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Modulation of cytokine expression in human macrophages by endocrine-disrupting chemical Bisphenol-A

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ABSTRACT

Exposure to environmental endocrine-disrupting chemical Bisphenol-A (BPA) is often associated with dysregulated immune homeostasis, but the mechanisms remain unclear. In the present study, the effects of BPA on the cytokines responses of human macrophages were investigated. Treatment with BPA increased pro-inflammation cytokines tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) production, but decreased anti-inflammation cytokines interleukin-10 (IL-10) and transforming growth factor-β (TGF-β) production in THP1 macrophages, as well as in primary human macrophages. BPA effected cytokines expression through estrogen receptor α/β (ERα/β)-dependent mechanism with the evidence of ERα/β antagonist reversed the expression of cytokines. We also identified that activation of extracellular regulated protein kinases (ERK)/nuclear factor κB (NF-κB) signal cascade marked the effects of BPA on cytokines expression. Our results indicated that BPA effected inflammatory responses of macrophages via modulating of cytokines expression, and provided a new insight into the link between exposure to BPA and human health.

1. Introduction

Endocrine disrupting chemicals (EDCs) are natural or synthetic compounds that have the ability within the body to alter endocrine functions often through mimicking or blocking endogenous hormones [1]. BPA, a common environmental endocrine disruptor, is widely used as a xenoestrogen product of epoxy resins, polycarbonate plastics, and flame retardants [2]. Recently, many studies showed that BPA can bioaccumulate in bodies of humans, and it has been detected in the urine of almost all adults and children, fetal serum during pregnancy, breast milk, follicular and amniotic fluid, human fetal livers, placental tissue, as well as blood serum [3–6]. BPA can directly bind to estrogen receptors and has estrogenic effects. Although BPA has a lower affinity for nuclear estrogen receptors relative to 17-beta estradiol (E2), its estrogenic potency is equal to E2 for responses mediated by non-nuclear estrogen receptors [7]. Furthermore, BPA can also act as an antiestrogen, blocking the estrogenic response by competing with endogenous E2 [8]. Therefore, because of its reported endocrine-disruptor activity, BPA widespread exposure and creating the concern that long-lasting adverse health effects may arise as a potentially consequence [9].

In recent years, some reports have been concern about a potential link between exposure to BPA and the occurrence of human diseases. For example, early exposure to BPA showed a linked with developmental and reproductive abnormalities in both sexes, including fertility, male sexual function, sperm quality, sex hormone concentrations, endometrial disorders, and polycystic ovary syndrome [10,11]. Accumulated studies also have revealed that BPA exposure may cause increased susceptibility to tumorigenesis and metabolic disease (i.e., type-2 diabetes, cardiovascular disease, liver function, and obesity) [12]. Among these health issues, increasing concern has been focused on the risk of BPA to immune function. General measures of immune function were shown to be negatively associated with BPA exposure [13]. Using animal model, many cell subsets of the murine immune system undergo extensive proliferation under BPA exposure [14].

Macrophages play a significant part in immunity and immune responses. These cells reside in every tissue of the body and their activations are the initial steps of inflammation [15]. Activation

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of macrophages secret inflammatory cytokines such as TNF-α that promotes an inflammatory response [16]. In this study, we investigated the effects of BPA on the generation of several regulatory cytokines in macrophage, as these cytokines are important in pro- and anti-inflammatory processes, respectively. The results herein provided evidence supporting the influence of BPA on the function of human macrophages, involving in activation of ERα/β/ERK/NF-κB signaling, with subsequent increased the expression of pro-inflammatory cytokines (TNF-α and IL-6), and decreased the expression of anti-inflammatory cytokines (TGF-β and IL-10) in human macrophages.

2. Materials and methods

2.1. Reagents

BPA, phorbol-12-myristate-13 acetate (PMA), G-1, G15, and Human AB Serum were purchased from Sigma–Aldrich (St. Louis, MO, USA). RPMI 1640 medium and fetal bovine serum (FBS) were purchased from GIBCO BRL (Grand Island, NY, USA). PDTC, U0126, and ICI182,780 were obtained from Beyotime Institute of Biotechnology (Beyotime, Jiangsu, China). Primary antibodies against p-AKT (Ser473), p-p38MAPK (Thr180/Tyr182), p-ERK (Thr202/204), p-JAK2 (Tyr1007/1008), p-STAT1 (Tyr701), p-STAT3 (Tyr705), p-IκB (Ser32), AKT, p38MAPK, ERK2, JAK2, STAT1, STAT3, IκB and β-actin were purchased from Cell Signaling Technology (Danvers, MA, USA). PE-conjugated CD68 antibody was from BD Bioscience (San Diego, CA, USA). Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA, USA). PrimeScript® RT reagent Kit and SYBR® Premix Ex Taq TM were products of TaKaRa (Otsu, Shiga, Japan). E.Z.N.Z® HP Total RNA Kit was bought from Omega Bio-Tek (Norcross, GA, USA). Vectors (pRL-TK and pNF-κB-luc) and dual-luciferase assay kit were purchased from Promega (Madison, WI, USA).

