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Trazodone treatment protects neuronal-like cells from inflammatory insult by inhibiting NF-κB, p38 and JNK

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ABSTRACT

Growing evidence suggests that alterations of the inflammatory/immune system contribute to the pathogenesis of major depression and that inflammatory processes may influence the antidepressant treatment response. Depressed patients exhibit increased levels of inflammatory markers in both the periphery and brain, and high comorbidity exists between depression and diseases associated with inflammatory alterations. Trazodone (TDZ) is a triazolopyridine derivative that belongs to the class of serotonin receptor antagonists and reuptake inhibitors. Although the trophic and protective effects of classic antidepressants have extensively been exploited, the effects of TDZ remain to be fully elucidated. In this study, the effects of TDZ on human neuronal-like cells were investigated under both physiological and inflammatory conditions. An *in vitro* inflammatory model was established using lipopolysaccharide (LPS) and tumour necrosis factor-α (TNF-α), which efficiently mimic the stress-related changes in neurotrophic and pro-inflammatory genes.

Our results showed that TDZ significantly increased the mRNA expression of both brain-derived nerve factor (BDNF) and cAMP response element-binding protein (CREB) and decreased the cellular release of the pro-inflammatory cytokine interferon gamma (IFN-γ) in neuronal-like cells.

In contrast, neuronal cell treatment with LPS and TNF-α decreased the expression of CREB and BDNF and increased the expression of nuclear factor kappa B (NF-κB), a primary transcription factor that functions in inflammatory response initiation. Moreover, the two agents induced the release of pro-inflammatory cytokines (*i.e.*, interleukin-6 and IFN-γ) and decreased the production of the anti-inflammatory cytokine interleukin-10. TDZ pre-treatment completely reversed the decrease in cell viability and counteracted the decrease in BDNF and CREB expression mediated by LPS-TNF-α. In addition, the production of inflammatory mediators was inhibited, and the release of interleukin-10 was restored to control levels.

Furthermore, the intracellular signalling mechanism regulating TDZ-elicited effects was specifically investigated. TDZ induced extracellular signal-regulated kinase (ERK) phosphorylation and inhibited constitutive p38 activation. Moreover, TDZ counteracted the activation of p38 and c-Jun NH_2-terminal kinase (JNK) elicited by LPS-TNF-α, suggesting that the neuro-protective role of TDZ could be mediated by p38 and JNK.

Overall, our results demonstrated that the protective effects of TDZ under inflammation in neuronal-like cells function by decreasing pro-inflammatory signalling and by enhancing anti-inflammatory signalling.

1. Introduction

Major depression is a common disorder that has a lifetime prevalence more than 15%. By 2020, major depressive disorder is estimated to be the second biggest contributor to the global burden of disease [1]. Over the last two decades, several lines of evidence have demonstrated that pro-inflammatory cytokines are involved in the pathophysiology of depression [2,3], suggesting that inflammatory processes may influence the antidepressant treatment response. When compared with non-depressed individuals, patients with major depression have been found to exhibit all of the cardinal features of inflammation, including increases in relevant inflammatory cytokines (*i.e.*, interleukin 6, IL-6; tumour necrosis factor, TNF-α) and their soluble receptors in the peripheral blood and cerebrospinal fluid [4,5]. Pro-inflammatory cytokines induce inflammatory signalling pathways including nuclear factor kappa B (NF-κB) and ultimately contribute to increased excitotoxicity, subsequently decreasing the production of relevant trophic factors [6]. Of note, the signalling pathways of pro-inflammatory cytokines can also contribute to the pathogenesis of depression. Indeed, mitogen-activated protein kinase (MAPK) pathways, including p38, c-Jun NH_2-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) 1/2, as well

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as inflammatory molecule gene expression, mediate the effects of cytokines on cell proliferation/differentiation and apoptosis [7–9]. Based on this evidence, the inhibition of neuro-inflammation has been postulated as a putative target in the treatment of neurodegenerative diseases and depressive disorders.

In this respect, the chronic administration of tricyclic antidepressants or agomelatine prevents stress-induced changes in cerebral metabolites, hippocampal volume, and cell proliferation in rats [10,11]. Several studies have demonstrated that selective serotonin reuptake inhibitors (SSRIs) and mood stabilizers not only decrease immunotherapy-induced depressive symptoms but also decrease the inflammatory response and lower pro-inflammatory factors (IL-2, IL-6, TNF-α, and interferon gamma (IFN-γ)) [12,13]. In addition, whereas the levels of brain-derived nerve factor (BDNF) and of the cAMP response element-binding protein (CREB) decrease in stress induced animal models of depression, their levels significantly increase after classic antidepressant treatment [10,14–17].

Trazodone (TDZ) is a triazolopyridine derivative that is structurally unrelated to the derivatives of other major classes of antidepressants. Its mechanism of action in the treatment of depression has not been fully elucidated primarily because of its affinity for a variety of receptors that may contribute to its clinical actions. TDZ is defined as a serotonin receptor antagonist and reuptake inhibitor (SARI) antidepressant. Unlike SSRIs, SARIs, such as TDZ, provide simultaneous inhibition of SERT, partial agonism of serotonin 5-HT_{1A} receptors, and antagonism of 5-HT_{2A} and 5-HT_{2C} receptors, thus avoiding the tolerability issues that are often associated with 5-HT_{2A} and 5-HT_{2C} receptor stimulation and improving treatment tolerability [18–20]. Moreover, TDZ exerts antagonistic properties against α1- and α2-adrenergic receptors and histamine H1 receptors with minimal anticholinergic effects. At low doses (25–100 mg), TDZ has therapeutic activity as a hypnotic [18,21]. The actions of several neurotransmitter systems including serotonin, noradrenaline, dopamine, acetylcholine and histamine are known to be involved in the arousal mechanism [18,22]. Thus, the pharmacological properties of TDZ are unique among antidepressants; its mixed serotonergic and adrenolytic activity makes TDZ an attractive off-label treatment option for multiple disorders, including insomnia, anxiety, behavioural disorders associated with dementia and Alzheimer's disease, substance abuse, schizophrenia, eating disorders and fibromyalgia [23].

Although the neuro-protective effects of TDZ have been investigated in animal models of depressive disorder [24] or of transient global ischaemia [25], the molecular and intracellular mechanisms of TDZ under *in vitro* neuro-inflammation remain to be fully elucidated.

In this study, an *in vitro* neuronal model system was established and used to investigate whether TDZ could exhibit neuro-protective effects during inflammation. For this purpose, H9-derived human neural precursor cells (NSCs) were differentiated to neuronal-like cells and then challenged with lipopolysaccharide (LPS) and TNF-α to establish a human *in vitro* model of neuroinflammation. The effects of TDZ under physiological conditions and under inflammatory stress exposure were assessed by measuring cellular viability and cytokine release, as well as the induction of pro-inflammatory genes and neurotrophic and pro-survival factors. Moreover, the possible intracellular cascades at the basis of the effects elicited by TDZ were investigated and correlated to its protective effects.

2. Materials and methods

2.1. Materials

H9-derived human NSCs were purchased from GIBCO (Life Technologies, Milan, Italy). ELISA kits for cytokines' determination were from Thermo Fisher Scientific, Rodano, Milan, Italy. All other reagents were obtained from standard commercial sources and were of the highest commercially available grade.

2.2. Cell culture and neuronal differentiation

H9-derived NSCs were cultured in complete medium consisting of KnockOut™D-MEM/F-12 with StemPro® Neural Supplement, 20 ng/ml of basic fibroblast growth factor (bFGF, Life Technologies, Milan, Italy), 20 ng/ml of epidermal growth factor (EGF, Life Technologies, Milan, Italy), and 2 mM L-glutamine at 37 °C in 5% CO_2.

For neuronal differentiation, H9-derived NSCs were plated on polyornithine and laminin-coated culture dishes, and switched into a defined Neurobasal serum-free medium, containing 2% B-27, 2 mM L-glutamine and 5 μM retinoic acid (RA, [26]) up to seven days.

2.3. Pharmacological treatments and neuronal inflammation

Trazodone (TDZ, Angelini Acraf S.p.A.) was diluted to different concentrations of stock solutions (10-fold of final concentration) by PBS. After plating, neuronal-like cells, differentiated from H9-derived NSCs, were treated with different concentrations of TDZ (1 nM–10 μM) for 24 or 72 h.

To set up the neuro-inflammation model, neuronal-like cells were incubated for 2, 6 or 16 h with LPS (50 μg/ml) and/or TNF-α (50 ng/ml), commonly used as inflammation inductors [27,28]. To verify the protective effects of TDZ, neuronal-like cells were treated with TDZ for 24 h or 72 h; following incubation time, cells were washed and incubated with LPS and TNF-α for 16 h. In some experiments, the effects of a 5-HT_2 receptor stimulation was assessed using the agonist (*R*)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane [(*R*)-DOI], which displays high and comparable affinities toward 5-HT_{2A} and 5-HT_{2c} serotonin receptors (3.36 ± 0.91 nM and 3.38 ± 0.66 nM respectively) [29]. Neuronal-like cells were incubated for 20 min with 30 nM (*R*)-DOI, before the addition of TDZ (1 nM–10 μM) for 24 or 72 h.

2.4. Cell proliferation/viability assays

Neuronal-like cells, differentiated from H9-derived NSCs, were treated as described above. Following incubation time, cell viability was determined using the MTS assay according to the manufacturer's instruction. The dehydrogenase activity in active mitochondria reduces the 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxy methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) to the soluble formazan product. The absorbance of formazan at 490 nM was measured in a colorimetric assay with an automated plate reader. Within an experiment, each condition was assayed in triplicate and each experiment was performed at least three times. The results were calculated by subtracting the mean background from the values obtained from each test condition, and were expressed as the percentage of the control (untreated cells).

The effects of TDZ on neuronal-like cells were also evaluated using the trypan blue-exclusion assay. Neuronal-like cells, treated as described above, were collected and centrifuged at 300 ×*g* for 5 min. The harvested cells were mixed with an equal volume of 0.4% trypan-blue dye, and the blue (dead cells) and white (living cells) cells in each well were manually counted. The number of live cells for each condition was reported as the number of living and dead cells in each well.

2.5. Annexin V and 7-AAD staining

Dual staining with Annexin V conjugated to fluorescein-isothiocyanate (FITC) and 7-amino-actinomycin (7-AAD) was performed using the commercially available kit (Muse Annexin V and Dead Cell Kit; Merck KGaA, Darmstadt, Germany), as previously reported [30]. Neuronal-like cells were treated with medium alone (control), or 1 μM TDZ for 72 h. After TDZ removal, cells were incubated with LPS and TNF-α for an additional 16 h. At the end of the treatment periods, both floating and adherent cells were collected, centrifuged at 300 ×*g* for 5 min and suspended in cell culture medium. Then, a

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100 μl aliquot of cell suspension (about 5×10^4 cell/ml) was added to 100 μl of fluorescent reagent and incubated for 10 min at room temperature. After incubation, the percentages of living, apoptotic and dead cells were acquired and analysed by a Muse™ Cell Analyser in accordance with the manufacturer's guidelines. In cells undergoing apoptosis, Annexin V binds to phosphatidylserine, which is translocated from the inner to the outer leaflet of the cytoplasmic membrane. Double staining is used to distinguish between viable, early apoptotic, and necrotic or late apoptotic cells. Annexin V-FITC positive and 7-AAD negative cells were identified as early apoptotic. Cells, which were positive for both Annexin V-FITC and 7-AAD, were identified as cells in late apoptosis or necrosis.

2.6. RNA extraction and real time PCR analysis

H9-derived NSCs were differentiated up to seven days with Neurobasal medium and RA. For the analysis of stemness and neuronal markers, cells were collected at zero, four and seven days of differentiation.

Neuronal-like cells were treated with medium alone (control), or TDZ (100 nM or 1 μM) for 24 or 72 h. In some experiments, after TDZ removal, cells were incubated with LPS and TNF-α for an additional 16 h. At the end of treatments, cells were collected, and total RNA was extracted using the RNeasy® Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and briefly as previously reported [30]. Purity of the RNA samples was determined by measuring the absorbance at 260:280 nm. cDNA synthesis was performed with 500 ng of RNA using the i-Script cDNA synthesis kit (BioRad, Hercules, USA) following the manufacturer's instructions. Primers used for RT-PCR were designed in intron/exon boundaries to ensure that products did not include genomic DNA. RT-PCR reactions consisted of 25 μl Fluocycle® II SYBR® (Euroclone, Milan, Italy), 1.5 μl of both 10 μM forward and reverse primers, 3 μl cDNA, and 19 μl of H_2O. All reactions were performed for 40 cycles using the following temperature profiles: 98 °C for 30 s (initial denaturation); T °C (see Table 1) for 30 s (annealing); and 72 °C for 3 s (extension). β-Actin was used as the housekeeping gene. mRNA levels for each sample were normalized against β-actin mRNA levels, and relative expression was calculated by using the Ct value. PCR specificity was determined by both the melting curve analysis and gel electrophoresis.

2.7. Cytokine assays

Neuronal-like cells were treated with medium alone (control), or TDZ (1 nM–10 μM) for 24 or 72 h. In some experiments, after TDZ removal, cells were incubated with LPS and TNF-α for an additional 16 h. The amount of IL-6, IL-10 and IFN-γ released into the culture medium was measured using ELISA kits (Thermo Fisher Scientific, Rodano, Milan, Italy) following the manufacturer's instructions. Culture supernatants were collected and stored at −80 °C until assayed for cytokine content.

2.8. Phosphorylation assays

Neuronal-like cells were treated with medium alone (control), or TDZ (1 nM–10 μM) for 24 or 72 h. In some experiments, after TDZ removal, cells were incubated with LPS and TNF-α for an additional 16 h. At the end of treatments, cells were rapidly fixed with 4% formaldehyde to preserve activation of specific protein modification. Levels of total and phosphorylated p38 and ERK1/2 were determined by ELISA assays, as previously reported [31]. Briefly, the cells were washed three times with wash buffer (0.1% Triton X-100 in PBS) and 100 μl of quenching buffer (1% H_2O_2; 0.1% sodium azide in wash buffer) was added and incubation was protracted for another 20 min. The cells were washed with PBS twice, and then 100 μl of blocking solution (1% BSA; 0.1% Triton X-100 in PBS) was added for 60 min. After blocking, cells were washed three times with wash buffer and the specific primary antibodies (anti-phospho-p38, 1:500, MABS64, Merck Millipore, Darmstadt, Germany; anti-p38, 1:500, sc-7972 Santa Cruz Biotechnology, Inc. Dallas, Texas U.S.A.; anti-phospho ERK1/2, 1:500, sc-7383 Santa Cruz Biotechnology; anti-ERK1/2, 1:500, #4695 Cell Signaling Technology; anti-phospho JNK, 1:300, sc-6254 Santa Cruz Biotechnology; anti-JNK, SAB4200176, 1:750, Sigma Aldrich, Milan, Italy) were added o.n. at 4 °C. Subsequent incubation with secondary HRP-conjugated antibodies and developing solution allowed a colorimetric quantification of total and phosphorylated levels. Blanks were obtained processing wells without cells in the absence of the primary antibody. The relative number of cells in each well was then determined using Crystal Violet solution. The results were calculated by subtracting the mean background from the values obtained from each test condition; values were normalized to the number of cells in each well, and were expressed as the percentage of the control (untreated cells).

