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The dynamics of the LPS triggered inflammatory response of murine microglia under different culture and *in vivo* conditions

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Abstract

Overall, the inflammatory potential of lipopolysaccharide (LPS) *in vitro* and *in vivo* was investigated using different omics technologies. We investigated the hippocampal response to intracerebroventricular (i.c.v) LPS *in vivo*, at both the transcriptional and protein level. Here, a time course analysis of interleukin-6 (IL-6) and monocyte chemotactic protein-1 (MCP-1) showed a sharp peak at 4 h and a return to baseline at 16 h. The expression of inflammatory mediators was not temporally correlated with expression of the microglia marker F4/80, which did not peak until 2 days after LPS injection. Of 480 inflammation-related genes present on a microarray, 29 transcripts were robustly up-regulated and 90% of them were also detected in LPS stimulated primary microglia (PM) cultures. Further *in vitro* to *in vivo* comparison showed that the counter regulation response observed *in vivo* was less evident *in vitro*, as transcript levels in PM decreased relatively little over 16 h. This apparent deficiency of homeostatic control of the innate immune response in cultures may also explain why a group of genes comprising *tnf receptor associated factor-1*, *endothelin-1* and *schlafen-1* were regulated strongly *in vitro*, but not *in vivo*. When the overall LPS-induced transcriptional response of PM was examined on a large Affymetrix chip, chemokines and cytokines constituted the most strongly regulated and largest groups. Interesting new microglia markers included *interferon-induced protein with tetratricopeptide repeat (ifit), immune responsive gene-1 (irg-1) and thymidylate kinase family LPS-inducible member (tyki)*. The regulation of the former two was confirmed on the protein level in a proteomics study. Furthermore, conspicuous regulation of several gene clusters was identified, for instance that of genes pertaining to the extra-cellular matrix and enzymatic regulation thereof. Although most inflammatory genes induced *in vitro* were transferable to our *in vivo* model, the observed discrepancy for some genes potentially represents regulatory factors present in the central nervous system (CNS) but not *in vitro*.

Keywords: Microglia; Inflammation; TNF-α; CNS; Transcriptomics; Proteomics

Abbreviations: AD, Alzheimers disease; CNS, central nervous system; aCSF, artifical cerebrospinal fluid; FBS, fetal bovine serum; i.c.v., intracerebroventricular; IL, interleukin; LPS, lipopolysaccharide; MCP-1, monocyte chemotactic protein-1; MIAME, minimum information about a microarray experiment; NO, nitric oxide; PM, primary microglia; PGE-2, prostaglandin E2; TNF-α, tumor necrosis factor alpha.

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1. Introduction

Microglia are the resident macrophage-like cells of the central nervous system (CNS) with a broad role in the brain's innate immunity and inflammatory neuropathologies (Nelson et al., 2002). They show great functional plasticity when activated, with the potential to take over both the roles known for macrophages and dendritic cells in the periphery

(Santambrogio et al., 2001). Microglia are equipped with a broad range of pattern-recognition receptors of the toll-like receptor family (TLR family) to detect microbial intruders (Jack et al., 2005). Upon stimulation, TLRs trigger a signaling cascade involving MyD88 as universal upstream adapter and activation of a set of transcription factors such as NF-κB and AP-1 (Takeuchi and Akira, 2001; Jones et al., 2001). Due to the complexities of the down-stream response, the eventual response pattern triggered by TLR stimulation is very dynamic. Most work on microglial TLR signaling has been performed *in vitro*, frequently by using cell lines such as N9 (Bi et al., 2005) and BV-2 (Lee et al., 2004). Data on primary microglia (PM), isolated from brain cultures of neonatal pups (Giulian and Baker, 1986) or directly from adult mice (Baker et al., 2002), are more restricted due to the limited yield of biological material.

From studies examining individual signaling pathways, it appears that one given cell type, such as a microglia, can assume many activation states. Basic microglial gene expression has for instance been described for murine (Re et al., 2002) and rat primary cells (Duke et al., 2004) in addition to a murine cell line (Inoue et al., 1999). It has also been described how the transcriptional profile of primary rodent microglia is changed in different environments, such as after exposure to IFN-γ (Moran et al., 2004), TGF-β (Paglinawan et al., 2003), fractalkine (Leonardi-Essmann et al., 2005) or colony-stimulating factors (Re et al., 2002). For inflammatory stimulation, data can be found for human microglia (Walker et al., 2001) or the murine BV-2 (Gan et al., 2004) cell line exposed to amyloid β peptide and for primary microglia exposed to Gram-positive bacteria (Kielian et al., 2002). Data on *in vivo* responses are still scarce, but ex vivo analyses of microglia isolated from disease models are becoming more feasible. For instance a study on prion-infected mice provides valuable data that focus solely on the microglial contribution of inflammatory mediators in the model and hereby bridges *in vivo* with *in vitro* studies (Baker and Manuelidis, 2003).

We decided here to study the LPS response pattern in different biological settings. The endotoxin LPS is a ligand for TLR-4 (Hoshino et al., 1999) and by far the most frequently used model stimulus for inflammatory signaling and pharmacology experiments. The response of peripheral macrophages to this stimulus is well-described (Rosenberger et al., 2000), and at least some data exist for stimulation of acutely isolated adult microglia (Baker and Manuelidis, 2003). However a comprehensive and comparative transcriptional profiling to this stimulus, *in vitro* and *in vivo*, has to our knowledge not been carried out. Initially, we focused on 480 inflammation-related genes, analyzed by custom spotted microarray analysis, to compare the LPS response of primary microglia cultures with that of a hippocampal model for cerebral inflammation. The outcome of these studies was correlated with data from proteomics analysis and genome spanning Affymetrix chips, to evaluate the influence of the methodology, and to obtain additional information on regulations of non-inflammatory genes and proteins.

2. Materials and methods

2.1. Materials and chemicals

Tissue culture material was obtained from Nunc (Roskilde, Denmark), media, phosphate buffered saline (PBS), antibiotics and fetal bovine serum (FBS) were obtained from GIBCO (Invitrogen, Taastrup, Denmark) and LPS (*Salmonella abortus equi*) was purchased from BioCloth (Aidenbach, Germany).

2.2. Animals and in vivo experimentation

All experimental procedures were carried out in accordance with national (directive of the Danish National Committee on Animal Ethics) and international laws and policies (EEC Council Directive 86/609, OJ L 358, 1, Dec. 12, 1987; Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996). Pregnant C57BL/6J and male C57BL/6J mice (3 months of age) were purchased from M&B (Lille Skensved, Denmark).

2.3. i.c.v. injection of LPS

Adult male C57BL/6J mice were anesthetized with sevoflurane and fixed in a homemade frame. Artificial cerebrospinal fluid (aCSF), or LPS dissolved in aCSF, was administered i.c.v. in 2.5 μL into the right ventricle using a micro pump equipped with a 100 μL Hamilton syringe (VWR, Martinsried, Germany) through a 5/8 in. 25-gauge needle at a flow rate of 1.7 μL/min. The electrolyte composition of aCSF was 140 mM NaCl, 3 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 1.2 mM Na_2HPO_4, adjusted to pH 7.4. Mice were sacrificed either 4 or 16 h after the i.c.v. injection by cervical dislocation. Both hippocampi were dissected free and stored in RNAlater (Invitrogen, Taastrup, Denmark) until RNA extraction (see below). For cytokine measurements the hippocampi were rotor-stator homogenized in 500 μl of Tris buffered saline (pH 7.4) with a 1× cocktail of "Complete" protease inhibitors from Roche. The lysates were cleared at 20,000×*g*, before ELISA analysis.

2.4. Primary murine microglia culture

Microglia cultures were prepared as initially described by Giulian and Baker (1986) using the following adaptations: Pups (1–3 days postpartum) were decapitated and the cerebral hemispheres were transferred to DMEM with 20% heat inactivated FBS, supplemented with antibiotics (penicillin 100 U/ml, streptomycin 100 μg/ml). This medium was used for all work related to PM, only the FBS concentration was varied. Following removal of the meninges, the tissue was triturated using a 10 ml pipette to obtain a homogeneous cell suspension and passed through a 70 μm cell strainer (sieve). The cell suspension was plated at a density of 3 brains/185 cm^2 flask and cultured undisturbed for 7 days.

Then, new medium with reduced serum concentration (10% FBS) was added. After 7 additional days of culturing, microglial cells were selectively detached by shaking (300 rpm, 5 h). Suspended microglia were pelleted at 180×*g*, before being seeded. Purity was always >95% as determined by routine staining with FITC-labeled lectin from *Bandeiraea simplicifolia* BS-I from Molecular probes (Leiden, the Netherlands).

2.5. Standard cell incubation scheme for array (PM and BV-2) and proteomics experiments (BV-2)

All incubations were performed at 37 °C, 5% CO_2, 95% relative humidity. Suspended PM (see above) were seeded at 3 million cells/Petri dish (surface area 20 cm^2) in 3 ml medium. After 25 min of incubation, loosely adherent cells were removed by tapping the sides of the dish, followed by two washes in PBS. After overnight incubation, cells were washed once in PBS followed by addition of 3 ml medium (1% FBS). Cells were stimulated with LPS (100 ng/ml) for 4 or 16 h, and 100 μL of the supernatant was sampled for cytokine analysis before the cells were harvested for RNA extraction.

BV-2 cells (murine microglia, kindly provided by Blasi, Perugia) (Blasi et al., 1990) were maintained in RPMI supplemented with 10% FBS and antibiotics (penicillin 100 U/ml, streptomycin 100 μg/ml). BV-2 cells were treated as described for PM except that only half the number of cells was plated. For proteomics analysis the BV-2 cells were stimulated with LPS (100 ng/ml) for 24 h, washed twice with PBS, and then lysed in 2% SDS with 0.1 M Tris (pH 8.8).

