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Curcumin is a potent modulator of microglial gene expression and migration

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Abstract

Background: Microglial cells are important effectors of the neuronal innate immune system with a major role in chronic neurodegenerative diseases. Curcumin, a major component of tumeric, alleviates pro-inflammatory activities of these cells by inhibiting nuclear factor kappa B (NFkB) signaling. To study the immuno-modulatory effects of curcumin on a transcriptomic level, DNA-microarray analyses were performed with resting and LPS-challenged microglial cells after short-term treatment with curcumin.

Methods: Resting and LPS-activated BV-2 cells were stimulated with curcumin and genome-wide mRNA expression patterns were determined using DNA-microarrays. Selected qRT-PCR analyses were performed to confirm newly identified curcumin-regulated genes. The migration potential of microglial cells was determined with wound healing assays and transwell migration assays. Microglial neurotoxicity was estimated by morphological analyses and quantification of caspase 3/7 levels in 661W photoreceptors cultured in the presence of microglia-conditioned medium.

Results: Curcumin treatment markedly changed the microglial transcriptome with 49 differentially expressed transcripts in a combined analysis of resting and activated microglial cells. Curcumin effectively triggered anti-inflammatory signals as shown by induced expression of *Interleukin 4* and *Peroxisome proliferator activated receptor α* . Several novel curcumin-induced genes including *Netrin G1*, *Delta-like 1*, *Platelet endothelial cell adhesion molecule 1*, and *Plasma cell endoplasmic reticulum protein 1*, have been previously associated with adhesion and cell migration. Consequently, curcumin treatment significantly inhibited basal and activation-induced migration of BV-2 microglia. Curcumin also potently blocked gene expression related to pro-inflammatory activation of resting cells including *Toll-like receptor 2* and *Prostaglandin-endoperoxide synthase 2*. Moreover, transcription of *NO synthase 2* and *Signal transducer and activator of transcription 1* was reduced in LPS-triggered microglia. These transcriptional changes in curcumin-treated LPS-primed microglia also lead to decreased neurotoxicity with reduced apoptosis of 661W photoreceptor cultures.

Conclusions: Collectively, our results suggest that curcumin is a potent modulator of the microglial transcriptome. Curcumin attenuates microglial migration and triggers a phenotype with anti-inflammatory and neuroprotective properties. Thus, curcumin could be a nutraceutical compound to develop immuno-modulatory and neuroprotective therapies for the treatment of various neurodegenerative disorders.

Background

Microglial cells are resident macrophages of the nervous system with pivotal roles in innate immune regulation and neuronal homeostasis [1,2]. They are cells of the mononuclear phagocyte lineage but their unique localization within the nervous system and their morphological

features clearly distinguish them from other macrophage populations [3]. Ramified microglial cells actively scan their environment with their long protrusions [4,5] and continuous inhibitory signals from neurons prevent microglial toxicity [6,7]. Disconnection of the microglia-neuron cross-talk [8], local danger signals such as released ATP [9], or neurotransmitter gradients [10] can lead to a functional transformation of microglial populations with a variety of effector functions. Consequently, alarmed microglia and reactive microgliosis have been identified in a variety of neurodegenerative diseases including Alzheimer's

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disease [11], Parkinson's disease [12], amyotrophic lateral sclerosis [13], multiple sclerosis [14], and inherited photoreceptor dystrophies [15]. The concept of a microglia-targeted pharmacotherapy to prevent neurodegeneration in the brain and the retina is therefore a promising approach under active investigation [16,17].

There is a growing interest in the identification of natural compounds that limit neuroinflammation and simultaneously support neuronal survival [18,19]. Among the naturally occurring immuno-modulators, curcumin ((E, E)-1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione), a major constituent of tumeric, is a herbal medicine used for centuries in India and China [20]. Curcumin has a wide range of pharmacological activities including anti-inflammatory, anti-microbial, antioxidant, and anti-tumor effects [21]. Curcumin is a particularly potent immuno-regulatory agent that can modulate the activation and function of T-cells, B-cells, neutrophils, natural killer cells and macrophages [22].

Curcumin treatment effectively inhibits the activation of microglial cells by diminishing the production of nitric oxide [23] and reducing the secretion of pro-inflammatory cytokines such as IL1 β , IL6 and TNF [24]. Moreover, curcumin blocks the LPS-mediated induction of cyclooxygenase-2 (COX2) via inhibition of the transcription factors nuclear factor kappa B (NF κ B), activator protein 1 (AP1), and signal transducers and activators of transcription (STATs) [25,26]. Recent experiments have also demonstrated that curcumin protects dopaminergic neurons against microglia-mediated neurotoxicity [27], limits brain inflammation [28], and rescues retinal cells from stress-induced cell death [29].

The inhibitory role of curcumin on pro-inflammatory gene expression in microglia is well documented. However, this information is limited to only a few well-studied examples including pro-inflammatory cytokines, Nos2 and COX2. In a genome-wide search for target genes, we investigated the transcriptomic effects of curcumin in resting and LPS-activated BV-2 microglial cultures using DNA-microarrays. Furthermore, we validated the curcumin-regulated expression of microglial transcripts with qRT-PCR and studied the related microglial migration and neurotoxicity.

Methods

Reagents

Curcumin and *E.coli* 0111:B4 lipopolysaccharide were purchased from Sigma Aldrich (Steinheim, Germany). Curcumin 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.

Cell culture

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. BV-2 cells were stimulated with 100 ng/ml LPS, 20 μ M of curcumin, or DMSO as control for 6 h. These stimulation conditions were adapted from previously published experiments [24,30]. MTT assays revealed that 100 ng/ml LPS, 20 μ M curcumin, or a combination of both had no cytotoxic effects on BV-2 cells (data not shown). 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 [31].

