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Research Article

# Polychlorinated Biphenyls (PCB 101, 153, and 180) Impair Murine Macrophage Responsiveness to Lipopolysaccharide: Involvement of NF-κB Pathway

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## ABSTRACT

Non-dioxin-like (NDL) polychlorinated biphenyls (PCBs) are persistent organic pollutants, associated with a range of adverse health effects, including interference with the immune system. In this study, we investigate the capability of NDL-PCBs 101, 153, and 180, 3 of the 6 NDL-PCBs defined as indicators, to impair the immune response in lipopolysaccharide (LPS)-activated J774A.1 and primary murine macrophages. Our results clearly demonstrate that the exposure of J774A.1 and primary macrophages to NDL-PCB 153 or 180 or all NDL-PCBs mixtures causes a significant reduction in LPS-induced cytokine/chemokine synthesis, such as tumor necrosis factor-α and interleukin-6, together with monocyte chemoattractant protein-1, involved in cell recruitment. Moreover, PCBs were found to suppress LPS-stimulated NO production, and to reduce cyclooxygenase-2 and inducible nitric oxide synthase expression in J774A.1 and primary macrophages. At mechanistic level, PCBs significantly counteract the LPS-driven toll-like receptor (TLR) 4 and CD14 upregulation, therefore inhibiting downstream nuclear factor-κB (NF-κB) activation in J774A.1. Furthermore, PCBs determine a significant loss of macrophage endocytic capacity, a prerequisite for efficient antigen presentation. Taken together, these data indicate that NDL-PCBs reduce macrophage responsiveness, particularly when they are combined at concentrations per se inactive, impairing the capability to orchestrate a proper immune response to an infectious stimulus, disrupting TLR4/NF-κB pathway.

**Key words**: non-dioxin-like polychlorinated biphenyls; lipopolysaccharide; NF-κB, toll-like receptor 4; immune suppression; endocytosis

## Abbreviations:

| | |
|---|---|
| PCBs, | polychlorinated biphenyls; |
| NDL, | non-dioxin-like; |
| LPS, | lipopolysaccharide; |
| EDCs, | endocrine disrupting chemicals; |
| DL, | dioxin-like; |
| AhR, | aryl hydrocarbon receptor; |
| TLR, | toll-like receptor; |
| NF-κB, | nuclear factor-κB; |
| COX, | cyclooxygenase; |
| iNOS, | inducible nitric oxide synthase; |
| TNF, | tumor necrosis factor; |
| IL, | interleukin; |
| MCP, | monocyte chemoattractant protein; |
| DMSO, | dimethyl sulphoxide; |
| DMEM, | Dulbecco's modified Eagle medium; |
| FBS, | fetal bovine serum; |
| MTT, | 3 -(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; |
| GAPDH, | glyceraldehyde 3-phosphate dehydrogenase; |
| HRP, | horseradish peroxidase. |

Polychlorinated biphenyls (PCBs), in addition to well-known polychlorinated dibenzo-*p*-dioxins and polychlorinated dibenzofurans, belong to the group of persistent organic pollutants and are currently considered as endocrine-disrupting chemicals (EDCs) (Marques-Pinto *et al.*, 2013). Since the endocrine and immune systems share several common intracellular signaling pathways, PCBs are also considered potential toxic agents in influencing host defense system against foreign pathogens.

Actually, several epidemiological studies show that PCB exposure is associated with modification of innate and adaptive immunity, including effects on immune cells (i.e. monocytes, polymorphonuclear, and natural killer cells). Such effects could lead to increased incidence of infections, insufficient antibody response to vaccination and alterations in immune organs, lymphocyte subsets, and functions (Belles-Isles *et al.*, 2002; Heilmann *et al.*, 2006, 2010). Notably, childhood is the age of greatest sensitivity to the toxic effects of these chemicals, since PCB exposure has been associated with an increased incidence of respiratory and ear infections, influenza, and chicken pox in healthy Dutch (Weisglas-Kuperus *et al.*, 2000, 2004) and Inuit preschoolers (Dallaire *et al.*, 2006; Dewailly *et al.*, 2000). Consistently, experimental studies indicate a failing of immune defenses after long-term PCB exposure due to disease resistance or increased susceptibility to infections, such as septicemic disease caused by *Flavobacterium psychrophilum* in rainbow trout *Oncorhynchus mykiss* eggs (Ekman *et al.*, 2004) or subcutaneous abscess by *Staphylococcus aureus* in mice (Imanishi *et al.*, 1984).

Furthermore, *in vitro* molecular studies demonstrated that dioxin-like (DL)-PCBs, differently from non-dioxin-like (NDL)-PCBs, exert strong immune suppressive effects binding to the aryl hydrocarbon receptor (AhR) (Ferrante *et al.*, 2011; Kerkvliet, 2009; Levin *et al.*, 2005a). Indeed, the immunotoxicity exerted by NDL-PCBs has been related to signaling interference via the neural-immune axis, calcium homeostasis, or serotonergic system alteration (Duffy-Whritenour *et al.*, 2010; Pessah *et al.*, 2010). Moreover, Levin *et al.* (2005a) showed that exposure of healthy human neutrophils and monocytes to the NDL-PCBs 138, 153, and 180, alone or in combination, resulted in reduced phagocytosis activity. In contrast, leukocyte exposure to the DL-PCB 169 or to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin did not affect phagocytosis, strengthening a different immunotoxic mechanism among DL- and NDL-PCBs (Levin *et al.*, 2005b). In addition, we showed that NDL-PCBs 101, 153, and 180 induce macrophage apoptosis by activating the intrinsic pathway (Ferrante *et al.*, 2011).

In macrophage, immune response by lipopolysaccharide (LPS), one of the most potent innate immune-activating stimuli, involves engagement of the toll-like receptor (TLR) 4, a signal-transducing integral membrane protein (Janeway and Medzhitov, 2002). After the binding of the endotoxin to the TLR4, the downstream activation of nuclear factor (NF)-κB occurs, inducing the transcription of cytokine and chemokine genes that collectively ramp up the host's immune defense mechanisms (Beyaert, 2011). Moreover, LPS is able to induce rapid alterations in cellular immediate-early gene expression, leading to the *de novo* synthesis of cyclooxygenase (COX)-2 (D'Acquisto *et al.*, 1997; Ferrante *et al.*, 2008) and inducible nitric oxide synthase (iNOS) (Meli *et al.*, 2000), whose coinduction has been shown in several cell types, including murine macrophages (Akarasereenont *et al.*, 1994; Salvemini *et al.*, 1993; Swierkosz *et al.*, 1995).

In this study, we have investigated the effects of PCB 101, 153, or 180, alone or mixed, on LPS-activated peritoneal macrophages and J774A.1 murine cells. We focused on these congeners, since (1) they are 3 of the 6 PCB indicators proposed as markers of PCB contamination, (2) they are the most frequently detected, and (3) they are revealed at high concentrations in human tissues (Corsolini *et al.*, 1995; Duarte-Davidson *et al.*, 1994; Malarvannan *et al.*, 2013) and food of animal origin (Domingo and Bocio, 2007; Ferrante *et al.*, 2010; Törnkvist *et al.*, 2011). In this study, we assessed the effect of NDL-PCBs on macrophage immune response by the alteration of LPS-induced transcription of pro-inflammatory cytokines and monocyte chemoattractant protein (MCP)-1. Moreover, the effect of PCBs on TLR4/CD14 transcription and the modulation of underlying pathway was also determined through the evaluation of NF-κB activation and the expression of its related pro-inflammatory genes.

## MATERIALS AND METHODS

*Chemicals and reagents*. 2,2′,4,5,5′-Pentachlorobiphenyl (PCB 101), 2,2′,4,4′,5,5′-Hexachlorobiphenyl (PCB 153), and 2,2′,3,4,4′,5,5′-Heptachlorobiphenyl (PCB 180) (99% purity) were obtained from Sigma Aldrich (St. Louis, Missouri). All PCBs were dissolved in dimethyl sulphoxide (DMSO) in a 10-mM stock solution. Dulbecco's modified Eagle's medium (DMEM) without red phenol, fetal bovine serum (FBS), and supplements were purchased from Lonza (Walkerville, Maryland). Escherichia coli LPS (serotype 0111:B4) was purchased from Fluka (Milan, Italy). The antibody against COX-2 was obtained from Cayman Chemical (Ann Arbor, Michigan). The antibody against iNOS was acquired by BD Biosciences Transduction Laboratories (Lexington, Kentucky), and the antibodies against IκB-α and p65 nuclear factor (NF)-κB were purchased by Santa Cruz Biotechnology (Santa Cruz, California). Unless stated otherwise, all reagents and compounds were obtained from Sigma Chemicals Company (Sigma, Milan, Italy).

*J774A.1 and primary peritoneal macrophages culture conditions*. Resident macrophages were obtained from male Wistar rats (200 g) by peritoneal lavage with sterile saline containing penicillin (100 U/ml), and streptomycin (100 μg/ml). After centrifugation at 2400 rpm for 10 min, macrophages were suspended in red phenol free DMEM supplemented with 2 mM glutamine, 25 mM HEPES, penicillin (100 U/ml), streptomycin (100 μg/ml), 10% FBS, and 1.2% sodium pyruvate. Cell culture were maintained at 37°C in a humidified chamber containing 5%$CO^2$ in air. Macrophages ($3 \times 10^{-6}$/P60 plate) were obtained from 2 donors for each independent experiment, and incubated for 3 h. Thereafter, nonadherent cells were removed by washing, and adherent macrophages were starved in the same medium at 5% FBS. After 2 h, cells were stimulated with LPS (1 μg/ml) and incubated with PCB mixtures as follows, 150 nM PCBx + 150 nM PCBy (300 nM final concentration) for combination of 2 PCBs, and 100 nM PCB 101, 100 nM PCB 153, and 100 nM PCB 180 (300 nM final concentration) when used all together. After 24 h, cells were lysed as reported previously and iNOS and COX-2 were evaluated by western blot analysis. Nitrite accumulation and cytokine release were measured in cell supernatants.

