Estrogens Augment Cell Surface TLR4 Expression on Murine Macrophages and Regulate Sepsis Susceptibility *in Vivo*

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Gender-based differences exist in infectious disease susceptibility. In general, females generate more robust and potentially protective humoral and cell-mediated immune responses after antigenic challenge than their male counterparts. Furthermore, evidence is accumulating that sex may also influence the early perception of microbial challenges and the generation of inflammatory immune responses such as sepsis. These differences have previously been attributed to the actions of reproductive hormones. Whereas androgens have been shown to suppress acute host immune responses to bacterial endotoxin challenge, estrogens have been found to promote increased resistance to bacterial infections. However, the mechanisms by which estrogens exert immunoprotective effects have not been established. In this study, we investigated the *in vivo* effects of 17β-estradiol on endotoxin susceptibility in mice. Importantly, we have examined the actions of this female reproductive hormone on the expression of pattern recognition receptors that recognize bacterial endotoxin by key innate immune sentinel cells. We show that removal of endogenous estrogens decreases both pro- and antiinflammatory cytokine production, with a concomitant reduction in circulating levels of lipopolysaccharide-binding protein and cell surface expression of Toll-like receptor 4 on murine macrophages. Exogenous *in vivo* replacement of 17β-estradiol, but not progesterone, significantly elevates sera lipopolysaccharide-binding protein levels and cell surface expression of Toll-like receptor 4 and CD14 on macrophages. Furthermore, this effect corresponds with significantly higher inflammatory cytokine levels after *in vivo* lipopolysaccharide challenge and a marked increase in endotoxin-associated morbidity. Taken together, these data provide a potential mechanism underlying the immunoenhancing effects of estrogens. **(*Endocrinology* 150: 3877–3884, 2009)**

It has become increasingly apparent that sexual dimorphism exists in infectious disease susceptibility (as reviewed in Ref. 1). In general, females generate more robust and potentially protective humoral and cell-mediated immune responses after antigenic challenge than their male counterparts. For example, it has been found that female deer have lower parasite loads than males, and helminth infections are generally more severe in males than females (2, 3). In addition, influenza infection elicits greater severity and hospitalization in male patients (4), and females generate greater humoral and cell mediated responses to herpes simplex viruses (5) and cytomegalovirus (6). Furthermore, this sexual dimorphism in susceptibility to infectious disease extends to cases of sepsis, in which male patients exhibit a 70% mortality associated with sepsis, but females show only 26% associated mortality (7). Interestingly, women have been found to have higher circulating levels of IgM than men (8), and this difference is most apparent at puberty (9, 10), suggesting a role for reproductive hormones in the development of this gender bias. Estrogens have been found to promote increased resistance to streptococcal infections (11), whereas androgens have been shown to suppress acute host immune responses to bacterial endotoxin challenge (12). Indeed, high levels of estrogens can elevate immune responses to a point at which they are detrimental to the host. For example, 17β-estradiol- or estriol-treated rodents exhibit greater mortality after gonococcal infection (13) or endotoxin challenge (14), respectively, than untreated animals. Fur-

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Abbreviations: LBP, LPS binding protein; LPS, lipopolysaccharide; TLR, Toll-like receptor.

thermore, it has been found that sera levels of 17β-estradiol are increased in human sepsis patients, and the highest incidence and severity of sepsis is associated with high circulating levels of estrogens regardless of gender (7, 15–17).

The mechanisms underlying innate immune responses to bacterial endotoxins are only now becoming apparent. Lipopolysaccharide (LPS) binding protein (LBP) is a type I acute phase protein that catalyzes the monomerization of LPS and mediates its transfer to CD14 (18). CD14 serves as a coreceptor for LPS (19) and is expressed as a glycosylphosphatidylinositol-linked protein within the plasma membrane of key immune sentinel cells, such as macrophages. However, neither LBP nor CD14 possesses a cytoplasmic component through which cellular responses can be initiated. This is accomplished via Toll-like receptor (TLR)-4, a member of the novel Toll-like family that can elicit bacterial motif-specific immune cell activation without the need for prior exposure (as reviewed in Ref. 20). Ligation of TLR4, facilitated by CD14 and LBP, can activate transcription factors that lead to the elevated production of the inflammatory cytokines and chemokines that precipitate bacterial septic shock (as reviewed in Refs. 21 and 22). As such, it is feasible that reproductive hormone-based differences in the level of expression of these molecules could lead to significant differences in the magnitude of inflammatory host responses after endotoxin exposure.

We previously demonstrated the ability of testosterone to down-regulate the expression of TLR4 on macrophages both *in vitro* and *in vivo* (12). In the present study, we show that removal of endogenous estrogens decreases circulating levels of key inflammatory mediators after *in vivo* endotoxin challenge. Furthermore, we demonstrate that these changes are associated with decreased circulating levels of LBP and diminished cell surface expression of TLR4 on macrophages. In contrast to our findings with testosterone (12), we show that acute *in vitro* exposure to exogenous 17β-estradiol fails to elicit significant changes in pattern recognition receptor expression on a macrophage-like cell line or authentic primary macrophages. However, *in vivo* administration of supraphysiological levels of 17β-estradiol results in a marked increase in endotoxin susceptibility, and this effect is associated with significant increases in LBP levels and cell surface TLR4 and CD14 expression on macrophages. Taken together, these data provide a potential mechanism underlying the immuno-enhancing effects of estrogens.