2.6. Immunohistochemistry

At both 4 h and 1, 2, 4, 7 days after i.c.v. LPS injection, animals were deeply anesthetized by intraperitoneal injection of Avertin and perfused intracardially with phosphate buffered saline (PBS, pH 7.4) followed by 4% freshly prepared paraformaldehyde. The brains were rapidly removed, immersion-fixed at 4 °C for 16 h, dehydrated, embedded in paraffin, and cut into 4-μm-thick coronal sections. Before immunohistochemical staining, sections were deparaffinized and dehydrated. For antigen retrieval, sections were digested with trypsin (0.1% trypsin Sigma T-7409) and 0.1% $CaCl_2$ in H_2O, pH 7.8) for 30 min at 37 °C. Endogenous peroxidase was inhibited with 1% H_20_2 in PBS for 10 min. Nonspecific binding was blocked by incubation with 1% BSA and 0.1% Tween in PBS for 10 min before incubation with primary antibody (rat-anti F4/80, Serotec MCAP497) 1:20 in PBS containing 1% BSA and 0.1% Tween) overnight at 4 °C. Sections were thereafter incubated with biotinylated secondary antibody (goat anti-rat, Amersham RPN1005) diluted 1:200 in PBS containing 1% BSA and 0.1% Tween for 1 h, followed by avidin–biotin enzyme complex for 1 h (ABC-Elite; Vector Laboratories). Immunoreactivity was visualized with a solution containing 0.05% 3,3′-diaminobenzidine and 0.01% H_2O_2.

2.7. Cytokine and PGE-2 determination

The murine cytokines, interleukin-1, -6, -10, -18, monocyte chemotactic protein-1 (MCP-1) and tumor necrosis factor-α (TNF-α) were measured using murine specific OptEIA™ ELISA kits from Pharmingen (Brøndby, Denmark) in MaxiSorp plates from Nunc. Prostaglandin E-2 (PGE-2) was measured using a competitive immunoassay kit, Correlate-EIA™ (Assay Designs, Ann Arbor, USA), according to the manufacturer's instruction. The kit has cross-reactivity to PGE-1 (70%) and PGE-3 (16.3%). The cytokine micro array (TranSignal Raybio Mouse Cytokine Antibody Array) was purchased from Panomics (Saltsjobaden, Sweden). Cytokine array analysis was performed according to the manufacturer's instructions. In brief, hippocampal brain homogenates equivalent to 500 μg protein from either LPS or vehicle injected animals was added per membrane and incubated at room temperature for 1 h before the detection solution was added. Prior to ligand binding, membranes were soaked in blocking solution, and the membranes were washed between incubations to remove unspecific binding. Cytokine binding was visualized with ECL catalyzed blackening of a hyperfilm (Amersham, Birkerød, Denmark). Each spot absorbance (cytokine signal) was quantified using ImagePro 4.5 software (ImageHouse, Copenhagen) and a light table.

2.8. RNA extraction and RT-PCR

Stimulated cells were washed twice with PBS and then lysed with RLT buffer. Hippocampi were transferred from the RNAlater to the RLT buffer and homogenized using the rotor-stator principle. Total RNA was extracted using the RNeasy kit from Invitrogen according to the manufacturer's protocol. Purified RNA was DNase treated using a DNA-free™ kit (Ambion, Huntington, United Kingdom) according to the manufacturer's protocol. Total RNA (1 μg) was reverse transcribed with TaqMan RT-Reagent (Applied Biosystems, Nærum, Denmark), using random hexamers in a 100 μL reaction on a PTC-200 DNA Engine Thermal Cycler (VWR international, Albertslund, Denmark), using a program of 10 min annealing at 25 °C, 30 min reverse transcription at 48 °C, 5 min inactivation at 95°. The cDNA was quantified using the SYBR GREEN® PCR Master Mix kit (Applied Biosystems, Nærum, Denmark). Each reaction contained 2.5 μL cDNA of the 100 μL RT-product, 300 nM forward and reverse primers, 12.5 μL master mix and 7 μL water in a total volume of 25 μL. PCR amplification was run in a 96 well experimental plate format on an iCycler Thermal Cycler equipped with iCycler Optical System (BIORAD, Hercules, CA). The program set-up was 10 min at 95 °C, 40 cycles of 15 s at 95 °C/1 min at 60 °C. A melting curve was obtained to verify the measured signal and the product was run on a 2.5% agarose gel to verify the presence of only one amplified band. Quantification was performed as follows: Using the iCycler data analysis software (Bio-Rad), the threshold cycle (C_T) was

Chemokine and cytokine related	PM Neuroflame 4 h	PM Neuroflame 16 h	PM Affy 4 h
Caspase 1, casp1	2.2	1.7	1.9
Caspase 4/caspase 11	8.0	4.3	5.7
Chemokine (c-c motif) ligand 12, MCP-5, Scya12, Ccl12	33.2	18.7	93.2
Chemokine (c-c motif) ligand 2, MCP-1, Scya2, Ccl2	21.1	6.8	30.7
Chemokine (c-c motif) ligand 3, MIP-1alpha, Ccl3	23.3	10.3	4.7
Chemokine (c-c motif) ligand 4, MIP-1 beta, Ccl4	37.6	4.1	11.6
Chemokine (c-c motif) ligand 5, RANTES, Ccl5	25.1	2.6	13.0
Chemokine (c-c motif) ligand 7, MCP-3, Scya7, Ccl7	12.0	6.0	26.0
Chemokine (c-x-c motif) ligand 11, IP9, H174, ITAC, Cxcl11	4.0	7.0	31.8
Chemokine (c-x-c motif) ligand 2, MIP-2, Scyb2, MIP-2a, Cxcl2	92.6	35.4	38.7
Chemokine (c-x-c motif) ligand 5, Cxcl5	1.5	7.8	
Chemokine (c-x-c motif) ligand 9, Mig, Scyb9, crg-10, Cxcl9	27.8	6.8	87.9
Chemokine orphan receptor 1, Rdc1,	8.3	1.6	-
Chemokine (c-c motif) receptor 1, Ccr1	1.3	2.6	
Chemokine (c-c motif) receptor-like 2, Ccri2,	9.4	1.6	
Chemokine (c-c) receptor 2, Ccr2,	3.2	1.3	
Csf2 (granulocyte-macrophage) colony stimulating factor 2	12.3	2.0	53.9
Endothelin 1	48.7	6.3	35.8
IL12Rb2, Interleukin 12 receptor, beta 2	10.5	2.8	
IL1b, Interleukin 1 beta	141.9	10.8	41.8
IL-1ra, Interleukin 1 receptor antagonist	1.7	5.2	19.0
IL23a, p19, Interleukin 23, alpha subunit p19	64.3	1.0	81.2
Interleukin 15	18.4	1.9	14.1
Interleukin 18	3.4	2.6	3.9
Interleukin 6	56.7	24.5	266.1
IRAK3, Interleukin-1 receptor-associated kinase 3	4.2	5.5	
NGFb, Nerve growth factor, beta	0.7	2.3	-
Oncostatin M	2.1	2.0	
OsM-R, Oncostatin m receptor	4.7	1.6	-
SOCS3, Suppressor of cytokine signaling 3	28.6	19.9	37.2
Bacterial and viral defence			
C-3, Complement component 3	3.6	1.1	6.0
CAT2, Solute carrier family 7, member 2	7.5	5.1	16.8
Cd14 antigen	3.8	3.1	
Fc receptor, IgG, low affinity	2.9	7.7	2.7
GBP2, Guanylate nucleotide binding protein 2	28.8	3.0	19.4
Ifit1, Ifi56, Interferon-induced protein with tetratricopeptide repeats 1	23.2	6.9	22.5
Ifit3, Ifi49, Interferon-induced protein with tetratricopeptide repeats 3	25.2	3.6	15.6
IFNb, Interferon beta	12.0	0.8	45.2
IKBE nuc. fac. of kap. light polypep. gene enhan. in B-cells inhib., epsi.	1.8	2.1	12.2
Immunoresponsive gene 1	35.6	2.0	40.1
I(Kappa)B(alpha), NFkBia	4.8	4.8	3.8
iNOS, Nitric oxide synthase 2, inducible, Nos2	1.2	2.3	3.8
Interferon-inducible GTPase	17.9	3.4	42.8
Irf1, Interferon regulatory factor 1	6.8	1.4	9.3
Lipocalin 2	1.7	3.3	3.2
MSR1, Macrophage scavenger receptor 1	1.5	4.9	
Myeloperoxidase	3.0	2.1	
p49/p100, NFkB2	3.7	2.5	6.6
p5/p105, NFkB10	3.6	2.4	3.9
Serum amyloid a 2, SAA2	4.1	2.5	
SOD2, Superoxide dismutase 2, mitochondrial	7.1	6.2	4.8
Superoxide dismutase 1, soluble	2.5	1.8	
SAA3, Serum amyloid a 3	3.7	1.5	4.3
Toll-like receptor 1	2.3	3.5	5.1
Toll-like receptor 2	7.0	2.1	5.4
Glycoprotein 49a, gp49a	2.2	0.9	
Adaptive immunity			
Cd209a antigen	3.9	1.4	
Cd86 antigen cd86	2.9	2.6	3.0
H-2l q gene for class 1 MHC glycoprotein	1.8	4.3	
Proteasome (prosome, macropain) 28 subunit, beta	2.1	3.2	2.6
Psmb8, Lmp7, Proteosome (prosome, macropain) subunit, beta type 8	2.9	2.6	3.9
Psmb9, Proteosome subunit, beta type 9	2.2	1.7	4.5

