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Identification of Genes Preferentially Expressed by Microglia and Upregulated During Cuprizone-Induced Inflammation

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KEY WORDS
microglia; monocytes; spleen macrophages; DNA microarray; gene profiling; cuprizone; demyelination; G protein-coupled receptor

ABSTRACT
Microglia, monocytes, and peripheral macrophages share a common origin and many characteristics, but what distinguishes them from each other at the level of gene expression remains largely unknown. In this study, we compared the transcriptional profiles of freshly purified microglia, monocytes, and spleen macrophages using Affymetrix Mouse Genome arrays to identify genes predominantly expressed by microglia. Among tens of thousands of genes assayed, 127 potential candidates were found, including nine newly discovered genes encoding plasma membrane and extracellular proteins. In the brain, the latter were selectively expressed by microglia, as revealed by *in situ* hybridization. Three of them were confirmed to be exclusively (MSR2) or predominantly (GPR12, GPR34) expressed in the brain compared to the other tissues examined. Furthermore, all of these genes were upregulated in activated microglia after treatment with the demyelinating toxin cuprizone, suggesting that they play roles in neuroinflammation. In conclusion, this study reports the identification of new selective markers for microglia, which should prove useful not only to identify and isolate these cells, but also to better understand their distinctive properties.

INTRODUCTION

After being produced from hematopoietic stem cells in the bone marrow, monocytes enter the bloodstream to migrate to peripheral tissues where they differentiate into macrophages. The properties of macrophages can vary considerably depending on the organs and even the compartments of a given organ. For example, the resident macrophages of the CNS, microglia, differ from their peripheral counterparts and other populations of cerebrospinal macrophages by a stellate morphology and lower expression of cell surface markers, such as major histocompatibility complex Class II molecules (Aloisi, 2001; Perry, 1998). These characteristics are likely to be conferred by a variety of soluble, matrix-bound, and cell-attached factors, but the precise nature of these and how microglia respond to them are questions that remain largely unsolved.

As the primary immune effector cells of the CNS, microglia play detrimental or beneficial roles in many pathologies, such as multiple sclerosis (Heppner et al., 2005), Alzheimer's disease (Simard et al., 2006), and cerebral tumors (Villeneuve et al., 2005). Selective inhibition or activation of microglia could be useful in the treatment of these diseases, but our ability to modulate these cells with specificity is hindered by our limited understanding of the mechanisms that govern their development and function. The identification of signaling molecules expressed exclusively or preferentially by microglia could not only help to clarify how microglia are regulated, but also provide the opportunity to manipulate their fate for therapeutic purposes. Thus far, such molecules have not been firmly identified, except perhaps the triggering receptor expressed on myeloid cells-2 (TREM2), a protein found at the surface of microglia and osteoclasts (Cella et al., 2003; Schmid et al., 2002). In this study, we attempted to fill this gap by comparing the transcriptional profiles of freshly purified microglia, monocytes, and spleen macrophages using DNA microarrays. Among 127 candidate genes, 12 were chosen for further analysis, of which 9 were confirmed to be expressed by microglia. Herein, we report the differential expression of these genes *in vivo* and their upregulation in the cuprizone model of demyelination.

MATERIALS AND METHODS

Animals and Treatments

Male C57BL/6 mice purchased from Charles River Laboratories (Montréal, QC, Canada) were used at 10 weeks of

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age in accordance with current guidelines of the Canadian Council on Animal Care. To induce chronic demyelination and microgliosis, mice were fed ground mouse chow containing 0.2% cuprizone (Sigma-Aldrich, St. Louis, MO) for 5 weeks. Control mice were treated identically, except that they were not exposed to cuprizone. To induce the transient expression of inflammatory genes in microglia, additional mice received an intraperitoneal injection of 1 mg/kg lipopolysaccharide (LPS) from *Escherichia coli* O55:B5 (Sigma-Aldrich) dissolved in pyrogen-free saline. Control mice were treated identically, except that LPS was substituted for saline.

Cell Isolation

Twelve untreated mice were anesthetized with a mixture of ketamine and xylazine. Blood samples were collected into EDTA tubes by intracardiac puncture. To remove residual blood, the animals were perfused with Dulbecco's phosphate-buffered saline (DPBS; Wisent, Saint-Bruno, QC, Canada) containing 1 g/L D-glucose. The brains and spleens were harvested, minced with razor blades in DPBS supplemented with 2% goat serum, digested for 1 h at 37°C in HBSS (Invitrogen, Carlsbad, CA) containing 200 U/mL DNase, 2 U/mL dispase, 2.5 U/mL papain, 12.4 mM $MnSO_4$, and 5 mM L-cysteine, and filtered through 40-μm nylon mesh (Becton Dickinson, Cockeysville, MD). The samples were then enriched in target cells by centrifugation on density gradient (blood: Ficoll; brain and spleen: 35% Percoll) and by positive immunomagnetic selection with an EasySep FITC selection kit (StemCell Technologies, Vancouver, BC, Canada). For that purpose, microglia were labeled with FITC-conjugated anti-CD11b and Cy-chrome-conjugated anti-CD45 antibodies (BD Pharmingen, San Diego, CA), whereas monocytes and spleen macrophages were stained with FITC-conjugated anti-F4/80 (Serotec, Raleigh, NC) and Cy-chrome-conjugated anti-CD45 antibodies. After enrichment, microglia ($CD11b^+$ $CD45^{low}$), monocytes ($F4/80^+$ $CD45^+$) and spleen macrophages ($F4/80^+$ $CD45^+$) were sorted using an Epic Elite ESP flow cytometer (Beckman Coulter, Fullerton, CA) by excluding dead cells by propidium iodide staining. The samples were kept cold during sorting to minimize RNA degradation and cell death.

RNA Preparation and Microarray Analysis

Total RNA was extracted from each cell population with the RNeasy Micro kit (Qiagen, Valencia, CA) following the manufacturer's instructions. RNA integrity and yield were assessed by microcapillary electrophoresis (Bioanalyzer 2100, Agilent Technologies, Palo Alto, CA). Twelve nanograms of total RNA were converted to cDNA, which was amplified and transcribed to produce biotinylated cRNA using the Small Sample Labeling Protocol Version 2 (Affymetrix, Santa Clara, CA). Fifteen micrograms of fragmented cRNA were hybridized in duplicate to Affymetrix Mouse Genome 430 2.0 arrays for 16 h at 45°C with constant rotation at 60 rpm The arrays were washed and stained with streptavidin-phycoerythrin (10 μg/mL; Molecular Probes, Eugene, OR) and biotinylated goat anti-streptavidin (3 mg/mL; Vector Laboratories, Burlingame, CA) using the Affymetrix Fluidics Station 400 (protocol EukGE-WS2Av5), then read using the Affymetrix GeneChip Scanner 3000. Data were preprocessed using Bioconductor 1.7 software and the GC Robust Multiarray Average (GCRMA) method (Wu et al., 2003). Genes differentially expressed in microglia versus monocytes and spleen macrophages were identified by calculating an intensity-dependent *z*-score for each probe set and each comparison as previously described (Quackenbush, 2002).

Histological Preparation

Mice were anesthetized and transcardially perfused with 10 mL of saline, followed by ice-cold 4% paraformaldehyde in borate buffer, pH 9.5, over 10 min. The brains and spleens were removed, postfixed for 2 days at 4°C, and then cryoprotected overnight in the same fixative supplemented with 20% sucrose. Series of sections were cut at 30 μm using a freezing microtome, collected in cryoprotectant (30% ethylene glycol, 20% glycerol, 50 mM sodium phosphate buffer, pH 7.4), and stored at −20°C until histological analysis.

cDNA Cloning

Five micrograms of mouse brain total RNA (Stratagene, La Jolla, CA) was reverse-transcribed for 50 min at 42°C with Superscript II reverse transcriptase (Invitrogen). Partial cDNAs (~1.5 kb) were PCR amplified with Platinum Pfx DNA polymerase (Invitrogen) and the primers listed in Table 1. The PCR conditions consisted of an initial denaturation step (94°C, 2 min), followed by 30 cycles of denaturation (94°C, 15 s), annealing (63°C, 30 s) and extension (68°C, 100 s), and a final extension step (68°C, 2 min). Amplicons were cloned into the pCR-Blunt II-TOPO vector (Invitrogen) and automatically sequenced from both ends to confirm identity. Before riboprobe synthesis (see below), plasmids were linearized with restriction enzymes listed in Table 1 and purified with the QIAquick PCR purification kit (Qiagen).

