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Immunomodulatory effects of Bisphenol A, perfluorooctanoic acid, and tris(1,3-dichloro-2-propyl) phosphate on THP-1-derived macrophages

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ABSTRACT

Endocrine-disrupting chemicals (EDCs) are widespread environmental contaminants known to interfere with hormonal and immune functions. Among these, Bisphenol A (BPA), tris(1,3-dichloro-2-propyl) phosphate (TDCPP), and perfluorooctanoic acid (PFOA) are of significant concern due to their widespread human exposure and differing physicochemical profiles. Given that macrophages can represent early immune targets following EDC exposure, this study investigated the immunomodulatory effects of these structurally diverse compounds on THP-1-derived macrophage-like model. Cell viability was evaluated following 24 h exposure to a range of concentrations (1–100 μM), from which three doses (10, 50, 100 μM) were selected for functional assays. A follow-up viability assay at 48 h was performed to confirm the cellular impact of prolonged exposure. BPA and PFOA showed limited effects on cell viability, whereas TDCPP produced the most pronounced reduction. To assess immunomodulation, NFκB phosphorylation and cytokine production were evaluated under basal and LPS-stimulated conditions. BPA showed minimal effects across endpoints. TDCPP elicited modest NFκB activation, and under LPS stimulation, selectively attenuated specific cytokine responses. PFOA induced robust NFκB activation without corresponding changes in the measured cytokines, suggesting dissociation between upstream NFκB signaling and cytokine output in this model. Overall, these findings indicate compound-specific effects of BPA, TDCPP and PFOA in THP-1-derived macrophage-like cells and support the utility to this *in vitro* platform for comparative immunotoxicity screening and hypothesis generation. Future work integrating such data with quantitative *in vitro*-to-*in vivo* extrapolation (QIVIVIE/IVIVE) and more complex immune models may further improve human relevance.

Abbreviations: EDC, Endocrine Disrupting Chemicals; BPA, Bisphenol A; TDCPP, Tris(1,3-dichloro-2-propyl) phosphate; PFOA, Perfluorooctanoic acid; PFAS, Per- and polyfluoroalkyl substances; OPFR, Organophosphate flame retardant; EFSA, European Food Safety Authority; EU, European Union; TDI, Tolerable daily intake; TWI, Tolerable weekly intake; POP, Persistent organic pollutant(s); UTC, Unintentional trace contaminant(s); NHANES, National Health and Nutrition Examination Survey; CDC, Centers for Disease Control and Prevention; OECD, Organization for Economic Co-operation and Development; THP-1, Human monocytic leukemia cell line; PMA, Phorbol 12-myristate 13-acetate; LPS, Lipopolysaccharide; DMSO, Dimethyl sulfoxide; NFκB, Nuclear factor kappa B; MAPK, Mitogen-activated protein kinase; AP-1, Activator protein 1; STAT3, Signal transducer and activator of transcription 3; Sp1, Specificity protein 1; C/EBPβ, CCAAT/enhancer-binding protein beta; CREB, cAMP response element-binding protein; IκBα, Inhibitor of kappa B alpha; IKK, IκB kinase; TLR, Toll-like receptor; TLR4, Toll-like receptor 4; PKC, Protein Kinase C; PPARα, Peroxisome Proliferator-Activated Receptor alpha; PPARγ, Peroxisome Proliferator-Activated Receptor gamma; ER, Estrogen Receptor; ERα, Estrogen Receptor Alpha; ERK1/2, Extracellular signal-regulated kinase 1/2; GPER, G protein-coupled estrogen receptor 1; AhR, Aryl hydrocarbon receptor; RACK1, Receptor for activated C kinase 1; TNF-α, Tumor necrosis factor alpha; IL-6, Interleukin 6; IL-1β, Interleukin 1 beta; IL10, Interleukin 10; CCL2, C–C motif chemokine ligand 2; MCP-1, Monocyte chemoattractant protein-1; CXCL8, C-X-C motif chemokine ligand 8; IL8, Interleukin 8; ROS, Reactive oxygen species; LDH, Lactate dehydrogenase; ATCC, American Type Culture Collection; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; SDS-PAGE, Sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PVDF, Polyvinylidene difluoride; IVIVE, *In vitro*-to-*in vivo* extrapolation; QIVIVE, Quantitative *In vitro*-to-*in vivo* extrapolation; PBPK, Physiologically based pharmacokinetic modeling; NAM(s), New Approach Methodology/Methodologies.

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1. Introduction

Endocrine Disrupting Chemicals (EDCs) are a heterogeneous group of exogenous compounds, both natural and synthetic, that are widespread in the environment and capable of interfering with the hormonal regulation of physiological processes in living organisms, potentially leading to detrimental health outcomes (Ahn and Jeung, 2023). Their mechanisms of action include mimicking, blocking or altering the synthesis, transport, and degradation of endogenous hormones. Structurally and functionally diverse, EDCs encompass a wide range of substances with varying environmental persistence and metabolic behavior, complicating their toxicological evaluation (Diamanti-Kandarakis et al., 2009; Gore et al., 2015).

Among the many known EDCs, bisphenol A (BPA), perfluorooctanoic acid (PFOA), and tris(1,3-dichloro-2-propyl) phosphate (TDCPP) have been increasingly studied due to their widespread presence in the environment and growing evidence of their potential to disrupt immune and endocrine functions. BPA, commonly found in plasticizers, and extensively used in polycarbonate plastics and epoxy resins, acts as a xenoestrogen due to its structural resemblance to estradiol, allowing it to bind to estrogen receptors and disrupt endocrine signaling. Despite its rapid metabolism and excretion, widespread and continuous exposures have been associated with significant biological effects in humans (Cimmino et al., 2020). In contrast, PFOA – a member of the per- and polyfluoroalkyl substances (PFAS) group- used as a surfactant and polymer precursor, is highly persistent, both environmentally and biologically, and accumulates in water, soil, and living organisms (Liang et al., 2022). TDCPP is an organophosphate flame retardant (OPFR) commonly found in household products such as upholstered furniture and electronic equipment. Although it is metabolized relatively quickly, repeated and chronic exposure is common due to its presence in indoor dust and consumer goods (Cho et al., 2023; Van den Eede et al., 2011).

Exposure to these EDCs has been associated with adverse effects on multiple biological systems, including the endocrine, reproductive, and immune systems (Ahn and Jeung, 2023; Li et al., 2020; Liang et al., 2022; Tang et al., 2020). Recent evidence has emphasized the immunotoxic effect of EDCs across various model systems. For instance, BPA has been shown to impair innate and adaptive immune responses in zebrafish, with the transcription factor STAT3 implicated as a key mediator (Qian et al., 2023). In murine models, PFOA exposure results in decreased B cell activity and diminished antibody production following dermal administration (Shane et al., 2020). Likewise, *in vitro* studies using THP-1-derived macrophages have demonstrated that TDCPP increases inflammatory activity, including the upregulation of pro-inflammatory cytokines (Li et al., 2020). While these findings underscore the immunomodulatory capacity of EDCs, most available studies, including those using THP-1 cells, focus on general inflammatory endpoints, while the underlying molecular pathways have not been comprehensively characterized.