2.2. Cell culture

THP-1 cell lines were obtained from Chinese Academy of Sciences (Shanghai, China). THP-1 cells were cultured with RPMI1640 supplemented with 10% FBS and 2 mM L-glutamine under a humidified 5% CO_2 atmosphere at 37 °C in incubator. The THP-1 macrophages were maturated by exposure THP-1 cells to 320 nM PMA for 48 h. After maturation, THP-1 macrophages were cultured with the normal medium for further analysis.

Human peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-Plaque Plus (GE Healthcare Life Sciences, Europe) density gradient centrifugation from heparinized buffy-coats. Monocytes were isolated by CD14-positive selection using anti-CD14 magnetic beads (Miltenyi Biotech, Bergisch Gladbach, Germany). Monocytes were plated at 10^7 cells per 100 mm dish and cultured for 7 days at 37 °C and 5% CO_2 in RPMI-1640 supplemented with 10% FBS, 5% Human AB Serum, 1% Penicillin/Streptomycin, and 10 ng/ml Macrophage-Colony Stimulating Factor (M-CSF) to differentiate the monocytes into macrophages.

2.3. Cell viability assay

THP1 macrophages (1×10^4) were seeded into each well of a 96-well plate, and cells were subsequently treated with BPA. After treatment, cell viability was assessed using the Cell Counting Kit-8 (CCK-8) according to the manufacturer's instructions (Dojindo Molecular Technologies, Kumamoto, Japan).

2.4. Quantitative real-time PCR

THP1 macrophages were treated with BPA for 12 h. Total RNA isolated from cells was used E.Z.N.A.® HP Total RNA Kit (Omega Bio-tek, Doraville, GA, USA). The reverse transcription was performed with the PrimeScript® RT reagent Kit (TakaRa, Shiga, Japan). Quantitative Real-Time PCR reaction was performed on a Bio-Rad iQ5 Real-time PCR Detection System (Bio-Rad, Hercules, CA). Crossing threshold values for individual genes were normalized to GAPDH. Changes in mRNA expression were expressed as fold change relative to control. Gene-specific primer pairs used in this study were as follows, GAPDH-sense 5′-CACCCAGAAGACT GTGGATGG-3′ and GAPDH-antisense 5′-GTCTACATGGCAACTGTG AGG-3′; IL-4-sense 5′-AGCAGTTCCACAGGCA-CAAG-3′ and IL-4-antisense 5′-AGCAGTTCCACAGGCACACAG-3′; IL-10-sense 5′-TGGT GAAACCCCGTCTCTAC-3′ and IL-10-antisense 5′-CTGGAGTACA-GG GGCAGTAT-3′; TGF-β-sense 5′-TGGTTGAGCCGTGGAGGGGA-3′ and TGF-β-antisense 5′-CTCGGCGGCCGGTAGTGAAG-3′; IL-8-sense 5′-TAGCA-AAATTGAGGCCAAGG-3′ and IL-8-antisense 5′-AAACC AAGGCACAGTGGA-AC-3′; IL-2-sense 5′-TGCAACTCCTGTCTTGCA TT-3′ and IL-2-antisense 5′-GCCTTCTTGGGCATGTAAAA-3′; IL-12-sense 5′-GGACATCATCAAACCTGA-CC-3′ and IL-12-antisense 5′-AG GGAGAAGTAGGAATGTGG-3′; TNF-α-sense 5′-GAGCACTGAAAGC ATGATCC-3′ and TNF-α-antisense 5′-CGAGAAGA-TGATCTGAC TGCC-3′; IL-6-sense 5′-GGCACTGGCAGAAAACAACC-3′ and IL-6-antisense 5′-GCAAGTCTCCTCATTGAATCC-3′.

2.5. Measurement of cytokine production

THP1 macrophages were treated with BPA for 24 h. After treatment, cell culture supernatants were collected. Then, human IL-2, IL-4, IL-6, IL-10 and TNF-α secretion were quantified by CBA Human Th1/Th2 Cytokine kit (BD Biosciences, San Jose, CA, USA), as previously described in the literature [17]. CBA data was analyzed by flow cytometry. TGF-β, IL-6, IL-10 and TNF-α levels in the supernatants of cultured THP1 macrophages or primary human macrophages were also measured by a sandwich enzyme-linked immunosorbent assay (ELISA) using a commercially available ELISA kit as described by the manufacturer (eBioscience, San Diego, CA, USA).

