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Telmisartan Protects a Microglia Cell Line from LPS Injury Beyond AT1 Receptor Blockade or PPARγ Activation

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Abstract

The Angiotensin II Receptor Blocker (ARB) Telmisartan reduces inflammation through Angiotensin II AT1 receptor blockade and peroxisome proliferator-activated receptor gamma (PPARγ) activation. However, in a mouse microglia-like BV2 cell line, imitating primary microglia responses with high fidelity and devoid of AT1 receptor gene expression or PPARγ activation, Telmisartan reduced gene expression of pro-injury factors, enhanced that of anti-inflammatory genes, and prevented LPS-induced increase in inflammatory markers. Using global gene expression profiling and pathways analysis, we revealed that Telmisartan normalized the expression of hundreds of genes upregulated by LPS and linked with inflammation, apoptosis and neurodegenerative disorders, while downregulating the expression of genes associated with oncological, neurodegenerative and viral diseases. The PPARγ full agonist Pioglitazone had no neuroprotective effects. Surprisingly, the PPARγ antagonists GW9662 and T0070907 were neuroprotective and enhanced Telmisartan effects. GW9226 alone significantly reduced LPS toxic effects and enhanced Telmisartan neuroprotection, including downregulation of pro-inflammatory TLR2 gene expression. Telmisartan and GW9662 effects on LPS injury negatively correlated with pro-inflammatory factors and upstream regulators, including TLR2, and positively with known neuroprotective factors and upstream regulators. Gene Set Enrichment Analysis (GSEA) of the Telmisartan and GW9662 data revealed negative correlations with sets of genes associated with neurodegenerative and metabolic disorders and toxic treatments in cultured systems, while demonstrating positive correlations with gene sets associated with neuroprotection and kinase inhibition. Our results strongly suggest that novel neuroprotective effects of Telmisartan and GW9662, beyond AT1 receptor blockade or PPARγ activation, include downregulation of the TLR2 signaling pathway, findings that may have translational relevance.

Keywords Angiotensin receptor blockers · Inflammation · Neuroprotection, microglia · PPARγ · TLR2

Introduction

Dysregulated and excessive inflammation is a significant factor in the initial stages and development of many brain diseases, where resident microglia play fundamental roles [1–3]. Microglia have key roles to maintain homeostasis, support brain repair and remodeling when their anti-inflammatory M2 phenotype predominates [3]. Conversely, the microglia pro-inflammatory M1 phenotype is a major player and contributor to neurotoxicity, with excessive production of inflammatory cytokines that are considered important participants in inflammatory, traumatic and degenerative brain disorders [4, 5].

Excessive Angiotensin II activity is one important injury factor contributing to the development of brain inflammation [6–10]. Angiotensin II stimulates two receptor types, AT1 and AT2. Pathological effects on inflammation and neurotoxicity were identified as the consequence of brain AT1 receptor activation [6–10]. Consequently, treatment with selective Angiotensin II AT1 Receptor Blockers (ARBs) reduces inflammation, cell injury and apoptosis, demonstrated in neuronal, cerebrovascular endothelial, primary microglia and astrocyte cultures, and after oral administration in many rodent models representing inflammatory, traumatic and neurodegenerative brain disorders [8–15].

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We wished to better understand the ARB protective effects using microglia cultures. Lipopolysaccharide (LPS)-induced injury is a representative damaging, pro-inflammatory factor in microglia [16]. ARBs reduce the M1 pro-inflammatory phenotype while stimulating the M2 anti-inflammatory phenotype, documented in cultured primary rat microglia for Candesartan [13] and in cultures of primary mouse microglia and microglia-like BV2 cells for Telmisartan [17].

We selected Telmisartan, the most effective ARB in neuronal cultures [14] with the widest pleiotropic pharmacological profile, blocking AT1 receptors and stimulating the anti-inflammatory, pro-metabolic peroxisome proliferator-activated receptor gamma (PPARγ) [14, 17–19].

To reveal the relative role of AT1 receptor blockade and PPARγ activation in Telmisartan neuroprotection, we analyzed the effects of the full PPARγ agonist Pioglitazone [20] and two PPARγ antagonists, GW9662 and T0070907 [21, 22]. We selected immortalized mouse microglia-like BV2 cell line cultures, injured in vitro by exposure to the inflammatory factor LPS. BV2 cells are frequently used as a suitable model for in vitro studies on microglia and models of brain inflammation [17, 23–27]. Upon LPS exposure, BV2 cells mimic primary microglia responses with high fidelity [23–27]. We performed global gene expression analysis of selected experiments and confirmed the expression of several important pathways and key genes by qPCR.

Materials and Methods

BV2 Cell Culture

BV2 cells were obtained from William Rebeck, Ph.D. Department of Neurosciences, Georgetown University Medical Center, and a mouse Short Tandem Repeat (STR) profile for genotyping and interspecies contamination test was generated (IDEXX, Columbia, MO) (Supplemental Table 1).

BV2 cells were cultured in DMEM (1X) Dulbecco's Modified Eagle Medium [+] 4.5 g/l D-Glucose [+] L-Glutamine [−] Sodium Pyruvate, with addition of 5% penicillin/streptomycin (Gibco lot# 3304c238), 10% heat inactivated Fetal Bovine Serum (FBS), at 37 °C in an atmosphere of 5% CO2. When reached 80% confluence, 400,000 cells per well were seeded in 6-well plates for further experiments.

Mouse Frontal Cortex

Three individual samples of frontal cortex from C57BL/6J mice (Jackson Laboratories, Farmington, CT), were supplied by Sonia Villapol, Ph.D., Department of Neuroscience, Georgetown University Medical Center (protocol number 2016-1263, approved by the Georgetown University Animal Use and Care Committee (ACUC) and conducted following the NRC guide to the Care and Use of Laboratory animals. These mice were from one of our previous experiments [10]. They had been subjected to traumatic brain injury and treated with vehicle. We demonstrated that traumatic brain injury did not increase AT1 receptor gene expression in the cerebral cortex [10].

Experimental Design and Randomization to Confirm Microarray Results Using qPCR

Dimethylsulfoxide (DMSO), Telmisartan, Valsartan, Pioglitazone, GW9662, T0070907 and LPS were from Sigma-Aldrich (St. Louis, MO). All drugs were used at 10 μM, diluted in 1.5% DMSO, and LPS at 100 ng/ml, diluted in water. All treatments consisted of three individual independent samples per group, each sample analyzed in triplicates, and included 1.5% DMSO (Sigma-Aldrich, St. Louis, MO). Vehicle-treated groups received 1.5% DMSO for 3 h. Groups treated only with drugs (Telmisartan, Pioglitazone, GW9662 and T0070907) received the drugs for 3 h. Groups treated only with LPS, received vehicle for 2 h, followed by LPS for 1 h. Groups treated with drugs + LPS received drugs for 2 h followed by LPS for 1 h. All experiments were conducted for 3 h. The experiments were terminated by discarding the medium and treating each well with 350 μl of lysis buffer RLT from RNeasy Mini Kits (Qiagen, Valencia, CA). Researchers performing the experiments were blinded to the protocols with a third party concealing the treatments with individually coded vials.

Separate experiments were conducted to test the effects of:

1. Telmisartan, LPS and Telmisartan + LPS;
2. Pioglitazone and Pioglitazone + LPS;
3. Valsartan, LPS and Valsartan + LPS
4. GW9662, GW9662 + LPS, and GW9662 + LPS + Telmisartan;
5. T0070907, T0070907 + Telmisartan, and T0070907 + LPS + Telmisartan.