2.9. Western blotting analysis

H9-derived NSCs were differentiated up to seven days as described above. The cells (H9-NSCs, or RA-differentiated for four or seven days) were collected and then were lysed for 60 min at 4 °C using 200 μl of RIPA buffer (9.1 mM NaH_2PO_4, 1.7 mM Na_2HPO_4, 150 mM NaCl, pH 7.4, 0.5% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS, and a protease-inhibitor cocktail). Equal amounts of the cell extracts (40 μg of protein) were diluted in Laemmli sample solution, resolved using

Table 1
Nucleotide sequences, annealing temperature and product size of the primers utilized in real time PCR experiments.

Gene	Primer nucleotide sequences	Product size (base pairs)	Annealing temperature
CD133	FOR: 5′-TCCACAGAAATTTACCTACATTGG-3′ REV: 5′-CAGCAGTTCAAGACGCAGATGACCA-3′	251 bp	55 °C
MAP2	FOR: 5′-TTGGTGCCGAGTGAGAAGAA-3′ REV: 5′-GGTCATGCTGGCAGTGGTTGGT-3′	280 bp	55 °C
CREB	FOR: 5′-AAGCTGAAAGTCAACAAATGACA-3′ REV: 5′-CCTCTTTTCAGAAAAATTCAGGA-3′	240 bp	52 °C
BDNF	FOR: 5′-TACATTTGTATGTTGTGAAGATGTTT-3′ REV: 5′-TTACTCGCCCCGGACCCTCT-3′	131 bp	56 °C
NF-κB	FOR: 5′-GCTCCGGAGACCCCTTCCA-3′ REV: 5′-GGTTTGAGGTAGTTTCCCAGT-3′	198 bp	54 °C
mTOR	FOR: 5′-CCGTTCCATCTCCTTGTCACG-3′ REV: 5′-CCACTTACTCTGCAGTGTG-3′	209 bp	56 °C
NeuN	FOR: 5′-GCGGCTACACGTCTCCAACATC-3′ REV: 5′-ATCGTCCCATTCAGCTTCTCCC-3′	189 bp	56 °C
β-Actin	FOR: 5′-GCACTCTTCCAGCCTTCCTTCC-3′ REV: 5′-GAGCCGCCGATCCACACG-3′	254 bp	55 °C

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SDS-PAGE (8.5%), transferred to PVDF membranes and probed overnight at 4 °C using the following primary antibodies: anti-nestin (sc-20978, Santa Cruz Biotechnology, Heidelberg, Germany); anti-NeuN (ab177487, Abcam, Cambridge, UK); and glyceraldehyde-3-phosphate dehydrogenase, GAPDH (G9545, Sigma Aldrich, Milan, Italy). The primary antibodies were detected using the appropriate peroxidase-conjugated secondary antibodies, which were then detected using a chemioluminescent substrate (ECL, Perkin Elmer). Densitometric analysis of the immunoreactive bands was performed using ImageJ Software.

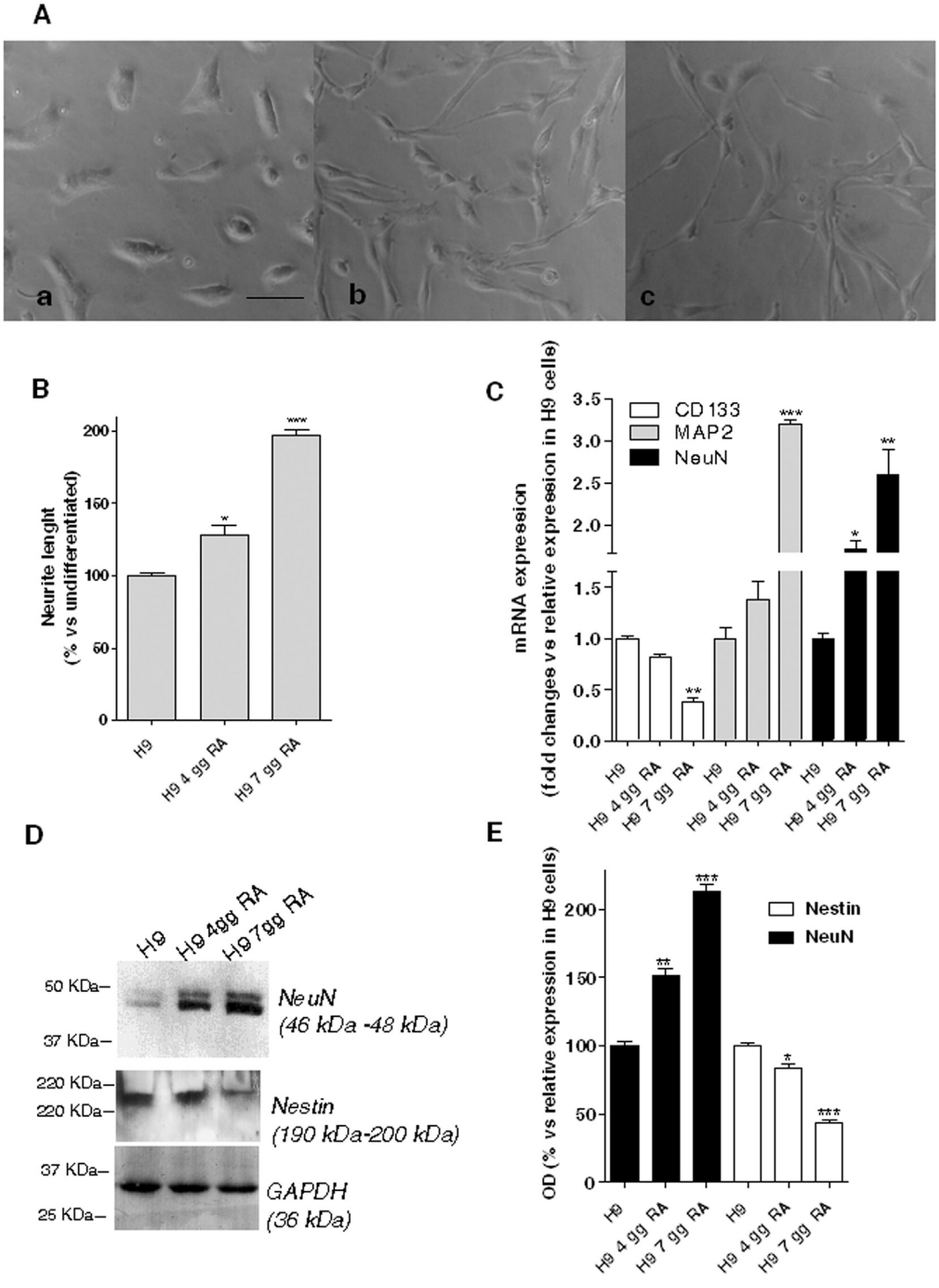

Fig. 1. Neuronal differentiation of H9-derived NSCs. H9-derived NSCs were switched into a defined serum-free Neurobasal medium, containing 2% B-27, 2 mM L-glutamine and 5 μM retinoic acid (RA) for up to seven days. Representative cell micrographs (panel A) and neurite length measurement (panel B) of H9-derived NSCs in growth medium (a) or after differentiation for four (b) or seven (c) days with RA. Panel C) The relative mRNA quantification of the neuronal markers (MAP2 and NeuN) and of the stem cell marker CD133 was performed by real-time RT-PCR as described in the Materials and methods section. The data are expressed as fold changes *versus* the relative expression in H9-derived NSCs, and are the mean ± SEM of three different experiments. Panels D, E) Cell lysates were prepared from undifferentiated H9-derived NSCs, or differentiated cells for four or seven days with RA. The protein levels of the neuronal marker NeuN and of the stem cell marker Nestin were evaluated by western blot analysis; GAPDH was the loading control. D) Representative Western blots. E) Densitometric analysis of the immunoreactive bands performed using ImageJ. The data are expressed as percentages relative to expression levels in H9-derived NSCs and are the mean values ± SEM of three different experiments. Statistical significance was determined with Student's t-test: $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* the relative expression in H9-derived NSCs. Scale bar, 20 μm.

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Neuronal-like cells were treated with medium alone (control), or TDZ (1 nM–10 μM) for 24 or 72 h. In some experiments, after TDZ removal, cells were incubated with LPS and TNF-α for an additional 16 h. At the end of the treatment period, the cells were collected and samples were evaluated by western blotting using the primary antibodies described in Section 2.8. The primary antibodies were detected using the appropriate peroxidase-conjugated secondary antibodies, which were then detected using a chemioluminescent substrate (ECL, Perkin Elmer). Densitometric analysis of the immunoreactive bands was performed using ImageJ Software.

2.10. Statistical analysis

The nonlinear multipurpose curve-fitting program Graph-Pad Prism (GraphPad Software Inc., San Diego, CA) was used for data analysis and graphic presentations. All data are presented as the mean ± SEM. Statistical analysis was performed by one-way analysis of variance (ANOVA) with Bonferroni's corrected t-test for post-hoc pair-wise comparisons. Student's t-test was used to evaluate whether differences between a single experimental group and the control were statistically significant. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Neuronal differentiation

H9-derived NSCs were switched into a defined serum-free Neurobasal medium containing 5 μM RA for up to seven days as reported previously [26]. Indeed, RA promotes the production of mature neurons, and these neurons express dopamine and/or serotonin receptors and form complex plexuses of neuronal processes [26]. As depicted in Fig. 1A and B, RA induced a neuron-like morphology starting from 4 days of treatment, showing a time-dependent increase in neurite length. Real-time RT-PCR (Fig. 1C) and western blot (Fig. 1D and E) analyses confirmed that RA-differentiated cells at seven days presented significantly higher levels of the neuronal markers MAP2 and NeuN and minor levels of the stem cell markers CD133 and Nestin compared with H9 NSCs. These results confirmed that serum-free

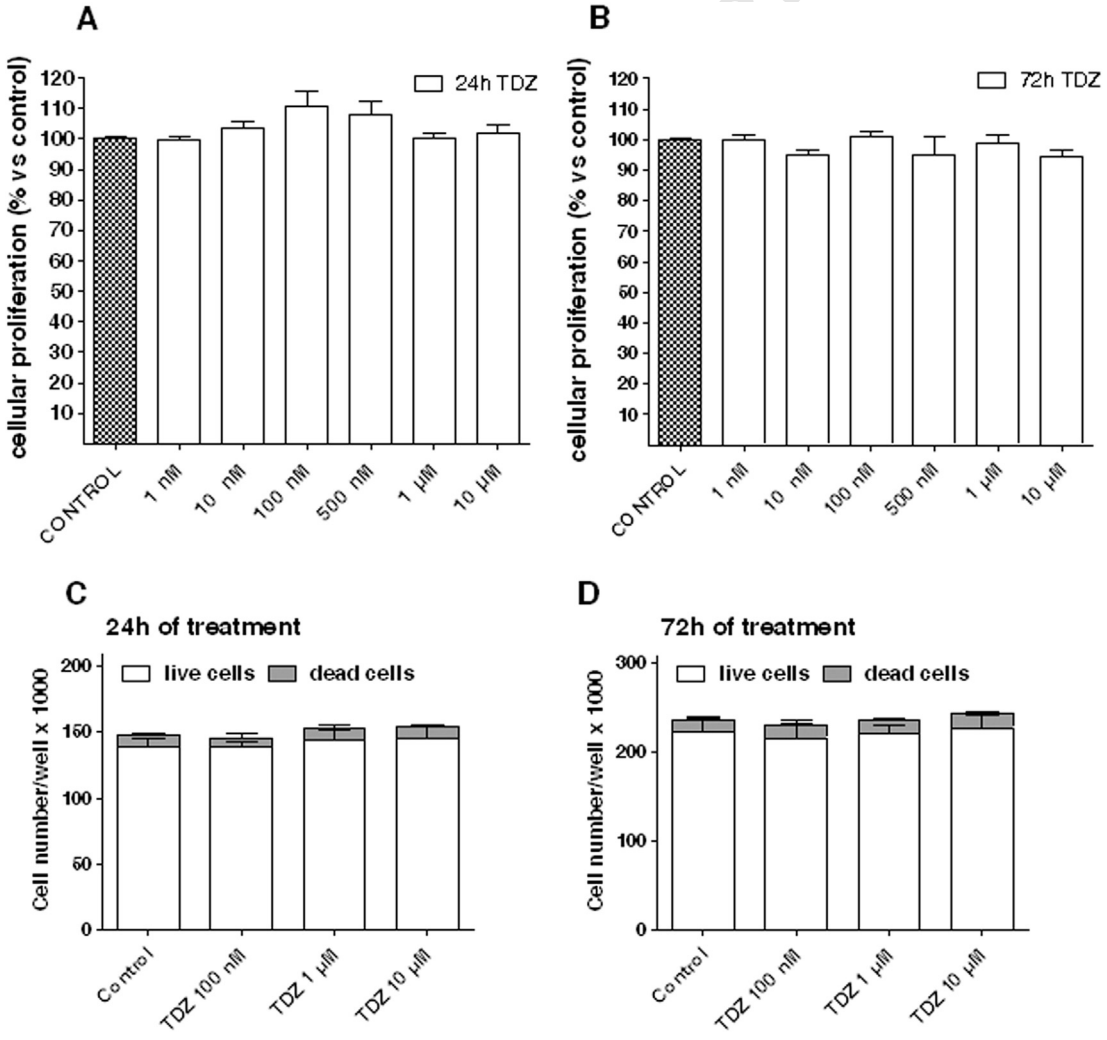

Fig. 2. Effect of TDZ on neuronal proliferation/viability. H9-derived NSCs were differentiated for seven days with Neurobasal-B27 and RA and then treated with different concentrations of TDZ (1 nM–10 μM) for 24 h (panel A) or 72 h (panel B). At the end of the treatments, cell proliferation was measured by MTS assay. The data are expressed as percentages relative to untreated cells (control), which were set at 100%, and represent the mean ± SEM of three independent experiments, each performed in triplicate. Statistical significance was determined using a one-way ANOVA followed by a Bonferroni post-test. Panels C, D) H9-derived NSCs were treated as in A and B. At the end of the treatment periods, living and dead cells were estimated using the trypan blue exclusion test. The data are expressed as the number of living or dead cells per well and are the mean values ± SEM of two independent experiments, each performed in duplicate. Statistical significance was determined using a one-way ANOVA followed by a Bonferroni post-test.

