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Chronic Estradiol Administration In Vivo Promotes the Proinflammatory Response of Macrophages to TLR4 Activation: Involvement of the Phosphatidylinositol 3-Kinase Pathway[1]

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Short-term exposure to 17β-estradiol (E2) in vitro has been reported to decrease the production of proinflammatory cytokines by LPS-activated macrophages through estrogen receptor α (ERα)-dependent activation of the PI3K pathway. In the present study, we confirm that in vitro exposure of mouse peritoneal macrophages to E2 enhanced Akt phosphorylation and slightly decreased LPS-induced cytokine production. In striking contrast, we show that chronic administration of E2 to ovariectomized mice markedly increases the expression of IL-1β, IL-6, IL-12p40, and inducible NO synthase by resident peritoneal macrophages in response to LPS ex vivo. These results clearly indicate that short-term E2 treatment in vitro does not predict the long-term effect of estrogens in vivo on peritoneal macrophage functions. We show that this in vivo proinflammatory effect of E2 was mediated through ERα. Although the expression of components of the LPS-recognition complex remained unchanged, we provided evidences for alterations of the TLR4 signaling pathway in macrophages from E2-treated mice. Indeed, E2 treatment resulted in the inhibition of PI3K activity and Akt phosphorylation in LPS-activated macrophages, whereas NF-κB p65 transcriptional activity was concomitantly increased. Incubation of macrophages with the PI3K inhibitor wortmanin enhanced proinflammatory cytokine gene expression in response to TLR4 activation, and abolishes the difference between cells from placebo- or E2-treated mice, demonstrating the pivotal role of the PI3K/Akt pathway. We conclude that the macrophage activation status is enhanced in vivo by E2 through ERα and, at least in part, by the down-modulation of the PI3K/Akt pathway, thereby alleviating this negative regulator of TLR4-signaling. *The Journal of Immunology,* **2008, 180: 7980–7988.**

I n addition to their pivotal role in sexual development and reproduction, estrogens have been reported to influence the incidence and the course of numerous pathophysiological processes, such as infections, and autoimmune and cardiovascular diseases (1–3). Furthermore, recent in vivo experimental studies demonstrated that 17β-estradiol (E2), the main endogenous estrogen, regulates several immune responses in mouse models (4–6). Most, if not all, of the effects of estrogens are mediated by two members of the nuclear receptor superfamily, estrogen receptor (ER)[3] α and β, encoded by 2 distinct genes, *Esr1* and *Esr2*.

Macrophages are crucial cells of the innate immune system that not only contribute to the first line of defense against pathogens, but also play an important role in directing adaptive immune responses. These cells express a broad range of pattern recognition receptors that mediate their interactions with natural and altered-self components of the host, as well as microorganisms (7). Among them, members of the TLR family recognize specific molecular patterns present in microbial components, leading to macrophage activation (8). One of the most potent inflammatory agents able to activate macrophages is LPS, a component of the outer membrane of Gram-negative bacteria that binds to CD14, a membrane anchored protein, and stimulates the TLR4-MD2 complex in macrophages. TLR4 signaling results in a rapid and strong production of inflammatory mediators and cytokines by macrophages, such as IL-1α, IL-1β, IL-6, IL-12p40, and TNF-α (9), mainly through the activation of the NF-κB and MAPK signaling pathway

[3] Abbreviations used in this paper: ER, estrogen receptor; iNOS, inducible NO synthase; qRT-PCR, quantitative real time-PCR; PLSD, protected least significant difference.

(10). Interestingly, TLR4 triggering induces the concomitant activation of the PI3K pathway that was recently shown to negatively regulate the production of IL-12 and IL-1β by dendritic cells and human monocytes, respectively (11–15).

Previous reports, most of them based on in vitro studies, suggested that E2 exerts anti-inflammatory properties on LPS-activated monocyte/macrophage cell lines or microglial cells, macrophage cells of the CNS (16–19). For instance, recent experiments have shown that short-term exposure to E2 prevented NF-κB nuclear translocation in response to LPS, leading to the inhibition of inducible NO synthase (iNOS) expression in the macrophage cell line RAW 264.7 and in microglial cells (16). This anti-inflammatory effect was mediated by ERα, leading to the subsequent activation of the PI3K pathway before TLR4 stimulation with LPS (16). In contrast, Soucy et al. (20) recently reported that long-term in vivo exposure to estrogens from endogenous or exogenous origin enhanced the LPS-induced transcription of proinflammatory cytokines, namely IL-12 and TNF-α, by microglial cells through ERα-dependent mechanisms. These divergent results could have been due to differences in cell type, duration (short-term vs long-term) of E2 exposure, and of course in vitro vs in vivo conditions. To further define the modulatory action of estrogens on macrophages in vivo, we studied the effect of E2 on the TLR4-dependent production of proinflammatory cytokines by murine resident peritoneal macrophages.

Materials and Methods

Mice and surgical procedure

Female C57BL/6J mice were purchased from Charles River Laboratories. ERα-deficient (ER$\alpha^{-/-}$) mice were bred in our animal facilities in heterozygotic state, and screened by PCR genotyping essentially as previously described (21). All mice were maintained under specific pathogen-free conditions, and procedures were performed in accordance with the recommendations of the European Accreditation of Laboratory Animal Care. Female mice were ovariectomized or sham-operated at 4 wk of age. Ovariectomized mice were given either placebo or E2 60-day time-release pellets (0.1 mg, 80 μg/kg/day; Innovative Research of America), implanted subcutaneously in the scapular region, as previously described (5). Mice were sacrificed 4 wk later by CO_2 inhalation, peritoneal cells were collected, and uterine weight was individually recorded to assess either the efficiency of ovariectomy or the exposure to E2 in vivo.

Resident peritoneal macrophages preparation and cell culture conditions

Resident peritoneal cells were harvested from mice peritoneal cavities by two peritoneal washes with phenol red-free serum-free X-Vivo 15 medium (Cambrex) containing heparin (20 UI/ml). After centrifugation (10 min, 328 $\times$ g), cells were resuspended in X-Vivo 15 medium supplemented with 50 mg/ml gentamicin (Invitrogen), 2.5 mg/ml amphotericin B (Biochrom), and 2 mM L-glutamine (Invitrogen), and macrophages were counted using a Neubauer slide in the presence of trypan blue (Eurobio). According to experimental design, cells were plated in either 96- (BD Biosciences), 24-, or 12-well plates (Costar), respectively, at 2 $\times$ 10^5, 5 $\times$ 10^5, or 10^6 macrophages per well. Peritoneal cells were incubated for 2 h (37°C, 5% CO_2) to allow macrophage adhesion, then, were washed three times to remove non-adherent cells. This protocol resulted routinely in a culture containing >95% macrophages. To study E2 effect in vitro, macrophages were incubated with 10 nM E2 (Sigma-Aldrich) diluted in DMSO (0.1% final), 2 h before and during activation with 20 ng/ml opsonized LPS from *Escherichia coli* 0111:B4 (Sigma-Aldrich). For in vivo studies, peritoneal macrophages from placebo- or E2-treated mice were harvested and stimulated in vitro with LPS in the absence or presence of E2 (1 nM), respectively. In some experiments, inhibition of PI3K activity was performed by incubating macrophages with 50 nM wortmannin (United States Biological) 1 h before and during LPS activation.