Scratch assay

500.000 BV-2 cells were grown in 6-well plates as 80% confluent monolayers and were wounded with a sterile 100 μ l pipette tip. Thereafter, the cells were stimulated with 100 ng/ml LPS, 20 μ M of curcumin, 100 ng/ml LPS + 20 μ M of curcumin, or DMSO as solvent control. Migration into the open scar was documented with microphotographs at different time points after wounding. The number of migrating cells was quantified by counting all cells within a 0.4 mm² region in the center of each scratch. A minimum of 5 individual cultures was used to calculate the mean migratory capacity of each cell culture condition.

Transwell migration assay

The Costar Transwell System (8- μ m pore size polycarbonate membrane) was used to evaluate vertical cell migration. 1 Mio BV-2 cells in 1.5 ml serum-free medium were added to the upper well, and 2.6 ml serum-free medium was added to the lower chamber. 100 ng/ml LPS, 20 μ M curcumin, 100 ng/ml LPS + 20 μ M curcumin, or DMSO as solvent control were added to the lower chamber medium. At the end of a 24 h incubation period, cells that had migrated to the lower surface were quantified by counting the migrated cells on the lower surface of the membrane using microscopy.

661W co-culture in microglia-conditioned medium and apoptosis assay

To test microglial neurotoxicity, a culture system of 661W photoreceptors with microglia conditioned medium was established. 661W cells were incubated for 48 h either in their own medium or with culture supernatants from unstimulated, 100 ng/ml LPS, 20 μ M curcumin, or 100 ng/ml LPS + 20 μ M curcumin treated microglial cells. The 661W cell morphology was assessed by phase contrast microscopy and apoptotic cell death 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. Relative luciferase units (RLU) reflect the level of apoptotic cell death in the different 661W cell cultures.

RNA isolation and reverse transcription

Total RNA was extracted from cultured microglial cells 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, Böblingen, 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

4 × 44 K microarrays (014868) (Agilent Technologies) were used for hybridization with three independent RNAs from non-stimulated BV-2 microglial cells or cultures treated for 6 h with 20 μM curcumin, 100 ng/ml LPS, or 20 μM curcumin + 100 ng/ml LPS, respectively. Briefly, 200 ng of total RNA were labeled with Cy3 using the Agilent Quick-Amp Labeling Kit - 1 color according to the manufacturer's instructions. cRNA was purified with the RNeasy Mini Kit (Qiagen) and labeling efficiency was determined with a NanoDrop ND-1000 photometer (PecqLab). The arrays were incubated with cRNAs in Agilent SureHyb chambers for 17 hours at 65°C while rotating. After washing, scanning was done with the Agilent G2565CA Microarray Scanner System and the resulting TIFF files were processed with Agilent Feature Extraction software (10.7.). Minimum information about a microarray experiment (MIAME) criteria were met [32]. The microarray dataset of this study is publicly available at the National Center for Biotechnology Information Gene Expression Omnibus <http://www.ncbi.nlm.nih.gov/geo/> as series record GSE23639.

Bioinformatic data analysis

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 [33]. 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. The Partek Genomics Suite (Partek Inc.) was used for ANOVA analysis and hierarchical clustering of normalized expression values. Differentially regulated transcripts in curcumin-stimulated versus non-treated and curcumin + LPS versus LPS-treated BV-2 cells, respectively, were retrieved with the Genomatix ChIPInspector program (Genomatix Software GmbH, Munich, Germany), applying the Significance Analysis of Microarray (SAM) algorithm using a false-discovery rate of 0.1%.

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 ProbeLibrary). 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 $\Delta\Delta C_t$ method for relative quantitation. A C_t (cycle threshold) < 35 was used as cutoff for estimating significantly expressed transcripts and cDNA samples with values > 35 were marked with n.e. for not expressed. C_t -values between 35 and 40 were solely used for calculation of relative expression differences in treated cells versus control cells. Primer sequences and Roche Library Probe numbers are listed in Table 1.

Statistical analyses

Statistical analyses were performed on $\Delta\Delta C_t$ data using the Mann-Whitney Rank Sum test and quantitative expression data are expressed as mean ± SD plotted at a logarithmic scale. Gene expression levels in control BV-2 cells were used as calibrators. The Student's t test or Mann-Whitney Rank Sum test were used for the comparison of experimental groups in cell migration assays and apoptosis assays as indicated. $p < 0.05$ was considered significant.

Results

Curcumin has a major impact on the microglial transcriptome

To determine the transcriptional profiles of resting and LPS-activated BV-2 microglial cells after treatment with 20 μM curcumin for 6 h, we performed DNA-microarray analyses from three independent stimulations. We first applied the Gene Expression Dynamics Inspector (GEDI) on the complete dataset to visualize the global patterns of gene expression in the four different conditions, untreated, curcumin-treated, LPS-treated, and curcumin + LPS-

Table 1 Primer pairs and Roche library probes for real-time qRT-PCR validation

Gene	F-Primer (5'-3')	R-Primer (5'-3')	Roche Library Probe
<i>Atp5b</i>	ggcacaatgcaggaaagg	tcagcaggcacatagatagcc	77
<i>C3</i>	accttacctcggcaagttct	ttgtagagctgctggtcagg	76
<i>Ccl2</i>	catccacgtgttggtca	gatcatcttgctggtgaatgagt	62
<i>Dll1</i>	ttcaactgtgagaagaagatggat	gccgaggtccacacactt	103
<i>Egr2</i>	ctaccgggtggaagacctc	aatgttgatcatccatctcc	60
<i>Il4</i>	catcggcattttgaacgag	cgagctcactctctgtggtg	2
<i>Il6</i>	gatggatgctacaaaactggat	ccaggtagctatggtactccaga	6
<i>Nos2</i>	ctttgccacggacgagac	tcattgtactctgaggctga	13
<i>Ntn1</i>	aggggcaagagaccaagg	agggatggtgtctatctctct	103
<i>Pecam1</i>	cggtgttcagcgagatcc	cgacaggatggaaatcacia	45
<i>Perp1</i>	tcatatgccggctcacct	atccactggcgtctggagt	110
<i>Pparaα</i>	ccgagggctctgtcatca	gggcagctgactgaggaa	11
<i>Ptgs2</i>	gatgctcttcgagctgtg	ggattggaacagcaaggattt	45
<i>Stat1</i>	aatgtgaaggatcaagtcatgtg	catcttgaattctctagggcttga	15
<i>Tlr2</i>	accgaaacctcagacaaagc	cagcgtttgctgaagagga	49

