Luteolin triggers global changes in the microglial transcriptome leading to a unique anti-inflammatory and neuroprotective phenotype

Konstantin Dirscherl1†, Marcus Karlstetter1†, Stefanie Ebert1†, Dominik Kraus1, Julia Hlawatsch1, Yana Walczak1, Christoph Moehle2, Rudolf Fuchshofer3, Thomas Langmann1*

Abstract

Background: Luteolin, a plant derived flavonoid, exerts a variety of pharmacological activities and anti-oxidant properties associated with its capacity to scavenge oxygen and nitrogen species. Luteolin also shows potent anti-inflammatory activities by inhibiting nuclear factor kappa B (NFkB) signaling in immune cells. To better understand the immuno-modulatory effects of this important flavonoid, we performed a genome-wide expression analysis in pro-inflammatory challenged microglia treated with luteolin and conducted a phenotypic and functional characterization.

Methods: Resting and LPS-activated BV-2 microglia were treated with luteolin in various concentrations and mRNA levels of pro-inflammatory markers were determined. DNA microarray experiments and bioinformatic data mining were performed to capture global transcriptomic changes following luteolin stimulation of microglia. Extensive qRT-PCR analyses were carried out for an independent confirmation of newly identified luteolin-regulated transcripts. The activation state of luteolin-treated microglia was assessed by morphological characterization. Microglia-mediated neurotoxicity was assessed by quantifying secreted nitric oxide levels and apoptosis of 661W photoreceptors cultured in microglia-conditioned medium.

Results: Luteolin dose-dependently suppressed pro-inflammatory marker expression in LPS-activated microglia and triggered global changes in the microglial transcriptome with more than 50 differentially expressed transcripts. Pro-inflammatory and pro-apoptotic gene expression was effectively blocked by luteolin. In contrast, mRNA levels of genes related to anti-oxidant metabolism, phagocytic uptake, ramification, and chemotaxis were significantly induced. Luteolin treatment had a major effect on microglial morphology leading to ramification of formerly amoeboid cells associated with the formation of long filopodia. When co-incubated with luteolin, LPS-activated microglia showed strongly reduced NO secretion and significantly decreased neurotoxicity on 661W photoreceptor cultures.

Conclusions: Our findings confirm the inhibitory effects of luteolin on pro-inflammatory cytokine expression in microglia. Moreover, our transcriptomic data suggest that this flavonoid is a potent modulator of microglial activation and affects several signaling pathways leading to a unique phenotype with anti-inflammatory, anti-oxidative, and neuroprotective characteristics. With the identification of several novel luteolin-regulated genes, our findings provide a molecular basis to understand the versatile effects of luteolin on microglial homeostasis. The data also suggest that luteolin could be a promising candidate to develop immuno-modulatory and neuroprotective therapies for the treatment of neurodegenerative disorders.
Background
Microglia, the resident macrophages of the nervous system, have important roles in immune regulation [1,2] and neuronal homeostasis [3,4]. Microglia belong to the mononuclear phagocyte system but their special localization in the fragile neuronal environment and their morphological features clearly distinguish them from other peripheral macrophages [5]. Ramified microglia perform a very active and continuous surveillance function with their long protrusions [6,7]. They receive permanent tonic inhibitory inputs from neurons to prevent microglial neurotoxicity [8,9]. Loss of microglia-neuron cross-talk [10], local danger signals such as extracellular ATP [11], or neurotransmitter gradients [12] rapidly lead to a functional transformation of ramified microglia with a variety of effector functions.

Microglia activation is a protective mechanism regulating tissue repair and recovery in the early phase of neurodegeneration [4]. However, excessive or sustained activation of microglia often contributes to acute and chronic neuro-inflammatory responses in the brain and the retina [2]. Activated microglia in the vicinity of degenerating neurons have been identified in a broad spectrum of neurodegenerative disorders including Alzheimer’s disease [13], Parkinson’s disease [14], amyotrophic lateral sclerosis [15], multiple sclerosis [16], and inherited photoreceptor dystrophies [17,18].

Macrophage heterogeneity and plasticity is very large and the set of marker combinations and sub-populations is essentially infinite [19]. To define a simplified conceptual framework, classification into polarized functional categories, called M1 and M2 macrophages has been proposed [20,21]. M1 or “classically activated” macrophages produce high levels of oxidative metabolites and pro-inflammatory cytokines but also cause damage to healthy tissue as side effect [22]. M2 or “alternatively activated” macrophages promote tissue remodeling and generally suppress destructive immune reactions. Informations on microglial subsets in the nervous system are relatively scarce compared to other tissue macrophages. Nevertheless, recent findings from in vitro cultures of the murine microglial cell line MGMT12 [23] and hippocampal microglia from the PS1xAPP Alzheimer’s mouse model [24] implicate that microglia have the ability to differentiate into M1 and M2 polarized phenotypes. A co-existence of neurotoxic M1 microglia and regenerative M2 microglia has been recently documented in the injured mouse spinal cord [25]. Microarray-based quantitation of M1 and M2 markers as well as functional tests on axonal regrowth after injury demonstrated that a transient anti-inflammatory and neuroprotective M2 response was rapidly overwhelmed by a neurotoxic M1 microglial response [25]. A similar but age-dependent switch from alternative to classical activation was shown in PS1xAPP Alzheimer’s mice [24], indicating a common phenomenon in neurodegenerative disorders. Compounds that induce the switch of microglia from inflammatory M1 type to anti-inflammatory M2 type could therefore be a potential therapeutic agent to attenuate neuronal inflammation and boost neuronal recovery [26].

Several anti-inflammatory drugs have been shown to diminish neuroinflammation, but only a few direct functional effects on microglial activity have been elucidated [27]. Among the naturally occurring immuno-modulators, the flavonoid luteolin (3’,4’,5,7-tetrahydroxyflavone), abundant in parsley, green pepper, celery, perilla leaf, and chamomile tea, exerts prominent anti-inflammatory and anti-oxidant activities [28]. Luteolin suppressed pro-inflammatory cytokine production in macrophages by blocking nuclear factor kappa B (NFkB) and activator protein 1 (AP1) signaling pathways [29] and inhibited the production of nitric oxide [30] and pro-inflammatory eicosanoids [31]. Luteolin also diminished the release of TNF and superoxide anions in LPS or interferon-γ treated microglial cell cultures [32,33] and reduced the LPS-induced IL6 production in brain microglia in vivo [34].

Although the inhibitory function of luteolin on NFkB and a few selected cytokines is well documented in macrophages, a genome-wide search for further molecular targets in microglia has not yet been published. Furthermore, the immuno-modulatory effects of luteolin related to the stimulation of distinct functional microglial phenotypes has not been investigated before. Therefore, this study investigated the global transcriptional effects of luteolin at near physiological concentrations [35] alone or in combination with LPS in pure BV-2 microglial cultures. We further validated the luteolin-regulated expression of novel pro- and anti-inflammatory microglial transcripts, analyzed microglial morphology, and studied the consequences of microglia-conditioned media for photoreceptor viability.

Methods
Reagents
Luteolin (3’,4’,5,7-tetrahydroxyflavone) and E. coli 0111: B4 lipopolysaccharide were purchased from Sigma Aldrich (Steinheim, Germany). Luteolin was dissolved in DMSO and added in concentrations that did not exceed 0.05% of the total volume in any of the cell culture experiments.

Animals
C57BL/6 mice were purchased from Charles River Laboratories. Mice were kept in an air-conditioned barrier environment at constant temperature of 20-22°C on
a 12-h light-dark schedule, and had free access to food and water. The health of the animals was regularly monitored, and all procedures were approved by the University of Regensburg animal rights committee and complied with the German Law on Animal Protection and the Institute for Laboratory Animal Research Guide for the Care and Use of Laboratory Animals, 1999.