Materials and Methods

Surgical ovariectomy and hormone replacement

Female C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME), 8 wk of age, underwent bilateral ovariectomy under inhalant isoflurane anesthesia as previously described (23) to remove the main endogenous source of estrogens. Groups of adult female mice were bilaterally ovariectomized or sham ovariectomized and allowed to recover for 5 wk. This time period not only ensures that estrogens produced by the ovary have been metabolized and are no longer present in the blood (23) but is also sufficient to allow the turnover of immune cells generated under the influence of this reproductive hormone. Mice in the sham-operated group underwent the same anesthesia and incision procedure but the ovaries were not excised. Hormone replacement was achieved essentially as described previously (24). Immediately after ovariectomy, SILASTIC brand tubing (Dow Corning, Midland, MI) containing 17β-estradiol (3.97 ± 0.18 mg/implant) and/or progesterone (4.74 ± 0.18 mg/implant; Sigma-Aldrich Co., St. Louis, MO) was implanted sc to deliver exogenous hormone throughout the 5-wk experimental period. Hormone packed in SILASTIC brand tubing implants (Dow Corning) have previously been shown to provide elevated levels of hormone for this time period (24). This resulted in five *in vivo* groups: intact sham operated animals (SHAM), ovariectomized animals (OVX), ovariectomized animals receiving progesterone implants (OVX+P_4), ovariectomized animals receiving 17β-estradiol implants (OVX+E_2), and ovariectomized animals receiving both progesterone and 17β-estradiol implants (OVX+E_2+P_4). All procedures were approved by the Institutional Animal Care and Use Committee of The University of North Carolina at Charlotte.

In vivo endotoxin challenge

Ovariectomized, sham-operated, and/or ovariectomized animals receiving 17β-estradiol and/or progesterone replacement were challenged with LPS (5 mg/kg, ip) isolated from *Escherichia coli* (>500,000 EU/mg; Sigma-Aldrich). At 8 or 20 h after treatment, animals were studied for behavior and appearance and then euthanized and analyzed for sera cytokine content. The severity of endotoxic shock was scored according to a system modified from that previously employed by Liu *et al.* (25), in which a score of 1 was given to mice with ruffled fur but no detectable behavioral differences compared with untreated mice, a score of 2 to mice with percolated fur and a huddle reflex but that were still active, a score of 3 to mice that were less active and were relatively passive when handled, a score of 4 to inactive mice that exhibited only limited response when handled, and a score of 5 to moribund mice.

Isolation of murine peritoneal macrophages

Elicited peritoneal macrophages were isolated as previously described by our laboratory (26–28). Briefly, mice from each treatment group received ip injections of 200 μl incomplete Freund's adjuvant (Sigma-Aldrich). Three days later, the peritoneal cavities were lavaged with RPMI 1640 (Cellgro, Washington, DC; 7 × 1.5 ml per animal) containing 10% fetal bovine serum (Atlanta Biologics, Norcross, GA) to remove the peritoneal macrophages. After washing twice in RPMI 1640, adherent macrophages were cultured in RPMI 1640 containing 2% fetal bovine serum and gentamicin.

Macrophage-like cell line culture

RAW 264.7 macrophage-like cells (CRL-2278; ATCC, Manassas, VA) were grown on Cellstar culture plates (Greiner Bio-one, Monroe, NC) in RPMI 1640 containing 2% NuSerum (BD Biosciences, Franklin Lakes, NJ) to minimize exposure to reproductive hormones. According to the determinations of NuSerum reproductive hormone content provided by the manufacturer, cells cultured under these conditions are exposed to less than 2.5×10^{-10} M 17β-estradiol and less than 3.1×10^{-10} M progesterone.

Quantification of TNF-α, IL-6, IL-10, and LBP production

Capture ELISAs were performed to quantify IL-6 levels as previously described (26, 28) using a commercially available capture antibody against IL-6 (clones MP5–20F3; BD Biosciences, San Diego, CA), a biotinylated antimouse IL-6 antibody (clones MP5-32C11; BD PharMingen), and streptavidin-horseradish peroxidase (BD PharMingen). A standard curve was constructed using varying dilutions of mouse recombinant IL-6 (BD PharMingen). TNF-α, IL-10, and LBP levels were quantified using commercially available ELISA kits according to the directions provided by the manufacturers (TNF-α and IL-10; R&D System, Minneapolis, MN; LBP; HyCult Biotechnology, Canton, MA). The minimum detectable levels in these assays were 4 pg/ml for IL-6, 16 pg/ml

for TNF-α, 8 pg/ml for IL-10, and 3 ng/ml for LBP. All determinations were made in duplicate from the indicted number of sera samples.