The J774A.1 cell line (BALB/c murine macrophages) was obtained from the European Collection of Animal Cell Cultures (Salisbury, Wiltshire, U.K.) and cultured as described previously (Ferrante *et al.*, 2011). The cells were mechanically scraped and plated. After 4 h to allow adhesion, cells were starved in 5% FBS Red Phenol free DMEM for 2 h and subsequently treated with PCB 101, or PCB 153, or PCB 180 (300 nM), when employed alone, otherwise 150 nM PCBx + 150 nM PCBy (300 nM final

concentration) for combination of 2 PCBs, and lastly 100 nM PCB 101, 100 nM PCB 153, and 100 nM PCB 180 (300 nM final concentration) when used all together. Cells were incubated with these pollutants alone or in the presence of LPS (10 ng/ml) for different times depending on the assay performed.

The final concentration of DMSO in all samples and in control cells was 0.1% (v/v). This DMSO percentage allows the optimal solubilization of PCBs in aqueous solutions and no effect on the measured parameters has been shown, through appropriate preliminary experiments performed with and without vehicle. The nanomolar concentrations of PCBs were chosen because they did not significantly modify cell viability. Higher concentrations of these chemicals were shown previously to cause cell death of this cell line (Ferrante *et al.*, 2011). These concentrations are in the range of serum concentrations detected after exposure to PCBs and residual levels evidenced in food of animal origins (Ferrante *et al.*, 2010).

*Determination of cell viability*. Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test, detecting functional mitochondria able to transform MTT to formazan salts, which can be measured with spectrophotometer (Ferrante *et al.*, 2011). J774A.1 macrophages ($3\times10^4$/well) were seeded on 96-well microtiter plates to a final volume of 150 µl. Cells were incubated with PCBs at increasing concentrations (100 nM-10 µM) in the presence of LPS (10 ng/ml) or with combined PCBs 2 by 2 (150 nM PCBx + 150 nM PCBy resulting in 300 nM final concentration for combination of 2 PCBs) or all together (100 nM PCB 101, 100 nM PCB 153, and 100 nM PCB 180, resulting in 300 nM final concentration) in the presence or absence of LPS. After 24 h of incubation at 37° C, 25 µl of MTT (5 mg/ml) were added to each well and the cells were incubated for an additional 3 h. Thereafter, cells were lysed with 100 µl of a solution containing 50% (v/v) N,N-dimethylformamide, 20% (w/v) sodium dodecyl sulphate (pH 4.5) to allow solubilization of dark blue crystals. Then, after 20-h incubation at 37°C, the optical density ($OD_{620}$) of the samples treated with the different serial dilutions of PCBs alone or in combination with LPS were compared with the OD of control wells to assess the cell viability, which was calculated as: % dead cells = 100 − (OD treated/OD control)×100.

*Real-time semi-quantitative PCR analysis*. After 4 h of incubation with PCBs, alone or mixed (final concentration, 300 nM), in the presence of LPS (10 ng/ml), total RNA was extracted by a modified method of Chomczynski and Sacchi (1987), using Trizol Reagent (Invitrogen Biotechnologies) in accordance with the manufacturer's instructions. cDNA was synthesized using a reverse transcription kit (Maxima First Strand cDNA Synthesized Kit, Fermentas, Ontario, Canada) from 2 µg total RNA. PCRs were performed with a Bio-Rad CFX96 Connect Real-time PCR System instrument and software (Bio-Rad Laboratories) as described previously (Ferrante *et al.*, 2014). The primer sequences are reported in Table 1.

The PCR conditions were 10 min at 95°C followed by 40 cycles of 2-step PCR denaturation at 95°C for 15 s and annealing extension at 60°C for 60 s. Each sample contained 1-100 ng cDNA in 2× Power SYBRGreen PCR Master Mix (Applied Biosystem) and 200 nmol/l of each primer (Eurofins MWG Operon, Huntsville, Alabama) in a final volume of 25 µl. The relative expression of each studied mRNA was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as housekeeping gene, and the data were analyzed according to the $2^{-\Delta\Delta CT}$ method. Before performing any reaction, the efficiency of primers was set through a standard curve in the cell sample analyzed, and an amplification efficiency of 100% was obtained.

*Cytokine determinations*. After 24 h of incubation with LPS in the presence or absence of PCBs, cytokines release from J774A.1 cells and peritoneal macrophages, was determined in cell culture supernatants. Levels of IL-6, TNF-α, and MCP-1 were measured using commercially available ELISA kits, according to the manufacturer's instructions (Thermo Scientific, Rockford, Illinois).

Cytokine concentrations were determined by linear regression from standard curves for each cytokine, generated with kit-supplied reference cytokine sample, and expressed as ng or pg/ml.

*Western blot analysis*. To evaluate COX-2 and iNOS expression by J774A.1 and peritoneal macrophages, after 24 h of incubation with LPS in the presence or absence of PCBs, cells were washed twice with ice cold PBS, harvested, and resuspended in 20 mM Tris–HCl (pH 7.5), 10 mM NaF, 150 mM NaCl, 1% Nonidet P-40, 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM sodium orthovanadate ($Na_3VO_4$), leupeptin, and trypsin inhibitor (10 µg/ml). After 1 h, cell lysates were obtained by centrifugation at 20 000 × g for 15 min at 4°C.

To determine the temporal effect of LPS in the presence or absence of PCBs on cytosolic IκB-α degradation and nuclear p65-NF-κB expression, J774A.1 cells were treated with LPS (10 ng/ml) for 0–5–15–30 min. In another set of experiments, cells were treated with PCBs, alone or mixed, in the presence of LPS for 30 min. At the determined times, cells were suspended in extraction buffer [0.32 M sucrose, 10 mM Tris–HCl pH 7.4, 1 mM ethyleneglycol-bis(β-aminoethyl)-N,N,N′,N′-tetraacetic acid (EGTA), 2 mM ethylenediaminetetraacetic acid (EDTA), 5 mM $NaN_3$, 10 mM 2-mercaptoethanol, 50 mM NaF, 0.2 mM PMSF, 0.15 µM pepstatin A, 20 µM leupeptin, 1 mM $Na_3VO_4$], scraped off, harvested cold, incubated for 15 min and then centrifuged at 1000 g for 10 min, 4°C. IkB-α degradation was evaluated in the cytosolic supernatant fraction. The pellets were resuspended in the supplied complete lysis buffer containing 1% Triton X-100, 150 mM NaCl, 10 mM Tris–HCl pH 7.4, 1 mM EGTA, 1 mM EDTA, 0.2 mM PMSF, 0.2 mM $Na_3VO_4$, and then centrifuged 30 min at 15 000 × g at 4°C to yield the nuclear fraction for the p65 NF-κB level determination.

Protein concentrations were estimated by the Bio-Rad protein assay using bovine serum albumin as standard. Equal amount of protein (cell lysates) were subjected to 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. The filter was, then, blocked with 1 × PBS, 5% nonfat dried milk and incubated with specific antibodies in 1 × PBS, 5% nonfat dried milk, 0.1% Tween-20 for 2 h at room temperature. We used the specific mAbs against COX-2 (1:500), iNOS (1:1000), IκB-α (1:2000) and p65 NF-κB (1:500) in 1 × PBS, 5% nonfat dried milk, 0.1% Tween-20 at 4°C, overnight. After incubation with the primary antibody, the filter was washed in 1 × PBS, 5% nonfat dried milk, 0.1% Tween, and incubated with the secondary antibody [IgG-horse radish peroxidise (HRP) conjugate; 1:2000 dilution] for 1 h at room temperature. Subsequently, the blot was extensively washed with 1 × PBS, developed using enhanced chemiluminescence detection reagents (Amersham Pharmacia Biotech, Piscataway, New Jersey) according to the manufacturer's instructions, and the immune complex visualized by Imag Quant. The protein bands were scanned and densitometrically analyzed with a model GS-

**TABLE 1.** Real-Time PCR Primer Sequences

| Target Gene | Forward Primer (5′→3′) | Reverse Primer (3′→5′) | Accession Number |
|---|---|---|---|
| IL-6 | 5′-ACAAGTGGGAGGCTTAATTACACAT-3′ | 3′-TTGCCATTGCACAACTCTTTTC-5′ | NM_031168.1 |
| TNFα | 5′-CATCTTCTCAAAACTCGAGTGACAA-3′ | 3′-TGGGAGTAGATAAGGTACAGCCC-5′ | NM_013693.3 |
| MCP-1 | 5′-CCCACTCACCTGCTGCTACT-3′ | 3′-TCTGGACCCATTCCTTCTTG-5′ | NM_011333.3 |
| TLR-4 | 5′-TGACAGGAAACCCTATCCAGAGTT-3′ | 3′-TCTCCACAGCCACCAGATTCT-5′ | NM_021297.2 |
| CD14 | 5′-GGTACTGAGTATTGCCCAAGC-3′ | 3′-CTAGTCCTTGCAGCTGTACCC-5′ | NM_009841.3 |
| GAPDH | 5′-AACTTTGGCATTGTGGAAGG-3′ | 3′-GGATGCAGGGATGATGTTCT-5′ | NM_008084.2 |

Abbreviations: MCP-1, monocyte chemoattractant protein; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

700 imaging densitometer (Bio-Rad Laboratories, Milan, Italy). To ascertain that blots were loaded with equal amounts of protein lysates, they were also incubated in the presence of the antibodies against GAPDH or β–actin (Sigma-Aldrich) or lamin A (Chemicon, Temecula, California) proteins.