**Cell culture**

Brain microglia were isolated and cultured as described earlier [36]. BV-2 microglia-like cells were provided by Professor Ralph Lucius (Clinic of Neurology, Christian Albrechts University, Kiel, Germany). BV-2 cells were cultured in RPMI/5% FCS supplemented with 2 mM L-Glutamine and 195 nM β-mercaptoethanol. Primary brain microglia or BV-2 cells were stimulated with 10 ng/ml or 50 ng/ml LPS and various concentrations of luteolin for 24 h. 661W photoreceptor-like cells were a gift from Prof. Muayyad Al-Ubaidi (University of Illinois, Chicago, IL) and the culture conditions have been described elsewhere [36].

**Phalloidin staining**

BV-2 cells were plated overnight on coverslips, fixed with 3.7% paraformaldehyde for 10 min at 37°C, permeabilized with 0.2% Triton X-100 for 5 min, blocked with 5% non-fat milk, 0.2% Triton X-100, and stained with DAPI for 10 min at room temperature (0.1 μg/ml in PBS, 4',6-diamidino-2-phenylindol, Molecular Probes). Filamentous actin was stained by addition of 1.5 μM TRITC-conjugated phalloidin (Sigma). The coverslips were mounted on microscopic glass slides and viewed with a Axioskop 2 fluorescence microscope equipped with an Eclipse digital analyzer (Carl Zeiss).

**NO measurement**

NO concentrations were determined by measuring the amount of nitrite secreted by BV-2 cells into the culture medium using the Griess reagent system (Promega). 50 μl cell culture supernatant was collected and an equal volume of Griess reagent was added to each well. After incubation for 15 min at room temperature, the absorbance was read at 540 nm on a BMG FluoStar Optima plate reader (Labtech, Offenburg, Germany). The concentration of nitrite for each sample was calculated from a sodium nitrite standard curve.

**Apoptosis assay**

Apoptotic cell death of 661W cells was determined with the Caspase-Glo® 3/7 Assay (Promega). Cells were lysed and incubated with a luminogenic caspase-3/7 substrate, which contains the tetrapeptide sequence DEVD. Luminescence was then generated by addition of recombinant luciferase and was proportional to the amount of caspase activity present. The luminescent signal was read on a BMG Fluostar Optima plate reader (Labtech, Offenburg, Germany). A blank reaction was used to measure background luminescence associated with the cell culture system and Caspase-Glo® 3/7 Reagent. The value for the blank reaction was subtracted from all experimental values. Negative control reactions were performed to determine the basal caspase activity of 661W cells.

**RNA isolation and reverse transcription**

Total RNA was extracted from cultured microglia according to the manufacturer’s instructions using the RNeasy Protect Mini Kit (Qiagen, Hilden, Germany). Purity and integrity of the RNA was assessed on the Agilent 2100 bioanalyzer with the RNA 6000 Nano LabChip® reagent set (Agilent Technologies, Büblingen, Germany). The RNA was quantified spectrophotometrically and then stored at -80°C. First-strand cDNA synthesis was performed with RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany).

**DNA microarray analysis**

Triplicate microarrays were carried out with three independent RNAs from non-stimulated BV-2 microglia or cultures treated for 24 h with 50 μM luteolin, 50 ng/ml LPS, or 50 μM LPS + 50 ng/ml LPS, respectively. Generation of double-stranded cDNA, preparation and labeling of cRNA, hybridization to Affymetrix 430 2.0 mouse genome arrays, washing, and scanning were performed according to the Affymetrix standard protocol. Minimum information about a microarray experiment (MIAME) criteria were met [37]. The microarray datasets of this study are publicly available at the National Center for Biotechnology Information Gene Expression Omnibus http://www.ncbi.nlm.nih.gov/geo/ as series record GSE18740.

**Bioinformatic data analysis**

The Affymetrix Expression Console Software Version 1.0 was used to create summarized expression values (CHP-files) from 3’ expression array feature intensities (CEL-files) using the Robust Multichip Analysis (RMA) algorithm. Integrative analysis of genome-wide expression activities from BV-2 cells was performed with the Gene Expression Dynamics Inspector (GEDI), a Matlab (Mathworks, Natick, MA) freeware program which uses self-organizing maps (SOMs) to translate high-dimensional data into a 2D mosaic [38]. Each tile of the mosaic represents an individual SOM cluster and is color-coded to represent high or low expression of the cluster’s genes, thus identifying the underlying pattern. Differentially regulated transcripts in 24 h luteolin stimulated versus non-treated and luteolin + LPS versus LPS-treated BV-2 cells, respectively, were retrieved with the Genomatrix ChipInspector program (Genomatrix Software GmbH, Munich, Germany), applying the Significance Analysis of Microarray (SAM) algorithm using a false-discovery rate of 0.1% and a minimum coverage of 3 independent probes.
Functional annotation of transcripts was performed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) [39] and the Bibliosphere pathway edition (Genomatix).

**Quantitative real-time RT-PCR**

Amplifications of 50 ng cDNA were performed with an ABI7900HT machine (Applied Biosystems) in triplicates in 10 μl reaction mixtures containing 1 × TaqMan Universal PCR Master Mix (Applied Biosystems), 200 nM of primers and 0.25 μl dual-labeled probe (Roche Probe-Library). The reaction parameters were as follows: 2-min 50°C hold, 30-min 60°C hold, and 5-min 95°C hold, followed by 45 cycles of 20-s 94°C melt and 1-min 60°C anneal/extension. Measurements were performed in triplicate. Results were analyzed with an ABI sequence detector software version 2.3 using the ΔΔCt method for relative quantitation. Primer sequences and Roche Library Probe numbers are listed in Table 1.

**Statistical analysis**

Statistical analysis were performed on ΔΔCt data using analysis of variance with a two-sample Student’s t test (P < 0.05) unless otherwise indicated. Quantitative data are expressed as mean ± SEM. The levels of gene expression in treated BV-2 cells are shown relative to control cells.

**Results**

**Effects of luteolin on selected pro-inflammatory markers**

As a basis to study the genome-wide transcriptional effects of luteolin on activated microglia and to validate our cell culture system, we initially performed a dose-response curve for luteolin. Four pro-inflammatory microglia

