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Estradiol Attenuates Lipopolysaccharide-Induced CXC Chemokine Ligand 8 Production by Human Peripheral Blood Monocytes

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Regulation of the inflammatory response is imperative to the maintenance of immune homeostasis. Activated monocytes elaborate a broad variety of proinflammatory cytokines that mediate inflammation, including CXCL8. Release of this chemokine attracts neutrophils to sites of bacterial invasion and inflammation; however, high levels of CXCL8 may result in excessive neutrophil infiltration and subsequent tissue damage. In this study, we demonstrate that 17β-estradiol (E2) attenuates LPS-induced expression of CXCL8 in human peripheral blood monocytes. Treatment of monocytes with estradiol before administration of LPS reduces CXCL8 message and protein production through an estrogen receptor-dependent mechanism, and luciferase reporter assays demonstrate that this inhibition is mediated transcriptionally. Importantly, the ability of estradiol-pretreated LPS-activated monocytes to mobilize neutrophils is impaired. These results implicate a role for estradiol in the modulation of the immune response, and may lead to an enhanced understanding of gender-based differences in inflammatory control mechanisms. ***The Journal of Immunology,*** **2007, 179: 6284–6290.**

Monocytes circulate throughout the periphery, mediating recognition and clearance of pathogens and cellular debris, and they extravasate into tissues where they differentiate into macrophages. As monocytes migrate from the vascular compartment to sites of inflammation, they become activated and secrete chemokines that recruit other immune effector cells. Secretion of CXCL8 by activated monocytes potently attracts neutrophils to infection sites. Neutrophils phagocytose invading microbes and release microbicidal enzymes from granules, including α-defensins, lysozyme, lactoferrin, and the cathelicidin hCAP-18 (1, 2). In addition to these functions, neutrophils can also elaborate a variety of immune mediators in response to infection, including cytokines such as IL-1β, TNF-α, and IL-12, as well as chemokines such as MIP-1α, MIP-1β, and IFN-γ-inducible protein of 10 kDa (1). However, although neutrophil recruitment is important for pathogen clearance and initiation of the inflammatory response, excessive neutrophil infiltration leads to the development of tissue damage under pathophysiological conditions. Thus, tight regulation of CXCL8 production and neutrophil mobilization is essential to mounting a controlled immune reaction.

Recent studies indicate that 17β-estradiol (E2) inhibits the production of proinflammatory cytokines, including IL-6 and macrophage inhibitory factor (3, 4). Furthermore, clinical reports demonstrate that premenopausal women have significantly lower LPS-induced TNF-α levels compared with men or postmenopausal women (5). In this regard, it is notable that sexually dimorphic responses to endotoxic shock have been reported, and that increased estrogen expression has been correlated with enhanced survival (6–9). These observations led us to postulate that estrogen attenuates LPS-induced CXCL8 expression as a potential mechanism for modulating neutrophil recruitment during inflammation. To test this hypothesis, human peripheral blood monocytes were pretreated with estradiol before activation with LPS, and CXCL8 message and protein production were analyzed. We determined that estradiol inhibits LPS-induced CXCL8 production by human monocytes in a dose-dependent manner, and that this effect is mediated through the estrogen receptor (ER).[2] Significantly, supernatants derived from monocytes that were treated with estradiol before stimulation with LPS are less chemotactic for neutrophils.

Materials and Methods

Isolation of human peripheral blood monocytes and neutrophils

PBMC were isolated with Ficoll-Hypaque (density = 1.077) from heparinized whole blood derived from female premenopausal donors that were not using hormonal contraception. Monocytes were positively selected from mononuclear cell fractions by positive selection with $CD14^+$ immunomagnetic beads (Miltenyi Biotec), as per the manufacturer's instructions. Monocyte purity was estimated at >98%, based on flow cytometric staining with anti-CD14 and -CD163 Abs.

For isolation of neutrophils, heparinized whole blood was mixed with Hetasep (Stem Cell Technologies) and allowed to stand for 30 min to sediment RBC. Discontinuous density gradients were prepared by layering an equal volume of Histopaque over Optiprep (1.095 g/ml; Axis Shield). The leukocyte-rich fraction from the Hetasep preparation was layered onto the density gradient. Neutrophils were recovered at the interface of the Histopaque and Optiprep layers. Neutrophils were >95% pure as assessed by both Wright-Giemsa staining and FACS analysis with anti-CD15 Ab.

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[2] Abbreviations used in this paper: ER, estrogen receptor; CM, conditioned medium; ARE, AU-rich element.