Assessment of TLR4/MD-2, CD14, and LPS binding by flow cytometry

Immunofluorescence analysis (FACSCalibur; Becton Dickinson, San Jose, CA) was performed to determine the presence of CD14 or TLR4 associated with the permissive molecule MD-2 on the surface of macrophages as previously described by our laboratory (26). Cells were isolated and a phycoerythrin-conjugated antibody directed against TLR4/MD-2 (clone MTS510; eBioscience) or a fluorescein isothiocyanate-conjugated antibody directed against CD14 (clone rmC5-3) was added for 45 min at 4 C. Cells were then washed and assayed by fluorescence-activated cell sorter analysis for the proportion of CD14 or TLR4/MD-2-positive cells relative to fluorescence obtained in cells stained with an fluorescein isothiocyanate- or phycoerythrin-conjugated antibody directed against an irrelevant peptide as appropriate. In some experiments, cells were permeabilized during immunofluorescent staining to assess total cellular TLR4 content using a CytoFix/CytoPerm kit according to directions provided by the manufacturer (BD PharMingen). Alexa Fluor 488 labeled LPS (*E. coli* serotype 055:B5; Invitrogen, Eugene, OR) was used to measure binding of endotoxin to isolated macrophages. Macrophages were cocultured with 400 ng/ml labeled LPS for 45 min before washing and assessment of the proportion of positive cells. In all cytometric analyses, a minimum of 50,000 cells were analyzed from at least three separate cell isolation procedures and results are presented as the geometric means of the fluorescence intensity.

Quantification of sera hormone levels

Sera 17β-estradiol and progesterone levels were quantified at the time the animals were killed using commercially available enzyme immunoassays according to the directions provided by the manufacturer (Oxford Biomedical Research, Oxford, MI).

Statistical analyses

Geometric means of immunofluorescence histogram plots were obtained using commercially available software (CellQuest; Becton Dickinson). Geometric means of immunofluorescence histograms or mean cytokine levels were tested statistically against values for these parameters obtained from untreated cells or cells derived from sham-operated animals using Student's *t* test or a one-way ANOVA as appropriate using commercially available software (SAS version 9.1.3; SAS Institute Inc., Cary, NC). In all experiments, results were considered statistically significant when $P < 0.05$ was obtained. Results are presented as the mean or the geometric mean of fluorescence intensity ± SEM.

Results

Endogenous estrogens are required for maximal *in vivo* cytokine responses to endotoxin administration

To begin to determine the *in vivo* effects of female reproductive hormones, ovariectomized animals that are largely devoid of endogenous estrogens and sham ovariectomized animals were challenged with a sublethal dose of LPS (5 mg/kg, ip). Sera levels of 17β-estradiol were decreased by 61.4% in ovariectomized animals. At 20 h after treatment the severity of endotoxic shock was assessed according to appearance and behavior using a scoring system modified from that used by Liu *et al.* (25). Animals were then euthanized and sera isolated for cytokine content by specific capture ELISAs. As shown in Fig. 1, ovariectomized animals exhibited markedly lower LPS-induced sera levels of IL-6 (Fig. 1A) and TNF-α (Fig. 1B) than age-matched intact females

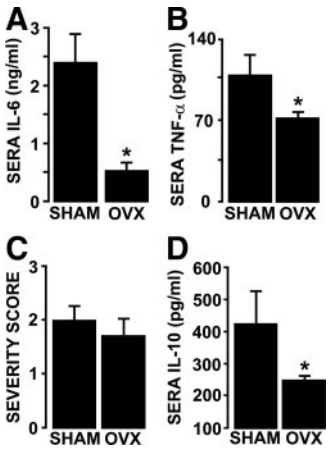

FIG. 1. Removal of endogenous estrogen reduces circulating levels of cytokines resulting from *in vivo* endotoxin challenge. Mice were sham ovariectomized (SHAM: n = 6) or ovariectomized (OVX: n = 7) and received an ip challenge with LPS (5 mg/kg). At 20 h after challenge, sera was isolated and assayed for the presence of IL-6 (A), TNF-α (B), or IL-10 (D) by specific capture ELISA. C, Severity of endotoxic shock assessed according to appearance and behavior is shown and is reported as a severity score for each animal in the three treatment groups. Severity was scored from 1 (no detectable behavioral differences) to 5 (moribund). *, Significant difference from sham-ovariectomized animals.

(n = 6–7 animals/group; $P < 0.05$). It is important to note that we did not observe significant differences in endotoxic shock severity scores between these groups (Fig. 1C). However, this observation could be explained by our finding that sera levels of the antiinflammatory cytokine, IL-10, were also significantly higher ($P < 0.05$) in sham-ovariectomized animals than in gonadectomized mice (Fig. 1D). Together these data show that removal of endogenous estrogens results in reduced host cytokine responses to endotoxin challenge.

