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Pro-inflammatory properties for thiazolidinediones

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Abstract

Thiazolidinediones (TZDs) are pharmacological ligands of the peroxisome proliferator-activated receptor (PPAR)-γ that are extensively used in the treatment of type II diabetes. Recently, an anti-inflammatory potential for TZDs has been suggested, based on observations that these compounds may inhibit pro-inflammatory cytokine expression in vitro and may attenuate the inflammatory response in vivo. Here, we show that the TZDs rosiglitazone (RSG) and troglitazone (TRO) do not inhibit the inflammatory response to tumor necrosis factor (TNF)-α in various epithelial cell types. On the contrary, both RSG and TRO significantly potentiated TNF-α-induced production of granulocyte/macrophage-colony-stimulating factor, interleukin (IL)-6 and/or IL-8 in these cells. This increase in pro-inflammatory cytokine expression was functionally significant as supernatants from cells co-treated with TNF-α and TZDs displayed increased neutrophil pro-survival activity when compared with supernatants from cells treated with TNF-α alone. Additionally, it was shown that TZDs enhance cytokine expression at the transcriptional level, but that the pro-inflammatory effects of TZDs are independent on PPARγ, nuclear factor κB or mitogen-activated protein kinase activation. Our study shows that TZDs may potentiate the inflammatory response in epithelial cells, a previously unappreciated effect of these compounds.

Keywords: Cytokines; Inflammation; Mitogen-activated protein kinases; Nuclear factor-κB; Peroxisome proliferator-activated receptor γ; Thiazolidinediones

1. Introduction

Thiazolidinediones (TZDs) are pharmacological agents that improve glucose homeostasis in type 2 diabetes by increasing insulin sensitivity. They were shown to reduce cardiovascular risk factors associated with this condition [1]. The TZDs are a group of structurally related compounds characterised by a thiazolidinedione ring, to which divergent molecular moieties are attached. Troglitazone (TRO) was the first TZD approved for treating type 2 diabetes but was withdrawn from the market due to hepatic toxicity [2]. Two other TZDs, rosiglitazone (RSG, also referred to as BRL49653) and pioglitazone, are now available and show no hepatic side effects [3,4]. Most of the beneficial effects of TZDs in the treatment of type 2 diabetes were attributed to the potential of these molecules to activate the nuclear receptor Peroxisome proliferator-activated receptor γ (PPARγ), a critical regulator of lipid metabolism and glucose homeostasis [5–10].

More recently, PPARγ has been suggested to play a downregulatory role in inflammatory processes, raising the hypothesis that PPARγ ligands, such as the TZDs, could be efficient in the treatment of inflammatory disorders [11,12]. The first support to the hypothesis of an

Abbreviations: AP-1, activator protein-1; HBEC, human bronchial epithelial cells; c/EBP, CAAT enhancer binding protein; CREB, cAMP responsive element binding protein; ERK, extracellular signal-regulated protein kinase; GM-CSF, granulocyte/macrophage-colony-stimulating factor; IL, interleukin; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; NF-AT, nuclear factor of activated T cells; NF-κB, nuclear factor-κB; PPARγ, peroxisome proliferator-activated receptor γ; RSG, rosiglitazone; TNF, tumor necrosis factor; TRO, troglitazone; TZD, thiazolidinedione

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anti-inflammatory potential for TZDs was provided by the observation that they inhibit monocyte/macrophage activation and expression of inflammatory molecules, i.e. interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF-α), inducible nitric oxide synthase (iNOS) and gelatinase B [13,14]. Later, these observations were extended to other inflammatory molecules (e.g. IL-2, IL-8, interferon-γ) and cell types (e.g. endothelial cells, colon cells, lymphocytes) in vitro [15–17]. Beneficial effects were also reported in vivo in animal models of human inflammatory disorders like inflammatory bowel disease [15], rheumatoid arthritis [18], multiple sclerosis [19] and asthma [20], suggesting a general anti-inflammatory potential for TZDs.

Here, we show that the TZDs RSG and TRO do not inhibit the cytokinic response to the potent pro-inflammatory cytokine TNF-α in various epithelial cell types. On the contrary, we show that both RSG and TRO may strongly increase TNF-α-induced pro-inflammatory cytokine expression. Insights into the mechanisms mediating this previously unappreciated effect of TZDs are also provided.

2. Materials and methods

2.1. Cell culture and reagents

A549, Hct-116, and OVCAR cells were obtained from the German Collection of Microorganisms and Cell Cultures. The cells were cultured in either Ham F-12 (A549 cells), McKoy (Hct-116 cells) or RPMI 1640 (OVCAR cells), supplemented with 10% FCS, 1% glutamine, 50 μg/ml streptomycin, and 50 IU/ml penicillin (GIBCO BRL). BEAS-2B cells from the American Type Culture Collection Cells and Human Bronchial Epithelial Cells (HBEC) from primary human explants were prepared and cultured as previously described [21]. Human blood neutrophils were obtained from buffy coats (Transfusion Center of Liege, Belgium). Neutrophils were separated from mononuclear cells by density centrifugation (Histopaque, Sigma). Contaminating erythrocytes were removed from the neutrophil fraction by hypotonic lysis. Neutrophil purity, as determined by counting of cytospin preparations stained with Diff-Quick (Dade Behring), was always >95%. Recombinant human TNF-α was purchased from Roche. Rosiglitazone was a generous gift from Laboratories Servier (Paris, France) and troglitazone was obtained from Biomol. Neutralizing antibodies directed against human GM-CSF or IL-6 were from R&D, actinomycin D was from Sigma and GW9662 was from Cayman Chemicals.

2.2. Immunoassays

The concentration of IL-8, IL-6, and GM-CSF in cell supernatants was measured using ELISA kits (Biosource).

2.3. Cell proliferation assays and detection of apoptosis and necrosis

Cell proliferation was assayed using the Cell Proliferation Reagent WST-1 (Roche) according to the manufacturer's instructions. Apoptosis and necrosis were assessed by staining with annexin-V-FITC and propidium iodide using the annexin-V-FLUOS staining kit (Roche), following the recommendations of the manufacturer. Flow cytometry analyses were performed with a FACStar Plus® (Becton Dickinson).

2.4. Quantitative polymerase chain reactions

Total RNA was extracted from cells using the Rneasy Mini kit according to manufacturer's instructions (Qiagen). Poly(A) RNA was primed with oligo(dT) and random hexamers (Roche) and reverse transcribed with the AMV reverse transcriptase (Roche) for 1 h at 42 °C. Sequences of the primers (Eurogentec) used in subsequent PCR were as follows—GM-CSF: cagcctcaccaagctcaag and ctgggttgcacaggaagttt; IL-6: cagccactcacctcttcaga and tcttgttacatgtctcctttctca; IL-8: tcaaagaactgagagtgattgaga and gagctctcttccatcagaaagc. Primer sequence was determined using the Primer 3 software [22]. Amplification reactions were performed in a final volume of 25 μl using SybrGreen reaction mix (Eurogentec) in the presence of 300 nM of the adequate primers and 0.5 μl of total cDNA. Real time PCR and fluorescence quantification were performed in a Lightcycler GeneAmp 5700 (Applied Biosystems). The level of β-actin mRNA was used as an internal control for normalization.