Quantitative PCR

To compare the relative gene expression of AT1 receptor and PPARγ, total RNA was extracted from three different BV2 cell stocks and three different mouse frontal cortex samples using 1 ml TRIzol reagent (Invitrogen, Carlsbad, CA) followed by purification using the RNeasy Mini Kit (Qiagen, Valencia, CA), according to manufacturer's instructions. To test microarray results on selective gene expression, total RNA was extracted from cultured BV2 cells as described above. Synthesis of complementary DNA (cDNA) was performed with 0.6 μg of total RNA and Super-Script III first-Strand Synthesis Kit (Invitrogen, Carlsbad, CA). The remaining reagents for RNA isolation and reverse transcription were

from Invitrogen. Quantitative real-time PCR reactions were performed using an Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA) with PowerUp™ SYBR® Green Master Mix (Thermo Fisher). qPCR was performed in a 10 μl reaction mixture containing 8 μl SYBR Green PCR Master Mix, 2 μl cDNA and 0.3 μmol/l of each primer for specific target (Supplemental Table 2). Amplification conditions consisted of 1 denaturation/activation cycle at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 60 s. Serial dilutions of cDNA from the same source as samples were used to obtain a standard curve. Individual targets for each sample were quantified by determining the cycle threshold and comparison with the standard curve (ΔΔCt method). The relative amount of the target mRNA was normalized with the housekeeping gene GAPDH.

Statistical Analysis for qPCR Samples

Data in Fig. 1 were expressed as fold-change relative to CTX after correction for GAPDH expression and were analyzed by one-way ANOVA followed by Duncan test. Multiple group comparisons for data obtained for all other qPCR experiments were performed by ANOVA followed by Newman-Keuls post-test. Statistical significance was determined using GraphPad Prism 5 Software (GraphPad Software, San Diego, CA, USA). In all cases, data are expressed as means ± SEM and considered statistically significant given a probability value of ≤0.05.

Gene Expression Analysis

Total RNA was extracted from groups of cultured BV2 cells; each group consisted of three independent experiments, with investigators blinded to the protocol. Experiment # 1 consisted on separated groups treated with vehicle (DMSO, 1.5%), Telmisartan, LPS, and Telmisartan + LPS. Experiment # 2 consisted on separate groups treated with vehicle, Telmisartan, GW9662, LPS, Telmisartan + LPS, GW9662 + LPS, and GW9662 + Telmisartan + LPS. Treatment times were identical to those used for qPCR experiments: Telmisartan and/or GW9662 were administered 2 h before LPS, and the experiments terminated 1 h after LPS administration. Standard procedures for labeling, hybridization, washing, staining and scanning were as per manufacturer's recommendation (Affymetrix, Santa Clara, CA) and as described in detail [28]. The RNA was purified using a RiboPure Kit (Ambion, Austin, TX, USA) according to manufacturer's protocol. The quality and quantity of RNA were ensured using the Bioanalyzer (Agilent, Santa Clara, CA) and NanoDrop (Thermo Scientific, Waltham, MA), respectively. All analyses were performed using Partek Genomics Suite (Chesterfield, MO). Data were considered statistically significant at a p value < 0.05 and included with a cutoff of 1.2-fold-change. For gene expression analysis and microarray data mining and dataset description, we used Ingenuity pathway analysis (IPA) http://www.ingenuity.com. To identify whether sets of genes may have an association with known functional pathways or disease phenotypes, we performed gene set enrichment analysis (GSEA) http://software.broadinstitute.org/gsea/ [28, 29].

The raw data has been submitted to Gene Expression Omnibus (GEO) under accession numbers GSE108669 and GSE108670.

Results and Discussion

Expression of AT1, AT1A, AT1B, AT2 Receptor and PPARγ Genes in BV2 Cells

Angiotensin II activates two receptor types, AT1 and AT2 receptors. While in humans only one AT1 is expressed, in rodents AT1 receptors are expressed as two different receptor subtypes, the AT1A and AT1B receptors. These receptor subtypes have 98% homology in their coding regions [30] and for this reason both receptor subtypes are inhibited by ARBs. The subtypes may only be distinguished by their gene expression, using primers directed to untranslated regions that are not homologous for AT1A and AT1B [30]. On the other hand the gene expression of both subtypes may be simultaneously detected by using primers directed to their common coding region [30]. We used primers directed to the common AT1A and AT1B coding regions to demonstrate expression of these receptor subtypes simultaneously (Supplemental Table 2).

In contrast with the clear expression of AT1 receptor gene in the mouse frontal cortex, AT1 receptor genes were not expressed in our BV2 cell line (Fig. 1a). Microarray analysis confirmed these results, revealing that genes for all Angiotensin II receptors (*Agtr1* encoding the AT1 receptor type, *Agtr1a* encoding the AT1A receptor subtype, *Agtr1b* encoding the AT1B receptor subtype, and *Agtr2,* encoding the AT2 receptor type) were not significantly expressed in any of the groups tested (Supplemental Table 3).

The expression of AT1 and AT2 receptors in primary microglia and BV2 cell lines has been controversial. AT1 gene expression was expressed in BV2 cell lines of different origin than the one used in our experiments and in unstimulated primary rat microglia [31, 32]. Conversely, AT1A, AT1B and AT2 gene expression in isolated cortical mouse microglia was not higher than background noise [33]. We did not find AT1 or AT2 receptor gene expression in a BV2 cell line of different origin [17] and in the human macrophage cell line HTP-1 or in human circulating monocytes [34]. Analysis of a complete transcriptome conclusively demonstrated that AT1A, AT1B and AT2 genes are not expressed in human or mouse microglia [35].

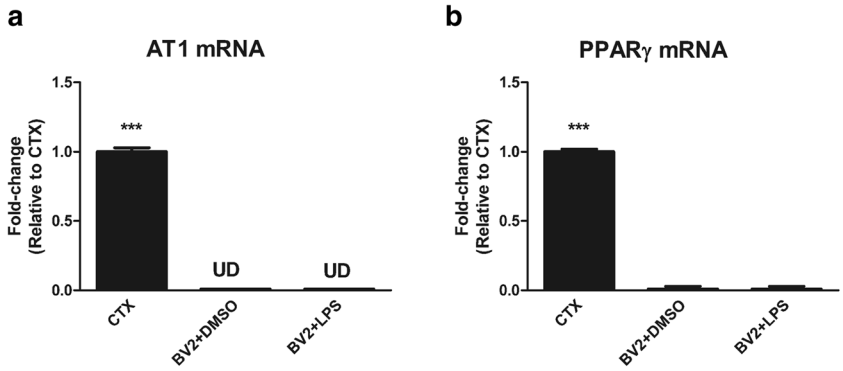

Fig. 1 Expression of AT1 and PPARγ genes in mouse cortex, BV2 cells treated with DMSO and BV2 cells treated with DMSO + LPS. In contrast to the mouse frontal cortex (CTX) BV2 cells do not express the AT1 receptor gene, whether treated with LPS or not (A). PPARg gene expression is extremely low in BV2 cells when compared to mouse frontal cortex and does not change whether treated with LPS or not. Results are means of three samples analyzed independently. Data are expressed as fold-change relative to CTX after correction for GAPDH expression and were analyzed by one way ANOVA followed by Duncan test. ***$p < 0.0001$ vs all others

The neuroprotective effects of Telmisartan in BV2 cells lacking AT1 receptor expression described here agree with prior observations demonstrating that in some systems, ARBs, including Telmisartan, can be neuroprotective beyond AT1 receptor blockade [36–38].

It has been reported that the AT1 receptor gene, not present in unstimulated primary microglia, was expressed only after 6 h of LPS injury [31]. We have not detected AT1 gene expression in our BV2 cell line after 1 h of LPS injury (Fig. 1a) and these results were confirmed in our microarray analysis (Supplemental Table 3).

The function of AT2 receptors in the brain is controversial, has not been clarified, and most of the evidence indicates that they do not play a significative role in Angiotensin II-induced brain toxicity [8, 9, 13, 39]. AT2 receptors are not present in mouse or human microglia [33, 35] or in BV2 cells [17] and were not expressed in our microarray analysis (Supplemental Table 3).

There is clear evidence of PPARγ gene expression in primary microglia and in several BV2 cell lines, and its stimulation is a major protective factor [2, 14, 35, 40–42]. It was also established that part of Telmisartan neuroprotective effects are the consequence of PPARγ activation [12, 14–18, 43]. However, there are also some previous indications that in some systems, the mechanisms of Telmisartan neuroprotection may not only be beyond AT1 receptor blockade but also unrelated to PPARγ activation [22, 37].