Quantitative real time-PCR (qRT-PCR)

After stimulation of peritoneal macrophages for 4 h with LPS in 12-well plates, total mRNA were extracted with Trizol reagent according to the manufacturer's instructions (Invitrogen) and purified with a mammalian total RNA miniprep kit, Genelute (Sigma-Aldrich). mRNA quantity and quality were checked with a spectrophotometer (ND-1000; NanoDrop Technologies). Reverse transcription was performed with 500-1000 ng of total mRNA, using High Capacity DNA Archive kit (Applied Biosystems). A total of 25–50 ng of reverse transcribed RNA were used as template for qRT-PCR, set up in 96-well plates using PCR Mastermix (Power SYBR Green; Applied Biosystems). Gene expression level was quantified using ABI Prism 7900 sequence detection system (Applied Biosystems). Primers were as follows; IL-1β: sense 5′-CAACCAACAAGTGATATTCTC CATG-3′, antisense 5′-GATCCACACTCTCCAGCTGCA-3′; IL6: sense 5′-GAGGATACCACTCCCAACAGACC-3′, antisense 5′-AAGTGCAT CATCGTTGTTCATACA-3′;IL12p40:sense5′-GGAAGCACGGCAGCA GAATA-3′, antisense 5′-AACTTGAGGGAGAAGTAGGAATGG-3′; TNF-α: sense 5′-CATCTTCTCAAAATTCGAGTGACAA-3′, antisense 5′-TGGGAGTAGACAAGGTACAACCC-3′; iNOS: sense 5′-CAGCTGG GCTGTACAAACCTT-3′, antisense 5′-CATTGGAAGTGAAGCGTTT CG-3′; TLR4: sense 5′-CCTGACACCAGGAAGCTTGAA-3′, antisense 5′-TCCATGCATTGGTAGGTAATATTAGG-3′; and GAPDH: sense D3: 5′-AGGTCGGTGTGAACGGATTTG-3′, antisense D3: 5′-TGTAGAC CATGTAGTTGAGGTCA-3′. Primers for IL-1β, TNF-α, IL-12p40, IL-6, and iNOS have been previously published (22). MyD88 primers were designed by Qiagen (Mm_MyD88_1_SG QuantiTect Primer assay). Results were analyzed using the SDS program, version 2.2 (Applied Biosystems). The relative expression of target transcripts in each sample was normalized to GAPDH and compared with the expression in a stimulated or nonstimulated control according to the ΔCt method: $\Delta Ct_{sample} = Ct_{sample}-Ct_{GAPDH}$, $\Delta\Delta Ct_{sample} = \Delta Ct_{sample}-\Delta Ct_{control}$, Relative expression$_{sample}$ = $2^{-\Delta\Delta Ctsample}$.

Cytokine production by peritoneal macrophages

Macrophages were stimulated with 20 ng/ml LPS for 24 h, and supernatants were collected and stored at -80°C until IL-6 and TNF-α quantification. For IL-1β, 5 mM ATP was added to culture medium during the last 3 h of stimulation to convert pro-IL-1β to IL-1β, as previously described (23, 24). IL-1β, IL-6, and TNF-α were quantified using specific enzyme-linked immunosorbent assays (BD Biosciences), with a sensitivity of 15 pg/ml. Results were expressed for individual mice as cytokine amounts (ng) normalized to 10^6 input macrophages.

Flow cytometry analysis and Abs

Before staining, cells (5–10 $\times$ 10^5) were incubated 15 min at room temperature with blocking buffer (PBS with 1% SVF, 3% normal mouse serum, 3% normal rat serum, 5 mM EDTA, and 1% NaN$_3$) containing 5 μg/ml anti-CD16/CD32 (2.4G2; BD Biosciences) to block Fcγ III/II receptors. Cells were then incubated for 30 min with FITC-, PE-, or allophycocyanin-conjugated mAbs diluted at the optimal concentration in FACS buffer (PBS, 5% SVF, 5 mM EDTA, and 1% NaN$_3$). Total peritoneal cells were stained with anti-CD11b allophycocyanin (M1/70.15; BD Biosciences), with anti-CD19 PE (1D3; BD Biosciences), and with anti-F4/80 FITC (BM8; eBioscience) or anti-MHC class II FITC (M5/114.15.2; eBioscience), or anti-CD14 FITC (mC5–3; BD Biosciences). Then, cells were washed three times and data were collected on a FACSCalibur flow cytometer (BD Biosciences). Expression of F4/80, MHC class II, and CD14 on macrophages was analyzed after gating on CD11b^{high}CD19^{negative} cells by using the CellQuest software (BD Biosciences).