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Neurobasal medium supplemented by RA is able to induce the neuronal differentiation of H9-derived NSCs.

3.2. Effect of TDZ treatments on neuronal proliferation, viability and apoptosis

To investigate the putative effects of TDZ alone on neuronal proliferation/viability, neuronal-like cells were treated with different concentrations of TDZ (1 nM–10 μM) for 24 and 72 h. The results (Fig. 2A and B) showed that TDZ did not induce any significant effects on neuronal proliferation at all tested concentrations after 24 or 72 h. Moreover, trypan blue exclusion assays demonstrated that TDZ did not significantly affect the numbers of living and dead cells (Fig. 2C and D).

Then, the effects of TDZ treatments on the experimental model of neuro-inflammation were investigated. Neuronal-like cells were

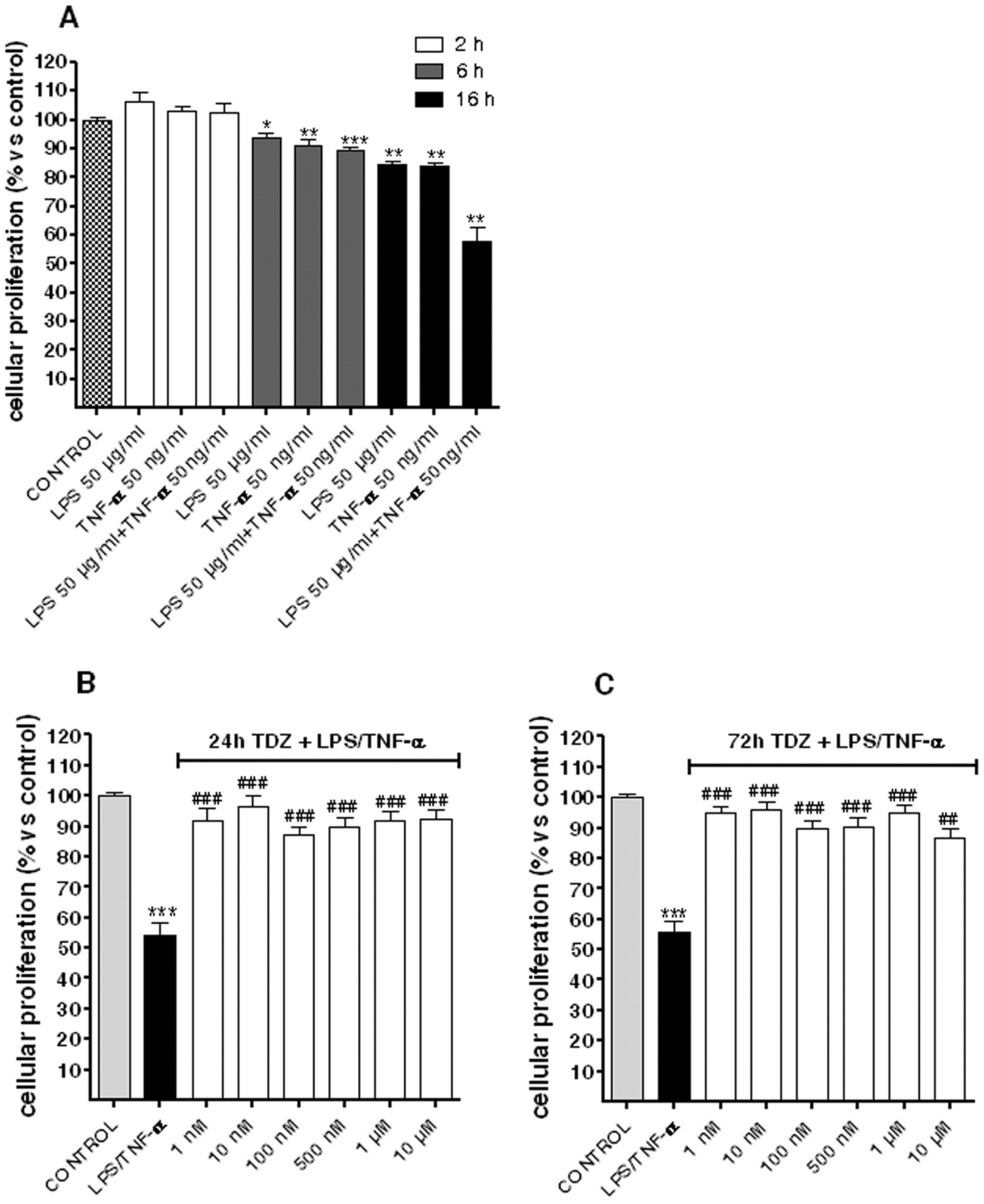

Fig. 3. Effect of TDZ treatments on an experimental model of neuroinflammation. Panel A) Neuronal-like cells were treated with 50 μg/ml LPS and 50 ng/ml TNF-α for different periods (2–16 h). Panels B, C) Neuronal-like cells were treated with different concentrations of TDZ (1 nM–10 μM) for 24 h (panel B) or 72 h (panel C); after TDZ removal, the cells were incubated with 50 μg/ml LPS and/or 50 ng/ml TNF-α for an additional 16 h. At the end of treatments, cell proliferation was measured by MTS assay. The data are expressed as percentages relative to untreated cells (control), which were set at 100%, and represent the mean ± SEM of three independent experiments, each performed in triplicate. Statistical significance was determined using a one-way ANOVA followed by a Bonferroni post-test: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ *vs* control; ##$P < 0.01$, ###$P < 0.001$ *vs* cells treated with LPS and TNF-α (LPS/TNF-α).

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treated with 50 μg/ml LPS and/or 50 ng/ml TNF-α for different periods. LPS activates signal transduction pathways similar to IL-1β and TNF-α [32]; thus, LPS is widely used as a strong inducer of inflammation.

LPS or TNF-α alone displayed modest but significant inhibition of neuronal proliferation starting from 6 h of incubation (Fig. 3A); significant and higher reduction of cell proliferation was observed when the two agents were used concomitantly for 16 h (Fig. 3A). This latter condition was thus chosen as an experimental model of neuroinflammation *in vitro*.

When neuronal-like cells were pre-treated with TDZ (1 nM–10 μM) for 24 h before the induction of neuroinflammation, significant enhancements of cellular proliferation were observed at all the examined concentrations (Fig. 3B), and incubation with LPS/TNF-α was not able to induce anti-proliferative effects. Similar results were obtained when cells were pre-treated for 72 h (Fig. 3C).

To verify whether our inflammatory model could be associated with neuronal apoptosis, annexin V staining was assessed. As depicted in Fig. 4, LPS and TNF-α induced slight but significant phosphatidylserine externalisation in the absence (early apoptosis) or presence of 7AAD binding to DNA (late apoptosis/death). Pre-treatment of neuronal-like cells with TDZ for 72 h prevented inflammatory-induced apoptosis (Fig. 4A and B).

Globally, these results demonstrated that TDZ was able to exert neuro-protective effects on cells in our experimental model of inflammation.

3.3. Effect of TDZ treatments on cytokine release

Cytokines are essential factors for neuronal cell activation, differentiation, survival, and apoptosis; under normal conditions and following excitotoxic lesion, both neurons and glia are indeed able to express a

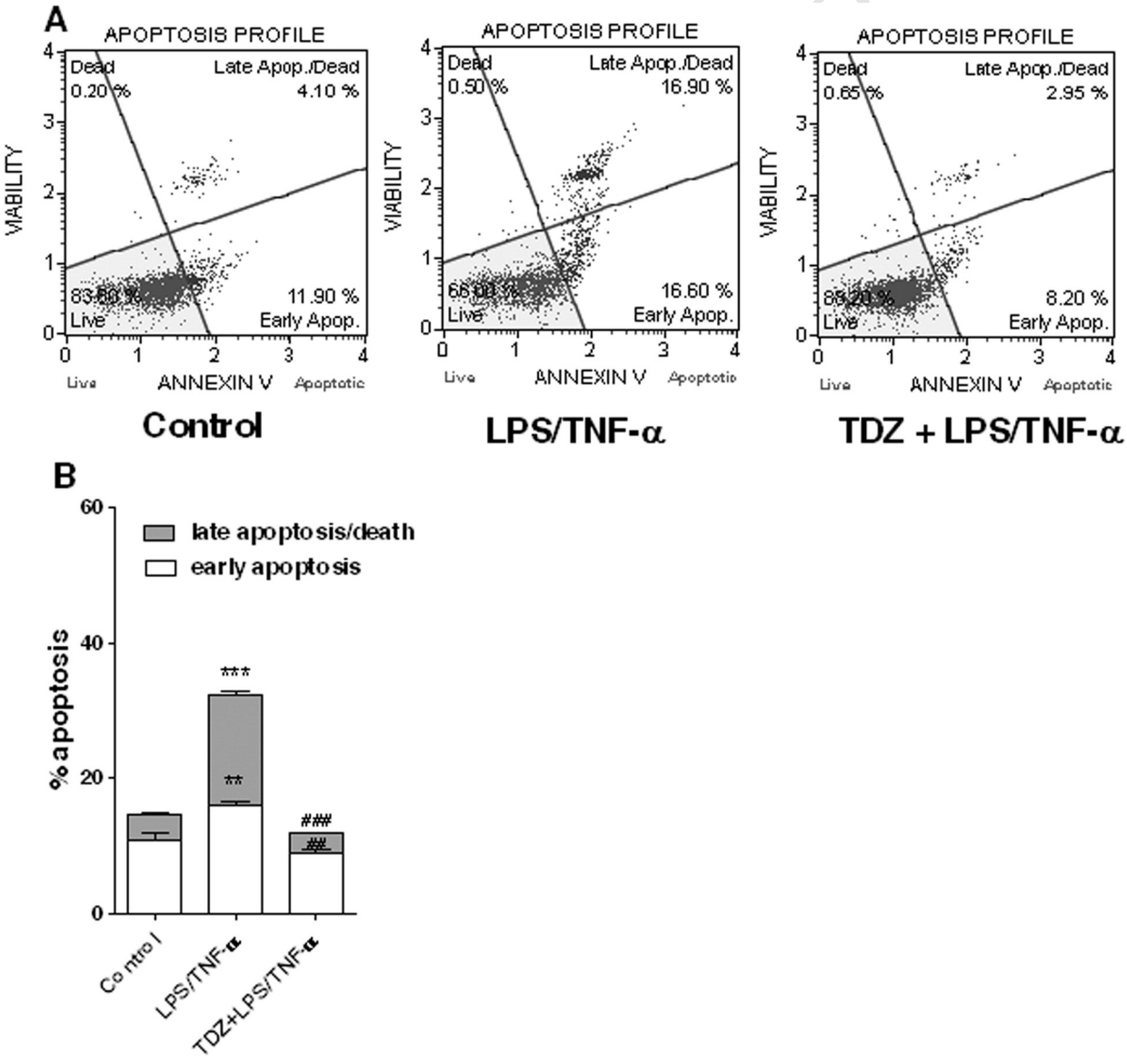

Fig. 4. Effect of TDZ treatment on inflammation-induced apoptosis of neuronal-like cells. Neuronal-like cells were treated with TDZ (1 μM) for 72 h; after TDZ removal, the cells were incubated with 50 μg/ml LPS and 50 ng/ml TNF-α for an additional 16 h. At the end of the treatment periods, the cells were collected, and the level of phosphatidylserine externalisation was evaluated using the annexin V-7AAD double staining protocol as described in the Materials and methods section. The data are expressed as the percentage of apoptotic cells (panel B; data for the early-stage apoptotic cells are shown in white, and data for the late-stage apoptotic/necrotic cells are shown in grey) *versus* the total number of cells. The data represent the mean ± SEM of three different experiments. The significance of the differences was determined using a one-way ANOVA followed by a Bonferroni post-test: $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* control; $\#\#P < 0.01$, $\#\#\#P < 0.001$ *vs* cells treated with LPS/TNF-α.

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variety of cytokines [33]. Thus, the effects of TDZ treatment alone on the release of both pro- and anti-inflammatory cytokines were assessed.

The results showed that treating neuronal-like cells with TDZ for 24 or 72 h did not significantly alter the release of IL-6 (Fig. 5A and B) and IL-10 (Fig. 5E and F) at all the tested concentrations. In contrast, in samples treated with TDZ for 24 h or 72 h significant decreases in the release of IFN-γ were noticed at all the tested concentrations or between 1 nM and 100 nM, respectively (Fig. 5C and D). These data suggested that TDZ alone was able to inhibit the release of pro-inflammatory cytokines.