We could only detect very low PPARγ gene expression (*Pparg*) in our BV2 cell line (Fig. 1b) and none in our microarray analysis (Supplemental Table 3). To test whether PPARγ could be activated in our system, we tested the effect of the PPARγ agonist pioglitazone and the PPARγ agonists GW9662 and T0070907. We found that activation of PPARγ with pioglitazone was not neuroprotective (Fig. 2). The PPARγ antagonists GW9662 (Fig. 3) and T0070907 (Supplemental Fig. 1) were neuroprotective and enhanced, rather than reduced Telmisartan neuroprotection, and there is evidence that T0070907 utilizes mechanisms beyond PPARγ activation [21]. In addition, Valsartan, an ARB without direct PPARγ stimulation [36, 43, 44] and in some systems acting beyond AT1 blockade [36], significantly reduced LPS activation of pro-inflammatory factors (Supplemental Fig. 2), showing that the neuroprotective mechanisms of Telmisartan neuroprotection in our system are not unique to this ARB.

In addition, we found that the expression of a number of PPARγ target genes (CD16, CD36, TAF12, CDKN3, MAGOH, GAPDH, STAT1, STAT6, CEBPB) [34, 45] and Supplemental Table 3) was not altered in our microarray analysis (Supplemental Table 3) and that the gene expression of ABCG1, significantly downregulated by Telmisartan in our array analysis (Supplemental Table 3) has been reported to be upregulated by PPARγ activation [14].

From the above we conclude that Telmisartan neuroprotection in our BV2 cells was unrelated to AT1 receptor blockade or PPARγ activation and that our BV2 cell line is an excellent model to characterize additional, novel mechanisms of Telmisartan neuroprotection from LPS.

We propose that apparently contradictory results obtained in different laboratories and including not only AT1 but also PPARγ receptor gene expression may be the result of alterations occurring in different BV2 cell lines over time. The BV2 cell line has been first produced in 1990 [24, 46, 47] and it may now be obtained from diverse sources around the World [17, 41, 42]. Cell lines may not remain as homogeneous clonal cells sharing a similar phenotype over time, and some established cell lines give rise to heterogenous progeny [48]. It is possible that phenotype changes in BV2 cell over time may explain apparently contradictory results. Unfortunately, the different BV2 cell lines from different laboratories have not been fully characterized and compared with each other. To facilitate replication and explain potential future discrepancies with our data, we performed, for the first time, a Cell Check including an STR profile of our BV2 cell line (Supplemental Table 1).

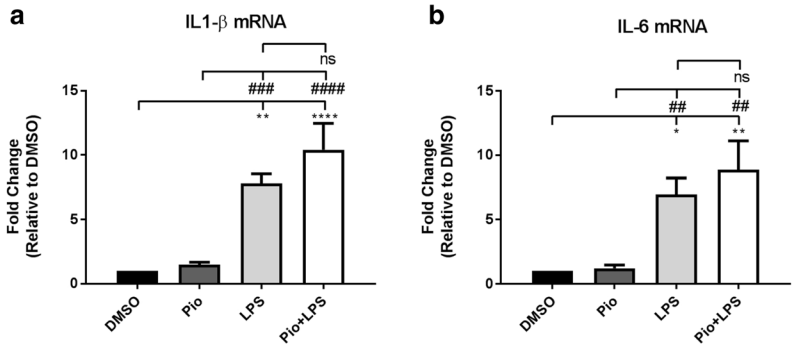

Fig. 2 The PPARγ agonist Pioglitazone does not reduce LPS-induced increase in IL-1β and IL-6 gene expression. **a** Pretreatment with the PPARγ full agonist Pioglitazone (Pio) 10 μM for 2 h does not decrease the enhanced IL-1β gene expression produced after 1 h of exposure to LPS (100 ng/ml). ANOVA F (3, 16) = 18.07, $p < 0.0001$. **b** Pretreatment with the PPARγ full agonist Pioglitazone (Pio) 10 μM for 2 h does not decrease the enhanced IL-6 gene expression produced after 1 h of exposure to LPS (100 ng/ml). ANOVA F (3, 16) = 9.578, $p = 0.0007$. Results are means ± SEM for three to five groups analyzed independently. Data were analyzed by one-way ANOVA with Newman-Keuls to correct for multiple comparisons. $^{****}p < 0.0001$, $^{**}p < 0.01$, $^{*}p < 0.05$ compared to DMSO; $^{\#\#\#\#}p < 0.0001$, $^{\#\#\#}p < 0.001$, $^{\#\#}p < 0.01$ compared to Pio; ns (not significant)

Administration of Telmisartan Alone to Uninjured BV2 Cells Is Neuroprotective, Downregulating Pro-injury and Upregulating Protective Gene Expression

Using global gene analysis, we discovered that when administered to cultured BV2 cells not injured by LPS, as compared with vehicle-treated samples, Telmisartan altered the expression of 492 genes (Supplemental Table 3), including downregulation of *Ccrl2*, *Dusp2, Dusp5, Csf1*, and *Bcl11b* expression (Supplemental Table 4). These genes have been demonstrated by others to markedly reduce inflammation [49–52]. Other downregulated genes included 11 *miRs,* such as *miR-874* and *miR-574* (Supplemental Table 4). These *miRs* have been previously reported to be involved in multiple functions as well as promoting stroke [53], neuronal vulnerability to injury [54] and cognitive impairment in pre-clinical models of Alzheimer's disease [55]. We found that *Cib1* was also downregulated (Supplemental Table 4). *Cib1* encodes CIB1, that activates kinase oncogenic pathways and pathological cardiovascular hypertrophy [56].

Conversely, Telmisartan upregulated numerous histone genes such as *Hist1h*, *Hist2h* and *Hist4h* (Supplemental Table 4). It has already been reported that these histone genes limit inflammation, [57]. *Ang4* was also found upregulated (Supplemental Table 4) and it has been demonstrated that *Ang4* encodes an angiogenin with microbicidal activity involved in innate immunity [58]. *Slc25a51* and *Usp17le*

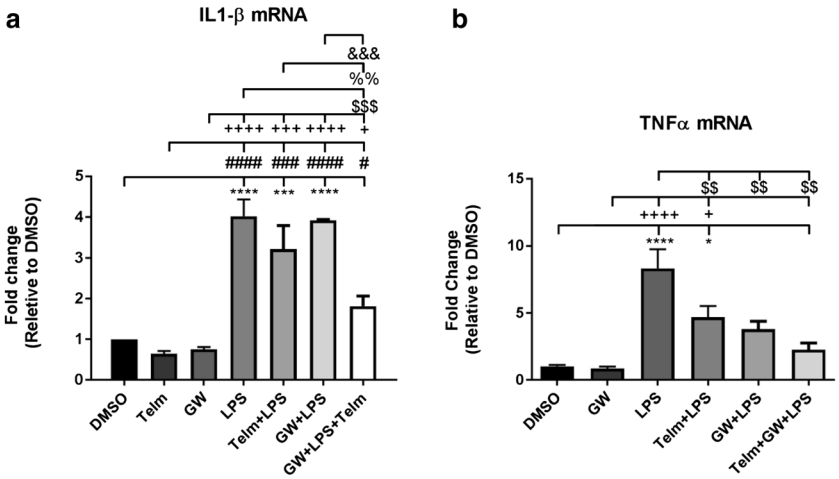

Fig. 3 The PPARγ antagonist GW9662 enhances the Telmisartan-induced reduction of LPS-induced increase in IL-1β gene expression and eliminates the LPS-induced increase in TNFα gene expression. **A** Exposure to the PPARγ antagonist GW9662 (GW) 10 μM of for 2 h potentiates the effect of Telmisartan to reduce the increase in IL-1β gene expression produced after 1 h of exposure to LPS (100 ng/ml). ANOVA F (6, 14) = 27.95, $p < 0.0001$. **B** Exposure to the PPARγ antagonist GW9662 (GW) 10 μM or Telmisartan (Telm) 10 μM alone for 2 h eliminates the increase in TNFα gene expression produced after 1 h of exposure to LPS (100 ng/ml). ANOVA F (5, 16) = 13.19, p < 0.0001 Results are means ± SEM for three to five groups analyzed independently. Data were analyzed by one-way ANOVA with Newman-Keuls to correct for multiple comparisons. $^{****}p < 0.0001$, $^{***}p < 0.001$, $^{*}p < 0.05$ compared to DMSO; $^{\#\#\#\#}p < 0.0001$, $^{\#\#\#}p < 0.001$, $^{\#}p < 0.05$ compared to Telm; $^{++++}p < 0.0001$, $^{+++}p < 0.001$, $^{+}p < 0.05$ compared to GW; $^{\$\$\$}p < 0.001$, $^{\$\$}p < 0.01$ compared to LPS; $^{\%\%}p < 0.01$ compared to LPS + Telm; $^{\&\&\&}p < 0.001$ compared to GW + LPS

(Dub3) were also upregulated by Telmisartan (Supplemental Table 4). *Slc25a51* is known to protect metabolism [59], and *Usp17le (Dub3*), encodes a deubiquitinating enzyme regulating multiple cellular processes [60].