The *in vivo* presence of estrogens elevate cell surface expression of key pattern recognition receptors by murine macrophages

Having established that removal of endogenous estrogens reduced *in vivo* cytokine responses to LPS, we next investigated the influence of these reproductive hormones on the expression of key molecules used in the perception of this microbial product. We determined the circulating levels of LBP in sera isolated from ovariectomized and sham-gonadectomized animals. As shown in Fig. 2A, circulating levels of LBP were significantly lower in female mice after the removal of endogenous estrogens (n = 8–12 animals; $P < 0.05$). To further determine the mechanisms underlying the effects of estrogens on LPS sensitivity, we investigated the expression of pattern recognition receptors on acutely isolated macrophages from intact and gonadectomized female animals. As shown in Fig. 2B, peritoneal macrophages isolated from ovariectomized mice demonstrated a reduced ability to bind fluorescently labeled LPS than their intact animal-derived counterparts (n = 7–8 animals, $P < 0.05$). Importantly, the diminished capacity of macrophages to bind LPS was mirrored by a significant reduction (n = 8–12 animals, $P < 0.05$) in the cell surface expression of TLR4 as determined by flow cytometry (Fig. 2C). Interestingly, this effect appears to be limited to TLR4 because the LPS coreceptor CD14 failed to demonstrate detectable differences in expression in the absence or presence of en-

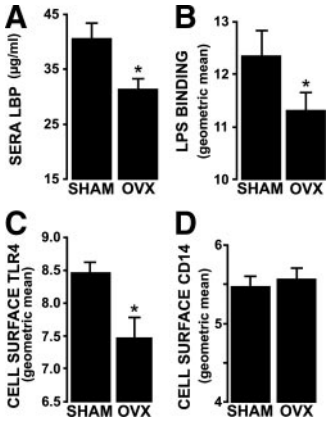

FIG. 2. Removal of endogenous estrogen decreases expression of key molecules necessary for the innate immune recognition of LPS. Mice were sham ovariectomized (SHAM) or ovariectomized (OVX) 5 wk before sera collection and isolation of peritoneal monocytes/macrophages. Sera levels of LBP were assessed by specific capture ELISA (A; SHAM n = 8, OVX n = 7). Total LPS binding (B; SHAM n = 7, OVX n = 8) and cell surface TLR4 (C; SHAM n = 11, OVX n = 12) and CD14 (D; SHAM n = 7, OVX n = 8) expression on isolated macrophages was determined by flow cytometry. Data are shown as the average fluorescence intensity (as geometric means) for each group. *, Significant difference from sham-ovariectomized animals.

dogenous estrogen (Fig. 2D). Together these data suggest that endogenous levels of estrogens in intact females may augment innate immune responses by elevating LBP production and cell surface TLR4 expression on a critical sentinel immune cell type.

To determine whether acute treatment of macrophages with 17β-estradiol can similarly elevate TLR4 expression on macrophages, we investigated the effects of exogenous 17β-estradiol on the macrophage-like cell line, RAW 264.7, and cultured primary macrophages derived from ovariectomized and sham ovariectomized animals. As shown in Fig. 3A, 24 h treatment with 17β-estradiol (1×10^{-8} and 1×10^{-10} M) failed to elicit significant effects on cell surface TLR4 expression on RAW 264.7 cells cultured in media containing 2% NuSerum (BD Biosciences) to minimize exposure to reproductive hormones. Similarly, TLR4 expression was not altered on primary macrophages derived from either ovariectomized or sham-ovariectomized animals (Fig. 3B). Consistent with the data presented in Fig. 2C, unstimulated cells from ovariectomized animals expressed significantly less cell surface TLR4 than cells derived from sham-operated animals (7.72 ± 0.47 arbitrary units of fluorescence intensity *vs.* 9.06 ± 0.17, respectively) ($P < 0.05$). Together these data indicate that acute *in vitro* exposure to physiological levels of 17β-estradiol is not sufficient to alter cell surface TLR4 expression on macrophages.

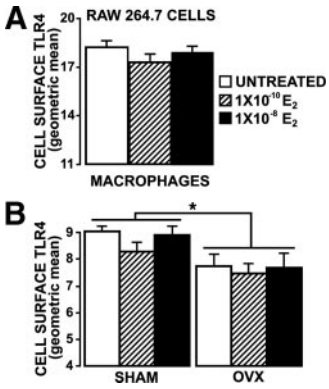

FIG. 3. Acute *in vitro* exposure to exogenous estradiol fails to augment cell surface TLR4 expression on a macrophage-like cell line and primary macrophages. A, RAW 264.7 cells were untreated or exposed to 17β-estradiol (E_2; 1×10^{-10} M or 1×10^{-8} M) for 24 h before analysis of cell surface TLR4 expression by flow cytometry (n = 18). B, Peritoneal macrophages derived from sham-ovariectomized (SHAM) or ovariectomized (OVX) mice (n = 8 animals in each group) were untreated or exposed to 17β-estradiol (E_2; 1×10^{-10} M or 1×10^{-8} M) for 24 h before analysis of cell surface TLR4 expression by flow cytometry. Data are shown as the average fluorescence intensity (as geometric means) for each group. *, Significant difference between the average of all ovariectomized mice and the average of all intact animals.