| Gene      | F-Primer (5’-3’) | R-Primer (5’-3’) | Roche Library Probe |
|-----------|------------------|------------------|---------------------|
| AA467197  | aaatggtggtatctactcaacc | gttgccctcgggactttt | 17                  |
| Blvrb     | tctctgattgcctcagctt   | gcaccgtctacactatactct | 81                  |
| C3        | acctctcgrcgacgatg    | tttgtagctgtgtgaccgtg | 76                  |
| CD36      | ttagaaagctgccccagagag | tcagacagacacagctgta | 6                   |
| CD83      | gcgtctctcgagcagcttg  | ggctctcgcatgctgggagc | 2                   |
| Cfbb      | ctgcaagtgcagttcagc   | tcaagtctgcgctgtcgtg | 1                   |
| Cst7      | atgcgacggacagccggta  | gttctctcgcatgctgggagc | 67                  |
| Cxcl10    | gcggctgcatctcttgctg  | tcaaggtgccccggtcattctc | 3                  |
| Ddit3     | cccacacaccgaagccggagagc | tcctcatacaggcttctctcagag | 33                  |
| Gbp2      | ttagagacagggtgccagag  | gataaggccatcctgctgctg | 62                  |
| Gbp3      | aagatgatgctgctgcatca | gaaacttcggaacacttttgcag | 73                  |
| Gclm      | tgaggcagtctagcctgaggt | caaaggccgtaaacttgggt | 18                  |
| Guibx     | gtggggtcggctcctgctctc | atttttgccggcggaaac | 25                  |
| Hmox1     | ctgctgtgctcggcttgagc | ccaacaggagggctggagttg | 25                  |
| Hp        | cctttgagctgctgctcacttc | ttgggagcttgcatctttc | 15                  |
| Hprt1     | tcctctctacgctctggcttt | cctgctctacgtgctggtccat | 95                  |
| Ifi44     | ctgacagcagcagctgcttc | aggcacacaccagctgcag | 78                  |
| Ifitm3    | acatcgtcgcagctgcttgctc | acacttcgctgttttgcag | 84                  |
| Ifitm6    | cgggtgacacagttcagttctc | catgtgtgcccccctactt | 27                  |
| IL-6      | gtggtagctcagagcagagag | caggtgtgcagctgctgttgta | 6                   |
| iNos      | cttggcagcgccgagcagac | tcaagctctgttctcgag | 13                  |
| If7       | cttggcagcctttctgcag | tgcgtaacagcaggctggagtc | 25                  |
| Kdr       | cagttgctgctgctgctgctg | acagcaatcgctggctgtttt | 68                  |
| Lcn2      | atgcattcttcgtgtctgctc | cctgtgctatctctttcaggctg | 1                   |
| Lpcat1    | aattttgagggcgctgctgctg | gcggctcttgccatgtgtctc | 81                  |
| Marco     | cagagggcagagcagcagccggagc | gcgggagcgacaaatcttttttgag | 20                  |
| Mpeg1     | cagaggggagagcagctgctgctgctg | gcgggagcgacaaatcttttttgag | 69                  |
| Nupr1-1F  | gattggaatgctgctgctgctg | qgtggcgagcatttggcagctgg | 64                  |
| Rnf14S    | cagagttgctgtgtctgctgctg | aatagggagtgttctgcagctgtttt | 67                  |
| Saa3      | atgtacgctggggagagagatg | acagccctctctggtctgctgctg | 26                  |
| Slpi      | ttgcagtccttgccacagcgac | cctgtgctacagcgtgctgcagctgctg | 69                  |
| Snx1      | gctatcgcgacagcagcagcagac | gttggagctctgtgtctgc | 33                  |
| Trb3      | gcgtacggccctgctctgct | acatgtgctggtggtcag | 98                  |
markers with different expression levels and ranges of induction were selected as positive controls for qRT-PCR analyses. Interleukin 6 (Il6) is a well known pro-inflammatory cytokine target of luteolin [34], chemokine (C-X-C motif) ligand 10 (Cxcl10), interferon-regulatory factor 7 (Irf7), and interferon-inducible protein 44 (Ifi44) are LPS-sensitive genes in microglia [36]. BV-2 microglia were pretreated with different concentrations of luteolin (0, 5, 10, 25, and 50 μM) for 1 h and then stimulated with LPS (10 ng/ml and 50 ng/ml) for a further 24 h period. Neither LPS nor luteolin changed the proliferation rate or cell survival at the concentration levels applied to the cells (data not shown). mRNA levels of Il6 (Fig. 1A), Cxcl10 (Fig. 1B), Irf7 (Fig. 1C), and Ifi44 (Fig. 1D), which were all induced by 10 ng/ml LPS and further increased by 50 ng/ml LPS, were dose-dependently reduced by luteolin (Fig. 1A-D). Luteolin’s effects on the four genes were most prominent at 50 μM (Fig. 1A-D) and therefore this concentration was used in all further experiments.

**Luteolin triggers global changes in the microglial transcriptome**

Our next goal was to capture and compare the transcriptional profile of non-activated and LPS-activated BV-2 microglia treated each with 50 μM luteolin for 24 h using Affymetrix Mouse Genome 430 2.0 GeneChips. Twelve microarray analyses from three independent stimulation experiments were performed and high stringency criteria with a minimal signal intensity of 50 fluorescence units were used. The complete microarray RMA datasets and all raw data (Affymetrix CEL-files) were stored in the NCBI GEO repository as record GSE18740. We used the Gene Expression Dynamics Inspector (GEDI) to determine the global patterns of gene expression in the four different conditions, untreated, luteolin-treated, LPS-treated, and luteolin + LPS-treated microglia. GEDI is based on self-organizing maps to identify genome-wide transcriptome activity via ‘gestalt’ recognition [38]. GEDI is sample-oriented rather than gene-oriented, which facilitates the identification of genome-wide patterns. Each mosaic tile in the GEDI map represents a gene group or cluster that is expressed at similar levels, with blue color indicating a low level and red corresponding to high expression. The four GEDI maps clearly show a dynamic regulation of gene expression in stimulated versus non-stimulated microglia (Fig. 2). Especially the upper right positions in the control dataset and more pronounced in the LPS-treated dataset display an inverse regulation of the gene clusters following luteolin stimulation (Fig. 2A, white circles). These results demonstrate that stimulation with luteolin has a major impact on the global pattern of gene expression notably in activated microglia and to a lower extend in resting cells.

**Figure 1** Dose-dependent suppression of LPS-induced pro-inflammatory gene expression in BV-2 microglia. BV-2 microglia were treated with the indicated concentrations of luteolin for 1 h and then stimulated with 10 ng/ml or 50 ng/ml LPS for 24 h. Gene expression levels of (A) interleukin 6 (Il6), (B) chemokine (C-X-C motif) ligand 10 (Cxcl10), (C) interferon-regulatory factor 7 (Irf7), and (D) interferon-inducible gene 44 (Ifi) were analyzed with real-time qRT-PCR. Expression was normalized to the control gene Gusb and mRNA levels (+/− SEM) are graphed relative to mock-treated control cells. Results are calculated from three independent experiments performed in triplicate measurements. * p ≤ 0.05, ** p ≤ 0.01, and *** p ≤ 0.001 for luteolin + LPS vs. LPS, respectively.

Dirscherl et al. Journal of Neuroinflammation 2010, 7:3
http://www.jneuroinflammation.com/content/7/1/3
We have previously shown that a single probe analysis of Affymetrix microarrays using the Genomatix ChipInspector tool with the Significance Analysis of Microarray (SAM) algorithm improves detection of regulated transcripts [40]. We therefore used ChipInspector to analyze our dataset applying a false discovery rate (FDR) of 0.1%, a minimal probe coverage of 3, and a minimum log2 ratio of 1 (fold change of 2.0). Thereby, 18 significantly regulated genes were identified in luteolin-treated versus non-treated microglia (Table 2) and 54 differentially expressed genes were detected in luteolin + LPS versus LPS-stimulated cells (Table 3). Comparison of the total number of differentially expressed genes indicated that the effects of luteolin were more pronounced in LPS-activated BV-2 cells than in resting microglia. Also, the overall fold change range was higher in the LPS-treated microglia than in the resting BV-2 cells when stimulated with luteolin. These findings correlate with the gene cluster patterns observed in GEDI analysis and implicate that luteolin is an effective counter-player of pro-inflammatory microglial activation.

**Luteolin regulates important immune pathways and changes the microglial transcriptional phenotype**

Our next aim was to put the newly identified luteolin-regulated expression patterns into a specific biological context. We used all differentially expressed genes to perform a classification into functional categories with the Database for Annotation, Visualization, and Integrated Discovery (DAVID). The major enriched functional categories in luteolin-treated resting microglia were anti-oxidants, phagocytosis, and anti-inflammation for up-regulated genes and pro-inflammation and apoptosis for down-regulated genes (Fig. 2B, upper part). These pathways indicate that luteolin stimulated antioxidant and anti-inflammatory transcriptional programs which could potentially reflect M2 macrophage polarization. Moreover, basal pro-inflammatory and pro-apoptotic gene expression was blocked in non-activated BV-2 microglia. In luteolin + LPS co-treated versus LPS-treated cells, 11 induced transcripts represented pathways of lipid metabolism, antigen presentation, and anti-oxidants (Fig. 2B, bottom left). Interestingly, a large number of down-regulated genes covered the pathway categories pro-inflammation, toll-like receptor (TLR) signaling, acute phase response, apoptosis, and complement factors (Fig. 2B, bottom right). These results implicate that luteolin-induced transcriptomic changes promote a stable anti-inflammatory, anti-oxidant, and anti-apoptotic microglial phenotype reminiscent of M2 macrophages. Our microarray data also identified a significant number of genes that could not be grouped into larger immune-related pathways and that have not been previously connected to microglial activation or flavonoid stimulation (Tables 2 and 3).