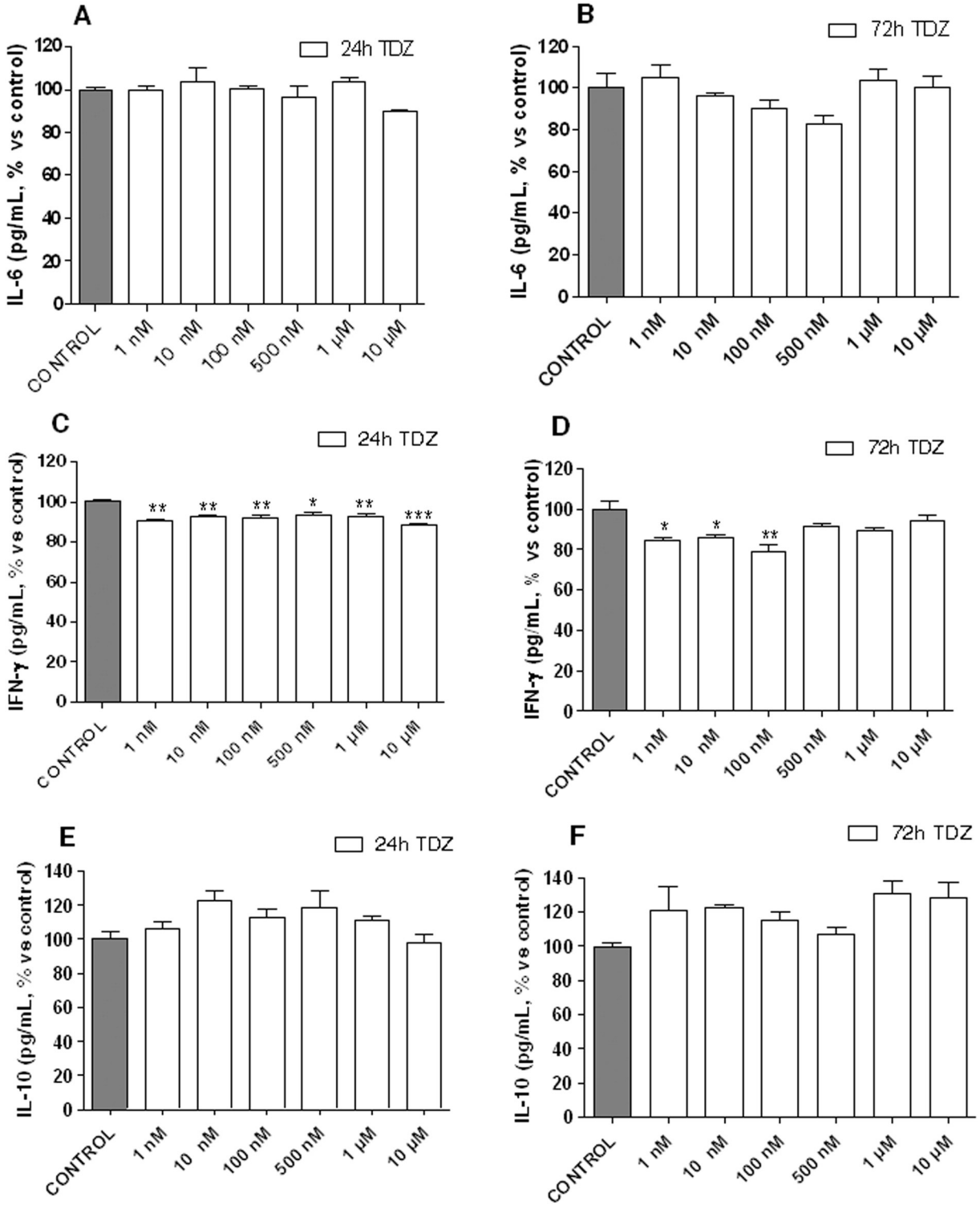

Fig. 5. Effect of TDZ on the release of cytokines. H9-derived NSCs were differentiated for seven days with Neurobasal-B27 and RA, and then treated with different concentrations of TDZ (1 nM–10 μM) for 24 h (panels A, C, E) or 72 h (panels B, D, F). At the end of treatments, culture supernatants were collected, and the amounts of IL-6 (panels A and B), IFN-γ (panels C and D) and IL-10 (panels E and F) released were measured using ELISA kits following the manufacturer's instructions. The data are expressed as percentages relative to untreated cells (control), which were set at 100%, and represent the mean ± SEM of two independent experiments, each performed in duplicate. Statistical significance was determined using a one-way ANOVA followed by a Bonferroni post-test: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ *vs* control.

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The effects of LPS/TNF-α treatment on the release of cytokines were then assessed. As expected, the induction of neuro-inflammation significantly enhanced the levels of the pro-inflammatory cytokines IL-6 and TNF-α and reduced the levels of the anti-inflammatory cytokine IL-10 (Fig. 6A–F), which is consistent with previously published results [34]. Challenging neuronal-like cells with TDZ for 24 h before the induced

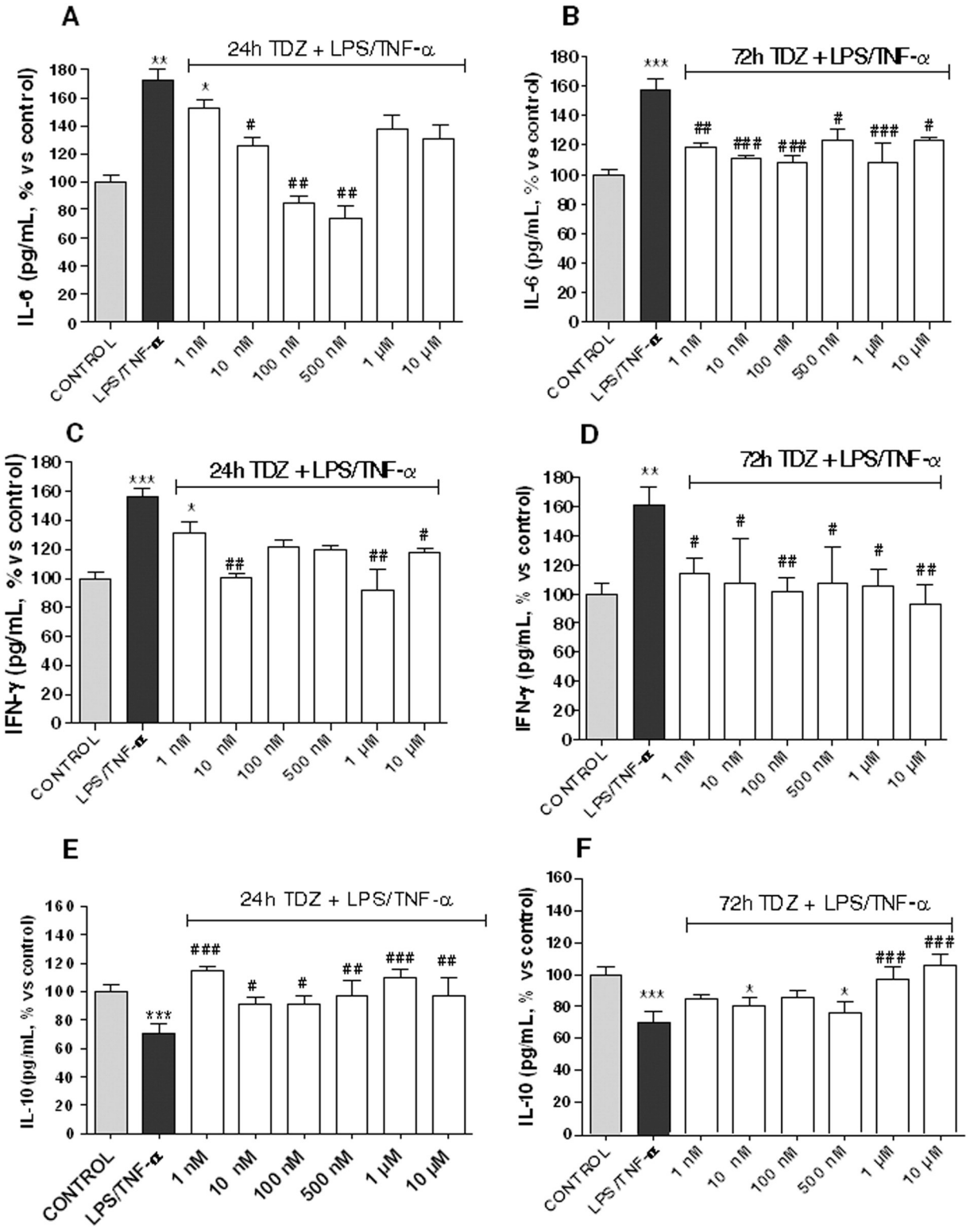

Fig. 6. Release of cytokines in an experimental model of neuroinflammation. Neuronal-like cells were treated with different concentrations of TDZ (1 nM–10 μM) for 24 h (panels A, C, E) or 72 h (panels B, D, F); after TDZ removal, the cells were incubated with 50 μg/ml LPS and 50 ng/ml TNF-α for an additional 16 h. At the end of the treatments, culture supernatants were collected, and the amounts of IL-6 (panels A and B), IFN-γ (panels C and D) and IL-10 (panels E and F) released were measured using ELISA kits according to the manufacturer's instructions. The data are expressed as percentages relative to untreated cells (control), which were set at 100%, and represent the mean ± SEM of two independent experiments, each performed in duplicate. Statistical significance was determined using a one-way ANOVA followed by a Bonferroni post-test: *P < 0.05, **P < 0.01, ***P < 0.001 *vs* control; #P < 0.05, ##P < 0.01, ###P < 0.001 *vs* cells treated with LPS/TNF-α.

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inflammatory damage was able to prevent the release of IL-6 significantly at 10 nM, 100 nM and 500 nM (Fig. 6A); likewise, the release of IFN-α was reduced in the samples pre-incubated with TDZ at 10 nM and 1 μM (Fig. 6C). Moreover, the levels of IL-10 were brought to those of the control at all tested TDZ concentrations (Fig. 6E). In a similar manner, a 72 h-pre-treatment with TDZ prevented the release of IL-6 and IFN-γ (Fig. 5B and D) in a significant manner at all tested concentrations. Moreover, TDZ counteracted the decrease in IL-10 levels induced by LPS + TNF-α (Fig. 6F), particularly for high concentrations of TDZ.

Overall, the results demonstrated that the pre-treatment of neuronal-like cells with TDZ for 24 or 72 h reduced the release of pro-inflammatory cytokines and counteracted the reduction of the anti-inflammatory cytokine.

3.4. Effects of TDZ treatments on the expression of proinflammatory genes and of neurotrophic and transcription factors

Antidepressant treatments can enhance the expression of several neurotrophic and transcription factors within hippocampal and cortical neurons [35,36]; thus, the effects of TDZ on the expression of BDNF, CREB and mammalian target of Rapamycin (mTOR) were investigated. Moreover, NF-κB, a primary transcription factor involved in the initiation of the inflammatory response [37], was also analysed.

Challenging neuronal-like cells with TDZ (100 nM or 1 μM) did not significantly affect NF-κB expression (Fig. 7A and B) after either 24 h or 72 h of incubation.

TDZ significantly enhanced the expression of mTOR, CREB and BDNF after both 24 and 72 h of cellular treatment. These results suggested that TDZ alone was able to induce the expression of neurotrophic and transcription factors starting from 24 h of treatment (Fig. 7A and B).

The effects of the induced inflammatory damage on the expression of neurotrophic and transcription factors were then determined. The treatment of neuronal-like cells with LPS/TNF-α resulted in a significant increase in NF-κB expression (Fig. 8A), which is consistent with the latter's role as a pro-inflammatory gene [37]. Significant decreases in the expression levels of CREB and BDNF were also observed, together with a decreasing trend in mTOR expression (Fig. 8A). These data suggested that LPS/TNF-α efficiently mimic the stress-related changes in the expression of neurotrophic and proinflammatory genes. Pre-incubation of the cells with TDZ for 24 h significantly decreased the expression of NF-κB (Fig. 8B), confirming the neuro-protective effect of TDZ. Moreover, although not significant, an increasing trend in mTOR expression was also observed, whereas, no significant influence on the expression of CREB and BDNF was observed (Fig. 8B). These results suggest that pre-treatment with TDZ for 24 h was not sufficient to completely reverse the effects of LPS/TNF-α on the transcriptional machinery.

Challenging neuronal-like cells with TDZ for 72 h completely counteracted the increased expression of NF-κB mediated by LPS/TNF-α (Fig. 8C) and enhanced the effect observed in the 24 h-pre-treated cells. Moreover, TDZ induced a significant increase in mTOR expression and blocked the decrease in CREB and BDNF expression mediated by the induced inflammatory damage (Fig. 8C). These data demonstrated that the pre-incubation of neuronal-like cells with TDZ for 72 h was able to prevent the reduced expression of neurotrophic and transcription factors caused by inflammation and the increased expression of the pro-inflammatory gene NF-κB.

3.5. Intracellular pathways associated with TDZ-mediated effects

The possible intracellular cascades at the basis of the effects elicited by TDZ were then investigated. Different signalling pathways have been demonstrated to play a pivotal role in neuronal cell proliferation, survival and apoptosis [38,39], including the MEK/ERK, p38, and JNK pathways. First, the effects of TDZ on the levels of total and phosphorylated ERK1/2, p38 and JNK under non-stressed conditions were investigated. Treating the neuronal-like cells with TDZ for 24 h or 72 h did not affect the total levels of ERK1/2 (Fig. 9B, C, D, F, G and H), p38 (Fig. 10B, C, D, F, G and H), or JNK (Fig. 11B, C, D, F, G and H) as demonstrated by both ELISA and western blot analyses. Concentration-dependent ERK1/2 activation was observed after 24 h and 72 h of incubation with TDZ; the percentages of ERK phosphorylation were significant at high concentrations of TDZ (Fig. 9A, C, D, E, G and H). In contrast, nanomolar concentrations of TDZ significantly inhibited p38 constitutive phosphorylation only after 24 h of TDZ treatment (Fig. 10A, C and D), while no significant changes were observed after 72 h of incubation (Fig. 10B, E, G and H). These data suggested that ERK1/2 and p38 activation could be implicated in TDZ-elicited effects under non-stressed conditions.

Finally, ELISA and western blot analyses showed that TDZ alone did not significantly affect the levels of phosphorylated JNK after either 24 h (Fig. 11A) or 72 h (Fig. 11E) of incubation with neuronal-like cells. Western blot analysis confirmed these results (Fig. 11C, D, G and H), suggesting that JNK is not affected by TDZ treatment under physiological conditions.

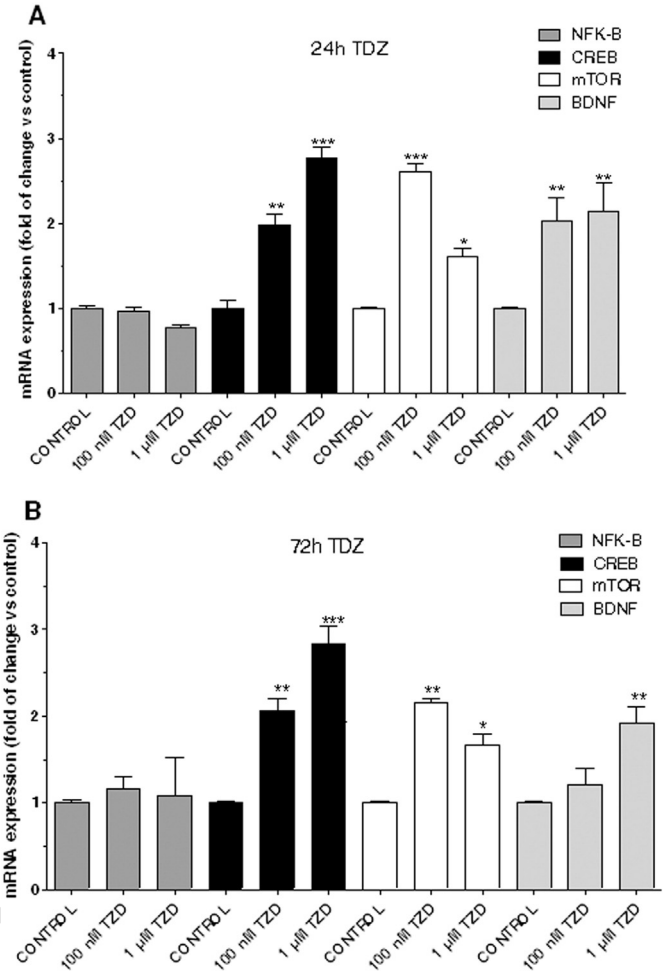

Fig. 7. Effect of TDZ on the expression of proinflammatory genes and of neurotrophic and transcription factors. H9-derived NSCs were differentiated for seven days with Neurobasal-B27 and RA and then treated with different concentrations of TDZ (1 nM–10 μM) for 24 h (panel A) or 72 h (panel B). At the end of the treatments, total RNA was extracted, and the relative mRNA quantification of NF-κB, CREB, mTOR and BDNF was performed by real-time RT-PCR. The data are expressed as fold changes *vs* control and represent the mean ± SEM of three different experiments, each performed in duplicate. Statistical significance was determined using a one-way ANOVA followed by a Bonferroni post-test: *P < 0.05, **P < 0.01, ***P < 0.001 *vs* control.

