Jorjani Biomedicine Journal
Online ISSN: 2645-3509

Cytotoxic effects of I3C and TCDD on macrophages: Role of aryl hydrocarbon receptor target genes

Delara Omrani [1], Saeed Mohammadi [2,3], Yaghoub Yazdani [4*]

1. Department of Immunology, Faculty of Medicine, Golestan University of Medical Sciences, Gorgan, Iran
2. Natural and Medical Sciences Research Center, University of Nizwa, P.O. Box 33, PC 616, Birkat Al Mauz, Nizwa, Sultanate of Oman
3. Golestan Research Center of Gastroenterology and Hepatology, Jorjani Clinical Sciences Research Institute, Golestan University of Medical Sciences, Gorgan, Iran
4. Laboratory Sciences Research Center, Golestan University of Medical Sciences, Gorgan Iran

* Correspondence: Yaghoub Yazdani. Laboratory Sciences Research Center, Golestan University of Medical Sciences, Gorgan Iran.
Tel: +981732450093; Email: yazdani@goums.ac.ir

Abstract

Background: Macrophages are central effectors of innate immunity and exhibit remarkable functional plasticity in response to environmental stimuli. The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that plays a critical role in regulating macrophage function, immune homeostasis, and xenobiotic metabolism. Two well-characterized AhR ligands, indole-3-carbinol (I3C), a dietary phytochemical derived from cruciferous vegetables, and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), a persistent environmental pollutant, exert divergent biological effects despite engaging the same receptor. However, their differential cytotoxic impact on macrophages and the extent to which they activate downstream AhR target genes remain poorly characterized. This study aimed to evaluate and compare the cytotoxic effects of I3C and TCDD on THP-1-derived macrophages and to assess the consequent transcriptional regulation of three canonical AhR target genes: CYP1A1, CYP1B1, and AhRR.

Methods: THP-1 human monocytic cells were differentiated into macrophages using phorbol 12-myristate 13-acetate (PMA) and 2-mercaptoethanol (2-ME). Differentiated macrophages were treated with I3C (10 ng/µL) or TCDD (100 nM), each dissolved separately in dimethyl sulfoxide (DMSO), for 24 and 48 hours. Lipopolysaccharide (LPS; 100 ng/mL) was used as a positive control. Cell viability was assessed using the MTT colorimetric assay. Gene expression of CYP1A1, CYP1B1, and AhRR was quantified by real-time quantitative RT-PCR (qRT-PCR) using SYBR Green chemistry, with GAPDH as the reference gene. Data were analyzed by one-way ANOVA followed by Tukey's post-hoc test; significance was set at P-Value < 0.05.

Results: TCDD demonstrated pronounced cytotoxicity, reducing cell viability by 25% at 24 hours (P-Value < 0.05) and by 40% at 48 hours (P-Value < 0.01) compared to untreated controls. I3C induced significantly milder cytotoxicity, with viability reductions of 10% and 15% at 24 and 48 hours, respectively (P-Value < 0.05). DMSO vehicle control did not significantly affect cell viability. Real-time qRT-PCR analysis revealed that both ligands significantly upregulated CYP1A1, CYP1B1, and AhRR. TCDD induced greater upregulation of CYP1A1 (5.5-fold at 48 h) and CYP1B1 (4.8-fold at 48 h) compared to I3C (3.2-fold and 2.7-fold, respectively; P-Value < 0.05 for all between-group comparisons). AhRR was upregulated to a similar extent by both ligands (~2.1-fold).

Conclusion: TCDD exhibited markedly higher cytotoxicity than I3C in THP-1-derived macrophages, consistent with potent and sustained AhR activation. I3C induced comparatively milder cytotoxic and transcriptional responses, suggesting more controlled AhR engagement. These results provide mechanistic insights into ligand-dependent AhR signaling in macrophages and underscore the immunotoxicological risks of TCDD exposure, while highlighting I3C as a candidate for future anti-inflammatory research.

Article Type: Research Article

Article History

Received: 2 December 2024
Received in revised form: 20 May 2025
Accepted: 20 June 2025
Available online: 29 December 2025
DOI: 10.29252/jorjanibiomedj.13.4.44

Keywords

Aryl Hydrocarbon Receptors
Aryl Hydrocarbon Receptor Repressor
Cytochrome P-450 CYP1B1
Cytochrome P-450 CYP1A1
THP-1 Cells
Indole-3-Carbinol
Tetrachlorodibenzo-p-dioxin
Protein
Macrophage Cytotoxicity
Immune Modulation

Highlights

What is current knowledge?