In Situ Hybridization

Each transcript was detected by radioisotopic *in situ* hybridization as previously described (Villeneuve et al., 2005).

Combined Immunohistochemistry and *In Situ* Hybridization

Free-floating sections were washed in 50 mM potassium phosphate-buffered saline (KPBS) and treated with an avidin-biotin blocking kit (Vector Laboratories, Burlingame,

TABLE 1. cDNAs Used for In Situ Hybridization

Symbol	Description	Primers[a] (5′→3′)	Size[b] (bp)	Linearization[c]		Transcription[d]	
				Antisense	Sense	Antisense	Sense
F11R	F11 receptor	Fwd: tccaggttcccgagaacgagtccat Rev: caaagggtccgtgcggtagttccag	1,460	Xho 1	Spe 1	SP6	T7
GPR12	G protein-coupled receptor 12	Fwd: atgctaaactgggagccgggcctct Rev: tgttcattcaagcagcaggcattttca	1,489	Spe 1	Eco RV	T7	SP6
GPR155	G protein-coupled receptor 155	Fwd: aagtgccagagagagccccacagga Rev: aggcttccatgcaaggtttcattttga	1,502	Eco RV	Spe 1	SP6	T7
GPR34	G protein-coupled receptor 34	Fwd: tggctttgctcctctcatggaatgc Rev: cactgacatttcaactcagtccccaga	1,500	Pst 1	Spe 1	SP6	T7
GPR56	G protein-coupled receptor 56	Fwd: tgcagtgcgtgttctgggttgaaga Rev: ggtttgcagaaaggctgggggagat	1,540	Eco RV	Spe 1	SP6	T7
GPR84	G protein-coupled receptor 84	Fwd: ctggaagcctgactgcccctcaaaa Rev: ctccttgggtcacacagggttgacg	1,472	Eco RV	Spe 1	SP6	T7
GPR86	G protein-coupled receptor 86	Fwd: gcacaagcggctggaggtgaaggt Rev: tgcctctgtggcttctgaggctatgg	1,453	Spe 1	Pst 1	T7	SP6
MSR2	Macrophage scavenger receptor 2	Fwd: gcaagacgtggtgacagtggctcct Rev: cgggctttctttggagctgttggaa	1,494	Eco RV	Spe 1	SP6	T7
Olfml3	Olfactomedin-like 3	Fwd: cttcagggacagcagcaccaccttg Rev: aagggagggagggcagctttgtctc	1,531	Eco RV	Spe 1	SP6	T7
PLXDC2	Plexin domain containing 2	Fwd: attctgccagccgggatctgtgg Rev: ctcctggcaagtccaccggagatgt	1,501	Eco RV	Spe 1	SP6	T7
Siglec-H	Sialic acid-binding glycoprotein lectin H	Fwd: tctgtgacggtgcaggagggtctgt Rev: tgggaggacagtgtggttcgctctt	1,512	Pst 1	Sac 1	SP6	T7
Stab1	Stabilin 1	Fwd: ggcgtgtgcctggatggtatacgtg Rev: aggctcagatcccagcactgcccta	1,473	Eco RV	Spe 1	SP6	T7
TNF	Tumor necrosis factor	Fwd: ccagaactccaggcggtgcctatgt Rev: tacaccccatcggctggcaccacta	1,384	Eco RV	Bam H1	SP6	T7

Abbreviations: Fwd, forward; Rev, reverse.
[a]Primer pair used for PCR amplification.
[b]Size of the PCR product that was inserted into the pCR-Blunt II-TOPO vector.
[c]Restriction enzyme used to linearize the plasmid for antisense or sense riboprobe synthesis.
[d]Polymerase used for *in vitro* transcription.

CA). The sections were then incubated sequentially for 2 h at room temperature in primary antibody (rabbit anti-Iba1, 1:1,000, Waco Chemicals, Richmond, VA; rat anti-galectin-3, 1:500, American Type Culture Collection, Rockville, MD) and secondary antibody (goat anti-rabbit or anti-rat IgG, 1:500, Jackson Immunoresearch, West Grove, PA) in KPBS supplemented with 2% heparin sulfate. After a 1-h incubation in ABC solution (Vector Laboratories), the staining was developed for 9 min in KPBS containing 0.5 mg/mL diaminobenzidine, 2 mg/mL β-D(+)-glucose, and 1 μL/mL glucose oxidase. Each of those steps was followed by four 5-min rinses in KPBS. Following staining, the sections were processed for *in situ* hybridization as described (Villeneuve et al., 2005), except that thionin counterstaining was omitted.

Imaging

Photomicrographs were taken using a Retiga EX monochrome camera (QImaging, Burnaby, BC, Canada) mounted on a Nikon E800 microscope. The images were adjusted for contrast, brightness, and sharpness using Photoshop 7 (Adobe Systems, San Jose, CA).

Optical Densitometry

In situ hybridization signals were quantified at the single-cell level by optical density readings. Individual cells were photographed (12-bit, grayscale) in dark-field microscopy using a 20× Plan objective. The intensity of the hybridization signals for 125–640 cells per region and per animal were analyzed with ImageJ software 1.36. The hybridization signals were circled using the round selection tool of a fixed dimension (50 × 50 pixels) and the mean optical intensity of that area was recorded. The values were subtracted from the average of six background measurements. Data were analyzed by Wilcoxon rank-sum test with JMP software (SAS Institute, Cary, NC).

Real-Time Quantitative RT-PCR (qRT-PCR)

Total RNA from leukocytes (separated by Ficoll density gradient centrifugation) and brains (exsanguinated by intracardiac perfusion with saline) was isolated using the GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich) following the manufacturer's protocol. RNA quantity and quality were assessed using the RNA 6000 Nano LabChip and Agilent Bioanalyser 2100 (Agilent, Mountain View, CA). About 1.25 μg of total RNA was reverse-transcribed for 10 min at 25°C and 120 min at 42°C using a random primer hexamer and Superscript II reverse transcriptase (Invitrogen). Quantitative PCR was conducted in duplicate in a final volume of 15 μL containing 1× Universal PCR Master Mix (Applied Biosystems, Foster City, CA), 10 nM *Z*-tailed forward primer, 100 nM untailed reverse primer, 100 nM Amplifluor Uniprimer (Chemicon, Temecula, CA), and 2 μL cDNA (40–50 ng/μL). The primer pairs were: 18S rRNA, 5′-tgcatgtctaagtacgcacgg-3′ and 5′-aatgagccattcgcagtttca-3′; F11R, 5′-tgggattctcctggattcaaa-3′ and 5′-ccacctcgtacggacaccat-3′; GPR12, 5′-gtcacagtgcaagggtggc-3′ and 5′-tgccggtgaaggtgagttg-3′; GPR34, 5′-acttcaggaaagcttcaactcagttc-3′ and 5′-tcaggccatacttcagaagctg-3′; GPR86, 5′-gagtctcttccaaaacaaagctgat-3′