treated cells. GEDI uses self-organizing maps to capture genome-wide transcriptome activity via 'gestalt' recognition [33]. GEDI facilitates the identification of genome-wide patterns with each mosaic tile in the map representing a gene cluster that is expressed at similar levels. The four GEDI maps, with blue color indicating low and red color high mRNA expression levels, show a dynamic regulation of gene transcription in the cultured microglial cells (Figure 1A). The major difference between curcumin-treated resting microglial cells and control cells was a region with higher expression at the bottom of the map (Figure 1A, white rectangles). In the LPS-treated condition, mimicking a highly activated state, curcumin elicited a largely converse expression pattern with a pronounced area of weakly expressed genes (Figure 1A, white circles). These data indicate that curcumin stimulates gene expression in resting, non-activated cells but mainly dampens activation-associated transcriptional programs in LPS-primed microglia.

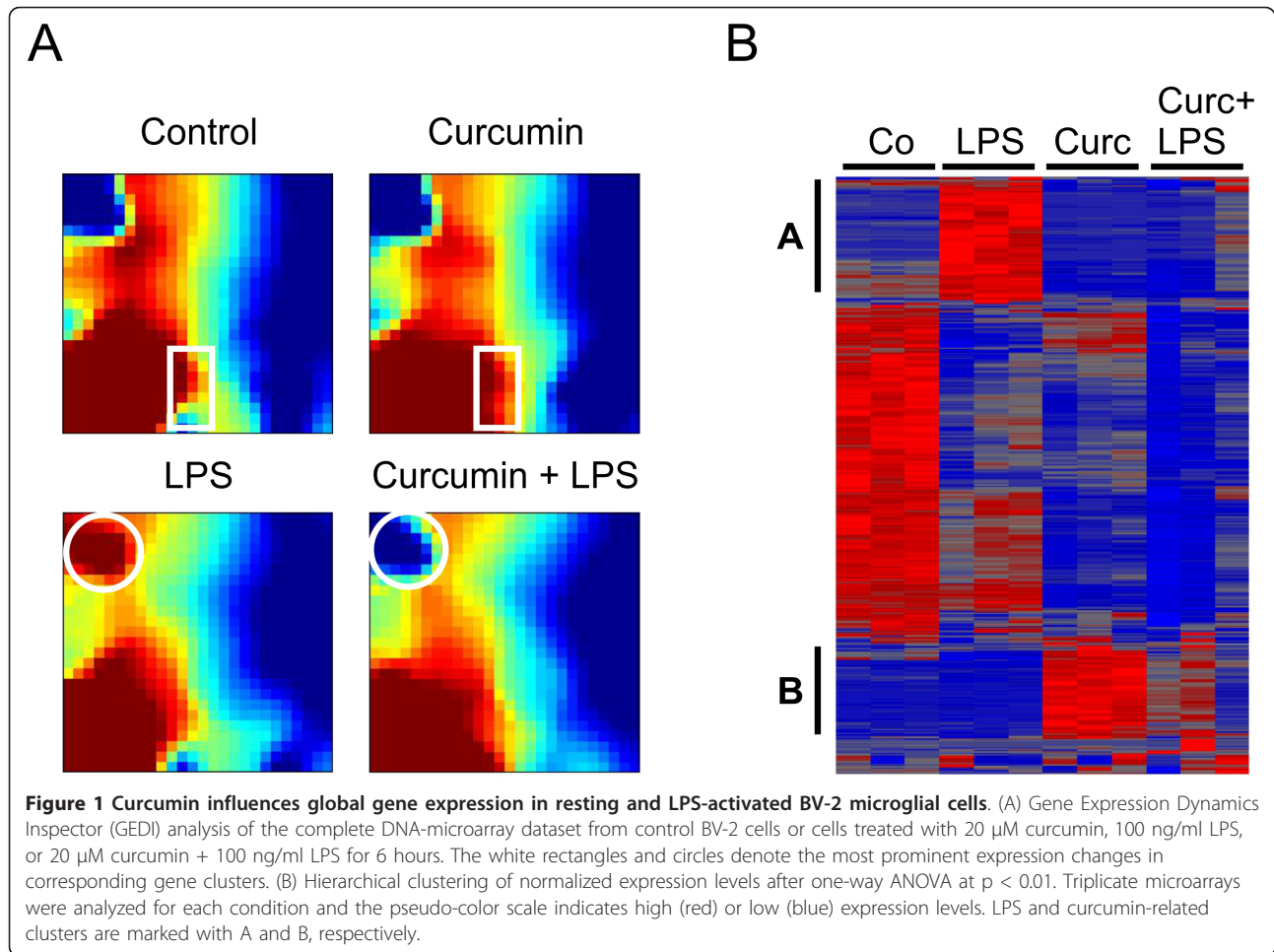
We next calculated hierarchical clusters of each individual microarray dataset after filtering for significantly altered gene expression using one way ANOVA at $p < 0.01$. This analysis showed a clear separation of the four different conditions with their own characteristic gene expression profiles (Figure 1B). The clustering revealed two distinct groups of inversely regulated genes. Group A (Figure 1B) contains LPS-induced genes, which are no longer up-regulated in the presence of curcumin. Group B (Figure 1B) represents genes selectively up-regulated by curcumin treatment of resting microglial cells. Together with the GEDI analysis, these results demonstrate that stimulation with curcumin impacts distinct patterns of gene expression in resting and LPS-activated microglial cells, respectively.