2.5. Transient transfections

The pcDNA3.1 (Invitrogen) expression vector coding for human PPARγ was generated by subcloning from a pSG5-PPARγ vector. The dominant negative form of the receptor (PPARγDN) was generated by deleting the 15-carboxy-terminal amino acids from the wild-type receptor [23]. The pTkpGL3 plasmid derived from a pGL3 vector (Promega) and luciferase reporter gene assays were previously described [24]. Transient transfection of A549 cells was performed using Fugene 6 (Roche) according to the manufacturer's instructions.

2.6. Nuclear protein extraction

Nuclear protein extracts were prepared as previously described [25]. Cytoplasmic buffer contained 10 mM Hepes, pH 7.9, 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 0.2% (v/v) Nonidet P-40, and 1.6 mg/ml protease inhibitors (Complete, Roche). Pelleted nuclei were resuspended in 20 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.63 M NaCl, 25% (v/v) glycerol, and 1.6 mg/ml protease inhibitors (nuclear buffer), incubated for 20 min at 4 °C

and centrifuged for 30 min at 14,000 rpm. Protein amounts were quantified with the Micro BCA protein assay reagent kit (Pierce).

2.7. Electrophoretic mobility shift assays (EMSAs)

Binding reactions were performed for 30 min at room temperature with 5 μg of nuclear proteins in 20 mM Hepes, pH 7.9, 10 mM KCl, 0.2 mM EDTA, 20% (v/v) glycerol, 1% (w/v) acetylated BSA, 3 μg of poly(dI–dC) (Amersham Pharmacia Biotech.), 1 mM DTT, 1 mM PMSF, and 100,000 cpm of [^{32}P]-labeled double-stranded oligonucleotide probes. Probes were prepared by annealing the appropriate single-stranded oligonucleotides (Eurogentec) at 65 °C for 10 min in 10 mM Tris, 1 mM EDTA, 10 mM NaCl, followed by slow cooling to room temperature. The probes were then labeled by end-filling with the Klenow fragment of *Escherichia coli* DNA polymerase I (Roche), with [α-^{32}P]-dATP and [α-^{32}P]-dCTP (Dupont-New England Nuclear (NEN) Life Science Products). Labeled probes were purified by spin chromatography on Sephadex G-25 columns (Roche). DNA–protein complexes were separated from unbound probe on 4% native polyacrylamide gels at 150 V in 0.25 M Tris, 0.25 M sodium borate, and 0.5 mM EDTA, pH 8.0. Gels were vacuum-dried and exposed to Fuji X-ray film at −80 °C for 12 h and the resulting signals were quantified by densitometry using the Quantity One software (Bio-Rad). To confirm specificity, competition assays were performed with a 50-fold excess of unlabeled wild-type probes and with mutated probes (data not shown). Binding of the non-inducible transcription factor Oct-1 was always used as an internal standard for normalization (data not shown). The sequences of the wild-type probes were as follows—nuclear factor (NF)-κB, 5′-CAA CGG CAG GGG AAT TCC CCT CTC CTT AGG TT-3′; activator protein (AP)-1, 5′-CGC TTG ATG AGT CAG CCG GAA-3′; cAMP responsive element binding protein (CREB), 5′-AGA GAT TGC CTG ACG TCA GAG AGC TAG-3′; CAAT enhancer binding protein (C/EBP), 5′-CTA GGC ATA TTG CGC AAT AT-3′; nuclear factor of activated T cells (NF-AT), 5′-TCG ACC AAA GAG GAA AAT TTG TTT CAT ACA GAG-3′; Oct-1, 5′-TGT CGA ATG CAA ATC ACT AGA A-3′.

2.8. Western blot analysis

Equal amounts of whole cell lysates were subjected to SDS/PAGE under reducing conditions, and proteins were electro-transferred to polyvinylidene difluoride membranes (Roche). The membranes were blocked for 1 h at room temperature with 5% milk in TBS 1× with 0.1%

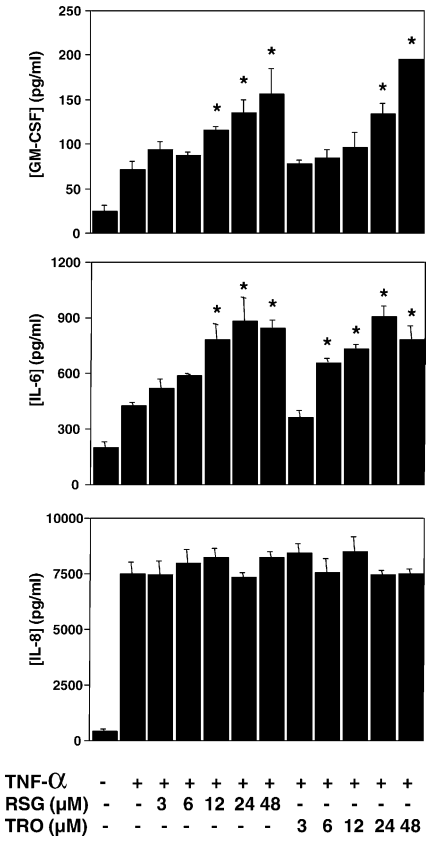

Fig. 1. RSG and TRO promote TNF-α-induced cytokine production in A549 cells. A549 cells were incubated for 2 h with RSG or TRO and then stimulated for 6 h with TNF-α (100 U/ml). IL-8, IL-6, and GM-CSF concentrations in cell supernatants were measured by ELISAs. Data are presented as means ± S.D. (*) Significantly different from the results obtained with supernatants from A549 cells treated with TNF-α alone.

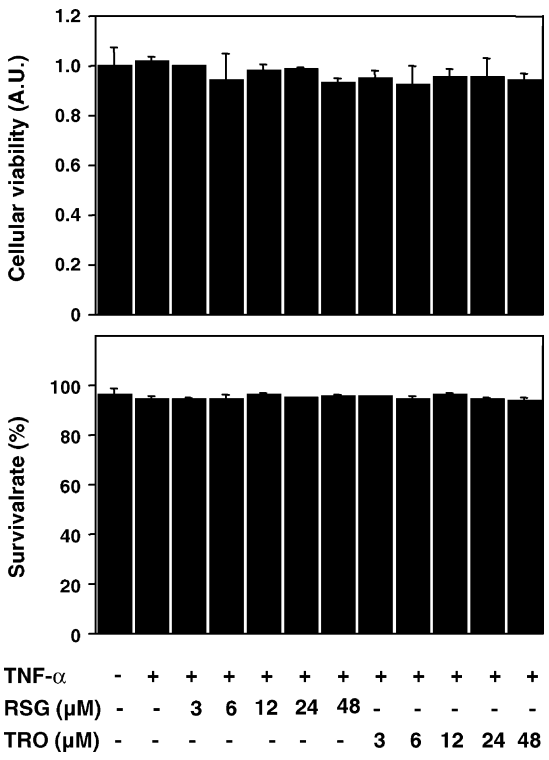

Fig. 2. RSG and TRO do not affect A549 cells survival at the concentrations used. A549 cells were incubated for 2 h with RSG or TRO and then stimulated overnight with TNF-α (100 U/ml). Cell survival and viability were then assessed using WST-1 (upper panel) and annexin-V-FITC/propidium iodide staining (lower panel), respectively. Data are presented as means ± S.D.