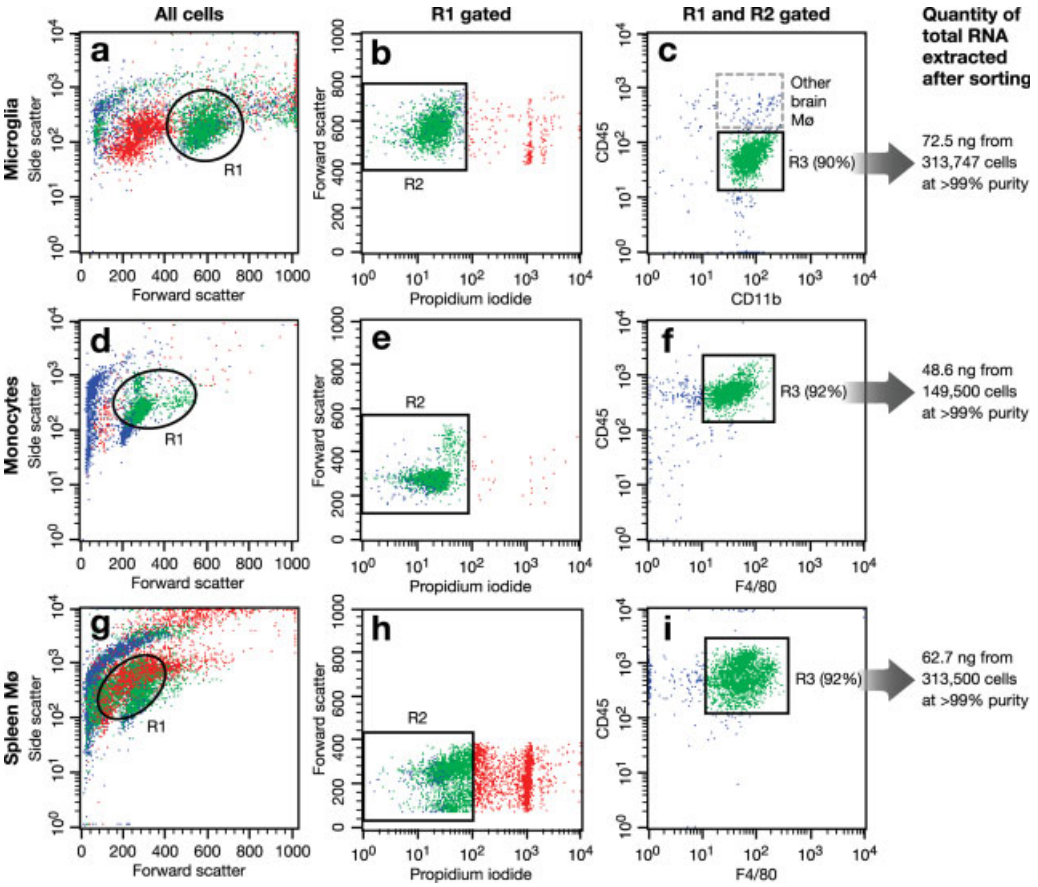

Fig. 1. Isolation of microglia, monocytes, and spleen macrophages (Mø) for total RNA extraction. (**a**, **d**, **g**) Cells harvested from mouse brains, blood, or spleens and enriched by positive immunomagnetic selection were analyzed by flow cytometry and gated (R1) using forward and side scatter. (**b**, **e**, **h**) Viable cells (R2) were identified by the lack of propidium iodide staining. (**c**) Microglia (R3) were sorted based on $CD11b^{+}$ and $CD45^{low}$ expression. (**f**, **i**) Monocytes and spleen macrophages (R3) were sorted based on $F4/80^{+}$ and $CD45^{+}$ expression. Purity was determined by reanalysis of sorted cells. Green, blue, and red dots represent target, contaminating and dead cells, respectively.

and 5′-tcccagtggtgttgattgtcc-3′; MSR2, 5′-gcttctggtcttcgctcctg-3′ and 5′-ctgatgctcaaccagtcgga-3′; Olfml3, 5′-atgagaaaatacgatatggtgacgga-3′ and 5′-tcacctgagcgactgtgtagct-3′; Siglec-H, 5′-gatctctgtgcatgtgacagacc-3′ and 5′-gtgggatgctgatgttggg-3′; Stab1, 5′-tccagtaacatgaatgcgacg-3′ and 5′-gacatgctgtcggcagagc-3′. The forward primers were flanked at their 5′-ends by the following sequence: 5′-actgaacctgaccgtaca-3′. Amplification was performed using the ABI PRISM 7900 sequence detector (Applied Biosystems) under the following conditions: 2 min at 50°C, 4 min at 95°C, followed by 55 cycles of 15 s at 95°C and 40 s at 55°C. Ribosomal 18S rRNA was used as an internal control to normalize the expression levels of each transcript. Data were acquired using SDS 2.0 software (Applied Biosystems) and analyzed by Student's *t* test with JMP software.

RESULTS

Microarray Analysis

To compare the transcriptomes of microglia, monocytes, and spleen macrophages, we extracted RNA from each of these cells after purification from normal mice by flow cytometry on the basis of well-established cell surface markers (Fig. 1). The RNA samples were analyzed in duplicate using oligonucleotide arrays interrogating over 39,000 transcripts. The raw data were normalized and quantified using the GCRMA algorithm (Supplementary Table 1), a stochastic-model-based procedure that takes into account probe sequence information, such as GC content, and that was shown to perform better than other widely used algorithms (Wu and Irizarry, 2004). As illustrated in Fig. 2, this method produced less experimental noise than Microarray Suite (MAS) 5.0, especially for low-expression genes.

By calculating an intensity-dependent *z*-score for each probe set, which represent the number of standard deviations a data point is from the local mean, we identified marked differences in the expression levels of several genes that had previously been found to be differentially regulated in microglia versus other cells of the monocytic lineage (Table 2). Notably, major histocompatibility complex class II genes were strongly expressed in monocytes and spleen macrophages, but not in microglia, which is in agreement with the concept that the latter are poorly equipped for antigen presentation under nonpathological conditions (Perry, 1998). Conversely, TREM2, a receptor that stimulates the migratory and phagocytic activity of microglia (Takahashi et al., 2005), was strongly expressed in these cells, but not in the other types examined, as anticipated from previous studies (Bouchon et al., 2000; Schmid et al., 2002). Interestingly, other members of the TREM family that serve to propagate the inflammatory response (Bleharski et al., 2003; Bouchon et al., 2000), namely TREM1 and TREM3, were regulated in an opposite way compared to TREM2, i.e., their transcripts were absent in microglia, but abundant in monocytes and