To narrow down the identified global gene clusters to a subset of genes with significantly different mRNA

expression in the different curcumin-treated conditions, we used the Genomatix ChipInspector tool applying the Significance Analysis of Microarray (SAM) algorithm at a false discovery rate of 0.1% and a minimum fold change of 2.0 [34]. Thereby, 35 significantly regulated transcripts were identified in curcumin-treated versus resting microglial cells (Table 2) and 30 differentially expressed genes were detected in curcumin + LPS versus LPS-stimulated cells (Table 3). Comparison of the total number of differentially expressed transcripts and considering overlapping gene sets revealed that curcumin affects both resting and LPS-activated BV-2 cells.

qRT-PCR confirmation of novel curcumin target genes in microglial cells

To validate selected differentially expressed genes identified by DNA-microarrays, real-time qRT-PCR assays were performed with RNA samples from three independent BV-2 stimulation series. We especially focused on genes which have not been previously shown to be curcumin targets. In the first set of experiments, mRNA levels of genes highly up-regulated by curcumin compared to control cells were assessed (Figure 2). Transcripts of Netrin G1 (*Ntn1*), Platelet endothelial cell adhesion molecule 1 (*Pecam1*), Delta-like 1 (*Dll1*), Plasma cell endoplasmic reticulum protein 1 (*Perp1*), Peroxisome proliferator activated receptor alpha (*PPAR α*), and Interleukin 4 (*Il4*) were all significantly increased by stimulation with curcumin (Figure 2). *Ntn1* showed the strongest expression difference with a change of more than 1800-fold. *Perp1* and *PPAR α* transcripts were not significantly expressed in resting microglia and were switched on to intermediate levels after curcumin treatment (Figure 2). All six transcripts after combined LPS/curcumin treatment remained similarly high as after curcumin stimulation alone, indicating



that the effects of curcumin persist in activated microglial cells.

In the next series of qRT-PCR experiments, down-regulated transcripts known to be involved in pro-inflammatory activation of microglial cells were analyzed. Toll-like receptor 2 (*Tlr2*), Early growth response 2 (*Egr2*), Prostaglandin endoperoxide synthase 2 (*Ptgs2*, alias *Cox2*), and Chemokine (C-C-motif) ligand 2 (*Ccl2*, alias *Mcp1*) showed diminished transcript levels in curcumin-treated resting BV-2 cells (Figure 3A). In the activated state, microglial cells also had the tendency to expressed lower amounts of these transcript but only *Ccl2* levels reached the level of statistical significance. When LPS-activated BV-2 cells were incubated in the presence of curcumin, transcription of Interleukin 6 (*Il6*), nitric oxide synthase 2 (*Nos2*, alias *iNos*), Signal transducer and activator of transcription 1 (*Stat1*), and Complement factor C3 (*C3*) were all repressed (Figure 3B).

Curcumin has an inhibitory effect on microglial migration

The induction of several transcripts related to cell motility and adhesion (*Ntn1*, *Pecam1*, and *Perp1*) prompted us to

study the effect of curcumin on microglial migration. We first cultured BV-2 microglia on plastic dishes until 80% confluence and then created a scratch with a pipette tip. 12 hours after stimulation of resting microglia or LPS-activated cells with 20 μ M of curcumin, migration into the cell-free scratch area was documented. Representative microscopic images clearly showed that curcumin-treated resting cells as well as activated BV-2 cells exhibit a highly reduced migratory potential (Figure 4A). The statistical analysis of five independent experiments revealed a significantly reduced number of migrating cells when curcumin was present in the culture medium (Figure 4B). As an independent measure of microglial cell motility and to study the long term effects of curcumin, we performed transwell migration assays over a period of 24 hours. Similar as in the scratch assays, the migratory capacity of BV-2 cells was not changed by the activation agent LPS alone (Figure 4C). In both, the resting and the activated microglial phenotype, curcumin caused a significant attenuation of microglial migration (Figure 4C). These results indicate that curcumin-mediated signaling events have functional consequences related to microglial motility.

Table 2 Differentially expressed transcripts after 6 h stimulation of BV-2 cells with 20 μM curcumin

Nr	ID	Symbol	Gene Name	FC
UP-REGULATED				
1	80883	Ntng1	Netrin G1	313.0
2	18613	Pecam1	Platelet/endothelial cell adhesion molecule 1	42.8
3	13388	Dll1	Delta-like 1	35.5
4	12824	Col2a1	Collagen, type II, alpha 1	21.6
5	14103	Fasl	Fas ligand (TNF superfamily, member 6)	12.8
6	12653	Chgb	Chromogranin B	11.9
7	66184	Rps4y2	Ribosomal protein S4, Y-linked 2	9.7
8	69816	Perp1	RIKEN cDNA 2010001M09 gene	9.7
9	16992	Lta	Lymphotoxin A	9.4
10	19206	Ptch1	Patched homolog 1	5.9
11	19013	Ppara	Peroxisome proliferator activated receptor alpha	4.6
12	109685	Hyal3	Hyaluronoglucosaminidase 3	4.5
13	20997	T	Brachyuri	4.2
14	17246	Mdm2	Transformed mouse 3T3 cell double minute 2	3.1
15	14102	Fas	Fas	2.8
16	14183	Fgfr2	Fibroblast growth factor receptor 2	2.7
17	13645	Egf	Epidermal growth factor	2.2
18	14179	Fgf8	Fibroblast growth factor 8	2.1
19	20655	Sod1	Superoxide dismutase 1	2.1
20	14526	Gcg	Glucagon	2.0
DOWN-REGULATED				
1	24088	Tlr2	Toll-like receptor 2	-6.9
2	18505	Pax3	Paired box gene 3	-6.6
3	14281	Fos	FBJ osteosarcoma oncogene	-4.4
4	11622	Ahr	Aryl-hydrocarbon receptor	-4.1
5	16835	Ldlr	Low density lipoprotein receptor	-3.8
6	13654	Egr2	Early growth response 2	-3.5
7	68010	Bambi	BMP and activin membrane-bound inhibitor	-3.3
8	12048	Bcl2l1	BCL2-like 1	-3.3
9	19225	Ptgs2	Prostaglandin-endoperoxide synthase 2	-3.0
10	20296	Ccl2	Chemokine (C-C motif) ligand 2	-2.8
11	12393	Runx2	Runt related transcription factor 2	-2.8
12	17311	Kitl	Kit ligand	-2.4
13	16869	Lhx1	LIM homeobox protein 1	-2.4
14	12977	Csf1	Colony stimulating factor 1	-2.4
15	20528	Slc2a4	Solute carrier family 2, member 4	-2.2

Significance analysis of triplicate microarrays was performed with a false discovery rate of 0.1%. ID, Entrez Gene ID; FC: Fold change.