Administration of high levels of exogenous 17β-estradiol exacerbates endotoxic shock susceptibility

To test the hypothesis that female reproductive hormones elevate the expression of key microbial pattern recognition receptor expression on innate immune sentinel cells *in vivo*, we tested the susceptibility of gonadectomized female mice to endotoxin challenge after long-term high-level 17β-estradiol and/or progesterone replacement. Mice were ovariectomized or sham ovariectomized and a group of the gonadectomized animals received SILASTIC brand implants containing 17β-estradiol, progesterone, or one implant of each 17β-estradiol and progesterone. Such implants have been previously been shown to continually release hormone for at least 35 d (24). In the present study, the implants were not depleted at 5 wk after ovariectomy. After the animals were killed, implants were removed and weighed, and the presence of hormone contents confirmed visually. On average, 17β-estradiol implant content weight decreased from 3.97 ± 0.18 to 2.83 ± 0.20 mg, and progesterone implant content weight decreased from 4.74 ± 0.18 to 1.99 ± 0.08 mg. Sera levels of 17β-estradiol were 390 ± 42 pg/ml at the time the animals were killed in animals receiving 17β-estradiol implants, far in excess of the sera levels of 17β-estradiol in intact females (56 ± 38 pg/ml). At the time the animals were euthanized, sera levels of progesterone were 7.31 ± 1.32 ng/ml in animals with progesterone implants, significantly higher than progesterone levels in intact females (3.35 ± 0.67 ng/ml).

Interestingly, mice with 17β-estradiol implants showed a significant increase in susceptibility to LPS challenge (5 mg/kg, ip) over that seen in either intact mice or ovariectomized animals that did not receive 17β-estradiol replacement. As shown in Fig. 4A, ovariectomized animals receiving exogenous 17β-estradiol showed significantly higher inflammatory cytokine levels at 8 h after endotoxin challenge than untreated gonadectomized or intact animals (n = 15, $P < 0.05$), an effect that was not seen in ovariectomized animals receiving exogenous progesterone treatment. Furthermore, ovariectomized animals receiving both 17β-estradiol and progesterone showed similar elevations in inflammatory cytokine levels (n = 7–15, $P < 0.05$). This difference in inflammatory cytokine expression was associated with a dramatic increase in endotoxin susceptibility as assessed by severity scoring with almost all animals in the 17β-estradiol treatment group, as well as animals receiving both 17β-estradiol and progesterone, becoming moribund as rapidly as 8 h after challenge (Fig. 4B). Whereas there was a trend for increased sera levels of the antiinflammatory cytokine IL-10 in animals receiving

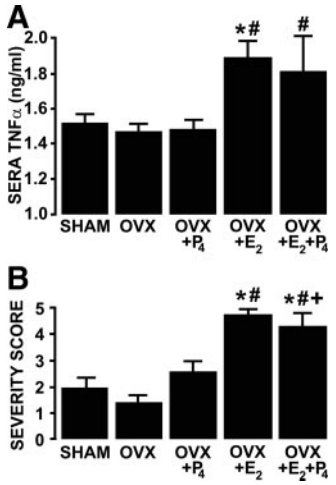

FIG. 4. Administration of high levels of exogenous estradiol renders females more susceptible to endotoxic shock. Mice were sham ovariectomized (SHAM; n = 15), ovariectomized (OVX; n = 15), ovariectomized and received exogenous progesterone replacement (OVX+P_4; n = 7), ovariectomized and received exogenous 17β-estradiol replacement (OVX+E_2; n = 15), and ovariectomized and received exogenous 17β-estradiol and progesterone replacement (OVX+E_2+P_4; n = 7), for 5 wk before LPS challenge (5 mg/kg, ip). At 8 h after challenge, sera was isolated and assayed for the presence of TNF-α (A) by specific capture ELISA. The severity of endotoxic shock was assessed according to appearance and behavior and is reported as a severity score for each animal in the three treatment groups (B). Severity was scored from 1 (no detectable behavioral differences) to 5 (moribund). *, Significant difference from sham-ovariectomized animals; #, significant difference from ovariectomized animals that did not receive hormone replacement; +, significant difference between progesterone-treated animals in the absence or presence of estradiol cotreatment. All data were analyzed statistically by one-way ANOVA with Tukey's *post hoc* analysis.

17β-estradiol treatment, this difference was not statistically significant.

