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Gender difference in tumor necrosis factor-α production in human neutrophils stimulated by lipopolysaccharide and interferon-γ

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ABSTRACT

The gender difference in tumor necrosis factor-α (TNF-α) production in human neutrophils stimulated by lipopolysaccharide (LPS) and interferon-γ (IFN-γ) was explored by using peripheral blood neutrophils from young men and women. As compared with female neutrophils, male neutrophils released greater amounts of TNF-α, and exhibited stronger activation of mitogen-activated protein kinases and phosphatidylinositol 3-kinase in response to LPS stimulation. LPS-induced TNF-α production was markedly enhanced by pretreatment of cells with IFN-γ, and IFN-γ-mediated priming in male neutrophils was significantly greater than that in female neutrophils. Male neutrophils showed higher expression of TLR4, but not IFN-γ receptors, than female neutrophils, and its expression was increased by stimulation with IFN-γ or IFN-γ plus LPS. These findings indicate that male neutrophils show higher responsiveness to stimulation with LPS and IFN-γ than female neutrophils, and suggest that the gender difference in neutrophil responsiveness to LPS and IFN-γ is partly responsible for that in the outcome of sepsis, in which premenopausal women show a favorable prognosis as compared with men.

1. Introduction

Accumulated evidences indicate that the gender difference exists in the clinical outcome of various inflammatory disorders, including cardiovascular diseases and sepsis [1,2]. As compared with age-matched men, pre-, but not post-, menopausal women have been shown to be protected against cardiovascular diseases, which may be associated with the vasoprotective and anti-inflammatory effects of estrogen (17β-estradiol) [1]. For example, estradiol strongly inhibits interleukin-1β (IL-1β)-induced up-regulation of E-selectin, vascular cell adhesion molecule-1, and intercellular adhesion molecule-1 in human umbilical vein endothelial cells [3]. In a murine model, estrogen-mediated suppression of IL-1β-induced P-selectin expression on endothelial cells may contribute to reduced leukocyte recruitment in females [4]. In regard to the outcome of sepsis, premenopausal women show a favorable prognosis as compared with men [2], which may be partly associated with the ability of whole blood to produce tumor necrosis factor-α (TNF-α) in response to stimulation with lipopolysaccharide (LPS); i.e., the TNF-α-producing ability of whole blood in premenopausal women is significantly less than that in postmenopausal women or men [5,6]. In fact, it has been reported that estradiol attenuates LPS-induced CXCL8 and TNF-α production in human peripheral blood monocytes when monocytes are pretreated with estradiol *in vitro* for 24 h before LPS stimulation, and that LPS-induced NF-κB activation in monocyte-derived macrophages is suppressed by pretreatment of cells with estradiol [7,8]. More recently, it has been reported that estradiol induces increased surface expression of annexin A1 on human neutrophils, and inhibits neutrophil adhesion to endothelial cells under the shear stress, which may also contribute to the anti-inflammatory effect of estradiol [9]. These previous reports suggest that estradiol exerts the anti-inflammatory effects by modulating the functions of vascular endothelial cells, monocytes/macrophages, and neutrophils [1–10].

In the present study, we examined the gender difference in TNF-α production in human neutrophils stimulated by LPS and interferon-γ (IFN-γ) by using peripheral blood neutrophils from young men and women. The results show that, as compared with female neutrophils, male neutrophils release greater amounts of TNF-α, and exhibit stronger activation of mitogen-activated protein kinases (MAPKs) and phosphatidylinositol 3-kinase (PI3K) in response to LPS stimulation. LPS-induced TNF-α production was markedly enhanced by pretreatment of cells with IFN-γ, and IFN-γ-mediated priming in male neutrophils was significantly greater than that in female neutrophils. Male neutrophils showed higher expression of TLR4, but not IFN-γ receptors, than female neutrophils, and its expression was increased by stimulation with IFN-γ or IFN-γ plus LPS. These findings suggest that male neutrophils show higher responsiveness to stimulation with LPS and IFN-γ than female neutrophils, which may be partly responsible for the gender difference in the outcome of sepsis [2].