*Measurement of nitrite in supernatants.* J774A.1 macrophages ($10^6$/P60 dish) or peritoneal macrophages ($3 \times 10^{-6}$/P60 dish) after 2 h of starvation in 5% FBS Red Phenol free DMEM, were treated with all NDL-PCBs tested, alone or differently associated (300 nM), in the presence of LPS (10 or 1 μg/ml, respectively). After 24-h exposure, nitrite ($NO^{2-}$), the stable end products of NO, was measured in the supernatant by Griess reaction. One hundred microliters of cell culture supernatant was added to 100 μl Griess reagent [0.1% (w/v) naphtylethylenediamine-HCl and 1% (w/v) sulphanilamide in 5% (v/v) phosphoric acid (vol.1:1)]. The $OD_{550}$ was measured using a microplate reader Titertek. Nitrite concentrations were determined by linear regression from a sodium nitrite standard curve freshly prepared in culture medium and expressed as micromolar unit.

*Endocytosis of HRP and its quantification.* In order to evaluate the effect of PCBs on the endocytic function, J774A.1 cells were plated $1.5 \times 10^6$ cells/P60 dish and incubated with PCBs at 300 nM, both alone and in association, in the presence or absence of LPS (10 ng/ml) for 24 h. The capacity of J774A.1 to internalize HRP was determined, as described previously (Ferrante *et al.*, 2002), by incubating the cells at 37°C with HRP (1 mg/ml) dissolved in DMEM containing 10% BSA for 80 min according to the method of Noble *et al.* (1994). Internalization was stopped by transferring the cells to 4°C and immediately rinsing in ice-cold 0.5% BSA in DMEM, followed by 4 washes of 5 min each in order to minimise non-cell bound HRP. Finally, the cells were lysed in lysis buffer (50 mM Tris–HCl, pH 7.8, 150 mM NaCl, 10 mM EDTA, 1% TX-100, 0.1% sodium deoxycholate and 1 mM PMSF). The HRP activity was assayed by the TMB method according to the manufacturer's instructions.

A standard curve was prepared using 0–33.3 ng/ml dissolved in 0.05% TX-100. Briefly, 10 μl of each HRP standard concentration or the same volume of sample dilutions (in triplicate) was added to each well of the 96-well plate. About 100 μl of the TMB complete substrate were then added and incubated until a brilliant blue colour developed. The reaction was terminated with 100 μl 2 M sulphuric acid and the absorbance read at 450 nm. The sample content of HRP was calculated from a linear regression analysis of the standard curve and expressed as μg of peroxidase/mg of proteins.

*Data analysis.* Data are reported as mean ± standard error mean (SEM) values of independent experiments, which were done at least 3 times, each time with 3 or more independent observations. Statistical analysis was performed by analysis of variance (ANOVA) test for multiple comparisons, followed by Bonferroni's test or, when appropriate, with Dunnet's test. Statistical significance was set at $P < .05$. Moreover, we performed a Kolmogorov-Smirnov test of normality, to test whether the residuals of the observations were normally distributed (which is one of the assumptions of the ANOVA). The results confirmed the null hypothesis of Gaussian distributed residuals, since the $P$ values were all $> 0.1$. Each analysis and calculations were performed by GraphPad Prism 5.

## RESULTS

### *Effects of PCBs, Alone or in Combination, on LPS-Induced iNOS and COX-2 Expression, Nitrite Production, and Cytokines Release in Peritoneal Macrophages*

We evaluated the impairment of macrophage responsiveness in primary rat peritoneal macrophages, focusing on pro-inflammatory enzyme induction (COX-2 and iNOS, and nitrite formation) and cytokine production. PCBs mixture incubation was performed on rat primary macrophages.The LPS-induced increase in iNOS expression was significantly reverted by all PCB mixtures ($P < .001$; Figs. 1A and 1B). Moreover, also COX-2 expression was markedly reduced in PCBs exposed macrophages, indicating a dampening of inflammatory response (Figs. 1A and 1C). Accordingly $NO^{2-}$ production showed a trend of reduction when macrophages were incubated with PCBs combined 2 by 2 and it became significantly lower compared with LPS-stimulated cells, when all PCBs were mixed, showing a synergistic effect ($P < .01$; Fig. 1D).

This effect was related to the suppression of NF-κB activation, through the reduction of p65 translocation into the nucleus in primary macrophages exposed to LPS and PCBs mixtures (data not shown).

The inhibitory effects of PCBs on LPS-driven macrophage activation were also strengthened by the results obtained with the evaluation of cytokine release in primary macrophage supernatants after 24-h exposure to LPS in the presence or absence of PCB combinations. Consistently with our findings, LPS significantly increased IL-6, TNFα and MPC-1 release ($P < .001$; Figs. 1E–G). PCB mixtures, 2 by 2 or all together at 300 nM final concentrations, strongly counteracted LPS effects on IL-6 and MCP-1. Conversely, regarding their effects on LPS-driven TNFα release, only the mixture of all PCBs led to a slight decrease of its production.

### *Effects of PCBs on Cell Viability of LPS-Stimulated J774A.1*

In another set of experiments performed on J774A.1 cell line, we confirmed the alteration of macrophage responsiveness and analyzed in more detail the mechanisms underlying PCBs immunotoxic effect.

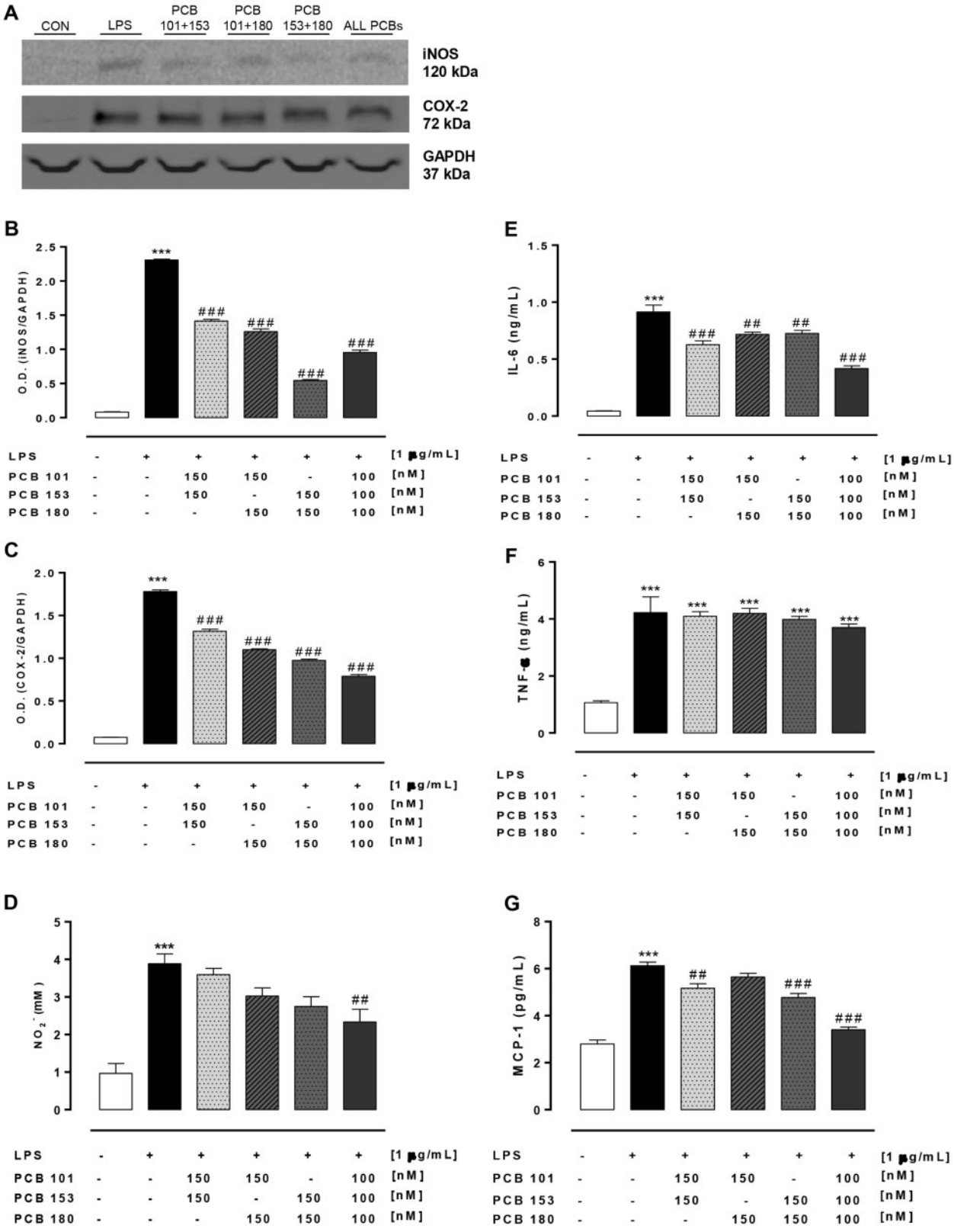


FIG. 1. Western blot analysis of inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 expression in lipopolysaccharide (LPS)-stimulated primary macrophages is reported (A). Densitometric analysis of protein bands of iNOS (B) and COX-2 (C) was performed on 3 separate experiments. GAPDH protein immunoblot was performed to ensure equal sample loading. $NO^{2-}$ (D), IL-6 (E), TNFα (F) and MCP-1 (G) levels were also reported. Lysates and supernatants were obtained from control and 24 h-treated cells with 300 nM polychlorinated biphenyls (PCBs), differently associated, in the presence of LPS (1 μg/ml) stimulus. $NO^{-2}$ values (μM) and cytokine levels (ng or pg/ml) are means ± SEM of 3 determinations. As specified in the Materials and Methods section, when PCBs were used in combination, final concentration of pollutants was always 300 nM. $^{***}P < .001$ versus control cells; $^{\#\#}P < .01$, and $^{\#\#\#}P < .001$ versus LPS-treated cells.