Tween-20 and incubated overnight at 4 °C with 1/1000 phosphospecific anti-p38, phosphospecific anti-c-Jun N-terminal kinase (JNK), or phosphospecific anti-extracellular signal-regulated kinase (ERK)1/2 Abs (New England Biolabs), or 1/10,000 anti-PPARγ Ab (Calbiochem-Merck). The blots were then incubated for 45 min with HRP-conjugated secondary Abs. Immunoreactive bands were revealed using the ECL detection method (ECL kit; Amersham Pharmacia Biotech.). Equal loading of proteins on the gel was always confirmed by probing the blots for α-tubulin (data not shown). Band intensities were estimated by densitometry using the Quantity One software (Bio-Rad) and normalized by probing the blots for α-tubulin (data not shown).

2.9. Statistical analysis

Data are presented as means ± S.D. The differences between mean values were estimated using either an ANOVA with subsequent Fisher's protected least significant difference tests or a Student's *t*-test for unpaired data. A value of $P < 0.05$ was considered significant. All presented results are representative of at least three similar experiments.

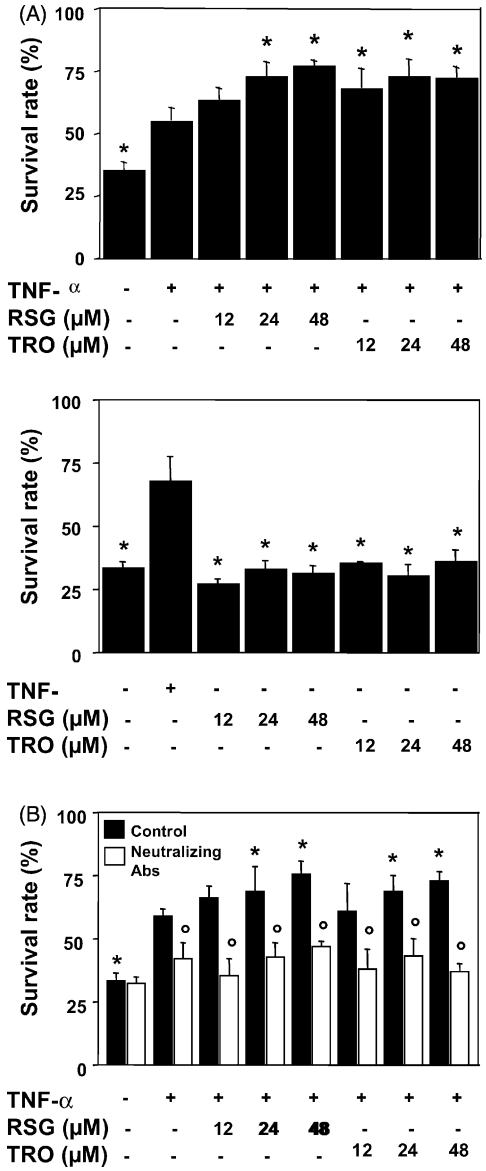

Fig. 3. RSG and TRO enhance neutrophil pro-survival activity of TNF-α-stimulated A549 cells through potentiation of IL-6 and GM-CSF production: (A) A549 cells were incubated for 2 h with 12, 24 or 48 μM RSG or TRO, and then activated for 6 h with TNF-α (upper panel). Alternatively, cells were incubated with TZDs alone for 8 h (lower panel). (B) A549 cells supernatants were incubated for 1 h with neutralizing anti-GM-CSF and anti-IL-6 antibodies (Abs) or left untreated before performing the same experiment as in A. Cell supernatants were assayed for neutrophil pro-survival activity as described in Section 2. Data are presented as means ± S.D. (*) Significantly different from the results obtained with supernatants from A549 cells treated with TNF-α alone; (o) significantly different from the results obtained with control supernatants from A549 cells.

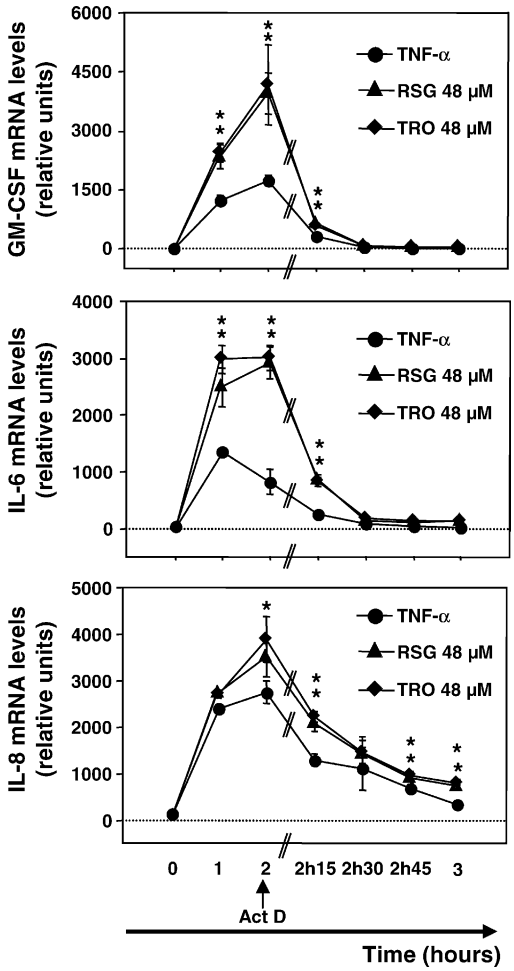

Fig. 4. RSG and TRO enhance cytokine expression at transcriptional level. A549 cells were incubated for 2 h with 24 or 48 μM RSG or TRO, and then activated for 1 or 2 h with TNF-α (100 U/ml) before being assayed for IL-6, IL-8 or GM-CSF mRNA expression by quantitative RT-PCR. The cells were treated with 5 μg/ml actinomycin D (Act D) 2 h after TNF-α stimulation, and assayed for cytokine mRNA expression 15–60 min later. The level of β-actin mRNA was used as an internal control for normalization. Data are presented as means ± S.D. (*) Significantly different from the results obtained with mRNA from A549 cells treated with TNF-α alone.