Human exposure occurs through various routes, such as ingestion of contaminated food or water, inhalation of indoor dust and air, and dermal absorption from consumer products (Cho et al., 2023; Ma et al., 2019; Post et al., 2012). Epidemiological data from biomonitoring studies, such as the U.S. National Health and Nutrition Examination Survey (NHANES), have consistently reported detectable EDCs like BPA and PFAS in urine and serum samples, indicating widespread human exposure across age groups and populations (CDC, 2024). These findings have led to regulatory actions, particularly in the European Union. For BPA, EFSA's most recent re-evaluation established a revised tolerable daily intake (TDI) of 0.2 ng/kg body weight/day (EFSA, 2023). Consistently, the EU has adopted a ban on BPA in food contact materials (Commission Regulation (EU) 2024/3190) (EU, 2024). For PFAS, EFSA established a group tolerable weekly intake (TWI) of 4.4 ng/kg body weight/week for the sum of PFOS, PFOA, PFNA and PFHxS, based on immune system effects as the critical endpoint (EFSA, 2020). In parallel, PFOA is regulated internationally and in the EU as a persistent organic pollutant (POP) under the Stockholm Convention and EU POP legislation (Regulation (EU) 2019/1021 (EU, 2019), including unintentional trace contaminant (UTC) limits of 0.025 mg/kg for PFOA and its salts and 1 mg/kg for PFOA-related compounds in substances, mixtures and articles (Commission Delegated Regulation (EU) 2020/784) (EU, 2020). Together, these regulatory measures underscore the high public health relevance of BPA and PFAS exposure and support the need for mechanistically informative experimental models to investigate their immunomodulatory potential.

Given their anatomical location and rapid responsiveness to environmental stimuli, macrophages are among the first immune cells to interact with ingested EDCs, making them a highly relevant target for immunotoxicology evaluation (Bai et al., 2025; Mowat and Bain, 2011; Wells et al., 2016). A key mechanism by which macrophages respond to environmental stimuli, including EDCs, is through the activation of pro-inflammatory signal pathways. Among these, the nuclear factor kappa B (NFκB) pathway is particularly relevant, as it regulates the expression of numerous cytokines involved in innate immune responses. When triggered by pathogens, chemical agents, or metabolic stress, this pathway initiates a signaling cascade, leading to the transcription of pro-inflammatory genes such as TNF-α, IL-6 and IL-1β (Biswas and Lewis, 2010; Mussbacher et al., 2023).

Recent expert reports emphasize the need for more mechanistically informative and standardized in-vitro systems, such as THP-1-derived macrophages, for assessing the immunotoxic effects of EDCs, especially given the limitations of conventional regulatory assays in capturing such complex responses (Demeneix and Slama, 2019). Consistently, the human leukemia monocytic cell line THP-1 is one of the most widely used *in vitro* models for evaluating immunotoxic responses (Li et al., 2020). When differentiated with phorbol 12-myristate 13-acetate (PMA), these cells acquire macrophage-like properties and exhibit cytokine production and activation profiles similar to those of primary macrophages, while offering advantages in reproducibility and standardization Almeida Roque et al., 2023; Li et al., 2020; Mohd Yasin et al., 2022). Although primary human macrophages provide physiological relevance, they are limited by donor variability, short lifespan, and technical challenges in culture. In contrast, THP-1-derived macrophages offer a practical alternative with consistent behavior across experiments (Chanput et al., 2014; Daigneault et al., 2010; Li et al., 2020).

Taking all this into account, this study primarily aims to explore the immunomodulatory effects of three representative environmental EDCs: BPA, TDCPP and PFOA using THP-1-derived macrophages. These compounds were selected to reflect distinct physicochemical and metabolic profiles, since we hypothesize that exposure to these structurally distinct EDCs elicits differential activation of inflammatory pathways in macrophages. BPA and PFOA were included as two of the most widely studied and regulated EDCs, and EFSA risk assessments have highlighted immune system effects as critical endpoints supporting stringent health-based guidance values and regulatory actions (EFSA, 2023, 2020, 2016). By contrast, TDCPP remains comparatively less uniformly regulated at the global level, although it has been subject to sector-specific regulatory scrutiny and restriction initiatives in some jurisdictions (ECHA, 2017).

From a mechanistic perspective, current evidence suggests that BPA can modulate macrophage inflammatory signaling through endocrine receptor pathways (e.g., ER/GPER) and downstream crosstalk with NFκB/MAPK signaling pathways, whereas PFOA is frequently linked to nuclear receptor-mediated effects, particularly involving PPARα-dependent immunomodulation (Buoso et al., 2021; Cai et al., 2017; Corsini et al., 2011; Gao et al., 2020; Pawlak et al., 2015). In contrast, TDCPP represents an emerging OPFR for which immunotoxic effects remain poorly characterized. OPFRs, including TDCPP, have been associated with oxidative stress and disruption of innate immune signaling pathways (e.g., TLR-related responses), which may contribute to altered inflammatory outcomes (Lin et al., 2023). Including compounds with contrasting regulatory status and mechanistic evidence

allows for a broader assessment of immune disruption potential across different chemical classes. A battery of assays was designed to assess changes in macrophage viability, NFκB activation and cytokine secretion, with the aim of providing insight into the immunotoxic potential of these environmentally relevant compounds.

By combining a standardized macrophage model with functional and signaling readouts, this study aims to contribute to a more nuanced understanding of EDC-induced immune modulation. The use of multiple compounds and exposure conditions helps capture a spectrum of potential effects, which may inform future *in vitro* testing strategies and support ongoing efforts to improve immune-related hazard identification.

2. Methods

2.1. Chemicals and stocks solutions

Bisphenol A (BPA), Perfluorooctanoic acid (PFOA), Tris(1,3-dichloro-2-propyl) phosphate (TDCPP), phorbol 12-myristate 13-acetate (PMA), and lipopolysaccharide (LPS) were purchased from Sigma-Aldrich (Darmstadt, Germany). Stock solutions (600 mM) were prepared in dimethyl sulfoxide (DMSO) and diluted to working concentrations in culture medium. Reagents for electrophoresis and immunoblotting were obtained from Bio-Rad (Basel, Switzerland). The following antibodies were used: rabbit monoclonal anti-p-NFκB p65 (Ser536) (93H1), and anti-NFκB p65 (D14E12) XP (R) from Cell Signaling Technology (Danvers, Massachusetts, USA); rabbit polyclonal anti-GAPDH (ab9485-100) from Abcam (Cambridge, UK).

2.2. Cell culture and chemical exposure

The human monocytic cell line THP-1 was obtained from the American Type Culture Collection (ATCC TIB-202; Manassas, VA, United States). Cells were cultured in RPMI 1640 medium supplemented with L-glutamine, HEPES, phenol red, 10% (v/v) Fetal Bovine Serum (FBS) and 5% (v/v) penicillin/streptomycin (all from Thermo Fisher Scientific, Eindhoven, Netherlands). Cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere. Differentiation into macrophage-like cells was induced by seeding cells at a density of $1x10^6$ cells/mL and treating with 25 nM of PMA for 72 h. This low-to-moderate PMA differentiation protocol is consistent with low-dose regimens reported to induce stable THP-1 macrophage-like differentiation while minimizing basal activation (Daigneault et al., 2010; Park et al., 2007). After differentiation, the PMA-containing medium was removed and replaced with fresh RPMI medium. Environmental chemicals were applied for 24 h or 48 h, unless otherwise specified (Loeffler et al., 2020). Morphological changes associated with PMA differentiation were documented by inverted microscopy, showing adherent macrophage-like THP-1 cells (Supplementary Fig. S1).