The Ingenuity Pathway Analysis (IPA) confirmed the protective effect of Telmisartan. The IPA's diseases and functions analysis with largest numbers of network molecules included activation, movement, and migration of vascular endothelial cells, inflammatory response and immunological disease (Supplemental Table 4). The upstream regulator analysis showed negative correlations with many inflammatory factors, such as LPS, IL-1β, TNF-α, TGF-beta 1, TLR4 and TLR7 (Table 1 and Supplemental Table 4). There were additional negative correlations with many known inflammation transcription regulators, although these correlations were not significant. Networks with largest numbers of molecules include cell death and survival, inflammatory response and many aspects of cell biology and cancer (Supplemental Table 4).

Geneset enrichment analysis revealed that genes upregulated by Telmisartan positively correlated with those upregulated in two aged mouse strains undergoing calories restriction diets (Supplemental Table 4). Calories restriction was associated with prolongation of life, favor lipid metabolism and protect from renal disease [61] (GSE75569). These effects were reported to be like those of ARB administration, and it was suggested that Telmisartan administration and calories restriction share common protective and anti-aging mechanisms [62].

In addition, GSEA demonstrated a strong correlation of genes downregulated by Telmisartan that were reported to be upregulated in a mouse model of neuronal ceroid lipofuscinoses (NCL), a severe monogenic neurodegenerative disease of childhood with widespread neuronal loss, demyelination, astrocytosis and microglial activation and without effective therapy [63] (GSE37643) (Supplemental Table 4). Whether Telmisartan administration may ameliorate NCL has not been yet considered.

The potential benefits of Telmisartan administration to uninjured cells support the proposal that this compound may prevent or delay brain injury when administered to populations vulnerable to brain diseases with strong inflammatory components, such as neurodegenerative disorders [8–10].

LPS, when Administered Alone, Upregulated Expression of Pro-inflammatory and Downregulated that of Protective Genes Associated to Multiple Mechanisms of Injury and Brain Disorders

Differential gene expression comparing results from LPS-treated BV2 cells with those of vehicle-treated cells yielded over 979 annotated transcripts significantly upregulated (534) or downregulated (445) by LPS (Supplemental Tables 3 and 5).

The response of the BV2 cells to LPS-induced injury was like that demonstrated in primary microglía [64], indicating that our BV2 cell culture was a reliable substitute for primary microglia cultures.

We confirmed and expanded the previously reported major upregulation of several genes by LPS in our BV2 cell cultures (Supplemental Tables 3 and 5). These genes included several pro-inflammatory cytokines such as IL1-β and IL-6, NF-κB and the TNF superfamily. The LPS-induced increase of IL1-β, IL-6, TNFα and NF-κB was confirmed by qPCR (Fig. 4). In addition, we found that the *miR-155*, *miR-221, Cxc110*, and *Ccrl2* genes were also upregulated by LPS (Supplemental Tables 3 and 5). All the genes mentioned have been defined as major players in inflammation, and their upregulation by LPS was previously reported [65]. They include the pro-inflammatory cytokines IL1-β [66], NF-κB [67], the TNF superfamily [68], NO and ROS production, *miR-155* and *miR-221* [69], *Cxc110* [70] and *Ccrl2* [49].

Several kinases and the olfactory receptors *Olfr887* and *Olfr97* were also upregulated (Supplemental Tables 3 and 5). Both the kinases and the olfactory receptors *Olfr887* and *Olfr97* have been previously associated with neurodegenerative disorders [71, 72].

Conversely, LPS downregulated genes including *Histh3b* and *Hist1h4c,* 8 *miRs*, including *miR-129*, 28 *snoRNA*s, including *Snora64*, *Snora20*, *Snord57* and *Snord69,* and *Sirt7* (Supplemental Tables 3 and 5). *Histh3b* and *Hist1h4c* were reported to reduce inflammation [73]. Downregulation of *miR-129* and *snoRNAs* has been reported to be associated with stress, oncological, neurodegenerative and viral diseases [74, 75]. *Sirt7* is a member of the sirtuin family proposed to attenuate aging [76].

The list of functionally/biologically annotated only genes (564 transcripts) was submitted to IPA analysis, confirming multiple injury mechanisms stimulated by LPS (Supplemental Table 5). As expected, and with great statistical significance, we identified major canonical pathways of neuroinflammation: IL-6, IL-1, NF-κB, TLR2, NO, ROS, glucocorticoid receptors, TREM1 and p38MAPK signaling. Diseases and functions prominently included inflammatory response (Supplemental Table 5). These major canonical pathways have been previously linked not only with inflammation, but also with Alzheimer's disease [77], atherosclerosis [78] and cancer, with a key role of protein kinase pathways [17, 79]. Consequently, analysis of diseases and functions (Supplemental Table 5) revealed a predominance of inflammation, and multiple cell functions including apoptosis and proliferation of blood cells [80].

Upstream regulator analysis of these LPS differentially expressed genes expanded our previous observations, including positive correlations with the well-known inflammatory cytokines (IL-1β, IL-6), IFNγ, TGF-beta 1, LPS, TLRs (TLR2, 3, 4, 7 and 9) and components of the TLR signaling pathway CD14, MYD88), NF-κB complex, LCN2, a biomarker for inflammatory and metabolic and neurodegenerative disorders [81, 82] and many kinases linking inflammation and cancer [83], including JNK, MAPK7, MAPK8, ERK, ERK1/2 and Pl3K (Table 1,

Table 1 IPA up-stream regulator's z-score comparisons in BV2 cells treated with LPS, LPS + Telmisartan and LPS + Telmisartan + GW9662. The genes differentially expressed between LPS/DMSO, between LPS/LPS + Telmisartan and between LPS + Telmisartan/LPS + Telmisartan+GW9662 were put in Ingenuity Pathway Analysis program upstream regulators to identify the transcriptional regulator genes/drugs that may be responsible for most of the differentially expressed genes in each comparison. The z-score values infer the activation (positive number) or inhibition (negative number) states of predicted transcriptional regulators. The list of all the common upstream regulators in the 3 comparisons were put in a table and sorted based on the LPS comparison z-score. Except for NUPR1, Forskolin and ADRB, all the others upstream regulators show a complete reversal of z-score when comparing LPS to LPS + Telmisartan or to LPS + Telmisartan+GW9662. On the other hand, the z-score of LPS + Telmisartan+GW9662 is in the same direction and always greater than the one with only LPS + Telmisartan