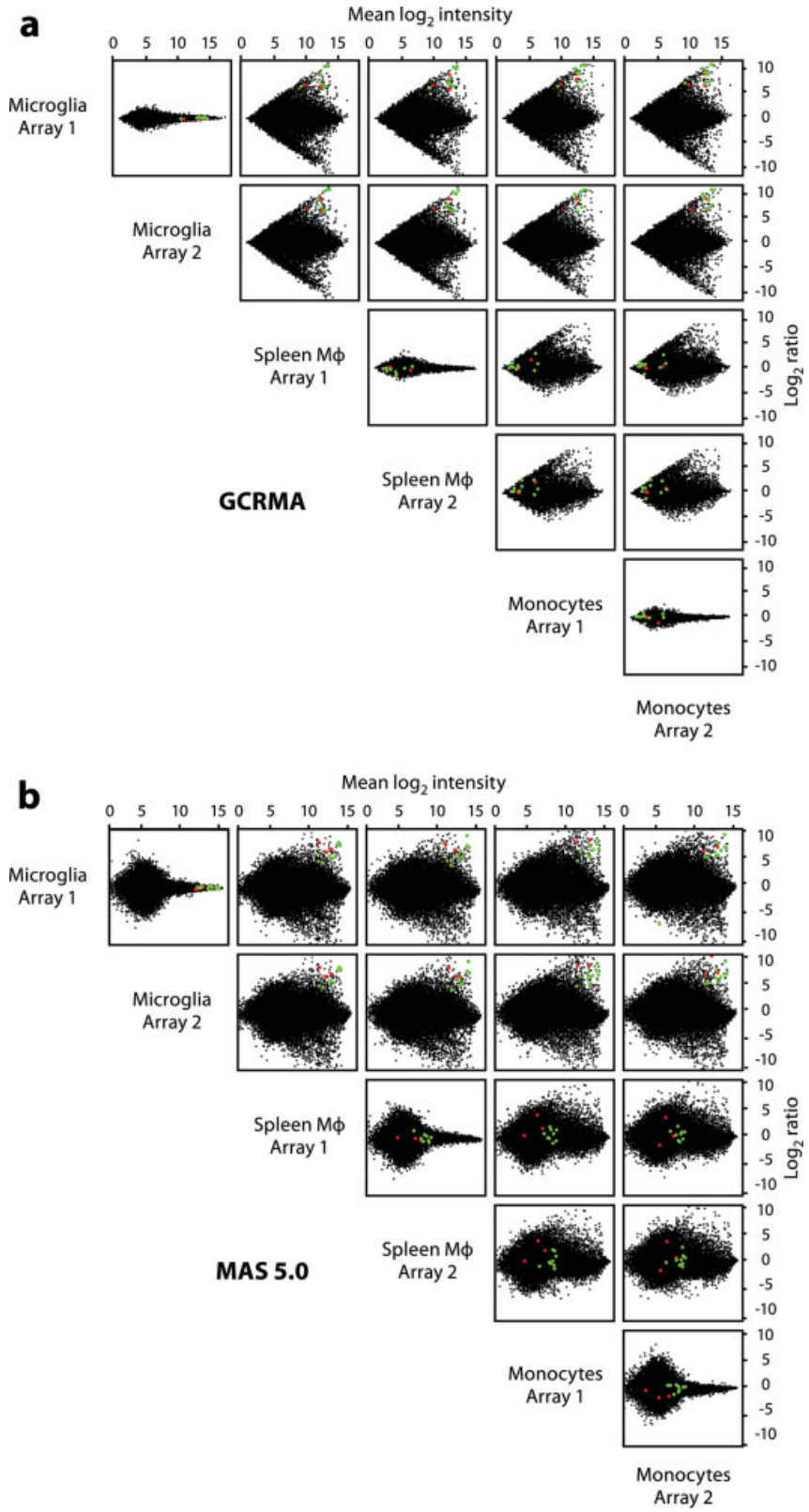

Fig. 2. Comparison of each microarray data set with each other after normalization with the GC Robust Multiarray Average (GCRMA) (**a**) or Microarray Suite (MAS) 5.0 algorithm (**b**). Green and red dots respectively represent transcripts that are microglia- or nonmicroglia-specific in the brain, as subsequently assayed by *in situ* hybridization. Note the reduction in noise level obtained with GCRMA compared to MAS 5.0.

spleen macrophages (Table 2). Furthermore, as expected, many monocytic markers, such as CD11b, CD14, F4/80, and macrophage colony-stimulating factor, were strongly expressed in the three cell subsets examined (Table 2), whereas markers of other lineages, such as glial fibrillary acidic protein (astrocytes), galactosylceramidase (oligo-

TABLE 2. Transcripts Known to be Expressed by Cells of the Monocytic Lineage and Detected by Microarray Analysis in Freshly Purified Microglia, Monocytes, and/or Spleen Macrophages (Mø)

Gene symbol[a]	Gene description[a]	Probe set	Average hybridization signal[b]			Expression change in microglia versus: Monocytes		Spleen Mø		References[c]
			Microglia	Monocytes	Spleen (Mø)	Ratio	*z*-score	Ratio	*z*-score	
CD11b (CR3, Itgam, Mac-1)	Integrin αM	1422046_at	2,313	1,825	1,795	1.3	0.3	1.3	0.3	–
CD11c (CR4, Itgax)	Integrin αX	1419128_at	11	328	2,189	0.0	−2.3	0.0	−4.2	Fischer and Reichmann, 2001
CD14	Lipopolysaccharide receptor	1417268_at	9,228	9,336	5,908	1.0	0.0	1.6	0.7	–
CD34	CD34 antigen	1416072_at	1,110	5	6	222.0	3.7	185.0	3.8	Ladeby et al., 2005a
CD45 (PTPRC, LCA)	Protein tyrosine phosphatase receptor C	1422124_a_at	10,614	17,289	19,677	0.6	−1.2	0.5	−0.9	Sedgwick et al., 1991
CD68	Macrosialin	1449164_at	7,874	5,406	1,600	1.5	0.6	4.9	2.2	–
CX3CR1	Fractalkine receptor	1450020_at	25,759	2,129	117	12.1	3.9	220.2	6.6	Sunnemark et al., 2003
F4/80 (EMR1)	EGF module-containing mucin-like hormone receptor 1	1451161_a_at	3,246	3,771	5,766	0.9	−0.2	0.6	−0.8	–
FcγR2β (CD32)	Fc γ receptor 2β	1435477_s_at	4,031	6,228	2,661	0.6	−0.6	1.5	0.6	–
FcγR3 (CD16)	Fc γ receptor 3	1448620_at	15,098	6,642	4,791	2.3	1.4	3.2	1.9	–
Gal-1 (LGALS1)	Galectin 1	1455439_a_at	87	9,339	5,113	0.0	−5.2	0.0	−4.4	–
Gal-3 (LGALS3, Mac-2)	Galectin 3	1426808_at	12	20,879	10,825	0.0	−7.6	0.0	−6.5	Reichert and Rotshenker, 1999
GM-CSFRβ (CSF2Rβ2)	Granulocyte-macrophage colony-stimulating factor receptor β	1449360_at	1,086	1,197	1,728	0.9	−0.1	0.6	−0.6	–
H2-Aα	Histocompatibility 2, class II, antigen A α	1452431_s_at	12	1,846	9,518	0.0	−3.9	0.0	−6.3	Perry, 1998
H2-Aβ1	Histocompatibility 2, class II, antigen A β 1	1450648_s_at	202	5,332	25,857	0.0	−3.7	0.0	−6.2	Perry, 1998
H2-Eβ1	Histocompatibility 2, class II, antigen E β	1417025_at	87	2,192	13,950	0.0	−3.2	0.0	−5.8	Perry, 1998
Iba1 (Aif1)	Ionized calcium-binding adapter molecule 1	1418204_s_at	17,401	2,213	1,452	7.9	3.1	12.0	3.6	Vallières and Sawchenko, 2003
IFNγR1 (CD119)	Interferon γ receptor 1	1448167_at	2,177	2,340	1,011	0.9	−0.1	2.2	0.9	–
IFNγR2	Interferon γ receptor 2	1423557_at	2,328	1,419	589	1.6	0.6	4.0	1.6	–
IL-10Rα	Interleukin 10 receptor α	1448731_at	2,873	1,345	1,239	2.1	0.9	2.3	1.1	–
IL-10Rβ	Interleukin 10 receptor β	1419455_at	2,499	885	862	2.8	1.2	2.9	1.3	–
IL-4Rα (CD124)	Interleukin 4 receptor α	1421034_a_at	545	1,125	479	0.5	−0.8	1.1	0.1	–
M-CSFR (CSF-1R, CD115)	Macrophage colony-stimulating factor receptor	1419873_s_at	25,671	7,637	4,060	3.4	2.2	6.3	3.2	–
MIF	Macrophage migration-inhibitory factor	1416335_at	799	640	1,905	1.2	0.2	0.4	−1.0	–
MRC1	Mannose receptor C, type 1	1450430_at	403	252	1,284	1.6	0.4	0.3	−1.3	–
SCARB2 (CD36)	Scavenger receptor, class B, member 2	1460235_at	2,236	2,111	1,029	1.1	0.1	2.2	0.9	–
TGFβR1	Transforming growth factor β receptor 1	1420895_at	17,984	1,560	790	11.5	3.6	22.8	4.3	–
TGFβR2	Transforming growth factor β receptor 2	1426397_at	8,538	1,806	1,876	4.7	2.1	4.6	2.1	–
TLR2	Toll-like receptor 2	1419132_at	1,084	3,027	797	0.4	−1.3	1.4	0.4	–
TNFR1 (CD120α, p55, TNFRSF1A)	Tumor necrosis factor receptor 1	1417291_at	913	1,678	512	0.5	−0.7	1.8	0.6	–
TNFR2 (CD120β, p75, TNFRSF1B)	Tumor necrosis factor receptor 2	1418099_at	3,529	5,635	1,930	0.6	−0.7	1.8	0.8	–
TREM1	Triggering receptor expressed on myeloid cells 1	1447284_at	15	3,013	2,226	0.0	−4.5	0.0	−4.1	–
TREM2	Triggering receptor expressed on myeloid cells 2	1421792_s_at	5,054	62	5	81.5	4.6	1010.8	5.5	Schmid et al., 2002
TREM3	Triggering receptor expressed on myeloid cells 3	1460271_at	5	2,222	980	0.0	−4.3	0.0	−3.8	–

[a]Annotations were obtained from the Affymetrix web site (www.affymetrix.com).
[b]Average signal of two microarrays normalized with the GCRMA algorithm.
[c]Reference that reports differential expression of the transcript in microglia versus other monocytic cells.