3. Results

3.1. Rosiglitazone and troglitazone potentiate the inflammatory response to TNF-α in A549 cells

First, using A549 epithelial cells we explored the effects of RSG and TRO on the production of three cytokines critical for initiating and maintaining the inflammatory process, namely IL-8, IL-6, and GM-CSF. In TNF-α-stimulated A549 cells, neither RSG nor TRO inhibited cytokine production, even at high concentrations (up to 48 μM) (Fig. 1). Rather, at concentrations ranging from 12 to 48 μM, both RSG and TRO significantly promoted TNF-α-induced cytokine expression with peak increase reached at 24 μM for IL-6 and 48 μM for GM-CSF (Fig. 1A). IL-8 production was not affected by RSG or TRO in TNF-α-stimulated A549 cells (Fig. 1A). When used at nanomolar concentrations (viz. 1–1000 nM), neither RSG

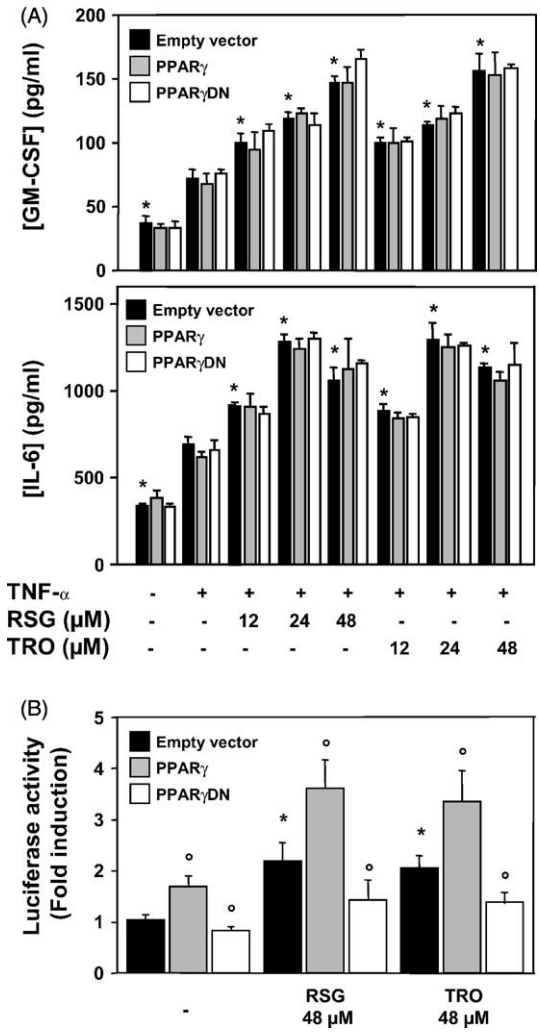

Fig. 5. RSG and TRO enhance cytokine expression in A549 cells independently of PPARγ activation: (A) modification of PPARγ activity in A549 cells does not affect RSG and TRO-mediated potentiation of cytokine expression. Cells were transfected with an expression vector coding for PPARγ or PPARγDN. After 24 h, the cells were treated for 2 h with 12, 24 or 48 μM RSG or TRO, and then stimulated with TNF-α (100 U/ml) for 6 h. IL-8, IL-6, and GM-CSF concentrations in cell supernatants were measured by ELISAs. Data are presented as means ± S.D. (*) Significantly different from the results obtained with supernatants from A549 cells treated with TNF-α alone. (B) Positive control for transfection efficiency and PPARγ and PPARγDN functionality. Following 24 h transfection with an expression vector coding for PPARγ or PPARγDN and pTkpGL3 reporter plasmid, A549 cells were treated for 8 h with 48 μM RSG or TRO before luciferase activity assessment. Data are presented as means ± S.D. (*) Significantly different from the results obtained with untreated A549 cells; (o) significantly different from the results obtained with empty vector-transfected A549 cells.

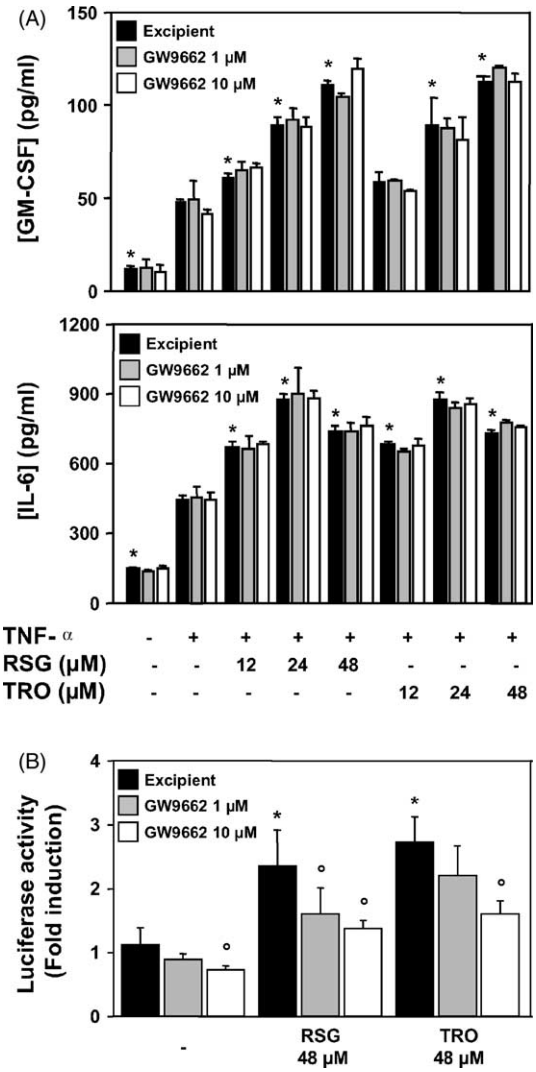

Fig. 6. Inhibition of PPARγ activity by a pharmacological antagonist (GW9662) does not affect RSG- and TRO-mediated potentiation of cytokine expression in A549 cells. (A) A549 cells were first treated with 1 or 10 μM GW9662. The 1 h later, the cells were treated for 2 h with 12, 24 or 48 μM RSG or TRO, and then activated for 6 h with TNF-α (100 U/ml). IL-8, IL-6, and GM-CSF concentrations in cell supernatants were measured by ELISAs. Data are presented as means ± S.D. (*) Significantly different from the results obtained with supernatants from A549 cells treated with TNF-α alone. (B) Positive control for GW9662 efficiency. Following 24 h transfection with pTkpGL3 reporter plasmid, A549 cells were treated with 1 or 10 μM GW9662 and then for 8 h with 48 μM RSG or TRO before luciferase activity assessment. Data are presented as means ± S.D. (*) Significantly different from the results obtained with untreated A549 cells; (o) significantly different from the results obtained with excipient-treated A549 cells.

nor TRO affected TNF-α-triggered cytokine production in A549 cells (data not shown). The TZD-elicited increase in cytokine production was a potentiation, as treatment of A549 with TZDs in the absence of TNF-α had no effect on IL-6, IL-8 or GM-CSF production (data not shown). The TZDs excipient, namely DMSO, had no effect on cytokine production. TZDs had no toxic effect on A549 cells at the concentrations used, as determined by proliferation and apoptosis/necrosis assays (Fig. 2).