Western blot analysis and Abs

Peritoneal macrophages were lysed with Laemmli buffer (25 mM Tris (pH 8), 200 mM glycine, and SDS 0.25%) supplemented with antiproteases (complete EDTA-free; Roche), 2 mM orthovanadate (Sigma-Aldrich), 1 mM PMSF (Sigma-Aldrich), and 1 mM NaF (Sigma-Aldrich). Protein extracts were sonicated and centrifuged (16,000 $\times$ g, 4°C, 15 min) to clarify supernatant. Proteins were quantified with the Bicinchoninic Acid technique (Interchim). Samples were heated to 95°C 2 min, with loading buffer (Tris (pH 8), 2% SDS, 10 mM EDTA, 0.01% bromphenol blue, 25 mM 1,4-dithio-DL-threitol, and 250 mM 2-ME) and 20 μg of proteins were separated by 10% SDS-PAGE. Proteins were then transferred on a 0.2 μm nitrocellulose membrane (Amersham Biosciences) in a transfer buffer (25 mM Tris (pH 8), 200 mM glycine, and 25% ethanol). Membranes were washed and blocked with 5% nonfat milk in TBST. Abs were incubated overnight at 4°C in TBST milk 5%. Membranes were washed three times for 10 min before addition of a secondary HRP-coupled Ab, in TBST milk 5% for 1 h at room temperature. Membranes were washed four times 15 min with TBST. Then ECL (Pierce) was added, and film (Kodak) was exposed and developed by Hyper Processor (Amersham
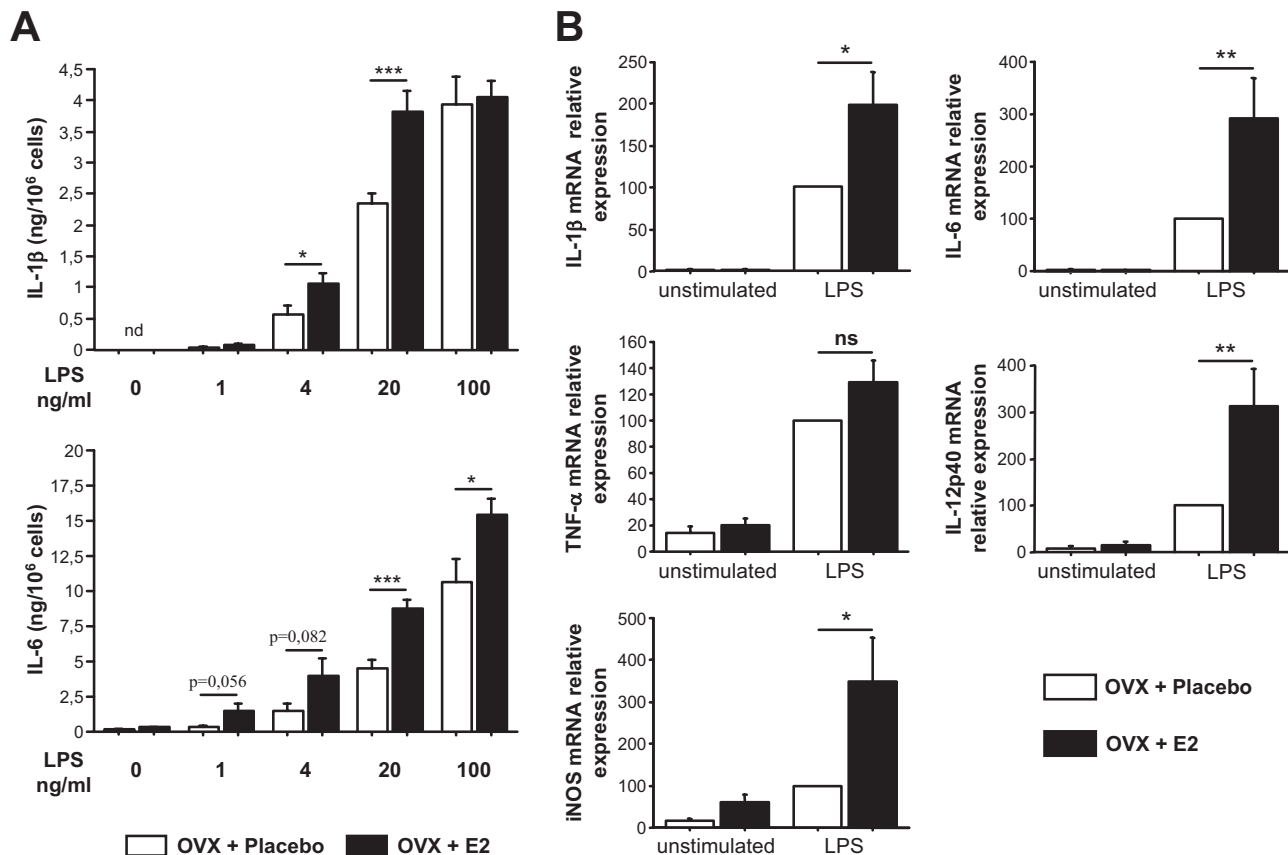
FIGURE 1. Chronic E2 treatment in vivo enhances the expression of several proinflammatory cytokines by resident peritoneal macrophages in response to LPS. Resident peritoneal macrophages from placebo- or E2-treated ovariectomized mice were activated with LPS: (*A*) for 24 h at indicated concentrations to evaluate cytokine production in culture supernatants by specific ELISA. At least eight individual mice from three independent experiments are considered in each group. Results are expressed as mean $\pm$ SEM of cytokine amounts (ng) normalized to 10^6 input macrophages; (*B*) for 4 h at 20 ng/ml to evaluate cytokines and iNOS mRNA expression by qRT-PCR. Results are normalized according to GAPDH mRNA level, referring to results obtained from LPS-activated macrophages from placebo-treated mice. Results are expressed as means $\pm$ SEM from three independent experiments, each including pools of fivw mice per group. *, $p < 0.05$; ** $p < 0.01$; ***, $p < 0.001$; ns = not significant; nd = not detectable.

Biosciences). Rabbit polyclonal anti-mouse C-terminal ERα (MC20; Santa Cruz Biotechnology) was used at 1/500. Goat polyclonal anti-mouse N-terminal ERβ (Y19; Santa Cruz Biotechnology) was used at 1/500. Monoclonal rabbit anti-phospho-Akt (serine 473) and anti-Akt2, rabbit polyclonal anti-Akt and anti-Akt3, and mouse monoclonal anti-Akt1 were all from Cell Signaling Technology and were used at 1/1,000. Mouse monoclonal anti-mouse β-actin (Sigma-Aldrich) was used at 1/5,000. Secondary polyclonal anti-mouse or -rabbit IgG HRP-coupled Abs were used at 1/5,000 to 1/20,000 (Amersham Biosciences).

PI3K activity assay

Macrophages were plated in 12-well plates and activated with LPS (20 ng/ml) for 10 min. For a negative control, wortmannin 200 nM was added before LPS stimulation. Cells were washed with PBS and lysis buffer was added (20 mM Tris-HCl (pH 8), 138 mM NaCl, 2.7 mM KCl, 5% glycerol, 1 mM MgCl$_2$, 1 mM CaCl$_2$, 1% NP40, 5 mM EDTA, 1 mM NaVO$_3$, 20 μM leupeptin, 20 μM aprotinin, and 20 mM NaF). Wells were scraped, then lysates were collected and centrifuged (15,000 $\times$ *g*, 10 min, 4°C) to clarify supernatant. A total of 5 μl of rabbit polyclonal Ab anti-PI3Kp85 (Millipore) was added, tubes were placed on wheel for 1 h at 4°C, then 5 mg of protA-Sepharose were added and tubes were kept on wheel 1 h at 4°C. Centrifugation was done to pellet the protein complex, which was washed once with the lysis buffer, twice with washing buffer 1 (0.1 M Tris-HCl (pH 7.4) and 0.5 M LiCl), and twice with washing buffer 2 (20 mM HEPES (pH 7.4) and 5 mM MgCl$_2$), to finally resuspend pellet with 40 μl of washing buffer 2. PI3K activity was performed in the presence of micelles of phosphatidylserine (2 mg/ml) and of phosphoinositol (1 mg/ml), 6 mM ATP, and 100 μCi/ml [γ-^{32}P]ATP in a final volume of 60 μl, at 37°C under agitation. Reac-

tion was stopped with 60 μl of 1 N HCl, and lipids were extracted with 200 μM methanol/CHCl$_3$ 1:1. Samples were deposed on a preactivated TLC plate by potassium oxalate solution. The plate was developed by chromatography in CHCl$_3$/MeOH/H$_2$O/NH$_4$OH 45:35:7:3.5. PI(3)^{32}P was detected by a phosphoimager, quantified by Imagequant and an exposed film was revealed as well.

NF-κB DNA binding assay

Macrophages (2.5 $\times$ 10^6 per well) from placebo- or E2-treated mice were stimulated with 20 ng/ml LPS for 30, 60, or 90 min, then nuclear protein extraction was performed using the NE-PER nuclear and cytoplasmic extraction reagents kit, according to the manufacturer's guidelines (Pierce). To quantify nuclear NF-κB p65 binding to a consensus NF-κB oligonucleotide, nuclear extracts (2.5, 5, or 10 μg) were analyzed with the NF-κB p65 EZ-TFA transcription factor assay kit (Millipore). Quantification was performed using the principle of a colorometric ELISA.