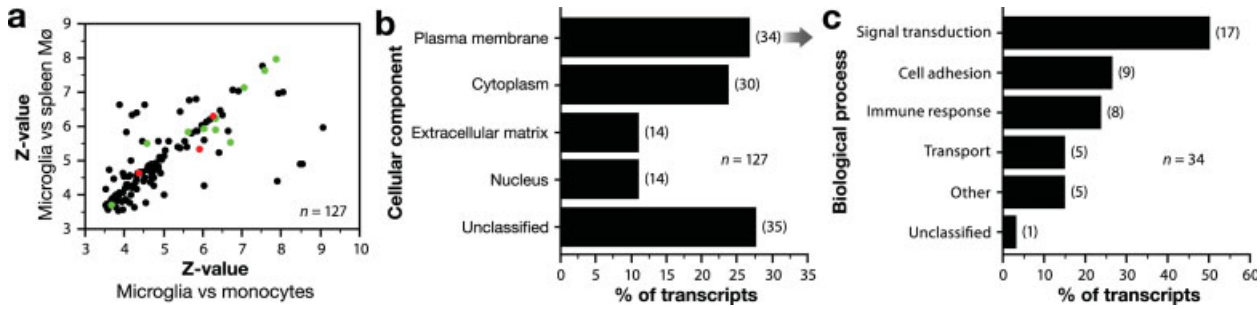

Fig. 3. Transcripts identified by microarray analysis as being enriched in freshly purified microglia compared to monocytes and spleen macrophages (Mø) with a *z*-score ≥3.5 for both of these comparisons. (**a**) Distribution of mRNAs according to their *z*-scores. Green and red dots represent, respectively, mRNAs confirmed or not to be expressed in microglia by *in situ* hybridization. (**b**) Classification of mRNAs according to the main cellular location of the encoded proteins using the Gene Ontology database. (**c**) Classification of mRNAs that encode plasma membrane proteins according to their biological functions. Data in brackets represent the total number of transcripts in each category.

TABLE 3. Summary of In Situ *Hybridization Results on the Expression of Selected Transcripts in the Brain and Spleen of Normal or Cuprizone-Treated Mice*

	Brain				Spleen		
Gene symbol	Basal distribution	Relative expression[a]	Colocalization with Iba1	Upregulation by cuprizone	Basal distribution	Relative expression[a]	Colocalization with Iba1
Genes predominantly expressed in microglia							
F11R	widespread (microglia, choroid plexus macrophages)	+++	yes	yes	–	–	–
GPR12 (P2Y12)	widespread (microglia)	+++	yes	yes	–	–	–
GPR34	widespread (microglia)	++	yes	yes	–	–	–
MSR2	widespread (microglia)	+++	yes	yes	–	–	–
Genes expressed in microglia and spleen							
GPR84	a few scattered cells (microglia)	+/−	yes	yes	red pulp	+/−	yes
GPR86 (P2Y13)	widespread (microglia)	+++	yes	yes	red and white pulp	+++	yes
Siglec-H (CD33)	widespread (microglia)	+++	yes	yes	red and white pulp	++	yes
Olfml3	widespread (microglia, leptomeningeal macrophages)	+++	yes	yes	predominantly red pulp	+++	some
Stab1	widespread (microglia, leptomeningeal and perivascular macrophages)	+[b]/+++[c]	yes	yes	predominantly red pulp	+++	some
Other genes							
GPR56	widespread (neurons, glia)	++	ND	ND	ND	ND	ND
GPR155	caudoputamen (predominant in neurons)	+	ND	ND	ND	ND	ND
PLXDC2	widespread (predominant in neurons)	+	ND	ND	ND	ND	ND

ND, not determined.
[a]Gene expression was graded as undetectable (−), very low (+/−), low (+), moderate (++), or strong (+++).
[b]Microglia.
[c]Leptomeningeal and perivascular macrophages.

dendrocytes), neurofilament light chain (neurons), fms-like tyrosine kinase (endothelia), and CD3e (T lymphocytes) were virtually undetectable (Supplementary Table 1). Altogether, these observations validate the overall quality of our data set and the approach undertaken here to identify microglia-enriched transcripts.

Confirmation of Microarray Results

To narrow our search for genes preferentially expressed in microglia versus monocytes and spleen macrophages, we filtered out probe sets that had a *z*-score <3.5 for at least one of the two comparisons. A total of 172 probe sets representing 127 different transcripts passed this filter (Supplementary Table 2, Fig. 3a). Interestingly, those encoding proteins known to be associated with the plasma membrane accounted for 27%, of which half-encoded molecules involved in signal transduction (Figs. 3b,c). Because of their potential scientific or pharmaceutical interest, 11 transcripts of this category, including six orphan G protein-coupled receptors, plus one additional transcript that encodes a secreted glycoprotein, Olfml3, were chosen for further analysis (Table 3).

To confirm the differential expression of the selected transcripts, brains and spleens harvested from healthy mice were analyzed by radioisotopic *in situ* hybridization. The results are shown in Figs. 4 and 5 and summarized in Table 3. Briefly, four transcripts (F11R, GPR12, GPR34, and MSR2) were strongly expressed in numerous microglia-like cells distributed throughout the brain and were undetectable in the spleen. Four other transcripts (Siglec-