IL-6 and GM-CSF delay neutrophil apoptosis, thus contributing to the persistence of the inflammatory response [26–28]. To determine whether elevated IL-6, and GM-CSF concentrations detected in the supernatants of A549 cells co-treated with TNF-α and TZDs have biological activity, we tested the ability of these supernatants to increase neutrophil viability. As illustrated in Fig. 3A (upper panel), supernatants from cells co-treated with TNF-α and TZDs displayed increased neutrophil pro-survival activity, when compared with supernatants from cells treated with TNF-α alone. TZDs treatment of A549 cells in the absence of TNF-α stimulation did not lead to any significant increase in the survival rate of neutrophils incubated in their supernatants (Fig. 3A, lower panel). Addition of neutralizing antibodies directed against IL-6 and GM-CSF led to abrogation of the neutrophil pro-survival activity of the supernatants from A549 cells treated with TNF-α alone or cotreated with TNF-α and TZDs (Fig. 3B). These experiments confirmed the functional significance of TZD-induced potentiation of IL-6 and GM-CSF expression in TNF-α stimulated A549 cells and established this potentiation as the main origin of the enhanced neutrophil pro-survival activity of TZDs-treated A549 cells supernatants.

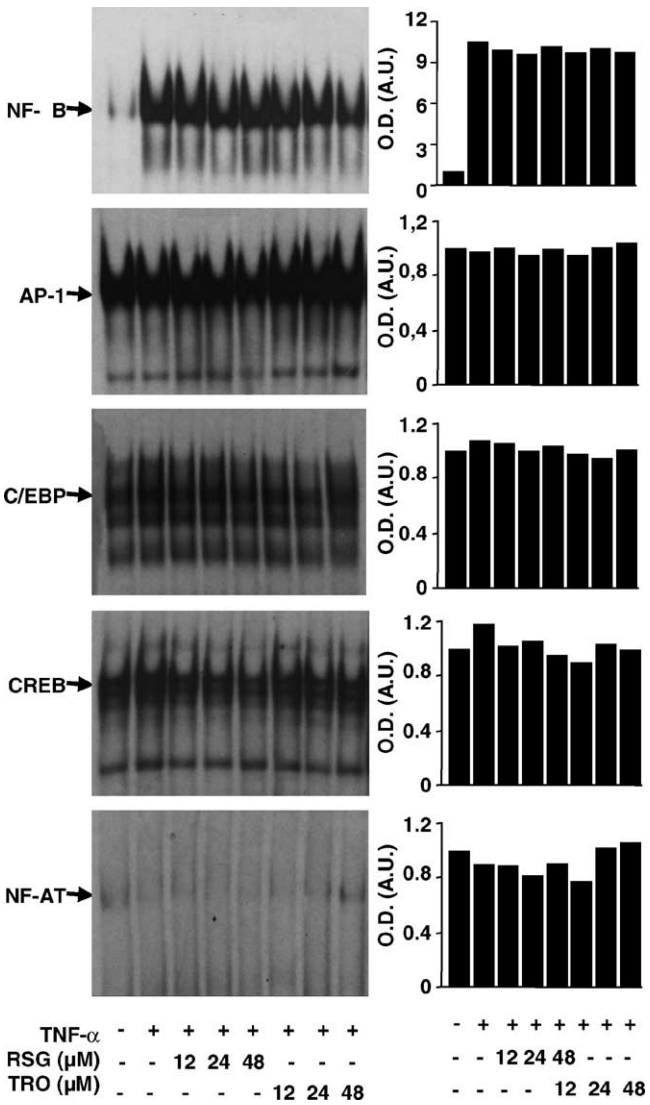

Fig. 7. RSG and TRO do not affect the activity of transcription factors implicated in IL-6, IL-8 and GM-CSF expression. A549 cells were incubated for 2 h with 12, 24 or 48 μM RSG or TRO. Cells were then stimulated for 30 min with TNF-α (100 U/ml), and nuclear protein extracts were assessed for NF-κB, AP-1, c/EBP, CREB and NF-AT DNA-binding activity by EMSAs. Band optical densities (O.D.) were estimated by densitometry and normalized by densitometric analysis of Oct-1 binding.

3.2. Rosiglitazone and troglitazone enhance pro-inflammatory cytokine expression in A549 cells at the gene expression level

To elucidate the molecular mechanisms by which TZDs potentiate cytokine expression in TNF-α-activated A549 cells, quantitative PCRs were performed. GM-CSF (Fig. 4, upper panel) and IL-6 (Fig. 4, middle panel) transcripts were significantly elevated in cells co-treated with TNF-α and RSG or TRO, as compared with cells treated with TNF-α alone, whereas IL-8 transcripts were only slightly elevated (Fig. 4, lower panel). As elevated transcript levels can be due to both transcriptional and post-transcriptional mechanisms implicating mRNA stabilization, stability of cytokine mRNA was assessed in similar experiments using actinomycin D, which blocks de novo RNA synthesis. The levels of IL-6, IL-8 and GM-CSF mRNA decreased promptly in TNF-α-treated cells after RNA synthesis was inhibited, even in the presence of TZDs (Fig. 4). No significant modification of mRNA half-life (approximately 4 min) was detected for any of the three cytokines, indicating that TZDs did not induce cytokine mRNA stabilization in TNF-α-treated A549 cells. Taken together, these results demonstrate that RSG and TRO potentiate IL-6 and GM-CSF expression at the transcriptional level, whereas they only moderately affect IL-8 gene expression in TNF-α-stimulated A549 cells.

3.3. Rosiglitazone and troglitazone enhance pro-inflammatory cytokine expression in A549 cells independent of PPARγ activation

TZDs are high affinity ligands of PPARγ [5,6]. RSG, the most potent TZD PPARγ agonist to date binds to the receptor with a K_d of 40 nM [5]. Although A549 cells express PPARγ [29], it seemed unlikely that PPARγ activation was implicated in TZD-mediated potentiation

of cytokine production considering the concentrations at which TZDs induced these effects. To solve this issue, A549 cells were transiently transfected with an expression vector coding for PPARγ or PPARγDN in order to increase or decrease PPARγ activity in the cells. Neither transfection with the expression vector coding for wild-type PPARγ, nor transfection with the vector coding for PPARγDN affected cytokine production in TNF-α-stimulated A549 cells following TZD treatment (Fig. 5A), indicating that the effects of RSG and TRO were independent on the level of PPARγ activity. Transfection efficacy was assessed by RT-PCR and immuno-blotting (data not shown). Reporter gene experiments further confirmed the functionality of the transfected PPARγ and PPARγDN (Fig. 5B). These results were further confirmed by the use of a chemical antagonist of PPARγ (GW9662) [30]. GW9662 at concentrations up to 10 μM had no effect on TZD-elicited potentiation of cytokine expression (Fig. 6A) although this inhibitor significantly inhibited PPAR-responsive element (PPRE)-driven reporter gene expression (Fig. 6B). Taken together, these results confirm that PPARγ activation is not implicated in the effects of TZDs on cytokine production in TNF-α-stimulated A549 cells.