AhR activation plays a key role in regulating immune responses, inflammation, and metabolic processes. TCDD, a potent environmental pollutant, is a high-affinity AhR ligand linked to immune suppression, chronic inflammation, and cellular toxicity. I3C, a naturally occurring AhR ligand found in cruciferous vegetables, possesses anti-inflammatory and immunomodulatory properties, though its effects on macrophages remain incompletely understood. The differential cytotoxic effects of these two compounds on macrophages, particularly in relation to their activation of AhR target genes such as CYP1A1 and CYP1B1, have not been fully explored.

What is new here?

This study demonstrates that TCDD induces significantly higher cytotoxicity in THP-1-derived macrophages compared to I3C, with a 40% reduction in cell viability after 48 hours of exposure to TCDD. In contrast, I3C shows milder effects on macrophage viability. Both compounds significantly upregulate AhR target genes, with TCDD inducing a stronger and more sustained transcriptional response. LPS was used as a positive control to validate macrophage inflammatory responsiveness. These findings highlight the distinct immunomodulatory and toxicological roles of I3C and TCDD in macrophage activation.

Introduction

Macrophages are versatile and highly specialized cells of the innate immune system, performing critical functions that include pathogen recognition and elimination, antigen presentation, tissue repair, and the orchestration of both pro-inflammatory and anti-inflammatory responses (1). These cells arise from circulating monocytes that migrate into tissues and undergo differentiation in response to microenvironmental signals. A hallmark of macrophage biology is phenotypic plasticity: in response to distinct stimuli, macrophages can polarize toward either the classically activated (M1) pro-inflammatory phenotype, characterized by the production of tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and reactive oxygen species, or the alternatively activated (M2) anti-inflammatory phenotype, which promotes wound healing and immune resolution (2). This polarization is not binary but exists along a spectrum and is regulated by a complex network of transcription factors that integrate metabolic and environmental signals.

Among the signaling pathways that modulate macrophage function, the aryl hydrocarbon receptor (AhR) pathway has emerged as a central regulator of immune homeostasis (3). AhR is a cytosolic ligand-activated transcription factor that belongs to the basic helix-loop-helix/Per-ARNT-Sim (bHLH-PAS) superfamily. In its unliganded state, AhR resides in the cytoplasm in association with chaperone proteins,

including Hsp90, AIP, and p23. Upon ligand binding, AhR undergoes a conformational change and translocates to the nucleus, where it dimerizes with the AhR nuclear translocator (ARNT). This AhR-ARNT heterodimer then binds to xenobiotic response elements (XREs) in the promoter regions of target genes, driving their transcription (4). Among the best-characterized AhR target genes are CYP1A1 and CYP1B1, which encode cytochrome P450 enzymes involved in xenobiotic metabolism, and the AhR repressor (AhRR), a feedback inhibitor that limits the duration of AhR activation.

Indole-3-carbinol (I3C) is a naturally occurring phytochemical produced from glucobrassicin upon hydrolysis in cruciferous vegetables such as broccoli, cauliflower, and Brussels sprouts (5). I3C and its main metabolite, 3,3′ -diindolylmethane (DIM), are recognized as AhR ligands with significantly lower receptor-binding affinity than halogenated aromatic hydrocarbons. Numerous preclinical studies have documented the anti-inflammatory, antiproliferative, and immunomodulatory properties of I3C. In THP-1 monocytes, I3C has been shown to induce G1 cell cycle arrest and apoptosis through AhR-dependent pathways and to inhibit LPS-stimulated inflammatory signaling by blocking the TRIF-dependent pathway in macrophages (6,7). These properties make I3C a promising candidate for dietary-based immune modulation strategies, particularly in the context of chronic inflammatory and autoimmune diseases (8).

In stark contrast, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is a highly toxic environmental contaminant and one of the most potent known AhR agonists. TCDD is a byproduct of industrial combustion processes, herbicide synthesis, and chlorine bleaching, and it persists in the environment because of its exceptional chemical stability and lipophilicity (9). TCDD-induced AhR activation in macrophages and other immune cells leads to a range of adverse immunological effects, including suppression of T-cell responses, alterations in cytokine profiles, impairment of phagocytic function, and induction of apoptosis. Previous studies using THP-1-derived macrophages demonstrated that TCDD significantly suppresses inflammatory gene expression and immune cell activity through AhR-mediated mechanisms (9). The capacity of TCDD to sustain prolonged AhR activation owing to its resistance to metabolic clearance distinguishes it mechanistically from rapidly metabolized ligands such as I3C.