2.3. Cell viability assay

Cell viability was assessed using the MTT assay (Sigma-Aldrich; Darmstadt, Germany). Differentiated THP-1 cells were seeded in 24- or 48-well plates (Thermo Fisher Scientific; Eindhoven, Netherlands), and exposed to BPA, TDCPP or PFOA at concentrations of 1, 10, 25, 50 or 100 μM, or to 0.1% DMSO (control vehicle) for 24 or 48 h. MTT solution was added, and plates were incubated for 2 h at 37°C and 5% CO_2. The supernatant was then replaced with DMSO, and the absorbance was measured at 560 nm. Cell viability was expressed as a percentage relative to the vehicle-treated control. The 24 h viability assay was initially performed to determine dose-dependent cytotoxicity and to identify non-toxic concentrations. Based on these results, three concentrations (10, 50 and 100 μM), were selected as representative low, intermediate and high exposures, and used in subsequent experiments assessing NFκB activation and cytokine production. Although these concentrations exceed typical human exposure levels, they fall within the 100–1000-fold range often used *in vitro* to compensate for low bioavailability, lack of metabolism, and the need to observe measurable responses within short exposure periods (Moreau et al., 2022; Stielow et al., 2023). The full range of concentrations (10–100 μM) was applied in exploratory screening assays, while 50 μM was maintained as the highest concentration in functionally relevant experiments to minimize potential cytotoxic effects. To confirm the absence of delayed cytotoxicity, a second viability assay was performed at 48 h using only the selected concentrations. These two time points (24 h and 48 h) were chosen to capture both early and sustained cellular responses to EDC exposure, while maintaining experimental feasibility and minimizing prolonged culture-associated artifacts.

2.4. Immunoblot analysis

THP-1 cell activation was evaluated via Western Blot analysis of phospho-NFκB p65. Cells were exposed 24 h to EDCs at 1, 10 or 50 μM, LPS (1 μg/mL), or DMSO (0.1%). Cells were washed twice with phosphate-buffered saline (PBS) and lysed on ice using RIPA lysis buffer supplemented with protease and phosphatase inhibitors. Lysates were centrifuged at 12500 rpm for 12 min at 4°C, and concentration was determined using the Lowry protein assay (Bio-Rad; Basel, Switzerland). Samples (25 μg protein) were mixed with 4X Laemmli buffer containing 1% β-mercaptoetanol, denatured at 95°C for 5 min, resolved by SDS-PAGE (10% or 12% gels), and transferred onto low-fluorescence PVDF membranes (Bio-Rad; Basel, Switzerland). Membranes were blocked for 1 h in 5% powder milk, 1X TBS (0.1% Tween-20) and incubated with primary antibodies diluted in blocking buffer (p-NFκB, 1:1500; t-NFκB, 1:1500; GAPDH, 1:2500). HRP-conjugated secondary antibodies were used (1:1750), and signal detection was performed with ECL Select reagents (Amersham, Little Chalfont, UK). Band intensities were quantified using Image Lab v5.1 software (Bio-Rad), and phospho-NFκB levels were normalized to GAPDH.

2.5. Cytokine quantification

To evaluate cytokine production, THP-1 cells were exposed to EDCs (10, 50, 100 μM), LPS (1 μg/mL), or 0.1% DMSO for 24 h and 48 h. Supernatants were collected and stored at −80°C. All wells were seeded in 24-well plates with equal numbers of differentiated THP-1 cells (10^6 cells/mL) and maintained under identical volume (0.8 mL) and treatment conditions; therefore, extracellular cytokine levels are reported as pg/mL of supernatant without further normalization. For intracellular cytokine measurement, cells were harvested after 24 h, washed twice in cold PBS, and lysed using ProcartaPlex™ Cell Lysis Buffer (EPX-99999-000; Thermo Fisher Scientific, Eindhoven, Netherlands) on ice for 5 min. Lysates were centrifuged at 14000 x g for 10 min at 4°C, and protein concentration was determined by the Lowry method. Samples were standardized to 8 mg/mL of total protein prior to analysis and stored at −80°C until processing. Cytokine levels (IL1β, IL6, IL10, TNFα) were quantified using a human-specific ProcartaPlex multiplex immunoassay (Thermo Fisher Scientific, Eindhoven, Netherlands) based on a magnetic bead format. These four cytokines were selected as representative markers of macrophage immune responses, encompassing key pro-inflammatory (IL1β, IL6, TNFα) and anti-inflammatory (IL10) mediators commonly evaluated in THP-1 cell models (Buoso et al., 2021; Corsini et al., 2011; Couleau et al., 2015). Measurements were conducted using a Luminex MAGPIX® System (Thermo Fisher Scientific, Eindhoven, Netherlands), following the manufacturer's protocol. Cytokine concentrations were determined by interpolation from standard curves generated for each analyte.

2.6. Statistical analysis

Data analysis was performed using IBM SPSS Statistics, version 22.0

(IBM Corp., Armonk, NY, USA). Data normality was assessed using the Shapiro-Wilk test and homogeneity of variances using Levene's test. Data are presented as mean ± standard deviation (SD) of at least n = 3 independent biological replicates. One-way analysis of variance (ANOVA) was used to evaluate group differences. Where applicable, post hoc comparisons were conducted using Tukey's HSD or Dunnett's multiple comparison tests. Statistical significance was defined as *p < 0.05, **p < 0.01 and ***p < 0.001.

3. Results

3.1. Cell viability

To evaluate the cytotoxic effects of environmental chemicals, we assessed the viability of differentiated THP-1 cells after 24 h and 48 h of exposure. Viability responses were analyzed across a range of concentrations for BPA, TDCPP and PFOA, revealing compound-specific and time-dependent patterns.

At 24 h (Fig. 1, Top), all tested concentrations of BPA, TDCPP, and PFOA maintained cell viability above the 70% threshold, indicating the absence of acute cytotoxic effects. BPA induced a general reduction in viability. Statistically significant decreases were observed at 1 μM (14.8% ± 7.6%; p < 0.0001), 10 μM (15.1% ± 5.8%; p < 0.0001) and 100 μM (16.5% ± 8.5%; p < 0.0001). No significant effects were detected at 25 μM and 50 μM. TDCPP exhibited the strongest cytotoxic response in a clear concentration-dependent manner, decreasing cell viability by 9.0% ± 4.0% at 10 μM, 14.3% ± 3.8% at 25 μM, 14.6% ± 3.0% 50 μM, and 26.3% ± 2.5% at 100 μM (all p < 0.0001). PFOA exposure produced a biphasic response. Cell viability was slightly reduced at 1 μM (6.3% ± 5.6%; p = 0.019) and 10 μM (6.8% ± 5.6%; p = 0.0095), followed by a significant increase at 100 μM (7.8% ± 6.7%; p = 0.0025).

At 48 h (Fig. 1, Bottom), BPA no longer reduced cell viability. Instead, a significant increase of 19.3% ± 6.0% was observed at 50 μM (p = 0.0004). TDCPP produced a more pronounced cytotoxic effect compared to 24 h, reducing viability by 11.3% ± 4.3% at 10 μM (p = 0.0046), 17.6% ± 3.2% at 50 μM (p = 0.0002) and 38.7% ± 3.7% at 100 μM (p < 0.0001). Although this highest concentration fell below the 70% threshold, it was included in the cytokine assay to assess potential immunomodulatory effects at higher exposure levels. Since no major alterations were observed in the inflammatory profile, it was retained in the analysis but interpreted with caution. PFOA maintained a viability-enhancing effect at 100 μM, with an increase of 11.1% ± 3.9% (p = 0.0015).

In summary, these results indicate that TDCPP consistently exerted the most substantial cytotoxic effect in a dose- and time-dependent manner. BPA decreased viability at earlier time points but showed a stimulatory effect after 48 h. PFOA displayed a mild biphasic effect, with low-concentration inhibition followed by enhanced viability at higher doses. These findings highlight compound-specific cytotoxic profiles.