Upstream regulator	LPS/DMSO z-score	LPS/LPS + Telmisartan z-score	LPS + Telmisartan/GW + LPS + Telmisartan z-score
TNF	8.83	−5.442	−3.088
CSF2	5.848	−4.438	−2.793
MYD88	5.816	−2.641	−2.818
TLR4	5.47	−3.434	−3.991
IL2	5.461	−2.31	−3.305
TLR3	5.329	−2.393	−3.928
TGFB1	5.226	−3.039	−3.051
TICAM1	5.216	−3.376	−3.451
TLR9	4.999	−2.008	−3.484
TLR7	4.58	−2.377	−3.533
TNF (family)	4.522	−2.96	−2.929
ERK1/2	4.43	−1.094	−2.174
APP	4.416	−2.077	−2.852
IL18	4.416	−2.04	−2.928
TLR2	4.416	−1.518	−2.575
IL6	4.226	−1.548	−2.39
STAT1	3.923	−2.422	−2.909
E. coli lipopolysaccharide	3.9	−2.092	−2.79
JUN	3.745	−0.827	−1.696
MAPK14	3.655	−1.614	−2.13
MAP3K7	3.518	−1.607	−2.938
IL7	3.514	−1.443	−1.709
NUPR1	3.182	−2	3.464
TNFRSF1A	3.056	−1.745	−0.102
CEBPB	3.017	−0.606	−1.978
OSM	2.791	−0.829	−2.107
IL4	2.684	−0.532	−2.528
IL33	2.584	−1.367	−2.404
ERBB2	2.255	−0.528	−2.184
IL21	2.001	−0.149	−1.709
TNFRSF1B	1.893	−1.095	−2.4
forskolin	1.783	0.487	−0.373
cycloheximide	1.589	−0.829	−1.937
PPARG	−1.202	1.49	1.54
dexamethasone	−1.238	0.368	1.597
MYC	−1.505	0.532	1.297
troglitazone	−1.559	1.749	2.759
CD28	−1.581	0.761	1.493
vorinostat	−1.736	0.067	0.522
NFE2L2	−1.741	1.734	3.905
APOE	−1.823	0.211	1.452
ADRB	−2.119	0.508	−1.941
caffeic acid phenethyl ester	−2.224	1.474	1.727
GW3965	−2.276	0.584	1.275
15-deoxy-delta-12,14 -PGJ 2	−2.402	2.268	2.863
rosiglitazone	−2.452	1.119	2.136
PTGER4	−2.454	1.577	3.058
TNFAIP3	−2.498	1.776	2.213
ZBTB16	−2.508	1.094	1.732

Table 1 (continued)

Upstream regulator	LPS/DMSO z-score	LPS/LPS + Telmisartan z-score	LPS + Telmisartan/GW + LPS + Telmisartan z-score
wortmannin	−2.588	0.601	1.356
resveratrol	−2.723	2.403	3.252
SOCS1	−2.809	0.303	2.771
VIP	−3.061	2.022	2.365
NR3C1	−3.279	2.413	2.511
PD98059	−4.49	1.259	1.622
CD3	−4.56	2.209	3.158
U0126	−4.679	1.214	1.91
LY294002	−5.419	2.264	1.992
SB203580	−5.623	1.207	1.486

Supplemental Table 5). Conversely, upstream regulator analysis revealed several kinase inhibitors with known neuroprotective properties, such as SB203580 [84], PD98059, LY294002, [85], U0126 [86] and GW3965 [87] (Table 1, Supplemental Table 5).

Network analysis comprised top diseases and functions: infectious diseases, cell cycle, death and survival, cell to cell signaling, cardiovascular and neurological disease, and lipid metabolism (Supplemental Table 5**).** These diseases and functions have been previously shown to significantly contribute to LPS-induced senescence in BV2 cells [88].

This information not only confirms the established multiple pro-inflammatory effects of LPS, but also reveals its participation in additional widespread mechanisms of injury. For example, the association with molecular mechanisms of cancer is not surprising, since inflammation has been earlier characterized as a major factor in the development and progression of malignancies [89, 90]. In turn, the association with the role of macrophages, fibroblasts and endothelial cells, supports the earlier report of LPS-induced direct injury to the neurovascular unit and the blood-brain barrier [91].

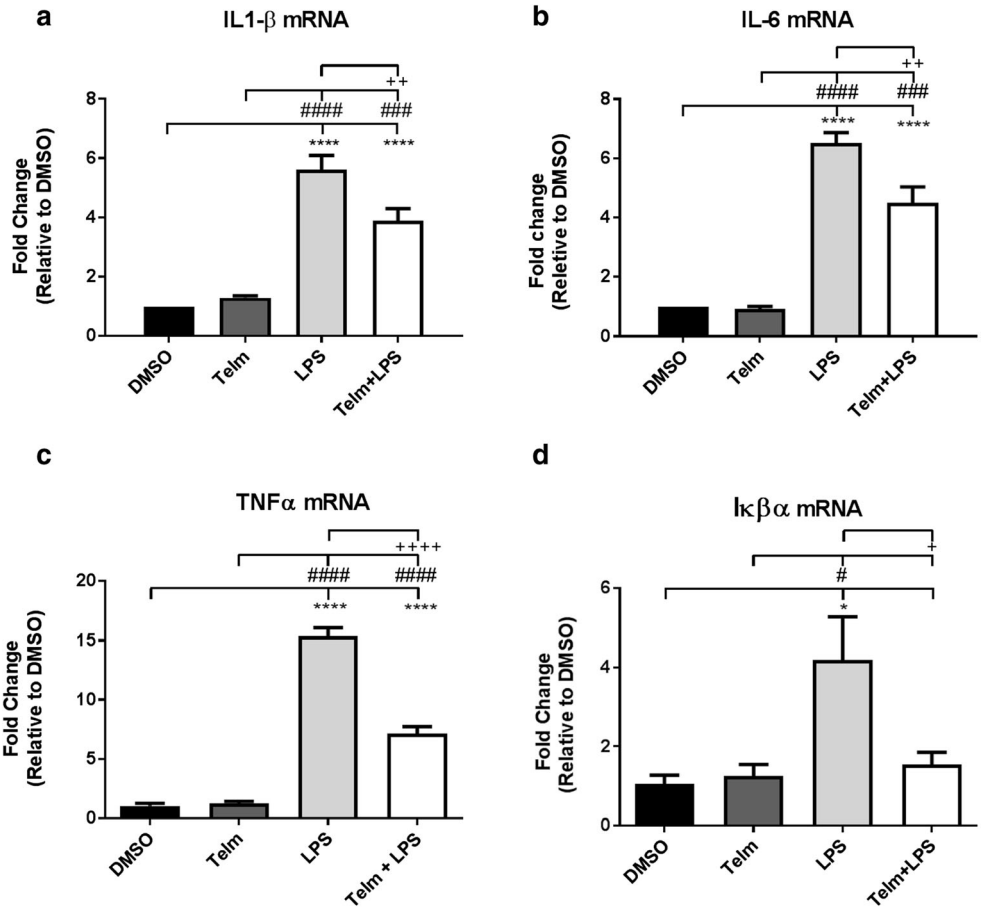

Fig. 4 Telmisartan significantly reduces LPS-induced IL-β, IL-6, TNFα and Iκβα gene expression. Pretreatment with Telmisartan 10 μM (Telm) for 2 h significantly reduces the increase in **a** IL-1β gene expression produced after 1 h of exposure to LPS (100 ng/ml). ANOVA F (3, 14) = 37.9 $p < 0.0001$. **b** IL-6 gene expression produced after 1 h of exposure to LPS (100 ng/ml). ANOVA F (3, 10) = 52.9 $p < 0.0001$. **c** TNFα gene expression produced after 1 h of exposure to LPS (100 ng/ml). ANOVA F (3, 8) = 196.4 $p < 0.0001$. **d** Iκβα gene expression produced after 1 h of exposure to LPS (100 ng/ml) F (3, 8) = 6.114 $p = 0.0182$. Results are means ± SEM for three to five groups analyzed independently. Data are expressed as fold-change relative to DMSO and were analyzed by one-way ANOVA followed by Newman-Keuls test for multiple comparisons. $^{****}p < 0.0001$, $^{*}p < 0.05$ compared to DMSO; $^{\#\#\#\#}p < 0.0001$, $^{\#\#\#}p < 0.001$, $^{\#}p < 0.05$ compared to Telm; $^{++}p < 0.01$, $^{+}p < 0.05$ compared to LPS

Telmisartan Normalized Expression of Multiple Pro-injury Genes Upregulated by LPS, and that of Protective Genes Downregulated by LPS

Differential gene expression comparing results from Telmisartan pretreatment followed by exposure to LPS injury for 1 h yielded over 572 well annotated transcripts significantly upregulated (335) or downregulated (237) by LPS (Supplemental Tables 3 and 6).