2.6. Western blot analysis

THP1 macrophages were lysed in cell lysis buffer as previously described [18]. Equal amounts of protein (30 μg) were run on 10% SDS polyacrylamide gels, and transferred to nitrocellulose membranes. Following blocking with 5% non-fat milk for 2 h, membranes were incubated with the primary antibody at 1:1000 dilution overnight at 4 °C and then incubated with a horseradish peroxidase-conjugated secondary antibody at 1:5000 dilution for 1 h at room temperature. Specific immune complexes were detected using Western Blotting plus Chemiluminescence Reagent (Life Science, Inc., Boston, MA).

2.7. NF-κB reporter assay

THP1 macrophages were transiently co-transfected with pNF-κB-luc (2 μg) and pRL-TK (0.5 μg). After 24 h, these cells were treated with BPA. Transcriptional activity was determined by the dual-luciferase reporter assay system. Results were calculated as the ratio between the activity of pNF-κB-luc and pRL-TK.

2.8. Statistical analysis

Results were expressed as Mean ± SD of three independent experiments. Data were analyzed by two-tailed unpaired Student's *t*-test between any two groups and bars represent SD. All calculations were carried out using GraphPad Prism Software Version 5.0 (GraphPad Software Inc., La Jolla, CA). *P*-value of <0.05 was considered statistically significant.

3. Results

3.1. Effects of BPA on the expression of cytokines in THP1 macrophages

THP-1 is a human monocytic leukemia cell line. After treatment with phorbol esters, THP-1 cells differentiate into macrophage-like cells which mimic native monocyte-derived macrophages in several respects [19], as macrophage specific marker CD68 showed a significant increased in phorbol esters PMA (320 nM) treatment THP1 cells (Fig. S1A and B).

To examine the potential effects of BPA on the function of macrophages, we first assessed the effects of BPA on cell proliferation of THP-1 macrophages. The results showed that the treatment of BPA, at concentrations ranging from 0 to 1 μM, exhibited no cytotoxic effects to THP1 macrophages during the 24 h incubation period (Fig. S1C). Then, we investigated the effects of BPA on the generation of several regulatory cytokines in human THP1 macrophages, as these cytokines are important in pro- and anti-inflammatory processes, respectively. The results showed that treatment of THP1 macrophages with BPA revealed significantly increased mRNA levels of pro-inflammatory cytokine TNF-α, and a moderate increased level of IL-6 (Fig. 1A). Concordant with the observed increase in mRNA expression, BPA treatment induced the release of TNF-α and IL-6 proteins from THP1 macrophages (Fig. 1B and C). In contrast, BPA significantly inhibited the expression of anti-inflammatory cytokine TGF-β (Fig. 1A and D). Furthermore, although lower concentration BPA appear to have no effect on levels of IL-10 expression, BPA with high concentration (0.1 μM) could inhibit the baseline level of IL-10 (Fig. 1A–C).

3.2. BPA modulated cytokine production in THP1 macrophages via the ERα/β

We have previously reported that G protein-coupled receptor 30 (GPR30) mediated estrogenic effects of BPA [11]. However, both GPR30 antagonist G15 and GPR30 agonist G1 have no effects on BPA-modulated cytokine production (Fig. S2). Because EDCs are able to serve as inflammatory inducers partly via the ER [20], ERα/β antagonist ICI182,780 were further used to examined whether ERα/β was involved in BPA-modulated cytokine production in macrophages. As shown in Fig. 2, the addition of ICI182,780 significantly decreased the levels of TNF-α and IL-6