While both I3C and TCDD engage the AhR pathway, their differential effects on macrophage viability and transcriptional activity have not been directly and comprehensively compared. Understanding these distinctions is essential for delineating the boundaries between beneficial immunomodulation, as potentially achieved by dietary ligands, and pathological immune suppression induced by environmental toxins. The THP-1 cell line is a well-established and widely used human monocytic leukemia model that, upon differentiation with PMA, acquires macrophage-like morphological and functional characteristics, making it an appropriate in vitro system for investigating AhR-mediated effects on human macrophage biology (10).

This study was designed to evaluate and compare the cytotoxic effects of I3C and TCDD on THP-1-derived macrophages and to characterize their differential capacity to transcriptionally activate AhR target genes (CYP1A1, CYP1B1, and AhRR) at two key time points (24 and 48 hours). The findings provide mechanistic insights into how ligand potency and receptor dwell-time shape macrophage responses and offer a foundation for distinguishing between immunoprotective and immunotoxic AhR engagement.

Methods

Cell culture and differentiation

THP-1 cells (A human monocytic leukemia cell line; ATCC TIB-202) were maintained in RPMI-1640 medium (Gibco, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Bioidea, Iran) and 1% penicillin-streptomycin (Bioidea, Iran) at 37°C in a humidified atmosphere containing 5% CO2. Cell density was maintained between 1×105 and 1×106 cells/mL, with regular passage every 2-3 days. For macrophage differentiation, THP-1 monocytes were seeded at a density of 5×105 cells/mL in 96-well plates (For the MTT assay) or 6-well plates (For RNA extraction). Differentiation into adherent macrophage-like cells was induced by co-treatment with 0.55 µg/mL phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, USA) and 0.05 mM 2-mercaptoethanol (2-ME; Bioidea, Iran) for 24 hours, as previously described (10). Following differentiation, cells were washed twice with phosphate-buffered saline (PBS) and allowed to rest in fresh complete medium for an additional 24 hours before ligand treatment.

Ligand preparation and treatment

I3C (Sigma-Aldrich, USA) and TCDD (Sigma-Aldrich, USA) were each dissolved separately in dimethyl sulfoxide (DMSO; Sigma-Aldrich, USA) to prepare concentrated stock solutions at 10 mg/mL and 1 mM, respectively. Working solutions were prepared by serial dilution of each stock in cell culture medium immediately before treatment. The final working concentrations used in all experiments were I3C at 10 ng/µL (Approximately 60 µM) and TCDD at 100 nM. The final DMSO concentration in the treatment media did not exceed 0.1% (v/v) in any condition. These concentrations were selected based on established dose-response data from prior studies and preliminary experiments conducted in our laboratory, which confirmed biological activity without overt solvent-induced toxicity (6,9).

Four experimental groups were established: (1) untreated cells (Negative control, receiving only culture medium), (2) DMSO vehicle control (Cells receiving 0.1% DMSO in culture medium, matched to the maximum solvent concentration in treatment groups), (3) I3C-treated cells (10 ng/µL), and (4) TCDD-treated cells (100 nM). In addition, lipopolysaccharide (LPS; 100 ng/mL; Sigma-Aldrich, USA) was used as a positive control for macrophage activation and inflammatory responsiveness. Cells were incubated with the treatments for 24 and 48 hours to capture both early and sustained responses. A 72-hour time point was not included in this study because preliminary observations revealed extensive cell detachment and non-specific cytotoxicity at that duration under the current treatment conditions, which would have confounded viability and gene expression measurements; future studies incorporating additional time points are warranted.

MTT assay for cell viability

Cell viability was assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay (Sigma-Aldrich, USA), a well-established method that measures the metabolic reduction of MTT to insoluble purple formazan crystals by mitochondrial dehydrogenases in metabolically active cells (11). THP-1-derived macrophages were seeded at 1×104 cells/well in 96-well flat-bottom plates. Following treatment with I3C, TCDD, DMSO, LPS, or medium alone for 24 or 48 hours, 30 µL of MTT solution (5 mg/mL in PBS) was added to each well. Plates were then incubated at 37°C for 4 hours to allow formazan crystal formation. The culture medium was carefully aspirated, and 100 µL of DMSO was added to each well to dissolve the formazan crystals with gentle agitation for 10 minutes at room temperature. The optical density (OD) was measured at 570 nm (Reference wavelength: 630 nm) using a Stat Fax 2200 microplate reader (Awareness Technology, USA). Cell viability was expressed as a percentage relative to the untreated control group and calculated as follows: % viability = (OD sample / OD control) × 100. Each condition was performed in triplicate, and experiments were repeated on at least three independent occasions.