3.2. NFκB expression

To investigate the potential activation of inflammatory signaling pathways by environmental contaminants, we assessed NFκB p65 phosphorylation levels in differentiated THP-1 cells following exposure to BPA, TDCPP and PFOA (Fig. 2). The phosphorylation of NFκB serves as an indicator of canonical NFκB pathway activation, associated with pro-inflammatory responses (Biswas and Lewis, 2010; Mussbacher et al., 2023). Normalization of phosphorylated NFκB levels to GAPDH was validated by confirming that total NFκB expression remained stable across treatments. Moreover, calculation of the phospho-NFκB/total-NFκB ratio supported that the observed differences were primarily due to increase phosphorylation rather than changes in total protein levels (Fig. 2C; Supplementary Fig. S2).

The results showed that BPA exposure did not induce a statistically significant change in NFκB phosphorylation levels compared to the control, although a slight upward trend was observed. TDCPP exposure led to increased phosphorylation across all tested concentrations, but without reaching statistical significance. PFOA was the only compound that elicited a statistically significant increase in NFκB phosphorylation. At 1 μM, a 309.8% ± 94.8% increase was observed (p = 0.003), at 10 μM, 580.4% ± 184.8% (p = 0.001); and at 50 μM, 486.3% ± 40.1% (p = 0.001). As expected, the positive control LPS induced a significant

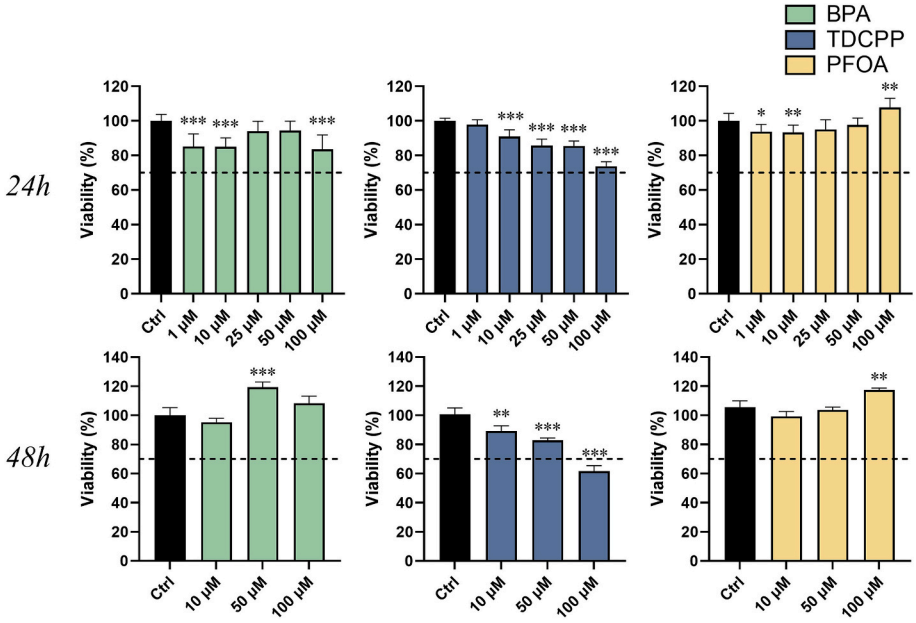


Fig. 1. MTT metabolism after 24 h (Top) and 48 h (Bottom) of exposure to BPA, TDCPP or PFOA. Cell viability is expressed as a percentage relative to the control (Ctrl). Cells were treated with increasing concentrations (1–100 μM) of each compound. Viability is expressed as a percentage relative to vehicle control (Ctrl). The threshold for cytotoxicity is indicated by a dotted line at 70% viability (Burkhardt et al., 2022). Data represents the mean ± standard deviation (SD) of n = 6 independent biological replicates, each tested in technical triplicate. Statistical significance was determined using Dunnett's multiple comparison test versus the untreated control group: *p < 0.05, **p < 0.01 and ***p < 0.001.

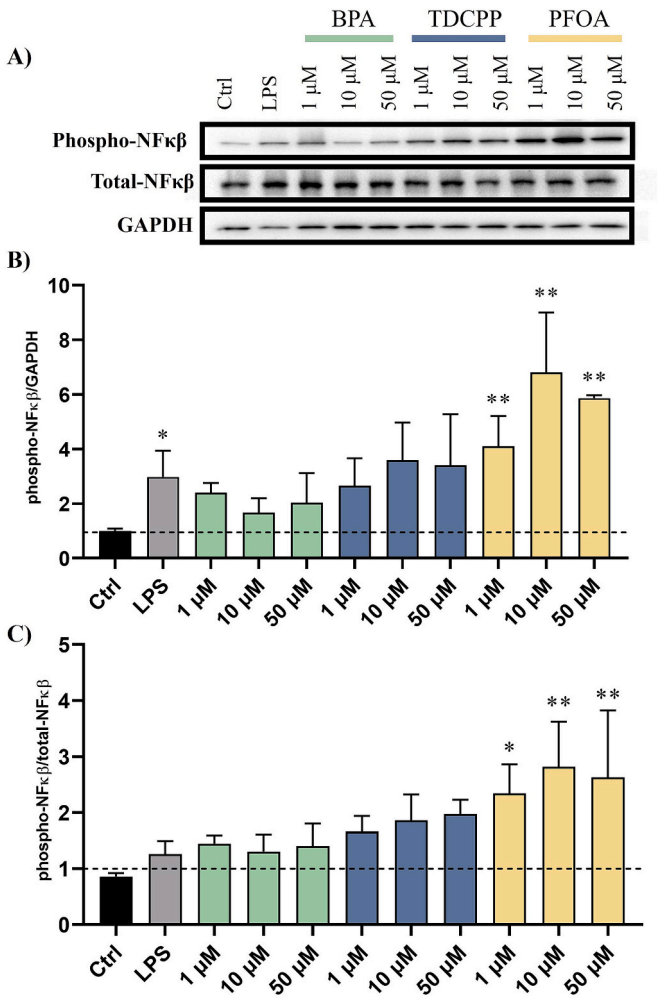


Fig. 2. Western Blot analysis of NFκB activation in differentiated THP-1 cells after 24 h exposure to 1 μg/ml LPS or 1–50 μM of BPA, TDCPP and PFOA. (A) Representative Western blots showing bands corresponding to phospho-NFκB p65, total-NFκB and GAPDH (used as loading control) in THP-1 cells treated with BPA, TDCPP or PFOA (1–50 μM) and LPS (1 μg/mL) for 24 h. (B) Densitometric quantification of phospho-NFκB levels normalized to GAPDH and expressed relative to the control. (C) Ratio of phospho-NFκB to total NFκB. In panels B and C, dashed horizontal lines are included for reference: in (B), the line indicates the control level; in (C), it marks the theoretical 1:1 ratio between phosphorylated and total NFκB. Data represents the mean ± standard deviation (SD) of at least n = 3 independent biological replicates (single measurement per condition). Statistical significance was determined using Dunnett's multiple comparison test versus the untreated control group: *p < 0.05, **p < 0.01 and ***p < 0.001.

197.8% ± 80.7% increase (p = 0.043).

In conclusion, PFOA induced a marked and statistically significant activation of NFκB signaling in THP-1 cells. TDCPP exhibited a non-significant increase in phosphorylation levels, while BPA had no measurable effect. These findings underscore the compound-specific potential of environmental chemicals to modulate inflammatory signaling.

3.3. Cytokine production

To investigate the immunomodulatory effects of BPA, TDCPP and PFOA, we evaluated both extracellular and intracellular cytokine levels in THP-1 cells following 24 h and their secretion after 48 h of exposure. In addition, we assessed the impact of co-exposure with LPS to determine whether these compounds modify the LPS-induced pro-inflammatory response. Four cytokines were analyzed: IL1β, IL6, IL10, and TNFα.