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2. Materials and methods

2.1. Reagents

Recombinant human IFN-γ was provided by Shionogi Pharmaceutical (Osaka, Japan). Re-extracted LPS from *Escherichia coli* was purchased from List Biological Laboratories (Campbell, CA). 17β-Estradiol was purchased from Wako Pure Chemical (Osaka, Japan). Phycoerythrin (PE)-conjugated monoclonal antibodies against human CD284 (TLR4; clone HTA125) and human CD119 (IFN-γ receptor α chain; clone GIR-94) were purchased from Biolegend (San Diego, CA). Rabbit polyclonal antibodies against extracellular signal-regulated kinase 1/2 (ERK1/2), Thr^{202}/Tyr^{204}-phosphorylated ERK1/2, p38 MAPK, Thr^{180}/Tyr^{182}-phosphorylated p38 MAPK, c-Jun amino-terminal kinase (JNK), Thr^{183}/Tyr^{185}-phosphorylated JNK, Akt, Ser^{473}-phosphorylated Akt, IκBα, Ser^{32}-phosphorylated IκBα, signal transducer and activator of transcription 1 (STAT1), Tyr^{701}-phosphorylated STAT1, and goat anti-rabbit IgG antibody conjugated with horseradish peroxidase were purchased from Cell Signaling Technology (Beverley, MA). SB203580 (p38 MAPK inhibitor), U0126 (MAPK/ERK kinase (MEK) inhibitor), and SP600125 (JNK inhibitor) were purchased from Calbiochem (San Diego, CA). LY294002 (PI3K inhibitor) was purchased from Sigma Chemical (St. Louis, MO). Conray was purchased from Mallinckrodt (St. Louis, MO). Ficoll and the enhanced chemiluminescence (ECL) Western blotting system were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK).

2.2. Preparation of cells

All donors that participated in the present experiments were healthy medical students. Before obtaining the peripheral blood, all donors were asked if they had any physical problems or were taking any medication, and we confirmed that all donors were absolutely healthy and taking no medication. The age of the donors primarily ranged from 21 to 25 years old with a few exceptions (range: 19–30 years old). For the comparative studies, the peripheral blood was obtained from 2 donors (a male and a female), and the samples were similarly processed in parallel to avoid any possible artifacts. Peripheral blood neutrophils were prepared as described previously [11], using dextran sedimentation, centrifugation with Conray–Ficoll, and hypotonic lysis of contaminating erythrocytes. Neutrophil fractions contained >98% neutrophils. Cells were suspended in RPMI 1640 supplemented with 10% autologous serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). The work described has been carried out in accordance with The Code of Ethics of the World Medical Association. The study protocol was approved by the Ethical Committee of Osaka City University, Graduate School of Medicine, and informed consent was obtained from all subjects.

2.3. Determination of TNF-α production

The neutrophil suspensions (3×10^6/ml) were placed in polypropylene tube (Falcon #2063; Falcon Labware, Becton Dickinson, NJ), and were cultivated with or without LPS (1 μg/ml) for 20 h in 5% CO_2/95% humidified air at 37 °C. When required, cells were pretreated with IFN-γ (1000 U/ml) or 17β-estradiol for 30 min at 37 °C before stimulation with LPS. After incubation, the amounts of TNF-α in the cell-free culture supernatants were determined by the TNF-α specific ELISA kits (R&D System, Mineapolis, MN), which can detect >1.6 pg/ml TNF-α.

2.4. Determination of TLR4 and IFN-γ receptor expression

Cells were stained with PE-conjugated antibody against human CD284 (TLR4) or CD119 (IFN-γ receptor α chain), and analyzed by flow cytometry with FACSCalibur (Becton Dickinson, Mountain View, CA) as described previously [12].