RNA extraction and Real-Time Quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from treated macrophages using TRIzol reagent (Sigma-Aldrich, USA) according to the manufacturer's instructions (6). Briefly, cells were lysed directly in culture wells, and the homogenate was subjected to chloroform phase separation, isopropanol precipitation, and ethanol washing. The RNA pellet was dissolved in RNase-free water. RNA quantity and purity were assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA); only samples with A260/A280 ratios between 1.8 and 2.0 and A260/A230 ratios ≥1.8 were used. RNA integrity was further confirmed by 1.5% agarose gel electrophoresis, with visualization of intact 28S and 18S ribosomal RNA bands.

Complementary DNA (cDNA) was synthesized from 1 µg of total RNA per sample using a cDNA synthesis kit (Yekta Tajhiz, Iran) according to the manufacturer's protocol, which included an initial denaturation step at 65°C for 5 minutes, followed by reverse transcription at 42°C for 60 minutes and enzyme inactivation at 70°C for 5 minutes. Real-time quantitative PCR (qRT-PCR) was performed using SYBR Green PCR Master Mix (Yekta Tajhiz, Iran) on an ABI StepOne Plus Real-Time PCR system (Applied Biosystems, USA). Each 20-µL reaction contained 10 µL SYBR Green master mix, 0.5 µL

each of forward and reverse primer (10 µM), 2 µL cDNA template, and 7 µL nuclease-free water. Thermal cycling conditions were as follows: initial denaturation at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Melt-curve analysis was performed at the end of each run to confirm amplicon specificity. Target gene expression was quantified using the 2-ΔΔCt method, with GAPDH as the reference housekeeping gene. All reactions were performed in triplicate, and the mean Ct values were used for calculations. No-template controls (NTCs) were included in every run. Primer sequences for all target and reference genes are provided in Table 1.

Table 1. Primer sequences used for real-time qRT-PCR amplification of AhR target genes (CYP1A1, CYP1B1, and AhRR) and the reference housekeeping gene GAPDH.

Gene	Primer type	Sequence (5′ → 3′)
CYP1A1	Forward	CTCCTCAACCTCCTGCTACC
CYP1A1	Reverse	GTCACTGATACCACCACATACC
CYP1B1	Forward	GCTCACCAGTGCGATTTCA
CYP1B1	Reverse	GCTTGCCTCTTGCTTCTTATTG
AhRR	Forward	CGGGATGTTGCCCAAAAGTG
AhRR	Reverse	AAGCGTGTGTCTTAGGACGG
GAPDH	Forward	GTCTCCTCTGACTTCAACAGCG
GAPDH	Reverse	ACCACCCTGTTGCTGTAGCCAA

Statistical analysis

All data are presented as mean ± standard deviation (SD) from a minimum of three independent experiments, each performed in triplicate. Statistical analysis was performed using GraphPad Prism version 9 (GraphPad Software, USA) and SPSS version 26 (IBM Corp., USA). Group comparisons were made using one-way analysis of variance (ANOVA), followed by Tukey's honestly significant difference (HSD) post-hoc test to account for multiple comparisons. A P-Value of < 0.05 was considered statistically significant. For gene expression data, the 2-ΔΔCt method was applied for relative quantification, and statistical comparisons were performed on ΔCt values to satisfy normality assumptions.

Results

Cytotoxic effects of I3C and TCDD on THP-1-derived macrophages

The cytotoxic effects of I3C, TCDD, DMSO (Vehicle control), LPS (Positive control), and untreated conditions were evaluated in THP-1-derived macrophages using the MTT assay at 24 and 48 hours. Statistical comparisons were performed by one-way ANOVA followed by Tukey's post-hoc test.

TCDD exhibited pronounced and time-dependent cytotoxicity. At a concentration of 100 nM, TCDD reduced cell viability by 25 ± 3.2% at 24 hours (P-Value < 0.05 vs. untreated control) and by 40 ± 4.8% at 48 hours (P-Value < 0.01 vs. untreated control; P-Value < 0.05 vs. 24-hour TCDD), demonstrating a significant temporal escalation in cytotoxic activity. In contrast, I3C (10 ng/µL) induced a significantly milder cytotoxic profile. Cell viability decreased by 10 ± 1.8% at 24 hours (P-Value < 0.05 vs. untreated) and by 15 ± 2.1% at 48 hours (P-Value < 0.05 vs. untreated), representing a statistically significant but considerably less severe reduction compared to TCDD at each corresponding time point (P-Value < 0.01 for TCDD vs. I3C at 48 hours). Cells treated with DMSO (Vehicle control) showed no significant change in viability at either time point (P-Value > 0.05), confirming that the cytotoxic effects were attributable to the active compounds rather than the solvent. LPS-treated cells-maintained viability comparable to controls, consistent with its role as an inflammatory activator rather than a cytotoxic agent. Untreated cells maintained stable viability across both time points, serving as the baseline reference. These data are illustrated in Figure 1.