3.3.1. Extracellular cytokine levels

Overall, exposure to BPA, TDCPP and PFOA induced limited changes in basal cytokine secretion under these experimental conditions, with only a small number of statistically significant alterations across endpoints, concentrations and time points. BPA exposure did not result in statistically significant alterations in extracellular levels of the four cytokines at either 24 h or 48 h (Fig. 3 A-H). In contrast, TDCPP induced significantly increased TNFα secretion at 100 μM after 24 h, (106.9% ± 53.6%; p = 0.03; Fig. 3E), and IL6 levels at 50 μM (30.7% ± 14.4%; p = 0.03; Fig. 3C). After 48 h, a significant elevation in IL6 was observed at 100 μM (p = 0.007; Fig. 3D). PFOA exposure did not produce statistically significant changes in extracellular cytokine levels at either time point.

3.3.2. Intracellular cytokine levels

BPA was the only compound that significantly altered intracellular cytokine levels, leading to a 77.4% ± 29.2% increase in TNFα at 10 μM after 24 h (p = 0.005; Fig. 4C). TDCPP and PFOA did not significantly affect intracellular cytokine levels under any tested condition (Fig. 4A-D).

3.3.3. Cytokine production under LPS co-exposure

LPS stimulation induced a marked increase in extracellular cytokine secretion compared to untreated controls (Fig. 5A–C). When cells were co-exposed to LPS and BPA, a significant reduction in IL10 secretion was observed (46.2% ± 13.9%; $p < 0.003$; Fig. 5C, green). No significant effects were detected for TNFα or IL1β. TDCPP co-exposure significantly suppressed the LPS-induced production of IL1β (40.4% ± 14.5%; $p = 0.007$; Fig. 5A, blue), and IL10 (74.2% ± 6.4%; $p < 0.0001$; Fig. 5C, blue). TNFα levels remained unaffected (Fig. 5B). PFOA did not significantly alter LPS-induced cytokine secretion.

In summary, basal cytokine secretion was only modestly affected by EDC exposure. BPA showed limited effect on cytokine production, with a modest impact in intracellular TNFα and a reduction in IL10 under LPS stimulation. TDCPP displayed the clearest immunomodulatory pattern, with modest increases in selected basal cytokine secretion but a pronounced suppression of LPS-induced responses. PFOA had no clear immunomodulatory effect in either basal or LPS-stimulated conditions, despite strong NFκB activation, suggesting additional regulatory mechanisms downstream of NFκB signaling.

4. Discussion

Macrophages play a fundamental role in both the first and second lines of immune defense. They are key components of the innate immune system but are also closely involved in the activation of adaptive immunity (Charles A. Janeway et al., 2001). In this study, we evaluated the effects of three of the most relevant endocrine-disrupting compounds (EDCs) to which humans are commonly exposed. Our results indicate compound-specific effects across the endpoints assessed (cell viability, NFκB activation, and cytokine production), with TDCPP showing the most pronounced modulation under the conditions tested, followed by BPA and, to a lesser extent, PFOA. These endpoints were selected to balance biological relevance with feasibility for standardized comparative screening across chemically distinct EDCs.

We observed that exposure to different EDCs differentially affects macrophage viability. While BPA and PFOA had limited impact on cell viability, TDCPP induced a significant concentration-dependent reduction. These findings are consistent with previous studies (Amstutz et al., 2024; Couleau et al., 2015; Gostner et al., 2015; Li et al., 2020; Liu et al., 2014). However, we also identified a modulatory factor influencing macrophage sensitivity to EDC-induced cytotoxicity. Differentiation of monocytes into macrophages was achieved via PMA treatment of THP-1

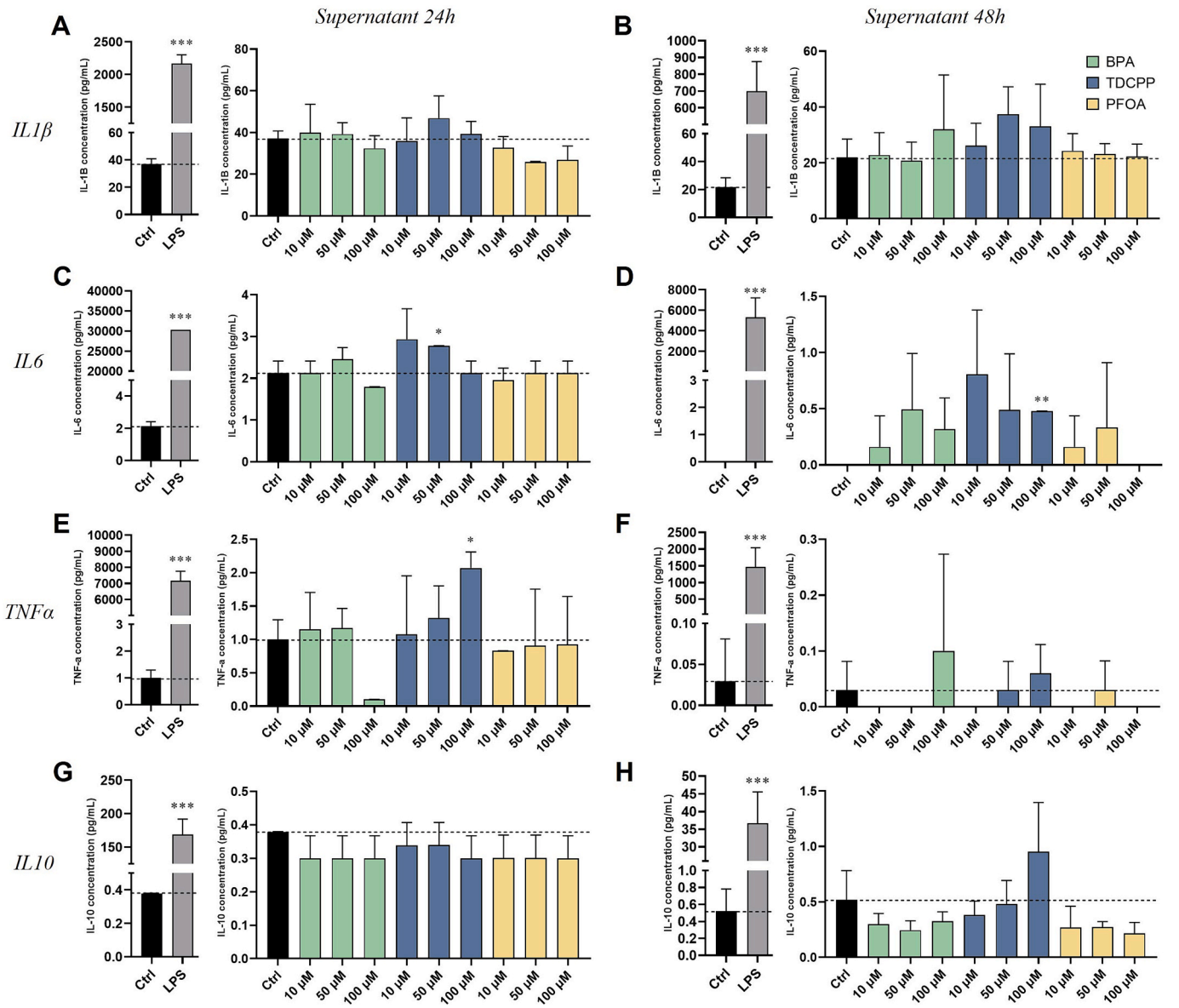


Fig. 3. Effect of chemical exposure on extracellular cytokine production in differentiated THP-1 cells at 24 and 48 h, quantified using the Luminex MAGPIX system. Cells were treated with 1 µg/mL LPS or increasing concentrations (10–100 µM) of BPA, TDCPP or PFOA. Panels A, C, E and G show cytokine levels in cell culture supernatants at 24 h; panels B, D, F and H correspond to 48 h. Cytokine concentrations (IL1β, IL6, TNFα, IL10) are expressed as pg/mL of supernatant. Dashed horizontal lines indicate the mean cytokine level in the vehicle control group. Data represents the mean ± standard deviation (SD) of at least n = 3 independent biological replicates analyzed in duplicates. Statistical significance was determined using Dunnett's multiple comparison test versus the untreated control group: *p < 0.05, **p < 0.01 and ***p < 0.001.