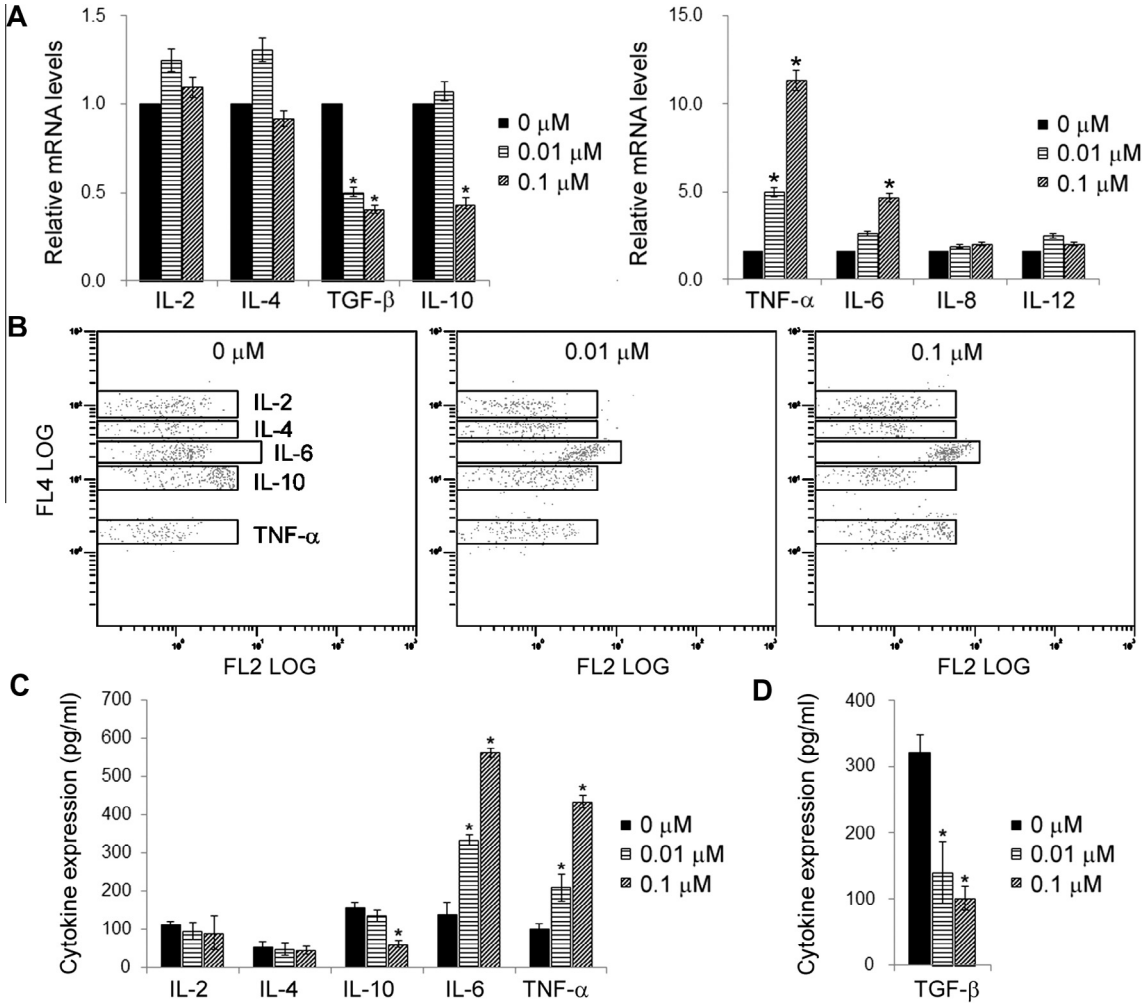

Fig. 1. Effects of BPA on production of cytokines in THP-1 macrophages. (A) Matured THP-1 macrophages were treated with the indicated concentrations of BPA for 12 h. IL-2, IL-4, TGF-β, IL-10, TNF-α, IL-6, IL-8, and IL-12 mRNA levels were determined by real-time RT-PCR using GAPDH as an internal control. (B) Matured THP-1 macrophages were treated with the indicated concentrations of BPA for 24 h. Supernatants were harvested and assayed to determine the concentration of different cytokines. The beads were conjugated with antibodies against corresponding cytokines. Secondary antibody conjugated with fluorescence dye PE was used as a detector. SSC versus FSC were used to locate the position of cytokine beads. The dots from top to bottom represent IL-2, IL-4, IL-6, IL-10, and TNF-α, respectively. FL4 versus FL2 were used to determine the concentration of cytokines. (C) Quantitative analysis of cytokines production using BD-Pharmingen CBA kit. (D) Matured THP-1 macrophages were treated with the indicated concentrations of BPA for 24 h. Supernatants were harvested and TGF-β production was detected by ELISA. Data were presented as means ± SD of three independent experiments. *Significant difference from control values with $P < 0.05$.

production, and increased the levels of TGF-β and IL-10 production in THP1 macrophages. These findings indicated that ERα/β, but not GPR30, was involving in BPA-modulated cytokine production in human macrophages.