Curcumin inhibits LPS-induced microglial neurotoxicity

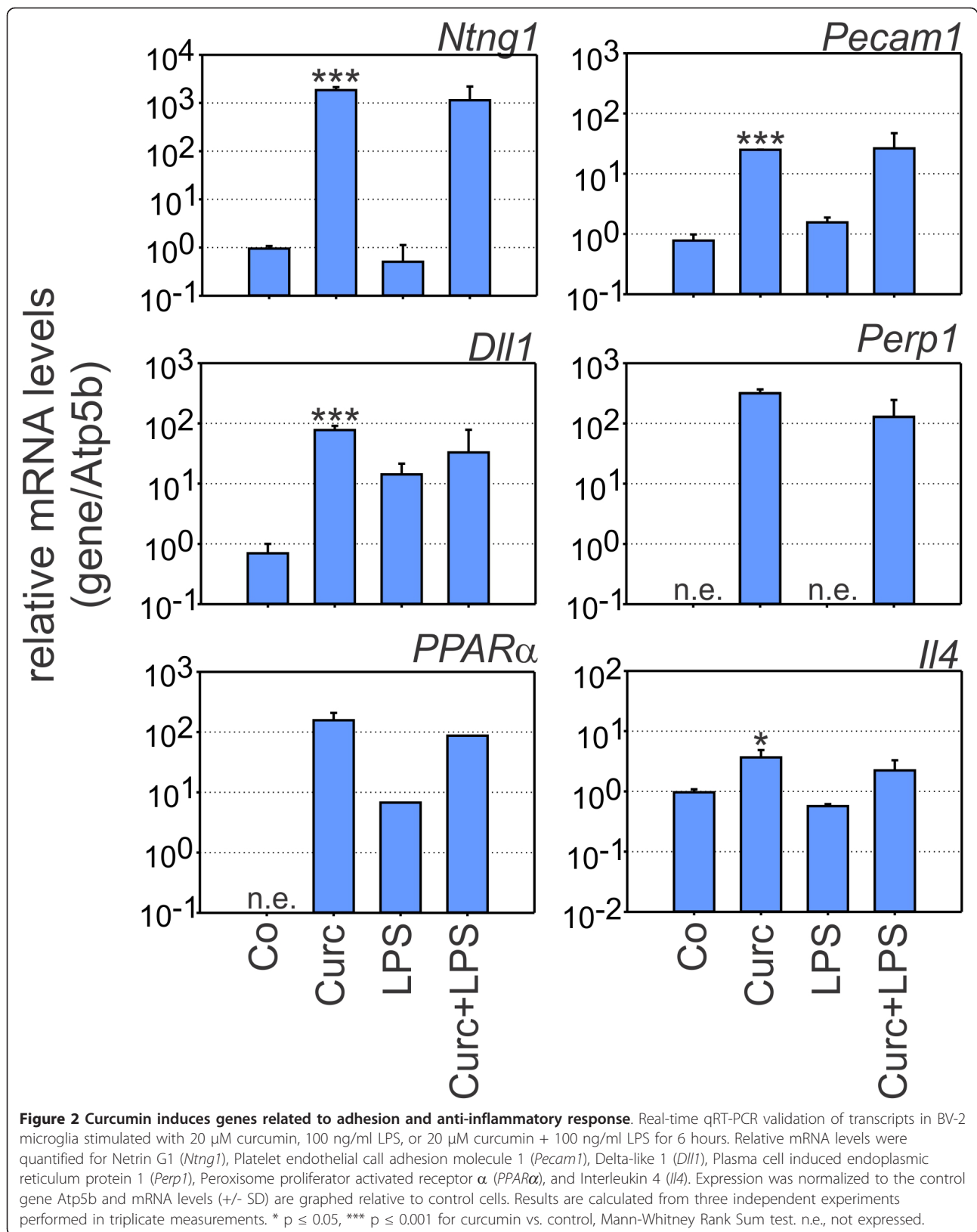
To test whether the transcriptomic changes in curcumin-stimulated cells influence microglial neurotoxicity, 661W photoreceptor cells were incubated with conditioned medium from BV-2 cells. 661W is a retinoblastoma-derived cell line, which represents an established model to study microglial neurotoxicity in the special context of retinal degeneration [30,31,35]. 661W cells were incubated for