Differential expression of AhR target genes induced by I3C and TCDD

Real-time qRT-PCR analysis demonstrated that both I3C and TCDD significantly upregulated the expression of the canonical AhR target genes CYP1A1, CYP1B1, and AhRR at both 24 and 48 hours compared to untreated controls (One-way ANOVA with Tukey's post-hoc test; P-Value < 0.05 for all comparisons). The magnitude of induction differed substantially between the two ligands and across time points.

TCDD treatment resulted in a 3.8 ± 0.4-fold increase in CYP1A1 expression at 24 hours (P-Value < 0.01) and a 5.5 ± 0.6-fold increase at 48 hours (P-Value < 0.001). I3C induced a 2.1 ± 0.3-fold upregulation of CYP1A1 at 24 hours (P-Value < 0.05) and a 3.2 ± 0.3-fold upregulation at 48 hours (P-Value < 0.01). The difference in CYP1A1 expression between TCDD and I3C was statistically significant at both time points (P-Value < 0.05 at 24 h and P-Value < 0.01 at 48 h).

The expression of CYP1B1 followed a similar pattern. TCDD induced a 3.2 ± 0.4-fold increase at 24 hours and a 4.8 ± 0.5-fold increase at 48 hours (P-Value < 0.001). I3C induced a 1.9 ± 0.3-fold upregulation at 24 hours (P-Value < 0.05) and a 2.7 ± 0.4-fold increase at 48 hours (P-Value < 0.01). At both time points, TCDD induced significantly greater CYP1B1 upregulation than I3C (P-Value < 0.05). These results further confirm the stronger transcriptional activity of TCDD through AhR.

In contrast, AhRR expression showed considerably less variability between the two ligands. TCDD induced a 1.7 ± 0.2-fold increase at 24 hours and a 2.1 ± 0.2-fold increase at 48 hours (P-Value < 0.01 vs. control). I3C similarly induced a 1.5 ± 0.2-fold upregulation at 24 hours and a 2.1 ± 0.3-fold increase at 48 hours (P-Value < 0.01 vs. control). Notably, there was no statistically significant difference in AhRR induction between TCDD and I3C at either time point (P-Value > 0.05), suggesting that the AhRR-mediated negative feedback circuit is engaged to a comparable degree by both ligands, regardless of their differential AhR binding potency. The progressive increase in all target genes from 24 to 48 hours was more pronounced with TCDD than with I3C, further reflecting the sustained receptor occupancy and transcriptional activity of TCDD. These results are summarized in Figure 2.

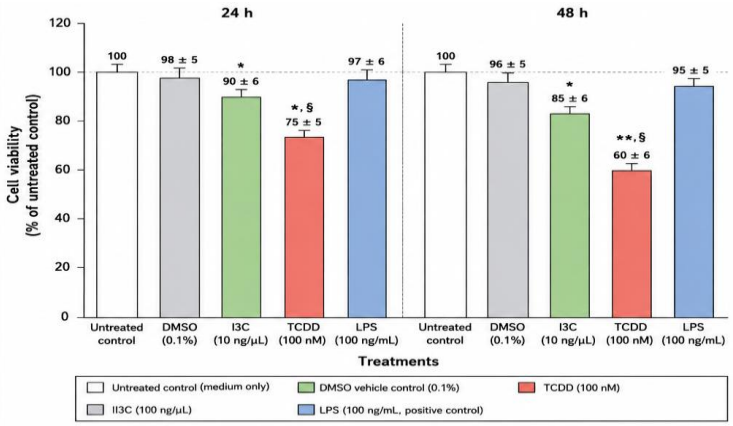

Figure 1. Cytotoxic effects of I3C and TCDD on THP-1-derived macrophages assessed by the MTT assay. Cell viability (%) is shown for each treatment group-untreated control, DMSO vehicle control (0.1%), LPS (100 ng/mL; positive control for macrophage activation), I3C (10 ng/µL), and TCDD (100 nM)-at 24 and 48 hours post-treatment. Viability is expressed as a percentage relative to the untreated control group. TCDD significantly reduced cell viability at both time points, with the greatest reduction (40%) observed at 48 hours. I3C induced a milder but statistically significant reduction in viability. LPS and DMSO did not significantly affect viability. Data represent mean ± SD from three independent experiments performed in triplicate. Statistical comparisons were performed using one-way ANOVA with Tukey's post-hoc test. * P-Value < 0.05, ** P-Value < 0.01 vs. untreated control; § P-Value < 0.05 TCDD vs. I3C at corresponding time points.