3.4. Insights into the transcriptional mechanisms by which rosiglitazone and troglitazone potentiate pro-inflammatory cytokine production in A549 cells

To further investigate the mechanisms responsible for the pro-inflammatory effects of TZDs in A549 cells, we next examined whether these compounds might modify the activity of transcription factors (viz. NF-κB, AP-1, CREB, C/EBP and NF-AT) or mitogen-activated protein kinases (viz. p38, JNK and ERK1/2), which all may affect inflammatory gene expression. TNF-α activated NF-κB, p38 and ERK1/2 but did not modulate AP-1, CREB, C/EBP, NF-AT and JNK activity in A549 cells, as demonstrated by EMSAs (Fig. 7) or western blot analyses (Fig. 8). RSG and TRO did not modify NF-κB, AP-1, CREB, C/EBP, and

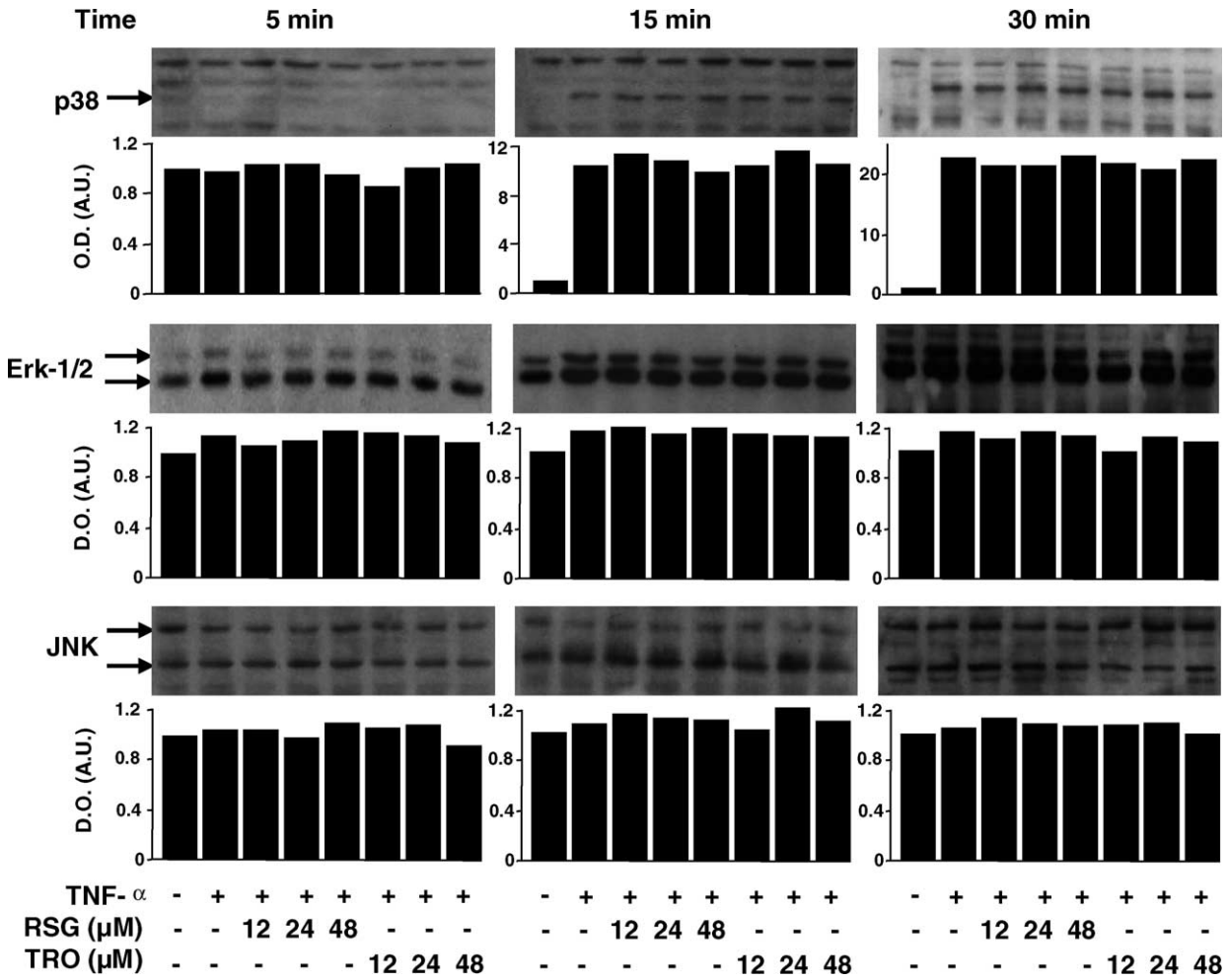

Fig. 8. RSG and TRO do not affect the activity of p38, JNK and ERK1/2. A549 cells were treated for 2 h with 12, 24 or 48 μM RSG or TRO, and subsequently activated for 5, 15 or 30 min with TNF-α (100 U/ml). Cell lysates were prepared and analyzed for p38, JNK and ERK1/2 activation by phospho-specific immunoblots. Band optical densities (O.D.) were estimated by densitometry and normalized by densitometric analysis of α-tubulin staining of the blots.

NF-AT DNA-binding activity in TNF-α-stimulated A549 cells, as assessed by EMSAs (Fig. 7). Moreover, neither RSG nor TRO could affect p38, JNK or ERK1/2 in these cells (Fig. 8).