2.5. Western blotting

Western blotting was performed as described previously [13]. Cells (5×10^6/ml) suspended in RPMI 1640 supplemented with 10% autologous serum were stimulated with LPS for indicated periods at 37 °C. The reactions were terminated by the addition of trichloroacetic acid (TCA). The final TCA concentration was 10%. The cells were washed with acetone containing 10 mM dithiothreitol, and were lysed with the 1× sample buffer, heated at 100 °C for 5 min, and then frozen at −20 °C until analysis. Samples were subjected to 5–20% gradient SDS gel electrophoresis. After electrophoresis, proteins were electrophoretically transferred from the gel onto a nitrocellulose membrane in a buffer containing 100 mM Tris, 192 mM glycine, and 20% methanol at 2 mA/cm^2 for 1.5 h at room temperature. Residual binding sites on the membrane were blocked by incubating the membrane in Tris-buffered saline (pH 7.6) containing 0.1% Tween 20 and 5% nonfat dry milk for 2 h at room temperature. The blots were incubated with appropriate primary antibody overnight at 4 °C. After washing, the membrane was incubated with appropriate secondary antibody conjugated with horseradish peroxidase, and the antibody complexes were visualized by the ECL detection system as directed by the manufacturer. Immunoreactive bands were quantified by a NIH Image program on a Macintosh computer.

2.6. Statistical analysis

An ANOVA followed by a multiple comparison test or the Student *t* test was used to determine statistical significance.

3. Results

Human neutrophils produced TNF-α in response to stimulation with LPS, but not IFN-γ, and LPS-induced TNF-α production was markedly enhanced by pretreatment of cells with IFN-γ [14,15]. The amounts of TNF-α produced by LPS-stimulated neutrophils were varied according to the samples, and this variation was found to be primarily attributed to the gender rather than the individuals. As shown in Fig. 1, male neutrophils produced significantly greater amounts of TNF-α than female neutrophils when stimulated with LPS, indicating that there is the gender difference in the ability of neutrophils to produce TNF-α in response to LPS stimulation. The gender difference in LPS-induced TNF-α production in neutrophils was further increased when cells were pretreated (or primed) with IFN-γ for 30 min before LPS stimulation (Fig. 1). Pretreatment of cells with IFN-γ enhanced LPS-induced TNF-α production in male and female neutrophils by 8.34 ± 1.16 and 4.24 ± 0.85 folds (means ± SEM), respectively; the fold increase in male neutrophils being significantly ($P < 0.001$) greater than that in female neutrophils. As a result, LPS-induced TNF-α production in IFN-γ-primed male neutrophils was much greater than that in female neutrophils (Fig. 1).

To explore the possible mechanisms underlying the gender difference in neutrophil responsiveness to LPS and IFN-γ, the surface expression of TLR4 and IFN-γ receptors on neutrophils was analyzed. As shown in Fig. 2A, male neutrophils showed higher expression of TLR4 than female neutrophils. The expression of TLR4 was up-regulated by stimulation with IFN-γ, but not LPS, and IFN-γ-mediated up-regulation of TLR4 was further enhanced by co-stimulation with LPS. As a result, the gender difference in TLR4 expression on neutrophils was increased when cells were stimulated with IFN-γ or IFN-γ plus LPS (Fig. 2A). On the other hand, no gender difference was observed in the expression of