The cytotoxicity of J774A.1 cells incubated for 24 h with increasing concentrations of all PCBs in the presence of LPS (10 ng/ml) was evaluated by MTT assay (Figs. 2A–C). We showed that PCB 101, 153, and 180, *per se* without LPS stimulus, did not modify cell viability up to 1 μM ($1 \times 10^{-6}$ M) (Ferrante *et al.*, 2011). We also performed experiments to assess the effects of PCB combinations in the presence or absence of LPS on cell viability (Fig. 2D). PCBs, 2 by 2 or all together at 300 nM final concentration, were not able to modify cell viability compared with untreated control cells. Moreover, all combinations tested in the presence of LPS did not modify cell viability compared with LPS-exposed cells.

As shown in Figure 2, all PCBs induced a concentration-dependent effect on cell death. In particular, PCB 101 and PCB 180 induced a significant increase in cell death at the highest concentration tested ($10^{-5}$ M) compared with untreated LPS-stimulated cells (Figs. 2A and 2C), while PCB 153 was significantly cytotoxic starting from $3 \times 10^{-6}$ M (Fig. 2B).

The PCB concentration of 300 nM ($3 \times 10^{-7}$ M) was chosen for further experiments on macrophage responsiveness to LPS, since this final concentration for all PCBs alone or in combination did not modify cell viability.

As reported in Table 2, the PCB 180 was the most cytotoxic showing an $IC_{50}$ value of $1.41 \times 10^{-5}$ M while the $IC_{50}$ values of PCB 101 and PCB 153 were $1.91 \times 10^{-3}$ M and $2.19 \times 10^{-3}$ M, respectively, when used alone without LPS challenge. Indeed, when cells were costimulated for 24 h with LPS, toxic effects of these pollutants were increased with $4.57 \times 10^{-4}$ M, $4.79 \times 10^{-5}$ M and $1.15 \times 10^{-5}$ M $IC_{50}$ values for PCB 101, 153, and 180, respectively.

### *PCBs Reduce Synthesis and Release of Proinflammatory Cytokines and MCP-1 in LPS-Activated Cells*

The pattern of cytokine expression after LPS challenge depends on time of exposure and concentration of bacterial endotoxin and it is related to the different regulation of TLR4 at transcriptional level (Huang *et al.*, 2012). Cytokines, such as IL-6 and TNFα, together with MCP-1 are involved in cell recruitment and play a key role in orchestrating innate immune response. As shown in Figure 3, LPS induced a significant increase in IL-6, TNF-α, and MCP-1 mRNA levels in J774A.1 cells 4 h after challenge. Among the PCBs analyzed, PCB 101 did not significantly modify cytokine/chemokine transcription. Conversely, PCB 153

**TABLE 2.** Effects of PCBs 101, 153, and 180, Alone (100 nM–10 μM) and in Combination with LPS (10 ng/ml), on J774A.1murine Macrophage Cell Line Viability

| Cell Treatment | $IC_{50}$ Value (M) |
|---|---|
| PCB 101 (100 nM–10 μM) | $1.91 \times 10^{-3}$ |
| PCB 153 (100 nM–10 μM) | $2.19 \times 10^{-3}$ |
| PCB 180 (100 nM–10 μM) | $1.41 \times 10^{-5}$ |
| PCB 101 (100 nM–10 μM) + LPS 10 ng/ml | $4.57 \times 10^{-4}$ |
| PCB 153 (100 nM–10 μM) + LPS 10 ng/ml | $4.79 \times 10^{-5}$ |
| PCB 180 (100 nM–10 μM) + LPS 10 ng/ml | $1.15 \times 10^{-5}$ |

The viability of control cells was designated as 100% and results were expressed as the concentration of NDL-PCBs able to induce the 50% of mortality in untreated or LPS-treated macrophages ($IC_{50}$). Results are expressed as mean ± SEM from at least 3 independent experiments. Abbreviations: LPS, lipopolysaccharide; PCBs, polychlorinated biphenyl.

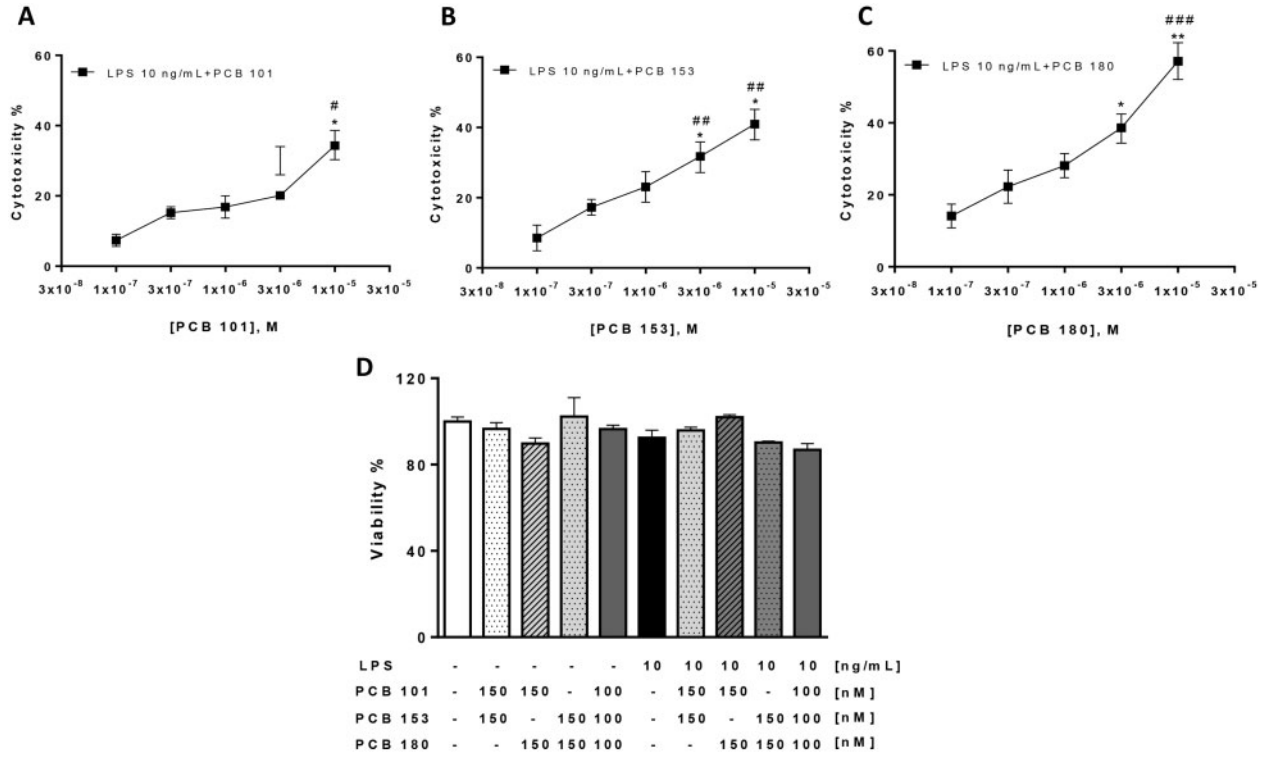


**FIG. 2.** Cytotoxic effects of polychlorinated biphenyl (PCB) 101 (A), 153 (B) and 180 (C) in combination with lipopolysaccharide (LPS) on macrophage J774A.1 cells. Cells were treated for 24 h with NDL-PCBs at increasing concentrations ($10^{-7}$–$10^{-5}$ M) in the presence of LPS (10 ng/ml). Cytotoxicity was then evaluated as described in Material and Methods section. The effect of PCB combinations in the presence or absence of LPS on cell viability is also shown (D). PCBs were combined 2 × 2 or all together, obtaining always 300 nM final concentration. Each per cent value is the mean ± SEM of 3 independent experiments. $^{*}P < .05$ and $^{**}P < .01$ versus untrated cells; $^{\#}P < .05$, $^{\#\#}P < .01$ and $^{\#\#\#}P < .001$ versus LPS stimulated cells.