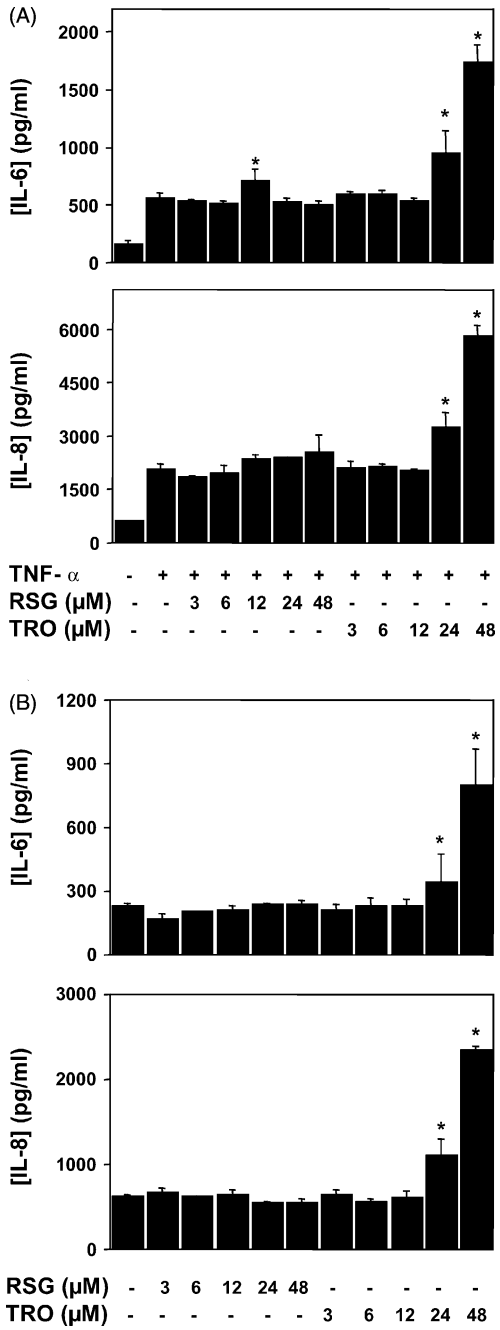

Fig. 9. (A) TRO, but not RSG, synergizes with TNF-α to enhance IL-6 and IL-8 expression in OVCAR cells. Cells were incubated for 2 h with the indicated concentrations of RSG or TRO, and then stimulated for 6 h with TNF-α (100 U/ml). IL-6 and IL-8 concentrations in cell supernatants were measured by ELISAs. (*) Significantly different from the results obtained with cells treated with TNF-α alone. (B) TRO alone, but not RSG, induces IL-6 and IL-8 expression in OVCAR cells. Cells were incubated for 8 h with the indicated concentrations of RSG or TRO. Afterwards, IL-6 and IL-8 concentrations were measured in cell supernatants using ELISAs. (*) Significantly different from the results obtained with untreated cells. Data are presented as means ± S.D.

3.5. Rosiglitazone and troglitazone also increase inflammatory cytokine production in OVCAR, Hct-116, BEAS-2B and human bronchial epithelial cells

To ascertain that the pro-inflammatory effects of TZDs were not restricted to A549 cells, the experiments performed with A549 cells were repeated using OVCAR and Hct-116 epithelial cells. OVCAR cells expressed IL-8 and IL-6, but not GM-CSF, in response to TNF-α. TRO considerably increased IL-6 and IL-8 production in TNF-α-stimulated OVCAR cells whereas RSG had no effect (Fig. 9A). For both cytokines, the maximum increase was observed at 48 μM TRO. TRO alone also induced IL-6 and IL-8 expression in OVCAR cells (Fig. 9B). However, the maximal levels of IL-6 and IL-8 reached following TRO stimulation were much lower than those recorded when TNF-α and TRO were combined. Hct-116 cells expressed IL-8, but not IL-6 and GM-CSF, in response to TNF-α (data not shown). TZDs did not potentiate TNF-α-induced IL-8 production in these cells (data not shown). However, both TZDs strongly stimulated GM-CSF secretion in Hct-116 cells (Fig. 10). Maximum induction was obtained at 48 μM RSG or 24 μM TRO and a significant induction was detectable at concentrations as low as 0.1 μM RSG or TRO. Finally, to ascertain that the observed effects of TZDs were not restricted to tumoral cells, we performed the same experiments using either the

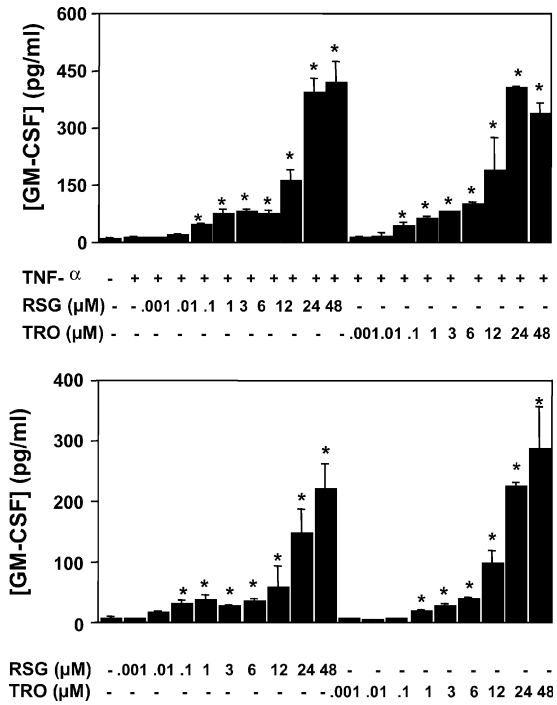

Fig. 10. RSG and TRO induce GM-CSF expression in Hct-116 cells. Cells were incubated for 8 h with the indicated concentrations of RSG or TRO. GM-CSF concentrations in cell supernatants were then measured by ELISAs. Data are presented as means ± S.D. (*) Significantly different from the results obtained with untreated cells.

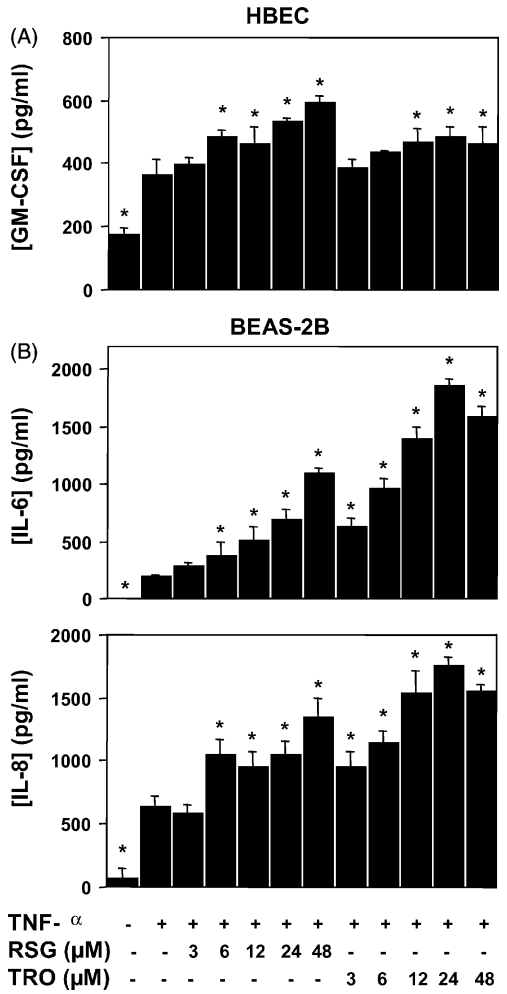

Fig. 11. RSG and TRO potentiate GM-CSF expression in HBEC (A) and IL-6 and IL-8 expression in BEAS-2B cells (B). Cells were incubated for 2 h with the indicated concentrations of RSG or TRO and then stimulated for 6 h with TNF-α. Cytokine concentrations in cell supernatants were then measured by ELISAs. Data are presented as means ± S.D. (*) Significantly different from the results obtained with cells treated with TNF-α alone.