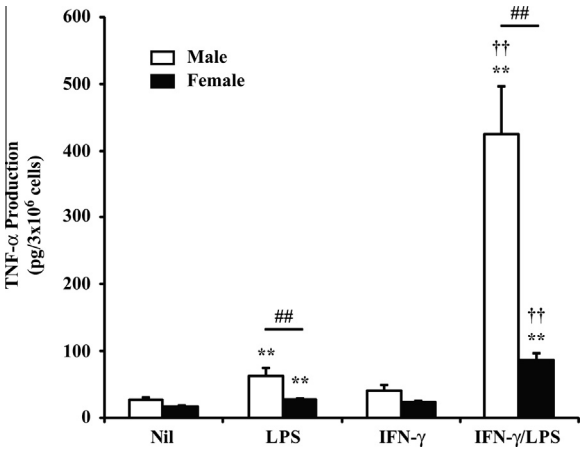

Fig. 1. TNF-α production in human neutrophils stimulated by LPS and IFN-γ. Neutrophils (3×10^6 cells) were cultivated in the presence of LPS (1 μg/ml), IFN-γ (1000 U/ml), or IFN-γ (1000 U/ml) plus LPS (1 μg/ml) for 20 h at 37 °C. When required, cells were pretreated with IFN-γ (1000 U/ml) for 30 min at 37 °C before LPS stimulation. After cultivation, the amounts of TNF-α in the culture supernatants were determined. The data are expressed as means ± SEM ($n = 12$ for males and $n = 16$ for females, respectively). **Significantly greater as compared with controls (Nil) (**$P < 0.01$). ##Significant gender difference (##$P < 0.01$). ††LPS-induced TNF-α production in both types of cells was significantly enhanced by pretreatment of cells with IFN-γ (††$P < 0.01$).

IFN-γ receptors (Fig. 2B). The expression of IFN-γ receptors was rather down-regulated by stimulation with IFN-γ, but not LPS, and IFN-γ-mediated down-regulation of IFN-γ receptors was unaffected by co-stimulation with LPS (Fig. 2B). These findings indicate that the surface expression of TLR4 and IFN-γ receptors on neutrophils may be differentially regulated by IFN-γ and LPS, and suggest that the gender difference in LPS-induced TNF-α production in neutrophils may be partly ascribed to that in the expression of TLR4 on resting and IFN-γ-primed neutrophils.

Since there is the gender difference in the expression of TLR4 on resting neutrophils, a comparative study in the magnitude of activation of the signaling pathways provoked by LPS stimulation was performed by using male and female resting neutrophils [15,16]. As shown in Fig. 3A, as compared with female neutrophils, male neutrophils exhibited greater activation of distinct signaling molecules, including ERK, p38, JNK, and Akt, in response to stimulation with a wide range of LPS concentrations. These findings are consistent with higher expression of TLR4 on male neutrophils. No apparent gender difference was observed in IκBα phosphorylation and degradation. The studies with various inhibitors show that, among the signaling molecules, ERK, p38, and PI3K/Akt, but not JNK, may be involved in TNF-α production in neutrophils stimulated by IFN-γ plus LPS, since this response was significantly inhibited by U0126 (MEK inhibitor), SB203580 (p38 inhibitor), and LY294002 (PI3K inhibitor), but not by SP600125 (JNK inhibitor) (Fig. 4A). These findings are consistent with our previous observations in LPS-induced IL-8 production in human neutrophils [15]. No gender difference was observed in the sensitivity to the inhibitory effects of these inhibitors. No apparent gender difference was observed in STAT1 phosphorylation in neutrophils stimulated by IFN-γ (Fig. 3B) [17]; a finding consistent with no gender difference in the expression of IFN-γ receptors. Pretreatment of cells with IFN-γ did not affect LPS-induced ERK, p38, JNK, Akt, and IκBα phosphorylation (Fig. 3C), suggesting that IFN-γ-mediated enhancement of LPS-induced TNF-α production is unlikely to be ascribed to enhanced activation of these signaling molecules.

A recent study has shown that annexin A1 is up-regulated by treatment of human neutrophils with estradiol for 30 min [9], indicating that neutrophil functions could be rapidly modulated