Table I. *Primer sequences for amplification of ER and β-actin*

Primer	Forward Sequence (5′–3′)	Reverse Sequence (5′–3′)
ERα	CGCGCAGGTCTACGGTCAG	CCACCCTGGCGTCGATTATCT
ERβ	CGCAGAAGTGAGCATCCCTCT	ACACCTGGGCACCTTTCTC
β-actin	TCGCCGCGCGCTCGTCGTC	TCTCTTGCTCTGGGCCTGGTC

Cell lines and cell culture

The T47D breast cancer cell line (obtained from American Type Culture Collection) and primary human monocytes were cultured in HEPES-buffered RPMI 1640 medium (Cellgro) supplemented with 10% FBS (HyClone) and 50 μg/ml gentamicin sulfate (Sigma-Aldrich) at a density of 1×10^6 cells/ml. Neutrophils were cultured in L-15 medium supplemented with 10% FBS (HyClone) and 50 μg/ml gentamicin sulfate (Sigma-Aldrich) at a density of 1×10^7/ml.

Cell viability assay

Positively selected monocytes were incubated with 10^{-7} M estradiol alone for 0, 24, or 48 h with and without 10 ng/ml LPS for 12 h, and cell viability was analyzed using the CellTiter 96 Assay (Promega) in accordance with the manufacturer's instructions. As positive control for apoptosis, monocytes were treated with 1.1 μM staurosporine. Monocytes were cultured with methanol as vehicle control for staurosporine treatment.

Estradiol and LPS treatments

Cells were treated with hormone in phenol red-free RPMI 1640 (Cellgro) supplemented with 10% charcoal dextran-stripped FBS (HyClone). Monocytes were preincubated with 17β estradiol (Calbiochem) as indicated for 24 h, and then treated or not with 10 ng/ml *Escherichia coli* LPS (Sigma-Aldrich) for an additional 12 h. For experiments in which ER binding was inhibited, monocytes were preincubated with 1×10^{-6} M of the pure ER antagonist ICI 182,780 (Tocris) for 1 h before treatment with 1×10^{-8} M estradiol and 10 ng/ml LPS. At the conclusion of these incubations, total RNA was extracted from these cells and supernatants were collected and analyzed for CXCL8 production by ELISA.

RNA extraction and RT-PCR

Total RNA was extracted from human peripheral blood monocytes using RNeasy mini columns (Qiagen). RNA samples were treated with RNase-free DNase I before amplification to eliminate genomic DNA contamination. RNA integrity and concentration were determined with the RNA6000 Nano LabChip kit (Agilent). Using 500 ng of RNA as template, first-strand cDNA was synthesized using random hexamers and SuperScript II Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies). PCR analysis was performed with *Taq*DNA polymerase for 35 cycles in a PTC-100 programmable thermocycler (MJ Research) using the primer pairs delineated in Table I. Cycling conditions were as follows: 2 min of initial denaturation at 95°C followed by 35 cycles, each of which consisted of 30 s at 94°C, 30 s at 57°C, and 45 s at 72°C, followed by a final extension at 72°C for 5 min. Reactions were amplified in the absence of reverse transcriptase as negative controls. A nontemplate control reaction was also included to ensure lack of DNA contamination. Ten microliters of PCR product were electrophoresed on a 1.5% agarose gel with 0.5% ethidium bromide, and photographed under UV light.

TaqMan PCR

Real-time TaqMan PCR was used to quantify mRNA expression of CXCL8. cDNA (0.5 μl/well) was transferred into 96-well format plates, and TaqMan Master Mix (Applied Biosystems) was added in accordance with the manufacturer's instructions. TaqMan-validated primers and CXCL8 TaqMan MGB probe (labeled with fluorescent reporter dye 6FAM) were used for amplification of CXCL8. Input cDNA was normalized with a validated predeveloped assay reagent β-actin primer probe pair (Applied Biosystems) as an internal control. Amplification was performed using an Applied Biosystems 7300 Real-Time PCR system with an optical unit that permits real-time monitoring of increased PCR product concentration. Threshold cycle number was determined with Opticon software, and CXCL8 mRNA expression levels were normalized to β-actin levels with the formula $2^{-(Et - Rt)}$, where Rt is the mean threshold cycle for the reference gene (*β-actin*), and Et is the mean threshold cycle for the experimental gene. Relative fluorescence units were assigned to these values, and these data were used to generate the expression profiles delineated in the text. Cycling conditions for TaqMan PCR consisted of 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Amplicon accumulation was measured during the extension phase. All reactions were performed in triplicate. Data were analyzed using ABI 7300 quantification software (Applied Biosystems).

Flow cytometric analysis

Surface expression of TLR4 was assessed using CellQuest analysis software on a FACSCalibur (BD Biosciences) flow cytometer. Monocytes were treated with E2 and/or LPS with concentrations indicated in Fig. 5. Cells were washed and incubated with normal human IgG (6 mg/ml) to block FcR-specific binding of mAbs and either 40 μg/ml biotinylated mouse IgG2a mAb HTA125 (AbD; Serotec) to detect TLR4 or an IgG2a biotin isotype control (AbD; Serotec). Following incubation with primary Abs, monocytes were washed and stained with streptavidin R-PE (AbD; Serotec). Flow cytometric analysis was performed on washed, unfixed cells immediately after staining.

