a “microglial” gate.
5. Plot cells within the microglia gate as Cx3cr1 vs CCR2 or P2ry12 vs CCR2, depending on which antibodies were used (Fig. 3a, b). The antibodies against P2ry12 and Cx3cr1 that are now available as well as other antibodies being developed will generate new tools for isolation and study microglia in a variety of diseases.

3.8 In Situ Hybridization

We use two RNAscope® in situ hybridization assays from Advanced Cell Diagnostics detection of hybridized custom target probe microglia in mouse brain sections.

We use a duplex chromogenic assay on formalin-fixed paraffin-embedded (FFPE) sections (5 μ m) of mouse brain, which allows hybridization and detection of two different probes that will appear as red and green dots in cells. For fixed-frozen sections, we use a multiplex fluorescent assay that allows three different probes to be hybridized and detected [1]. The manufacturer's guides (ACD) for performing both assay systems are very detailed and easy to follow so we will not give detailed protocols, but generally outline the multistep process.

3.8.1 RNAscope® 2.5 Duplex Assay and Detection (FFPE Tissue)

1. Remove mouse brains and fix the mouse in formalin overnight. If your lab is set up for paraffin embedding, then proceed using your protocol and cut tissue into 1–5 μ m sections onto slides or send the fixed tissue to your institutional Histology Core.

2. Bake slides at 60 °C for 1 h in a drying oven or slide heater.
3. Deparaffinize FFPE brain sections in xylene two times for 5 min each, and then incubate slides in 100% ethanol for two times for 1 min each.
4. Quench endogenous peroxidases for 10 min using kit-supplied hydrogen peroxide and rinse slides as directed.
5. Perform target retrieval using a steamer as directed in ACD protocol.
6. Treat tissue with protease as directed and rinse. Tissue sections are now ready for hybridization with probes.
7. Probe hybridization: follow manufacturer's protocol closely for successful probe hybridization and detection steps, which will take about 7 h to complete in the HybEZ oven. Cells that have hybridized with the probes will show as red or green dots or both colors may be present in the same cell. Figure 4a shows cohybridization of FFPE mouse sections with CD11b (green) and P2y12 (red).

3.8.2 RNAscope® Fluorescent Multiplex Assay on Fixed-Frozen Brain Sections

1. Remove mouse brains and fix in formalin 24–48 h.
2. Immerse sequentially in 10, 20, and 30% sucrose 18–24 h each solution until the brain sinks to the bottom of the tube.
3. Embed in OCT and cut 7–12 µm frozen sections onto Superfrost® Plus slides.
4. Air-dry tissue for 20 min.
5. Wash 5 min in PBS to remove OCT.
6. Following the ACD protocol, the next crucial steps are heated target retrieval and incubation with protease to permeabilize cells and prepare cellular RNA for optimal access to target probe.
7. Probe hybridization: follow manufacturer's protocol closely for successful probe hybridization and detection steps, which will take about 4 h to complete. Cells that have hybridized with the probes will show as red or green dots or both colors may be present in the same cell. Figure 4b shows cohybridization fixed-frozen mouse sections with CD11b (red, Atto 647) and Cx3cr1 (green, Alexa 488) against a background of DAPI-stained nuclei (blue).

4 Notes

1. We obtain 15–25% fewer cells with Percoll™ gradient method compared with myelin removal magnetic separation.
2. Using a small volume to initially resuspend the pellet results in better dissociation of the cell in the pellet.
3. A pilot experiment needs to be performed using single stains on cells for compensation on the flow cytometer you will be using. We use the FACS-Aria II

from BD Biosciences. Once the compensations are set, you usually do not need to do compensation each time unless you change the fluorescent tags on the antibodies or the lasers are readjusted.

4. When sorting, the sample flow rate should be maintained on a lowest setting to avoid shear force from rupturing cells during the sort.
5. Depending on the ages of the mice and genotypes, we usually obtain from 80,000 to 200,000 cells from the “microglia” gate.

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Fig. 1.

Flow diagram showing two protocols for isolating microglia from adult mouse brains. Brain is enzymatically and mechanically dissociated, and then myelin and debris are removed by one of the two methods: myelin removal with beads and magnetic separation (Miltenyi Biotech reagents) or by centrifugation over Percoll™ gradient to enrich for cells. Myelin-free cells are then stained with antibodies of choice and sorted using FACS

Fig. 2.