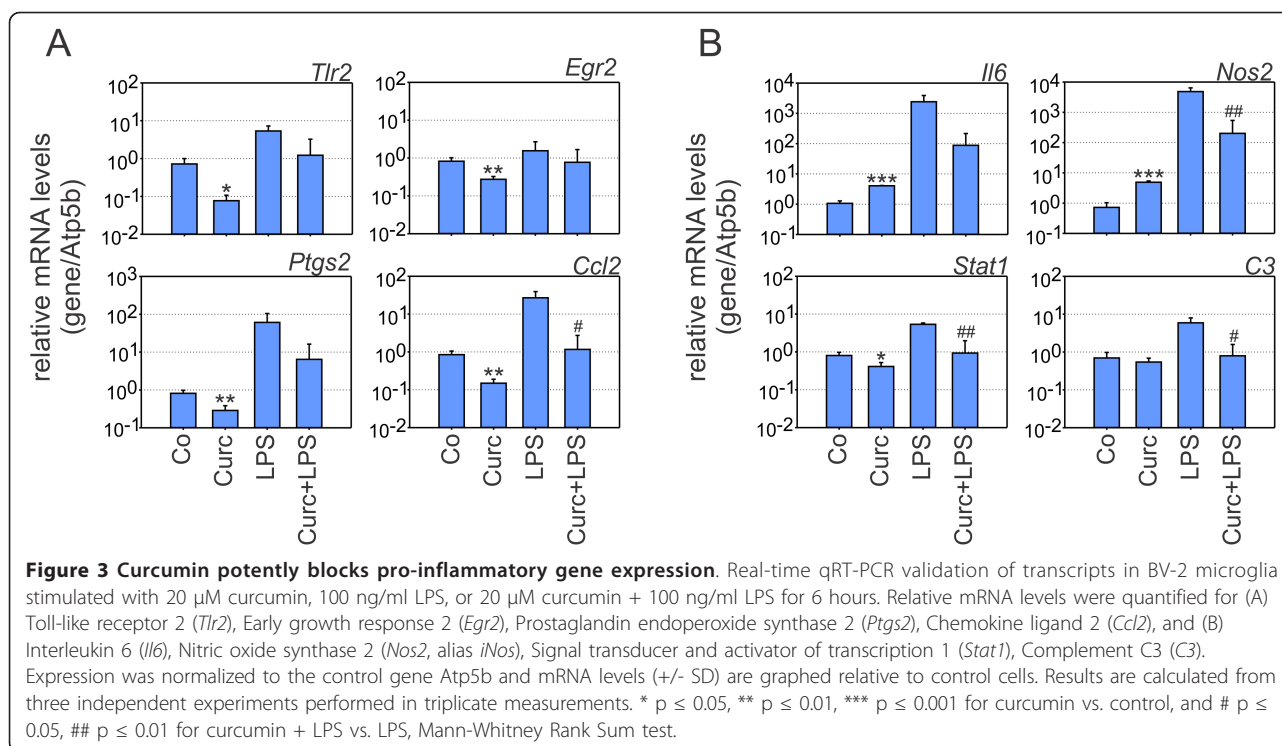
Table 3 Differentially expressed transcripts after 6 h stimulation with 20 μM curcumin + 100 ng/ml LPS versus 100 ng/ml LPS

Nr	ID	Symbol	Gene Name	FC
UP-REGULATED				
1	80883	Ntng1	Netrin G1	86.2
2	18613	Pecam1	Platelet/endothelial cell adhesion molecule 1	11.7
3	12824	Col2a1	Collagen, type II, alpha 1	9.0
4	12653	Chgb	Chromogranin B	8.1
5	14103	Fasl	Fas ligand (TNF superfamily, member 6)	6.9
6	19206	Ptch1	Patched homolog 1	6.5
7	69816	Perp1	RIKEN cDNA 2010001M09 gene	5.4
8	14526	Gcg	Glucagon	5.0
9	109685	Hyal3	Hyaluronoglucosaminidase 3	4.1
10	16189	Il4	Interleukin 4	3.8
11	20655	Sod1	Superoxide dismutase 1	3.5
12	19013	Ppara	Peroxisome proliferator activated receptor alpha	3.1
13	20997	T	Brachyuri	2.6
14	640627	Gm9789	ENSMUSG00000044227	2.6
15	16147	lhh	Indian hedgehog homolog	2.6
16	17246	Mdm2	Transformed mouse 3T3 cell double minute 2	2.5
17	99439	Duox1	Dual oxidase 1	2.3
18	257956	Olf1307	Olfactory receptor 1307	2.3
19	14179	Fgf8	Fibroblast growth factor 8	2.1
DOWN-REGULATED				
1	16193	Il6	Interleukin 6	-93.1
2	18126	Nos2	Nitric oxide synthase 2	-55.7
3	19225	Ptgs2	Prostaglandin-endoperoxide synthase 2	-17.6
4	20296	Ccl2	Chemokine (C-C motif) ligand 2	-14.7
5	12266	C3	Complement C3	-9.1
6	20846	Stat1	Signal transducers and activator of transcription 1	-7.4
7	12048	Bcl2l1	BCL2-like 1	-5.4
8	14281	Fos	FBJ osteosarcoma oncogene	-4.6
9	16835	Ldlr	Low density lipoprotein receptor	-4.1
10	16992	Lta	Lymphotoxin A	-3.5
11	17395	Mmp9	Matrix metalloproteinase 9	-2.2

Significance analysis of triplicate microarrays was performed with a false discovery rate of 0.1%. ID, Entrez Gene ID; FC: Fold change.

48 h with culture supernatants from unstimulated, curcumin-, LPS- or LPS + curcumin-treated BV-2 cells and 661W photoreceptor cell morphology was assessed by phase contrast microscopy. 661W cells in their own medium grew in a spindle-like shape with only few rounded apoptotic cells (Figure 5A). Conditioned media from control- or curcumin-treated microglial cells did not affect this morphology (Figure 5A). In contrast, 661W cells incubated with LPS-stimulated BV-2 supernatant appeared apoptotic, leading to larger cell-free areas in the culture (Figure 5A). When conditioned media from LPS + curcumin-stimulated BV-2 cells was used, a nearly normal





661W cell morphology was retained (Figure 5A). Direct incubation of 661W cells with curcumin, LPS, or both had no effects on the cell cultures (data not shown), demonstrating that the observed changes in 661W cell characteristics stem from secreted microglial compounds.