Preparation of lysates and immunoblot analysis

Cell pellets were resuspended in M-PER mammalian protein extraction reagent (25 mM bicine buffer (pH 7.6); Pierce) to generate whole cell lysates. Samples were placed in a shaking incubator for 10 min and subsequently centrifuged at 13,000 rpm for 15 min to pellet cell debris. Supernatants were harvested, aliquoted, and stored at −80°C. Protein concentrations were determined by BCA protein assay (Pierce). Total cellular proteins (50 μg) were resolved by 10% SDS-PAGE, and electrotransferred to nitrocellulose membrane in Tris-glycine buffer, with 20% methanol. Immunoblots were washed with 1× TBS, 0.1% Tween 20, and blocked in 5% milk for 1 h at room temperature. Membranes were then probed with mouse mAb 62A3 (Cell Signaling Technology) for detection of ERα, and protein expression of ERβ was evaluated using the mouse mAb ab16813 (Abcam), followed by goat anti-mouse HRP-conjugated secondary Ab (Bio-Rad). Blots were probed with anti-GAPDH mAb (clone 6C5; American Research Products) followed by goat anti-mouse HRP-conjugated secondary Ab to control for protein loading. Incubations with primary Ab were performed at 4°C overnight in 5% milk with rocking. Reactive Ags were visualized with Supersignal chemiluminescence substrate (Pierce).

ELISA

Supernatants were collected from cultured monocytes and expression of human CXCL8 was quantified using the human CXCL8 Quantikine ELISA kit (R&D Systems). Samples collected from cells treated with LPS were diluted 1/100 before analysis.

CXCL8 promoter reporter construct and luciferase assay

A 1.4-kb fragment of the CXCL8 promoter was cloned from human monocytes using 5′-CGGATCCGAATTCGAGTAACCCAGGCATTATT-3′ and 5′-CGGATCCAGCTTGTGTGCTCTGCTGTCTCTGAAA-3′ primers. This fragment, containing −1481/+44 bp of the *CXCL8* gene 5′ flanking region, was subcloned into the pGL3 basic vector with firefly luciferase as a reporter gene.

Transient transfection was performed using Lipofectamine 2000 (Invitrogen Life Technologies). RAW 264.7 cells were plated 18 h before transfection at a density of 2×10^5 cells/well in 24-well tissue culture dishes in phenol red-free RPMI 1640 and 10% charcoal dextran-stripped FCS. Cells were incubated with either 0.8 μg of reporter plasmid (CXCL8) or pGL3 basic as negative control, and cotransfected with 40 ng of pRL-TK *Renilla* luciferase vector to control for transfection efficiency. One day after transfection, cells were treated with 1×10^{-8} M estradiol for 24 h and/or stimulated with 10 ng/ml LPS for an additional 6 h. Cells were harvested and lysed according to the manufacturer's instructions (Promega). Supernatants were analyzed for firefly and *Renilla* luciferase activity using the dual-luciferase reporter assay system (Promega), and each transfection was repeated at least three times. Data are represented as relative light units.

Chemotaxis assay and quantification of migration

Neutrophil chemotaxis was assayed using a modification of the under-agarose method described by Nelson et al. (10, 11). Briefly, a 2.4% suspension of UltraPure agarose (Invitrogen Life Technologies) in sterile Hanks' buffered saline was solubilized by heating in a boiling water bath, cooled to 65°C, and immediately mixed with an equal volume of complete L-15 medium at 37°C, to yield a final agarose concentration of 1.2%. Agarose was aliquoted into Falcon 3001 petri dishes and allowed to solidify. Circular wells were cut into the agarose using a sterile stainless steel punch guided by a template, and gel plugs were removed with a sterile needle. The template generated one central well surrounded by three equidistant peripheral wells. The central well was filled with 23 μl of medium containing either rCXCL8 or monocyte conditioned medium (CM) and each peripheral well was filled with 23 μl of the neutrophil suspension. The dishes were incubated at 37°C, 5% CO_2 for 16 h and then fixed with 1 ml of 37% formaldehyde (Fisher Scientific) for 1 h at room temperature. Following fixation, the agarose layers were removed, the dishes were rinsed in distilled water, and the cells were stained with Coomassie brilliant blue (Sigma-Aldrich) for 30 min.

For quantification of chemotaxis, the area between each peripheral well (containing neutrophils) and the central well (containing attractant) was photographed using a Nikon Coolpix 5700 digital camera attached to an inverted microscope. Nondirectional migration was determined by photographing an equivalent area on the side of the peripheral well opposite the central well. Images were subsequently converted to bitmaps. Neutrophils that had migrated 1.7–2.4 mm from the edge of the peripheral well were counted using the NIH ImageJ Particle Analyzer program. Results are expressed as the mean and SD of triplicate neutrophil wells.