**Microarray validation by qRT-PCR confirms novel luteolin-regulated target genes**

To validate a significant portion of the DNA microarray results, real-time qRT-PCR analyses were carried out with RNA samples from three independent BV-2 replicate experiments. We focused on representative candidates from different biological pathway which have not been previously described as flavonoid targets. 22 genes
fulfilled these criteria and were analyzed with qRT-PCR. In the first set of experiments, mRNA levels of genes up-regulated by luteolin treatment were assessed (Fig. 3). Transcripts of Sulfirexodin 1 (Srxn1), Biliverdin reductase B (Blvrb), Heme oxygenase 1 (Hmox1), and Glutamate-cysteine ligase (Gclm) are components of the cellular anti-oxidant response [41-43] and were all induced by luteolin treatment (Fig. 3A-D). We could further validate increased levels of Lysophosphatidylcholine acyltransferase (Lpcat1), Ring finger protein 145 (Rnf145), Cd36 antigen, Kinase insert domain receptor (Kdr, alias Vegfr2), and Cd83 antigen. Lpcat1 catalyzes the inactivation of inflammatory lipids [44], whereas Cd36 and Kdr are surface receptors required for phagocytic uptake and chemotactic migration, respectively [45,46]. The function of Rnf145 is currently unknown.

In the next set of qRT-PCR experiments, down-regulated transcripts involved in pro-inflammatory activation and acute phase response were analyzed. Complement factor C3 (C3), Complement factor b (Cfb), Secreted leukocyte peptidase inhibitor (Slp), and the pro-inflammatory Guanylate binding proteins 2 (Gbp2) and Gbp3 [47] showed diminished mRNA levels in luteolin-treated samples (Fig. 4A-D). The same tendency was seen in the newly identified microRNA miR-147, which is involved in toll-like receptor signaling [48], the acute phase gene Haptoglobin (Hp), the stress-related gene Nuclear protein 1 (Nupr1, alias p8) [49], and Cystatin 7 (Cst7) (Fig. 4F-I).

As third group of genes validated by qRT-PCR, four luteolin-repressed genes related to apoptosis and microglial shape formation were studied (Fig. 5A-D). The apoptotic mediators DNA-damage inducible transcript 3 (Ddit3, alias Chop, Gadd153) and Tribbles homolog 3 [50,51] were down-regulated by luteolin in non-activated as well as LPS-activated microglia (Fig. 5A, B), indicating anti-apoptotic protection mechanisms elicited by luteolin. In line with these findings, we also observed reduced expression of the Macrophage receptor with collagenous structure (Marco) and Lipocalin 2 (Lcn2), two molecules with dual roles in apoptosis and dem-ramification of activated microglia [52,53].

### Table 2 Differentially expressed transcripts after 24 h stimulation of BV-2 cells with 50 μM luteolin

| Nr | ID    | Symbol | Gene Name                  | FC* | Cov |
|----|-------|--------|----------------------------|-----|-----|
| **UP-REGULATED** |       |        |                            |     |     |
| 1  | 74315 | Rnf145 | Ring finger protein 145    | 2.87| 11  |
| 2  | 76650 | Srxn1  | Sulfirexodin 1 homolog     | 2.38| 5   |
| 3  | 19252 | Dusp1  | Dual specificity phosphatase 1 | 2.35| 10  |
| 4  | 12267 | C3ar1  | Complement component 3a receptor 1 | 2.30| 22  |
| 5  | 12491 | Cd36   | CD36 antigen               | 2.28| 10  |
| 6  | 12475 | Cd14   | CD14 antigen               | 2.25| 10  |
| 7  | 233016| Blvrb  | Biliverdin reductase B     | 2.22| 9   |
| 8  | 15368 | Hmox1  | Heme oxygenase 1           | 2.17| 10  |
| 9  | 210992| Lpcat1 | Lysophosphatidylcholine acyltransferase 1 | 2.13| 11  |
| **DOWN-REGULATED** |       |        |                            |     |     |
| 1  | 13198 | Ddit3  | DNA-damage inducible transcript 3 | -5.21| 11 |
| 2  | 12862 | Cox6a2 | Cytochrome c oxidase, subunit VI a, polypeptide 2 | -2.89| 9 |
| 3  | 228775| Trib3  | Tribbles homolog 3         | -2.62| 22 |
| 4  | 56312 | Nupr1  | Nuclear protein 1          | -2.36| 15 |
| 5  | 13011 | Cst7   | Cystatin F                 | -2.19| 9 |
| 6  | 223920| Soat2  | Sterol O-acyltransferase 2 | -2.16| 8 |
| 7  | 16149 | Cd74   | CD74 antigen               | -2.10| 8 |
| 8  | 17064 | Cd93   | CD93 antigen               | -2.08| 11 |
| 9  | 213002| Il1mt6 | Interferon induced transmembrane protein 6 | -2.06| 8 |

Significance analysis of triplicate microarrays was performed with a false discovery rate of 0.1% and a minimum probe coverage of 3. ID, Entrez Gene ID; FC: Fold change; Cov: probe coverage; * validated by Taqman realtime RT-PCR

Luteolin changes microglial morphology and inhibits NO secretion

To assess whether the particular gene expression profiles measured in luteolin-stimulated microglia translate into detectable functional properties, phenotypic characterization was performed. The general activation state and morphological phenotype of microglia is particularly reflected by their cell shape and cytoskeletal organization. To detected morphological changes and for visualization of filopodia we performed phalloidin staining. Conventional BV-2 microglia cultures were low level activated cells with a flat shape and some filopodia (Fig. 6A). Culture of BV-2 cells in the presence of luteolin lead to considerable ramification and formation of long protrusions (Fig. 6B), indicating induction of a surveillance state. LPS-activation of BV-2 cells caused
Table 3  Differentially expressed transcripts after 24 h stimulation with 50 μM luteolin + 50 ng/ml LPS versus 50 ng/ml LPS