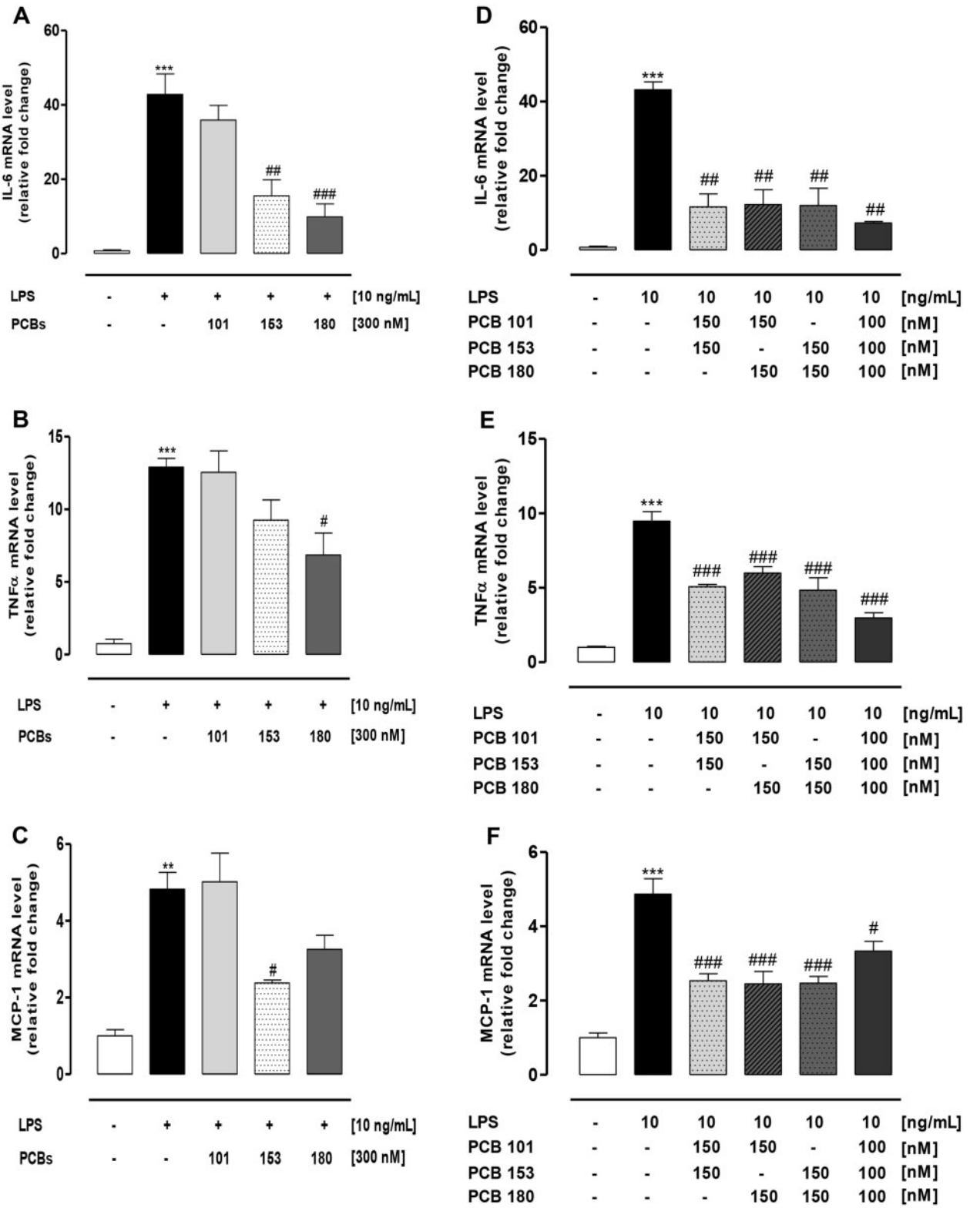


FIG. 3. Effects of polychlorinated biphenyl (PCB) 101, 153, and 180 alone (A–C) or in combination (D–F), on lipopolysaccharide (LPS)-induced mRNA expressions of IL-6 (panels A and D), TNFα (panels B and E), and MCP-1 (panels C and F). J774A.1 macrophages were exposed to PCBs at 300 nM, alone or mixed, in the presence of LPS (10 ng/ml) for 4 h. Therefore, cells were lysed for collection of RNA as described in Materials and Methods section. The mRNA expression levels were analyzed by real-time PCR. Data are means ± SEM of 3 independent experiments. As specified in the Materials and Methods section, when PCBs were used in combination, final concentration of pollutants was always 300 nM. $^{**}P < .01$ and $^{***}P < .001$ versus untreated cells; $^{\#}P < .05$; $^{\#\#}P < .01$; and $^{\#\#\#}P < .001$ versus LPS-treated cells.

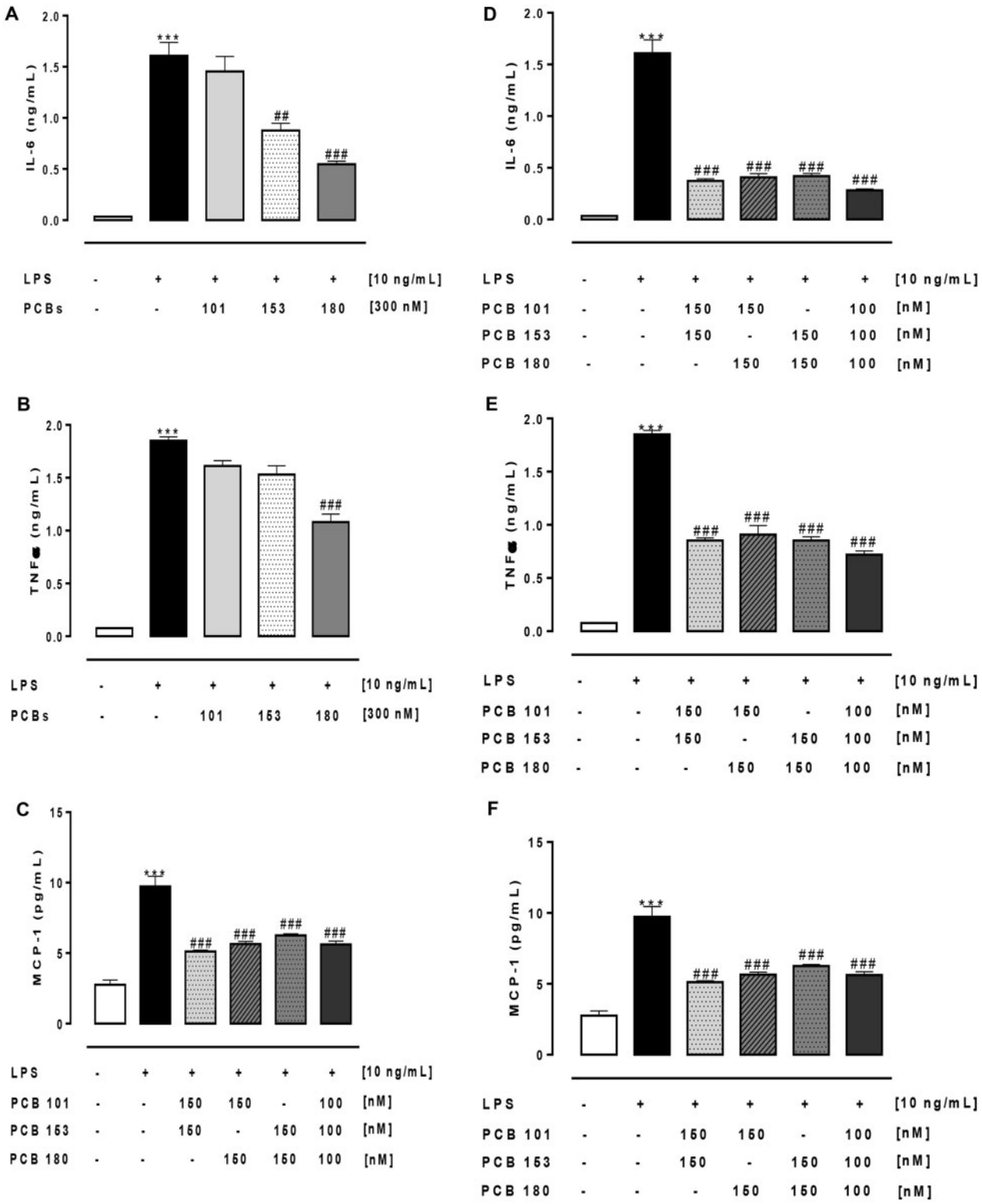


**FIG. 4.** Effects of polychlorinated biphenyl (PCB) 101, 153, and 180 alone (A–C) or in combination (D–F), on lipopolysaccharide (LPS)-induced release of IL-6 (A and D), TNFα (B and E), and MCP-1 (C and F). J774A.1 macrophages were exposed to PCBs at 300 nM, alone or mixed, in the presence of LPS (10 ng/ml) for 24 h. Therefore, supernatants were collected and cytokine levels were determined as described in Materials and Methods section. Data are means ± SEM of 3 independent experiments. As specified in the Materials and Methods section, when PCBs were used in combination, final concentration of pollutants was always 300 nM. $^{***}P < .001$ versus untreated cells; $^{\#\#}P < .01$ and $^{\#\#\#}P < .001$ versus LPS-treated cells.

and PCB 180 differently inhibited the LPS-induced expression of pro-inflammatory cytokines and MCP-1 (Figs. 3A–C, respectively). In particular, both PCB 153 and PCB 180 significantly suppressed LPS-induced IL-6 expression (Fig. 3A, $P < .01$ and $P < .001$, respectively). Solely PCB 180 significantly suppressed LPS-induced TNF-α expression (Fig. 2B), whilst PCB 153 reduced LPS-induced MCP-1 mRNA level (Fig. 3C; $P < .05$). It is noteworthy that all the combinations of PCBs synergistically reduced LPS-induced transcription of these proteins (Figs. 3D–F), while each single PCB at 100 or 150 nM did not modify cytokine transcription (data not shown).

Furthermore, these results regarding the capability of PCBs to suppress the LPS-driven cytokines and MCP-1 synthesis were confirmed and corroborated by the quantification of these immune mediators in J774.A1 supernatants after 24 h exposure to LPS in the presence or absence of PCBs, alone or in combination. As shown in Figure 4, LPS significantly increased the release of IL-6, TNFα and MCP-1 ($P < .001$), reaching concentrations shown in previous studies (Mazaleuskaya *et al.*, 2012; Sung *et al.*, 2015), and, PCBs, alone or mainly in combination, strongly counteracted the bacterial endotoxin effects, determining thus a reduction of the release of these essential immune mediators.

### *Effects of PCBs, Alone or in Combination, on LPS-Induced COX-2 Expression in J774A.1 Cells*

In order to confirm if PCBs were able to influence LPS-induced COX-2 expression, we evaluated the protein level in cellular lysates from J774A.1 macrophages exposed to these pollutants (300 nM) alone (Fig. 5A) or in combination (Fig. 5B). After 24 h LPS stimulation, a marked increase in COX-2 expression was observed compared with untreated cells ($P < .001$), that was unchanged by single PCBs (Fig. 5A). Conversely, when PCBs were combined 2 by 2 or alltogether, a strong reduction of COX-2 protein level was shown ($P < .001$; Fig. 5B).

### *Effects of PCBs, Alone or in Combination, on LPS-Induced iNOS Expression and Nitrite Production in J774A.1 Cells*

iNOS expression was evaluated by western blot analysis after 24 h LPS challenge. The bacterial endotoxin induced a significant increase in iNOS expression in J774A.1 cells (Figs. 6A and 6B), that was partially reverted only by PCB 180 exposure ($P < .05$, Fig. 6A). Conversely, all the combinations of PCBs markedly reduced LPS-induced iNOS expression ($P < .001$; Fig. 6B). Accordingly with iNOS induction, LPS significantly increased $NO^{2-}$ production (Figs. 6C and 6D), that was blunted solely by all PCB combinations, showing a synergistic effect ($P < .001$).