Statistical analysis

Data are represented as mean ± SD. As indicated, statistical analysis was performed with a paired *t* test, and statistical significance was achieved at $p < 0.05$.

Results

Estradiol attenuates LPS-induced CXCL8 production by primary human monocytes

Gender-based differences in the incidence of autoimmune disease suggest that estradiol modulates immune responses. In this regard, recent studies have demonstrated that physiologic levels of estradiol modulate proinflammatory cytokine production in innate immune effector cells, including epithelial cells and macrophages (12, 13). To determine whether estradiol mediates similar effects in primary human monocytes, $CD14^+$ monocytes were preincubated with estradiol at concentrations ranging from 10^{-11} to 10^{-7} M and then stimulated with LPS. Following activation with LPS, supernatants were harvested from cultures and total RNA was extracted from monocytes for real-time PCR analysis.

As demonstrated in Fig. 1, constitutive production of CXCL8 by human monocytes is minimal, and is not affected by estradiol pretreatment. In contrast, activation of monocytes with LPS induces production of proinflammatory CXCL8 message and protein. Preincubation of monocytes with estradiol attenuates LPS-induced CXCL8 synthesis in a dose-dependent manner (Fig. 1). This inhibition is specific for CXCL8, as we have previously shown that expression of IL-1β is not decreased by estradiol pretreatment (12).

ER expression in primary human monocytes

Many biological effects of estradiol are transduced through two known isoforms of the ER, ERα and ERβ. Recent reports have shown that many of the tissue-specific differences in estradiol activity are attributable to selective binding to each ER subtype (reviewed in Ref. 14). To ensure ER mRNA expression in primary human monocytes, RNA was extracted from isolated monocytes, and PCR amplification was performed using primers that distinguish between ERα and ERβ (Table I). As a positive control for expression of ERα and ERβ, RNA that had been isolated from T47D breast cancer cells was also analyzed (15). In accordance

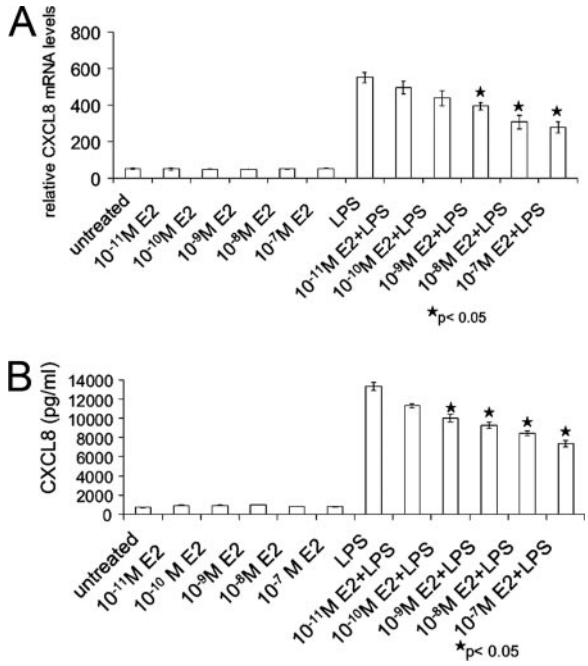

FIGURE 1. Estradiol attenuates LPS-induced CXCL8 production by primary human monocytes. $CD14^+$ monocytes were isolated using positive selection and pretreated with estradiol at the doses ranging from 10^{-11} to 10^{-7} M for 24 h. Cells were activated with the TLR4 agonist LPS (10 ng/ml) for an additional 12 h. *A*, CXCL8 mRNA expression was analyzed by TaqMan PCR. *B*, Supernatants were harvested from these cultures and CXCL8 secretion assessed by ELISA. Results are shown as mean ± SEM. Significantly different ($p < 0.05$) from LPS-treated cells.

with previous reports, we confirmed that both ER mRNA isoforms are constitutively expressed in human peripheral blood monocytes (Fig. 2*A*) (16, 17).

To confirm proper posttranscriptional processing and expression of ERα and ERβ, we performed immunoblot analysis of whole cell lysates that had been prepared from $CD14^+$ human peripheral blood monocytes. T47D cells that express both ER isoforms were included as controls for the ER Abs. Blots were also probed with anti-GAPDH Ab to control for total protein loading. As demonstrated in Fig. 2*B*, we confirmed that both ER isoforms are expressed in human monocytes, as previously reported by Ashcroft et al. (16). These data are significant in that estradiol has been shown to induce apoptosis in human myelomonocytic U937 cells that lack ERα expression (15). To further ensure that the observed inhibition of LPS-induced CXCL8 expression in monocytes was not due to estradiol-mediated induction of apoptosis, we performed cell viability assays (Fig. 2*C*). Monocytes were incubated in the presence or absence of 10^{-7} M E2 for 0, 24, or 48 h, and cultured with LPS for an additional 12 h as indicated. Cell viability was assessed using the CellTiter 96 proliferation assay. Monocytes were treated with staurosporine as positive control for induction of apoptosis. Incubation of cells with estradiol alone and estradiol followed by LPS treatment failed to induce apoptosis at any of the time points tested, indicating the reduced expression of CXCL8 that we observed in estradiol-treated monocytes was not attributable to cell death. To ensure that other concentrations of estradiol did not induce apoptosis, monocytes were subjected to pretreatment with estradiol concentrations ranging from 10^{-8} to 10^{-11} M followed by activation with LPS, and none of these concentrations resulted in loss of cell viability (data not shown).