| Nr | ID  | Symbol | Gene Name                                      | FC* | Cov |
|----|-----|--------|-----------------------------------------------|-----|-----|
|    | 74315 | Rnf145 | Ring finger protein 145                        | 3.27 | 11  |
| 1  | 210992 | Lpcat1 | Lysophosphatidylcholine acyltransferase 1      | 2.55 | 11  |
| 2  | 12522  | Cd83   | CD83 antigen                                   | 2.51 | 11  |
| 3  | 14630  | Gclm   | Glutamate-cysteine ligase, modifier subunit    | 2.46 | 10  |
| 4  | 16889  | Lipa   | Lysosomal acid lipase A                        | 2.31 | 6   |
| 5  | 56336  | B4galt5| UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 5 | 2.30 | 8   |
| 6  | 14950  | H13    | Histocompatibility 13                          | 2.30 | 7   |
| 7  | 14104  | Fasn   | Fatty acid synthase                            | 2.28 | 4   |
| 8  | 12125  | Bcl2l11| BCL2-like 11                                   | 2.16 | 14  |
| 9  | 16542  | Kdr    | Kinase insert domain protein receptor          | 2.16 | 9   |
| 10 | 216345 | Ccdc131| Coiled-coil domain containing 131              | 2.11 | 7   |
|    |        |        | **DOWN-REGULATED**                             |     |     |
| 1  | 66141  | Ifitm3  | Interferon induced transmembrane protein 3     | -13.74 | 9   |
| 2  | 433470 | AA467197| Expressed sequence AA467197, miR-147          | -9.32 | 9   |
| 3  | 16819  | Lcn2   | Lipocalin 2                                    | -6.87 | 10  |
| 4  | 14469  | Gbp2   | Guanylate binding protein 2                    | -6.41 | 16  |
| 5  | 14962  | Cfb    | Complement factor B                            | -5.94 | 8   |
| 6  | 13198  | Ddit3  | DNA-damage inducible transcript 3              | -5.58 | 11  |
| 7  | 16181  | Il1rn  | Interleukin 1 receptor antagonist               | -5.28 | 33  |
| 8  | 55932  | Gbp3   | Guanylate binding protein 3                    | -4.72 | 10  |
| 9  | 56312  | Nupr1  | Nuclear protein 1                              | -4.35 | 15  |
| 10 | 75345  | Slamf7 | SLAM family member 7                           | -4.14 | 9   |
| 11 | 20210  | Saa3   | Serum amyloid A 3                              | -3.78 | 7   |
| 12 | 17386  | Mmp13  | Matrix metallopeptidase 13                     | -3.63 | 11  |
| 13 | 15439  | Hp     | Haptoglobin                                    | -3.56 | 11  |
| 14 | 17167  | Marco  | Macrophage receptor with collagenous structure | -3.34 | 8   |
| 15 | 12266  | C3     | Complement component 3                        | -3.29 | 6   |
| 16 | 12870  | Cp     | Ceruloplasmin                                  | -3.05 | 5   |
| 17 | 13011  | Cst7   | Cystatin F                                    | -3.03 | 9   |
| 18 | 23882  | Gadd45g| Growth arrest and DNA-damage-inducible 45 gamma| -2.95 | 11  |
| 19 | 242341 | Atp6v0d2| ATPase, H+ transporting, lysosomal V0 subunit D2| -2.81 | 13  |
| 20 | 68774  | Moea6d | Membrane-spanning 4-domains, subfamily A, member 6D| -2.75 | 19  |
| 21 | 83673  | Snhg1  | Small nucleolar RNA host gene 1                | -2.75 | 24  |
| 22 | 12494  | Cd38   | CD38 antigen                                   | -2.73 | 6   |
| 23 | 19655  | Rbmx   | RNA binding motif protein, X chromosome        | -2.73 | 11  |
| 24 | 56405  | Dusp14 | Dual specificity phosphatase 14                | -2.68 | 10  |
| 25 | 213002 | Ifitm6  | Interferon induced transmembrane protein 6     | -2.57 | 8   |
| 26 | 12517  | Cd72   | CD72 antigen                                   | -2.55 | 9   |
| 27 | 14129  | Fcgr1  | Fc receptor, IgG, high affinity 1              | -2.55 | 11  |
| 28 | 14130  | Fcgr2b | Fc receptor, IgG, low affinity 1lb             | -2.48 | 27  |
| 29 | 231532 | Arhgap24| Rho GTPase activating protein 24               | -2.46 | 8   |
| 30 | 29818  | Hspb7  | Heat shock protein family, member 7 (cardiovascular) | -2.45 | 5   |
| 31 | 17476  | Mpeg1  | Macrophage expressed gene 1                    | -2.43 | 6   |
| 32 | 66222  | Serpinb1a| Serine peptidase inhibitor, clade B, member 1a| -2.41 | 10  |
| 33 | 78771  | Mctp1  | Multiple C2 domains, transmembrane 1           | -2.39 | 8   |
| 34 | 20568  | Slpi   | Secretory leukocyte peptidase inhibitor        | -2.39 | 9   |
| 35 | 12507  | Cds5   | CDS antigen                                   | -2.35 | 9   |
| 36 | 50778  | Rgs1   | Regulator of G-protein signaling 1             | -2.35 | 11  |
| 37 | 21897  | Tlr1   | Toll-like receptor 1                           | -2.20 | 5   |
Figure 3 Luteolin induces anti-oxidant, anti-inflammatory, and survival pathways. Real-time qRT-PCR validation of transcripts in BV-2 microglia stimulated with 50 μM luteolin, 50 ng/ml LPS, or 50 μM luteolin + 50 ng/ml LPS. Relative mRNA levels were quantified for (A) Sulfiredoxin 1 (Srxn1), (B) Biliverdin reductase b (Blvrb), (C) Heme oxygenase 1 (Hmox1), (D) Glutamate-cysteine ligase modifier subunit (Gclm), (E) Lysophosphatidylcholine acyltransferase 1 (Lpcat1), (F) Ring finger protein 145 (Rnf145), (G) Cd36 antigen (Cd36), (H) Kinase insert domain protein receptor (Kdr), and (I) Cd83 antigen (Cd83). Expression was normalized to the control gene Gusb and mRNA levels (+/- SEM) are graphed relative to mock-treated control cells. Results are calculated from three independent experiments performed in triplicate measurements. * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001 for luteolin vs. control and luteolin + LPS vs. LPS, respectively.
Figure 4 Luteolin inhibits pro-inflammatory and stress-related pathways. Real-time qRT-PCR validation of transcripts in BV-2 microglia stimulated with 50 μM luteolin, 50 ng/ml LPS, or 50 μM luteolin + 50 ng/ml LPS. Relative mRNA levels were quantified for (A) Complement component 3 (C3), (B) Complement factor B (Cfb), (C) Serine leukocyte peptidase inhibitor (Spli), (D) Guanylate binding protein 2 (Gnbp2), (E) Guanylate binding protein 3 (Gnbp3), (F) micro RNA 147 (miR-147), (G) Haptoglobin (Hp), (H) Nuclear protein 1 (Nupr1), and (I) Cystatin F (Cst7). Expression was normalized to the control gene Gusb and mRNA levels (+/- SEM) are graphed relative to mock-treated control cells. Results are calculated from three independent experiments performed in triplicate measurements. * p ≤ 0.05, ** p ≤ 0.01 for luteolin vs. control and luteolin + LPS vs. LPS, respectively.
formation of a round cell shape with retracted filopodia (Fig. 6C). In contrast, co-incubation of LPS-treated cells with luteolin nearly completely abolished NO secretion (Fig. 6I). These data indicate that luteolin favors the ramified surveillance state of microglia and effectively inhibits cytotoxic NO formation. Kim et al. previously reported that luteolin triggered a blockade of NO secretion in LPS-stimulated BV-2 microglia, which was mediated by inhibition of inducible NO synthase (iNos) protein expression [54]. To independently verify these data by qRT-PCR, we analyzed iNos mRNA levels in BV-2 cells treated with luteolin, LPS, or both simultaneously. LPS caused a more than 40-fold increase in iNos transcripts and luteolin co-treatment significantly reduced iNos levels more than 2-fold. Thus, our expression data corroborate the findings by Kim et al. and provide a reasonable explanation for reduced NO secretion levels.

To test the hypothesis that luteolin leads to decreased microglial neurotoxicity, a culture system of 661W photoreceptor cells with conditioned medium (CM) from BV-2 cells or primary microglia was established. 661W, a retina-derived cell line [55], represents a valuable model to study microglial neurotoxicity in the special context of retinal degeneration [36]. 661W cells were incubated for 24 h with culture supernatants from unstimulated, luteolin-, LPS- or LPS + luteolin-treated cells and 661W photoreceptor cell morphology was assessed by phase contrast microscopy. 661W cells in their own medium grew confluent after 24 h and the presence of CM from control- or luteolin-treated BV-2 cells did not affect cell morphology (Fig. 7A, B). As described previously [56], confluent 661W cells flattened out and often multiple cells were connected to each other through their projected extensions (Fig. 7A, B). In contrast, 661W cells co-incubated with LPS-stimulated BV-2 supernatant appeared elongated and smaller, leading to prominent cell-free areas present in the culture (Fig. 7C). When adding CM from LPS + luteolin-stimulated BV-2 cells, nearly normal cell characteristics were retained (Fig. 7D). Similar morphological changes of 661W cells were observed when cultured in the presence of primary microglia CM (Fig. 7E-H). Direct incubation of photoreceptor cells with LPS, luteolin, or both had no effects on cell morphology (data not shown), indicating that the observed changes in 661W cell growth arise from secreted microglial compounds.