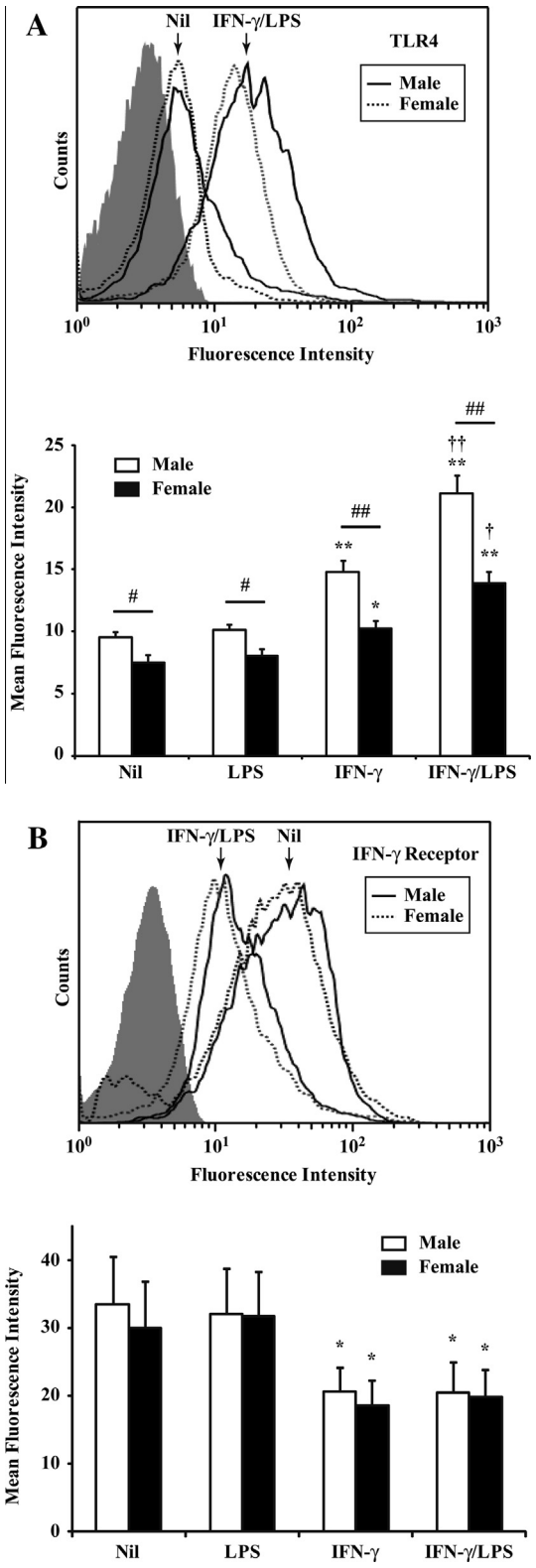

Fig. 2. Expression of TLR4 and IFN-γ receptors on human neutrophils. Neutrophils were cultivated with or without IFN-γ (1000 U/ml), LPS (1 μg/ml), or IFN-γ (1000 U/ml) plus LPS (1 μg/ml) for 20 h at 37 °C. After cultivation, the expression of (A) TLR4 and (B) IFN-γ receptors was analyzed by flow cytometry. (Upper panels for A and B) The histograms shown are representative of five independent experiments; each experiment done using 2 donors (a male and a female) for comparison. Unstained cells are indicated by shaded area. (Lower panels for A and B) The data are expressed as means ± SEM of five independent experiments. *,**Significantly (A) up- or (B) down-regulated by stimulation with IFN-γ or IFN-γ plus LPS (*$P < 0.05$, **$P < 0.01$). (A) #,##Significant gender difference (#$P < 0.05$, ##$P < 0.01$). †,††IFN-γ-induced up-regulation of TLR4 in both types of cells was significantly enhanced by co-stimulation with LPS (†$P < 0.05$, ††$P < 0.01$).