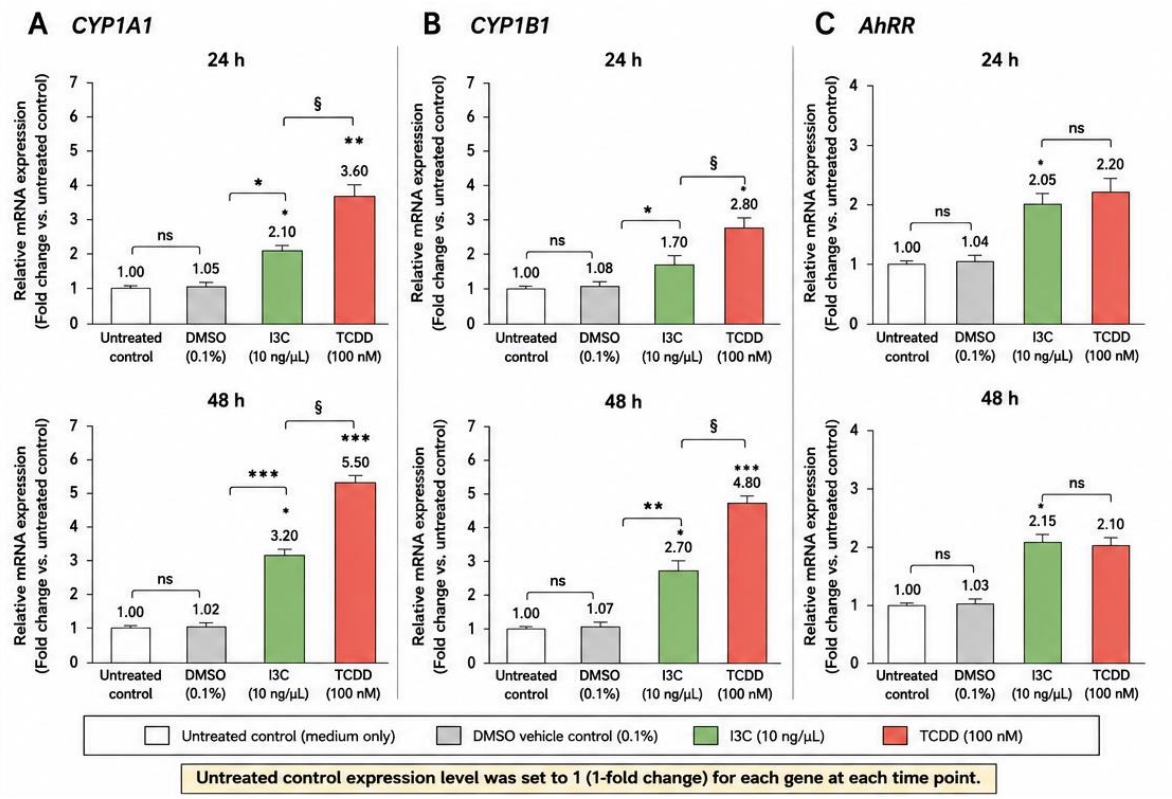

Figure 2. Differential expression of AhR target genes CYP1A1, CYP1B1, and AhRR in THP-1-derived macrophages following treatment with I3C or TCDD. Bar graphs depict relative mRNA expression levels (Fold change) for each target gene at 24 and 48 hours post-treatment with I3C (10 ng/µL) or TCDD (100 nM), compared to untreated control cells. Expression was quantified by real-time qRT-PCR using the 2-ΔΔCt method, with GAPDH as the reference housekeeping gene. TCDD induced markedly greater upregulation of CYP1A1 (5.5-fold) and CYP1B1 (4.8-fold) at 48 hours compared to I3C (3.2-fold and 2.7-fold, respectively). AhRR was upregulated to a similar extent by both ligands (~2.1-fold at 48 h). Data represent mean ± SD from three independent experiments. Statistical comparisons used one-way ANOVA with Tukey's post-hoc test. * P-Value < 0.05, ** P-Value < 0.01, *** P-Value < 0.001 vs. untreated control; § P-Value < 0.05 between TCDD and I3C at corresponding time points. ns: Not Significant.