To correlate the microscopic findings with functional data, we next studied the influence of microglia-secreted products on caspase-related apoptotic signaling in the neuronal cell model. 661W cells cultured in the presence of CM from LPS-stimulated BV-2 cells displayed a strong induction of caspase 3/7 activity (Fig. 7I). When CM from microglia co-treated with LPS + luteolin was used, 661W apoptosis was still present but was

Figure 5 Luteolin inhibits apoptosis-related pathways and reduces de-ramification genes. Real-time qRT-PCR validation of transcripts in BV-2 microglia stimulated with 50 μM luteolin, 50 ng/ml LPS, or 50 μM luteolin + 50 ng/ml LPS. Relative mRNA levels were quantified for (A) DNA-damage inducible transcript 3 (Ddit3), (B) Tribbles homolog 3 (Trib3), (C) Macrophage receptor with collagenous structure (Marco), and (D) lipocalin 2 (Lcn2). Expression was normalized to the control gene Gusb and mRNA levels (+/- SEM) are graphed relative to mock-treated control cells. Results are calculated from three independent experiments performed in triplicate measurements. * p ≤ 0.05 for luteolin vs. control and luteolin + LPS vs. LPS, respectively.
Figure 7 Luteolin inhibits microglial neurotoxicity on photoreceptors. Phase contrast micrographs showing morphological changes of 661W photoreceptor cultures treated with conditioned media from BV-2 cells (A-D) or primary brain microglia (E-H). The supernatant from control-stimulated (A, E), 50 μM luteolin-treated (B, F), 50 ng/ml LPS-treated (C, G), or 50 μM luteolin + 50 ng/ml LPS-treated cells (D, H) was added to 661W photoreceptor cells, respectively. The micrographs shown are from one representative experiment out of three independent experiments with the same tendencies. Scale bar, 50 μM. (I) Apoptosis-related caspase 3/7 activation in 661W photoreceptor cells incubated with conditioned media from control-stimulated, 50 μM luteolin-treated, 50 ng/ml LPS-treated, or 50 μM luteolin + 50 ng/ml LPS-treated BV-2 cells. Results are calculated from two independent experiments performed in triplicate measurements. ** p ≤ 0.01, * p ≤ 0.05 for LPS vs. control and luteolin + LPS vs. LPS, respectively.
significantly diminished (Fig. 7I). Culture media from BV-2 cells kept in the presence of luteolin alone had no effect on 661W apoptosis. These findings implicate that luteolin either stimulates microglia to produce less pro-apoptotic substances or actively promotes the release of survival signals.

**Discussion**

Like other plant-derived flavonoids, luteolin has a variety of biological activities including well-known anti-mutagenic and anti-tumorigenic properties [57]. Moreover, this flavone possesses direct anti-oxidant activity, which is attributed to structural features of all flavonoids, which favor scavenging of reactive oxygen and nitrogen species [58]. Although the anti-oxidant and anti-inflammatory activities of luteolin may be also useful in the treatment of many chronic inflammatory diseases including neurodegeneration, only little information is available about luteolin-mediated transcriptional mechanisms or molecular targets in microglia [59].

We have therefore performed the first genome-wide study of luteolin-mediated transcriptional effects in microglia. To our surprise, luteolin treatment did not only change expression levels of a few transcripts but had a broad and strong impact on the transcriptome of resting and particularly of LPS-activated microglia. The microarray dataset and the qRT-PCR validations revealed several luteolin-regulated pathways. Luteolin caused simultaneous up-regulation of four important anti-oxidant enzymes Srxn1, Blvrb, Gclm, and Hmox1. These data are consistent with earlier findings demonstrating increased Hmox1 transcription in RAW264.7 macrophages after luteolin treatment [60]. Stimulation with the flavonoid induced binding of the transcription factor NF-E2-related factor 2 (Nrf2) to anti-oxidant response elements (ARE) in the Hmox1 promoter region [60]. Luteolin is a potent activator of Nrf2 [61] and the majority of anti-oxidant enzymes contain ARE in their regulatory regions, including Srxn1 [62]. Moreover, mouse embryonic fibroblasts derived from Nrf2 -/- mice showed significantly lower Blvrb and Gclm mRNA levels upon Diquat induction [63]. Therefore, we speculate that increased microglial expression of Srxn1, Blvrb, and Gclm is also mediated by activation of Nrf2. This hypothesis is further corroborated by the protective functions of Nrf2 in several microglia-related neurodegenerative disorders [64].

Luteolin significantly enhanced mRNA synthesis of five other genes involved in different biological pathways. Lpca1 is a lysophospholipid acyltransferase implicated in anti-inflammatory responses by converting lysoplatelet activation factor (lyso-PAF) to PAF and lysophosphatidylcholine (lyso-PC) to PC [65]. LPC exerts considerable neuro-inflammatory reactivity in the brain and inhibition of LPC signaling in astrocytes and microglia confers neuroprotection [66]. Lpca1 is also highly expressed in the retina [44], indicating that luteolin-induced Lpca1 levels could lead to diminished LPC levels in retinal microglia. Rnf145 was also up-regulated by luteolin but the function of this protein remains to be determined. Cd36 and Kdr (alias Vegfr2) were also significantly induced by luteolin in non-activated as well as activated microglia. The pattern recognition receptor Cd36 signals to the actin cytoskeleton and regulates microglial migration and phagocytosis [67], whereas Kdr is involved in the chemotactic response of microglia [46]. We thus speculate that luteolin-mediated expression of both genes could result in increased phagocytic responses of microglia without inducing inflammation.

Several reports have demonstrated that luteolin inhibits pro-inflammatory cytokine expression in various cell types by blocking NFkB (reviewed in [28]). Our microarray data confirmed these findings and revealed further NFkB target genes including the recently discovered microRNA miR-147 [48]. Recently, Jang et al. showed that luteolin reduced Il6 production mainly by inhibiting JNK signaling and AP1 activation [34]. Luteolin did not affect IkB-α degradation or NFkB DNA binding in brain microglia, implicating that luteolin-mediated effects in microglia are not solely dependent on NFkB blockade [34]. In line with this notion, our luteolin-regulated expression profiles identified several genes with NFkB-independent promoter control. Likewise, luteolin down-regulated complement factor 3, which is regulated by AP1 [68] and blocked expression of Slpi, which is a target of interferon regulatory factor 1 (IRF1) [69]. Luteolin also diminished mRNA levels of the pro-inflammatory GTPase Gbp2 and the acute phase protein Haptoglobin, which are both regulated by signal transducers and activators of transcription (STATs) [70,71]. These data clearly show that luteolin dampens microglia activation by interfering with several divergent signaling pathways.