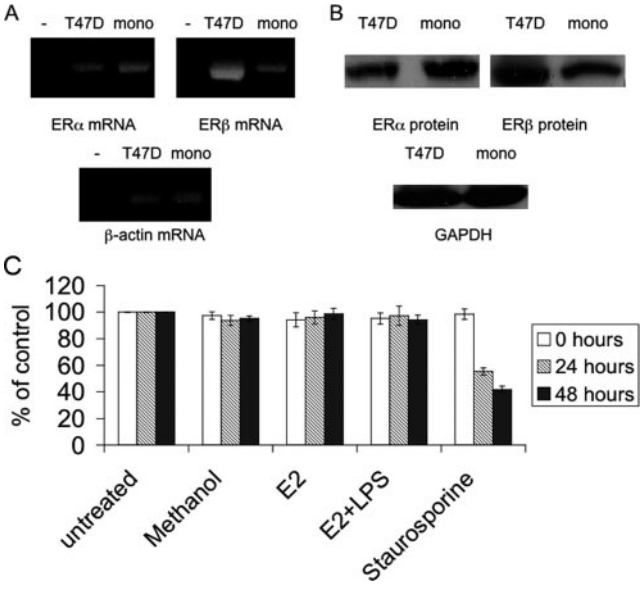

FIGURE 2. ER expression in primary human monocytes. Monocytes were isolated from peripheral blood of female premenopausal donors using immunomagnetic beads. T47D breast cancer cells were used as positive control for expression of both ER isoforms. *A*, Total RNA was extracted from monocytes and T47D cells and cDNA was prepared using random hexamers. Samples were also prepared in the absence of reverse transcriptase to control for genomic DNA contamination. mRNA expression of ERα, ERβ, and β-actin were analyzed using primers specific for these transcripts. *B*, Whole cell lysates were prepared from these cells, 50 μg of each lysate was run on 10% SDS-PAGE and subjected to immunoblot analysis with Abs that detect ERα, ERβ, and GAPDH. *C*, Estradiol does not induce apoptosis in cells that express both ER isoforms. Monocytes were incubated with 10^{-7} M estradiol for 0, 24, or 48 h (as indicated by □, ▨, and ■), and cultured with 10 ng/ml LPS for an additional 12 h as indicated, and cell viability was determined as described in *Materials and Methods*. Monocytes were treated with staurosporine as positive control for apoptosis, and methanol treatment was included as vehicle control for staurosporine. Data are graphed as percent of control (untreated) cells.

Inhibition of LPS-induced CXCL8 expression is ER dependent

To determine whether the effect of estradiol on LPS-induced CXCL8 production was mediated through estrogen receptors, monocytes were preincubated with estradiol and/or the pure ER antagonist ICI 182,780 (18), and subsequently stimulated with LPS for 12 h. In accordance with our earlier observations, pretreatment of monocytes with estradiol markedly attenuated LPS-induced CXCL8 production. However, CXCL8 expression was not inhibited in monocytes that had been pretreated with ICI 182,780 and subsequently stimulated with estradiol and LPS (Fig. 3). Addition of the ER antagonist did not affect constitutive expression of CXCL8. These data indicate that estradiol inhibits CXCL8 production through engagement of ERs on human peripheral blood monocytes.

Estradiol attenuates LPS-induced CXCL8 transcription

Previous studies have demonstrated that estradiol regulates cytokine gene expression through altered mRNA transcription (19). In this model, the binding of estradiol to intracellular ERs induces retention of the activated steroid-receptor complex in the nucleus, where it functions as a ligand-inducible transcription factor that augments or represses estradiol-specific gene expression (reviewed in Ref. 20). As we demonstrated that LPS-induced CXCL8 mRNA levels were decreased in estradiol-treated monocytes, we tested the hypothesis that this regulation occurs transcriptionally by transfecting ER-positive RAW 264.7 macrophages with a CXCL8 promoter-luciferase reporter construct. Following transfection, macrophages were pretreated with and without ICI 182,780 for 1 h and/or estradiol for 24 h and stimulated or not with LPS. Cells were transfected with the pGL3 basic plasmid as negative control. As demonstrated in Fig. 4, estradiol pretreatment attenuates LPS-induced CXCL8 promoter activity. Moreover, the ER antagonist ICI 182,780 abrogated the inhibition of CXCL8 promoter activity by estradiol (Fig. 4). These results indicate that estradiol regulates CXCL8 expression at the level of transcription, and confirm the requirement of the ER for CXCL8 down-regulation.