Statistical analysis

Results were expressed as means $\pm$ SEM. Statistical analyses were performed using Statview or Excel software. In RT-PCR experiments, the effect of either E2 or wortmaninn was considered to be significant when the confidence interval of the treated groups did not include the reference value (100%). For all the other experiments, mean differences between groups were evaluated using Fisher's protected least significant difference (PLSD) *t* test, values of $p < 0.05$ were considered statistically significant.

FIGURE 2. Effect of short-term exposure to E2 in vitro on the expression of cytokines by peritoneal macrophages in response to LPS. Resident peritoneal macrophages from ovariectomized mice were incubated with E2 (10 nM) or vehicle (DMSO) 2 h before and during the 4 h of LPS stimulation (20 ng/ml). Cytokine mRNA expression was evaluated by qRT-PCR, normalized by GAPDH, and compared with results obtained from LPS stimulated macrophages in presence of DMSO. Results are expressed as means ± SEM from three independent experiments, each including pools of five mice per group. The effect of E2 was considered significant when the confidence interval of the data did not include the reference value of 100%: *, $p < 0.05$; **, $p < 0.01$.
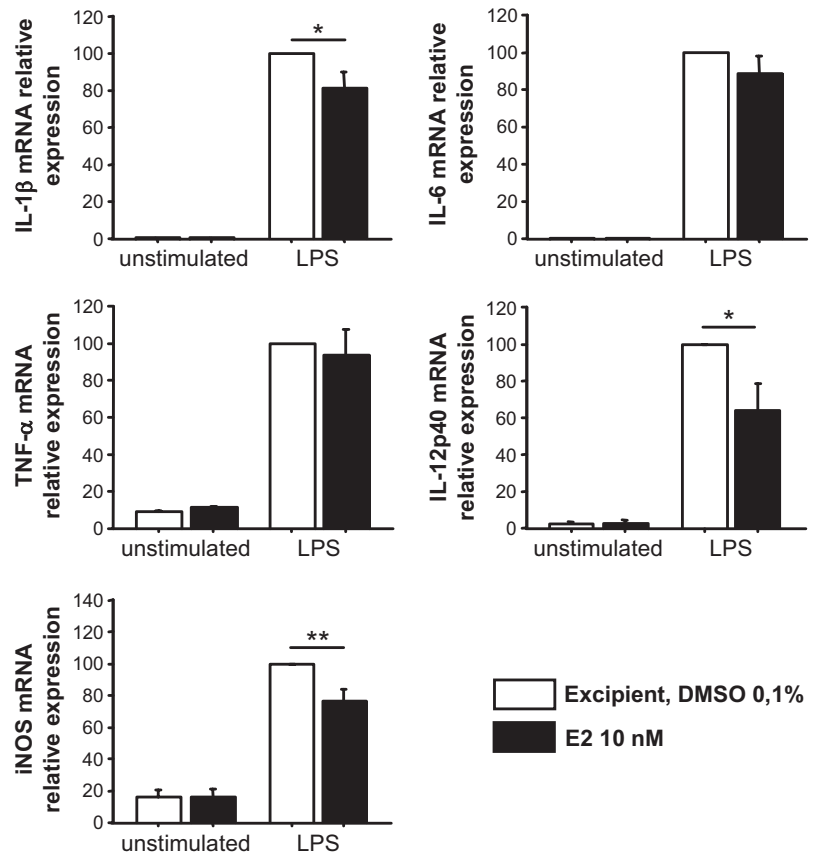
Results

In vivo chronic E2 administration enhances the production of proinflammatory cytokines by LPS-activated macrophages

As pivotal cells of the innate immunity, one of the main features of macrophages is to produce a diverse range of inflammatory mediators, including cytokines and NO, following the interaction of pathogen-associated molecular patterns with their surface cognate receptors, such as TLRs. To assess the influence of estrogens on macrophage effector functions, ovariectomized female mice were treated with either placebo or E2 for 4 wk, then, peritoneal resident macrophages were activated ex vivo with the TLR4 ligand LPS. Cytokine production was then measured in 24-h culture supernatants. As shown in Fig. 1A, macrophages from E2-treated mice exhibited an increase capacity to produce IL-1β and IL-6 as compared with macrophages from control ovariectomized mice. Although detectable at various levels of activation, this effect of E2 appeared to be maximal when macrophages were stimulated with 20 ng/ml LPS. In these experimental conditions, the production of IL-1β (1.7-fold, $p < 0.001$) and IL-6 (2.0-fold, $p < 0.001$) were significantly enhanced by E2 treatment (Fig. 1A), as well as that of IL-1α and IL-12p40, whereas LPS-induced TNF-α secretion was not altered by E2 administration (data not shown). To examine whether the proinflammatory effect of E2 resulted from an increase in gene transcription, cytokine mRNA abundance in macrophages was determined after a 4-h activation with LPS. As shown in Fig. 1B, in vivo E2-treatment induced a significant increase of IL-1β (2-fold increase, $p < 0.05$), IL-6 (2.9-fold, $p < 0.01$), IL-12p40 (3.1-fold, $p < 0.01$), and iNOS (3.5-fold, $p < 0.05$) mRNA levels in LPS-stimulated cells. By contrast, TNF-α mRNA was not significantly influenced by E2 treatment, in agreement with TNF-α quantification in supernatants.

Finally, we observed, in two independent experiments, a significant increase in the production of IL-1β (2.3-fold, $p < 0.0005$) and IL-6 (1.7-fold, $p < 0.001$) by peritoneal macrophages from sham-operated female mice as compared with macrophages from ovariectomized mice, suggesting that endogenous estrogens could also modulate proinflammatory cytokine production by macrophages. IL-10 was not detected in the supernatants in our experimental conditions (data not shown). Altogether, these data clearly demonstrate that chronic exposure to E2 in vivo enhances, at the transcriptional level, the production of numerous proinflammatory cytokines by peritoneal macrophages following TLR4-dependent activation by LPS.