The luteolin-regulated differential expression patterns also revealed genes involved in microglial apoptosis and ramifications. Microglia are more susceptible than macrophages to apoptosis [72] and recent evidence indicates that microglial apoptosis and senescence may precede neurodegeneration [73]. Ddit3 and Trib3, which are both induced by LPS and suppressed by luteolin, support stress and NO-mediated apoptosis [50,51]. We therefore hypothesize that luteolin could promote the survival of activated and stressed microglia in an environment of early neurodegeneration. Our expression data also revealed the unexpected finding that luteolin down-regulated Lcn2 and Marco, two molecules involved in microglial ramification and formation of filopodia. Lee et al. demonstrated that stable expression of Lcn in BV-2 microglia, the same cell line we used in our
experiments, induced a round cell shape with a loss of processes [52]. In line with this, over-expression of the scavenger receptor Marco in dendritic cells caused rounding of cells and down-regulated antigen uptake [53]. Thus, we hypothesized that the observed changes in mRNA levels of both genes might also translate into different morphological characters.

The morphological and functional assays fully supported the implications from gene expression profiles and revealed a direct effect of luteolin on the microglial phenotype. Luteolin stimulated the formation of filopodia and caused ramification of BV-2 cells and primary microglia even in the setting of strong LPS activation. Moreover, NO secretion was completely blocked in LPS-activated microglia upon co-incubation with luteolin. We studied the effects of conditioned media from microglia on cultured photoreceptor-like 661W cells and demonstrated that luteolin-treatment effectively protected 661W cells from LPS-induced microglial toxicity. Since NO and other reactive oxygen species are the major radicals secreted from microglia, we speculate that luteolin directly inhibits the secretion of these cytotoxic radicals. Our hypothesis is corroborated by recent data demonstrating that luteolin concentration-dependently attenuated LPS-induced dopaminergic neuron loss by blocking NO secretion from cultured rat microglia [32].

Conclusions

We have shown that the flavonoid luteolin is a potent modulator of microglial activation, cell shape, and effector functions. Luteolin induced global changes in the transcriptome of resting or LPS-activated microglia leading to a polarized M2-like phenotype with anti-inflammatory and neuroprotective characteristics. Luteolin’s mechanisms of action appear to target several independent pathways independent of NFKB. Our results provide a basis to develop immuno-modulatory and neuroprotective therapies for the treatment of neurodegenerative disorders.

Acknowledgements

This work was supported by grants from the German Research Foundation (FOR1075 Project 4), the Elite Network of Bavaria, and the Pro Retina Foundation. The authors thank Prof. Muayyad Al Ubaidi for providing the 661W photoreceptor cell line.

Author details

1Institute of Human Genetics, University of Regensburg, Franz-Josef-Strauss-Allee 11, 93053 Regensburg, Germany. 2Center of Excellence for Fluorescent Bioanalytics, University of Regensburg, Josef-Engert-Str 9, 93053 Regensburg, Germany. 3Institute of Human Anatomy and Embryology, University of Regensburg, Universitätsstr 31, 93053 Regensburg, Germany.

Authors’ contributions

KD, SE, and DK carried out all cell cultures and qRT-PCR experiments. MK, JH, and YW analyzed qRT-PCR and functional data. CM performed microarray hybridizations and raw data analyses. RF analyzed microarray data and critically read the manuscript. TL designed the study, obtained funding, carried out biostatistical analyses of microarrays and wrote the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Received: 2 December 2009
Accepted: 14 January 2010
Published: 14 January 2010

References

1. Hanisch UK: Microglia as a source and target of cytokines. Glia 2002, 40:140-155.
2. Hanisch UK, Kettenmann H: Microglia: active sensor and versatile effector cells in the normal and pathologic brain. Nat Neurosci 2007, 10:1387-1394.
3. Streit WJ: Microglia as neuroprotective, immunocompetent cells of the CNS. Glia 2002, 40:133-139.
4. Streit WJ: Microglia and neuroprotection: implications for Alzheimer’s disease. Brain Res Brain Res Rev 2005, 48:234-239.
5. Giulian D, Li J, Bartel S, Broker J, Li X, Kirkpatrick JB: Cell surface morphology identifies microglia as a distinct class of mononuclear phagocyte. J Neurosci 1999, 15:7712-7726.
6. Davalos D, Grutzendler J, Yang G, Kim JY, Zuo Y, Jung S, Littman DR, Dustin ML, Gan WB: ATP mediates rapid microglial response to local brain injury in vivo. Nat Neurosci 2005, 8:752-758.
7. Nimmesgabn A, Kirchhoff F, Helmchen F: Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. Science 2005, 308:1314-1318.

8. Broderick C, Hoek RM, Forrester JV, Liversidge J, Sedgwick JD, Dick AD: Constitutive retinal CCL20 expression regulates resident microglia and activation state of inflammatory cells during experimental autoimmune uveoretinitis. Am J Pathol 2002, 161:1669-1677.
9. Cardona AE, Pioro E, Sasse ME, Kostenko V, Cardona SM, Dijkstra IM, Huang D, Kidd G, Dombrovski S, Dutta R, Lee JC, Cook DN, Jung S, Lira SA, Littman DR, Ransohoff RM: Control of microglial neurotoxicity by the fractalkine receptor. Nat Neurosci 2006, 9:917-924.
10. Dick AD, Carter D, Robertson M, Broderick C, Hughes E, Forrester JV, Liversidge J: Control of myeloid activity during retinal inflammation. J Leukoc Biol 2003, 74:161-166.
11. Haynes SE, Hollópeter G, Yang G, Kuprus D, Dalley ME, Gan WB, Julius D: Control of myeloid activity during retinal inflammation. Nat Neurosci 2006, 9:1512-1519.
12. Ransohoff RM, Perry VH: Microglial physiology: unique stimuli, specialized responses. Annu Rev Immunol 2009, 27:119-145.
13. El Khoury J, Luster AD: Mechanisms of microglia accumulation in Alzheimer’s disease: therapeutic implications. Trends Pharmacol Sci 2008, 29:626-632. 14. Orr CF, Rowe DB, Halliday GM: An inflammatory review of Parkinson disease. Prog Neurobiol 2002, 68:325-340. 15. Sargsyan SA, Monk PN, Shaw PJ: Microglia as potential contributors to motor neuron injury in amyotrophic lateral sclerosis. Glia 2005, 51:241-253. 16. Ravich G, Banati R: Brain microglia and blood-derived macrophages: molecular profiles and functional roles in multiple sclerosis and animal models of autoimmune demyelinating disease. Brain Res Brain Res Rev 2004, 46:261-281. 17. Langmann T: Microglia activation in retinal degeneration. J Leukoc Biol 2007, 81:1345-1351. 18. Schuetz E, Thanos S: Microglia-targeted pharmacotherapy in retinal neurodegenerative diseases. Curr Drug Targets 2004, 5:619-627. 19. Hartle DK: Differentiation and heterogeneity in the mononuclear phagocyte system. Mucosal Immunol 2008, 1:432-441. 20. Gordon S, Taylor PR: Monocyte and macrophage heterogeneity. Nat Rev Immunol 2005, 5:953-964. 21. Moiier DM: Monocyte and macrophage heterogeneity. J Leukoc Biol 2003, 73:209-212. 22. Martinez FO, Sica A, Mantovani A, Locati M: Macrophage activation and polarization. Front Biosci 2008, 13:453-461. 23. Michelucci A, Heutax T, Grandbarbe L, Morga E, Heuschling P: Characterization of the microglial phenotype under specific pro-
inflammatory and anti-inflammatory conditions: Effects of oligomeric and fibrillar amyloid-beta. J Neuroimmunol 2009, 210:3-12.

24. Jimenez S, Baglietto-Vargas D, Caballero C, Moreno-Gonzalez I, Torres M, Sanchez-Varo R, Ruano D, Vizuete M, Gutierrez A, Vitorica J. Inflammatory response in the hippocampus of PS1M146L APPPS151M mouse model of Alzheimer's disease: age-dependent switch in the microglial phenotype from alternative to classic. J Neurosci 2008, 28:11650-11661.