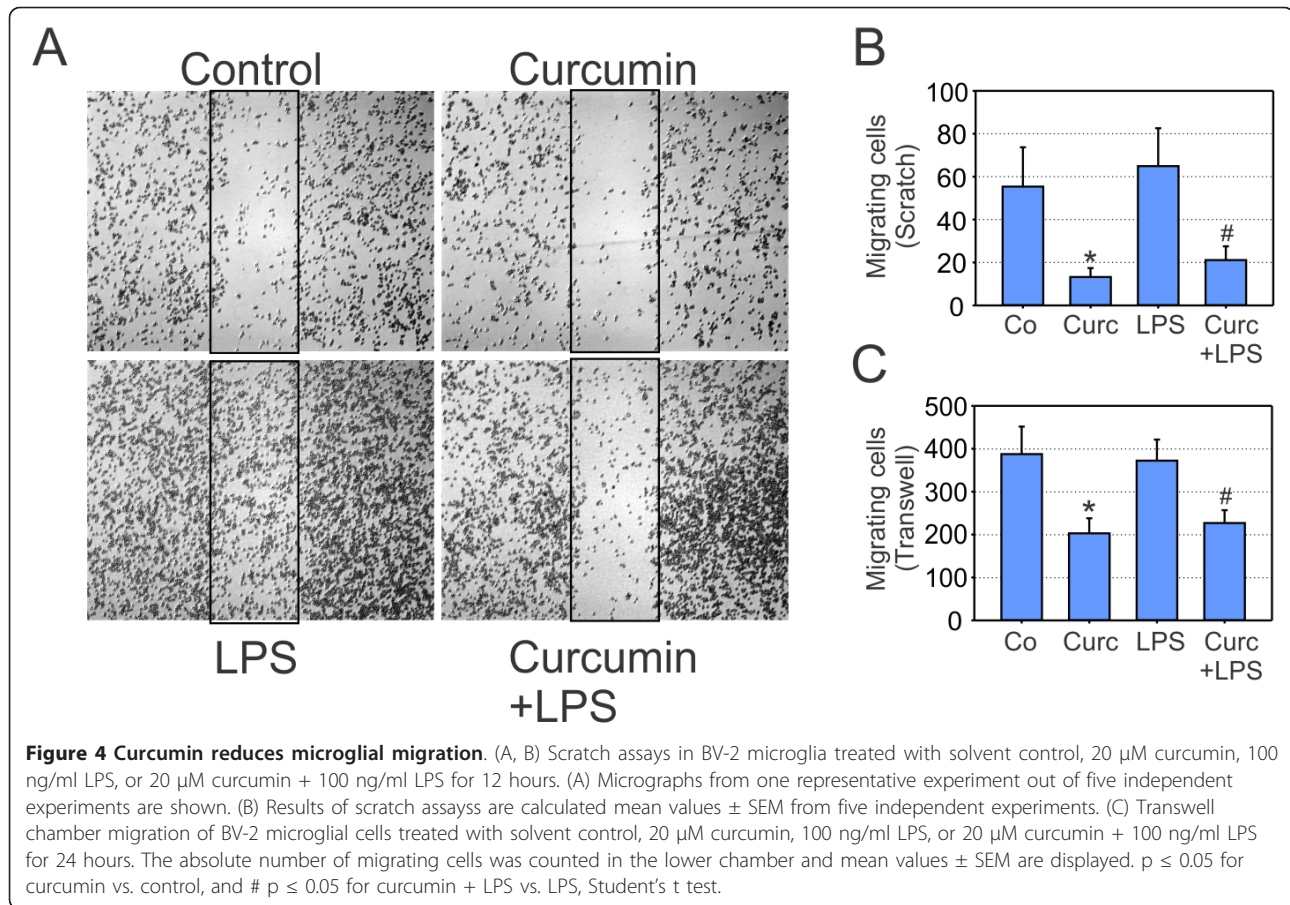
To corroborate these morphological findings with further functional data, we analyzed the influence of microglia-derived products on caspase-related apoptotic cell death. 661W cells cultured with supernatants from LPS-stimulated BV-2 cells showed a significant induction of caspase 3/7 activity (Figure 5B). When using conditioned media from microglial cells co-treated with LPS + curcumin, 661W apoptosis was still present but was significantly diminished (Figure 5B). These data clearly implicate that curcumin may limit the production of pro-apoptotic compounds in activated microglial cells or even promote the release of neurotrophic factors.

Discussion

Oxidative stress and neuroinflammation are major factors in the pathogenesis of neurodegenerative disorders [36]. Therefore, antioxidant and anti-inflammatory compounds like curcumin may be treatment options for this group of diseases [37]. However, only few experimental data are available that report on curcumin-triggered transcriptional mechanisms and direct signaling targets in microglia.

Our transcriptomic analysis in BV-2 cells sheds some light on target genes and potential signaling mechanisms. We identified a prominent transcriptional response of

resting as well as LPS-activated microglial cells after curcumin treatment. Distinct gene clusters were detected that reflect up-regulated and suppressed transcripts in both microglial phenotypes. We identified and validated six genes that were consistently induced in resting as well as activated BV-2 cells that have not been described as curcumin targets before. Among these, four curcumin target genes are related to cell migration. Netrin G1 is a lipid-anchored protein that is structurally related to the netrin family of axon guidance molecules [38]. It regulates synaptic interactions between neurons by binding to transmembrane netrin G ligands [39]. Interestingly, the related Netrin 1 molecule is a broad inhibitor of leukocyte chemotaxis [40] and Netrin G1 may have a similar function in microglia. The adhesion molecule PECAM1 is also directly involved in monocyte/macrophage migration [41]. Another migration-related gene induced by curcumin is Plasma cell endoplasmic reticulum protein 1. PERP 1 is a molecular chaperone required for proper folding and secretion of immunoglobulins in B-cells [42,43]. Related to our study, a recent report linked PERP 1 (alias MZB1) to calcium signaling, activation of integrins and cell adhesion [44]. Expression of the Notch-ligand Delta-like 1 has been demonstrated in BV-2 cells and primary rat brain microglial cells, where Notch-1 signaling negatively regulates TNF release [45]. Our data show that basal Dll1 expression in resting microglial cells can be potently induced by curcumin, which could potentially trigger Notch-signaling to prevent migration associated with pro-inflammatory priming of BV-2 cells.