When followed by exposure to LPS, Telmisartan pretreatment completely reversed 145 of the 534 genes upregulated by LPS when administered alone, and the remaining genes showed a partial downregulation or normalization not reaching statistical significance (Supplemental Tables 3 and 6).

The genes upregulated by LPS and downregulated by Telmisartan include the pro-inflammatory cytokines IL1-β and IL-6, NFκB and its activator *miR221*, *miR155*, the TNF superfamily, TLR2, *Adamts1* and *miR129–2* (Supplemental Tables 3 and 6). These genes, including NFκB and its activator *miR221* [92], *miR155* [93], the TNF superfamily [94], TLR2 [95], *Adamts1* [96] and *miR129–2* [74] have been previously reported to be strongly associated with inflammation.

In addition, Telmisartan normalizes LPS-induced upregulation of *miR-146,* several *olfactory receptors, ABCA1, BCB2-like 11* and *ptgs2* (Supplemental Tables 3 and 6). *miR-146*, [97], the olfactory receptors [98] and *ABCA1* [99] are genes identified by other groups to promote neurodegenerative disorders; *BCB2-like 11* has been associated to apoptosis [100] and *ptgs2* with diabetes [101].

Only two genes, *Olfr700* and *Dynap*, remained upregulated after Telmisartan treatment of LPS-injured BV2 cells (Supplemental Table 3).

The effect of Telmisartan on gene expression of inflammatory factors upregulated by LPS was confirmed by qPCR analysis; Telmisartan significantly reduces IL-1β, IL-6, TNF-α and Iκβα gene expression enhanced by LPS (Fig. 4a, b, c, d). Another ARB, Valsartan, not directly stimulating PPARγ [43, 44] similarly reduced LPS-induced increase in IL-1β and IL-6 gene expression indicating that reduction of LPS effects is not restricted to the ARB Telmisartan (Supplemental Fig. 1).

Conversely, Telmisartan pretreatment significantly reversed the expression of 141 (fold change above 1.2) and normalized 262 (fold change between 1.19 and 1.00) of the 445 genes downregulated by LPS**.** The remaining 42 genes have a fold change between −1.0 and − 1.19 not reaching significance (Supplemental Table 3). Only one gene (*Fv1*) remained downregulated after Telmisartan pre-treatment, but with a *p* value of 0.089 (Supplemental Table 3).

Telmisartan upregulated many genes that were downregulated by LPS, including multiple *snoRNA*s, *Eid2b*, *Hist1h4m* and *Hist1h2bh* (Supplemental Table 3). These genes have been demonstrated to be protective, including multiple *snoRNA*s [75], the interacting inhibitor of differentiation *Eid2b*, protecting against oxidative stress, repressing glucocorticoid-dependent transcription [102], endogenous suppressor of TGF-β signaling [103] and *Hist1h4m* and *Hist1h2bh* inhibiting inflammation and vascular apoptosis [57, 104]. Only one gene, the antiretroviral restriction factor Fv1 [105] remained still downregulated after Telmisartan is administered before LPS (Supplemental Table 3).

Taken together, these results indicate that Telmisartan exerts multiple protective mechanisms, not only by downregulating gene expression enhanced by LPS and associated with cell injury and brain diseases, but also by normalizing protective genes downregulated by LPS (Supplemental Table 6).

Consequently, IPA analysis revealed canonical pathways previously identified to be related to neuroinflammation, NF-kB, HMGB1 [106]**,** glucocorticoid receptor, and many networks involving the immune response and infection, cellular function, movement, signaling, maintenance, assembly, organization, death and survival, development of neurological diseases [107] and a cancer signaling network [108] (Supplemental Table 6).

In turn, the IPA examination revealed that Telmisartan administration involved many upstream transcription regulators associated with LPS injury (Supplemental Table 6). As expected, the influence of Telmisartan administration prior to LPS treatment shows a negative z-score (or inhibition) for pro-inflammatory, cellular stress, neurodegenerative and metabolic disorders associated genes that are upregulated by LPS and downregulated by Telmisartan such as those encoding IL-1β, TNF-α, TLR3-TRIF (TICAM1)-TRAF-INF-β and the TLR3-TRIF (TICAM1)-NF-κB pathways, the TICAM1 pathway including HMGB1, TLR4, TLR2, MyD88, and several MAP kinases; ERK1/2, Map3k7, P38 MAPK (Supplemental Table 6).

Conversely, several up-stream regulators with positive z-score were found for genes downregulated by LPS and upregulated by Telmisartan, such as *Smad7, Tnfaip3, ATF4,* RARA, ZFP36, the kinase inhibitors U0126, SB203580, LY294002, SP600125 and PD98059, and compounds such as N-acetyl-L-cysteine, simvastatin, trichostatin A and thapsigargin (Table 1, Supplemental Table 6). These up-stream regulators have been previously reported to be protective, including *Smad7* [109], *Tnfaip3* [110], *ATF4* [111], RARA [112], ZFP36 [113], genes encoding the PI3K complex, U0126 [114] (GSE6675), SB203580 [84], LY294002 [115], SP600125 [116], PD98059 [117], N-acetyl-L-cysteine [118], simvastatin [119], trichostatin A [120, 121] and thapsigargin [122].

Networks with the highest number of molecules include cell death, survival and organization, lipid and carbohydrate metabolism and cancer (Supplemental Table 6).

To see whether the genes regulated by Telmisartan when administered alone overlap with genes regulated by Telmisartan followed by LPS treatment, we looked up the 622 genes upregulated by Telmisartan administered before LPS injury. Off these 622 genes upregulated by Telmisartan+ LPS, only 15 genes are also upregulated by Telmisartan only (Supplemental Table 3). On the other hand, there are only 14 genes downregulated by Telmisartan alone out of the 399 genes downregulated by Telmisartan+ LPS. (Supplemental Table 3). This means that the effect of Telmisartan on uninjured BV2 cells, although associated with neuroprotection, is vastly different from the neuroprotection that Telmisartan offers when the BV2 cells are injured with LPS.

GW9662, a PPARγ Antagonist, Protected from LPS-Induced Injury and Enhanced Telmisartan Neuroprotective Effects

To further clarify PPARγ participation on Telmisartan neuroprotective effects, we treated BV2 cells, in separate experiments, with the PPARγ antagonist GW9662, administered alone, added prior to LPS, and together with Telmisartan, prior to LPS injury. We expected that GW9662 administration would significantly reduce Telmisartan protective effects. Surprisingly, we found the opposite response, a significant increase in Telmisartan protective effects, such as a reduction in the LPS-induced increase in TNFα gene expression, confirmed by qPCR analysis (Fig. 3a and b).

The finding that GW9662 exerts protective effects unrelated to PPARγ antagonism is supported by previous reports of GW9662 inhibition of tumor growth and promotion of the anticancer effects of the PPARγ agonist rosiglitazone, independently of PPARγ activation [123]. In another study, GW9662 antagonism of PPARγ was not complete, since it only partially antagonized rosiglitazone neuroprotection from NMDA-induced neurotoxicity in cultured hippocampal slices [124]. Moreover, the report of GW9662 neuroprotection in our BV2 cells is not unique, since we found similar neuroprotective effects of another PPARγ antagonist, T0070907 (Supplemental Fig. 2A and 2B).

Treatment of BV2 cells with the PPARγ antagonist GW9662 and Telmisartan followed by LPS showed 211 downregulated and 135 upregulated genes compared to those expressed in BV2 cells treated only with Telmisartan and LPS (Supplemental Tables 3 and 7).