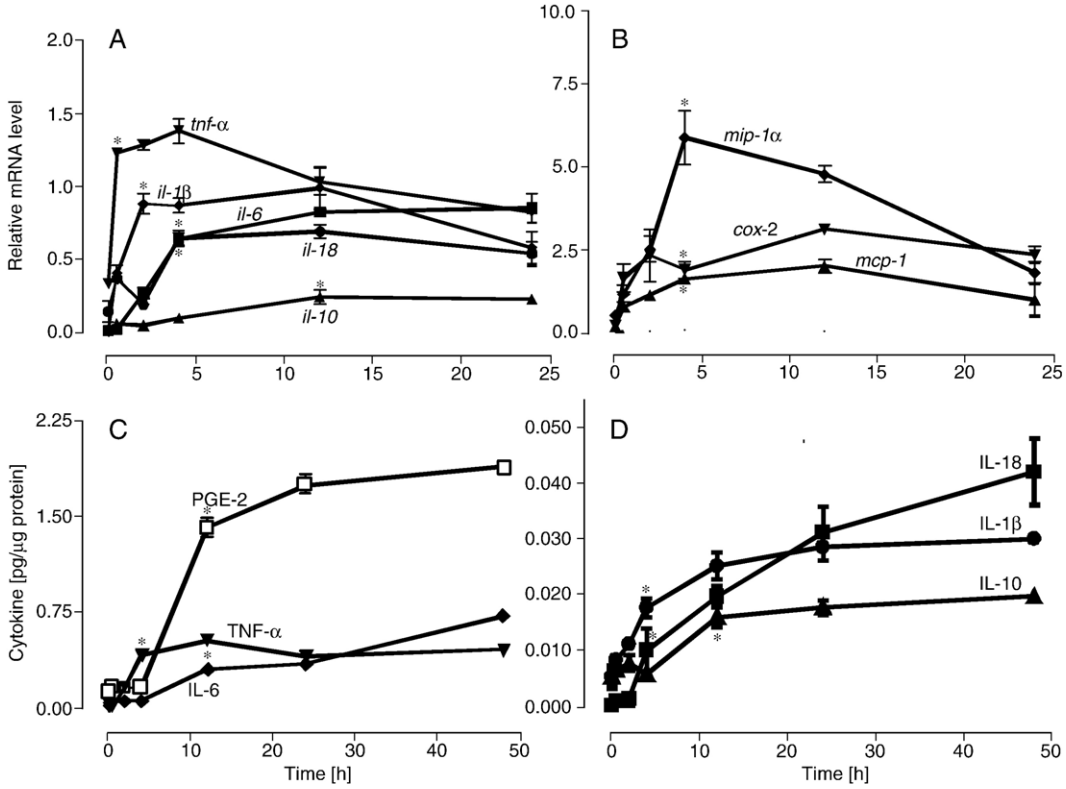


Fig. 2. Correlation of transcriptional changes and cytokine expression in primary microglia exposed to LPS. Primary microglia were stimulated with LPS (100 ng/ml) for various times. At each time point, culture supernatants were used for determination of inflammatory mediators (by ELISA), and mRNA was prepared from cells for quantification of gene expression (by RT-PCR). A, B. Data are presented as relative mRNA levels standardized to the amount of *gapdh* mRNA. C, D. Data are presented as mediator concentration (in pg) relative to the amount of protein in the cells of the corresponding culture well (in µg). One-way ANOVA analysis was used to test if gene expression/cytokine production was induced above control levels after LPS stimulation. Experiments were performed three times in triplicates of which one representative outcome is presented (mean±SD). * indicates first time point, at which data were significantly different from control ($p<0.05$) in all three experiments.

determined for each sample. C_T was defined as the cycle at which the level of fluorescence increased linearly. Each sample was run in two reactions, one with the primer set of interest and one with a GAPDH primer set. The mRNA levels were compared among different groups using the delta–delta method as described by Biocompare (http://www.biocompare.com/pcr/tutorial/qpcr/static/delta.asp). Primers were designed using DNA-star software package (DNASTAR inc, Madison, USA) and scrutinized to minimize secondary structures, self-complementarity, optimal melting temperature, etc. All primers were analyzed using BLASTn to ensure primer specificity for the gene of interest (www.ncbi.nlm.nih.gov/BLAST/). Primers used were *gapdh* sense (NM_008084): 5′-TGC ACC ACC AAC TGC TTA G-3′, anti-sense: 5′-GGA TGC AGG GAT GAT GTT C-3′. *tnf-α* sense (NM_013693): 5′-CTA TGG CCC AGA CCC TCA CAC TCA-3′, anti-sense: 5′-CAC TCC AGC TGC TCC TCC ACT TG-3′. *mcp-1* sense (NM_011333): 5′-CAT GCT TCT GGG CCT GCT GTT C-3′, anti-sense 5′-CCT GCT GCT GGT GAT CCT CTT GTA G-3′. *il-1α* sense (NM_002983): 5′-CCC GAC TGC CTG CTG CTT CTC-3′, anti sense: 5′-GAT CTG CCG GTTTCT CTT AGT CA-3′. *socs-3* sense (NM_003955): 5′-ACT TGT TTG CGC TTT GAT TTG GTT T-3′, anti sense: 5′-GTT GGG CAG TGG GAG TGG TTA TTT-3′. *il-6* sense (NM_000600.1): 5′-GGA GCC CAC CAA GAA CGA TAG TCA-3′, anti-sense: 5′-GAA GTA GGG AAG GCC GTG GTT GTC-3′. *schlafen-1* sense (NM_011407,1): 5′-TGG GGA TGT TGG AAG GGT TTT T-3′, anti-sense 5′-GGG TTG GGG GTG GGT TTT TTA C-3′.

Fig. 1. Up-regulation of genes by LPS in primary microglia. Primary murine microglia (PM) were stimulated with LPS (100 ng/ml) for 4 or 16 h, before isolation of total RNA. Transcriptional changes were examined by chip analysis using Neuroflame (NF) and Affymetrix (Affy) arrays. Genes up-regulated significantly in PM on NF arrays were selected for display and sorted according to gene ontology classes. Regulations for these genes are also displayed for PM analyzed on an Affymetrix chip. All genes regulated for three inflammation/immunity-relevant classes are shown here as examples. Gene induction was considered significant when exceeding a ratio ≥1.8 in 3 out of 4 Neuroflame hybridizations (average ratio displayed). For the Affymetrix hybridization biological material was pooled from 16 independent experiments and analyzed by one Affymetrix chip per condition. A ratio was then calculated using GeneSifter RMA analysis. No shading indicates non-regulated genes. Light gray shading indicates up-regulations of 1.8–5 fold. Dark gray shading indicates up-regulations greater than 5-fold. Empty fields indicate that the gene was not spotted on the Affymetrix array. "–"=more than 1.8 fold down-regulated.

tnf receptor associated factor-1 sense (NM_009421,1): 5′-GCC AGC CTC CGA AGC CAG C-3′, anti-sense 5′-GGG CGG TAG CGT CCT TGG G-3′. *il-23* (p19) sense (NM_031252,1): 5′-CAT GCA CCA GCG GGA CAT ATG-3′, anti-sense 5′-CAG ACC TTG GCG GAT CCT TTG-3′. *endothelin-1* (NM_010104): 5′-CTT GTT CAG ACG GGC AGG ACC A-3′, anti-sense 5′-CCC GCC CTG CTG TGG AAT C-3′. *oxidized low density lipoprotein receptor-1* (NM_138648): 5′-GCC TCC TGT TGC TGC ATG AAA G-3′, anti-sense 5′-CTC GGA CGA GCT TTG CCT TTG-3′. *ifnβ* fibroblast sense (NM_010510,1): 5′-CTT CTC CAC CAC AGC CCT CTC C-3′, anti-sense 5′-CCA CCC AGT GCT GGA GAA ATT G-3′.

2.9. Transcript analysis by oligonucleotide hybridization analysis (Neuroflame)

A list of mouse cytokines, apoptotic mediators, and inflammatory factors was compiled. For each of these genes one oligonucleotide (40–50mer) was designed by MWG (MWG, Ebersberg, Germany) using their proprietary Oligo4array software and CodeSeq database, which selects the oligos preferentially from the 3′-region of each coding sequence. Furthermore, each oligomer was scrutinized to meet physicochemical parameters (like melting temperature, self-complementarity, secondary structure, etc.) and extensively tested to minimize cross-hybridization to other sequences of the mouse genome *in silico*. All oligos were synthesized using MWG's HPSF technology followed by MALDI-TOF quality control.

The oligomeres were spotted onto activated glass slides (Pan Epoxy, MWG or CodeLink, Amersham) using a 417 Affymetrix (ring and pin) spotter. Sample preparation and labeling was carried out as described by Joseph DeRisi (www.microarrays.org/protocols.html), a protocol, derived from Hughes et al. (2001). In brief, 12–15 μg of total RNA was reverse transcribed using random hexameres, dT16 primers and Superscript II reverse transcriptase (Invitrogene), incorporating amino-allyl dUTP into the 1st strand cDNA. After the cDNA synthesis, the remaining RNA was hydrolyzed and after a clean-up step (Microcon-30 spin filters, Millipore), Cy-3 or Cy-5 dye esters, respectively, were coupled to the cDNA samples. Excessive dye and buffer were removed with QiaQuick PCR purification columns (Qiagen) and the eluates were concentrated with Microcon-30 spin filters. The hybridization mixture contained the Cy-labeled cDNAs in hybridization buffer (50%

A

LPS induced regulation of proteins (BV-2)	Protein ratio	Identification	cDNA: BV-2 Neuroflame ratio (4/16 h)	cDNA: PM Affymetrix ratio (4 h)	MW [kD]	pI	Protein bank ID
immune-responsive protein 1 (IRG-1)	>10	s	8.3/11.0	40.1	57	7.0	P54987
immune-responsive protein 1 (IRG-1)	>10	s	8.3/11.0	40.1	57	7.1	P54987
interferon-induced protein with tetratricopeptide repeats-3 (IFIT-3)	>10	s	3.0/9.7	15.6	48	5.8	NP_034631
superoxide dismutase-2 (SOD-2)	2.1	s+p	4.0	4.8	22	7.5	NP_038699
aldo-keto reductase family-1, member A4	2.0	s+p	-	1.5	37	7.2	NP_067448
peroxiredoxin-1	3.7	s+p	1.0/1.3	1.5	22	8.6	P35700
peroxiredoxin-1	2.1	s+p	1.0/1.3	1.5	22	8.4	P35700
vimentin	>10	s	0.8/1.0	1.3	27	4.9	p20152
elongation factor-2 (EF-2)	1.5	s+p	-	1.2	102	6.8	P58252
gluthatione S-transferase	1.7	s+p	-	1.2	26	8.1	CAA72413
prohibitin	2.1	s+p	-	1.0	30	5.7	CAI24279
nudix-type motif 5	<0.1	s	0.9/0.9	0.9	34	5.4	NP_058614
phosphatidylinositol-4-phosphate 5-kinase type 1 alpha (PIP5K)	7.7	s+p	1.0/1.0	1.0	20	5.6	NP_032872
similar to glyceraldehyde-3-phosphate	3.8	p	-	-	45	7.5	AAH85275
unnamed protein product similar to trypsinogen	2.4	s	-	-	11	6.6	CAA24581