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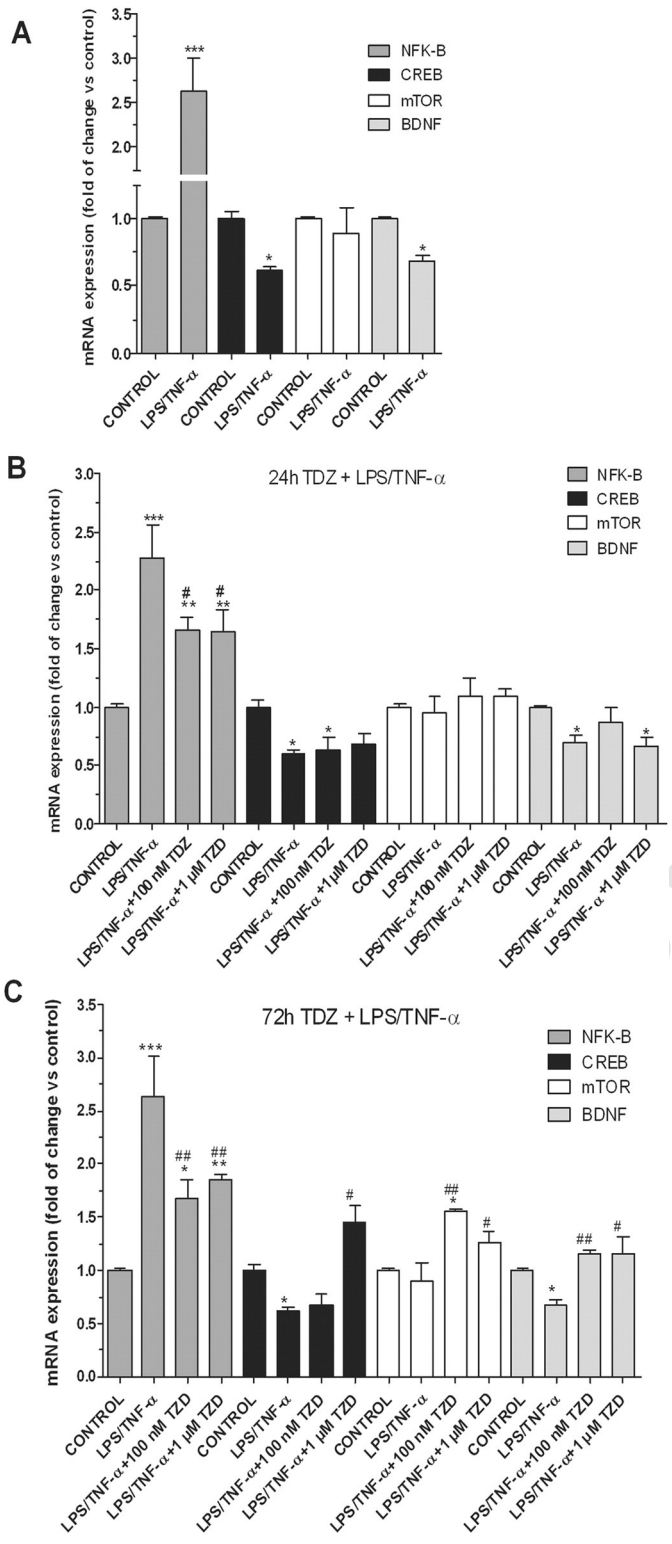

Fig. 8. Expression of proinflammatory genes, neurotrophic and transcription factors in an experimental model of neuroinflammation. Panel A) Neuronal-like cells were treated 50 μg/ml LPS and 50 ng/ml TNF-α for 16 h. Panels B, C) Neuronal-like cells were treated with different concentrations of TDZ (1 nM–10 μM) for 24 h (panel B) or 72 h (panel C); after TDZ removal, cells were incubated with 50 μg/ml LPS and 50 ng/ml TNF-α for an additional 16 h. At the end of treatments, total RNA was extracted, and the relative mRNA quantification of NF-κB, CREB, mTOR and BDNF was performed by real-time RT-PCR. The data are expressed as fold changes *vs* control and represent the mean ± SEM of three different experiments, each performed in duplicate. Statistical significance was determined using a one-way ANOVA followed by a Bonferroni post-test: $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *vs* control; $^{\#}P<0.05$, $^{\#\#}P<0.01$ *vs* cells treated with LPS/TNF-α.

When neuronal-like cells were incubated with LPS/TNF-α for 16 h, no significant changes in total or activated ERKs were observed (Fig. 12A–H). Pre-treatment of neuronal-like cells with micromolar concentrations of TDZ for 24 h remained able to improve the levels of phosphorylated (Fig. 12A, C and D) and total (Fig. 12B, C and D, Suppl. Fig. 1) ERK1/2, confirming the involvement of the MAPK pathway in TDZ-mediated effects.

ELISA and western blot analyses demonstrated significant enhancement in the levels of both phosphorylated and total p38 (Fig. 13A–H, Suppl. Fig. 2). Moreover, JNK was significantly activated after LPS/TNF-α treatment (Fig. 14A, C, D, E, G and H), without any changes in the total levels of MAPK (Fig. 14B, C, D, F, G and H). These data confirmed the roles of p38 and JNK in the neuro-inflammatory process [9,40].

TDZ counteracted the augmentation of phosphorylated (Fig. 13A, C, D, E, G and H, Suppl. Fig. 2) and total (Fig. 13B, C, D, F, G and H, Suppl. Fig. 2) p38 levels elicited by LPS/TNF-α after both 24 h and 72 h of TDZ pre-treatment. Moreover, challenging neuronal-like cells with TDZ (100 nM–1 μM) for 24 h (Fig. 14A, C and D) or 72 h (Fig. 14E, G and H) before the induction of inflammation significantly reduced the percentage of phosphorylated JNK. These results suggest that the protective effects of TDZ against neuro-inflammation involve the p38 and JNK pathways.

3.6. Contributions of the 5-HT_2 receptor to TDZ-elicited effects

To dissect the putative contribution of 5-HT_2 receptors to the neuroprotective effects elicited by TDZ, viability experiments were repeated in the presence of the selective 5-HT_2 serotonin receptor agonist (R)-DOI. Neuronal-like cells were incubated with TDZ (1 nM–10 μM) for 24 or 72 h in the absence or presence of 30 nM (R)-DOI before the addition of LPS/TNF-α. As depicted in Fig. 15, R-DOI was partially able to counteract the effects on neuronal viability elicited by TDZ pre-treatment for 24 h or for 72 h. These results suggested that the neuroprotective actions of TDZ partially involved the antagonism at 5-HT_2 serotonin receptors.

4. Discussion

The present study showed the effects of the multi-action drug TDZ on human neuronal-like cells under physiological conditions and in an experimental model of inflammation. In particular, TDZ, without affecting cell proliferation, was able to decrease the cellular release of the pro-inflammatory cytokine IFN-γ and increase the mRNA expression of neurotrophic and transcription factors such as CREB, BDNF and mTOR. Most importantly, the TDZ pre-treatment of LPS/TNF-α-treated cells was able to completely reverse the decrease in cell viability induced by the insult; significantly reduce the expression levels of inflammatory mediators such as TNF-α, IL-6, p38, JNK and NF-κB; and counteract the LPS/TNF-α-mediated decreases in the expression levels of the anti-inflammatory cytokine IL-10 and neurotrophic and transcription factors.

Neuronal-like cells were obtained by differentiating H9-derived NSC in a specific medium with RA. Indeed, RA promotes the differentiation of precursors into neurons, and these neurons express dopamine and/or serotonin receptors and form complex plexuses of neuronal processes [26]. Following TDZ neuronal-like cell treatment for 72 h, cell proliferation was not affected but reduced IFN-γ release was observed. This cytokine has been demonstrated to induce neuronal damage in mouse cortical neurons [41] and in other cellular models [42,43]. TDZ also increased the mRNA expression levels of CREB, BDNF and mTOR after both 24 h and 72 h of treatment. These data together suggest a pro-survival/neurotrophic effect of TDZ under physiological conditions. Consistent with our findings, recent studies have demonstrated that chronic antidepressant administration (classic tricyclic drugs, SSRIs, and valproate) increases the expression and function of the

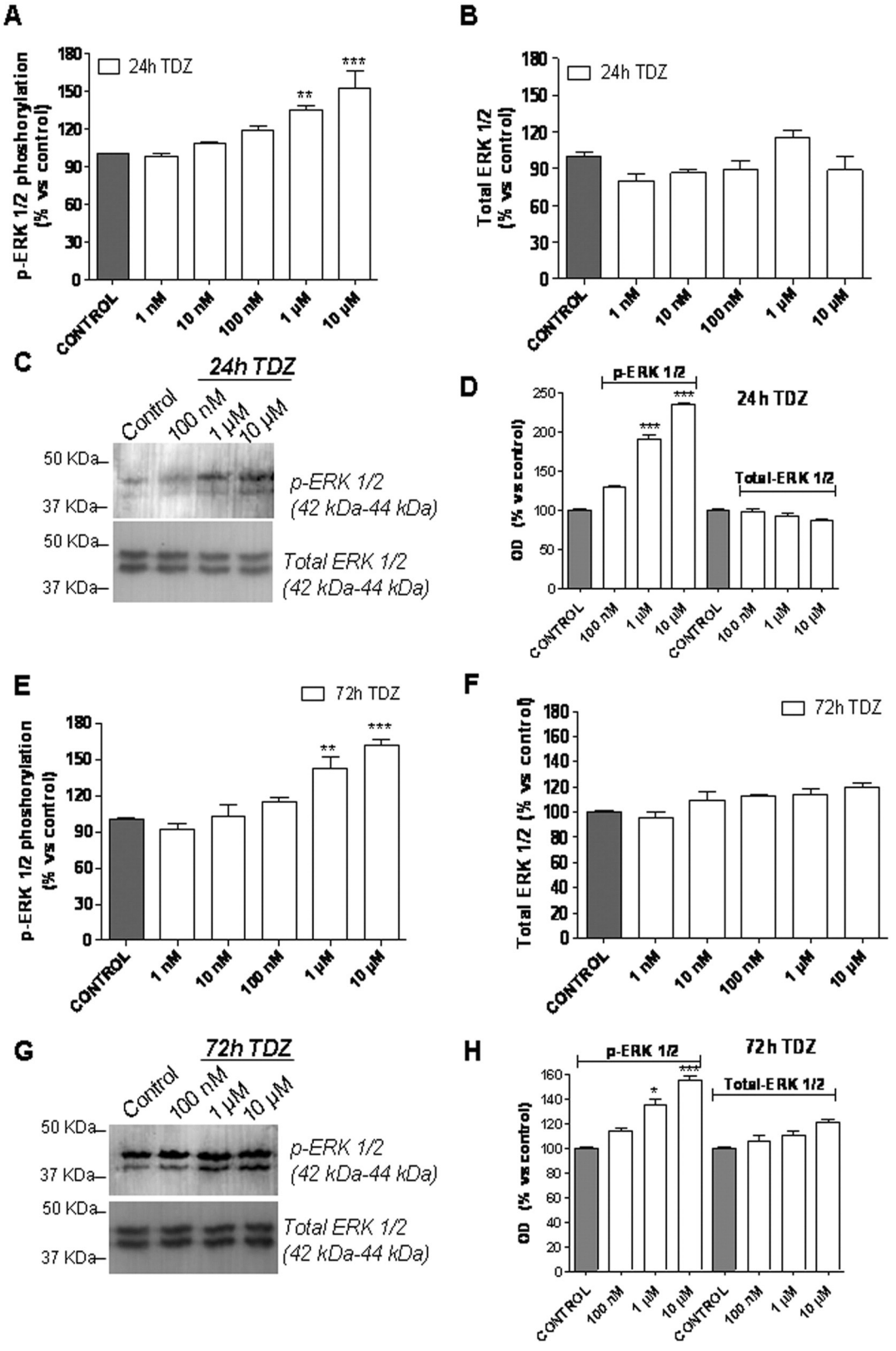

Fig. 9. Levels of total and phosphorylated ERK1/2 after TDZ treatment. Neuronal-like cells were treated with medium alone (control) or with the indicated concentrations of TDZ for 24 h (panels A and B) or 72 h (panels E and F). Following incubation, the levels of phosphorylated (panels A and E) or total (panels B and F) ERK1/2 were evaluated using an ELISA kit as described in the Materials and methods section. The data are expressed as percentages of phosphorylated or total ERK1/2 relative to untreated cells (control), which were set at 100%, and are the mean ± SEM of two independent experiments performed in triplicate. The significance of the differences was determined using a one-way ANOVA followed by a Bonferroni post-test: $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* control. Neuronal-like cells were treated with medium alone (control) or with the indicated concentrations of TDZ for 24 h (panels C and D) or 72 h (panels G and H). Following incubation, the protein levels of phosphorylated or total ERK1/2 were evaluated by western blot analysis. Panels C, G) Representative western blots. Panels D, H) Densitometric analysis of the immunoreactive bands was performed using ImageJ. The data are expressed as the percentage of optical density of the immunoreactive band relative to that of the control, which was set at 100%, and are the mean values ± SEM of three different experiments. The significance of the differences was determined using a one-way ANOVA followed by a Bonferroni post-test: $^{*}P < 0.05$, $^{***}P < 0.001$ *vs* control.