Gene analysis expanded our findings, demonstrating a negative correlation between GW9662 effects and inflammatory genes, drugs and toxins inducing inflammation. When we compared the gene expression resulting from the addition of GW9662 to BV2 cells pretreated with Telmisartan and injured by LPS, we found significant further downregulation of genes previously associated with inflammatory, autoimmune, neurodegenerative, microvascular and metabolic disorders, including 25 genes encoding histones, that are also the most downregulated genes in our study, *Edn1* and various chemokines such as *Ccl7*, *Ccl10 and Ccl2*. Histones participate in tumor progression and activate TLR receptors and the NLRP3 inflammosome, contributing to cerebrovascular injury [125, 126]. *Edn1*, encoding the vasoconstrictor endothelin, was proposed as an important factor in the cerebrovascular dysfunction in Alzheimer's disease [127]. *Ccl2* was reported to participate in a several neurological disorders and autoimmune disease [128].

These findings were confirmed by qPCR analysis. GW9662 significantly enhanced the Telmisartan-mediated reduction of LPS-induced increase in IL-1β (Fig. 3a) and reduced the increase in TNF-α gene expression produced by LPS (Fig. 3b). TLR2 was significantly upregulated by LPS (+1.44), down by LPS + Telimsartan (−1.18) and still down by LPS + Telmisartan+GW9662 (−1.13) (Supplemental Table 3).

Conversely, addition of GW9662 to BV2 cells pretreated with Telmisartan followed by LPS upregulated *Plin2, Hmox1,* and *Srxn1* (Supplemental Table 3). *Plin2* [129], *Hmox1* [130] and *Srxn1* [131] have been previously demonstrated to be neuroprotective, to reduce inflammation and to offer beneficial effects on metabolism.

Canonical pathways with larger numbers of associated genes include DNA methylation and transcriptional regulation/repression signaling, including multiple histone genes, inflammation, and oxidative stress response (Supplemental Table 7). Networks with the largest number of molecules include post-translational modifications, cardiovascular disease and cell death and survival, signaling, interaction and development (Supplemental Table 7).

At the disease and function level, the GW9662 treatment of BV2 cells treated with both Telmisartan and LPS included a decrease in inflammatory response, cell-to-cell signaling/interaction, cellular movement and macrophage activation. Several functions associated with cardiovascular disease and diabetes were also statistically significant. Interestingly, organism survival showed the highest number of upregulated genes at the disease level (Supplemental Table 7).

The IPA analysis of upstream regulators for the genes differentially expressed between BV2 cell treated with Telmisartan and LPs, and BV2 cells treated with Telmisartan and GW9662 followed by LPS revealed negative correlations with IL-1β and TNF-α, NF-κB, several TLRs, INF, *poly rl:rC*, enterotoxinB, cardiotoxin and APP (Table 1 and Supplemental Table 7). These factors were demonstrated to induce inflammation and autoimmune diseases normally activated by LPS, including the pro-inflammatory cytokines IL-1β and TNF-α, NF-κB, the TLRs, INF and the interferon-inducible gene *poly rl:rC* [132], and enterotoxin B [133]. Cardiotoxin was found to exhibit anti-neoplastic properties [134] and APP is a multifunctional protein associated with Alzheimer's disease [135]. Additionally, there was a negative

correlation with ERK1/2 and p38MAPK pathways, including MEK (MAP2K1) and with Raf1 (MAP3K) which acts upstream of MEK and ERK (Table 1 and Supplemental Table 7),

Of interest is the TGF-beta 1 pathway, as the most downregulated pathway after LPS and TNFα, with over 90 genes upregulated by LPS, 69 of them are downregulated by Telmisartan and 32 by GW9662 (Supplemental Table 7). TGF-beta 1 was reported to increase microglial p38 MAPK and AKT phosphorylation [136] that is impaired by SB203580 and LY294002, two kinase inhibitors [84, 137] that we found to have positive correlation with GW9662 (Supplemental Table 7).

Conversely, we found that genes upregulated by GW9662 are positively correlated with upstream regulators such as genes, drugs, statins and other neuroprotective compounds (Table 1 and Supplemental Table 7). There was a positive correlation with factors previously reported to protect from oxidative stress, inflammation and age-related disorders such as cancer, metabolic and cardiovascular diseases. These include the transcription regulator NFE2l2 [138, 139] the nuclear receptor NR3C1 [140], several kinase inhibitors including LY294002, PD98059, SB203580 and U0126, statins such as fluvastatin, cerivastatin, atorvastatin and simvastatin, resveratrol [141] and curcumin [142, 143]. When comparing the IPA's upstream regulator's z-score activation/inhibition for LPS, LPS + Telmisartan and LPS + Telmisartan + GW9662, we found a striking reversal action of Telmisartan over LPS and that reversal is even enhanced with the addition of GW9662 (Table 1).

Using GSEA, we compared the results obtained after treating the BV2 cell culture with the PPARγ antagonist GW9662 in the presence of Telmisartan and LPS with published genesets. We found several remarkable positive correlations between GW9662 effects with those reported for inhibitors of the MAP kinase pathways. These include a positive correlation with genes reported to be expressed in astrocytes activated with FGF2 and then treated with the MEK inhibitor U0126 (Supplemental Table 8), [114] (GSE6675) a compound that reduces apoptosis and protects from ischemia [144, 145]. Supplemental Table 8 reveals that GW9662 and U0126 share over 100 genes associated with neuroprotection and reduction of inflammation (Fig. 5a and Supplemental Table 8). Conversely, the effects of GW9662 were negatively correlated with upregulated genes in mouse primary microglia cells treated with IL-4 [146] (GSE49329) (Fig. 5b) and with genes upregulated with excitotoxic glutamate concentrations and down regulated by Candesartan in rat primary cerebellar neurons treated with glutamate [14] (GSE67036), (Fig. 5c). There was a positive correlation between GW9662 effects with the antidyskinetic effect of PD98059 in a pre-clinical model of Parkinson disease [147] (GSE93695), (Fig. 5d and Supplemental Table 8). These GW9662 analyses demonstrated also correlations with the effects of the kinase inhibitors PD98059, SB203580 and LY294002 as revealed by our IPA analysis (Supplemental Table 8).

GW9662 effects negatively correlated with a number of gene signatures indicating cellular injury, such as the neuroblastoma cell line SH-SY5Y treated with PDGF and pretreated with the ERK inhibitors U0126 [117] (GSE7403) (Fig. 5e), and another negative correlation with a gene signature of upregulated genes in MCF-7 cell lines stably overexpressing growth factor signaling, constitutively active EGFR [148] (Fig. 5f and Supplemental Table 8), MEK (MAP2K1) [148] (Fig. 5g and Supplemental Table 8) or Raf1 [148] (Fig. 5h and Supplemental Table 8).

In addition to our findings using GW9662, we found additional evidence of the paradoxical effect of PPARγ inhibition of LPS-induced alterations in gene expression. Administration of another PPARγ antagonist, T0070907, enhanced the Telmisartan reduction of LPS-induced gene expression of inflammatory cytokines; T0070907, administered alone, abolished the IL-1β gene expression enhanced by LPS and significantly reduced that of IL-6 (Supplemental Fig. 2A and 2B). Furthermore, PPARγ activation with the full agonist Pioglitazone did not alter the LPS-induced increase in IL-1β and IL-6 gene expression (Fig. 5).

Hypothesis

In our BV2 mouse cell model, we have found that LPS significantly upregulated, and that Telmisartan and GW9662 significantly downregulated TLR2 gene expression (Supplemental Table 3). Mouse microglia strongly expresses most of TLRs [3] and LPS upregulates TLR2 and TLR4 gene expression in microglia [149]. LPS was positively, and Telmisartan and GW9662 were negatively, correlated with several TLR upstream regulators including TLR2, and with several components of the TLR signaling pathways (Table 1). This makes downregulation of the TLR2 pro-inflammatory gene and signaling pathway a candidate for a novel neuroprotective mechanism for Telmisartan and GW9662 in microglia, beyond AT1 receptors or PPARγ.

The association of AT1 receptor blockade and TLR downregulation is well established in the literature. AT1 receptor stimulation enhances TLR gene expression in microglia and Telmisartan and several other ARBs reduce gene expression of several TLRs in many in vitro and in vivo models [13, 36, 150–157]. In addition, ARBs downregulate gene expression of many members of the TLR down-stream pathways, including CD14 [13], MyD88 [158, 159], IRAK1 and TRAF6 [160] and MMP-2 activation [153]. Whether the novel mechanism postulated here has a role in other conditions remain to be determined.