3.3. BPA modulated cytokine production in THP1 macrophages via the ERK/NF-κB signaling

We next sought to identify the downstream signal involved in a BPA-modulated cytokine production. To this end, we used total and phospho-specific antibodies to monitor the expression levels and activation statuses, respectively, of key members of several signal transduction pathways. We found that BPA did not affect phosphorylation levels in JAK2, AKT, p38MAPK, and STAT-1. However, IκB and ERK phosphorylation level was significantly increased in a dose-dependent manner after BPA treatment, whereas STAT-3 phosphorylation level was significantly decreased at 0.1 μM BPA treatment (Fig. 3A). Since phosphorylation of IκB resulting in the dissociation of IκB/NF-κB complex, following the nuclear translocation of NF-κB for target genes transcription [21], activation of NF-κB by BPA was further verified by dose-dependently enhancing NF-κB promoter activities in THP1 macrophages (Fig. 3B). Previous study has demonstrated that NF-κB translocate into the cell nucleus where, either alone or in combination with STAT, induce pro-inflammatory gene expression [22]. Then we investigated the effects of PDTC, a relatively specific inhibitor of the activation of NF-κB [23], on BPA-modulated cytokine production in THP1 macrophages. As shown in Fig. 3B, PDTC significantly inhibited BPA-induced NF-κB activation, and inhibition of NF-κB pathway decreased the levels of TNF-α and IL-6 production (Fig. 3C). Furthermore, we showed that ERK inhibitor U0126 significantly inhibited NF-κB promoter activities (Fig. 3D), as well as decreased the levels of TNF-α and IL-6 production (Fig. 3E). These results suggested that ERK/NF-κB pathway were involved in BPA-modulated cytokine production. In addition, ERα/β antagonist ICI182,780 significantly inhibited BPA-induced activation of ERK (Fig. 3F) and NF-κB (Fig. 3G), which suggested that BPA activates ERK/NF-κB signaling via ERα/β.

3.4. BPA modulated cytokine production in primary human macrophages

We further investigated the effect of BPA on the cytokines production in primary human macrophages. After incubated with or without BPA for 24 h, the cytokines production in primary human macrophages were analyzed by ELISA. The results also showed that BPA significantly increased the expression of TNF-α and IL-6, but decreased the expression of IL-10 and TGF-β, whereas ERα/β antagonist ICI182,780, ERK inhibitor U0126, and NF-κB inhibitor PDTC revised BPA-mediated cytokine expression (Fig. 4).