cells for either 48 or 72 h. PMA mimics diacylglycerol and activates the protein kinase C (PKC) pathway, leading to the increased expression of several macrophage surface receptors (Lin et al., 2011). The variability in PMA concentration and exposure time employed across different studies likely contributes to discrepancies in cell viability outcomes. Specifically, studies using higher PMA concentrations tend to report increased macrophage resistance to toxic effects (Couleau et al., 2015). This is particularly evident in studies where undifferentiated THP-1 monocytes are used, which show higher cytotoxicity at lower EDC concentrations (Gostner et al., 2015). Similarly, studies employing pre-differentiated macrophage cell lines report stronger reductions in viability at lower concentrations than those observed in PMA-differentiated THP-1 cells (Huang et al., 2018; 2019). Thus, PMA-induced differentiation may exert a protective effect against EDC exposure, possibly through PKC-mediated activation of cytoprotective and antioxidant pathways.

In our study, exposure to various concentrations of EDCs resulted in increased activation of the transcription factor NFκB. The activation of NFκB is essential for the synthesis of pro-inflammatory cytokines and mediates feedback mechanisms that regulate inflammation, including the induction of IL-10 (Saraiva et al., 2020). PFOA elicited the strongest NFκB activation, whereas TDCPP showed a consistent but non-significant increase across concentrations, and BPA did not induce measurable changes. Despite the activation of this transcription factor, we did not observe a corresponding increase in cytokine production. This suggests that, in this model, EDC-induced NFκB phosphorylation is not necessarily sufficient to elicit a downstream cytokine secretion profile, highlighting additional regulatory checkpoints downstream of NFκB. While TDCPP induced a slight rise in the secretion of TNF-α, BPA and PFOA had negligible effects. These findings align with Li et al. (2020), who reported that PMA-differentiated THP-1 cells exposed to 1–50 µM TDCPP for 24 h showed increased TNF-α secretion (Li et al.,

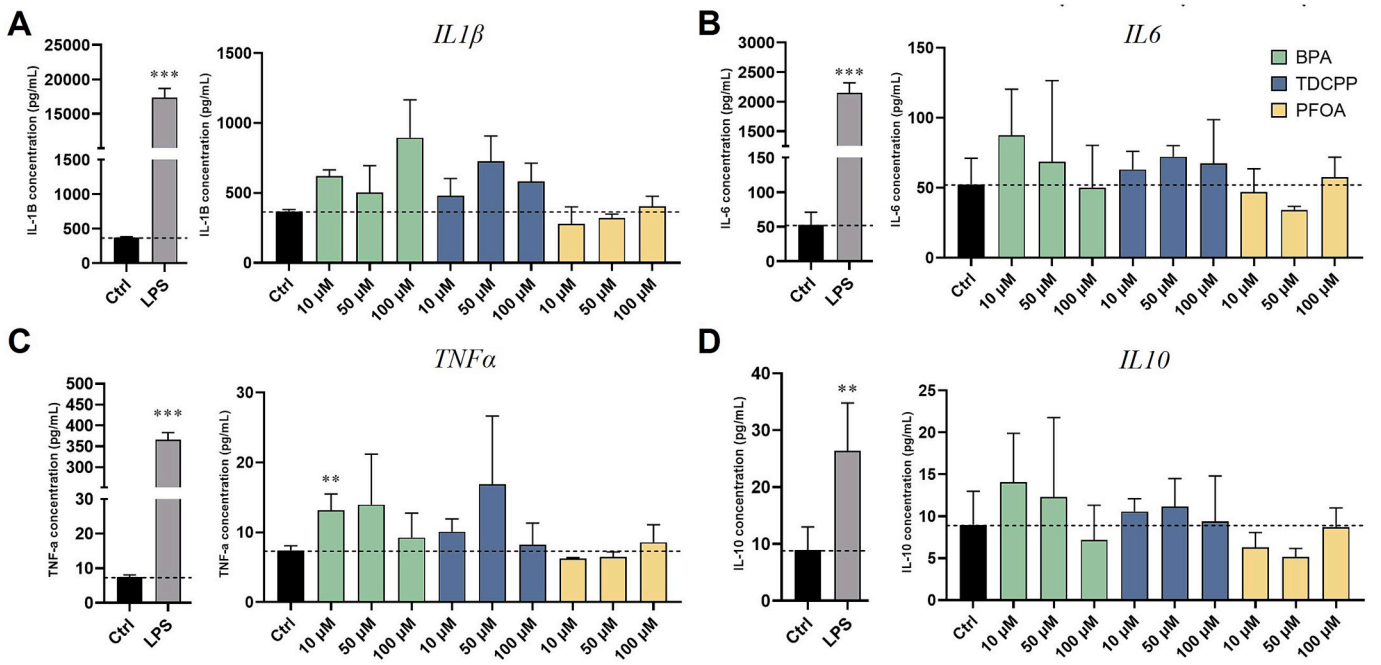


Fig. 4. Effect of chemical exposure on intracellular cytokine levels in differentiated THP-1 cells after 24 h, measured using the Luminex MAGPIX system. Cells were treated with 1 µg/mL LPS or increasing concentrations (10–100 µM) of BPA, TDCPP or PFOA. Panels show cytokine concentrations in cell lysates: (A) IL1β, (B) IL6, (C) TNFα and (D) IL10. Results are expressed as pg/mL of lysate. Dashed horizontal lines indicate the mean cytokine level in the vehicle control group. Data represents the mean ± standard deviation (SD) of at least n = 3 independent biological replicates analyzed in duplicates. Statistical significance was determined using Dunnett's multiple comparison test versus the untreated control group: *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

2020). This effect may be caused by enhanced oxidative stress or the release of damage-associated molecular patterns, which can indirectly stimulate TLR and amplify inflammatory signaling (Zhou et al., 2011). However, TDCPP-induced cytokine levels remained substantially lower than those observed in response to LPS.

The lack of correlation observed between NFκB upregulation and cytokine secretion suggests that additional regulatory layers may act downstream of NFκB phosphorylation, limiting cytokine output under basal conditions. In our study, neither extracellular nor intracellular cytokine levels showed consistent increases after 24 h or 48 h exposure, indicating that NFκB activation alone was not sufficient to trigger a measurable cytokine secretion profile within the tested time window. This contrasts with previous findings reporting enhanced pro-inflammatory cytokine secretion in THP-1 cells after BPA exposure (Liu et al., 2014). A key methodological difference between their protocol and ours is the PMA concentration used for differentiation. Compared to the 320 nM PMA used in their protocol, we used 25 nM. PMA activates NFκB by stimulating the PKC pathway, which phosphorylates and activates the IKK complex, leading to phosphorylation and degradation of IκBα, an NFκB inhibitor. This allows NFκB to translocate to the nucleus and initiate gene transcription (Jones et al., 2007). A higher PMA concentration could result in a higher basal activation state in THP-1 cells, rendering them more responsive to secondary stimuli in terms of cytokine release. Moreover, although EDCs increased NFκB levels, they may also affect other transcription factors such as AP-1. Jang et al. (2020) demonstrated that BPA decreased the binding activity of both AP-1 and NFκB in human bladder cells (Jang et al., 2020).