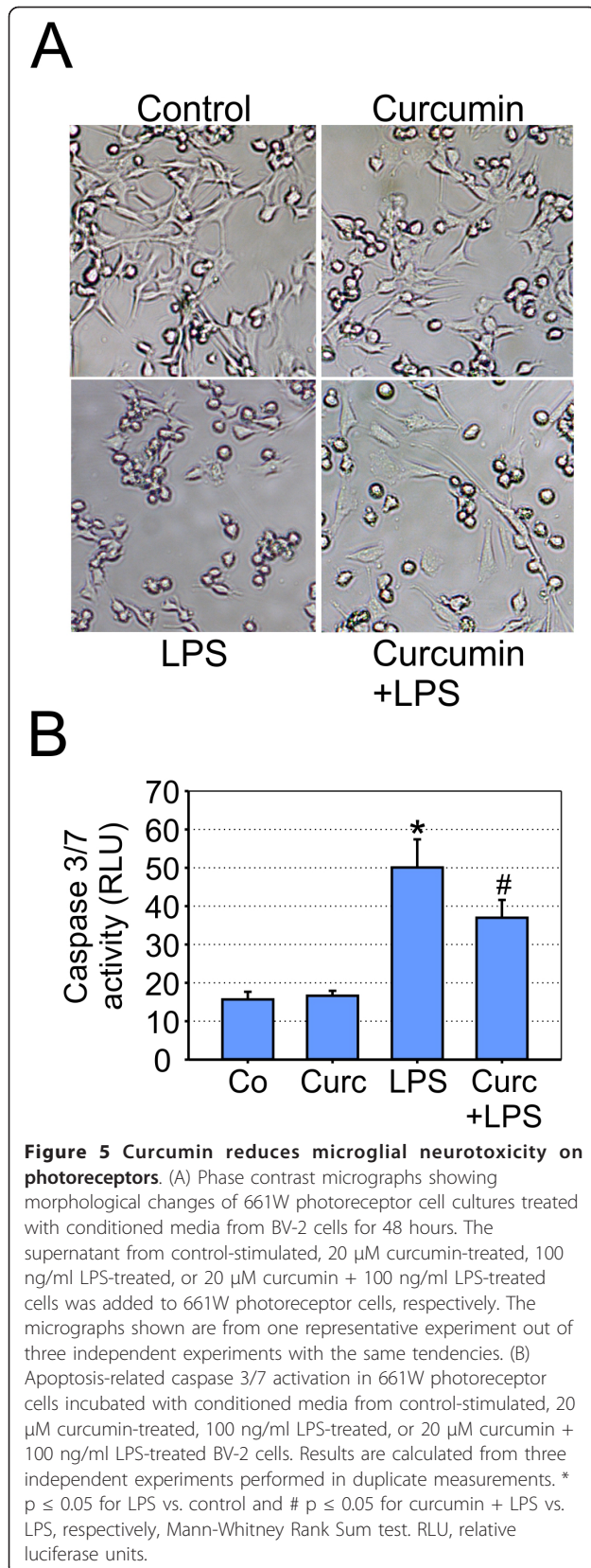


These transcriptomic data of curcumin-treatment promoted us to analyze its effects on microglial motility. Both types of assays, the wound healing assays and the transwell migration experiments, showed that BV-2 cell migration was significantly inhibited by 20 μ M curcumin over a period of 12 hours to 24 hours. These findings are in good agreement with papers reporting reduced migration of tumor cells, endothelial cells, and dendritic cells after treatment with comparable doses of curcumin [46-48]. In the homeostatic state, microglia constantly scan their environment with their long protrusions without movement of the somata [5]. In contrast, migration of microglial cells is a hallmark of pro-inflammatory and chronic activation during early phases of neurodegeneration. Thus, curcumin may support the homeostatic state of microglia and prevent their early and excessive transformation into migrating phagocytes.

It is well known that curcumin broadly inhibits pro-inflammatory gene expression by targeting different signal pathways and transcriptional regulators including NF κ B, AP1, EGR1, and STAT3 [49]. Our microarray data corroborate these findings especially in LPS-activated BV-2 cells by showing curcumin-triggered suppression of Ptg2, Ccl2, Il6, and Nos2, which are NF κ B, AP1, and

STAT3 target genes [21]. Moreover, the curcumin-regulated transcriptomic profiles revealed lower gene expression of toll-like receptor 2 in resting microglia and complement factor 3 in activated cells. These two factors broadly support the conversion of microglial cells to the pro-inflammatory state [50,51] and hence curcumin signaling may abrogate both pathways. Our data also showed diminished mRNA expression of the transcription factors Egr2 and Stat1 following curcumin-treatment. This indicates that curcumin may further dampen microglial activation by interfering with two other key transcription factors expressed in activated microglial cells. In addition to its inhibitory effects on pro-inflammatory signaling, two well known anti-inflammatory molecules, PPAR α and IL4, were significantly induced by curcumin. PPAR α and IL4 both specifically inhibit pro-inflammatory activation of microglial cells [52,53] and some of the immune-dampening effects of curcumin may be mediated via this signaling axis.

The cell culture experiments with conditioned media from BV-2 cells showed that curcumin significantly reduced LPS-triggered microglial neurotoxicity on 661W photoreceptor cells. We hypothesize that the strong suppression of LPS-induced Nos2 transcription by curcumin



is a major pathway responsible for this phenomenon. In this context, Mandal *et al.* have recently demonstrated that curcumin protects 661W cells from hydrogen peroxide-induced cell death [29]. This effect is very likely mediated by the antioxidant and radical-scavenging capacity of curcumin. In a model of light-induced retinal degeneration, curcumin also suppressed inflammatory marker expression *in vivo* [29], which could be potentially mediated by its attenuating effect on retinal microglia.

Conclusions

We have shown that curcumin triggered global changes in the transcriptome of resting and LPS-activated microglial cells. In addition to its known function in blocking pro-inflammatory gene expression via interference with NF κ B signaling, curcumin induced novel anti-inflammatory targets in microglia. Curcumin also significantly inhibited microglial migration and cytotoxicity, which are key features of neuroinflammation. Our publicly available dataset provides a basis to understand the pleiotropic beneficial effects of curcumin on microglia as key innate immune cells of the nervous system. Moreover, the results of this study also underscore the importance of curcumin as a promising dietary compound for the treatment of various neurodegenerative disorders associated with inflammation.

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Authors' contributions

MK, EL, and YW carried out cell culture stimulations and qRT-PCR experiments. MK and EL analyzed qRT-PCR and functional data. CM performed microarray analysis. AA performed scratch assays. MM critically read and corrected the paper. 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.

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