Gating strategy for isolation of microglia with flow cytometry. After processing of brain tissue, staining for CD11b, CD45, and CCR2 allows to separate microglia from recruited peripheral monocytes and isolation of pure microglial populations. Panel (a) shows the microglial gate. Panel (b) shows the CCR2-negative population of cells sorted

Fig. 3.

Sorting of microglia using antibodies to sense proteins Cx3cr1 or P2ry12. Cells from the microglial gate ($\text{Cd}11\text{b}^+/\text{CD}45^+$ population) described in Fig. 2 were stained for with antibodies to $\text{CX}_3\text{CR}1$ (a) or P2RY_{12} (b) plotted vs CCR2 expression

Fig. 4.

In situ hybridization of mouse brain sections with sense probes. (a) Microglia from FFPE mouse brain sections (5 μm) cohybridizing with CD11b (green) and P2ry12 (red) probes using chromogenic detection against the background of hematoxylin-stained brain cells. (b) Probes for CD11b (red, Atto 647) and Cx2Cr1 (green, Alexa 488) cohybridizing on a background of DAPI-stained cells (blue) using fluorescent detection in fixed-frozen mouse brain sections (12 μm)

Table 1

The microglial sensome genes with their accession number and potential ligands Table 2

Genbank link	Gene names	Ligand
<i>Purinergic and related receptors</i>		
NM_027571	P2ry12 = P2Y purinoceptor 12	Nucleotides
NM_028808	P2ry13 = P2Y purinoceptor 13	Nucleotides-ADP
NM_183168	P2ry6 = P2Y purinoceptor 6	Nucleotides-UDP
NM_011823	Gpr34 = probable G-protein coupled receptor 34	Nucleotides also Lysophosphatidylserine
NM_001174169	Adora3 = adenosine receptor A3	Adenosine
NM_009848	Entpd1 = ectonucleoside triphosphate diphosphohydrolase	Nucleotides-ATP
NM_028261	Tmem173 = transmembrane protein 173 = STING	Bacterial Cyclic Di-GMP
NM_001122596	Purinergic receptor P2Y G-protein coupled	Nucleotides
<i>Cytokine receptors</i>		
NM_001037859	Csf1r = macrophage colony-stimulating factor 1 receptor	m-CSF, IL-34
NM_007782	Csf3r = granulocyte colony-stimulating factor receptor	g-CSF
NM_009370	Tgfr1 = TGF-beta receptor type-1	Tgf-β
NM_009371	Tgfr2 = TGF-beta receptor type-2	Tgf-β
NM_010511	Ifngr1 = interferon gamma receptor 1 precursor	Tgf-γ
NM_008348	Il10ra = interleukin-10 receptor subunit alpha	IL-10
NM_010559	Il6ra = interleukin-6 receptor subunit alpha precursor	IL-6
NM_021887	Il21r = interleukin-21 receptor	IL-21
NM_011608	Tnfrsf17 = tumor necrosis factor receptor superfamily	BAFF
NM_011610	Tnfrsf1b = tumor necrosis factor receptor superfamily	TNF
<i>Chemokine and related receptors</i>		
NM_009987	Cx3cr1 = CX3C chemokine receptor 1	cx3cl1
NM_009917	Ccr5 = C-C chemokine receptor type 5	Ccl3, Ccl4, Ccl8, RANTES
NM_009779	C3ar1 = C3a anaphylatoxin chemotactic receptor	C3a
NM_001081211	Ptafr = platelet-activating factor receptor	PAF
NM_001146005	Gpr77 = C5a anaphylatoxin chemotactic receptor C5L2	C5a
NM_008153	Cmklr1 = chemokine-like receptor 1	The chemokine chemerin
NM_021476	Cysl1r = cysteinyl leukotriene receptor 1	LTD4
NM_017466	Ccr12 = C-C chemokine receptorlike 2	Chemerin
NM_026036	Cmtm6 = CKLF-like MARVEL transmembrane domain-containing	May bind cxcl7
NM_007577	C5ar1 = C5a anaphylatoxin chemotactic receptor	AKA CD88 C5a, Staph. Aureus
<i>Fc receptors</i>		
NM_010188	Fcgr3 = low affinity immunoglobulin gamma Fc region	Fc portion of IgG
NM_010185	Fcer1g = high affinity immunoglobulin epsilon receptor	Fc portion of IgE
NM_010187	Fcgr2b = low affinity immunoglobulin gamma Fc region	Fc portion of IgG-Low affinity
NM_010186	Fcgr1 = high affinity immunoglobulin gamma Fc receptor I	Fc portion of IgG-high affinity
NM_133978	Cmtm7 = CKLF-like MARVEL transmembrane domain	IgM?
NM_178165	Fcrl1 = Fc receptor-like protein 1	IgG ₃ ?
NM_144559	Fcgr4 = Fc receptor IgG low affinity IV	Binds Fc fragment of IgG