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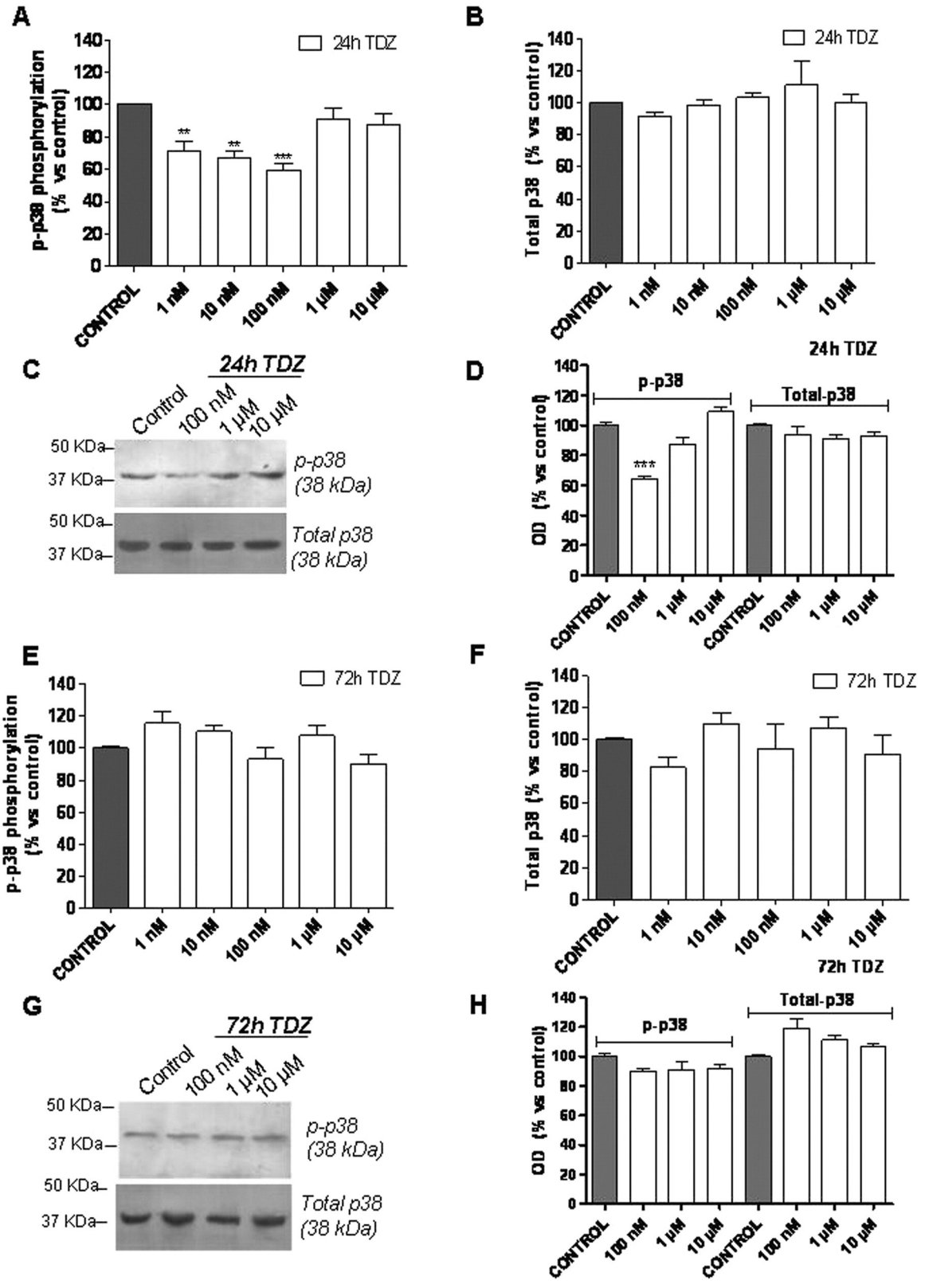

Fig. 10. Levels of total and phosphorylated p38 after TDZ treatment. Neuronal-like cells were treated with medium alone (control), or with the indicated concentrations of TDZ for 24 h (panels A and B) or 72 h (panels E and F). Following incubation, levels of phosphorylated (panel A and E) or total (panel B and F) p38 were evaluated using an ELISA kit as described in the Materials and methods section. The data are expressed as percentages of phosphorylated or total p38 relative to untreated cells (control), which were set at 100%, and are the mean ± SEM of two independent experiments performed in triplicate. The significance of the differences was determined using a one-way ANOVA followed by a Bonferroni post-test: $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* control. Neuronal-like cells were treated with medium alone (control), or the indicated concentrations of TDZ for 24 h (panel C and D) or 72 h (panels G and H). Following incubation, the protein levels of phosphorylated or total p38 were evaluated by western blot analysis. Panels C, G) Representative western blots. Panels D, H) Densitometric analysis of the immunoreactive bands was performed using ImageJ. The data are expressed as the percentage of optical density of the immunoreactive band relative to that of the control, which was set at 100%, and are the mean values ± SEM of three different experiments. The significance of the differences was determined using a one-way ANOVA followed by a Bonferroni post-testned with a one-way ANOVA with Bonferroni post-test: $^{***}P < 0.001$ *vs* control.

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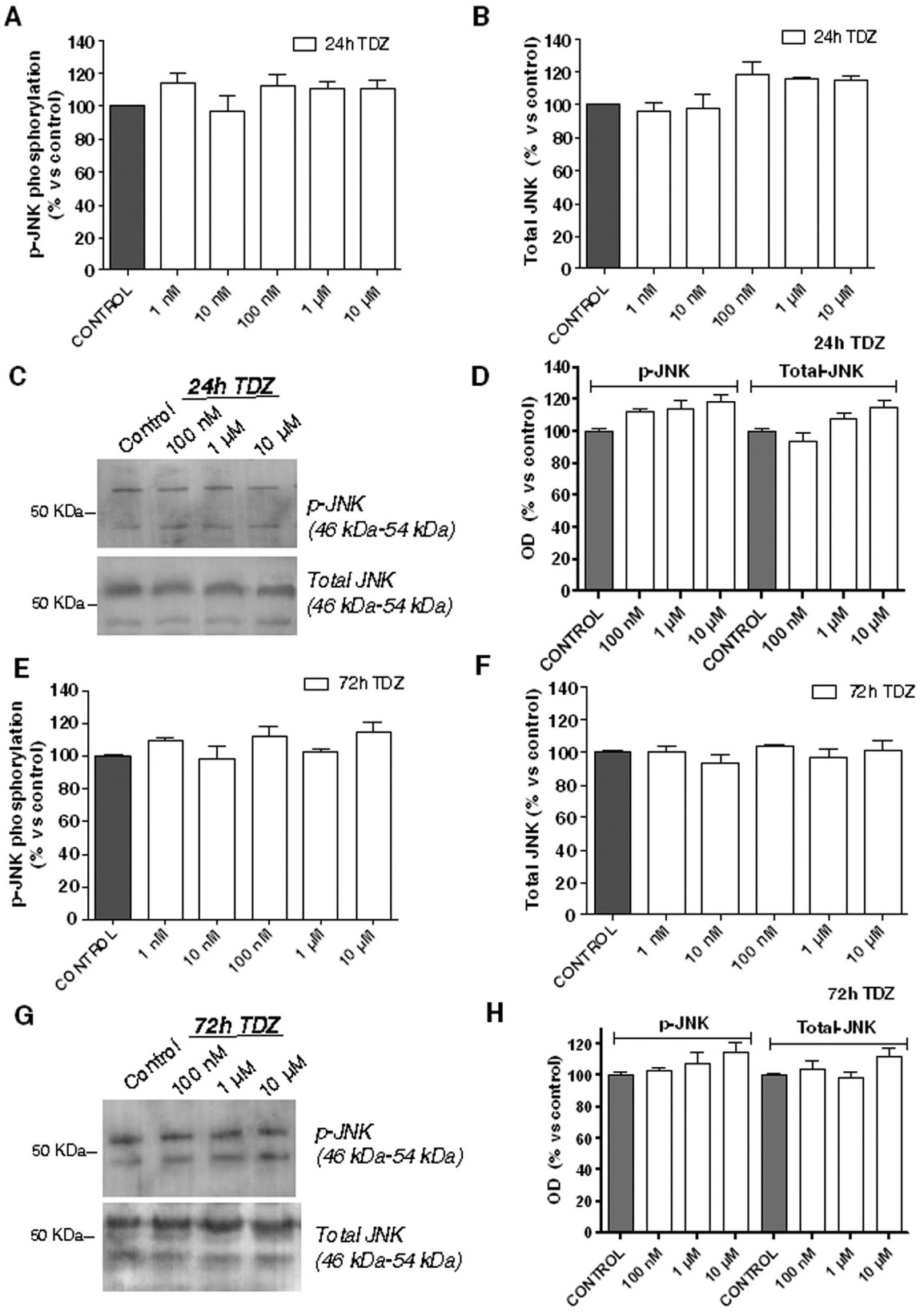

Fig. 11. Levels of total and phosphorylated JNK after TDZ treatment. Neuronal-like cells were treated with medium alone (control), or with the indicated concentrations of TDZ for 24 h (panel A and B) or 72 h (panel E and F). Following incubation, levels of phosphorylated (panel A and E) or total (panel B and F) JNK were evaluated using an ELISA kit as described in the Materials and methods section. The data are expressed as percentages of phosphorylated or total JNK relative to untreated cells (control), which were set at 100%, and are the mean ± SEM of two independent experiments performed in triplicate. The significance of the differences was determined using a one-way ANOVA followed by a Bonferroni post-test. Neuronal-like cells were treated with medium alone (control), or the indicated concentrations of TDZ for 24 h (panels C and D) or 72 h (panels G and H). Following incubation, the protein levels of phosphorylated or total JNK were evaluated by western blot analysis. Panels C, G) Representative western blots. Panels D, H) Densitometric analysis of the immunoreactive bands was performed using ImageJ. The data are expressed as the percentage of optical density of the immunoreactive band relative to that of the control, which was set at 100%, and are the mean values ± SEM of three different experiments. The significance of the differences was determined using a one-way ANOVA followed by a Bonferroni post-test.

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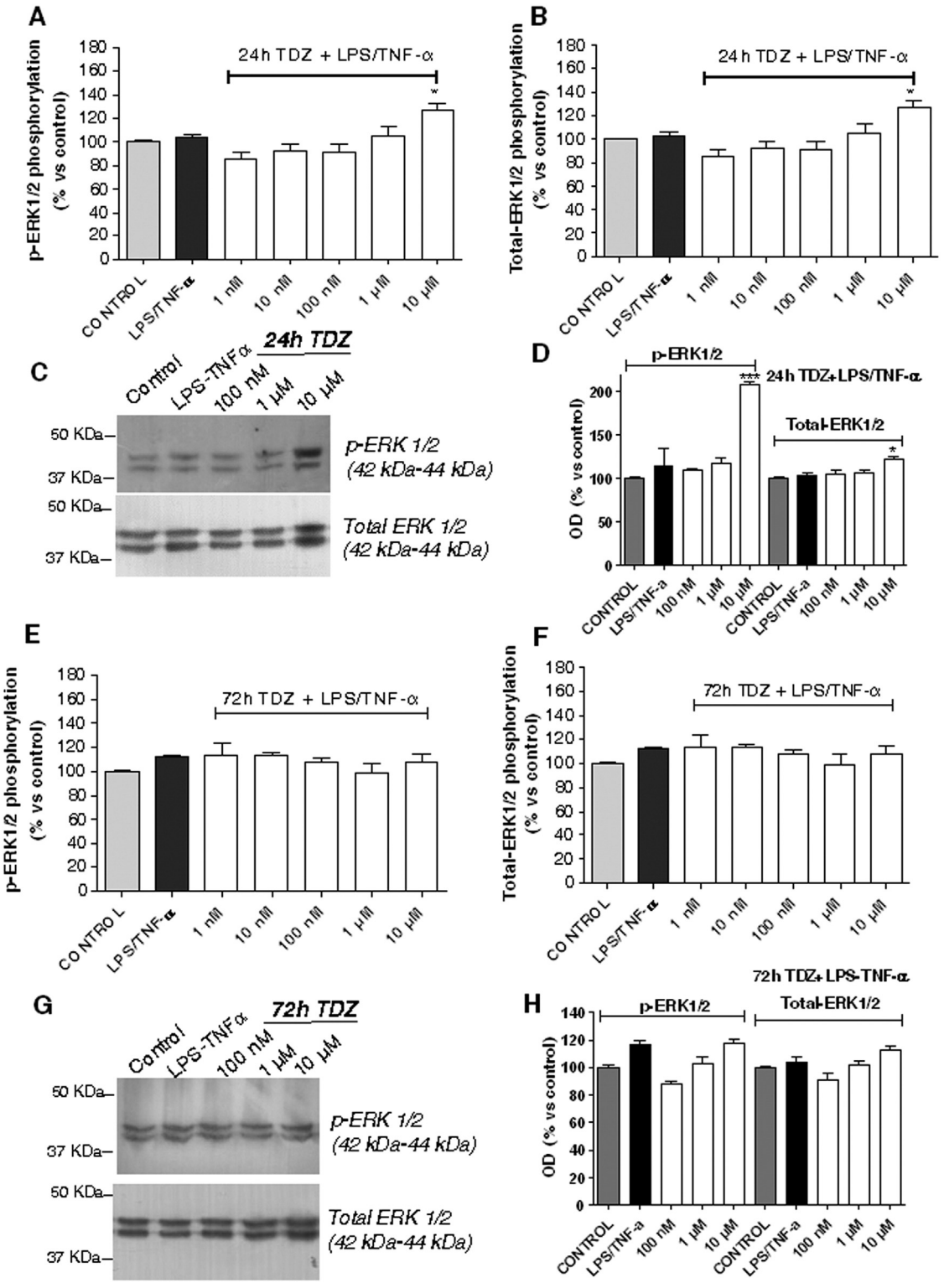

Fig. 12. Levels of total and phosphorylated ERK1/2 in an experimental model of neuroinflammation. Neuronal-like cells were treated with medium alone (control), or the indicated concentrations of TDZ for 24 h (panels A and B) or 72 h (panels E and F); after TDZ removal, cells were incubated with 50 μg/ml LPS and 50 ng/ml TNF-α for an additional 16 h. Following incubation, levels of phosphorylated (panel A and E) or total (panel B and F) ERK 1/2 were evaluated using an ELISA kit as described in the Materials and Methods section. The data are expressed as percentages of phosphorylated or total ERKs relative to untreated cells (control), which were set at 100%, and are the mean ± SEM of two independent experiments performed in triplicate. The significance of the differences was determined using a one-way ANOVA followed by a Bonferroni post-test: *$P < 0.05$ *vs* control. Neuronal-like cells were treated with medium alone (control), or with the indicated concentrations of TDZ for 24 h (panels C and D) or 72 h (panels G and H); after TDZ removal, cells were incubated with 50 μg/ml LPS and 50 ng/ml TNF-α for an additional 16 h. Following incubation, the protein levels of phosphorylated or total ERK1/2 were evaluated using by western blot analysis; GAPDH was used as the loading control (see Suppl. Fig. 1). Panels C, G) Representative western blots. Panels D, H) Densitometric analysis of the immunoreactive bands performed using ImageJ. The data are expressed as the percentage of optical density of the immunoreactive band relative to that of the control, which was set at 100%, and are the mean values ± SEM of three different experiments. The significance of the differences was determined using a one-way ANOVA followed by a Bonferroni post-test: *$P < 0.05$, ***$P < 0.001$ *vs* control.