Discussion

This study provides a comprehensive comparison of the cytotoxic and transcriptional effects of two structurally and functionally distinct AhR ligands-I3C and TCDD-on THP-1-derived macrophages. Our findings clearly demonstrate that TCDD exerts markedly greater cytotoxicity and transcriptional activation of AhR target genes compared to I3C, underscoring the profound immunotoxicological consequences of exposure to dioxin-class environmental pollutants and highlighting the relative safety and controlled biological activity of dietary AhR ligands.

The pronounced cytotoxicity of TCDD observed in this study, as evidenced by a 40% reduction in cell viability at 48 hours, is consistent with its well-established role as an ultrapotent AhR agonist with high receptor-binding affinity and exceptional metabolic resistance (9). Several prior investigations using THP-1-derived macrophage models have similarly reported that TCDD suppresses immune cell viability, impairs phagocytic activity, and dysregulates inflammatory gene networks (9,12). The cytotoxic mechanism of TCDD in immune cells is thought to be multifactorial, involving AhR-mediated induction of oxidative stress, generation of reactive oxygen species (ROS), mitochondrial membrane depolarization, DNA strand breaks, and activation of intrinsic apoptotic pathways (13). Furthermore, TCDD-activated AhR has been shown to interfere with NF-κB signaling, p53-dependent cell cycle checkpoints, and anti-apoptotic Bcl-2 family members, collectively resulting in a pro-apoptotic cellular environment (12). The sustained receptor occupancy of TCDD-attributable to its high lipophilicity and resistance to CYP1A1-mediated biotransformation-prolongs AhR signaling and amplifies downstream cytotoxic cascades over time, explaining the progressive increase in cytotoxicity observed between 24 and 48 hours in the present study.

In contrast, I3C exhibited significantly milder cytotoxicity, with only a 15% reduction in cell viability at 48 hours. This attenuated response is consistent with I3C's considerably lower AhR binding affinity, rapid hepatic metabolism, and propensity to undergo acid-catalyzed condensation in the gut to form oligomeric products with variable AhR activity (5,13). Our findings align with earlier reports in THP-1 cells demonstrating that I3C induces G1 cell cycle arrest and moderate apoptosis via AhR-dependent mechanisms, without the catastrophic cytotoxicity associated with halogenated dioxins (9). In macrophages specifically, I3C has been shown to attenuate LPS-induced inflammatory signaling by inhibiting TRIF-dependent activation of IRF3 and downstream interferon-β production (10). The mild cytotoxicity observed with I3C in the present study may therefore represent a beneficial form of cellular stress that promotes controlled immune modulation rather than pathological toxicity. It is important to note that framing I3C as a "safer alternative" to TCDD does not imply that they serve the same biological purpose; rather, it reflects their contrasting impacts on macrophage viability when both are studied as AhR ligands in the same model system. TCDD is unequivocally a toxic environmental pollutant, while I3C is a dietary phytochemical with reported health-promoting properties. The comparison is therefore mechanistic and toxicological in nature (14).

The transcriptional data further reinforce the mechanistic divergence between the two ligands. TCDD drove substantially greater upregulation of both CYP1A1 (5.5-fold) and CYP1B1 (4.8-fold) at 48 hours, compared to I3C (3.2-fold and 2.7-fold, respectively). The CYP1A1 and CYP1B1 genes encode cytochrome P450 enzymes that are among the most sensitive and reliable biomarkers of AhR activation (15). Their differential induction in this study directly mirrors the differences in AhR binding affinity and receptor dwell time between the two compounds. TCDD's higher and more sustained transcriptional output likely reflects prolonged nuclear AhR-ARNT complex formation and persistent XRE occupancy (16). The progressive increase in fold change observed from 24 to 48 hours for both genes, especially for TCDD, supports the concept of cumulative transcriptional activity driven by continuous receptor engagement. Notably, CYP1B1 upregulation in macrophages may also contribute to the production of immunomodulatory lipid mediators via arachidonic acid metabolism, potentially linking AhR activation to downstream inflammatory pathways (12).