BEAS-2B transformed bronchial epithelial cell line or primary HBEC cultures. BEAS-2B cells expressed IL-6 and IL-8 but not GM-CSF in response to TNF-α stimulation. Both RSG and TRO significantly potentiated IL-6 and IL-8 production in TNF-α-stimulated BEAS-2B (Fig. 11). Maximum induction was obtained at 48 μM RSG or 24 μM TRO. A more pronounced effect was observed for TRO compared to RSG. HBEC expressed GM-CSF, IL-6, and IL-8. TZD treatment had no effect on IL-6 and IL-8 production in TNF-α-stimulated HBEC (data not shown). However, both RSG and TRO significantly promoted GM-CSF expression in these cells, with a maximum potentiation of cytokine expression reached for 48 μM RSG or 24 μM TRO (Fig. 11). In HBEC, the most significant effect was observed with RSG treatment. In both BEAS-2B and HBEC, TZDs alone had no effect on cytokine production (data not shown).

4. Discussion

TZDs have recently been proposed as potential new anti-inflammatory agents. Indeed, most of the studies regarding the effects of TZDs in inflammation models have shown both in vitro and in vivo that these compounds could downregulate the inflammatory response [13–20]. In this study, we explored the effects of two TZDs, RSG and TRO, on the cytokinic response to TNF-α. Surprisingly, we show that TZDs do not inhibit but may significantly promote TNF-α-induced cytokine (i.e. IL-6, IL-8 and/or GM-CSF) production in A549, OVCAR, BEAS-2B and HBEC cells. Moreover, we report that TZDs alone are able to induce cytokine production in OVCAR and Hct-116 cells. Lastly, we demonstrate that TZD-mediated potentiation of cytokine expression may be biologically relevant as supernatants from A549 cells co-treated with TNF-α and TZDs displayed increased neutrophil prosurvival activity when compared with supernatants from cells treated with TNF-α alone.

Treatment of A549, OVCAR, Hct-116, BEAS-2B and HBEC cells with TZDs alone and/or in combination with TNF-α led to a significant increase in the expression of IL-6, IL-8 and/or GM-CSF, three cytokines essential for initiating and maintaining the inflammatory process. This is the first report of a positive effect of TZDs on GM-CSF production. This effect was particularly marked in Hct-116 cells, in which a 50-fold induction of GM-CSF expression was observed following treatment with TZDs alone. Most of the current data report an inhibitory effect of TZDs on IL-6 and IL-8 expression. An inhibition of IL-8 expression following TZD treatment was observed in IL-1β-stimulated colon cancer cells [15], in synoviocytes from rheumatoid arthritis-affected patients [31], and in LPS-stimulated endometrial tissue [32]. An inhibition of IL-6 expression was reported in a number of cases, e.g. in activated macrophages [13,14], in LPS-stimulated hepatocytes [33], in synoviocytes from rheumatoid arthritis-affected patients [31], and in LPS-stimulated gestational tissue [32]. Here, we show that TZDs alone or in combination with TNF-α do not inhibit but rather may lead to increased expression of IL-6 and IL-8. These results are consistent with two other studies in which increased circulating IL-6 levels were recorded in LPS-treated mice following TZD treatment [34] and elevated expression of IL-6 and IL-8 was noticed in TZD-treated endometrial tissue [35]. These apparently divergent effects of TZDs on pro-inflammatory cytokine expression could be attributable to cell type or stimulus-dependent specificity, or to the nature of the TZDs used. In support of this, the effects of TZDs in our cellular models were dependent on both the cell type and the TZD used, as RSG increased cytokine expression in A549, Hct-116, BEAS-2B and HBEC cells but had no effect in OVCAR cells and was generally less potent than TRO, while TRO promoted cytokine expression in the five cell lines, but had a much weaker effect than

RSG in HBEC. Moreover, TZDs increased IL-8 production in OVCAR and BEAS-2B cells but not in A549, Hct-116, and HVEC cells. The molecular mechanisms by which TZDs exert these divergent effects are presently unknown.

Quantitative PCRs performed in A549 cells indicated that RSG and TRO potentiate IL-6 and GM-CSF expression at the transcriptional level. TZDs are high affinity ligands of the transcription factor PPARγ [5,6] and it is assumed that most of the metabolic and anti-inflammatory actions of these compounds are mediated through ligation of this receptor. We therefore tested whether TZD-mediated potentiation of inflammatory cytokine expression was dependent on PPARγ activation in TNF-α-activated A549 cells, although this seemed unlikely considering the concentrations at which the effects of RSG and TRO on cytokine expression were observed. Indeed, RSG binds to PPARγ with a K_d of 40 nM and PPARγ-specific effects are generally observed at concentrations ranging from 1 nM to 1 μM [5,6], far below the concentration of 12–48 μM at which potentiation of cytokine expression occurred. Both transfection experiments and the use of a potent PPARγ antagonist confirmed that the pro-inflammatory effects of RSG and TRO were independent on PPARγ activation.

A growing body of evidence indicates that TZDs can mediate diverse PPARγ-independent effects. For example, TRO has anti-oxidative potential [36]. We attempted to identify the PPARγ-independent mechanisms by which TZDs potentiate IL-6 and GM-CSF expression in TNF-α-stimulated A549 cells. Induction of *il6* and *gmcsf* gene transcription critically depends on NF-κB activity [37]. However, maximum response requires additional transcription factors, including AP-1, CREB, c/EBP and NF-AT [38,39]. In A549 cells treated with TNF-α, RSG and TRO failed to enhance NF-κB, AP-1, CREB, c/EBP, and NF-AT activity indicating that these compounds do not potentiate *il6* and *gmcsf* gene expression through activation of the most important transcription factors involved in activation of these genes. Another mechanism by which inflammatory genes are upregulated is through activation of members of the MAP kinases family, namely p38, JNK and ERK1/2 [39–43]. However, although TZDs may activate MAP kinases [44–46], neither RSG nor TRO activated MAP kinases in TNF-α-treated A549 cells. The identification of the precise mechanism by which TZDs upregulate inflammatory gene expression thus requires further studies.

An anti-inflammatory potential for TZDs has been suggested, based on observations that these compounds may attenuate the inflammatory responses in vitro and in vivo. In the present study, we demonstrate that these compounds are unable to inhibit TNF-α-induced pro-inflammatory cytokine expression in epithelial cells. On the contrary, we show that RSG and TRO may increase the inflammatory response to TNF-α, thus shedding light on previously unappreciated effects of these compounds.

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