### *Effects of PCBs on LPS-Induced TLR4 and CD14 Upregulation in J774A.1 Cells*

Activation of TLR4 by LPS induces the activation of NF-κB, finally resulting in the release of pro-inflammatory cytokines and enzymes (Kagan and Medzhitov, 2006). To further investigate the mechanism underpinning the immune suppressive effects of NDL-PCBs, the expression of TLR4 was determined by real-time PCR in J774A.1 macrophages. The results showed that PCBs 101 and 180 down-regulated the expression of LPS-induced TLR4 in (Fig. 7A). Similar effect was shown for all PCB mixtures, except the combination of PCB 101 with PCB 153 (Fig. 7B). Consistently, in LPS-stimulated cells the upregulation of the co-receptor CD14 was blunted by single or combined PCBs (Figs. 7C and 7D).

### *Effect of NDL-PCBs 101, 153, and 180 on NF-κB Activation in J774A.1 Cells*

In order to determine the target pathways underlying the immune suppression caused by NDL-PCB exposure, we investigated their ability to inhibit LPS-induced NF-κB activation in J774A.1 macrophages. LPS induced a time-dependent IκB-α degradation and, accordingly, an increase in p65 NF-κB content in nuclear lysates (Figs. 8A and 8B), which was significant 30 min after LPS stimulation ($P < .001$). At this time, PCBs alone did not significantly modify LPS-induced effects (Figs. 8C and 8D);

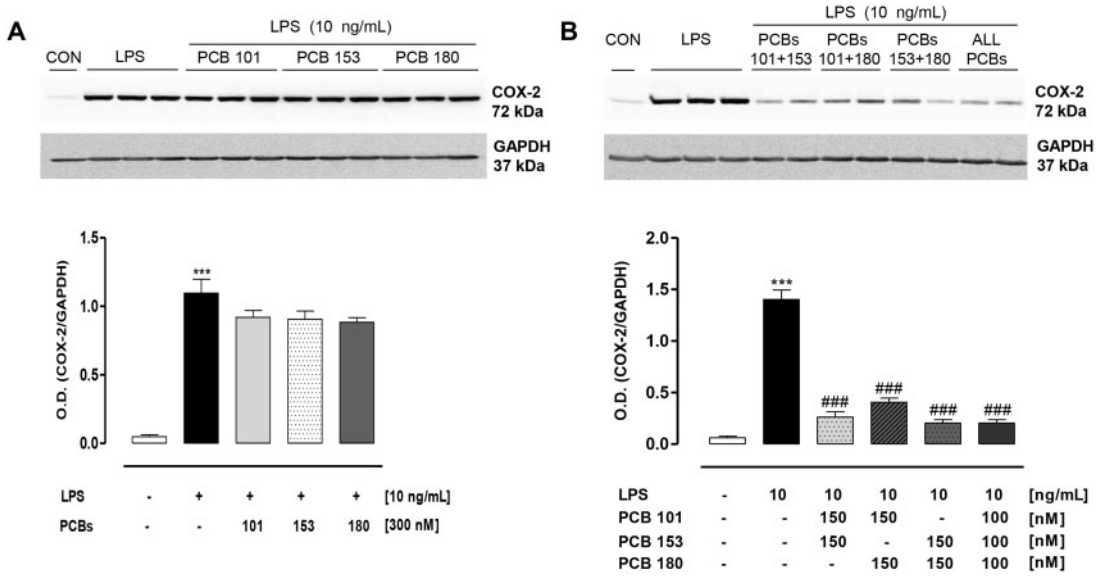


**FIG. 5.** Effects of polychlorinated biphenyl (PCB) 101, 153, and 180, alone (A) or in combination (B), in the presence of lipopolysaccharide (LPS) stimulus, on COX-2 protein expression. Lysates were obtained from control and 24 h-treated cells with PCBs at 300 nM, alone or differently combined, in the presence of LPS (10 ng/ml). Representative immunoblots are shown. Densitometric analysis of protein bands was performed on 3 separate experiments. GAPDH protein immunoblot was performed to ensure equal sample loading. As specified in the Materials and Methods section, when PCBs were used in combination, final concentration of pollutants was always 300 nM. $^{***}P < .001$ versus control cells; $^{###}P < .001$ versus LPS-treated cells.

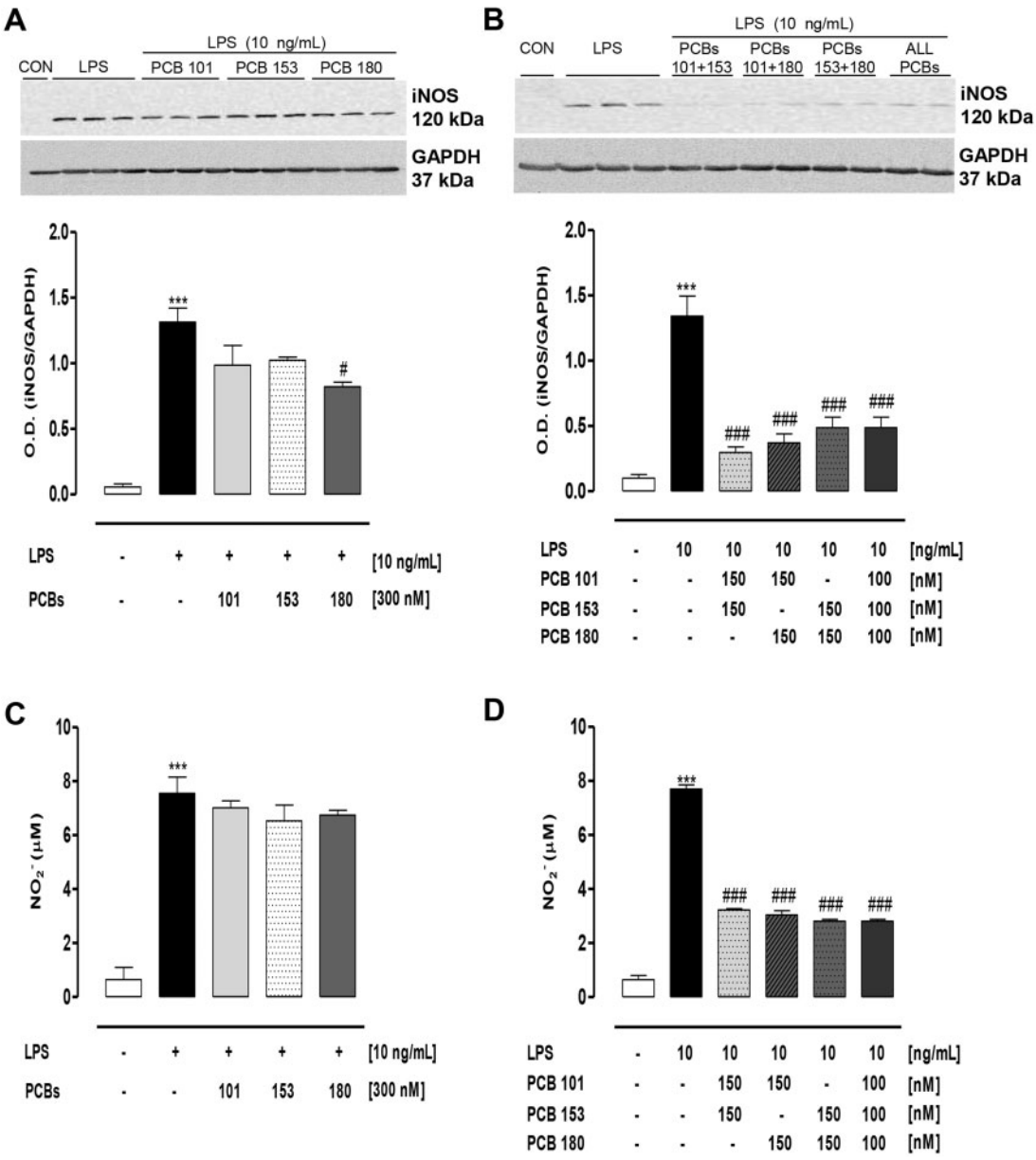


FIG. 6. Effects of polychlorinated biphenyl (PCB) 101, 153, and 180, alone (A and C) or in combination (B and D), on lipopolysaccharide (LPS)-induced iNOS protein expression and $NO^{2-}$ production by J774A.1 cells. Lysates and supernatants were obtained from control and 24 h-treated cells with PCBs examined at 300 nM, alone or differently associated, in the presence of LPS (10 ng/ml) stimulus. Representative immunoblots are shown. Densitometric analysis of protein bands was performed on 3 separate experiments. GAPDH protein immunoblot was performed to ensure equal sample loading. $NO^{-2}$ values (μM) are means of 3 determinations ± SEM. As specified in the Materials and Methods section, when PCBs were used in combination, final concentration of pollutants was always 300 nM. $^{***}P < .001$ versus control cells; $^{\#}P < .05$, and $^{\#\#\#}P < .001$ versus LPS-treated cells.

interestingly, IκB-α degradation and p65 translocation into the nucleus were obviated by PCB combinations (Figs. 8 E and 8F).

### *Effects of PCBs on HRP-Endocytosis in J774A.1 Cells*

Another approach in the present study was to evaluate the endocytic function after PCB exposure, using HRP, a fluid phase marker as tracer, to add functional data in support of the molecular modifications of macrophage in response to LPS stimulation. As reported in Figure 9, LPS significantly increased HRP endocytosis ($P < .001$) performed at 80 min and its effect was prevented in the presence of PCBs alone (Fig. 9A) or in combination ($P < .001$; Fig. 9B).