Importantly, these differences cannot be attributed to treatment-induced changes in body weight. Before surgery, there was no significant difference among groups (18.0 ± 0.3, 18.2 ± 0.3, 18.3 ± 0.3, 18.3 ± 0.2, 17.9 ± 0.4 g in SHAM, OVX, OVX+P_4, OVX+E_2, OVX+E_2+P_4, respectively). Whereas ovariectomized animals weighed significantly more than sham-ovariectomized animals (20.6 ± 0.2 *vs.* 22.1 ± 0.3 g in SHAM and OVX, respectively, $P < 0.05$), there was no difference between ovariectomized groups, regardless of hormone treatment (22.5 ± 0.4, 22.0 ± 0.3, 22.3 ± 0.4 g, in OVX+P_4, OVX+E_2, and OVX+E_2+P_4, respectively). It is interesting to note that neither of the groups receiving exogenous 17β-estradiol demonstrated a decrease in body weight after long-term ovariectomy. Whereas this was somewhat unexpected, it is possible that this result may be due to the long-term administration of high doses of this hormone. Indeed, others have reported that ovariectomy and long-term exposure to estrogens either do not alter body weight or elicit only transient changes (29, 30).

Administration of exogenous 17β-estradiol elevates cell surface expression of pattern recognition receptors for LPS on murine macrophages

To investigate the mechanisms underlying the increased susceptibility of mice receiving high levels of exogenous 17β-estradiol to endotoxin challenge, we assessed the relative expression of critical innate immune receptors for LPS in these animals. Mice were ovariectomized, sham ovariectomized, or ovariectomized and given SILASTIC brand implants containing 17β-estradiol, progesterone, or both 17β-estradiol and progesterone before isolation of sera and peritoneal macrophages. As shown in Fig. 5A, circulating levels of LBP were significantly higher in gonadectomized mice receiving exogenous 17β-estradiol than those in mice that did not receive this hormone or intact females (n = 3–8 animals; $P < 0.05$: ANOVA with Tukey's *post hoc* analysis). Furthermore, peritoneal macrophages isolated from ovariectomized mice receiving 17β-estradiol replacement demonstrated a significantly greater ability to bind fluorescently labeled LPS (n = 4–8 animals, $P < 0.05$: ANOVA with Tukey's *post hoc* analysis) than those derived from animals that did not receive implants (Fig. 5B). Importantly, the increased ability of macrophages to bind LPS was mirrored by significant increases in the cell surface expression of CD14 (Fig. 5C) and TLR4 (Fig. 5D) as determined by flow cytometry (n = 4–12; $P < 0.05$: ANOVA with Tukey's *post hoc* analysis). Interestingly, this difference was not due simply to increased total cellular TLR4 expression, as evidenced by the absence of such effects in permeabilized cells (Fig. 5E). Rather, these differences were due to a reduction in the relative proportion of these receptors on the cell surface.

An ability of exogenous 17β-estradiol to reverse, and indeed augment, the susceptibility of ovariectomized female mice to endotoxin challenge suggests that this hormone alone mediates gonadectomy-induced effects on LPS sensitivity. However, these data do not preclude the involvement of other female reproductive hormones including progesterone. To investigate the role of this hormone on LPS receptor expression on a critical sentinel immune cell type, we also examined the effects of progesterone replacement on ovariectomy-induced changes in macrophage pattern recognition receptor expression. As shown in Fig. 5, the presence of progesterone-containing implants failed to augment sera levels of LBP (Fig. 5A), levels of LPS binding to macrophages (Fig. 5B), cell surface levels of CD14 on these cells (Fig. 5C), or either cell surface TLR4 expression or total TLR4 content in macrophages (Figs. 5, D and E, respectively). Similarly, coadministration of progesterone with 17β-estradiol failed to significantly effect expression of most of these parameters compared with 17β-estradiol treatment alone (Fig. 5). Whereas coadministration of progesterone with 17β-estradiol did significantly increase LPS binding to acutely isolated macrophages (Fig. 5B), it is important to note that no significant changes in cellular CD14 or TLR4 expression were observed, so the mechanisms underlying this effect are unclear.

Discussion

It has recently been recognized that gender may influence host responses to infectious organisms. In general, females generate more robust and potentially protective humoral and cell-mediated immune responses after antigenic challenge than their male counterparts (as reviewed in Ref. 1). Studies have found that females generate greater immune responses and exhibit less