B

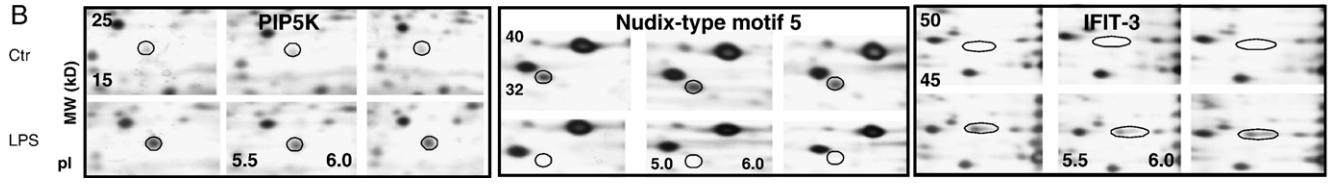


Fig. 3. Proteomics analysis of LPS-stimulated BV-2 cells. BV-2 cells were stimulated for 24 h with saline or LPS (100 ng/ml) before preparation of protein samples, and subsequently separated on 2D gels. For spot quantification, samples were run on triplicate silver gels (s), or they were analyzed by the proteotope (p) method (differential labeling with iodine-isotopes, remixing before the 2D run and ratio-imaging of each spot). Differentially expressed proteins were identified by mass spectrometric sequencing. A. Total protein from 4 independent biological experiments was pooled and run in triplicates. All statistically significantly regulated proteins identified are shown with their regulation factor (protein ratio), the method of identification and their gel coordinates. The table also indicates the regulation factors of their cognate mRNAs as determined by chip analysis using Neuroflame (BV-2) or Affymetrix (primary microglia). The data for SOD-2 on Neuroflame are for PM (non-regulated in BV-2). "–" symbolizes absence of a gene on the array. B. Three examples of regulated proteins and the corresponding silver-stained gels with coordinates of molecular weight (MW) and isoelectric point (pI) indicated at the edges of each gel clip: phosphatidylinositol-4-phosphate 5-kinase (PIP5K), nudix-type motif 5, and interferon-induced protein with tetratricopeptide motifs-3 (IFIT-3).

Fold down-regulation of extracellular matrix regulating genes by LPS		
biglycan	13.5	BC019502
chondroitin sulfate proteoglycan 2	13.5	BI692925
connective tissue growth factor (Ctgf)	22.7	NM_010217
connective tissue growth factor related protein WISP-1 (Wisp1)	8.2	AF100777
decorin (Dcn)	5.1	NM_007833
fibrillin 1 (Fbn1)	11.9	NM_007993
fibulin 2	6.3	NM_007992
FVBN collagen pro-alpha-1 type I chain	89.7	gb:U08020
laminin B1	5.1	M15525
nidogen 1 (Nid1)	27.7	NM_010917
procollagen C-proteinase enhancer protein	68.5	NM_029620
procollagen lysine, 2-oxoglutarate 5-dioxygenase 2	18.6	NM_008788
procollagen, type I, alpha 2	34.2	NM_011961
procollagen, type III, alpha 1	48.2	NM_007743
procollagen, type IV, alpha 1	26.0	NM_007742.2
procollagen, type IV, alpha 6	26.1	gb:J04694
procollagen, type IX, alpha 3	35.9	NM_009936
procollagen, type V, alpha 1	44.8	NM_015734
procollagen, type V, alpha 2	31.2	NM_007737
procollagen, type V, alpha 3	6.6	NM_016919
procollagen, type VI, alpha 1	10.8	NM_009933
procollagen, type XI, alpha 1	62.4	NM_007729
secreted acidic cysteine rich glycoprotein	13.2	NM_009242
Fold down-regulation of extracellular matrix protective genes by LPS		
Oserine (or cysteine) proteinase inhibitor, clade F	24.2	NM_011340
serine (or cysteine) proteinase inhibitor clade H	33.2	NM_009825
serine protease inhibitor 17	13.2	NM_009250
serine protease inhibitor 2-2 (Spi2-2)	19.3	NM_009252
serpine2	13.9	NM_009255
serpinh1	13.0	NM_009825
similar to tissue inhibitor of metalloproteinase 1	13.0	NM_011593
similar to tissue inhibitor of metalloproteinase 3	35.8	NM_011595
tissue inhibitor of metalloproteinase 2	4.9	NM_011594
tissue inhibitor of metalloproteinase 3	15.4	NM_011595
tissue inhibitor of metalloproteinase 4	6.7	NM_080639
Fold up-regulation of matrix degrading factors		
MMP-13, matrix metalloproteinase 13, collagenase 3	9.5	NM_008607

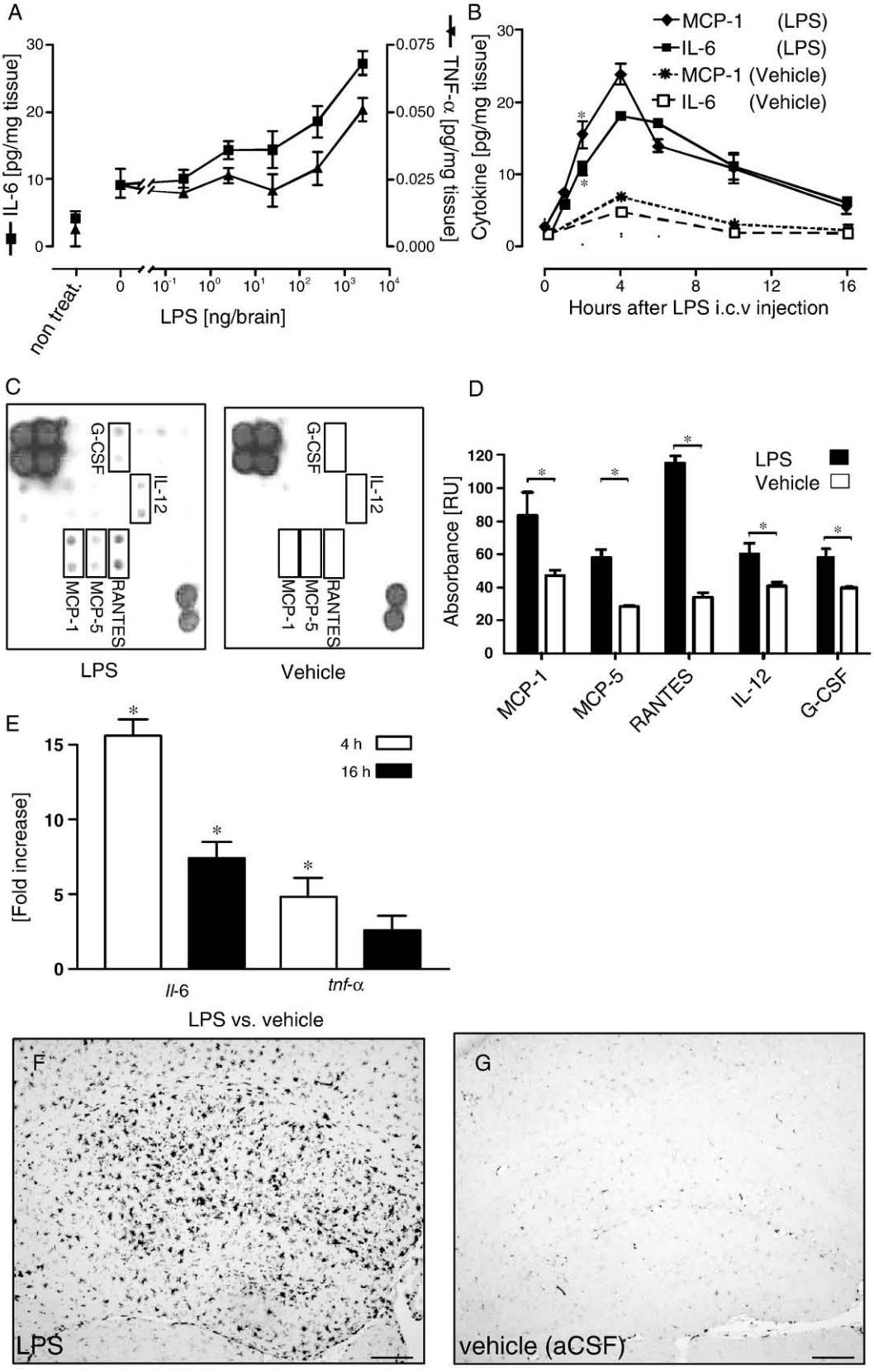
Fig. 4. LPS regulated expression of genes affecting the extracellular matrix. Primary microglia were stimulated with LPS for 4 h. Hereafter total RNA was pooled from 16 independent experiments and analyzed by one Affymetrix chip per condition and a ratio was calculated using GeneSifter RMA analysis. Genes regulated >5-fold were selected. From these, genes involved in extra-cellular matrix re-modeling were listed together with their regulation factors.

formamide, 6× SSC, 5× Denhardts, 0.5% SDS and 50 mM sodium phosphate, pH=8) and was denatured for 5 min before incubation on the slides for 16 h at 42 °C. Washing was carried out in three steps of increasing stringency: 2× SSC, 0.1% SDS followed by 1× SSC, 0.01% SDS and 0.5× SSC (all solutions were preheated to 30 °C). Finally, each slide was spun dry and scanned in a 428 Affymetrix confocal laser scanner at three different intensities (photo multiplier gains).

The micro-arrays were analyzed using ImaGene 4.2 (BioDiscovery) for spot location, array alignment and background subtraction. Signal intensities for individual spots were adjusted for local background. Microsoft Excel was used for further statistical analysis of the ImaGene output files, e.g. Cy3/Cy5 ratio normalization was carried out by multiplying each ratio value with a scaling factor, which was defined as the ratio of the overall signal intensity of the Cy5 versus Cy3 channel (Knudsen, 2002). Each microarray experiment was performed at least twice independently. To further account for bias introduced by dye bleaching or labeling, each experiment was carried out as dye-swap experiment with the resulting ratio value being the arithmetical mean from two slides of opposite labeled sample pairs.