Effect of in vitro short-term exposure to E2 on cytokine production by LPS-activated macrophages

Noteworthy, previous in vitro studies reported that estrogens exert an anti-inflammatory effect on macrophage functions, contrasting with our present observation following in vivo chronic administration. Therefore, we next investigated the influence of a short-term in vitro exposure to E2 on TLR4-dependent cytokine production in our model of resident peritoneal macrophages. In these experimental settings, a modest reduction of IL-1β (−20%, $p < 0.05$), IL-12p40 (−37%, $p < 0.05$), and iNOS mRNA (−24%, $p < 0.01$) expression was observed when the cells were incubated in the presence of 10 nM E2 2 h before and during LPS stimulation (Fig. 2). In contrast, in vitro exposure to E2 had no significant effect on LPS-induced expression of IL-6 and TNF-α at the mRNA level (Fig. 2). At the protein level, after 24 h of LPS activation, we observed a weak reduction in IL-1β (−22%, $p = 0.20$) and IL-6 (−21%, $p < 0.05$) under E2 in vitro treatment, whereas IL-12 and TNF-α secretions were not altered (data not shown). Altogether,
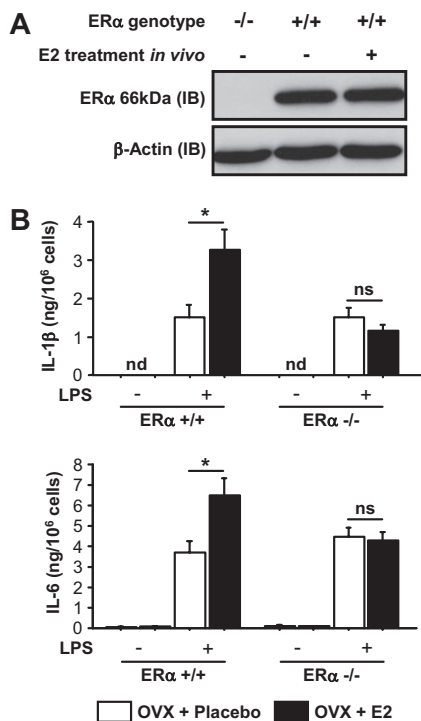
FIGURE 3. ERα mediates the effect of chronic E2 treatment in vivo on LPS-induced cytokine production by macrophages. *A*, ERα expression was analyzed by Western blot in protein extracts from peritoneal resident macrophages from placebo- or E2-treated wild-type mice. Macrophages from nonovariectomized ERα-deficient mice were used as a negative control. *B*, Resident peritoneal macrophages from placebo- or E2-treated ovariectomized wild-type and ERα-deficient mice were stimulated by LPS 24 h. IL-1β and IL-6 concentrations were quantified in culture supernatants by specific ELISA. Seven individual mice from two separate experiments are considered in each group. Results (mean ± SEM) are expressed as in Fig. 2. Fisher's PLSD *t* test: ∗, $p < 0.05$, ns = not significant, nd = not detectable.

these results show that short-term in vitro exposure to E2 has a marginal anti-inflammatory effect on peritoneal macrophages, contrasting with the strong increase in proinflammatory cytokine production induced by in vivo E2 administration.

ERα mediates the effect of in vivo E2 treatment on macrophages

Since ERα was recently reported to mediate several effects of estrogens on the immune system, we investigated its role in the proinflammatory effect of E2 on macrophages. The expression of the full length 66 kDa ERα isoform was demonstrated by Western blot in peritoneal macrophages and was not altered by in vivo E2 treatment (Fig. 3*A*). In contrast, we failed to detect the expression of the ERβ protein in these cells (data not shown). To assess the contribution of ERα to the effect of estrogens on peritoneal macrophages, wild-type and ERα-deficient ovariectomized female mice were treated with either placebo or E2 for 4 wk, then IL-1β and IL-6 production was measured in culture supernatants of LPS-activated macrophages. Whereas E2 increased the production of both cytokines by macrophages from wild-type mice, this effect was totally abolished in ERα-deficient mice, demonstrating that ERα mediates the proinflammatory effect of estrogens on macrophages (Fig. 3*B*).

The expression of TLR4 by peritoneal macrophages is not altered by in vivo E2 administration

To explain the enhanced TLR4-dependent production of proinflammatory cytokines by macrophages from E2-treated mice, one
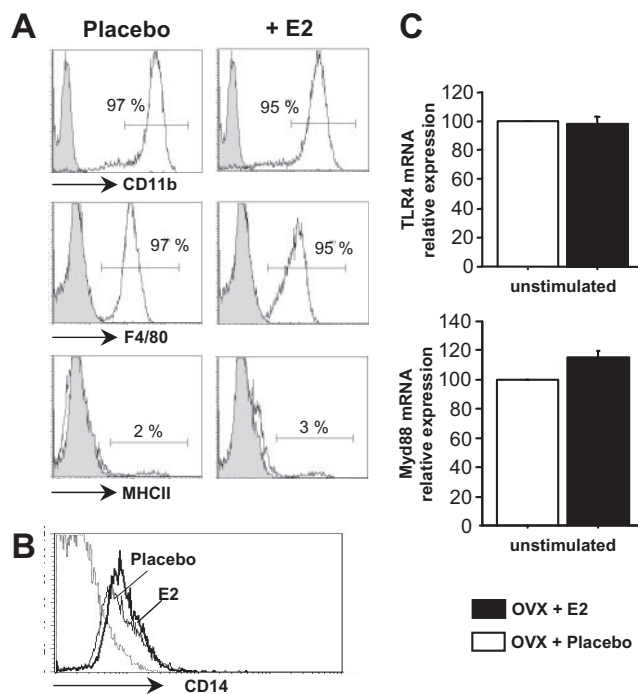

FIGURE 4. Chronic E2 treatment in vivo does neither alter surface expression of MHC class II and CD14, nor TLR4 mRNA expression in peritoneal macrophages. *A*, Resident peritoneal macrophages from placebo- or E2-treated mice show high expression level of CD11b and F4/80 markers and are characterized by low MHC class II surface expression as determined by flow cytometry. *B*, Histograms of CD14 surface expression on CD11b + macrophages from placebo- or E2-treated mice. *C*, Total mRNA of peritoneal resident macrophages from placebo- or E2-treated mice were subjected to qRT-PCR to assess TLR4 and MyD88 expression. Results are normalized by GAPDH expression, referring to the placebo-treated condition. Results are expressed as means ± SEM from four independent experiments, each including pools of five mice per group.

hypothesis could be that in vivo chronic exposure to E2 modulates the activation status or the expression level of surface molecules involved in LPS recognition, namely CD14 and TLR4. Peritoneal resident macrophages were identified on the basis of forward and side light scatter parameters, CD19-negative and CD11b-positive expressions by flow cytometry analysis. Macrophages from control or E2-treated mice expressed similar levels F4/80 (Fig. 4*A*). We found a low level of CD14 expression by resident peritoneal macrophages from both control (mean fluorescence intensity = 9.44) and E2-treated mice (mean fluorescence intensity = 9.81) (Fig. 4*B*). In addition, macrophage activation status, as assessed by MHC class II expression, was not influenced by E2 (Fig. 4*A*). As assessed by specific mRNA quantification, the expression of TLR4 and MyD88, a crucial adaptor protein in the initial signal transduction pathway induced by this receptor, were not influenced by in vivo E2 treatment in resident peritoneal macrophages (Fig. 4*B*). Altogether, these observations do not support the hypothesis that E2 increases in vivo the LPS-induced synthesis of proinflammatory cytokines by up-regulating the expression of components of the LPS-recognition complex.

In vivo E2 treatment inhibits the activation of the PI3K pathway in LPS-activated macrophages

Short-term exposure to E2 of RAW 264.7 macrophages in vitro has been shown to inhibit proinflammatory cytokine production upon LPS activation (16). This inhibitory effect of E2 has been attributed to its capacity to induce a rapid and persistent activation

of the PI3K/Akt pathway, resulting in the inhibition of NF-κB activity (16). Thus, we decided to evaluate the respective effects of in vitro and in vivo exposure to E2 on the PI3K activation status in peritoneal macrophages following LPS stimulation. To this end, we first measured the phosphorylation status of Akt, a direct target of PI3K activity through the generation of phosphatidylinositol-3,4,5-triphosphate (PIP3) and activation of PDK1 at the plasma membrane.