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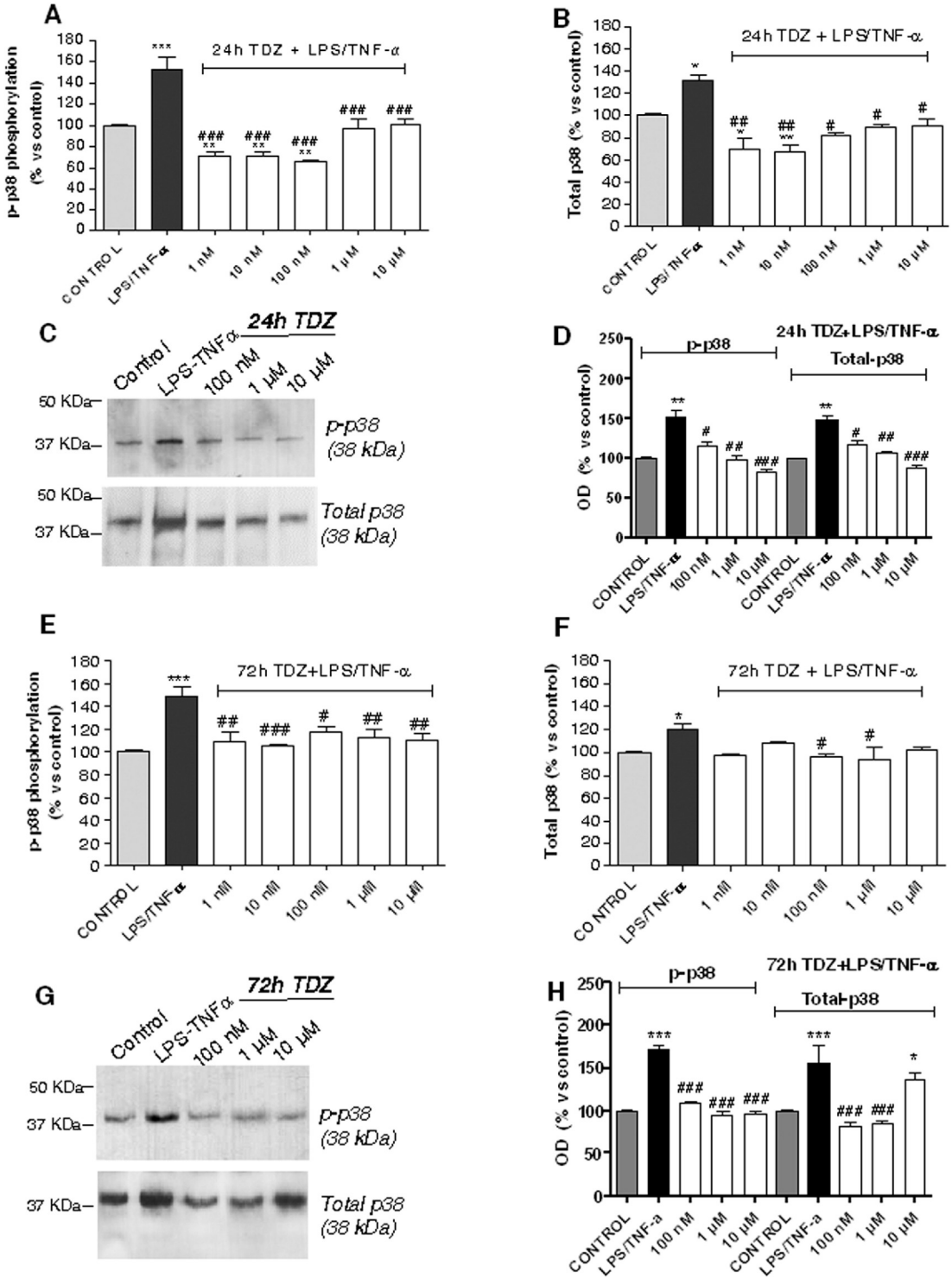

Fig. 13. Levels of total and phosphorylated p38 after TDZ treatment in an experimental model of neuroinflammation. Neuronal-like cells were treated with medium alone (control), or the indicated concentrations of TDZ for 24 h (panels A and B) or 72 h (panels E and F); after TDZ removal, cells were incubated with 50 μg/ml LPS and 50 ng/ml TNF-α for an additional 16 h. Following incubation, levels of phosphorylated (panel A and E) or total (panel B and F) p38 were evaluated using an ELISA kit as described in the Materials and methods section. The data are expressed as percentages of phosphorylated or total p38 relative to untreated cells (control), which were set at 100%, and are the mean ± SEM of two independent experiments performed in triplicate. The significance of the differences was determined using a one-way ANOVA followed by a Bonferroni post-test: *P < 0.05, **P < 0.01, ***P < 0.001 *vs* control; #P < 0.05, ##P < 0.01, ###P < 0.001 *vs* cells treated with LPS/TNF-α. Neuronal-like cells were treated with medium alone (control), or the indicated concentrations of TDZ for 24 h (panels C and D) or 72 h (panels G and H); after TDZ removal, cells were incubated with 50 μg/ml LPS and 50 ng/ml TNF-α for an additional 16 h. Following incubation, the protein levels of phosphorylated or total p38 were evaluated using by western blot analysis; GAPDH was the loading control (Suppl. Fig. 2). Panels C, G) Representative western blots. Panels D, H) Densitometric analysis of the immunoreactive bands performed using ImageJ. The data are expressed as the percentage of optical density of the immunoreactive band relative to that of the control, which was set at 100%, and are the mean values ± SEM of three different experiments. The significance of the differences was determined using a one-way ANOVA followed by a Bonferroni post-test: *P < 0.05, **P < 0.01, ***P < 0.001 *vs* control; #P < 0.05, ##P < 0.01, ###P < 0.001 *vs* cells treated with LPS/TNF-α.

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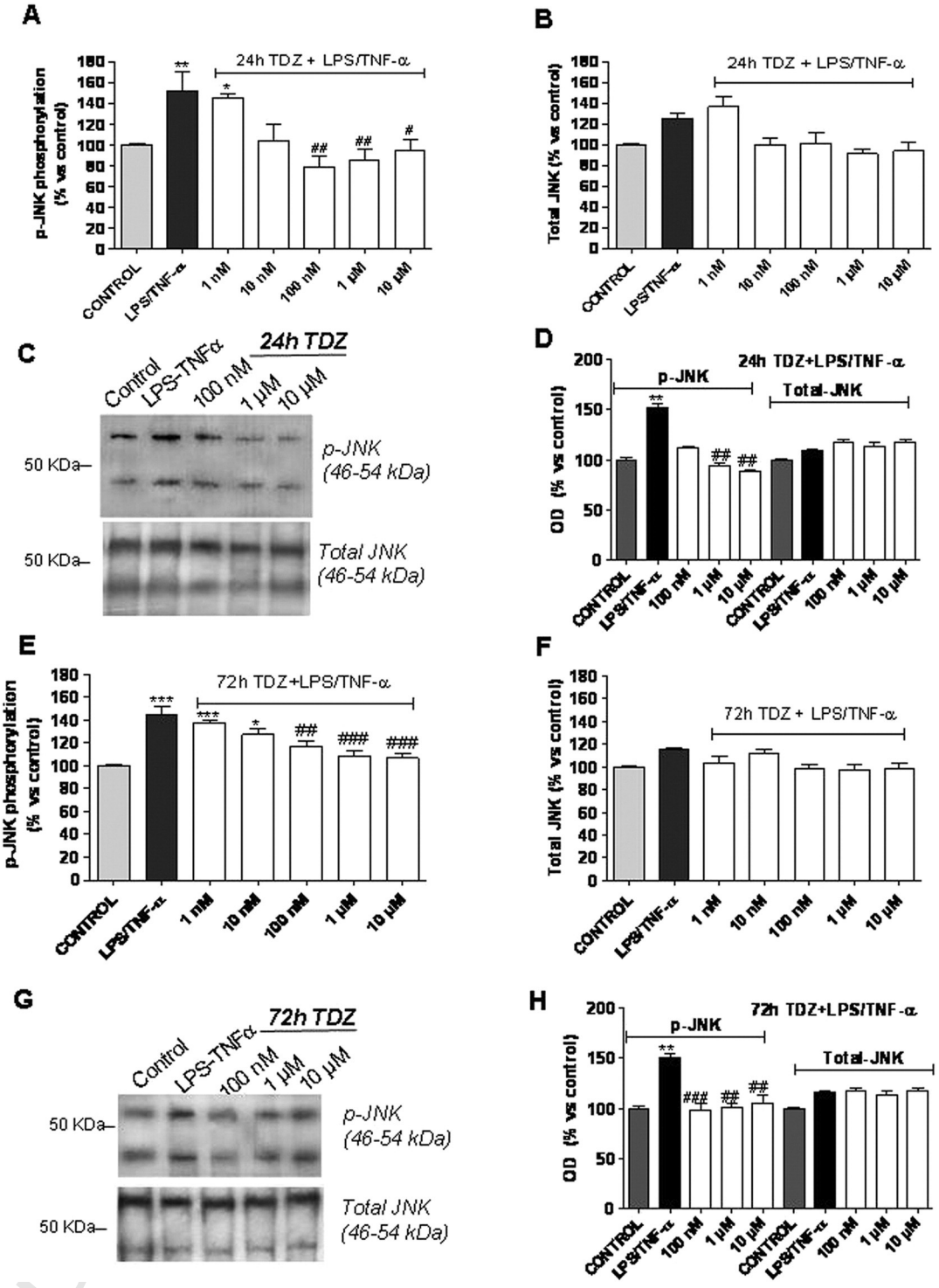
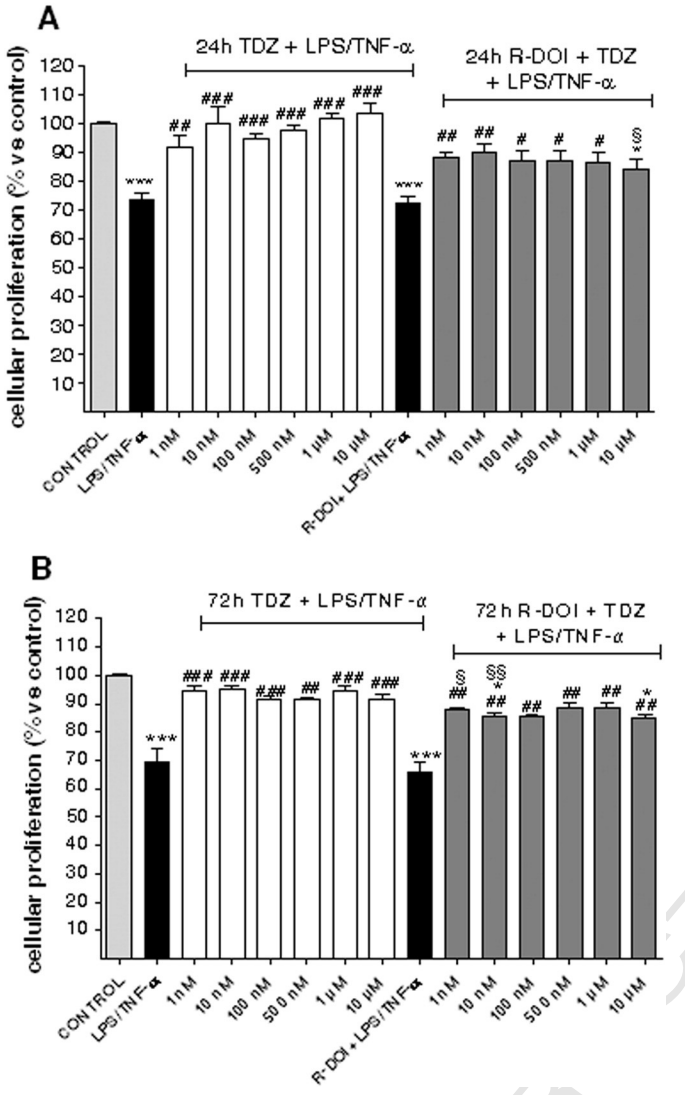


Fig. 14. Levels of total and phosphorylated JNK after TDZ treatment in an experimental model of neuroinflammation. Neuronal-like cells were treated with medium alone (control), or the indicated concentrations of TDZ for 24 h (panels A and B) or 72 h (panels E and F); after TDZ removal, cells were incubated with 50 μg/ml LPS and 50 ng/ml TNF-α for an additional 16 h. Following incubation, levels of phosphorylated (panel A and E) or total (panel B and F) JNK were evaluated using an ELISA kit as described in the Materials and methods section. The data are expressed as percentages of phosphorylated or total JNKs relative to untreated cells (control), which were set to 100%, and are the mean ± SEM of two independent experiments performed in triplicate. The significance of the differences was determined using a one-way ANOVA followed by a Bonferroni post-test: *P < 0.05, **P < 0.01, ***P < 0.001 *vs* control; #P < 0.05, ##P < 0.01, ###P < 0.001 *vs* cells treated with LPS/TNF-α. Neuronal-like cells were treated with medium alone (control), or with the indicated concentrations of TDZ for 24 h (panels C and D) or 72 h (panels G and H); after TDZ removal, cells were incubated with 50 μg/ml LPS and 50 ng/ml TNF-α for an additional 16 h. Following incubation, the protein levels of phosphorylated or total p38 were evaluated by western blot analysis. Panels C, G) Representative western blots. Panels D, H) Densitometric analysis of the immunoreactive bands performed using ImageJ. The data are expressed as the percentage of optical density of the immunoreactive band relative to that of the control, which was set at 100%, and are the mean values ± SEM of three different experiments. The significance of the differences was determined using a one-way ANOVA followed by a Bonferroni post-test: **P < 0.01 *vs* control; ##P < 0.01, ###P < 0.001 *vs* cells treated with LPS/TNF-α.

transcription factor CREB, subsequently leading to the up-regulation of specific target genes, including the neurotrophic factor BDNF [44–46]. Moreover, some antidepressant drugs (*i.e.*, escitalopram and paroxetine) promote dendritic outgrowth and increase synaptic protein levels *via* mTOR signalling [47,48], subsequently resulting in a rapid antidepressant-like effect in rats [49,50].