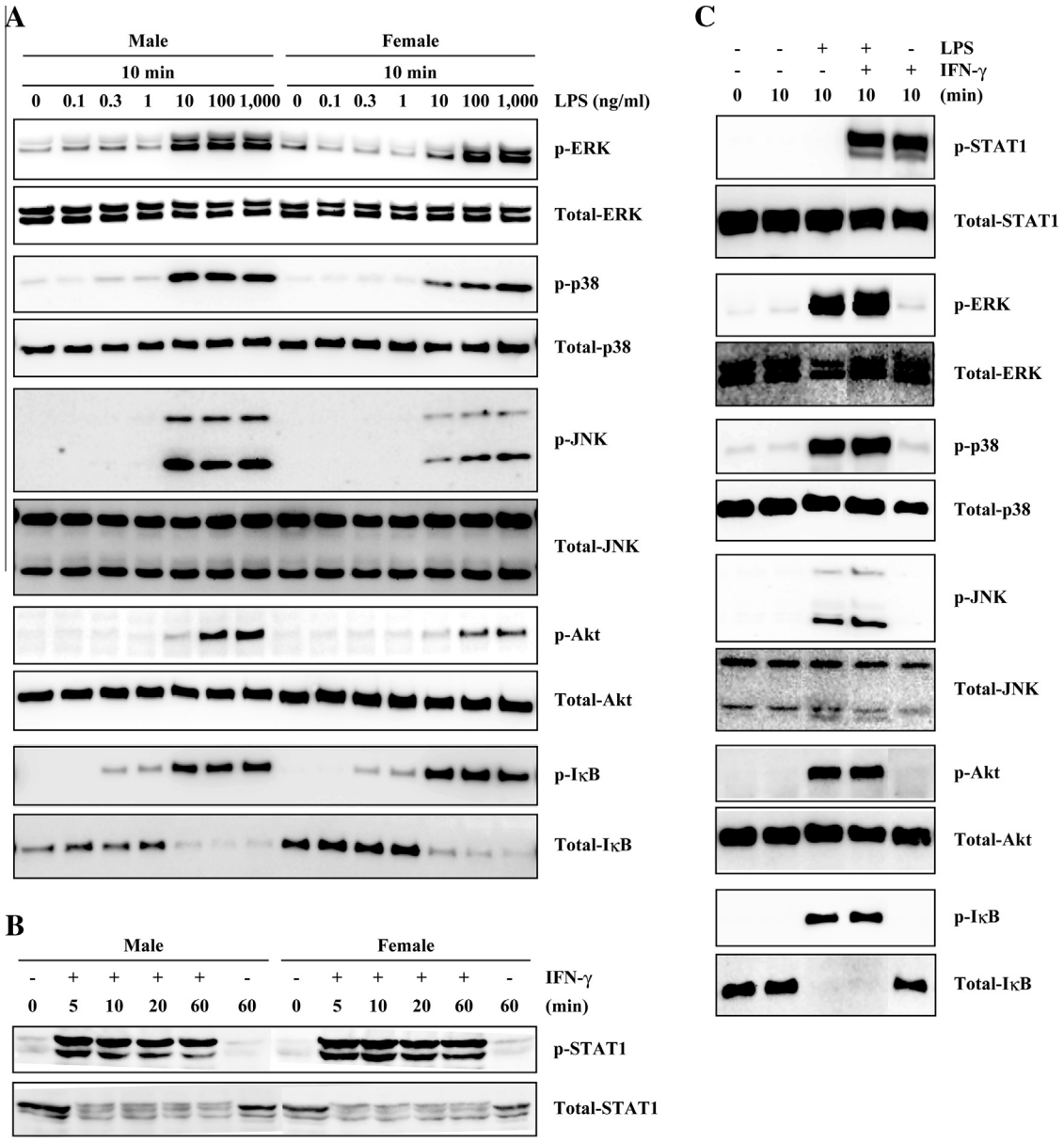

Fig. 3. Activation of the signaling molecules in human neutrophils stimulated by LPS and/or IFN-γ. (A) Neutrophils were stimulated with indicated concentrations of LPS for 10 min at 37 °C. (B) Neutrophils were stimulated with IFN-γ (1000 U/ml) for indicated periods at 37 °C. (C) Neutrophils were pretreated with or without IFN-γ (1000 U/ml) for 30 min at 37 °C, and thereafter stimulated with LPS (1 μg/ml) for 10 min at 37 °C. Immunoblotting was performed using antibodies against phosphorylated or unphosphorylated form of each protein. The cell lysates equivalent to 5×10^5 cells were loaded onto each lane. The results shown are representative of 3–5 independent experiments.

in vitro by estradiol. Then, we studied the effect of estradiol on TNF-α production in male neutrophils stimulated by IFN-γ plus LPS. As shown in Fig. 4B, TNF-α production induced by IFN-γ plus LPS was unaffected by pretreatment of cells with 17β-estradiol (10^{-10}–10^{-7} M) for 30 min at 37 °C. The expression of TLR4 on male neutrophils was also unaffected by treatment of cells with 17β-estradiol (10^{-7} M) for 20 h (data not shown).