## DISCUSSION

In this study, we investigated the capability of NDL-PCBs 101, 153, and 180 to impair the innate immune response in macrophage after LPS challenge. Interestingly, we addressed their synergistic immunotoxic effect, mimicking the environmental condition, where organisms are, at the same time, potentially exposed to different PCB mixtures and bacteria.

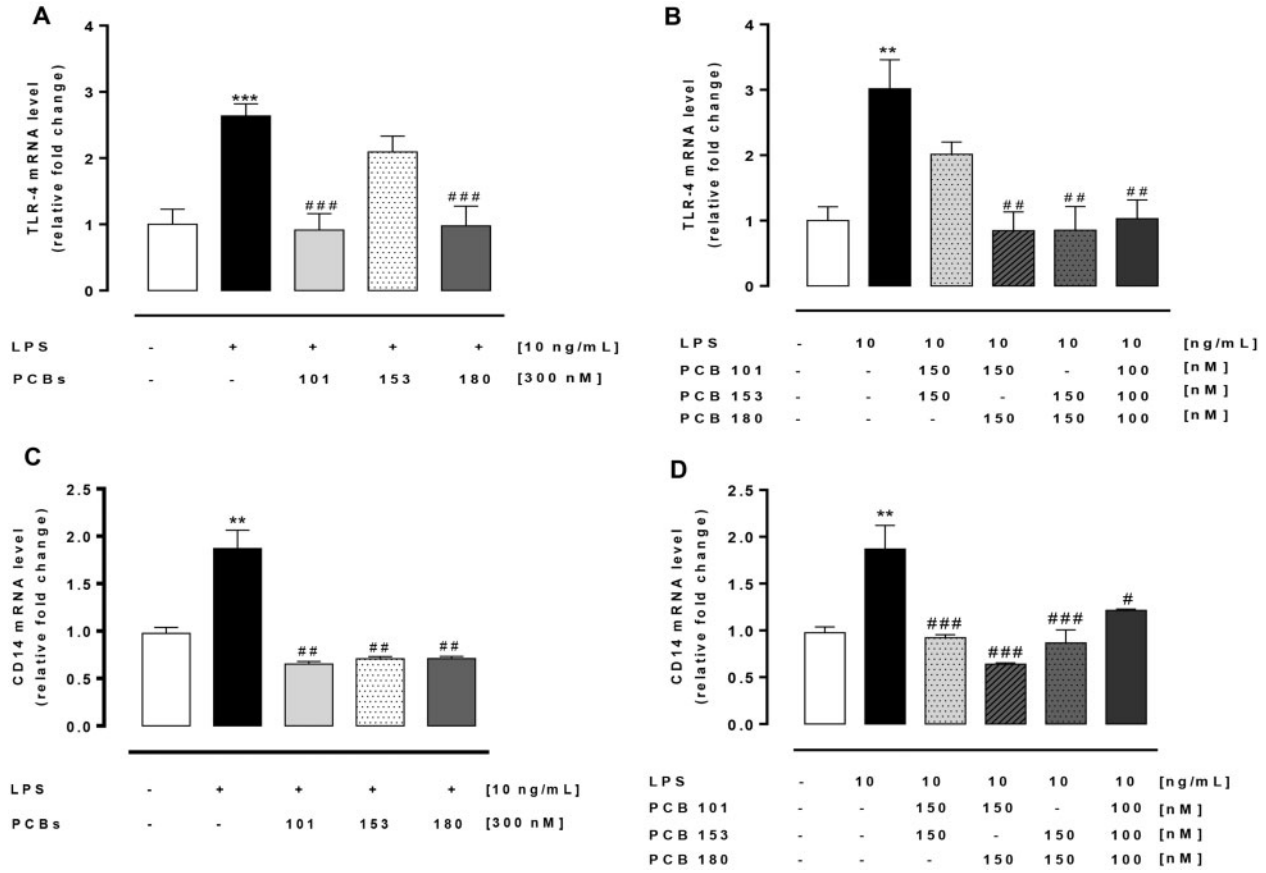


FIG. 7. Effects of polychlorinated biphenyl (PCB) 101, 153, and 180 alone (A and C) or in combination (B and D), on lipopolysaccharide (LPS)-induced TLR4 and CD14 mRNA expression. J774A.1 macrophages were exposed to PCBs at 300 nM, alone or mixed, in the presence of LPS (10 ng/ml) for 4 h. Therefore, cells were lysed to collect RNA, as described in Materials and Methods section. The mRNA expression levels were analyzed by real-time PCR. Data are means ± SEM of 3 independent experiments. As specified in the Materials and Methods section, when PCBs were used in combination, final concentration of pollutants was always 300 nM. $^{**}P < .01$ and $^{***}P < .001$ versus untreated cells; $^{\#}P < .05$; $^{\#\#}P < .01$ and $^{\#\#\#}P < .001$ versus LPS-treated cells.

Among pollutants, PCBs have a possibility to exacerbate infectious diseases because they disturb the human immune system also interfering with endocrine balance (Ferrante *et al.*, 2014; Igarashi *et al.*, 2006). In particular, PCBs affect lipid metabolism, endocrine function, and they can indirectly modulate or accelerate the metabolic alterations related to the pathogenesis of cardiovascular disease (Perkins *et al.*, 2015).

To date, there has been growing evidence suggesting the ability of NDL-PCBs, frequently detected in human samples, to weaken the immune response. Indicator PCBs, including PCB 101, 153, and 180, are known to be the predominant congeners in biotic and abiotic matrices (Storelli and Perrone, 2010). Moreover, the sum of the 6 indicator NDL-PCBs is, on average, about 5 times higher than the sum of the 12 DL-PCBs (EFSA 2010) that result a relatively minor component of the total body burden in humans (Cave *et al.*, 2010).

Here, we provide evidence that macrophage exposure to NDL-PCBs alters cell responsiveness, reducing the inflammatory innate immune response and showing their synergistic effects. Therefore, our study indicates the potential of NDL congeners, i.e. PCB 101, 153, and 180, in increasing the susceptibility to bacterial infection.

Our previous findings showed that these 3 NDL congeners not only were able to induce macrophage apoptosis in a concentration-dependent manner (Ferrante *et al.*, 2011), but were also more cytotoxic at concentrations that were inactive by themselves, showing a synergistic effect in inducing cell death. Here, we use these compounds at nanomolar concentrations, unable to modify macrophage viability, in order to identify a specific immune suppressive effect independently from cell death.

The final concentration of 300 nM, obtained by the mixture of all 3 NDL-PCBs and used to treat macrophages, corresponds to about 108 ppb (ng/ml). This concentration, as well as those of the single PCBs or combined 2 by 2, was close to those found in the serum (De Felip *et al.*, 2008; Pieters and Focant, 2014) and adipose tissue (Malarvannan *et al.*, 2013; Schiavone *et al.*, 2010; Tan *et al.*, 2008) of exposed people and/or present in food of animal origin, mirroring the exposure of humans and animals to NDL-PCBs.

It is well known that macrophages play an important role in the mechanisms of host's immune defense, recognizing patterns on foreign biological substances through the activation of pattern-recognition receptors, such as TLRs (Meli *et al.*, 2014). In particular, macrophages are activated by LPS, the major molecular component of the outer membrane of Gram-negative bacteria and a potent natural immune stimulator (Fujihara *et al.*, 2003), which was identified as a ligand of TLR4. LPS/TLR4 interaction initiates various signaling pathways leading to production of proinflammatory cytokines, enzymes and chemokines (Rodriguez-Vita and Lawrence, 2010), that collectively ramp up the host's immune defense. Here, on the basis of transcription