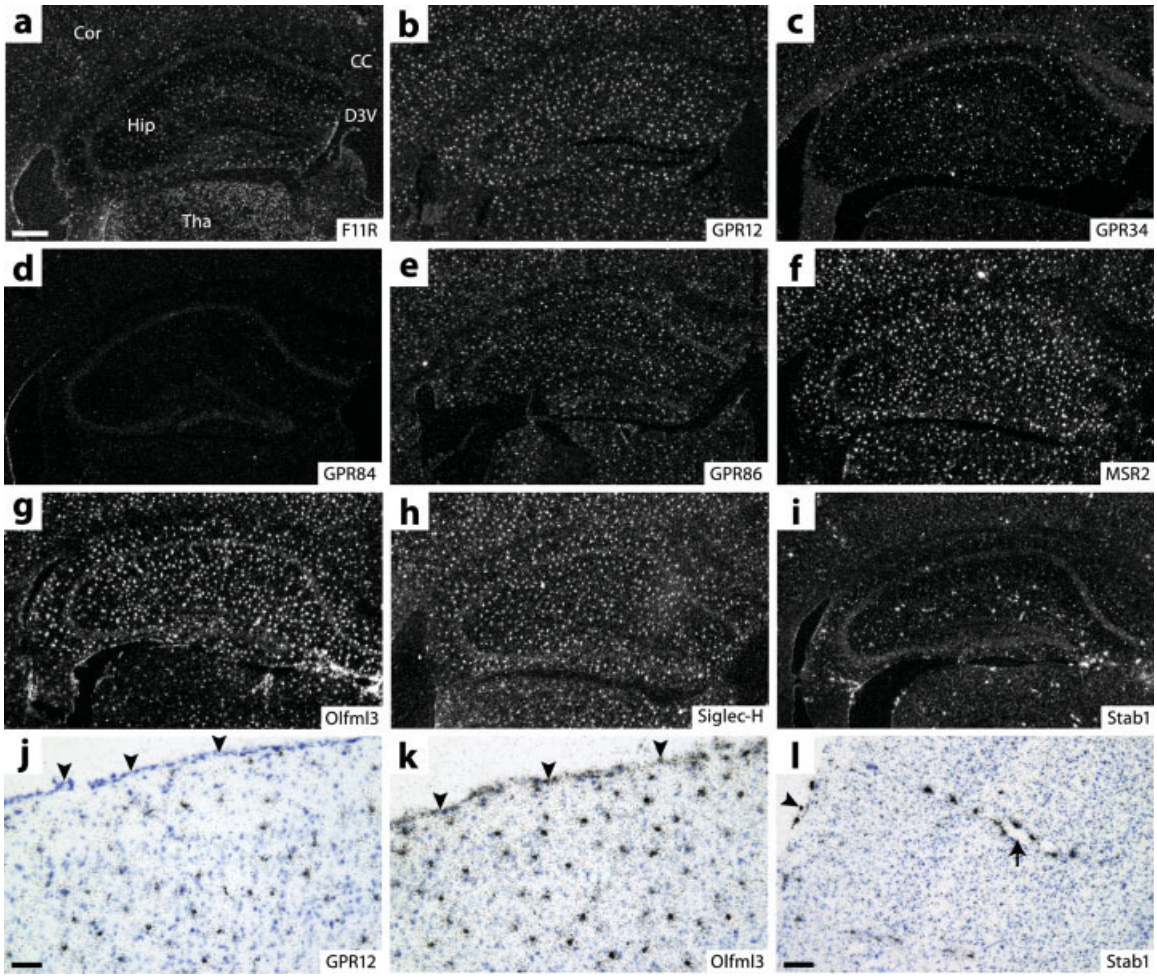

Fig. 4. Transcripts found to be expressed in microglia-like cells by *in situ* hybridization. (**a–i**) Dark-field photomicrographs of brain sections taken at the level of the dorsal hippocampus showing strong hybridization signals (white grains) for all transcripts, except GPR84 (**d**). Note that only the hippocampus is shown here as a representative example, but that the distribution and intensity of the signals were similar in the other regions. (**j–l**) Bright-field images showing hybridization signals (black grains) at higher magnifications in the cerebral cortex. Note the presence or absence of positive cells in the leptomeninges (arrowheads) and around the vasculature (arrow) depending on the transcript. Blue, thionin counterstaining. Abbreviations: CC, corpus callosum; Cor, cerebral cortex; D3V, dorsal third ventricle; Hip, hippocampus; Tha, thalamus. Scale bars: a–i, 250 μm; j, k, 50 μm; l, 100 μm.

H, GPR86, Olfml3, and Stab1) were also expressed in a large proportion of microglia-like cells, but were detectable in subpopulations of spleen cells. One transcript, GPR84, was detected in a very small number of cells both in the brain and the spleen. Finally, three transcripts (GPR56, GPR155, and PLXDC2) were predominantly expressed in neurons and were thus excluded from the study at this point, although we cannot reject the possibility that microglia express these genes under basal conditions or after stimulation. Adjacent brain and spleen sections hybridized with sense riboprobes exhibited no positive signal (e.g. in Figs. 8u–x), confirming the specificity of the method.

We next sought to identity the cellular source of each transcript by a combination of *in situ* hybridization and immunohistochemistry. In the brain, the nine transcripts described above as being expressed in microglia-like cells colocalized with the pan-monocytic marker Iba1 (e.g. in Fig. 6), a calcium-binding protein involved in membrane ruffling and phagocytosis (Ito et al., 1998; Kanazawa et al., 2002). All these transcripts were predominantly expressed by parenchymal microglia, except Stab1, which was more abundant in perivascular and leptomeningeal macrophages (Figs. 4i and 6e,f). In the spleen, three of the five mRNAs seen in this organ, Siglec-H, GPR84, and GPR86, clearly colocalized with Iba1 (e.g. in Figs. 6g,h). In contrast, Olfml3 was expressed by both $Iba1^{+}$ and $Iba1^{-}$ cells, whereas Stab1 was mainly found in $Iba1^{-}$ cells, often localized along the vasculature (Fig. 6). Interestingly, while numerous macrophages are found in every compartment of the spleen, these five transcripts were not expressed evenly across this organ (Fig. 5 and Table 3), suggesting that they were differentially expressed among subsets of spleen macrophages. Altogether, the results identify F11R, GPR12, GPR34, MSR2, Olfml3, and Stab1 as being enriched in microglia compared to spleen macrophages, and identify GPR86 and Siglec-H as being produced by both of these cell populations at levels indistinguishable by the technique used here.

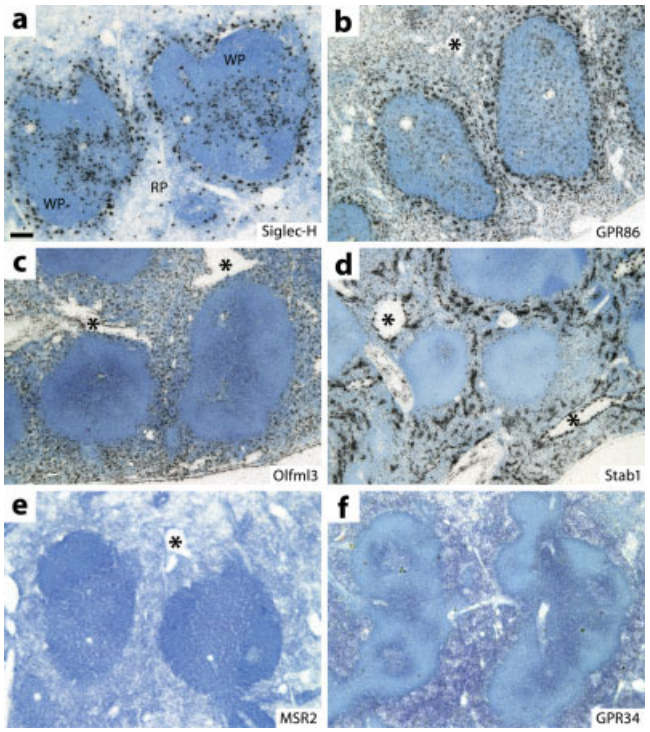

Fig. 5. *In situ* hybridization for different transcripts in the spleen. (**a–d**) Bright-field photomicrographs of spleen sections showing strong hybridization signals (black grains) distributed differently depending on the transcript. (**e–f**) No hybridization signal was detected for MSR2 and GPR34 mRNAs. Blue, thionin counterstaining. *Blood vessel lumen. Abbreviations: RP, red pulp; WP, white pulp. Scale bar: a–d, 100 μm.

To determine whether the nine transcripts confirmed to be expressed in microglia were more abundant in the brain compared to blood, RNA was isolated from exsanguinated brains and Ficoll-separated leukocytes, then analyzed by qRT-PCR. This approach was used because our *in situ* hybridization protocol cannot be used for blood. Furthermore, cells were not purified to minimize the introduction of bias in gene expression during the isolation procedure. As shown in Fig. 7, the majority of the transcripts, except F11R and GPR84, were expressed more abundantly in the brain than in blood, supporting our microarray results. The lack of difference in F11R mRNA levels between the brain and blood is possibly due to the fact that different types of blood cells express this transcript, as previously reported (Williams et al., 1999).

Regulation of the Transcripts Under Inflammatory Conditions

To determine whether the expression of the nine genes identified as being expressed in microglia are modulated during inflammatory processes, brain sections of mice treated with cuprizone or LPS were analyzed by *in situ* hybridization. Cuprizone is a neurotoxicant that induces demyelination and microgliosis when given chronically (Matsushima and Morell, 2001), whereas LPS is a bacterial endotoxin that stimulates the transient expression of inflammatory genes after a single injection (Rivest, 2003). In mice treated for 5 weeks with cuprizone, stronger hybridization signals were detected in demyelinated areas of the brain compared to gray matter regions such as the cerebral cortex (Figs. 8a–t). In demyelinated regions, the transcripts colocalized with galectin-3 (e.g. in Fig. 8y), a marker for activated microglia (Reichert and Rotshenker, 1999). In contrast, no increase in their expression was noted 3 and 6 h after LPS injection, except for GPR84, whose regulation has been examined in greater depth in another study to be published separately (Bouchard B, Pagé J, Bédard A, Tremblay P, and Vallières L, unpublished observations). As a positive control, we hybridized adjacent sections with a probe for TNF mRNA, and many signals were detected throughout the brains of all the animals (data not shown), confirming that LPS was active and able to induce microglia activation.