2.10. Transcript analysis by oligonucleotide hybridization analysis (Affymetrix)

Affymetrix array expression analysis was performed according to the Affymetrix expression analysis manual at the Swegene facility in Lund, Sweden. Briefly, double-stranded cDNA was synthesized from total RNA and an *in vitro* reaction was performed to produce biotin-labeled cRNA from the cDNA. The cRNA was fragmented and hybridized to Affymetrix GeneChips (MG-430 2,0) and a ratio was calculated for comparison of the two conditions (LPS *vs.* vehicle) using GeneSifter (RMA analysis). GeneSifter is available at http://www.genesifter.net/web/. Both Neuroflame and Affymetrix data are filed according to the MIAME standard with the accession number E-MEXP-420 and are


A
IL-6 [pg/mg tissue]
TNF-α [pg/mg tissue]
non treat.
LPS [ng/brain]
B
MCP-1 (LPS)
IL-6 (LPS)
MCP-1 (Vehicle)
IL-6 (Vehicle)
Cytokine [pg/mg tissue]
Hours after LPS i.c.v injection
C
G-CSF
IL-12
MCP-1
MCP-5
RANTES
LPS
Vehicle
D
LPS
Vehicle
Absorbance [RU]
MCP-1
MCP-5
RANTES
IL-12
G-CSF
E
4 h
16 h
[Fold increase]
Il-6
tnf-α
LPS vs. vehicle
F
LPS
G
vehicle (aCSF)

A

LPS up-regulated genes *in vivo* and *in vitro* across platforms

	i.c.v (4 h)		i.c.v (16 h)		PM 4 h		PM 16 h	
	Neuroflame	Affymetrix	Neuroflame	Affymetrix	Neuroflame	Affymetrix	Neuroflame	
Bcl2A1a	2.7				10.0	4.2	4.5	NM_009742
Caspase 4/ caspase 11	2.3	2.5			8.0	5.7	4.3	NM_007609
Ccaat/enhancer binding protein (c/ebp), delta	1.9	2.6				2.2.	2.9	NM_007679
Cd14 antigen, cd14	2.1	3.4	1.9				3.1	NM_009841
cdkn1a, Cyclin-dependent kinase inhibitor 1a (p21)	2.0	2.4					1.9	NM_007669
Chemokine (c-c motif) ligand 12, MCP-5, Scya12, Ccl12	2.1	3.3			32.0	93.2	14.0	U50712
Chemokine (c-c motif) ligand 4, MIP-1 beta, Ccl4	3.7	2.5	2.8		37.6	11.6	4.1	NM_013652
Chemokine (c-c motif) ligand 5, RANTES, Ccl5	4.4	4.9	2.4		25.1	13.0		NM_013653
Chemokine (c-x-c motif) ligand 1, Cxcl1	4.3		3.2		45.1	45.7	17.5	NM_008176
Chemokine (c-x-c motif) ligand 11, Cxcl11	2.2	5.5			4.0	31.8	7.0	NM_019494
Chemokine (c-x-c motif) ligand 2, MIP-2, Scyb2, MIP-2a , Cxcl2	8.6	12.8	4.8		92.6	38.7	35.4	NM_009140
Chemokine (c-x-c motif) ligand 9, Cxcl9	3.3	3.1			27.8	87.9	6.8	XM_2897 86
Chi3l1, Chitinase 3-like 1	2.1	2.8	2.1					NM_007695
Cytochrome p450, family 2	2.3				17.1		6.5	NM_007815
GBP2, Guanylate nucleotide binding protein 2	2.5	4.1			28.8	19.4	3.0	NM_010260
Interferon-induced protein with tetratricopeptide repeats 1/3*	3.0	3.0			23.2	22.5	6.9	NM_008331
Interferon-inducible GTPase	2.6	4.1			17.9	42.8	3.4	NM_021792
Interleukin 12 receptor, beta 2	2.8				10.5		2.8	NM_008354
Interleukin 6	3.7	11.7			56.7	266.1	24.5	NM_031168
Lipocalin 2			1.9			3.2	3.3	XM_1301 71
MMP3, Matrix metalloproteinase 3	3.7	4.7			5.3		11.9	NM_010809
Psmb8, Lmp7, Proteosome subunit, beta type 8	2.1	2.2				3.4		XM_2898 58
ptx3, pentaxin related gene	2.5	4.9	2.6			-	7.0	X83601
Serum amyloid a 2, SAA2	3.7	1.9			4.1			NM_011314
Serum amyloid a 3, SAA3	3.3	4.5				4.3		NM_011315
SOCS3, Suppressor of cytokine signaling 3	2.6	3.2			28.6	37.2	19.9	BC052 031
TDK1, Thymidylate kinase family lps-inducible member	5.5	3.0			45.2	16.6	3.4	NM_020557
Toll-like receptor 2	1.9				7.0	5.4		BC014 693
Zinc finger protein 36	2.4	2.6			6.7			XM_2826 39

Fig. 6. LPS regulated genes *in vivo*. Mice were injected i.c.v. with LPS (2.25 μg/brain) or vehicle. After 4 or 16 h total hippocampal RNA was purified and expression-profiled on arrays. All genes significantly regulated on the Neuroflame array are listed. The data columns indicate the ratio of up-regulation. For purposes of easier comparability, the table also lists the data obtained *in vitro* from primary microglia (PM), as well as the data obtained for the different systems using the Affymetrix array. Biological material was obtained from at least two independent cell or animal experiments ($n=6$ animals/group) and was analyzed by 4 independent chip hybridizations. Neuroflame regulations were considered significant if a gene was regulated ≥1.8 fold in 3 out of 4 hybridizations. Light grey shading indicates regulations of 1.8–5 fold. Dark grey shading indicates regulations greater than 5-fold. *Data displayed are for *ifit-1*, but *ifit-3* up-regulation was confirmed *in vivo* by PCR and *in vitro* by chip.

freely available at http://www.ebi.ac.uk/miamexpress/login.html.

2.11. Array statistics (Neuroflame and Affymetrix)

2.11.1. Neuroflame

Two independent biological experiments (6 animals per condition) were performed for each time point (4 and 16 h). Total RNA extracted from a group (aCSF or LPS treated) was pooled before hybridization. Since each experiment included a dye-swap a total of 4 hybridizations (data points) were obtained per time point. Genes regulated ≥1.8 fold (up or down) in 3 out of 4 hybridizations were considered regulated but only if they had a signal intensity more than 5 fold above background.

Equivalent numbers of hybridizations and threshold values were used to identify gene regulations in BV-2 and primary microglia cells. The material for a competitive hybridization (LPS *vs*. ctr) was pooled from 8 independent experiments per condition. Neuroflame values displayed in Figs. 1 and 6 are the arithmetic means of the 4 ratios pertaining to a given experiment.

2.11.2. Affymetrix

Aliquots from the biological material used for Neuroflame hybridizations were subsequently used for Affymetrix analysis. For analysis of RNA obtained from *in vivo* experiments, 4 chips were used per time point (2 chips for aCSF and 2 for LPS). For primary microglia, the material used for Neuroflame analysis was pooled (thereby averaging 16 independent experiments) and analyzed by one chip per condition.

Threshold ratios of 1.5 and 5 were used for *in vivo* and *in vitro* experiments, respectively, and were calculated using

Fig. 5. An *in vivo* model for LPS-triggered inflammation. A. Adult male C57BL/6 mice were injected i.c.v. with increasing doses of LPS. TNF was determined after 2 h, and IL-6 after 4 h in brain homogenates by ELISA. B. Mice were injected i.c.v. with LPS (2.25 μg/animal) or vehicle. The hippocampi were collected 0–16 h after the injection and homogenised. The contents of IL-6 and MCP-1 were determined by ELISA. C. Pooled samples from B (4 h) were run on an antibody-based protein micro array detecting cytokines on duplicate spots. Intense black spots represent positive controls. The positions for positively hybridising samples are labelled with the cytokine names. D. Quantification of the samples from C by scanning. E. RT-PCR evaluation of hippocampal mRNA expression for *il*-6 and *tnf*-α at 4 and 16 h after LPS (2.25 μg/brain) injection into the right ventricle (±S.D). F, G. Hippocampal expression of the microglial activation marker F4/80 2 days post LPS injection. Length of scale bar, 50 μM. Experiments were performed three times (cytokine micro array experiment only once) and one representative outcome is shown. Group size equals 6 animals. * indicates $p<0.05$ compared to respective control.

GeneSifter (RMA analysis) software. GeneSifter is available at http://www.genesifter.net/web/. Both Neuroflame and Affymetrix data are filed according to the MIAME standard and are freely available at http://www.ebi.ac.uk/miamexpress/login.html (accession number E-MEXP-420).

2.12. Proteomics analysis

The differential and quantitative protein expression analysis was performed by the ProteoTope method (Cahill et al., 2003), which is based on radio-iodination, 2D-PAGE and high sensitivity radio imaging. In brief, small amounts of each sample were labeled with ^{125}I and ^{131}I for differential pattern control. The signals from these two isotopes were distinguished in one 2D-PAGE gel to generate a quantitative multicolor differential display of proteins. A direct comparison of integrated spot intensities for the samples run on one gel was used for further analysis. In parallel, silver stained gels were produced for spot picking and protein identification and for complementary quantification. In general, a spot was selected for further analysis if the *t*-test probability was higher than 95% and the expression ratio higher than 1.5. At that point the image quality was checked to assure that the spot was consistently detected on all images. Protein identification was based on different mass spectrometric methods. Briefly, gel plugs of selected protein spots were excised and the proteins contained in the gel plugs digested using trypsin. The resulting solution was analyzed first with a high throughput peptide mass fingerprint procedure based on MALDI-TOF-MS. For those spots where no unambiguous identification was achieved a fragment ion analysis based on LC-ESI-IonTrap-MS/MS was added (Cahill et al., 2003). For the identification of the proteins the peptide masses extracted from the mass spectra were searched against the NCBI non-redundant protein database (www.ncbi.nlm.nih.gov) using MASCOT software version 1.9 (Matrix Science, London).

3. Results

3.1. The pattern of inflammatory genes triggered by LPS in murine primary microglia

The transcriptional response of LPS-activated murine PM was evaluated using a custom spotted array (Neuroflame) capable of analyzing the expression of 480 genes (listed in Supplementary figure 1) broadly representative of inflammation and cell death pathways. The time points for analysis were chosen at 4 h to cover the primary response and 16 h to detect potential secondary responses and counter-responses. Of the 118 genes up-regulated by LPS, 43 were regulated only early and 19 only late (Fig. 1 and Supplementary figure 2). Thirty-six genes showed reduced transcription — most of them at the limit of significance and none exceeding more than a 5-fold change (Supplementary figure 3).