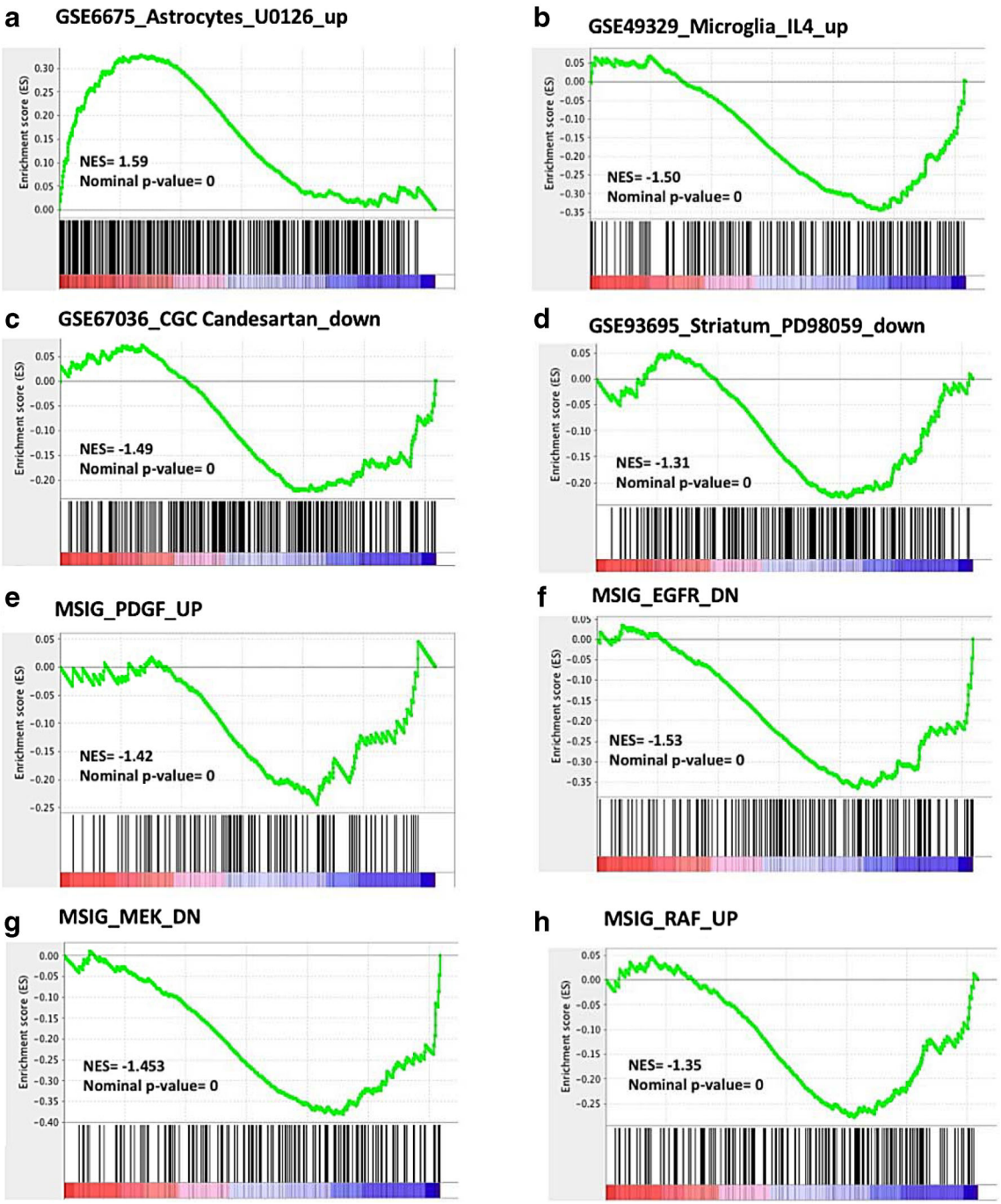

Fig. 5 Gene Set Enrichment Analysis (GSEA) of BV2 cells treated with Telmisartan + LPS versus Telmisartan + LPS + GW9662. Gene signatures (vertical bars) from GEO (NCBI) and MSIG (Broad Institute) were overlaid on the ranked list of genes from our microarray data (red and blue bar). In mouse astrocytes, genes activated by FGF2 and up regulated by U0126 correlate with genes upregulated by GW9662 (Fig. 4a) [114]. Genes up regulated by IL-4 in murine microglia are negatively correlated with genes up-regulated by GW9662 (Fig. 4b, GSE49329, [146]). Genes upregulated by glutamate and down regulated by Candesartan in rat cerebellar cortical neurons correlate with genes downregulated by GW9662 (Fig. 4c, GSE67036 [14]). In the striatum of dyskinetic rats treated with L-DOPA, genes down regulated by the anti-dyskinesia MEK inhibitor (PD98059) are also down-regulated by GW9662 (Fig. 4d, GSE93695 [147]). Genes up-regulated by PDGF in the neuroblastoma cell line SH-SY5Y pre-treated by the MEK inhibitor U0126 are negatively correlated with genes up-regulated by GW9662 (Fig. 4e [117, 144]). In the MCF7 breast cancer cell line stably overexpressing ligand-activable EGFR, genes down-regulated by EGFR are also down-regulated by GW9662 (Fig. 4f [148]). In the MCF7 breast cancer cell line stably overexpressing constitutively active MAP2K1 (MEK), genes down-regulated by MEK are also down-regulated by GW9662 (Fig. 4g [148]). In the MCF7 breast cancer cell line stably overexpressing constitutively active RAF1, genes upregulated by RAF1 are downregulated by GW9662 (Fig. 4h GSE3542 [148])

Conclusions

We revealed Telmisartan neuroprotection in a culture of microglia-like BV2 cells with a response to LPS like that of primary microglia, and not expressing AT1A receptors or PPARγ genes.

This indicated that in our system Telmisartan effects were the consequence of novel neuroprotective mechanisms, beyond its canonical AT1 receptor blockade and PPARγ activation.

Unexpectedly, in our system, administration of PPARγ inhibitors significantly decreased LPS-induced injury and enhanced, rather than reduced, Telmisartan neuroprotective effects.

Telmisartan and the PPARγ inhibitor GW9662 protective effects are widespread, including normalization of the expression of many pro-injury genes upregulated and that of many protective genes downregulated by LPS.

IPA analysis uncovered many associated pathways, diseases and functions and upstream regulators reducing cell toxicity and promoting protection.

GSEA analysis revealed multiple gene sets associated with several disease phenotypes, validating IPA analysis and homing in on common pathways of major clinical interest. They included not only a reduction of inflammation and a positive correlation with neuroprotective kinase inhibitors, but also indicate possible protection against cardiovascular, metabolic and neurodegenerative disorders and malignancies, where inflammation plays determinant roles.

Our results may have translational significance. Telmisartan neuroprotection in uninjured cells supports the hypothesis that this compound may have a role in preventing or delaying neurodegenerative and age-related disorders. The strong association of Telmisartan effects with that of kinase inhibitors is promising, since this class is increasingly considered for the treatment of many disorders. The protective effects of GW9662 indicate that novel derivatives of this compound with translational value may be developed. In addition, future drug development may result in Telmisartan and/or GW9662 derivatives with enhanced downregulating properties on the TLR signaling pathways.

The present study is not without limitations. Our results have been obtained using a microglia cell line, and whether they may be replicated in primary microglia remains to be determined. This study has been restricted to determine changes in gene expression without analysis of protein expression or direct cellular effects. The molecular mechanisms involved in the Telmisartan and GW9662 downregulation of the TLR signaling pathways have not been determined.

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Authors' Contributions AE performed the microarrays, conducted and analyzed the genome wide study, interpreted the results, and contributed to writing the manuscript.

YR, SA and EW performed the cells culture and qPCR experiments, interpreted the results and contributed to writing the manuscript.

JMS conceived the project, supervised the cell culture and qPCR experiments, interpreted the results, and contributed to writing the manuscript.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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