In accordance to previous observations, short-term in vitro exposure to E2 significantly enhanced Akt phosphorylation at the Serine 473 site in macrophages activated or not with LPS (Fig. 5A). In striking contrast, in vivo E2 treatment led to a significant inhibition of Akt phosphorylation, following TLR4 stimulation of peritoneal macrophages (Fig. 5B). This inhibitory effect of E2 was observed as early as 30 min after LPS activation (data not shown). We next assessed whether the decrease in phosphorylated Akt level in macrophages from E2-treated mice was a consequence of a reduced expression of Akt. As shown in Fig. 5C, the expression of total Akt, nor Akt1, Akt2, or Akt3, in peritoneal macrophages was not altered by in vivo E2 treatment, suggesting that PI3K activity might be directly inhibited by chronic estrogen administration. To test this assumption, resident peritoneal macrophages from either control or E2-treated mice were harvested for in vitro PI3K activity measurement. Data in Fig. 5D clearly show that E2 treatment in vivo led to an inhibition of PI3K activity not only in response to LPS, but also in nonactivated resident macrophages.

Interestingly, PI3K activation in response to TLR4 stimulation was recently demonstrated to negatively regulate LPS-induced cytokine expression by cells of the monocyte/macrophage lineage, namely dendritic cells and monocytes (11–13, 15). Thus, our results suggest that, by inhibiting PI3K activity, in vivo E2 treatment enhances signaling pathways that favor cytokine gene transcription. Among the major signaling events involved in TLR4-mediated inflammatory responses, p38 and p42/44 MAPK activation were not altered in LPS-activated macrophages from E2-treated mice (data not shown). In contrast, NF-κB activity, assessed by the measurement of nuclear p65 DNA binding, was enhanced in both unstimulated and LPS-activated macrophages from E2-treated mice (Fig. 5E). Altogether, these data demonstrate that in vivo exposure to E2 regulates the signaling events involved in the TLR4-mediated activation of macrophages.

Inhibition of the PI3K pathway by E2 contributes to its proinflammatory effect on LPS-activated macrophages

To the best of our knowledge, no data were available regarding the influence of PI3K on the production of cytokines by murine peritoneal macrophages in response to TLR4 activation. To test this hypothesis, these cells were incubated in the presence of the pharmacological inhibitor of PI3K family wortmannin 1 h before and during LPS stimulation. Loss of PI3K activity resulted in a strong increase of LPS-induced IL-1β (2.1-fold, $p < 0.001$), IL-6 (6.3-fold, $p < 0.001$), IL-12p40 (5.4-fold, $p < 0.001$), and iNOS (4.9-fold, $p < 0.001$) mRNA levels in macrophages, and TNF-α mRNA expression in a lesser extent (1.3-fold, $p < 0.01$) (Fig. 6). We confirmed at the protein level the enhancing effect of wortmannin on the production of IL-1β and IL-6, as shown in Fig. 7. Similar results were obtained using LY294002, another PI3K inhibitor (data not shown), confirming that, in our experimental settings, the activation of the PI3K pathway inhibits the TLR4-dependent expression of proinflammatory cytokines by resident peritoneal macrophages.

This observation prompted us to study whether the inhibition of this signaling pathway by E2 contributes to the enhancement of proinflammatory cytokine production. To this end, macrophages
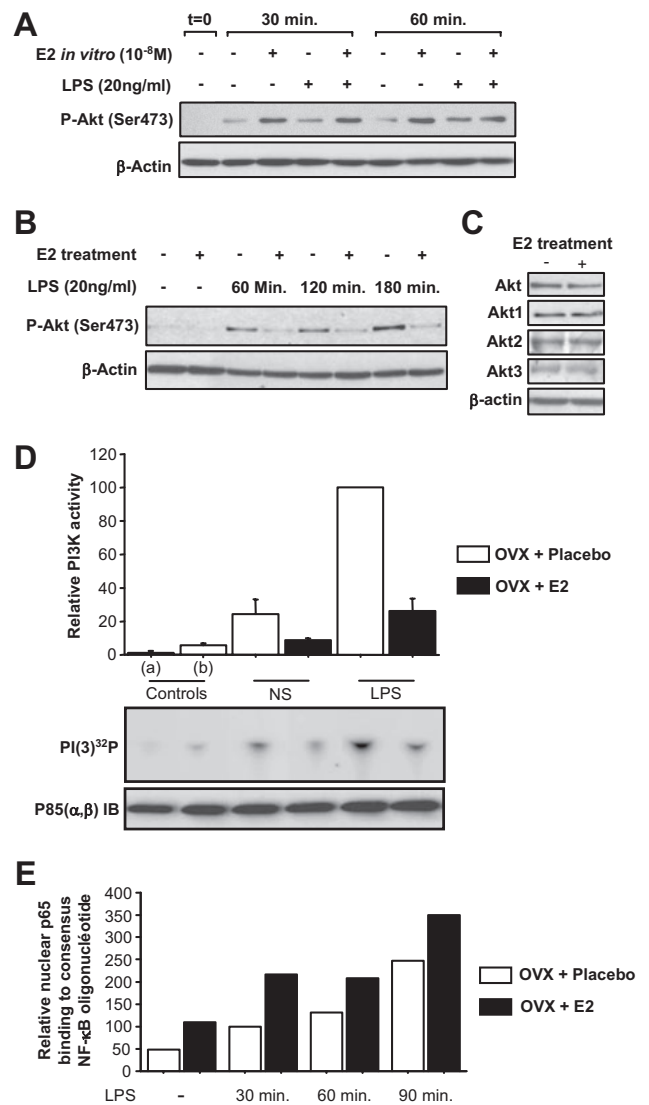

FIGURE 5. Chronic E2 treatment in vivo inhibits the activation of the PI3K/Akt pathway and enhances NF-κB activity in LPS-activated macrophages. *A*, Resident peritoneal macrophages were stimulated by E2 10^{-8} M or vehicle (DMSO 1/10000) with or without LPS (20 ng/ml) for 30 and 60 min. Then, cells were lysed and protein extracts were analyzed by Western blotting for the phosphorylation of Akt at the Serine 473 site and the expression of β-actin (as loading control). Results are representative of three independent experiments. *B*, Resident peritoneal macrophages from placebo- or E2-treated ovariectomized mice were stimulated with LPS (20 ng/ml) for indicated times. The phosphorylation state of Akt and the expression of β-actin were analyzed as described above. Results are representative of five independent experiments. *C*, Nonactivated peritoneal macrophages from placebo- or E2-treated ovariectomized mice were used to analyze the expression of Akt (panAkt), its isoforms (Akt1, Akt2, and Akt3) and β-actin (as loading control). Results are representative of two independent experiments. *D*, PI3K activity measurement was assessed in peritoneal macrophages from placebo- or E2-teated mice after stimulation with LPS (20 ng/ml) for 10 min, as described in *Materials and Methods*. PI(3)^{32}P, products of PI3K enzymatic reaction, were visualized with a phosphoimager and quantified with Imagequant. Absence of the anti-PI3Kp85(α,β) Ab during the immunoprecipitation step (a), and incubation with wortmannin (200 nM) before cell stimulation (b) were used to assess the specificity of PI3K activity. Results are representative of two independent experiments and are expressed in reference to the placebo-treated LPS condition. *E*, Peritoneal macrophages from placebo- or E2-treated mice were activated with LPS (20 ng/ml) for 30, 60, or 90 min and lysed to collect nuclear extract. Nuclear NF-κB p65 DNA binding was then measured. Results are representative of two independent experiments and are expressed in reference to the placebo-treated LPS (30 min) condition.