Among the induced genes were some that have not previously been associated with murine microglial activation (e.g. *endothelin-1, ifit-3, thymidylate kinase family lps-inducible member (tyki), guanylate nucleotide binding protein-2 (gbp-2)* and *lipocalin-2*) and we got interested in whether this discovery would hold true *in vivo* (see below). A further conspicuous finding was that the up-regulated inflammatory genes were dominated by chemokines and their receptors as the largest and most strongly regulated single class. As expected and serving as array validation, the "usual suspects" like *il-1β, il-6, gm-csf, myeloperoxidase, caspase-1, cox-2, I-kB, mmp-3, sod-2 and socs-3* were amongst the regulated transcripts, while e.g. *il-2, e-nos, n-nos* and *ifn-γ* were not regulated.

We chose the group of 62 regulated genes related to "bacterial and viral defense", "adaptive immunity", and "chemokine and cytokine-related genes" for detailed display of regulations in Fig. 1 since the regulation of inflammatory mediators was of primary interest. All other regulations are shown in Supplementary figure 2.

3.2. Time course of transcriptional and protein responses

Some key inflammatory markers detected on the Neuroflame chip (*il-1β, il-6, il-18, mip-1α, cox-2* and *mcp-1*) were chosen for analysis of the temporal profile of expression using RT-PCR. In addition *tnf-α* and *il-10* were included as well-established microglia cytokines. All transcripts for the inflammatory markers investigated were induced at 4 h post LPS stimulation, confirming the correct choice of this time point for chip analysis. At 16 h, some transcripts were further up-regulated, while others had reduced expression levels. Overall, the general temporal pattern of LPS induced up-regulations identified by Neuroflame analysis was verified (Fig. 2A,B).

In order to examine the correlation between gene regulation and protein expression, the time dependent release of mediators (TNF-α, IL-6, IL-10, IL-18, IL-1β, and PGE-2) was examined for PM stimulated with LPS for 0–48 h. A significant increase was determined for all cytokines investigated (Fig. 2C,D). The increase in COX-2 activity was confirmed indirectly by measurement of the concentration of prostaglandin E2 (PGE-2) that accumulated significantly over time (Fig. 2C).

3.3. Proteomics analysis of intracellular protein changes

Since the expression levels of proteins are not always regulated transcriptionally we also used the direct approach of proteomics analysis to further characterize the microglial response to LPS. For these experiments, we chose the microglial cell line BV-2 which was stimulated for 24 h before preparation of protein extracts. Thorough transcriptional examination of BV-2 cells (Neuroflame) showed that 90% of genes induced in these cells by LPS were also regulated in primary microglia. The absolute number of genes regulated in the two systems did however differ as primary microglia up-regulated 118 genes compared to 22 for BV-2 cells (data not shown). To maximize the chance of

identifying regulated proteins, two different methods of analysis were applied (relying on silver-staining (s) and differential iodine labeling (p) of proteins, respectively). In total, 15 intracellular, soluble protein species were found to be regulated by LPS and two of the proteins (immune responsive protein-1 (IRG-1) and peroxiredoxin-1) were detected twice, reflecting different post-translationally modified isoforms or possibly splice variants (Fig. 3). Of the 13 unique proteins, 10 were "new" compared to the microarray approach. The regulation of three proteins, IRG-1, interferon-induced protein with tetratricopeptide repeats-3 (IFIT-3), and inducible superoxide dismutase (SOD-2)) correlated well with mRNA expression in BV-2 or PM. Thus, IFIT-3 and IRG-1 were confirmed on the protein level as potentially new markers for activated microglia. Of all regulated proteins only one was down-regulated (nudix-type motif 5) while the ratio for up-regulated proteins ranged from 1.5 to >10 fold (Fig. 3). Some of the regulated proteins (or their genes) had previously been shown in the literature to be related to macrophage responses such as: phagocytosis/cytoskeletal remodeling [phosphatidylinositol-4-phosphate 5-kinase (Coppolino et al., 2002) (PIP5K) and vimentin (Graeber et al., 1988)], oxidative stress response [SOD-2 (Sugaya et al., 1997), glutathione *S*-transferase (Xue et al., 2005) and peroxiredoxin (Hess et al., 2003)], protease activity (unnamed protein product similar to trysinogen), general transcriptional response to LPS stimulation [immune-responsive protein-1 (Lee et al., 1995), IFIT-3 (Smith and Herschman, 1996), and nucleotide regulation/interaction (nudix-type motif 5 (Kraft et al., 2004)] and LPS binding [prohibitin (Sharma and Qadri, 2004)]. Overall, the proteomics analysis identified several inductions/reductions at the protein level not previously described for activated microglia/BV-2 cells (PIP5K, prohibitin, IFIT-3, nudix-type motif 5 and elongation factor-2) and not readily detectable by transcriptional profiling (except for *ifit-3*).

3.4. Affymetrix analysis of LPS regulated genes in primary microglia

As a second approach to address LPS-induced regulations in PM, an aliquot of the RNA preparation first analyzed by Neuroflame hybridization was subsequently examined using the Affymetrix technology platform containing 20,000 genes. Overall, the total number of up-regulated genes detected by Affymetrix was 355. In a first approach to compare the correlation of the methods (at the 4 h time point), we focused on those genes that we had found to be regulated by Neuroflame analysis (103 genes present on both platforms). Sixty eight percent of the genes regulated (=70) were also identified by the Affymetrix chip (Fig. 1 and Supplementary figure 2). This corroboration of the Neuroflame data also correlates with our proteomics/BV-2 data since the regulation of *irg-1*, *sod-2* and *ifit-3* in primary microglia was confirmed using the Affymetrix platform (Fig. 3).

Regarding new information gained from the additional genes on the Affymetrix chip, we noticed a consistent pattern of regulations for transcripts assigned to proteins having a role in the composition of the extracellular matrix (ECM). In total 23 ECM genes, the majority being procollagen related (*procollagen type Iα2, IIIα1, IVα1/α6, IXα3, Vα1/α2/α3, VIα1, XIα1*), were down-regulated strongly (23-fold on average). Besides the procollagen family, other ECM down-regulated genes were *chondroitin sulfate proteoglycan 2*, *biglycan*, *laminin B1*, *fibrilin*, *fibulin*, and *decorin*. In addition to genes directly constituting the ECM, modulators such as inhibitors of various proteases were also down-regulated. These included *proteinase inhibitor clade F, H*; *serine protease inhibitor 17*, *2-2; serpine 2,1*; *similar to*

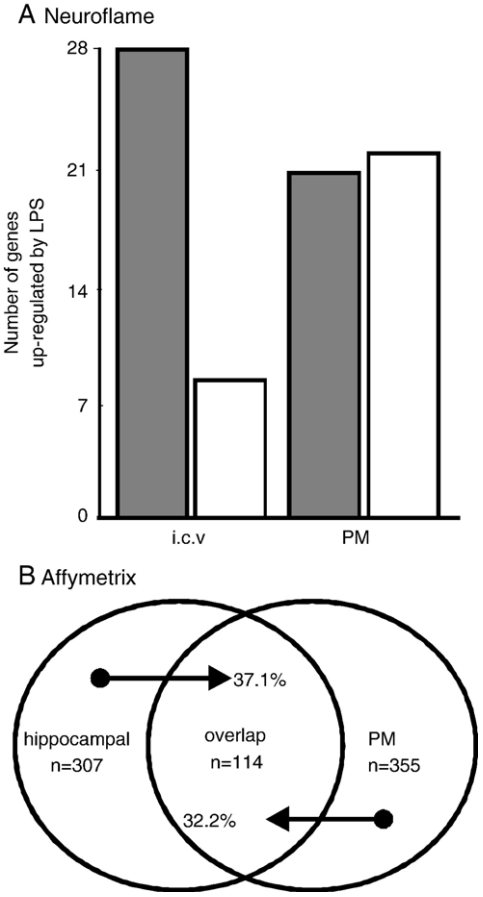


Fig. 7. Overlap of regulations in different systems. Quantitative display of genes regulated by LPS *in vivo* and *in vitro*. Mice (hippocampal regulation) and primary microglia were treated with LPS as described in Figs. 1 and 6. A Neuroflame based quantification of the temporal response of gene regulations in primary microglia and in the hippocampus. B. Affymetrix based quantification of genes regulated in primary microglia, the hippocampus, or both systems. The percentage number with right-pointing arrow indicates that the percentage of the genes to the left are also regulated in the system on the right. The percentage number with left-pointing arrow indicates that the percentage of the genes to the right are also regulated in the system on the left. Genes were considered regulated (*n*) when they exceeded ≥1.8 (Neuroflame), ≥5 (PM, Affymetrix), and 1.5 (hippocampal, Affymetrix) fold induction in their respective systems at least at one time point.

tissue inhibitor of metalloproteinase 1, 3; and *tissue inhibitor of metalloproteinase-2, 3, 4*. Furthermore, one protease (*mmp-13*), known to degrade the ECM, was up-regulated 10-fold. Overall, this profile points towards a scenario where LPS activated microglia down-regulated the production of ECM constituents, and up-regulated the corresponding degrading activities (Fig. 4).

3.5. Validation of an in vivo model for hippocampal inflammation

Having established a baseline for the response pattern of primary microglia to LPS *in vitro*, we subsequently examined the LPS response in an *in vivo* model. First, increasing doses of LPS (0–2.25 μg/brain) were injected i.c.