4. Discussion

The present experiments show that male neutrophils produced significantly greater amount of TNF-α than female neutrophils when stimulated with LPS or IFN-γ plus LPS. Male neutrophils also exhibited higher sensitivity to IFN-γ than female neutrophils when assessed on the basis of IFN-γ-mediated priming on LPS-induced TNF-α production and IFN-γ-mediated up-regulation of TLR4. The gender difference in neutrophil responsiveness to LPS may be, at least in part, responsible for that in the outcome of sepsis, in which premenopausal women show a favorable prognosis as compared with men. The remarkable gender difference in LPS-induced TNF-α production in IFN-γ-primed neutrophils may be physiologically relevant and important, since neutrophils could be exposed to both IFN-γ and LPS at the inflammatory sites.

The gender difference in LPS-induced TNF-α production in neutrophils may be partly ascribed to that in the expression of TLR4; i.e., the expression of TLR4 on male neutrophils is higher than that on female neutrophils. These findings are consonant with the previous reports demonstrating that the gender difference exists in human monocyte responsiveness to LPS stimulation [18,19]. It has been reported that the expression of TLR4 on male monocytes is higher than that on female monocytes, and LPS-induced cytokine

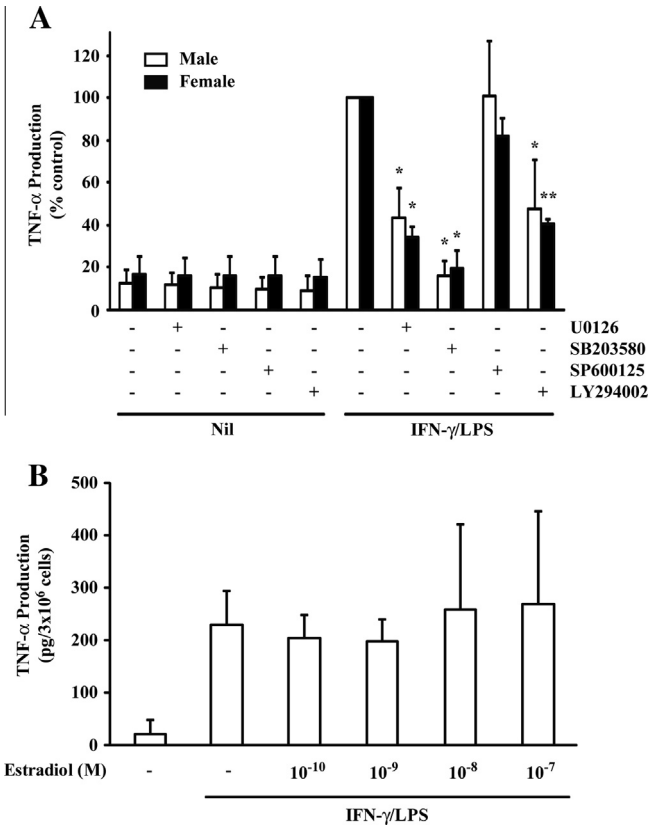

Fig. 4. Effects of various inhibitors and estradiol on TNF-α production in human neutrophils stimulated by IFN-γ plus LPS. (A) Neutrophils (3×10^6 cells) were pretreated with U0126 (10 μM), SB203580 (10 μM), SP600125 (25 μM), or LY294002 (10 μM) for 30 min at 37 °C, and thereafter cultivated with IFN-γ (1000 U/ml) plus LPS (1 μg/ml) for 20 h. The data are expressed as means ± SEM of three independent experiments; each experiment done using 2 donors (a male and a female) for comparison. *,**Significantly inhibited by each inhibitor (*$P < 0.05$, **$P < 0.01$). (B) Male neutrophils (3×10^6 cells) were pretreated with indicated concentrations of 17β-estradiol for 30 min at 37 °C, and thereafter cultivated with IFN-γ (1000 U/ml) plus LPS (1 μg/ml) for 20 h. After cultivation, the amounts of TNF-α in the culture supernatants were determined. The data are expressed as means ± SEM of three independent experiments.

production in male monocytes is greater than that in female monocytes [18]. It have been also reported that the percentage of peripheral blood mononuclear cells expressing intracellular TLR4 protein is higher in young men as compared with young women, and LPS-induced TNF-α and IL-10 mRNA production in mononuclear cells is greater in male than female donors [19]. The expression of TLR4 on human neutrophils was not altered by the short term incubation of cells with 17β-estradiol, although it has been reported that annexin A1 on neutrophils is up-regulated by 17β-estradiol under the same conditions [9]. These findings suggest that the gender difference in the expression of TLR4 on human neutrophils may be caused by the *in vivo* effect of sex hormones on maturation process of neutrophils.