FIGURE 6. Inhibition of the PI3K pathway increases the expression of proinflammatory cytokines by macrophages in response to LPS. Resident peritoneal macrophages from ovariectomized mice were incubated with wortmannin (50 nM) or excipient (DMSO) 1 h before and during the 4 h of stimulation by LPS (20 ng/ml). Total mRNA were extracted to test proinflammatory cytokines and iNOS mRNA expression by qRT-PCR. Cytokine mRNA level expression was normalized by GAPDH mRNA expression and expressed as fold induction compared with the condition of LPS stimulation with DMSO. Results were expressed as means ± SEM from four independent experiments, each including pools of five mice per group. The effect of E2 was considered significant when the confidence interval of the data did not include the reference value of 100%: **, $p < 0.01$; ***, $p < 0.001$; ns = not significant.
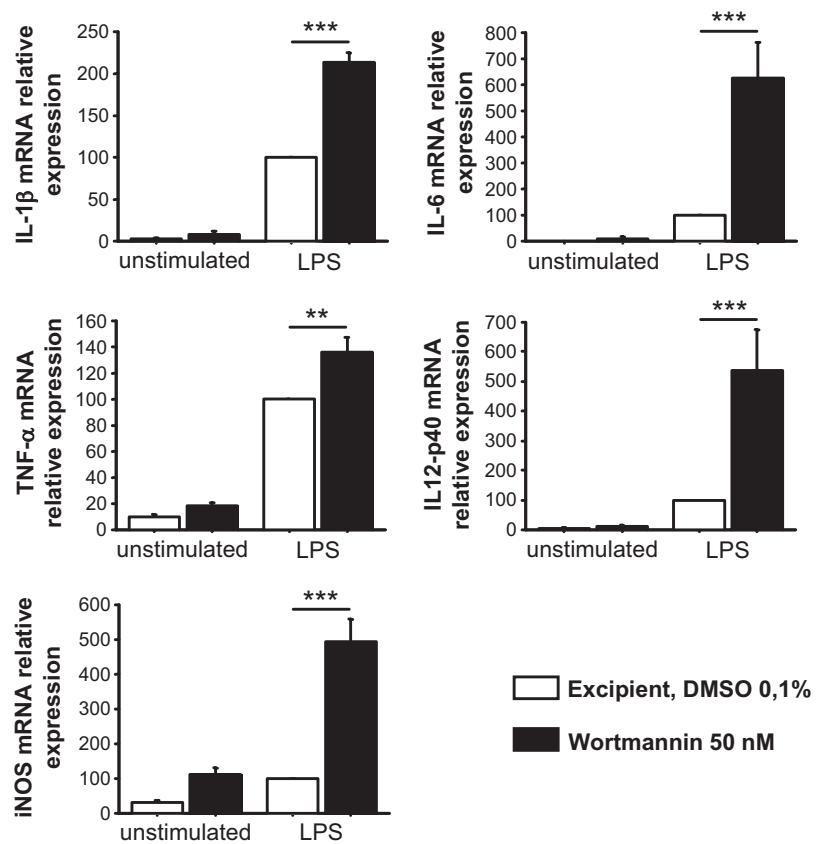
from placebo- or E2-treated mice were exposed to wortmannin 1 h before and during the stimulation by LPS. Whereas E2 treatment alone increased LPS-induced IL-1β (1.8-fold, $p < 0.001$) and IL-6 (1.7-fold, $p < 0.005$) secretion as expected, this effect was no longer observed in the presence of wortmannin (Fig. 7). Altogether, these data suggest that the inhibitory effect of E2 on the PI3K/Akt pathway in response to TLR4 activation contributes, at least in part, to the increased production of proinflammatory cytokines by macrophages, observed after an in vivo chronic exposure to estrogens.
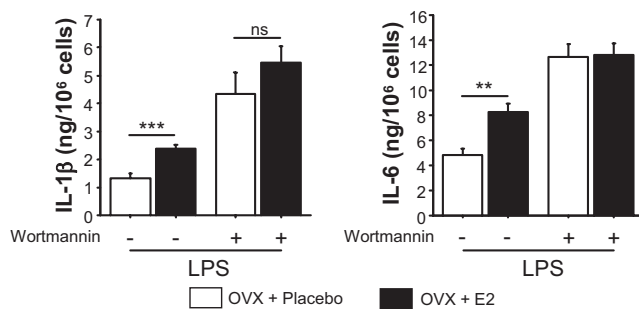
FIGURE 7. Pharmacological inhibition of the PI3K pathway alters the effect of chronic E2 treatment on cytokine production by LPS-activated macrophages. Resident peritoneal macrophages from either placebo- or E2-treated ovariectomized mice were incubated with wortmannin (50 nM) or excipient (DMSO 0.1%) 1 h before, and then during the 24 h of stimulation by LPS (20 ng/ml). IL-1β and IL-6 concentrations were quantified in culture supernatants by specific ELISA. At least six mice per group from two separate experiments were considered. Results (mean ± SEM) are expressed in ng cytokine per 10^6 input macrophages. Fisher's PLSD t test: **, $p < 0.005$; ***, $p < 0.001$; ns = not significant.

Discussion

Understanding the effects of estrogens on macrophage functions will provide important new insights into the mechanisms by which these sexual steroid hormones affect immune and inflammatory responses in women. The present study demonstrates that chronic exposure to E2 in vivo (4 wk) enhances the production of several cytokines, namely IL-1β, IL-6 and IL-12, as well as the expression of iNOS, by murine peritoneal resident macrophages in response to TLR4 activation with LPS ex vivo.