To demonstrate that the increases in signal intensities observed after cuprizone treatment were due, at least in part, to increases in gene expression and not only to increases in microglia density, hundreds of hybridization signals were analyzed at the single-cell level by optical density readings. Although this method does not provide an exact estimate of gene expression, it permits a meaningful relative comparison of the intensity of hybridization signals found in different brain regions. As shown in Fig. 8z, the results confirm that all the genes examined were modestly upregulated in microglia located in demyelinated areas compared to those of the cerebral cortex. Overall, our study suggests that these genes play potentially important roles in microglia biology and neuroinflammatory processes.

DISCUSSION

Although microglia, monocytes, and peripheral macrophages share a common origin and exert similar functions, each of these cells has distinctive properties and morphologies (Aloisi, 2001; Ladeby et al., 2005b; Rock et al., 2004). What may account for these differences at the level of gene expression is an issue that remains largely unexplained. Many genes, such as those coding for MHC antigens and CD45 (Aloisi, 2001; Sedgwick et al., 1991), have been shown to be downregulated in microglia compared to their peripheral counterparts and other cerebrospinal macrophages, supporting the general view that microglia are maintained in a relatively resting state (Aloisi, 2001; Ladeby et al., 2005b). In contrast, very few genes have been found, thus far, to be predominantly expressed in microglia, with TREM2 being a typical example (Cella et al., 2003; Schmid et al., 2002). The present work not only reveals the existence of other such genes, but provides comprehensive gene expression profiles that can help investigate the monocyte-macrophage system and its relation to the CNS.

Previous gene profiling studies on microglia were typically designed to identify genes induced by inflammatory stimuli in cultured microglia (Albright and Gonzalez-

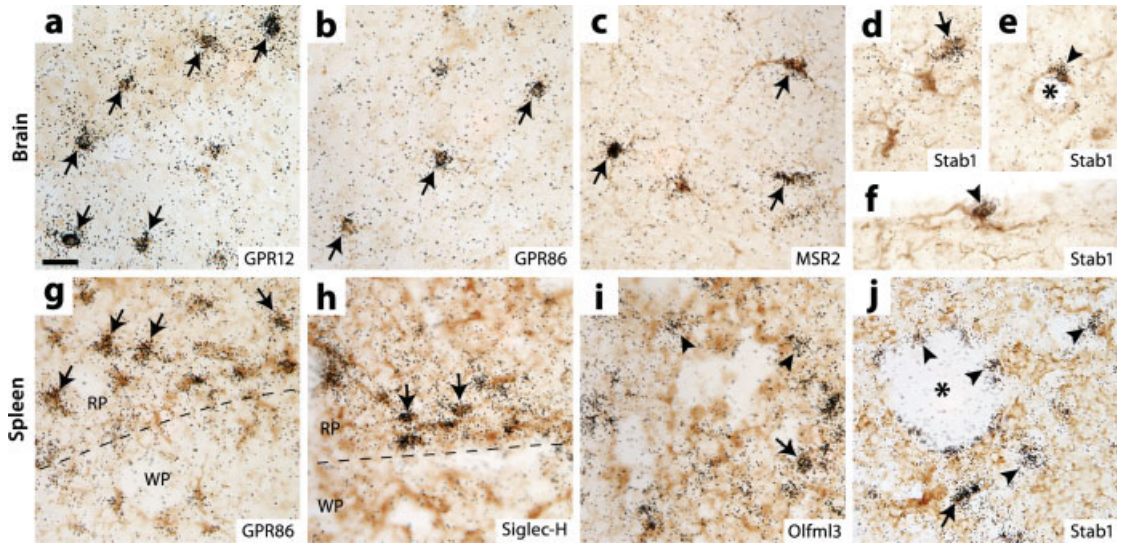

Fig. 6. Transcripts confirmed to be expressed in microglia and spleen macrophages by a combination of *in situ* hybridization and immunohistochemistry. (**a**–**f**) Photomicrographs of brain sections showing double labelings for different transcripts (black grains, *in situ* hybridization) and the pan-monocytic marker Iba1 (brown, immunoperoxidase staining). Note that all transcripts were found in parenchymal microglia (a–d; arrows), and that Stab1 mRNA was found, in addition, in perivascular (e) and leptomeningeal (f) macrophages (arrowheads). (**g**–**j**) Photomicrographs of spleen sections showing double labelings for different transcripts and Iba1. Note that GPR86 and Siglec-H were expressed only by $Iba1^+$ macrophages (arrows), whereas Olfml3 and Stab1 were also expressed by $Iba1^-$ cells (arrowheads). Abbreviations: RP, red pulp; WP, white pulp. *Blood vessel lumen. Scale bar: a–j, 20 μm.

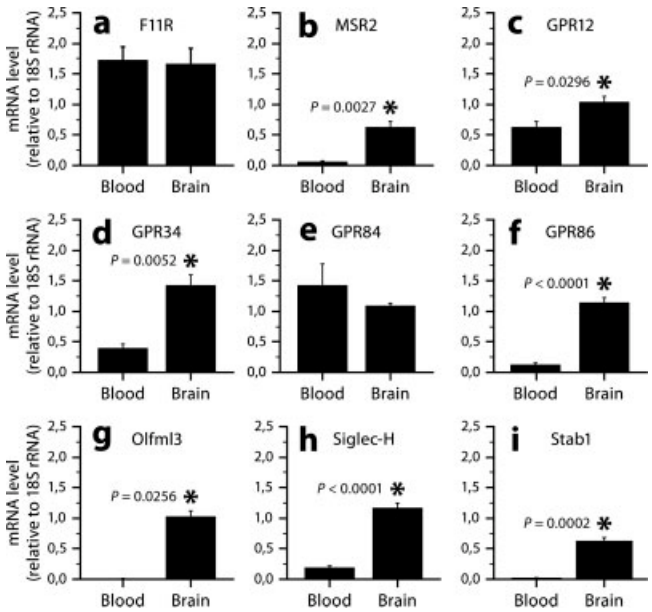

Fig. 7. Transcripts differentially expressed in the brain compared to blood. (**a**–**i**) Quantitative RT-PCR analysis revealed that mRNA levels of all transcripts, except F11R and GPR84, were higher in the brain than in Ficoll-purified leukocytes. Data are expressed as ratio to 18S rRNA (mean ± SE). *Student's *t* test, $P < 0.05$.

Scarano, 2004; Baker and Manuelidis, 2003; Donnou et al., 2005; Duke et al., 2004; Mahe et al., 2001; Moran et al., 2004; Rock et al., 2005; Schmid et al., 2002; Thomas et al., 2006). In contrast, the present study mainly aimed to identify constitutively expressed genes and used freshly purified cells to avoid phenotypic changes in culture. As expected, all the genes that we confirmed as being expressed in microglia *in vivo*, except GPR84, were transcriptionally active under basal conditions and can thus be considered as constitutive. Nevertheless, our results indicate that all these genes can be modestly upregulated during chronic stimulation with cuprizone, but not during acute endotoxemia. These observations suggest that the corresponding proteins are not primarily regulated at the transcriptional level, but that an increase in transcription is not excluded when microglia are strongly activated over a prolonged period, perhaps to compensate for an increase in protein turnover. Whether these genes can be positively or negatively regulated by other inflammatory stimuli or therapeutic drugs is an issue that remains to be addressed.