The greater ability of male neutrophils to produce TNF-α in response to IFN-γ plus LPS stimulation may be ascribed not only to higher expression of TLR4 but also to higher sensitivity to IFN-γ. The higher sensitivity to IFN-γ in male neutrophils may be ascribed to the intracellular cross-talk of the signaling molecules activated by IFN-γ and LPS stimulation, since no gender difference was observed in the expression of IFN-γ receptors and IFN-γ-induced STAT1 phosphorylation in neutrophils. LPS-induced ERK, p38, JNK, Akt, and IκBα phosphorylation in neutrophils was not affected by pretreatment of cells with IFN-γ, suggesting that IFN-γ affects the pathway downstream or independent of these signaling molecules. It has been proposed that the synergistic induction of CXCL10 by LPS and IFN-γ in human neutrophils may be caused by the cooperative activation of NF-κB and STAT1 by LPS and IFN-γ, respectively [20], and the similar mechanisms may be also functioning in granulocyte–macrophage colony-stimulating factor (GM-CSF)-mediated enhancement of TLR2 or TLR4 agonist-induced cytokine (TNF-α and IL-8) production in human neutrophils [15]. In this regard, it is of interest that the expression of TLR4 on neutrophils was up-regulated by IFN-γ, but not GM-CSF, and enhancement of LPS-induced TNF-α production by IFN-γ (approximately 4–8 folds) was much greater than that by GM-CSF (approximately 2 folds) [15], suggesting that IFN-γ-mediated up-regulation of TLR4 may play a role in IFN-γ-mediated enhancement of LPS-induced TNF-α production. Our previous studies indicate that granulocyte CSF (G-CSF) negatively regulates TLR agonist-induced cytokine production in neutrophils via activation of STAT3, and G-CSF does not affect TLR agonist-induced phosphorylation of ERK, p38, JNK, Akt, and IκBα [15]. These findings taken together suggest that G-CSF, GM-CSF, and IFN-γ may regulate LPS-induced cytokine production in neutrophils via activation of STAT1 or STAT3 without affecting activation of MAPKs and PI3K/Akt. The precise mechanisms for STAT1- and STAT3-mediated regulation of LPS-induced cytokine production remain to be determined.

Engagement of TLR4 with LPS results in activation of two major intracellular signaling pathways, the MyD88-dependent and independent pathways. The former is primarily involved in the expression of inflammatory cytokines such as TNF-α and IL-6, and the latter is primarily involved in the expression of type I IFNs [21]. Accumulated evidences suggest that the MyD88-dependent pathway may lead to the expression of various inflammatory cytokines by coordinated action of NF-κB, activator protein-1 (AP-1), and interferon regulatory factor 5 (IRF5), and AP-1 is activated by MAPKs [21]. In fact, LPS-induced TNF-α production in human neutrophils was inhibited by specific inhibitors of MEK and p38. These findings and the present experiments taken together suggest that the gender difference in LPS-induced TNF-α production in neutrophils may be partly ascribed to different activation of the MAPK/AP-1 pathway, since no apparent gender difference was observed in IκBα phosphorylation and degradation, and apparent gender difference was detected in activation of MAPKs and PI3K.

In summary, the present experiments show that there is the gender difference in TNF-α production in human neutrophils stimulated by LPS or IFN-γ plus LPS. The lower sensitivity of female neutrophils to LPS and IFN-γ, in concert with the anti-inflammatory effects of estradiol on vascular endothelial cells and monocytes/macrophages, may contribute to the favorable outcome of premenopausal women in case of sepsis as compared with men.

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