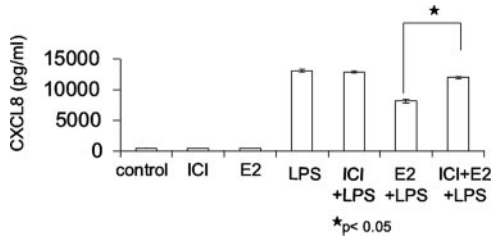

FIGURE 3. Inhibition of LPS-induced CXCL8 expression is ER dependent. CD14$^+$ monocytes were incubated with and without the pure ER antagonist ICI 182,780 (10^{-6} M) for 1 h before treatment with estradiol (10^{-8} M) for 24 h. Cells were subsequently activated with 10 ng/ml LPS as indicated for an additional 12 h. Conditioned medium was collected from these cells and CXCL8 production was analyzed by ELISA.

Estradiol does not alter TLR4 expression on human monocytes

TLR signal transduction is a key component of the innate immune response to pathogenic challenge. Of the 10 TLRs expressed in humans, TLR4 plays an integral role in the recognition of bacterial endotoxin (LPS) and mediates many monocyte and macrophage responses to inflammation (reviewed in Ref. 21). Given the importance of TLR4 in regulating monocyte immune function, we investigated the hypothesis that estradiol inhibits monocyte TLR4 expression, which results in a blunted response to LPS and consequently attenuated CXCL8 production. To test this possibility, monocytes were preincubated with estradiol followed by activation with LPS. Stimulation of monocytes with LPS induced TLR4 surface expression, as corroborated by previous reports (22). In contrast, neither constitutive nor LPS-induced surface expression

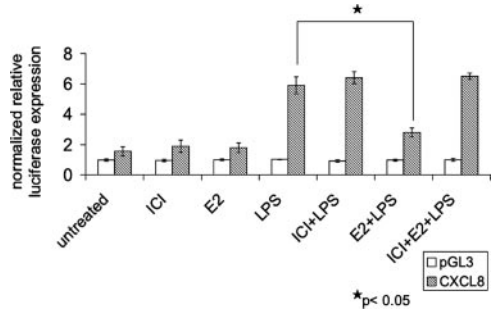

FIGURE 4. Estradiol attenuates LPS-induced CXCL8 transcription. RAW 264.7 cells were transiently transfected with CXCL8 promoter luciferase reporter constructs (CXCL8) or empty vector (pGL3) and pretreated with and without ICI 182,780 for 1 h and/or 10^{-8} M estradiol for 24 h. Following treatment with hormone, cells were stimulated with LPS (10 ng/ml) as indicated for 6 h. Firefly luciferase signal was normalized to *Renilla* expression to control for transfection efficiency. Data are graphed as normalized relative luciferase expression mean ± SD. Data are representative of three separate experiments.

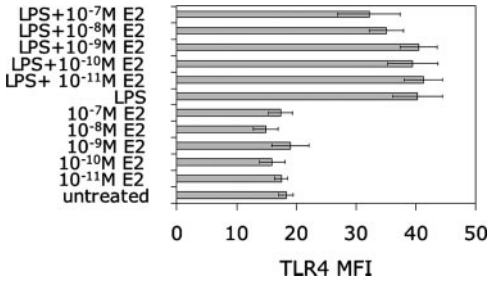

FIGURE 5. Estradiol does not alter TLR4 expression on human monocytes. Primary human monocytes were preincubated E2 (10^{-11}M–10^{-7} M) for 24 h, and activated or not with 10 ng/ml LPS for an additional 12 h. Cells were stained for TLR4 surface expression. Values are mean ± SD of triplicates. Data are representative of three experiments. MFI, Mean fluorescence intensity.

of TLR4 was altered by estradiol treatment (Fig. 5). Because biochemical and genetic evidence indicate that complex formation with the coreceptor CD14 is necessary for maximal TLR4 signaling in monocytes (23), we also monitored expression of CD14 by flow cytometry and real-time TaqMan PCR. Similar to our findings with TLR4, we failed to demonstrate an effect of estradiol on CD14 levels (data not shown). These data indicate that estradiol inhibition of monocyte CXCL8 expression is not attributable to blunted TLR4 expression.