An important question in neuroimmunology is whether there are markers that could be used to distinguish between microglia derived from newly recruited monocytes and those derived from pre-existing microglia. In this study, seven genes were potentially identified as such markers, because they were constitutively expressed in microglia and were undetectable or significantly less expressed in blood. However, all these genes were also abundantly expressed by activated microglia in regions demyelinated by cuprizone exposure. As revealed by the use of chimeric mice (McMahon et al., 2002), these regions are populated by a mixture of activated, morphologically indistinguishable microglia derived from both sources. Altogether, these observations suggest that monocytes, under the influence of molecules present in the neural environment, adopt a microglia phenotype soon after infiltration, questioning the existence of reliable markers to distinguish centrally from peripherally derived microglia.

An important contribution of the present study is the identification of several new factors involved in the biology

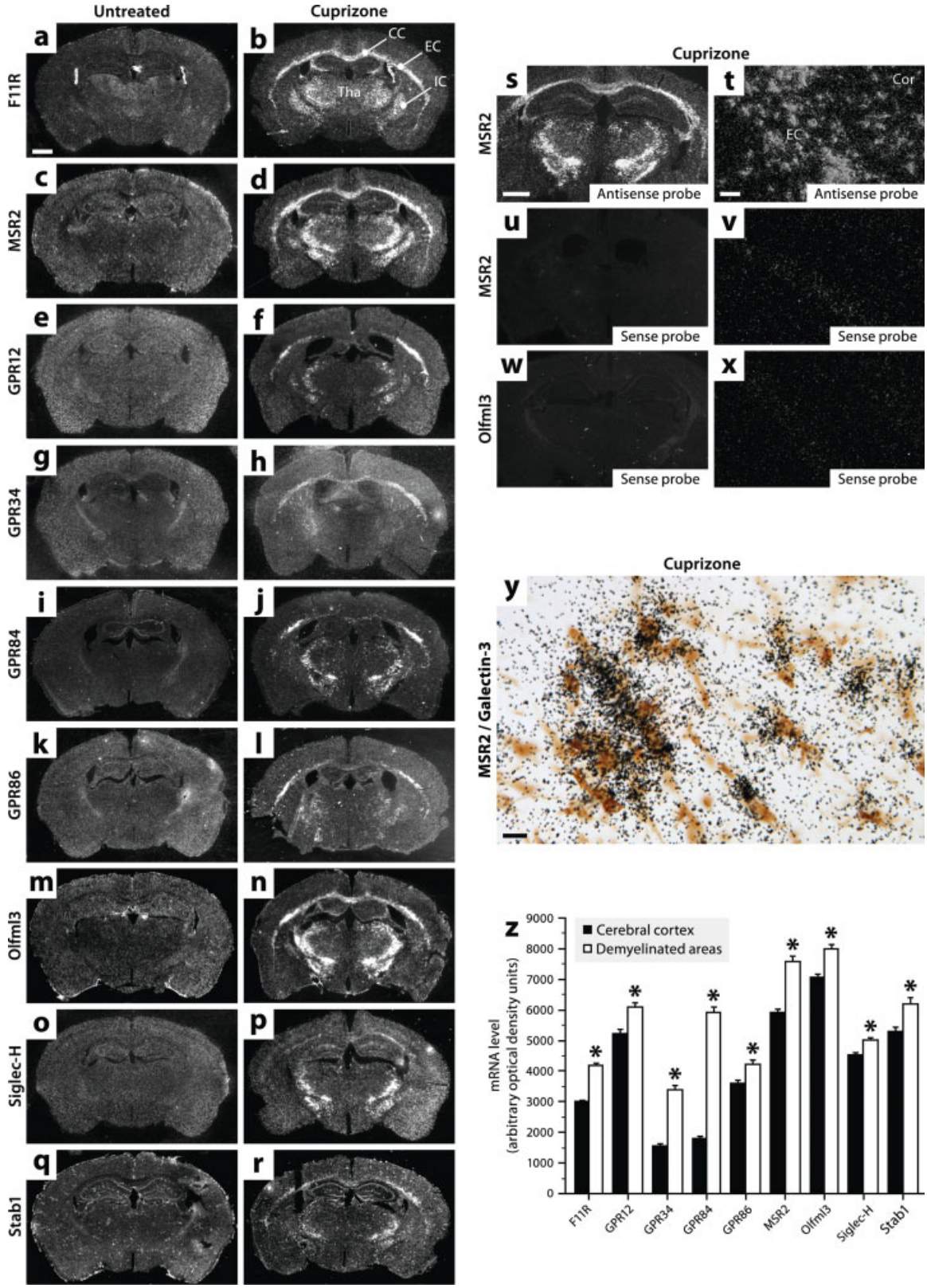

Fig. 8. Transcripts upregulated in activated microglia after cuprizone treatment. (**a**–**r**) Dark-field images of brain sections from mice treated or not with cuprizone for 5 weeks showing *in situ* hybridization signals for different transcripts. Note that expression of these transcripts was predominantly increased in the corpus callosum and other white matter regions. (**s**–**t**) Higher magnifications of hybridization signals for MSR2 mRNA. (**u**–**x**) Adjacent sections showing no signal after hybridization with sense riboprobes for MSR2 and Olfml3 mRNAs. (**y**) Photomicrograph showing microglia in the thalamus doubly labeled for MSR2 mRNA (black grains, *in situ* hybridization) and the microglial activation marker galectin-3 (brown, immunoperoxidase staining). (**z**) Optical density readings revealed that all the transcripts examined were more abundantly expressed in microglia associated with demyelinated regions (white bars) compared with those of the cerebral cortex (black bars). Data are mean ± SE ($n = 4$). *Wilcoxon rank-sum test, $P \leq 0.002$. Abbreviations: CC, corpus callosum; Cor, cerebral cortex; EC, external capsule; IC, internal capsule; Tha, thalamus. Scale bars: a–r, 250 μm; s, u, w, 1 mm; t, v, x, 50 μm; y, 10 μm.

of microglia. All these genes code for plasma membrane or extracellular proteins putatively involved in signal transduction. For example, GPR34 is an orphan receptor that exhibits structural features characteristic of the P2Y family that includes the ADP receptors GPR12 (P2Y12) and GPR86 (P2Y13), which have been implicated in microglia chemotaxis (Nasu-Tada et al., 2005). GPR84 is another orphan receptor recently discovered whose expression is strongly induced in microglia under inflammatory conditions, such as endotoxemia and experimental autoimmune encephalomyelitis (Bouchard B, Pagé J, Bédard A, Tremblay P, and Vallières L, unpublished observations). MSR2 is a scavenger receptor for which very little information is available and whose specific expression in microglia is intriguing, since scavenger receptors are normally widely expressed in monocytic cells (Murphy et al., 2005). Siglec-H is a sialic acid-binding lectin that, like TREM2, interacts with the signaling adaptor protein DAP12 (Blasius et al., 2006). Finally, the scavenger receptor Stab1, the immunoglobulin-like adhesion molecule F11R and the secreted glycoprotein Olfml3 are other molecules whose functions in the context of microglia remain to be elucidated.

It is important to note that the genes identified as being preferentially expressed in microglia compared to monocytes and spleen macrophages may also be constitutively expressed in peripheral tissues by other cell types or be upregulated under conditions not examined in the present study. For example, MSR2 has recently been identified in tumor-associated macrophages (Biswas et al., 2006), whereas F11R and GPR34 have been detected in endothelial cells (Martin-Padura et al., 1998) and mast cells (Sugo et al., 2006), respectively. In the normal brain, however, it is clear that these genes are expressed only by phagocytes and not by neural cells nor the vasculature.

In conclusion, this study reports new markers for microglia that can help not only to identify and isolate these cells, but also to clarify their distinctive properties. In future studies, it will be important to validate the differential expression of other candidate genes, to determine their role and importance in neuroinflammation (e.g., whether they are engaged in processes that are beneficial or detrimental to the nervous system), and to examine whether the encoded proteins could be targeted for therapeutic purposes. Specific inhibitors of key proteins involved in microglia function could prove more effective and less compromising than the broad-range immunosuppressants commonly used today.

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