Although EDC exposure alone had a minimal effect on cytokine secretion in THP-1 cells, co-exposure with LPS resulted in reduced LPS-induced cytokine production for both BPA and TDCPP. These results suggest that exposure to these compounds may interfere with proper LPS-induced signaling, thereby compromising macrophage immune responses to external agents. Several studies have shown that BPA modulates immune responses through receptor signaling and exerts immunomodulatory effects. In undifferentiated THP-1 cells, BPA was shown to significantly downregulate RACK1, a scaffolding and modulatory protein involved in PKC signaling, via a GPER-dependent mechanism. This downregulation was associated with reduced NFκB activation and decreased production of pro-inflammatory cytokines such as TNF-α, suggesting an immunosuppressive profile (Buoso et al., 2021). Similarly, in differentiated THP-1 cells, BPA has been shown to activate PPARγ, a receptor with well-established anti-inflammatory functions in macrophages, likely contributing to the suppression of NFκB signaling (Bouhlel et al., 2007; Cai et al., 2017; Gao et al., 2020; Jiang et al., 1998). Some studies have reported contrasting findings, likely due to differences in experimental design. For example, Couleau et al. (2015) observed an increase in TNFα and IL1β levels following sequential exposure of cells to BPA (10 µM for 24 h) and LPS (10 ng/mL for 24 h). This pro-inflammatory effect was associated with ERα downregulation and reduced ERK1/2 phosphorylation, which may contribute to enhanced NFκB activation (Couleau et al., 2015; Cvoro et al., 2008; Kalaitzidis and Gilmore, 2005; Vermeulen et al., 2002). These seemingly mild or context-dependent effects may stem from the biphasic action of BPA on ER signaling. BPA can act as either an agonist or antagonist of ERα, depending on dose and context, potentially promoting or suppressing cytokine response (Acconcia et al., 2015; Bruno et al., 2019; Su et al., 2025). Additionally, BPA has been reported to modulate oxidative stress pathways in immune cells, where low to moderate ROS levels may activate NFκB, while higher oxidative stress could suppress cytokine production (Gao et al., 2021; Nowak et al., 2021; Wang et al., 2021). These factors may contribute to the variability observed across studies. In the case of PFOA, distinct mechanisms have been proposed, particularly involving PPARα-mediated immunomodulation. Corsini et al. (2011) reported that PFOA reduced pro-inflammatory cytokine production, such as TNF-α, and inhibited NFκB activation in LPS-stimulated, PMA-differentiated THP-1 cells via PPARα (Corsini et al., 2011). Other authors have reported comparable findings (DeWitt et al., 2009; Schlezinger et al., 2020). In contrast, in our model, co-exposure to PFOA and LPS did not significantly affect cytokine production, although a slight increase in IL-1β was noted. These

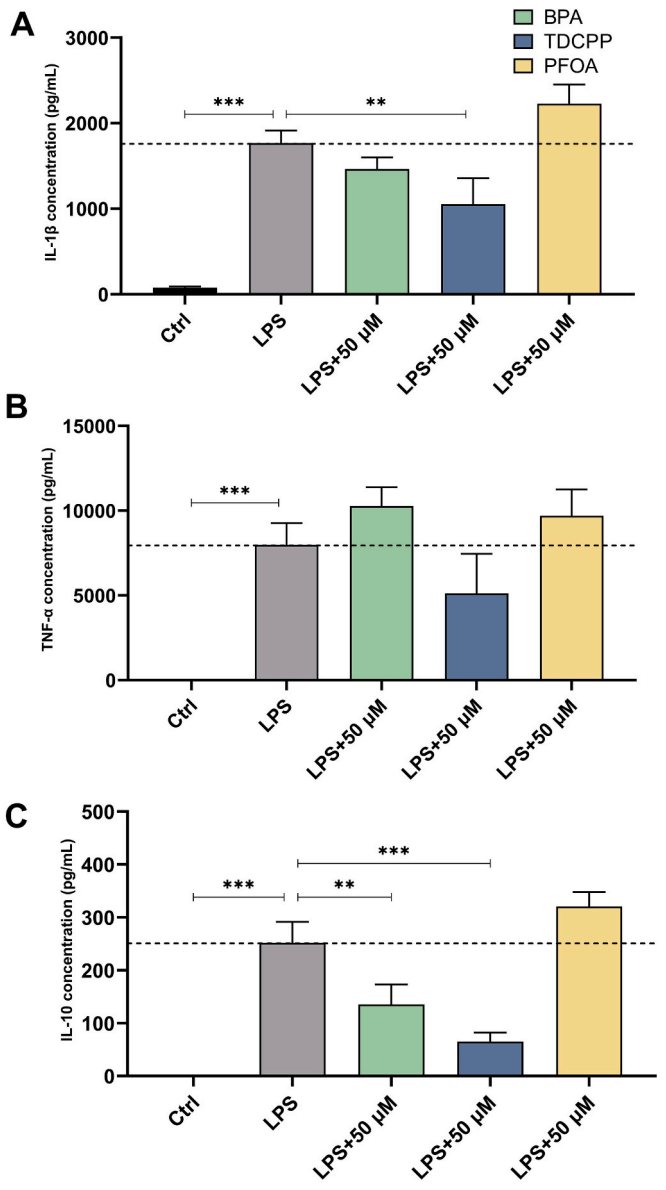


Fig. 5. Effect of co-exposure with LPS and EDCs on extracellular cytokine production in differentiated THP-1 cells after 24 h, measured using the Luminex MAGPIX system. Cells were treated with 1 μg/mL LPS alone or in combination with 50 μM BPA, TDCPP or PFOA. Cytokine concentration in cell supernatants is shown for: (A) IL1β, (B) TNFα and (C) IL10 and are expressed as pg/mL. Data represents the mean ± standard deviation (SD) of at least n = 3 independent biological replicates analyzed in duplicates. Dashed horizontal lines indicate the mean cytokine level in the LPS group. Statistical significance was determined using Tukey's HSD multiple comparison test. For clarity, only comparisons versus the LPS-only group are indicated in the figure: *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

discrepancies could stem from differences in cell differentiation status, exposure protocols, and inflammatory timing. It is possible that, in our system, PPARα-mediated immunosuppression was insufficient to counteract the strong pro-inflammatory stimulus induced by LPS. Moreover, the slight increase observed in IL-1β could be indicative of additional, PPARα-independent mechanisms, as suggested in previous reports (DeWitt et al., 2009). As previously noted, PFOA induced strong NFκB activation in our model without eliciting a concomitant increase in cytokine secretion. This dissociation could stem from several layers of regulation acting downstream of NFκB phosphorylation. For instance, PPARα is known to interfere with NFκB-driven transcription through transrepression mechanisms, effectively limiting cytokine gene expression despite upstream signaling (Bougarne et al., 2009; Pawlak et al., 2015). These mechanisms may help explain why increased NFκB phosphorylation was not translated into elevated extracellular cytokine levels in our system.

On the other hand, there is limited information available regarding the disruption mechanisms induced by TDCPP. Under basal conditions, TDCPP induced modest pro-inflammatory changes (including a significant increase in IL6 at 50 μM and a clear induction of TNFα at 100 μM) together with a non-significant increase in NFκB activation. However, co-exposure with LPS led to a marked reduction of selected cytokines, particularly IL1β and IL10. Notably, TDCPP also affected cellular metabolic activity at this concentration, suggesting that cellular fitness may have partially contributed to the attenuated cytokine output (Foster et al., 2007; Medvedev et al., 2007; Zerdan et al., 2021). In fact, organophosphate flame retardants such as TDCPP and triphenyl phosphate (TPHP) have been reported to disrupt upstream components of the TLR4 signalling cascade and impair mitochondrial energy metabolism, both of which are essential for effective cytokine production (Le et al., 2021; Lin et al., 2023). These additional mechanisms may contribute to the attenuated inflammatory responses observed under co-exposure scenarios. Notably, the suppression of IL-10 by BPA and TDCPP under LPS co-exposure conditions may further suggest disruption of regulatory nodes required for sustained IL10 transcription. In macrophages, IL10 induction requires coordinated activity of transcription factors such as STAT3, Sp1, C/EBPβ, or CREB, since NFκB activation alone is not sufficient to drive optimal IL10 expression (Benkhart et al., 2000; Brightbill et al., 2000; Ernst et al., 2019).