The observation that AhRR was upregulated to a statistically indistinguishable extent by both TCDD and I3C (~2.1-fold at 48 hours) is particularly noteworthy. AhRR is a bHLH-PAS protein that forms inactive heterodimers with ARNT and competes with AhR for XRE binding, thereby functioning as a classical negative feedback regulator of AhR activity (17). The similar AhRR induction observed with both ligands, despite their vastly different potencies for activating CYP1A1 and CYP1B1, suggests that the AhRR circuit operates as a threshold-gated dampener rather than a linear sensor of AhR activity. This interpretation aligns with studies by Schanz et al. (18) and Vogel and Haarmann-Stemmann (17), demonstrating that dietary AhR ligands can engage AhRR expression to a degree comparable to potent synthetic agonists, likely through partially overlapping transcriptional mechanisms. The functional consequence of equivalent AhRR induction may be that the feedback circuit is insufficient to fully counteract the sustained AhR activation driven by TCDD, resulting in the net greater cytotoxicity and gene expression responses observed in TCDD-treated cells compared to I3C-treated cells.

The present study has several limitations that merit consideration. First, the experiments were conducted using a single concentration of each compound, which precludes a full characterization of dose-response relationships. Future studies should incorporate concentration gradients to define IC50 values and map dose-dependent transcriptional responses. Second, the use of the THP-1 cell line, while convenient and reproducible, does not fully recapitulate the complexity of primary human macrophages, which exhibit greater heterogeneity in AhR expression and ligand metabolism. Extending these findings to primary monocyte-derived macrophages from human donors would strengthen their translational relevance. Third, the mechanisms of cytotoxicity were not directly interrogated; future investigations should include flow cytometric analysis of apoptosis and necrosis (e.g., Annexin V/PI staining), caspase activation assays, and mitochondrial membrane potential measurements to fully characterize the mode of cell death. Fourth, the functional consequences of AhR target gene induction on macrophage polarization, cytokine secretion profiles (e.g., IL-6, TNF-α, IL-10), and phagocytic capacity remain to be examined in this model, and these represent high-priority directions for subsequent research (19,20).

Taken together, these findings contribute to a growing body of evidence that AhR ligands with distinct physicochemical properties and biological origins can engage the same receptor yet produce fundamentally different immunological outcomes. The differential cytotoxicity and gene regulation observed with I3C and TCDD illustrate the principle of ligand-selective AhR modulation, which may have important implications for the rational design of AhR-targeting therapeutic strategies in inflammatory and autoimmune diseases, as well as for the toxicological risk assessment of dioxin-class environmental contaminants (3,4).

Conclusion

This study demonstrates that AhR ligands of distinct origins and binding potencies exert markedly different effects on THP-1-derived macrophage viability and transcriptional activity. TCDD, a potent environmental dioxin, induced significant cytotoxicity and substantially greater upregulation of CYP1A1 and CYP1B1 compared to I3C, reflecting its high-affinity and sustained AhR engagement. I3C, a dietary phytochemical, activated AhR in a milder and more controlled manner, resulting in limited cytotoxicity. Both ligands induced comparable AhRR upregulation, suggesting a shared negative feedback ceiling. These findings underscore the immunotoxicological risks of chronic TCDD exposure and highlight the mechanistic contrast between pathological AhR overstimulation and physiologically relevant dietary AhR modulation. Future studies incorporating primary macrophages, extended time courses, dose-response analyses, and functional immunological readouts are warranted to build upon these findings.

Acknowledgement

The authors would like to thank the Immunology Laboratory at Golestan University of Medical Sciences for technical support and financial assistance.

Funding sources

This study was funded by Golestan University of Medical Sciences, grant number: 11884.

Ethical statement

This study was conducted using commercially available cell lines and did not involve human or animal subjects. All procedures complied with applicable institutional and national ethical guidelines.

Conflicts of interest

The authors declare no conflicts of interest related to this study.

Author contributions

Delara Omrani performed the experimental work, including THP-1 cell culture, macrophage differentiation, ligand treatment, MTT assay, RNA extraction, and qRT-PCR analysis. Saeed Mohammadi contributed to study conceptualization, experimental design, data interpretation, manuscript drafting, scientific revision, and supervision of the immunological aspects of the study. Yaghoub Yazdani contributed to study conceptualization, project supervision, funding acquisition, laboratory resources, data interpretation, and critical revision of the manuscript. All authors reviewed and approved the final version of the manuscript and agreed to be accountable for all aspects of the work.

Data availability statement

N/A.

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Cite this article as:

Omrani D, Mohammadi S, Yazdani Y. Cytotoxic effects of I3C and TCDD on macrophages: Role of aryl hydrocarbon receptor target genes. *Jorjani Biomedicine Journal.* 2025;13(4):44-9. http://dx.doi.org/10.29252/jorjanibiomedj.13.4.44