Ability of monocytes to attract neutrophils is attenuated by estradiol

Monocytes recruit neutrophils to sites of inflammation through the release of chemokines, notably CXCL8. Rapid mobilization of neutrophils in response to infection is critical to pathogen clearance, but excessive neutrophil infiltration can also contribute to the pathogenesis of sepsis (reviewed in Ref. 24). Thus, modulation of neutrophil recruitment is critical to the maintenance of immune homeostasis. Given our observation that estradiol inhibits LPS-induced CXCL8 production in monocytes, we hypothesized that the ability of estradiol to attenuate CXCL8 production would consequently result in a decreased ability of monocytes to attract neutrophils. To test this hypothesis, monocytes were preincubated with and without estradiol for 24 h and/or 10 ng/ml LPS for an additional 12 h. CM were collected from these cultures and used to attract neutrophils as described in *Materials and Methods*. rCXCL8 served as positive control for induction of chemotaxis. In accordance with estradiol inhibition of LPS-induced CXCL8 production, CM derived from estradiol and LPS-stimulated monocytes induced less neutrophil chemotaxis than CM from monocytes that had been activated with LPS in the absence of estradiol (Fig. 6). These data suggest that exposure of monocytes to estradiol results in reduced ability of these cells to induce directed neutrophil migration to sites of inflammation.

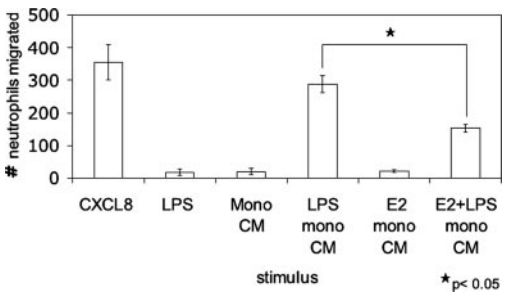

FIGURE 6. Estradiol treatment of monocytes impairs ability to attract neutrophils. Human monocytes were pretreated or not with estradiol (10^{-8} M) for 24 h, and then activated with 10 ng/ml for an additional 12 h. CM were collected from these cells, and used as attractant for neutrophils in chemotaxis assays as described in *Materials and Methods*. Results are representative of three separate experiments. rCXCL8 served as positive control.

Discussion

Both animal studies and gender-based differences in the incidence of autoimmune disease and susceptibility to sepsis demonstrate sexual dimorphism in the immune response. Although the mechanism responsible for these differences is unclear, these observations implicate sex hormones as modulators of immune function. In this study, we demonstrate that estradiol attenuates the ability of primary human monocytes to produce proinflammatory CXCL8 in response to endotoxin challenge. This inhibition is mediated directly through the ER, as treatment with pure ER antagonist ICI 182,780 abrogates this effect. Significantly, the ability of LPS-challenged monocytes to attract neutrophils is markedly inhibited by pretreatment of monocytes with estradiol. These data imply a role for estrogen in the regulation of inflammation through modulation of neutrophil recruitment.

Estradiol has been shown to modulate proinflammatory cytokine production in a variety of cell types, including monocytes and macrophages. Using a murine model of combined ethanol and burn injury, Messingham et al. (4) demonstrated that estradiol reduces macrophage production of proinflammatory IL-6 and enhances survival following bacterial challenge. Furthermore, estradiol has been shown to facilitate cutaneous wound healing through attenuation of macrophage migration inhibitory factor, and to down-regulate proinflammatory TNF-α production in activated macrophages (3, 16, 25). More recently, estrogen has been shown to inhibit expression of keratinocyte-derived chemokine, the murine homolog of CXCL8, in activated splenocytes (26). These reports corroborate our findings that estradiol mediates anti-inflammatory functions in stimulated human monocytes through suppression of proinflammatory cytokine synthesis and secretion.

In this regard, it is notable that sepsis-related morbidity and mortality is higher in men than premenopausal women (6, 9). Intriguingly, the apparent female survival advantage is abrogated in postmenopausal women, in which both incidence and sepsis-related mortality rates increase to levels equivalent to those in age-matched men (7, 8). These data imply that estradiol confers protection against excessive inflammation; one potential mechanism by which this occurs is through modulation of neutrophil mobilization. As key innate effector cells, neutrophils mediate host defense against infection through pathogen uptake and release of antimicrobials, including oxidants, proteinases, and cationic peptides. Under pathophysiological conditions, these molecules may be released into the extracellular space, resulting in host tissue damage (reviewed in Ref. 27). Significantly, neutrophils have been implicated as mediators of tissue injury in lung, renal, and intestinal inflammation (24). Considered in this context, it may be advantageous to limit neutrophil recruitment to sites of inflammation. In this report, we now show that estradiol attenuates monocyte production of LPS-induced proinflammatory CXCL8. The estradiol-mediated down-regulation of this potent chemokine may represent an important means of regulating inflammatory responses and facilitating host survival.