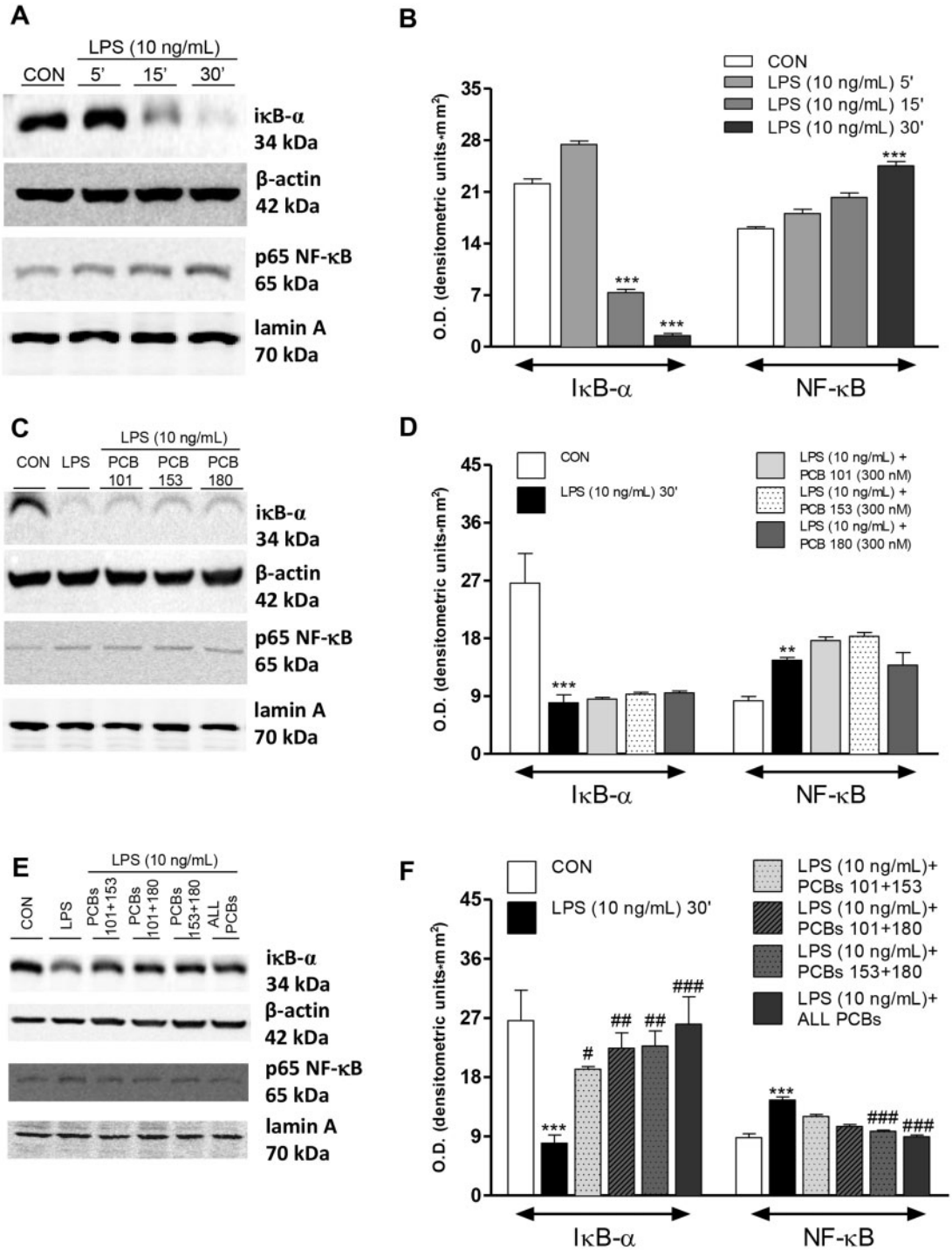


FIG. 8. Time course of IkB-α degradation and nuclear p65 NF-κB content after lipopolysaccharide (LPS) stimulation (A and B) and polychlorinated biphenyl (PCBs) effect alone (C and D) or in combination (E and F). Lysates of cells were obtained from control and LPS-treated (10 ng/ml) macrophages for 5-15-30 min (A and B) or from PCBs (300 nM) and LPS (10 ng/ml) costimulated macrophages for 30 min (C–F). Representative immunoblots are shown. Densitometric analysis of protein bands was performed on 3 separate experiments. β-Actin or lamin A protein immunoblot was performed to ensure equal sample loading. As specified in the Materials and Methods section, when PCBs were used in combination, final concentration of pollutants was always 300 nM. $^{**}P < .01$ and $^{***}P < .001$ versus control cells; $^{\#}P < .05$, $^{\#\#}P < .01$ and $^{\#\#\#}P < .001$ versus LPS-treated cells. < AQ4/>

timing of pro-inflammatory factors (Huang *et al.*, 2012), we found that NDL-PCBs showed a strong synergistic effects, dampening LPS-induced IL-6, TNFα,, and MCP-1 gene expression 4 h after challenge. Indeed, PCB 101, the penta-chlorinated congener, was the less immunotoxic among PCB analyzed, whilst both PCB 153 and PCB 180 (hexa-chlorinated and hepta-chlorinated congeners, respectively) significantly suppressed LPS-induced IL-6 expression. PCB 153 also reduced LPS-induced MCP-1 mRNA level and PCB 180, on the other hand, suppressed TNF-α expression. These latter results are consistent with those

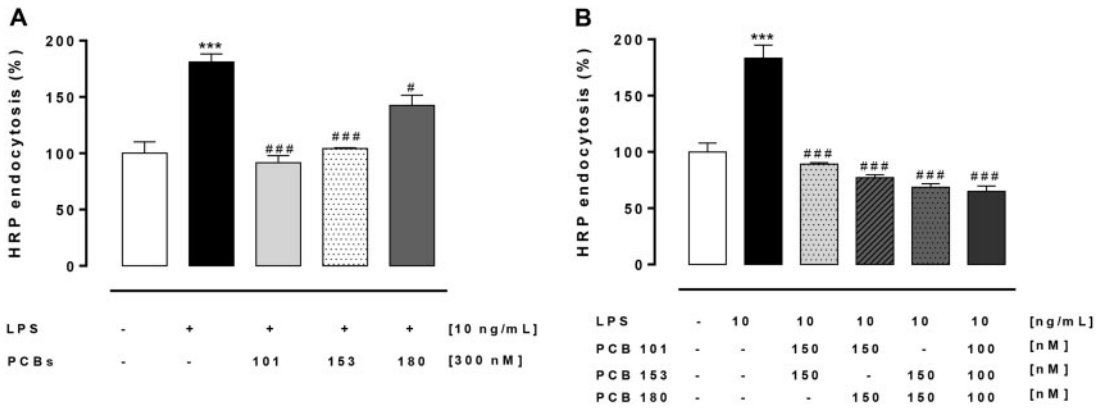


**FIG. 9.** Endocytosis of HRP (1 mg/ml) after 80 min of exposure of J774A.1 cells pretreated for 24 h with polychlorinated biphenyl (PCBs) (300 nM), alone (A) or in combination (B), in the presence of lipopolysaccharide (LPS) (10 ng/ml). The values are expressed as % of HRP endocytosis of control cell lysates and are the means ± SEM of 3 independent experiments. As specified in the Materials and Methods section, when PCBs were used in combination, final concentration of pollutants was always 300 nM. $^{***}P < .001$ versus control cells; $^{\#}P < .05$ and $^{\#\#\#}P < .001$ versus LPS-treated cells.

by Hong *et al.* (2004), showing the ability of other EDCs to reduce or counteract the LPS-induced expression of TNFα in macrophage cell line RAW 264.

During inflammation and immune response, such as the process of host defense, endotoxins and cytokines induce rapid alterations in cellular immediate-early gene expression, leading to the *de novo* synthesis of COX-2 (Emami *et al.*, 2010; Ferrante *et al.*, 2008) and iNOS (Meli *et al.*, 2000). Our data clearly demonstrated that macrophages exposed to NDL-PCB mixtures were less able or unable to orchestrate a proper immune response against LPS, since LPS-induced COX-2 and iNOS expression was suppressed by the simultaneous incubation of this cells with PCB combinations and LPS. As known, macrophages have a wide repertoire of chemical signals to communicate with other immunocompetent cells, for example, LPS-stimulated macrophages generate excess NO through the action of iNOS (Zhou *et al.*, 2014). In our experimental conditions, NDL-PCBs, especially in combination, strongly inhibit LPS-induced $NO^{-2}$ production. Our data are consistent with those by Hong *et al.* (2004) and Yoshitake *et al.* (2008), which highlighted the ability of several EDCs to suppress NO production in *in vitro* LPS-stimulated macrophages, suggesting that they interfere with NO-mediated signaling and host defense system against foreign pathogens.

Since the activation of NF-κB is essential for the induction of inflammatory cytokines, enzymes, and mediators, we investigated the effects of NDL-PCBs on this pathway. We showed that NDL-PCBs alone did not significantly modify LPS-induced degradation of cytosolic IκB-α and the expression of nuclear p65 NF-κB, while all the examined PCB combinations reverted the effects on NF-κB activation on J774A.1. The reduction of NF-κB activation was also determined in primary macrophages (data not shown). Therefore, all these data suggest that PCBs mixture exposure suppress the production of inflammatory enzymes by modulating their expression at transcriptional level. Other researchers, in agreement with our data, demonstrated that other EDCs inhibit bacteria-induced activation of NF-κB, highlighting their potential to exacerbate infectious diseases (Igarashi *et al.*, 2006; Ohnishi *et al.*, 2008). Moreover, we also investigated whether the immune suppressive effects exerted by PCBs could involve transcription level of TLR4, which is the main target of LPS. Our results showed that NDL-PCBs, especially in combinations, inhibited the LPS-induced TLR4 expression, and conceivably the activation of NF-κB. These data were strengthened by the parallel reduction of CD14 expression which is reported to be necessary not only for LPS recognition, but also for TLR4 signal transduction (Zanoni and Granucci, 2013). Nevertheless, CD14-deficient mice are highly resistant to LPS-induced shock and monocytes derived from CD14-deficient mice are insensitive to LPS as determined by a decrease in IL-6 production, suggesting the essential role for CD14 in binding to LPS (Haziot *et al.*, 1996).

Additionally, Levin *et al.* (2005a) showed a positive correlation between the NDL-PCB exposure and the reduction in phagocytosis in healthy human leukocytes. Epidemiological and toxicological data on PCBs exposure and non-Hodgkin lymphoma occurrence were reviewed by Kramer *et al.* (2012), addressing the role on immune dysregulation. In particular, the authors highlight the impairment of immune cells in identifying foreign antigens, limiting the early cytokine and chemokine production and signaling, that initiate an effective and complete immune response. Our study demonstrates that all PCBs alone and their combinations decrease endocytosis. In macrophages HRP is taken up by fluid-phase endocytosis (Steinman and Cohn, 1972), involving the uptake of macromolecules from extracellular fluid into membrane-bound endocytic vesicles (Silverstein *et al.*, 1977). Neither membrane receptors nor cell surface adsorption appear to be implicated in this process and as a result, uptake is concentration dependent. In our experiments the internalization of HRP is clearly modified by cell exposure to PCBs, causing a reduction of cell endocytosis capability.

Disturbance of well-orchestrated immune response may results in the development of serious infectious diseases. In agreement, several epidemiological studies demonstrated a positive correlation between long-term PCB exposure and a weakening of immune defenses and increased incidence of ear or respiratory infections (Dewailly *et al.*, 2000; Heilmann *et al.*, 2006; Weisglas-Kuperus *et al.*, 2000, 2004), reduced functional capacity of lymphocytes, as indicated by decreased responses to mitogen stimulation (Belles-Isles *et al.*, 2002) and insufficient antibody response to vaccination (Heilmann *et al.*, 2006, 2010).

Taken together, our data show that NDL-PCB mixtures reduce the ability of macrophages to respond properly to noxious stimuli, such as LPS, by interfering with TLR4/NF-κB pathway. Thus, NDL-PCB mixtures might cause, as other immunotoxic pollutants, a nonphysiological response to bacterial infections accordingly with the observed direct correlation between PCB exposure and the increased occurrence of bacterial diseases shown in epidemiological studies.

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