Genbank link	Gene names	Ligand
<i>Pattern recognition and related receptors</i>		
NM_009151	Selplg = P-selectin glycoprotein ligand 1	Enterovirus 71, hand foot and mouth disease AKA CD162
NM_010745	Ly86 = lymphocyte antigen 86 precursor	AKA MD1 LPS
NM_009853	Cd68 = macrophage	Ox LDL, SR
NM_031254	Trem2 = triggering receptor expressed on myeloid cells 2	Apoptotic neurons, Lipids, A β
NM_008533	Cd180 = CD180 antigen	LPS AKA rp105
NM_011905	Tlr2 = toll-like receptor 2	Pathogens and endogenous ligands
NM_007645	Cd37 = leukocyte antigen CD37	P-glucan? interacts with dectin-1
NM_133211	Tlr7 = toll-like receptor 7	Pattern recognition receptor
NM_009841	Cd14 = monocyte differentiation antigen CD14	Pattern recognition receptor
NM_001204241	Clec4a3 = dendritic cell inhibitory receptor 3	HIV AKA DCIR3
NM_021297	Tlr4 = toll-like receptor 4	Bacterial, fungal and parasitic ligands
NM_205820	Tlr13 = toll-like receptor 13	Bacterial 23 s rRNA
NM_021364	Clec5a = C-type lectin domain family 5 member A	Japanese encephalitis virus dengue virus
NM_134250	Havcr2 = hepatitis A virus cellular Hepatitis A virus AKA Tim3 also binds galectin 9 receptor 2 homolog	
NM_020008	Clec7a = C-type lectin domain family 7 member A	β glucan and yeast
NM_023158	Cxcl16 = C-X-C motif chemokine 16	G+ and G-bacteria, OxLDL, MTB
NM_007649	Cd48 = CD48 antigen	MTB, E-coli
NM_008522	Ltf = lactotransferrin	AGEs, Bacteria
NM_010545	Cd74 = H-2 class II histocompatibility antigen gamma	Bacteria (<i>H. pylori</i>), HIV
NM_178924	Upk1b = uropod protein 1b	Bacteria (<i>E. coli</i>)
NM_205823	Tlr12 = toll-like receptor 12	Parasites (<i>Taenia solium</i>) ⁶
NM_030682	Tlr1 = toll-like receptor 1	Bacterial, fungal and parasitic ligands
NM_153510	Pilra = paired immunoglobulin-like type 2 receptor alpha	CD99, HSV-1, NPDC1, COLEC12, PANP (neural tissue)
NM_011604	Tlr6 = toll-like receptor 6	Microbial and endogenous ligands
NM_001033632	Ifitm6 = interferon induced transmembrane protein 6	May regulate viral entry
<i>ECM receptors</i>		
NM_001082960	Itgam = integrin alpha-M	Fibrinogen other ECMs and amyloid β
NM_008404	Itgb2 = integrin beta-2	CD18 binds amyloid β and ECM
NM_010580	Itgb5 = integrin beta-5	Adenovirus, Vitronectin
NM_010130	Emr1 = EGF-like module-containing mucin-like hormone	AKA F4/80, hormones? ECM adhesion ⁷⁸ ?
NM_001033141	Ecsr = endothelial cell-specific chemotaxis regulator	ECM? Intracellular filamin
NM_178611	Lair1 = leukocyte-associated immunoglobulin-like	Collagens
NM_178706	Siglech = sialic acid binding Ig-like lectin H	Sialic acid
NM_175316	Slco2b1 = solute carrier organic anion transporter family	Organic anions sensing/uptake/transport
NM_019741	Slc2a5 = solute carrier family 2, facilitated glucose	Glucose AKA Glut5
NM_001159301	Lgals9;Lgals9 = galectin-9	Urate, glycans
NM_183031	Gpr183 = G-protein coupled receptor 183	Oxysterols
NM_019432	Tmem37 = voltage-dependent calcium channel gamma-like	Inorganic cations?
NM_021293	Cd33 = myeloid cell surface antigen CD33	Sialic acid sensing AKA siglec 3
NM_030720	Gpr84 = G-protein coupled receptor 84	Medium chain fatty acids