Our results confirm the findings of Ashcroft et al. (16), which showed that primary human monocytes express ER. We now demonstrate that estradiol regulates LPS-induced CXCL8 expression in monocytes directly through the ER, as preincubation with the pure ER antagonist ICI 182,780 abrogates the ability of estradiol to

attenuate CXCL8 message and protein. Moreover, our data suggest that the mechanism by which estradiol regulates *CXCL8* gene expression is at least partly transcriptional. Although the CXCL8 promoter lacks canonical ER elements, it does contain AP-1 and NF-κB-binding sites (28). Estradiol has been shown to alter proinflammatory gene expression by binding to both of these transcription factors (28). Significantly, estradiol has also been shown to modulate NF-κB subcellular localization in the RAW 264.7 macrophage cell line (29). In accordance with previous studies, these investigators determined that stimulation of macrophages with LPS activates NF-κB, resulting in its nuclear translocation. However, when RAW cells were treated with estradiol before activation with LPS, nuclear translocation of the p65 subunit of NF-κB was inhibited, thereby precluding its ability to bind DNA and presumably induce activation of proinflammatory genes (29). This mechanism may account for our results, in which estradiol pretreatment of monocytes inhibits LPS-induced *CXCL8* gene expression. In this model, stimulation of monocytes with LPS induces nuclear translocation of NF-κB, which subsequently binds to its cognate site in the CXCL8 promoter, resulting in increased CXCL8 production. Estradiol pretreatment blocks the ability of NF-κB to transcriptionally activate CXCL8 expression by preventing its nuclear translocation, resulting in attenuation of LPS-induced CXCL8 production.

Furthermore, our report that treatment of monocytes with the specific estrogen receptor antagonist ICI 182,780 abrogates estradiol effects on CXCL8 supports a role for estradiol in the direct modulation of CXCL8 transcription. ICI 182,780 completely attenuates the ability of the ER to activate or inhibit transcription in a ligand-dependent or -independent manner (18). Binding of ICI 182,780 to the ER results in increased ER degradation and impaired ER dimerization and nuclear translocation (30–32). This activity reduces binding of the ICI 182,780/ER complex to the ER elements of target genes and impedes the function of AF1 and AF2 domains of the ER (reviewed in Ref. 33). When E2 is bound to the ER, AF1 and AF2 recruit transcriptional coactivators or corepressors to the promoters of ER-responsive genes. However, binding of ICI 182,780 to the ER inactivates AF1 and AF2, thereby impeding their ability to engage these proteins in the general transcription complex. Therefore, possible mechanisms by which ligand-bound ER regulates LPS-induced CXCL8 production in monocytes are through recruitment of transcriptional corepressors and/or competitive inhibition of transcriptional activators to the CXCL8 promoter (34). The CXCL8 promoter contains C/EBP and NF-κB-binding sites, and C/EBP and the p65 subunit of NF-κB have been shown to cooperatively activate CXCL8 transcription through complex formation on the CXCL8 promoter (35). Recent studies suggest that E2/ER complexes may bind these transcription factors, thus changing their conformation and ability to bind DNA and activate transcription (34, 36). It will be of considerable interest to determine whether C/EBP and/or NF-κB binding to the CXCL8 promoter are altered in estradiol-treated monocytes.

Additionally, it is entirely possible that estradiol also mediates posttranscriptional regulation of *CXCL8* gene expression posttranscriptionally. The 3′ UTR of CXCL8 contains reiterations of RNA-destabilizing AU-rich elements (AREs) (37). These sequences have been shown to rapidly and transiently modulate chemokine and cytokine gene expression. Selective binding of proteins to AREs confers specificity to the regulation of mRNA turnover, and studies have shown that the p38 MAPK-signaling pathway regulates stress-activated CXCL8 mRNA expression in an ARE-dependent manner (38, 39). Thus, it is possible that estradiol mediates direct effects on CXCL8 mRNA turnover, or through altered protein binding or activation of p38 signal transduction.

Because these studies focused on the influence of estradiol on monocyte immune function, they were conducted using cells derived from premenopausal female donors. Women using hormonal contraception were excluded to limit confounding results due to exogenous hormone treatment. However, it is notable that we have observed that estradiol attenuates LPS-induced CXCL8 production in monocytes derived from male donors as well. In this report, we have shown that the magnitude of the inhibition is dose dependent. These data suggest that estradiol suppresses LPS-induced CXCL8 production in both men and women, and that the effect may be enhanced during ovulation or pregnancy, coincident with elevated serum estradiol levels and reduced in postmenopausal women. We are currently studying whether E2 regulates expression of other chemokines such as MCP-1.

In conclusion, we have demonstrated that estradiol attenuates LPS-induced CXCL8 production in human monocytes. This inhibition is dose dependent, as demonstrated by reduced levels of CXCL8 message and protein. The ability of estradiol to blunt CXCL8 production in the context of LPS is transduced through the ER, as treatment with the ER antagonist ICI 182,780 prevents this effect. Luciferase reporter assays demonstrate that estradiol regulates LPS-induced *CXCL8* gene expression at the level of transcription. Significantly, estradiol inhibition of CXCL8 production results in a reduced ability of monocytes to attract neutrophils. Collectively, these data demonstrate a role for estradiol in the regulation of inflammation and may lead to an enhanced understanding of gender differences in innate immune responses.

Disclosures

The authors have no financial conflict of interest.

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