25. Kigerl KA, Gensel JC, Ankency DP, Alexander JK, Donnelly DJ, Popovich PG. Identification of two distinct macrophage subsets with divergent effects causing either neurotoxicity or regeneration in the injured mouse spinal cord. J Neurosci 2009, 29:13435-13444.

26. Zhang Z, Zhang ZY, Schluensener HJ. Compound A, a plant origin ligand of glucocorticoid receptors, increases regulatory T cells and M2 macrophages to attenuate experimental autoimmune neuritis with reduced side effects. J Immunol 2009, 183:3081-3091.

27. Lleo A, Galea E, Sastre M. Distribution and biological activities of the flavonoid luteolin. Mini Rev Med Chem 2005, 64:1403-1418.

28. Lopez-Lazaro M. Distribution and biological activities of the flavonoid luteolin. Mini Rev Med Chem 2009, 9:31-59.

29. Chen CY, Peng WH, Tsai KD, Hsu SL. Luteolin and luteolin-7-O-glucoside from dandelion flower suppress iNOS and COX-2 in RAW264.7 cells. Mol Cell Biochem 2004, 265:107-113.

30. Chen CY, Peng WH, Tsai KD, Hsu SL. Luteolin suppresses inflammation-associated gene expression by blocking NF-kappab and AP-1 activation pathway in mouse alveolar macrophages. J Cell Sci 2007, 81:1602-1614.

31. Harris GK, Qian Y, Leonard SS, Sbarra DC, Shi X. Chondroitin sulfate disaccharide stimulates microglia to adopt a novel regulatory phenotype. J Neuroinflammation 2008, 5:41.

32. Chongdon T, Kettler S, Johnson RW. Luteolin reduces IL-6 production in microglia by inhibiting JNK phosphorylation and activation of AP-1. Proc Natl Acad Sci USA 2008, 105:7534-7539.

33. Shiroma K, Okada H, Furugomori M, Gods T, Takase S, Suzuki M, Hara Y, Yamamoto H, Kinia H. Intestinal absorption of luteolin and luteolin 7-O-beta-glucoside in rats and humans. FEMS Lett 1998, 438:220-224.

34. Ebert S, Schoebel T, Walczak Y, Stoeckel K, Stemml T, Moehle C, Weber BH, Langmann T. Chondroitin sulfate disaccharide stimulates microglia to adopt a novel regulatory phenotype. J Leukoc Biol 2008, 84:736-740.

35. Brazma A, Hingamp P, Quackenbush J, Sherlock G, Spellman P, Stoeckert C, Aach J, Ansorge W, Ball CA, Causton HC, Gaasterland T, Glisson P, Holstege FC, Engert F, van Hartevelt PJ, Vilo J, Bittner M, Saldanha J, Lashkari D, Nolan G, Conklin BR, Fu Y, Stumpf M, Yang Y, Zody MC, Deb K, Moch R, Doerks T, Ebisuya M, Ramage D, thiell G, Tavare S, Shymra J, Kololo H, Donninger T, Jin U, Billker O, Halbeisen D, Haunstein S, Tabb D, Kepler T, Yu A, Vingron M. Minimum information about a microarray experiment (MIAME)-toward standards for microarray data. Nat Genet 2001, 29:365-371.

36. Eichler GS, Huang S, Ingber DE. Gene Expression Dynamics Inspector (GEDI); for integrative analysis of expression profiles. Bioinformatics 2003, 19:2301-2302.

37. Dennis G Jr, Sherman BT, Hesterman OY,Comparative pathway-driven antioxidant response through phosphatidylinositol 3-kinase signaling. Arch Pharm Res 2007, 30:1590-1598.

38. Elserer GM, Merfort I, Schirrmeier MM. Anti-oxidant, anti-inflammatory and anti-allergic activities of luteolin. Planta Med 2008, 74:167-177.

39. Elserer GM, Merfort I, Schirrmeier MM. Anti-oxidant, anti-inflammatory and anti-allergic activities of luteolin. Planta Med 2008, 74:167-177.

40. Elserer GM, Merfort I, Schirrmeier MM. Anti-oxidant, anti-inflammatory and anti-allergic activities of luteolin. Planta Med 2008, 74:167-177.

41. Elserer GM, Merfort I, Schirrmeier MM. Anti-oxidant, anti-inflammatory and anti-allergic activities of luteolin. Planta Med 2008, 74:167-177.

42. Elserer GM, Merfort I, Schirrmeier MM. Anti-oxidant, anti-inflammatory and anti-allergic activities of luteolin. Planta Med 2008, 74:167-177.

43. Elserer GM, Merfort I, Schirrmeier MM. Anti-oxidant, anti-inflammatory and anti-allergic activities of luteolin. Planta Med 2008, 74:167-177.

44. Elserer GM, Merfort I, Schirrmeier MM. Anti-oxidant, anti-inflammatory and anti-allergic activities of luteolin. Planta Med 2008, 74:167-177.

45. Elserer GM, Merfort I, Schirrmeier MM. Anti-oxidant, anti-inflammatory and anti-allergic activities of luteolin. Planta Med 2008, 74:167-177.
64. de Vries HE, Witte M, Hondius D, Rozemuller AJ, Drukarch B, Hoozemans J, van Horssen J. Nrf2-induced antioxidant protection: a promising target to counteract ROS-mediated damage in neurodegenerative disease. Free Radic Biol Med 2008, 45: 1375-1383.
65. Harayama T, Shindou H, Ogasawara R, Suwabe A, Shimizu T. Identification of a novel noninflammatory biosynthetic pathway of platelet-activating factor. J Biol Chem 2008, 283: 1097-1106.
66. Sheikh AM, Nagai A, Ryu JK, McLarnon JG, Kim SU, Masuda J. Lysophosphatidylcholine induces glial cell activation: role of rho kinase. Glia 2009, 57: 898-907.
67. Stuart LM, Bell SA, Stewart CR, Silver JM, Richard J, Goss JL, Tseng AA, Zhang A, El Khoury JB, Moore KI. CD36 signals to the actin cytoskeleton and regulates microglial migration via a p130Cas complex. J Biol Chem 2007, 282: 27392-27401.
68. Martin CB, Ingersoll SA, Martin BK. Transcriptional control of the C3a receptor gene in glial cells: Dependence upon AP-1 but not Ets. Mol Immunol 2007, 44: 709-712.
69. Nguyen H, Teskey L, Lin R, Hiscott J. Identification of the secretory leukocyte protease inhibitor (SLPI) as a target of IRF-1 regulation. Oncogene 1999, 18: 5455-5463.
70. Ramsauer K, Farlik M, Zupkovitz G, Seiser C, Krogner A, Hauser H, Decker T. Distinct modes of action applied by transcription factors STAT1 and IRF1 to initiate transcription of the IFN-gamma-inducible gbp2 gene. Proc Natl Acad Sci USA 2007, 104: 2849-2854.
71. Zauberman A, Lapter S, Zipori D. Smad proteins suppress CCAAT/enhancer-binding protein (C/EBP) beta- and STAT3-mediated transcriptional activation of the haptoglobin promoter. J Biol Chem 2001, 276: 24719-24725.
72. White CA, McCombe PA, Pender MP. Microglia are more susceptible than macrophages to apoptosis in the central nervous system in experimental autoimmune encephalomyelitis through a mechanism not involving Fas (CD95). Int Immunol 1998, 10: 935-941.
73. Streit WJ, Braak H, Xue QS, Bechmann I. Dystrophic (senescent) rather than activated microglial cells are associated with tau pathology and likely precede neurodegeneration in Alzheimer’s disease. Acta Neuropathol 2009, 118: 473-485.

Cite this article as: Dirsehrl et al. Luteolin triggers global changes in the microglial transcriptome leading to a unique anti-inflammatory and neuroprotective phenotype. Journal of Neuroinflammation 2010 7:3.