Q2 **Fig. 15.** Contribution of 5-HT_2 receptor in TDZ-elicited effects. Neuronal-like cells were treated with medium alone (control), 30 nM (*R*)-DOI and/or TDZ (1 nM–10 μM) for 24 h (panel A) or 72 h (panel B); after TDZ removal, cells were incubated with 50 μg/ml LPS and 50 ng/ml TNF-α for an additional 16 h. At the end of treatments, cell proliferation Q3 was measured using MTS assay. Data are expressed as percentage with respect to untreated cells (control), set to 100%, and are the mean ± SEM of three independent experiments, each performed in triplicate. The significance of the differences was determined using a one-way ANOVA followed by a Bonferroni post-test: *P < 0.05, ***P < 0.001 *vs* control; #P < 0.05, ##P < 0.01, ###P < 0.001 *vs* cells treated with LPS/TNF-α; §P < 0.05, §§P < 0.01 *vs* cells not treated with R-DOI.

Of note, in the pathology of depression, inflammation has emerged as a potentially important factor, and the efficacy of add-on anti-inflammatory treatments for depressive episodes was recently proposed [51]. Indeed, in recent years, several findings have demonstrated that pro-inflammatory cytokines may influence the exacerbation of depression [2,3,52].

In this study, an *in vitro* inflammatory model was established, and LPS and TNF-α were chosen for their ability to mimic the stress-related changes in neurotrophic and pro-inflammatory genes efficiently, even if the cellular activation induced by LPS/TNF-α could not be fully comparable with that occurring in *in vivo* depression models. In contrast, LPS/TNF-α treatments of neurons and glial cells are largely used as *in vitro* models of neuro-inflammation for testing the potential neuro-protective effects of drugs [26]. Interestingly, several findings have demonstrated that TNF-α and LPS administration can induce depression-like behaviours in animals [52–54].

Here, LPS/TNF-α cell treatment decreased the cellular viability of neuronal-like cells and induced significant release of the pro-inflammatory cytokines IL-6 and IFN-γ, together with a decrease in the anti-inflammatory cytokine IL-10. Consistent with the activation of the inflammatory cascade, challenging neuronal-like cells with LPS/TNF-α resulted in significant increases in the levels of NF-κB expression. According to our findings in an *in vitro* neuronal model, cellular exposure to IL-1β and TNF-α caused robust induction of a large number of inflammatory mediators by a mechanism involving NF-κB [55]. A significant decrease in the expression of CREB and BDNF was also observed, which is consistent with decreases in these neurotrophic factors in stress-induced animal models of depression [10, 14–16,56]. Following neuronal-like cell pre-treatment with TDZ for 24 h before neuro-inflammation induction, significant enhancement of cellular viability at all the tested concentrations was observed. Moreover, TDZ pre-treatment for 72 h completely prevented the inflammatory-induced apoptosis of neuronal-like cells, demonstrating that TDZ was able to exert neuro-protective effects. To the best of our knowledge, this study is the first to investigate and to demonstrate the positive and protective effects of TDZ at the molecular level using a human neuronal-like cell model before and following inflammatory insult.

The observed effects partially involved antagonism at 5HT-$_{2A}$ and 5HT-$_{2C}$ receptors as demonstrated by the partial reduction of TDZ-mediated effects on neuronal viability in the presence of the 5HT-$_2$ receptor agonist R-DOI.

Neuronal-like cell pre-treatment with TDZ for 24 or 72 h also significantly reduced the release of pro-inflammatory cytokines and counteracted the decrease in anti-inflammatory cytokines as demonstrated previously for a tricyclic anti-depressant in animal models [11,12,57,58]. Moreover, challenging neurons with TDZ for 72 h completely counteracted the increased expression of NF-κB mediated by LPS/TNF-α treatment. Finally, TDZ induced a significant increase in mTOR expression and blocked the decrease in the expression of CREB and BDNF mediated by the inflammatory damage. Thus, activated CREB has been proposed to directly inhibit NF-κB activation; in contrast, mTOR seems to upregulate anti-inflammatory cytokines and to inhibit pro-inflammatory cytokines, thereby limiting pro-inflammatory responses as demonstrated previously in human dendritic cells [59,60].

Because TDZ provides the simultaneous inhibition and stimulation of several different cellular receptors and transporters and because the use of R-DOI demonstrated only a partial reduction of TDZ-mediated effects, the possible intracellular cascades at the basis of the effects elicited by TDZ were explored to investigate a common node downstream of multiple pathways. Different signalling pathways have been demonstrated to play pivotal roles in neuronal cell proliferation, survival and apoptosis [38,39,61,62], including the MEK/ERK, p38 and JNK pathways. Our results demonstrated that TDZ alone induced concentration-dependent ERK1/2 activation and inhibited the constitutive phosphorylation of p38 in the nanomolar range, suggesting that both ERK1/2 and p38 could be implicated in TDZ-elicited effects under non-stressed conditions.

Data from the literature support the effects elicited by TDZ in our experimental model. Specifically, ERK1/2 activation and p38 pathway inhibition have been reported to involve agonistic effects at the $5HT_{1A}$ serotonin receptor [63,64], inhibition of the $5HT_{2A}$ serotonin receptor [63], and inhibition of SERT [65,66]. The aforementioned putative routes between TDZ and p38/ERK signalling are reported in Fig. 16.

The drug-mediated antagonism at other receptor populations should be mentioned in the complex mechanism of action of TDZ. In contrast to the observed TDZ-mediated activation of ERK, the inhibition of serotonin $5HT_2$ [64], histamine H_1 [68–70] and α- Q26 adrenergic receptors [71–73] has been related to the inhibition of ERK phosphorylation. We speculate that ERK phosphorylation is

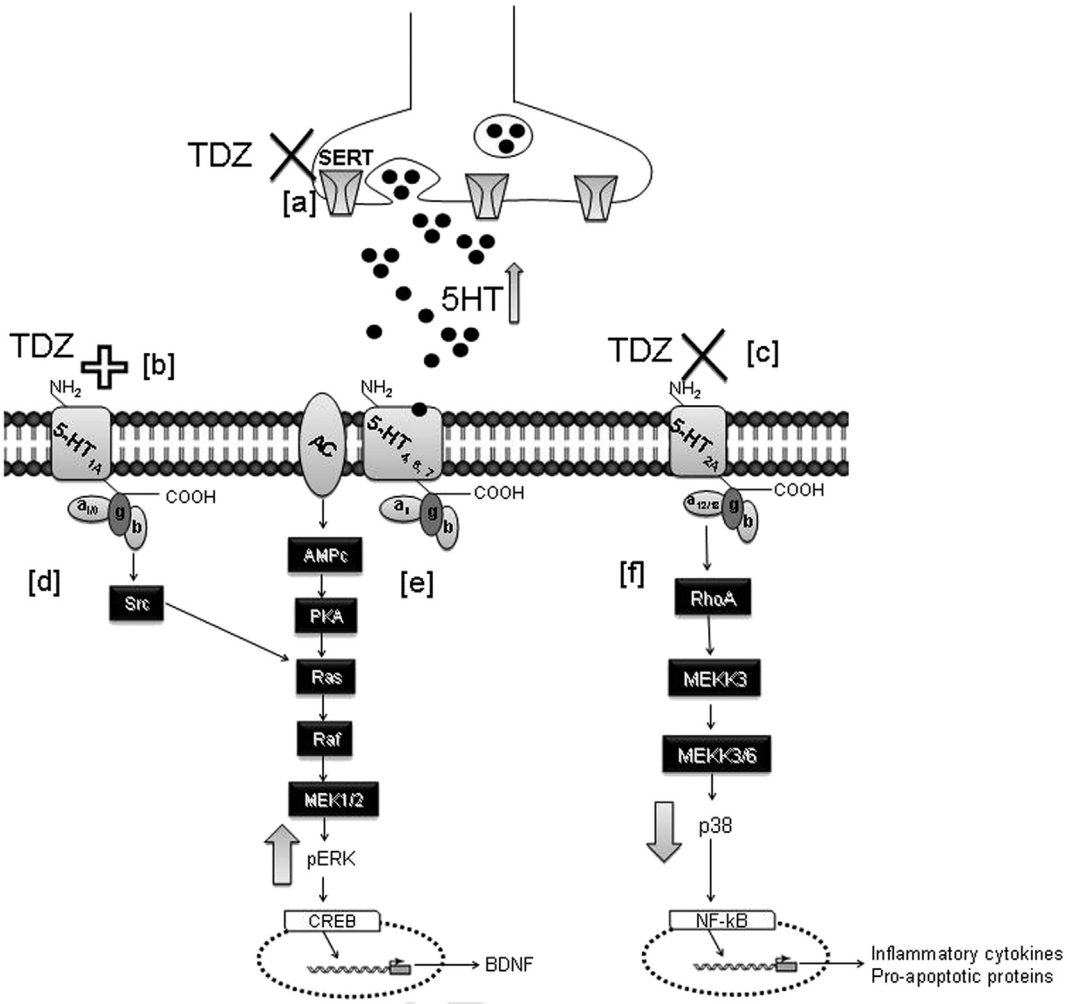


Fig. 16. The possible intracellular route between TDZ and p38/ERK signalling in H9-derived neuronal-like cell. Schematic overview of the possible TDZ/p38/ERK signalling pathway in our experimental model is depicted. In neurons, the 5-HT_{1A} receptor is coupled to Gαi/o proteins; its activation increases ERK phosphorylation *via* the Src/Ras pathway ([a], [63,64,78]). TDZ, *via* its agonistic activity at the 5-HT_{1A} receptor, promotes ERK phosphorylation (grey arrow). TDZ blocks monoamine reuptake by inhibiting SERT. This inhibition leads to the regulation of postsynaptic serotonin receptors, which couple to a variety of second messenger systems ([b] [66,67,78]). In particular, serotonin $5HT_4$, $5HT_6$, and $5HT_7$ receptors are positively coupled to AC; their stimulation activates PKA, leading to ERK phosphorylation (grey arrow). Serotonin 5-HT_{2A} receptors may couple to $G\alpha_{12/13}$ proteins, which activate p38 and subsequently induce the expression of inflammatory cytokines and pro-apoptotic proteins ([c], [64,65,78]). TDZ, showing an antagonistic activity on $5HT_{2A}$ receptors, reduces p38 activation (grey arrow). Abbreviations: AC: adenylyl cyclase; cAMP: cyclic adenosine monophosphate; PKA: protein kinase A; CREB: cAMP response element binding protein; Src: tyrosine kinase (src from sarcoma); Ras, Raf, RhoA: small GTPases; MEKK: MAP kinase kinase; (p)-ERK: (phospho)-extracellular-signal-regulated kinase; BDNF: brain-derived nerve factor; SERT: serotonin transporter; NF-κB: nuclear factor kappa B.

prevalent in our experimental neural model, most likely *via* TDZ agonism at $5HT_{1A}$ serotonin receptors. Further studies are needed to investigate the specific intracellular pathways associated with specific TDZ receptor targets and in relation to the different neuronal populations.

When human neuronal-like cells were incubated with LPS/TNF-α, significant enhancements in the levels of both phosphorylated and total p38 were observed, together with significant JNK activation. The transduction cascades of p38 and JNK are well-established intracellular signals that regulate pro-inflammatory cytokine production [9,40]. Microglial p38 MAPK deficiency has been demonstrated to rescue neurons and to reduce synaptic protein loss *via* suppressing LPS-induced TNF-α overproduction [73]. Furthermore, IL-1β and TNF-α have been shown to increase serotonin re-uptake in rat brain synaptosomes *via* p38 MAPK activation [7]. In addition to p38, JNK plays a key role in nerve cell apoptosis and is closely correlated with depression [61,62,74]. In the adult mouse, inflammatory cytokines, brain injury and ischaemic insult, or exposure to psychological acute stressors induces hippocampal JNK activation [9].

Interestingly, pre-treatment with TDZ significantly counteracted the augmentation of phosphorylated and total p38 levels elicited by LPS/TNF-α. We speculate that these effects could involve both serotonin receptors and SERT because p38 has been demonstrated to be an essential mediator of stress-induced adverse behavioural responses by regulating serotonergic neuronal functioning and transport [75]. Moreover, challenging neuronal-like cells with TDZ for 24 h or 72 h before the induction of inflammation significantly reduced the percentage of phosphorylated JNK, similar to data previously reported for fluoxetine or a Chinese natural antidepressant [76].

These data suggest that the neuro-protective role of TDZ could be mediated by the p38 and JNK pathways. Consistent with these findings, the inhibition of p38 and JNK, but not of ERK1/2, partially protects neurons from glia-induced death [77].

Altogether, our results shed light on the mechanism of the protective effects of TDZ under inflammatory conditions in human neuronal-like cells; TDZ decreases pro-inflammatory signalling (*i.e.*, IL-6, IFN-γ, NF-κB, p38 and JNK) and enhances anti-inflammatory signalling (*i.e.*, IL-10, BDNF, and CREB).

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5. Conclusions

In summary, the effect of TDZ on neuronal-like cells under both physiological condition and inflammatory insult has been determined. This study has shown that TDZ alone:

i) decreased the cellular release of the pro-inflammatory cytokine IFN-γ;
ii) increased the mRNA expression of neurotrophic and transcription factors;
iii) activated ERK1/2 and inhibited p38 constitutive phosphorylation.

Most importantly, a pre-treatment with TDZ before the inflammatory insult:

i) completely reversed the decrease of cell viability, through a mechanism that partially involved an antagonism at 5-HT_2 serotonin receptors;
ii) inhibited inflammation-induced production of inflammatory mediators, such as IL-6 and IFN-γ production in LPS/TNF-α stimulated neuronal cells;
iii) counteracted the decrease of neurotrophic and transcription factors mediated by LPS/TNF-α;
iv) counteracted the activation of p38 and JNK elicited by LPS/TNF-α.

The results obtained at molecular level demonstrated that the antidepressant agent TZD was able to modulate cellular pathways, activated by inflammatory insults, confirming the suggested link between depression and inflammation.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cellsig.2015.04.006.

Conflict of interest

The authors declare no conflict of interest.

Contributors

Daniele S. carried out most of the biological experiments, elaborated results and also made a significant contribution in the writing of the manuscript; Da Pozzo E. designed the study and experiments, and also help in the writing of the manuscript; Zappelli E. carried out real time PCR experiments and elaborated results; Martini C. contributed in the design of the study, played a fundamental role as the supervisor of experimental protocols, and an important helpful in writing article discussion section. All authors contributed to and have approved the final manuscript.

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