Noteworthy, this chronic effect of E2 in vivo contrasts with previous studies reporting anti-inflammatory actions of estrogens on macrophages in vitro. Indeed, an acute (only few hours) in vitro treatment of the macrophage cell line RAW 264.7 as well as splenic macrophages by E2 led to a significant decrease in the expression of inflammatory mediators following TLR4 activation (16, 25). In the present study, the short-term in vitro incubation of peritoneal macrophages with E2 2 h before and during LPS stimulation resulted in a modest decrease in the production of several proinflammatory cytokines, in accordance to these previous reports. To the best of our knowledge, this study is the first to clearly demonstrate opposite effects between short-term in vitro and long-term in vivo exposure to E2 on peritoneal macrophage function. It is in line with the observed effect of E2 on microglial cells, the resident macrophages of the brain (16, 25). It is important to note that our in vivo and in vitro experiments were all performed with physiological concentrations of the hormone (1–10 nM), as were those used in previous studies (16). Furthermore, supporting a critical role of endogenous estrogens for a proper innate immune response in intact females, we observed a significant increase in the production of proinflammatory cytokines, namely IL-1β and IL-6, by LPS-activated macrophages from sham-operated female mice, compared with those from ovariectomized mice. Altogether, our

data extend previous observations in microglial cells (20), and support the conclusion that in vivo exposure to endogenous or exogenous estrogens enhances the production of inflammatory mediators by TLR4-activated macrophages.

E2 treatment in vivo does not appear to modulate the phenotype of peritoneal macrophages but rather seems to regulate either directly or indirectly their intrinsic capacity to respond to TLR4-dependent triggering. We, therefore, propose that E2 may influence cytokine production in macrophages by regulating downstream signaling events rather than surface expression of molecules involved in LPS recognition, such as CD14 or TLR4. Studying the pivotal players involved in this TLR4-mediated activation process, we found that, whereas the expression of MyD88 and the phosphorylation status of the p38 and p42/44 MAPKs were not influenced by E2 treatment, the transcriptional activity of nuclear NF-κB p65 was significantly enhanced in macrophages from E2-treated mice. This enhanced NF-κB activation could be a consequence of the reduced PI3K activity we observed in LPS-stimulated macrophages from E2-treated mice. Several lines of evidence support this conclusion. First, we demonstrate here that the LPS-induced activation of the PI3K/Akt pathway negatively regulates the activation of proinflammatory cytokine genes in peritoneal macrophages. Second, the activation of the PI3K/Akt pathway in response to TLR4 signaling is inhibited in macrophages from E2-treated mice. Third, the stimulatory effect of E2 on LPS-induced IL-1β production by macrophages is no longer observed in presence of the PI3K/Akt inhibitor wortmannin. Interestingly, Ghisletti et al. (16) reported that the anti-inflammatory effect induced by short-term exposure to E2 in vitro on RAW 264.7 macrophages was associated with a rapid and persistent activation of the PI3K/Akt pathway that inhibits DNA binding and transcriptional activity of p65 NF-κB by preventing its nuclear translocation. Thus, it appears that short-term in vitro and chronic in vivo exposure to estrogens lead to opposite effects on the PI3K/Akt pathway, and this effect could largely contribute to the divergent modulation of the production of inflammatory mediators by TLR4-activated macrophages. This latter observation definitely demonstrates that in vitro studies do not appear to be predictive of the effect of estrogens on macrophage functions in vivo.

Although the underlying molecular mechanisms that specifically control the production of pro- and anti-inflammatory cytokines following TLR stimulation are not fully understood, recent studies suggested the critical involvement of the PI3K/Akt pathway in this process. This regulatory effect of PI3K was first established in dendritic cells since both pharmacological and transgenic approaches demonstrated that LPS-induced PI3K activation inhibits the expression of IL12p40 (11, 12). This negative regulatory effect of the PI3K/Akt pathway on cytokine production following TLR4, but also TLR2 and TLR5, activation was recently confirmed in human monocytes and macrophage cell lines (15, 26), and our data extend this observation to TLR4-activated peritoneal murine macrophages. Interestingly, we showed that the pharmacological inhibition of PI3K induced a strong increase in the expression of IL-1β, IL-6, IL-12p40, and iNOS in response to LPS, whereas the expression of TNF-α was only slightly enhanced, in striking concordance with the effect of chronic in vivo E2 administration.

The precise molecular mechanisms leading to the modulation of the PI3K/Akt by estrogens are still uncertain. In addition to macrophages, the activation of the PI3K/Akt pathway by acute exposure to E2 in vitro has been reported in different cell types, including endothelial cells and cortical neurons (27–29). In these experimental settings, beside the classical regulation of gene transcription, the activation of ERα was recognized to modulate signal transduction pathways through a nongenomic activity that involves the interaction of ERα with cytoplasmic kinases, including p85 PI3K, in membrane-bound complexes in caveolae (27, 29, 30). However, the pathophysiological relevance of these acute effects of estrogens on signaling events remains to be determined in vivo. We show here that peritoneal macrophages express ERα, which is required for the proinflammatory effect of E2 in vivo. However, whether chronic exposure to estrogens inhibits the activation of the PI3K/Akt pathway in these cells through direct or indirect mechanisms remains to be elucidated. Our experiments indicate that exposure to E2 in vivo exerts an inhibitory effect on PI3K activity as demonstrated in both activated and nonactivated macrophages, suggesting that E2 could alter the expression of molecular actors of this pathway. However, neither the expression of the p85 PI3K subunit, nor that of phosphatases involved in the PI3K pathway (PTEN, SHIP-1, and SHIP-2) was altered in macrophages after E2 treatment (data not shown).

One naturally occurring question is whether the enhanced cytokine production by macrophages from E2-treated mice is restricted to LPS activation or reflects a more general higher capacity to produce proinflammatory cytokines. In agreement with this latter hypothesis, we observed that in vivo E2 treatment significantly increased the production of proinflammatory cytokines by peritoneal macrophages in response to TLR2 and TLR5 activation: IL-1β and IL-6 under Pam3-CSK4 (TLR2 ligand) activation, IL-12p40 under SAC/IFN-γ (TLR2 ligand) stimulation, and IL-6 following exposure to flagellin (TLR5 ligand) (unpublished data). Interestingly, as indicated above, the PI3K/Akt pathway was recently demonstrated to negatively regulate TLR2 and TLR5 signaling in monocytes/macrophages (15, 26), and could thus represent one key molecular target mediating the proinflammatory effect of estrogens in these stimulation conditions. In addition, we previously reported that in vivo estrogens could modulate the activation and effector functions of Ag-specific CD4$^+$ T (6) cells and NKT lymphocytes (5), resulting in a strong increase in IFN-γ production in each lymphocyte subpopulation. Altogether, these observations support the idea that estrogens promote inflammatory responses by enhancing the capacity of professional APCs to produce Th1 polarizing cytokines, in accordance with recent observations in murine microglial cells (20) and dendritic cells (4, 31).

Unexpectedly, despite their proinflammatory potential, estrogens exert protective effects in several inflammatory disease models, preventing the inflammatory processes in target organs or tissues (32, 33). Although these effects of estrogens appear paradoxical, recent experiments in ERα chimeric mice revealed that the protective effect of E2 on experimental autoimmune encephalomyelitis was mediated through ERα signaling in nonhematopoietic cells (33). Thus, the inflammatory potential of the actors of the immuno-inflammatory system can be balanced by the action of E2 on extramedullar cell targets, which should be now investigated. Understanding the inflammatory immune "reshaping" elicited by changes in the estrogen status, such as through contraception, pregnancy, menopause as well as its associated hormone therapy, will be crucial to understand the pathophysiology of diseases influenced by sex hormones.

Disclosures

The authors have no financial conflict of interest.

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