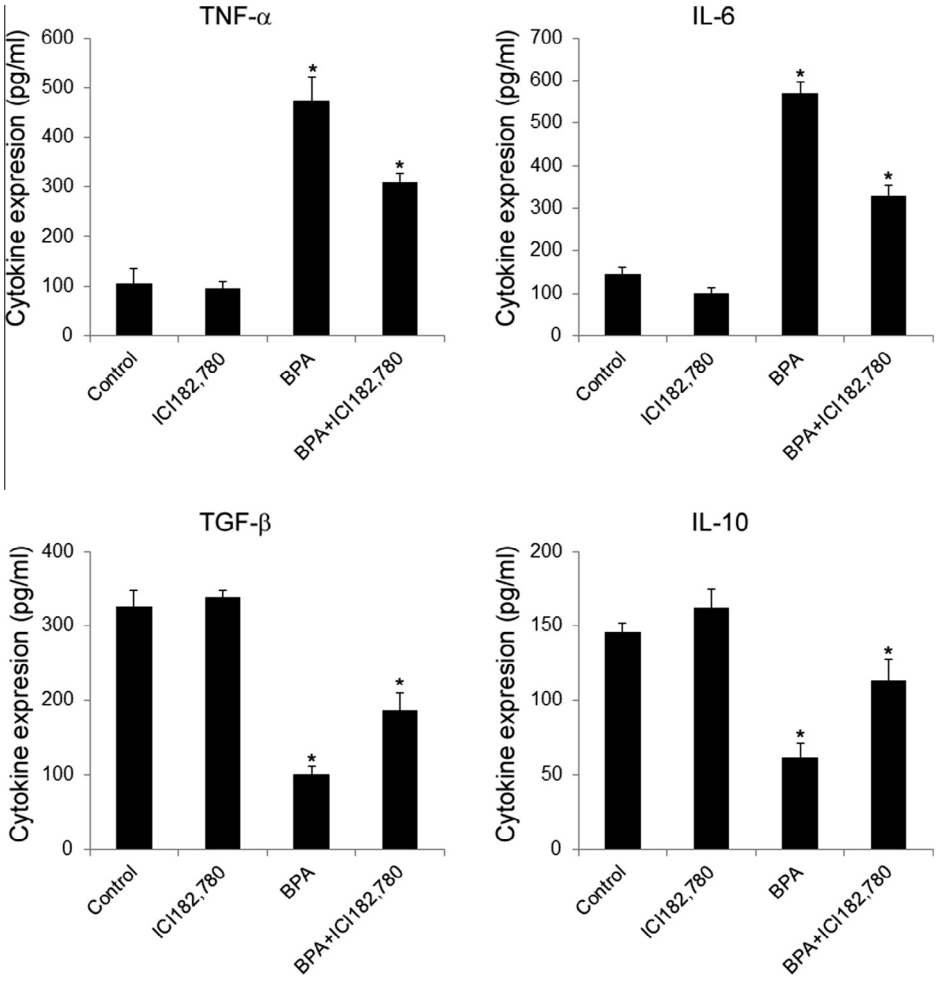

Fig. 2. Effects of ERα/β antagonist ICI1182,780 on BPA-mediated expression of cytokines. Matured THP-1 macrophages were treated with BPA in the presence or absence of ERα/β antagonist ICI1182,780 (1 μM). Supernatants were harvested and TNF-α, IL-6, TGF-β and IL-10 production was detected by ELISA. Data were presented as means ± SD of three independent experiments. *Significant difference from control values with $P < 0.05$.