Genbank link	Gene names	Ligand
NM_011405	Slc7a7 = Y + L amino acid transporter 1	Amino acids
NM_013706	Cd52 = CAMPATH-1 antigen precursor	C1q
NM_145581	Siglec5 = sialic acid-binding Ig-like lectin 5	Sialic acid
NM_008339	Cd79b = B-cell antigen receptor complex-associated	Involved in antigen binding, a component of the BCR complex
NM_030696	Slc16a3 = monocarboxylate transporter 4	Monocarboxylate
<i>Cell-cell interactions</i>		
NM_010493	Icam1 = intercellular adhesion molecule 1	LFA-1, rhinoviruses
NM_023892	Icam4 = intercellular adhesion molecule 4	β2 Integrins
NM_013489	Cd84 = SLAM family member 5 precursor	CD84 (Homophilic)
NM_008479	Lag-3 = lymphocyte activation gene 3	Binds MHC II
NM_019388	Cd86 = T-lymphocyte activation antigen CD86 precursor	Binds CD28 on T cells
NM_011210	Ptprc = receptor-type tyrosine-protein phosphatase C	Galectin-1 involved in cell-cell interactions, CMV
NM_011662	Dap12 = TYRO protein tyrosine kinase-binding protein	Lipids from membranes AKA CD300L
NM_021349	Tnfrsf13b = TNF receptor superfamily member AKA TACI	BLyS
NM_011608	Tnfrsf17 = TNF receptor superfamily member 17 AKA BCMA	BAFF
NM_001043317	Cd22 = B-cell receptor CD22	CD45, Sialic acids, IgM
<i>Potential sensors but no known ligands</i>		
NM_146162	Tmem119 = transmembrane protein 119 precursor	Regulation of osteoblast function-AKA OBIF, TM protein so potential sensor
NM_007651	Cd53 = leukocyte surface antigen CD53	Shown in a microglial cell line tetraspanin no known sensing function
NM_001159572	Platelet receptor Gi24	Unknown function. Not known to be expressed on microglia aka Dies1
NM_029612	Slamf9 = SLAM family member 9 precursor	Unknown but measles virus and bacterial ligands bind Slamf1
NM_001033780	Transmembrane protein C1orf162 homolog	Unknown function. Not known to be expressed on microglia
NM_001170332	Clec4a2 = C-type lectin domain family 4 member A2 AKA Dcir	Unknown ligand. Deficiency causes autoimmune disease due to excess expansion of dendritic cells
NM_001190310	Clec4b1 = C-type lectin domain family 4, member b1 isoform	Unknown ligand but associates with Fcgr ¹¹⁵
NM_001081239	Lilra5 = leukocyte immunoglobulin-like receptor	Natural ligand unknown but cross-linking on macrophages upregulates TNF, IL1b, IL6
NM_025376	Tmem8c = transmembrane protein 8C	Unknown function. Not known to be expressed on microglia
NM_027965	Gpr160 = probable G-protein coupled receptor 160	Unknown function. Not known to be expressed on microglia
NM_001099332	Cd101 = immunoglobulin superfamily member 2	Natural ligand unknown, involved in T cell activation

Table 2

Validated qPCR primer sets for easy quantification of CD11b, CX₃CR1, P2RY₆, P2RY₁₂, P2RY₁₃, and TREM2

Gene	Forward	Reverse	Amplicon (bp)
CD11b	GTGTGACTACAGCACAAGCCG	CCCAAGGACATATTCACAGCCT	75
Cx3cr1	ACCGGTACCTTGCCATCGT	ACACCGTGCTGCACTGTCC	64
P2ry6	CTGCGTCTACCGTGAGGATT	GCAATGACGCAGATGTTTCA	96
P2ry12	CACGGATTCCCTACACCCTG	GGGTGCTCTCCTTCACGTAG	87
P2ry13	AACAAAGCTGATGCTCGGGA	GTGTCATCCGAGTGTCCTG	89
Trem2	GCCTTCCTGAAGAAGCGGAA	GAGTGATGGTGACGGTTCCA	73