A Cytokine and chemokine related	Affym. *In vivo*	Neurof. *In vivo*	Affym. *In vitro*	Neurof. *In vitro*	Gene Identifier
Ccl5/RANTES	4.9	+	13.0	+	NM_013653
Chitinase 3-like 1	2.8	+			BC005611
Cxcl11	5.5	+	31.8		AF136449
Cxcl5	2.2				NM_009141
Cxcl9	3.1	+	87.9		NM_008599
Interleukin-6	11.7	+	266.1	+	NM_031168
MCP-5/Ccl12	3.3	+	93.2	+	U50712
MIP-1 beta/Ccl4	2.5	+	11.6	+	AF128218
MIP-2/Cxcl2	12.8	+	38.7	+	NM_009140
Suppressor of cytokine signaling 3	3.2	+	37.2	+	NM_007707
Caspase 11	2.5	+	5.7	+	NM_007609
Chemokine orphan receptor 1	1.6			-	BC015254
Eotaxin/Ccl11	1.9				NM_011330
Interleukin 1 receptor antagonist	4.6		19.0		M57525
Interleukin 15	2.3		14.1	+	NM_008357
Interleukin-1 receptor-associated kinase 3	1.8		-	+	AV228493
Leukemia inhibitory factor	2.6		5.1		BB235045
MCP-1/Ccl2	5.1		30.7	+	AF065933
MCP-3/Ccl7	2.8		26.0	+	AF128193
MIP-1 alpha/Ccl3	3.8			+	NM_011337
Oncostatin M receptor	2.7		-	+	NM_011019
Ccl19	2.2				NM_011888
Ccrl2	2.8		24.8		AJ318863
Colony stimulating factor 3/G-CSF	6.2		18.9		NM_009971
Epstein-Barr virus induced gene 3	1.7		12.2		NM_015766
IL-13 receptor alpha chain	1.6				S80963
Interleukin 15 receptor, alpha chain	1.6		14.7		NM_133836
IP-10/Cxcl10	3.5		24.6		NM_021274
MIP-1 gamma/Ccl9	2.4				NM_011338
B Interferon regulated					
Interferon-inducible GTPase 1	4.1	+	42.8	+	BM239828
Interferon-induced protein with tetratricopeptide repeats 1	3.0	+	22.5	+	NM_008331
Guanylate nucleotide binding protein 2	4.1	+	19.4	+	NM_010260
Interferon regulatory factor 1	1.8		9.3	+	NM_008390
Interferon-induced protein with tetratricopeptide repeats 3	2.8		15.6	+	NM_010501
ATP-dependent interferon responsive	1.8		6.0		AV290846
Guanylate nucleotide binding protein 4	3.6		20.9		NM_018734
Interferon activated gene 203	1.8		16.1		BC008167
Interferon activated gene 205	3.8		7.0		AI481797
Interferon gamma induced GTPase	2.7		10.9		NM_019440
Interferon gamma inducible protein, 47 kDa	3.2		22.5		NM_008330
Interferon induced transmembrane protein 3	2.1				BC010291
Interferon induced with helicase C domain 1	3.8		9.3		AY075132
Interferon inducible protein 1	2.9		5.6		NM_008326
Interferon regulatory factor 7	2.4		9.2		NM_016850
Interferon-induced protein 35	2.3		6.7		BC008158
Interferon-induced protein 44	3.1		8.5		BB329808
Interferon-induced protein with tetratricopeptide repeats 2	4.0		15.4		NM_008332
Interferon-stimulated protein (15 kDa)	3.4		28.8		AK019325
Interferon-stimulated protein (20 kDa)	6.6		40.2		BC022751
Similar to guanylate nucleotide binding protein 1	2.6		9.6		BC010229
Torsin family 3, member A	2.5		5.9		AK009693

Fig. 8. Overlap of *in vivo* and *in vitro* regulation of inflammatory genes on basis of Affymetrix analysis. Mice were injected i.c.v. with LPS (*in vivo*) or primary microglia (*in vitro*) were exposed to LPS (100 ng/ml), and after 4 h mRNA was prepared and used for expression profiling on an Affymetrix array. All cytokine/interferon-related genes, that were up-regulated (≥ 1.5 fold) *in vivo* are presented with their regulation factors. For the same genes, the regulation data *in vitro* are also given. For comparison, the data on these genes obtained with Neuroflame arrays are included in the table where available. Empty fields indicate that the gene was not spotted on the Neuroflame. Blanks: a gene was induced less than 1.5 fold. "–", gene down-regulated ≥ 1.5 fold. "+", significantly regulated on Neuroflame.

v., resulting in an increasing production of IL-6 and TNF-α protein in the brain region investigated (hippocampus) (Fig. 5A). A dose of 2.25 μg LPS injected into the right ventricle was chosen for subsequent studies to monitor the temporal dynamics of IL-6 and MCP-1 concentrations. Both interleukins were found to peak at 4 h and then return almost to basal levels at the end of the experiment (16 h) (Fig. 5B). TNF-α peaked 2 h after i.c.v. injection of LPS and was not detectable by ELISA 4 h later (data not shown). The brain cytokine levels 4 h after LPS i.c.v. injection were further explored using a protein array capable of analyzing the expression of 20 cytokines. This experiment identified G-CSF, MCP-5, RANTES, and IL-12 as being up-regulated by LPS in addition to MCP-1 as already described. Non-detected cytokines were GM-CSF, IL-2, -3, -4, -5, -9, -10, -13, -17, IFN-γ, thrombopoetin, and vascular endothelial growth factor (VEGF) (TNF-α was only borderline regulated as it peaked after 2 h) (Fig. 5C,D). The return of cytokine concentrations (TNF-α, IL-6) towards the baseline correlated with a decrease in their respective mRNA levels over time, and suggests reduced production rather than increased removal as the regulating factor of cytokine production (Fig. 5E). To correlate the expression profile of inflammatory mediators with a microglial activation marker (F4/80) a parallel histological study was carried out. Interestingly, the abundance of F4/80 peaked 2 days after LPS injection when cytokine production was no longer detectable (Fig. 5F,G) (only data for the 2 day time point is shown).

3.6. In vivo transcriptional response triggered by i.c.v. injection of LPS (Neuroflame vs. Affymetrix)

In order to thoroughly examine the inflammatory response obtained *in vivo* after i.c.v. injection of LPS, mice were sacrificed either 4 or 16 h after the injection and hippocampal cDNA was analyzed via two different arrays. Using the Neuroflame microarray, injection of LPS was found to induce a robust expression of 29 genes (Fig. 6). In striking difference to the *in vitro* systems, the response was mostly transient, as only one quarter of the genes found at 4 h were still up-regulated at 16 h (Fig. 7A). Only one gene qualified as being solely up-regulated at the late time point (*lipocalin-2*). The regulated genes comprised chemokines as the dominant group, the acute phase proteins *saa-2* and *saa-3*, *il-6*, *mmp-3* and a group of conspicuous nucleotide-interacting genes (*interferon-inducible gtpase, gbp-2, tyki*), that have been described earlier as inflammation-linked, but have not been examined in brain tissue. The latter, together with *ifit-3* were all confirmed by RT-PCR in an independent experiment (not shown). In total, 90% of the genes up-regulated by LPS *in vivo* (4+16 h) were also induced by LPS stimulated primary microglia *in vitro* (4+16 h) (Fig. 6). It therefore appears that the inflammatory response to an i.c.v. bolus injection of LPS to a wide extent comprises genes induced in LPS stimulated microglia *in vitro*. In order to strengthen our Neuroflame findings, an aliquot of the same pool of cDNA (hippocampal tissue 4 h and 16 h) was re-analyzed using Affymetrix chips. Overall, 307 genes were found to be up-regulated by LPS, and 16 down-regulated (Supplementary figures 4 and 5). Of the 28 genes identified by Neuroflame to be up-regulated after 4 h, 23 (=82%) were confirmed on the Affymetrix chip. The group comprising "cytokine and chemokine related molecules" was expanded by *g-csf*, and *ccl-9* as additional hits compared to Neuroflame, and the group of nucleotide-interacting factors and *ifit-3* were confirmed (Fig. 6 and Supplementary figure 4). No genes qualified as being regulated 16 h after stimulation according to the inclusion criteria for the Affymetrix chip, corroborating again the transient nature of the *in vivo* response (Fig. 6).

3.7. In vivo–in vitro correlations

We used the Affymetrix data to further elucidate the relation of *in vitro* and *in vivo* transcriptional response to LPS. Of the 29 cytokine and chemokine related genes up-regulated *in vivo* by LPS, 19 (65%) were also up-regulated by LPS *in vitro* (Fig. 8A). For some subgroups, like the *ifn*-related genes, the overlap was up to 95% (Fig. 8B), whereas the percentage of overlap was 37% when all up-regulated genes (including the non-inflammatory ones) were compared indiscriminately (Fig. 7B). Since microglia *in vitro* up-regulated a large number of additional genes, beyond those observed *in vivo* (compare Fig. 1 with Fig. 6), microglia *in vivo* may be subjected to regulations restricting the inflammatory response. The presence of these factors may also be reflected by the strong transient nature of gene induction *in vivo*, which is not seen *in vitro* (Fig. 7A). To verify the lack of induction of inflammatory mediators *in vivo*, we identified

LPS *in vitro* hyper responsive genes	*In vivo* Affymetrix	*In vitro* Affymetrix	*In vivo* Real-time PCR	*In vitro* Real-time PCR	Gene ID
Schlafen-1	-	++++	-	++++	NM_011407
TNF receptor-associated factor	-	+++	-	+++	BB218245
IL-23 (p19)	-	++++	+	++++	NM_031252
Endothelin-1	-	++++	+	++++	D43775
Interferon beta-1, fibroblast	-	++++	+	++++	NM_010510

Fig. 9. LPS hyper responsive genes *in vitro*. RNA isolated from either primary microglia stimulated with LPS (4 h) or hippocampi of i.c.v. injected animals (4 h) was analyzed on both, the Affymetrix and Neuroflame platforms (as previously described in Fig. 1+6). The five inflammatory genes showing the highest induction in LPS stimulated primary microglia without being induced by i.c.v. injection of LPS were filtered out for display in the left side of the table. Using RT-PCR the material was reanalyzed and the ratios hereby determined are shown in the right side of the table. Gene induction (fold change) was translated as follows; − (0 ≤ 1.5); + (>1.5 ≤ 5.0); ++ (>5.0 ≤ 15); +++ (>15 ≤ 100.0); ++++ (>100).

genes highly induced *in vitro*, but not *in vivo*. We focused on 5 inflammation-related genes (*schlafen-1*, *tnf receptor-associated factor*, *il-23* (α *subunit p19*), *endothelin*, and *interferon β-1*) highly induced by LPS *in vitro* (80–450 fold) but regulated less than 1.2 fold *in vivo*. The expression of the above mentioned genes was re-evaluated by RT-PCR. *Schlafen-1* and *tnf receptor associated factor-1* were indeed not regulated at all *in vivo*, whereas the other three were slightly regulated *in vivo*. Overall these results corroborate our initial array findings that at least some inflammatory genes are more prone to be induced by LPS *in vitro* than *in vivo* (Fig. 9).