Taken together, the distinct response patterns observed for BPA, TDCPP and PFOA may reflect compound-specific engagement of endocrine-related pathways and their crosstalk with immune signalling networks. The strong NFκB activation induced by PFOA in the absence of a measurable cytokine response suggests the presence of regulatory checkpoints downstream of NFκB phosphorylation, potentially including PPAR-dependent transrepression mechanisms described for PFAS (Bougarne et al., 2009; Corsini et al., 2011; Pawlak et al., 2015). The non-monotonic viability profile observed for BPA is consistent with the context- and dose-dependent behaviour of endocrine-active signalling (including estrogen-related pathways) and its interaction with inflammatory pathways (Acconcia et al., 2015; Bruno et al., 2019; Su et al., 2025). Finally, TDCPP co-exposure phenotype (attenuated LPS-induced cytokine output) highlights potential interference with TLR4-related signalling and/or altered cellular state under co-exposure conditions (Le et al., 2021; Lin et al., 2023). Although these mechanistic considerations are supported by prior literature, they should be viewed as hypothesis-generating and warrant targeted validation in future studies.

Despite these findings, several limitations of the present study should be acknowledged. First, cell viability was assessed solely using the MTT assay, which measures mitochondrial metabolic activity rather than absolute cell number. While this is a commonly used proxy for viability, it may not distinguish between reduced proliferation and early stages of cell death. Complementary assays such as LDH release or Annexin V staining could provide more precise insights in future studies. Importantly, interpretation of TDCPP co-exposure effects at 50 μM should be made cautiously. TDCPP at this concentration induced a modest but statistically significant reduction in MTT metabolic activity (14.6% ± 3.0%). Because metabolic activity/viability was not directly assessed under the combined TDCPP (50 μM) + LPS condition, a partial contribution of reduced metabolic activity to the attenuated cytokine output under co-exposure cannot be excluded. Future studies should directly assess viability under co-exposure conditions using complementary assays (e.g., LDH release or Annexin V/PI staining) and evaluate immunomodulatory responses at lower TDCPP concentrations under minimal impact on cell metabolism. Second, macrophage differentiation was assessed based on morphological changes and adherence, but it was not

further validated using macrophage-associated markers (e.g., CD14, CD16/CD32) or additional functional assays (e.g., phagocytosis/chemotaxis). Third, although NFκB signaling is a well-established pathway in immune activation, other molecular targets potentially involved in EDC-induced immunomodulation – such as estrogen receptors (ERs), aryl hydrocarbon receptor (AhR), peroxisome proliferator-activated receptors (PPARs), and Toll-like receptors (TLRs) – were not assessed and may contribute to the observed effects. Moreover, the cytokine panel was limited to four markers; future studies could expand immune mediator coverage to include chemokines (e.g., CCL2/MCP-1, CXCL8/IL8) and assess chemokine-driven functions using co-cultures and/or migration/chemotaxis assays. Fourth, while the concentrations of EDCs used in this study (up to 100 μM) are higher than typical human plasma levels – generally in the nanomolar range – such doses are often required in *in vitro* systems to elicit detectable effects within a limited time window. This discrepancy stems from multiple factors, including reduced compound uptake, lack of metabolism, and the absence of systemic interactions in simplified monoculture models (Bansal et al., 2017; Kuo et al., 2012; Schjenken et al., 2021).

Additionally, plasma biomonitoring concentrations may not fully represent internal exposure under occupational scenarios or local tissue microenvironments (Bousoumah et al., 2021). In occupational settings, repeated higher-intensity exposures may lead to an increased cumulative internal dose (body burden), whereas short-lived compounds may still generate recurrent peak exposures that are not well captured by single-timepoint plasma measurements (National Research Council, 2006; OECD, 2022). Localized concentrations in exposure-relevant target tissues or microenvironments can differ substantially from circulating plasma levels (Bousoumah et al., 2021). Therefore, although the present doses exceed typical systemic biomonitoring levels, they were selected for mechanistic *in vitro* screening and should be interpreted cautiously when extrapolating to human exposure scenarios. Importantly, quantitative *in vitro*-to-*in vivo* extrapolation (QIVIVE/IVIVE), including reverse dosimetry supported by toxicokinetic or PBPK modeling, could help translate *in vitro* effect concentrations into external exposure scenarios that better reflect occupational settings (Chang et al., 2022; Louisse et al., 2016). In addition, longer-term or repeated-dose *in vitro* exposure designs would be valuable to better approximate chronic occupational exposures and potential bioaccumulation dynamics in future studies.

Furthermore, this study evaluated the effects of each EDC individually; future work should address combined exposure scenarios (binary and multi-compound mixtures), and assess potential additivity, synergy or antagonism using established mixture-effect approaches (e.g., concentration addition/independent action). Finally, although THP-1-derived macrophages provide a useful and standardized *in vitro* platform for mechanistic screening, monoculture conditions cannot fully recapitulate the complexity of immune regulation *in vivo*. Accordingly, further validation in more physiologically relevant models and/or *in vivo* studies would strengthen extrapolation to real-world immune outcomes. In this context, integrating standardized *in vitro* immune models within new approach methodologies (NAMs), together with *in silico* toxicokinetic modeling and QIVIVE/IVIVE frameworks, represents a promising strategy to support immune-related hazard identification and reduce uncertainty in human relevance assessment. Such integrated approaches can help prioritize chemicals and guide targeted follow-up studies in more complex experimental systems.

In summary, our findings demonstrate that EDC exposure has a compound-specific effect on macrophage function, with TDCPP showing the strongest impact. This compound reduced cell viability and induced NFκB activation without consistent cytokine release. BPA and PFOA triggered milder NFκB responses and had limited effects on cytokine secretion. Notably, co-stimulation with LPS and either TDCPP or BPA led to reduced cytokine release, suggesting that these compounds may impair macrophage inflammatory responses under stimulatory conditions.

5. Conclusion

This study highlights the compound-specific immunomodulatory potential of three environmentally relevant endocrine-disrupting chemicals −BPA, TDCPP, and PFOA- in THP-1-derived macrophages. Overall, cytokine secretion was only modestly affected under basal conditions, whereas TDCPP and BPA selectively modulated cytokine production under inflammatory stimulation. TDCPP emerged as the most bioactive compound, significantly reducing cell viability and being associated with a marked suppression of LPS-induced IL1β and IL10 secretion under co-exposure conditions. BPA and PFOA produced milder effects, with limited impact on cytokine secretion despite increased NFκB activation. Collectively, these findings underscore the heterogeneous nature of EDC immunotoxicity and support the use of THP-1-derived macrophages as a useful *in vitro* platform for comparative immunotoxicity screening and mechanistic hypothesis generation.

CRediT authorship contribution statement

Oscar Sabuz: Writing – original draft, Visualization, Methodology, Investigation, Data curation, Conceptualization. **Jordi Blanco:** Writing – original draft, Validation, Software, Methodology, Investigation, Conceptualization. **Deepika Deepika:** Writing – review & editing, Conceptualization. **Marta Schuhmacher:** Writing – review & editing. **Vikas Kumar:** Writing – review & editing, Visualization, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2026.110321.

Data availability

Data will be made available on request.

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