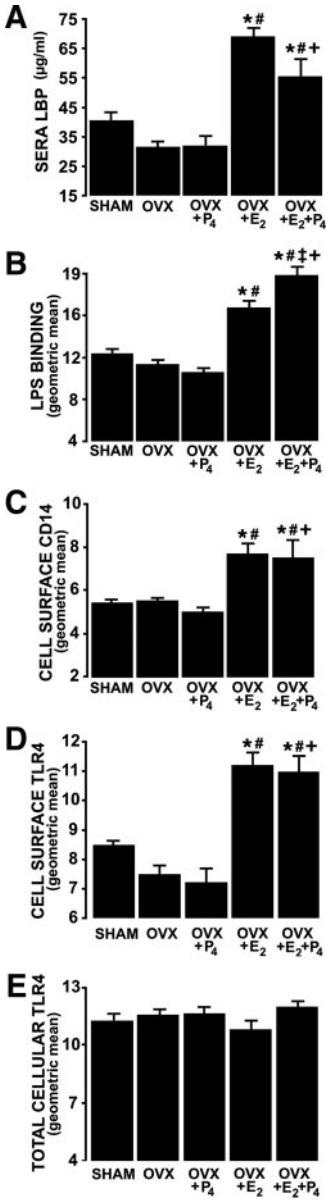

FIG. 5. Administration of exogenous estradiol elevates cell surface expression of pattern recognition receptors for LPS on murine macrophages. Mice were sham ovariectomized (SHAM), ovariectomized (OVX), or ovariectomized with 17β-estradiol (OVX+E_2) or progesterone (OVX+P_4) replacement or received replacement of both hormones (OVX+E_2+P_4) before sera collection and isolation of peritoneal monocytes/macrophages. Sera levels of LBP were assessed by specific capture ELISA (A; SHAM n = 8, OVX n = 7, OVX+P_4 n = 3, OVX+E_2 n = 3, OVX+E_2+P_4 n = 3). Total LPS binding (B; SHAM n = 7, OVX n = 8, OVX+P_4 n = 4, OVX+E_2 n = 4, OVX+E_2+P_4 n = 4), and cell surface CD14 (C; SHAM n = 7, OVX n = 8, OVX+P_4 n = 4, OVX+E_2 n = 4, OVX+E_2+P_4 n = 4) and TLR4 (D; SHAM n = 11, OVX n = 12, OVX+P_4 n = 8, OVX+E_2 n = 7, OVX+E_2+P_4 n = 7) expression on isolated macrophages was determined by flow cytometry. E, Peritoneal macrophages were permeabilized and total cellular content of TLR4 was assessed by flow cytometry (SHAM n = 7, OVX n = 8, OVX+P_4 n = 4, OVX+E_2 n = 4, OVX+E_2+P_4 n = 4). Data are shown as the average fluorescence intensity (as geometric means) for each group. *, Significant difference from sham-ovariectomized animals; #, significant difference from ovariectomized animals; ‡, significant difference between estradiol-treated animals in the absence or presence of progesterone cotreatment; +, significant difference between progesterone-treated animals in the absence or presence of estradiol cotreatment. All data were analyzed statistically by one-way ANOVA with Tukey's *post hoc* analysis.

hospitalization associated with viral infections (4–6). Furthermore, males have been found to have more severe parasitic infections than their female counterparts (2, 3). Importantly, many of these differences become apparent at puberty (9, 10), suggesting a role for reproductive hormones in their development, and this hypothesis has been supported by the finding that receptors for reproductive hormones have been found in a variety of immune cells types (as reviewed in Ref. 31). Estrogens have been demonstrated to increase resistance to streptococcal infections (11), whereas the removal of endogenous estrogens have been shown to markedly increase the severity of *Mycobacterium avium* infections, an effect that can be reversed after17β-estradiol replacement (32). However, the role played by estrogens and other female reproductive hormones in susceptibility to acute infection and/or sepsis has not been defined.

In the present study, we demonstrate that removal of endogenous estrogen results in reduced production of cytokines after endotoxin challenge. Whereas circulating levels of the inflammatory cytokines, IL-6, and TNF-α are decreased after estrogen removal, levels of the antiinflammatory cytokine, IL-10, are also reduced. This finding could account for an apparent lack of significant effects of ovariectomy on endotoxin susceptibility as assessed by severity scoring. Indeed, it has been suggested that the immunosuppressive effects of IL-10 limit the lethal sequela associated with excessive proinflammatory cytokine production and protect against endotoxemia (33–35). IL-10-deficient mice exhibit increased bacteriemia, increased inflammatory TNF-α secretion, and increased mortality associated with bacterial infection (33).

Importantly, this decreased cytokine response to endotoxin administration after removal of estrogens occurs in association with a corresponding decrease in the cell surface expression of a key microbial pattern recognition receptor for LPS, TLR4, and diminished sera levels of the permissive protein LBP. These findings are consistent with the recent observation that ovariectomy results in lower expression of another TLR family member, TLR2, in the brain after LPS challenge compared with intact females (36). As such, the reduced expression of critical molecules used in the recognition of LPS provides a potential mechanism underlying diminished *in vivo* cytokine responses after endotoxin administration in gonadectomized females. Furthermore, these data suggest that physiological levels of estrogens augment innate immune pattern recognition receptor expression on this important sentinel immune cell type.

We recently demonstrated that acute *in vitro* application of exogenous testosterone can reduce both the cell surface and total cellular expression of TLR4 in RAW 264.7 macrophage-like cells and primary macrophages derived from androgen depleted mice (12). In this study, we assessed the acute *in vitro* effects of 17β-estradiol on pattern recognition receptor expression on innate immune sentinel cells. We report that this female reproductive hormone fails to exert demonstrable effects on TLR4 levels on either RAW 264.7 cells or primary macrophages. This is consistent with previous studies showing that acute estriol treatment of Kupffer cells (14), 17β-estradiol treatment of macrophage-like cell lines (37, 38), or LPS challenged human monocytes (39) does not significantly alter TLR4 or CD14 expression. The ac-

tions of endogenous *in vivo* 17β-estradiol on macrophage pattern recognition receptor expression in the absence of acute effects *in vitro* suggests either that this hormone exerts delayed and sustained changes, such as those that may be envisaged to occur via genomic effects or, alternatively, occur as a consequence of an as-yet-undetermined intermediary effect *in vivo*. Such indirect secondary effects may include estrogen influences on macrophage progenitor cells during hematopoiesis or effects on other cell types that could in turn alter macrophage function. These questions are currently the focus of ongoing studies in our laboratory.