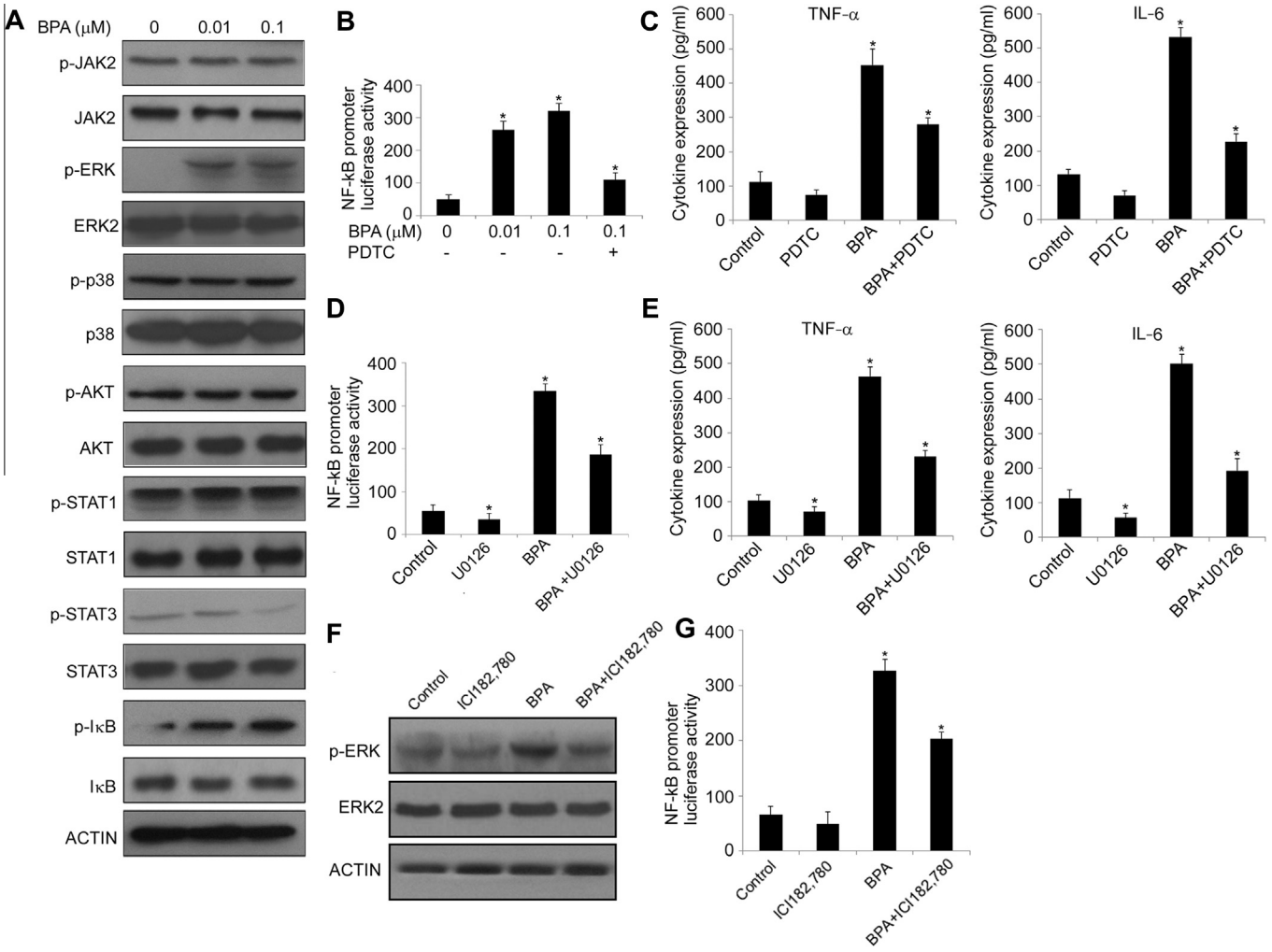

Fig. 3. Effects of BPA on cell signal transduction pathways. (A) Matured THP-1 macrophages were treated with BPA for 24 h. Protein lysates were prepared. The phospho-AKT (Ser473), AKT, phospho-p38MAPK (Thr180/Tyr182), p38MAPK, phospho-ERK (Thr202/204), ERK2, phospho-JAK2 (Tyr1007/1008), JAK2, phospho-STAT1 (Tyr701), STAT1, phospho-STAT3 (Tyr705), STAT3 phospho-IκB (Ser32), and IκB levels were analyzed by Western blot. (B) Matured THP-1 macrophages were co-transfected with pNF-κB-luc and pRL-TK. After 24 h, these cells were treated with BPA for the indicated concentration. NF-κB reporter activities were calculated as the ratio between the activity of pNF-κB-luc and pRL-TK. (C) Matured THP-1 macrophages were treated with BPA in the presence or absence of NF-κB inhibitor PDTC (20 μM). Supernatants were harvested and TNF-α and IL-6 production was detected by ELISA. (D and E) Matured THP-1 macrophages were treated with BPA in the presence or absence of ERK inhibitor U0126 (20 μM), NF-κB reporter activities were calculated (D), TNF-α and IL-6 production was detected by ELISA (E). (F and G) Matured THP-1 macrophages were treated with BPA in the presence or absence of ERα/β antagonist ICI1182,780 (1 μM), phospho-ERK (Thr202/204) levels was analyzed by Western blot (F), NF-κB reporter activities were calculated (G). Data were presented as means ± SD of three independent experiments. *Significant difference from control values with $P < 0.05$.

4. Discussion

Macrophages have been recognized as a critical effectors and regulators of inflammation and the innate immune response, and macrophages exert their regulatory functions, in part, through the release of cytokines. In the present study, we investigated the possible stimulative effects of BPA on human macrophages. The results demonstrated that BPA could increase cytokines TNF-α, IL-6 secretion, and inhibit cytokines TGF-β, IL-10 secretion. In addition, we found that BPA targets and regulates cytokine responses of macrophages through mechanisms involving in activation of Rα/β/ERK/NF-κB signal cascade. These results suggested that the existence of a BPA-cytokines axis in the regulation of macrophages function.

Dysregulated TNF-α and IL-6 production from macrophages at local disease sites have been considered to be the major contributors to the development of inflammatory diseases [16], whereas TGF-β and IL-10 is recognized as an anti-inflammatory cytokine [24]. Therefore, the functional effect of BPA in inducing TNF-α and IL-6, while decreasing TGF-β and IL-10 production in macrophages, suggested its pro-inflammatory function. Similar to our results, treatment of BPA increased the expression tumor necrosis factor-alpha and infer a stimulatory activity of BPA on macrophages [25]. However, using high concentrations, BPA may suppress LPS-induced production of TNF-α, and downregulate macrophage activities [26]. Thus, although complex, BPA alters immune responses and may thus affect the acquisition or progression of inflammatory disorders.

BPA has been reported to have estrogenicity and bind to estrogen receptor α [27]. Previous studies demonstrated that the activation of this nuclear receptor *in vivo* mediates the pro-inflammatory effect of estrogens on resident peritoneal macrophages [28]. From the present study, induction or inhibition of cytokine production by BPA in human macrophages was mediated through the ERα/β. Alternatively, recent evidences also indicated that BPA specifically binds GPR30, a novel non-classical membrane ER, by which estrogenic compounds might induce biological effects in different cell types [11,29]. However, both GPR30 antagonist G15 and GPR30