4. Discussion

4.1. The inflammatory transcriptome of LPS activated primary microglia in vitro

Here, we characterized and compared the time course and pattern of inflammatory gene regulation in murine microglia and *in vivo*. Using primary cultures, we found that LPS induced regulations define a unique activation state of microglia compared to other published studies on microglial transcriptomic changes (growth factors, IFN-γ, fractalkine and Aβ). For instance, several LPS induced gene-clusters were markedly different from those observed after stimulation of microglia with either GM-CSF or M-CSF (Re et al., 2002). Treatment with M-CSF increased the transcription of insulin-like growth factor binding protein II/III and several ECM constituents, while the expression was reduced by LPS treatment. Conversely, LPS induced the production of chemokines, interferon related molecules, and interleukins, while the CSFs did not. In another study using primary rat microglia, IFN-γ stimulation predominantly and in a coordinated fashion induced genes involved in antigen processing (proteasome subunits) and presentation (*mhcI/II*), but not cytokines and chemokines (Moran et al., 2004). The transcriptional response of rat microglia to the chemokine fractalkine, secreted from neurons, resulted in regulation of only eight genes of which one, milk-fat globule EGF factor-8, is hypothesized to facilitate microglial phagocytosis of apoptotic neurons (Leonardi-Essmann et al., 2005). In contrast, LPS stimulation predominantly induced chemokines, while genes involved in antigen presentation and processing were only sparsely and weakly induced. The published activation profile most closely resembling LPS stimulated microglia is that of amyloid precursor protein-derived peptide (Aβ1-42) challenged human microglia. The most prominently regulated clusters were cytokines/chemokines, matrix metalloproteinases, and NF-κB subunits (Walker et al., 2001). Assuming that the main findings of all these studies are comparable despite the different technical platforms, a picture is emerging, where distinct microglia activation profiles are stimulus dependent and composed of common as well as unique transcriptional features. For instance, simultaneous induction of a gene cluster composed of *gbp-3*, *superoxide dismutase-2*, *ifit-3*, *mmp-13*, *mcp-5*, *endothelin-1*, *g-csf*, and *lipocalin-2* constitutes an LPS induced inflammation-relevant transcriptional signature as opposed to the transcriptional profile triggered with growth factors (GM-CSF, G-CSF), IFN-γ, or Aβ1-42. Another interesting recent study, examining the response of human microglia to TNF-α, indicated that human microglia may enter rather heterologous activation states upon stimulation (Meeuwsen et al., 2005). Whether the observed response pattern of human microglia is linked to the variability of the donor material (such as difference in age, sex, disease history and drug exposure) or is a true species difference is intriguing but requires additional research.

On the other hand, the transcriptional analysis across various studies reveals also striking similarities. For instance, we observed the up-regulation of *phosphodiesterase 4b* (*pde4b*) amongst many other enzyme up-regulations (Supplementary figure 4B). Since also microglia stimulated with IFN-γ (Moran et al., 2004) or Aβ1-42 (Sebastiani et al., 2005) up-regulated *pde4b*, there is a stronger likelihood for a generalized biological significance, and indeed, very recent work indicates a role of this regulation in the activation of macrophages (Jin et al., 2005).

A common pattern of stimulation via TLR-4 and TLR-2 seems to be indicated by data from a study using whole gram-positive *S. aureus* bacteria as stimulus. More than 90% of the regulated genes overlap with the profile induced by LPS in the present work. However, this comparison is based on a small data basis, since in the *S. aureus* study only ~120 inflammatory genes were examined. For examination of specific transcriptional signatures of very related stimuli, genome spanning chip studies may be required.

4.2. Comparison of array data across platforms and experimental procedures

The comparability of data across array platforms may be affected by differences in design and experimental procedures. However, our own comparative validation of two very different array platforms (Affymetrix *vs*. our custom-array), showed that the regulation of several gene clusters such as chemokines, interleukins, NF-κB regulation, and the interferon response was robustly picked up independent of the platform. Gene cluster comparisons of microglial responses therefore seem feasible across platforms, whereas comparison of individual gene-regulations can only be used for hypothesis generation and requires independent verifications. Both platforms generated some obvious false-negative results for individual genes. This is best exemplified by our transcriptional data for *tnf-α*, a well-known hallmark of LPS stimulated microglia, which was regulated clearly both according to the Affymetrix array and RT-PCR, but not according to the Neuroflame read out. Here, the specific oligo chosen was found to have a very low hybridization efficiency.

4.3. Two examples for conspicuous LPS induced gene regulations in microglia

One of the less examined areas in brain inflammation is the interaction of microglia with the extracellular matrix (ECM). However, our data show a very consistent and

interesting co-regulation of a large cluster of matrix-regulated genes. The LPS induced activation profile of microglia indicates a potential degradation and down-regulation of ECM constituents, as the basis for proteolytic activity was increased (*mmp-3* and *mmp-13* up-regulated, protease inhibitors down-regulated) while the production of ECM components was reduced. Possibly, increased mobility is essential for some microglial activation states and requires remodeling of the surrounding stromal environment. One may speculate that chronically activated microglia (as seen in Alzheimer's and Parkinson's disease) contribute to degenerative events by degrading parts of the ECM, which normally provides physical and trophic support for other cell types. LPS activated microglia in our study were for instance shown to reduce the transcription of biglycan (a neurotrophic signal for neurons (Koops et al., 1996; Kappler et al., 1997)).

Besides potential degradation of the ECM, microglial migration is with certainty dependent on chemo-attractant factors. Best evaluated are CCL21 (de Jong et al., 2005), CXCR3 (Rappert et al., 2004), and CCR5 (Carbonell et al., 2005) which have been demonstrated to control microglial recruitment to sites of trauma-induced neuronal injury. The ligands and receptors investigated did however not fully account for all the motility. It therefore seems likely that other chemo-attractants are at play and in this context we identified here a cluster of additional chemokines (c–c motif 4–5–12 and c–x–c motif 1–2–9–11), which were highly regulated both *in vitro* and *in vivo*. This information may be valuable for future research addressing microglial locomotion.

4.4. Protein markers for LPS induced microglial activation

We found that the genes induced in BV-2 cells by LPS have a very high degree of similarity (90%) with those in PM, although the induction was weaker. An explanation for the more narrow induction profile of BV-2 cells is most likely multifactorial and might for instance be related to the v-raf/v-myc induced immortalization of BV-2 cells (Blasi et al., 1990). Thus, LPS activated BV-2 cells may allocate relatively more resources for cellular division compared to primary microglia.

Since the regulations that were induced by LPS in BV-2 overlapped very well with those in primary microglia, the cell line was chosen for a proteomics study requiring protein amounts on a milligram scale. In the proteomic study we identified intracellular LPS-regulated proteins as inflammatory markers novel to microglial activation (e.g. PIP5K, prohibition and nudix type motif 5). Some of the identified proteins correlated with the transcriptional changes and some had previously been reported regulated on the mRNA level in LPS stimulated macrophages [e.g. IFIT-3 (Lee et al., 1994; Smith and Herschman, 1995) and IRG-1 (Lee et al., 1995)].

Though the published information on most of the proteins identified is limited, it appears as if several of them might control microglial activation and pose potential new targets for pharmacological modulation. Mutated PIP5K (kinase activity deleted) has for instance been shown to impair macrophage phagocytosis (Coppolino et al., 2002), and its up-regulation by LPS might indicate an increased phagocytic capacity of activated microglia. Also, the observed elimination of nudix type motif 5 might control microglial activation. This enzyme catalyses the degradation of ADP-ribose and since ADP-ribose facilitates opening of the cation channel TRPM-2 (Kraft et al., 2004) the removal of nudix type motif 5 may lead to increased ADP-ribose levels and thereby increased TRPM-2 currents which are essential for the microglial activation process.

4.5. In vivo transcriptional response triggered by i.c.v. injection of LPS

A major part of this study dealt with the characterization and validation of an *in vivo* system allowing the profiling of a microglial response. i.c.v. injection of LPS induced a robust expression of several inflammatory gene-clusters also identified *in vitro*. However, the temporal dynamics of the cerebral inflammation was distinct from that observed *in vitro*. The response peaked after 4 h and then returned almost to the baseline after 16 h, while a continuous up-regulation was observed for primary microglia *in vitro*. This was evident both at the protein (secreted cytokines) and at the transcriptional level. The rapid down-regulation of inflammatory mediators at all levels is intriguing, since i.c.v. LPS injection has been studied in many laboratories, and histological data (Kalehua et al., 2000) generally indicate that the accumulation of CD11b-positive microglia peaks as late as 48 h after LPS administration. Our histological analysis, using F4/80 as microglia marker confirms these observations. This discrepancy between microglial activation, when comparing histology to gene expression, indicates that extrapolations from commonly used microglial surface markers to actual inflammatory states, appear to be more complex than assumed. In this context some of the proteins identified as up-regulated by LPS stimulation may prove of interest as new staining markers with better correlation to the actual inflammatory state of the cells, and merit further investigation.

Although a striking correlation was found for the transcripts induced by LPS *in vitro* and *in vivo* (after 4 h) for some families of inflammatory mediators, this does not hold true in general. For instance, there was a very high correlation for the class of interferon related proteins when comparing *in vitro* with *in vivo* (95%), whereas other mediators such as *il-23 (p19)*, *endothelin-1*, and *interferon β-1* were not detected *in vivo* but highly up-regulated *in vitro*. Since microglia only constitute 5–15% of the total cell population in the CNS, one might expect that the signal for certain inflammatory mediators simply was too diluted to be detected by conventional microarray analysis. Indeed, by reanalyzing the samples with the highly sensitive method of RT-PCR we found that some of the mediators initially thought to be non-regulated were indeed slightly regulated. However, regulations were around 5 fold *in vivo vs*. more than 1000 fold *in vitro* (see Fig. 9). Some genes, such as *schlafen 1* and *tnf receptor associated factor-1* were confirmed to be non-regulated *in vivo* and highly regulated *in*

vitro. This has two major implications. First, chip evaluation of the inflammatory response *in vivo* is prone to yield false-negative results. Therefore it may be useful for further studies of inflammogens and pharmacological modulators to pick a representative set of markers to be analyzed by RT-PCR. Our study provides a good basis for the selection of such an indicator set covering *in vitro* and *in vivo* responses as well as different signaling pathways and response modes. Second, some genes seem indeed to be differentially regulated by LPS *in vivo* and *in vitro*. Such differences may for instance be explained by microglial modulatory factors present in the CNS (Hoek et al., 2000). The presence of such factors is an exciting aspect of the innate immune response of the brain and will require more focus on *in vivo* microglial activation studies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jneuroim.2006.07.007.

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