The decreased level of pattern recognition receptors for LPS in ovariectomized mice implies an ability of estrogens to augment the expression of these molecules. To further test this hypothesis, we assessed the effects of exogenous 17β-estradiol replacement on the level of expression of innate receptors for LPS. We demonstrated that supraphysiological levels of 17β-estradiol after hormone replacement resulted in markedly higher sera levels of LBP and cell surface TLR4 and CD14 expression on macrophages that were associated with greater inflammatory cytokine secretion and dramatically higher endotoxin susceptibility. These findings concur with previous studies showing that *in vivo* administration of estriol elevates expression of mRNA encoding CD14 and LBP in Kupffer cells and that this effect corresponds with increased LPS-associated mortality in rats (14).

Whereas 17β-estradiol treatment resulted in increased TNF-α secretion and greater susceptibility to endotoxin, it did not result in significantly higher sera levels of the antiinflammatory cytokine, IL-10. Because IL-10 provides protection against sepsis (33–35), lower levels of IL-10 secretion would be expected to correlate with increased susceptibility to endotoxin challenge. Consistent with this notion, pregnant mice that have high circulating estrogen levels demonstrate increased TNF-α expression, suppressed IL-10 levels, and increased mortality after LPS challenge (40).

Our findings indicate that 17β-estradiol treatment alters cell surface expression of TLR4 but not total protein levels of this pattern recognition receptor. This is consistent with previous studies that fail to detect changes in cellular TLR4 levels in whole-cell protein isolates from peritoneal macrophages after chronic *in vivo* 17β-estradiol treatment (41). As such, it appears that 17β-estradiol does not alter production of TLR4 at the protein level but rather affects trafficking of this receptor to the cell surface by an as-yet-unknown mechanism.

In the present study, our 17β-estradiol implants achieved a 8-fold increase in sera levels of this estrogen, whereas our progesterone implants resulted in only a 2-fold increase in sera levels of this hormone. Whereas we have been unable to elicit similar fold-increases in progesterone in our studies, it is possible that our sera concentrations are an underestimation of the release of this hormone from the implants due to high conversion of progesterone to other steroid hormones. In addition, it should be noted that in absolute terms our implants resulted in an approximately 340 pg/ml increase in the 17β-estradiol levels compared with an almost 4000 pg/ml increase in progesterone. Furthermore, previous studies have demonstrated that 17β-estradiol treatment can increase cellular progesterone receptor expression (42) that would be anticipated to increase sensitivity to this hormone. As such, the inability of progesterone replacement, alone or in combination with high levels of exogenous 17β-estradiol, to significantly elevate pattern recognition expression leads us to suggest that the ability of 17β-estradiol to increase TLR4 expression and/or sepsis susceptibility is specific to the actions of this female reproductive hormone. However, we acknowledge that we cannot currently dismiss the possibility that similar fold increases in progesterone hormone levels to those achieved with the 17β-estradiol implants might exert effects on pattern recognition receptor expression or sepsis susceptibility.

That being said, it is interesting to note that macrophages isolated from animals that received replacement of both 17β-estradiol and progesterone demonstrated higher total LPS binding capacity than those that received 17β-estradiol alone without significantly different levels of TLR4 or CD14 expression. As such, we suggest that progesterone in concert with high levels of 17β-estradiol may be capable of augmenting LPS binding in an as yet undetermined manner.

In general, estrogens have been considered to be immunoprotective. However, this term fails to delineate whether estrogens act as immunoenhancers to combat bacterial infection or are immunosuppressors and protect against the overactive and damaging immune response associated with sepsis. The present study sheds light on this paradox by showing that the effects of estrogens have to be considered in the context of both duration and level of exposure. At physiological levels seen during the menstrual cycle, we suggest that estrogens are protective and contribute to a more robust immune response to bacterial endotoxin challenge compared with their ovariectomized counterparts. However, such immune responses do not render these mice more susceptible to endotoxic shock, perhaps due to a concurrent increase in antiinflammatory cytokine levels. In contrast, animals that have supraphysiological levels of estrogens demonstrate sensitized innate immune cells, resulting in a more rapid and elevated inflammatory response after endotoxin challenge and hence greater sepsis severity. Such a hypothesis is supported by recent studies in human patients showing that the probability of septic shock mortality is lowest when sera estrogen levels are within the normal physiological range but is significantly higher in nonsurviving sepsis patients, regardless of sex (16, 17).

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