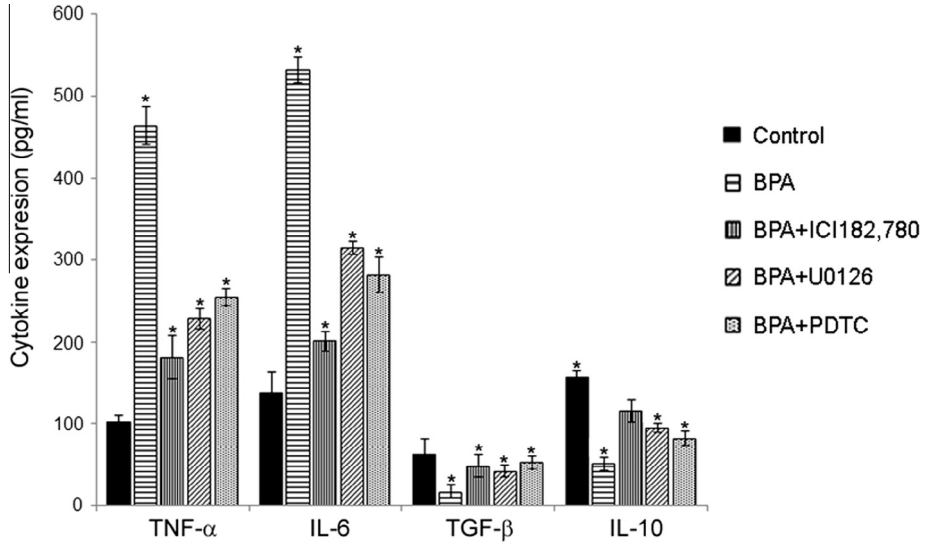

Fig. 4. Effects of BPA on production of cytokines in human primary macrophages. PBMC were isolated using Ficoll-Plaque Plus density gradient centrifugation from heparinized buffy-coats. Monocytes were isolated by CD14-positive selection using anti-CD14 magnetic beads and cultured for differentiation into macrophages, and then treated with BPA in the presence or absence of ERα/β antagonist ICI182,780 (1 μM), NF-κB inhibitor PDTC (20 μM), or ERK inhibitor U0126 (20 μM). Supernatants were harvested and TNF-α, IL-6, TGF-β and IL-10 production was detected by ELISA. Data were presented as means ± SD of three independent experiments. *Significant difference from control values with $P \leqslant 0.05$.

agonist G1 have no effects on BPA-modulated cytokine production, suggested that GPR30 does not involved in BPA-modulated cytokine production in human macrophages.

Estrogen receptor signaling can activate a kinase cascade, which in turn regulation of gene transcription [30]. Thus, diverse estrogenic compounds could activate ser kinases. It has been reported that BPA altered the activation of JAK [31], mitogen-activated protein kinases (MAPK) [32] and PKA [33] in a different manner. In the present study, we showed that BPA can activate ERK, and inhibition of ERK reversed BPA-medicated regulation of cytokines expression, indicated that activation of ERK plays an important role in BPA-induced expression of cytokines in human macrophages. Moreover, ERα/β antagonist inhibited BPA-induced activation of ERK, suggested that BPA activates ERK via ERα/β.

Nuclear factor-κB (NF-κB) transcription factor has been considered the central mediator of the inflammatory process and a key participant in innate and adaptive immune responses [34]. NF-κB regulates the transcription of pro-inflammatory innate immune genes, including TNFα, IL-6, iNOS, IL-1β and MCP-1 [35]. In our results, BPA stimulated the NF-κB signaling, and activation of NF-κB resulted in an increase in production of cytokines. As evidence, the cells pretreated with PDTC for inhibition of NF-κB had a decreased TNF-α and IL-6 production. Several studies pointed towards a crucial role of the interaction between NF-κB and STAT dependent signaling for the regulation of cytokines expression [22]. NF-κB signaling suppresses the activation of STAT-3 [36], while activation of STAT-3 induce TGF-β and IL-10 expression in macrophages. Therefore, the effect of activation of NF-κB may further suppress STAT-3 activation and inhibit TGF-β and IL-10 expression in human macrophages. Furthermore, BPA-induced NF-κB activation was prevented in the presence of ERK inhibitor, suggested that activation of the ERK/NF-κB pathway was involved in BPA-induced cytokines production.

In conclusion, this study presented evidence supporting a role of BPA in modulation of macrophage function through their ability to enhance TNF-α and IL-6 expression, but suppress TGF-β and IL-10 release, and this effect involved in the ERα/β/ERK/NF-κB signaling. TNF-α and IL-6 play an important role in the expression of inflammatory responses, whereas TGF-β and IL-10 are recognized as an anti-inflammatory cytokine. Therefore, the functional effects of BPA in inducing TNF-α and IL-6 production, and inhibiting TGF-β and IL-10 production in human macrophages, suggested its pro-inflammatory function. Our study gives an insight into the immunotoxicity of BPA, which is likely to impact on the human health.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.08.031.

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