MyD88 and Src Are Differentially Regulated in Kupffer Cells of Males and Proestrus Females Following Hypoxia

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Hypoxia produces sex dimorphic immune responses in males and proestrus females. Because Kupffer cells are the major source of proinflammatory cytokines, studies were conducted to discern IL-6 production in mouse Kupffer cells following hypoxia. Hypoxia enhances TLR4 expression in Kupffer cells irrespective of sex. However, MyD88 and Src expression in Kupffer cells decreased significantly after hypoxia in proestrus females, whereas Src protein expression and phosphorylation increased in males in concurrence with differences in IL-6 production. 17β-Estradiol administration elevated MyD88 and Src expression in males to levels in normoxic proestrus females. Administration of Src inhibitor in hypoxic males prevented increased IL-6 production. Thus, differential regulation of MyD88 and Src in males and females plays an important role in sex-specific immune response following hypoxia.
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INTRODUCTION

Trauma-hemorrhage remains a major health crisis in the United States. The trauma-related mortality rate is greater for males (9%) than females (4%). An epidemiological study carried out at the University of Alabama at Birmingham (UAB) predicted a statistically higher risk of death for males (up to 50 y old) compared with premenopausal adult females following blunt trauma (1). Although the etiology of mortality induced by traumatic injury in males and females remains unknown, the depression of immune functions leading to sepsis and multiple organ dysfunctions strongly correlates with high mortality (2-8). One major consequence of injury following trauma-hemorrhage is hypoxia (9,10). Hypoxia promotes undesirable proinflammatory responses in young male mice; however, such an adverse effect of hypoxia is not observed in proestrus females (10,11). Severe depression in immune function following hypoxia or trauma-hemorrhage is evidenced by altered macrophage (Mϕ) functions, the release of TNF-α and IL-6, and their increased levels in the circulation (12-17). Among cytokines, high levels of plasma IL-6 after the onset of sepsis were a good predictor of mortality (18).

The toll-like receptors (TLRs) are cell-surface components that participate in innate immune recognition (19). Studies have identified more than 10 different TLRs, each specific for a ligand (14,19-23). Upon binding of the ligand, TLR initiates host responses through a series of signaling events. In general, the cascade of events involves the activation of a common set of adaptor proteins and the protein kinases (19,24). Studies have shown that the toll/interleukin-1 receptor (TIR), recruitment of MyD88, and subsequent downstream signaling that include activation of mitogen-activated protein kinase (MAPK) and transcription factors NF-κB constitute a signaling network, which is critical to the production of proinflammatory mediators, IL-6 and TNF-α (19,22-25).

MyD88 is an adaptor protein that is shared by all TLR pathways. Upon activation of TLR, MyD88 is recruited to TLR receptor domains and links TLR with the downstream intracellular signaling cascades (26,27). Recent studies indicate that activation of MAPK and NF-κB also occurred in cells from MyD88 knockout mice, implying the presence of an alternate MyD88-independent signaling pathway (28-31). In the MyD88-independent pathway, TIR domain-containing adaptor protein (TIRAP) is responsible for activating intracellular signaling. Furthermore, studies have also shown that Src tyrosine kinases play a positive regulatory role in TLR4-mediated iNOS expression in a MyD88-independent (TIRAP-dependent) manner. Thus, both MyD88-dependent and MyD88-independent signaling pathways are implicated in innate immunity (28-33).

Although Mϕ from spleen, peritoneal cavity, or liver contribute significantly to immune depression following hypoxia or trauma-hemorrhage, the largest Mϕ pop-

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ulation is the Kupffer cells, which are located in the liver (34). Furthermore, Kupffer cells are the major source of TNF-α and IL-6 production, and their enhanced release following hypoxia is evidenced by the increased levels of TNF-α and IL-6 levels in the circulation following injury (11,35). The increased release of these immunomodulators is significant and correlates with immunosuppression in males, but not in proestrus females, following hypoxia (11). Earlier studies have demonstrated the expression of TLR in macrophages and their involvement in the intracellular signaling cascade for the generation of immune modulators, TNF-α, IL-6, and iNOS (6,7,36,37). In this study, we examined the expression of TLR4, the downstream signaling pathway, and release of the cytokine IL-6 by Kupffer cells from male and proestrus female mice to determine whether intracellular signaling differs significantly and reflects a divergent hypoxic effect. Our results demonstrate that hypoxia increased Kupffer cell TLR-4 expression in both males and proestrus females. However, MyD88 and Src expression were downregulated in Kupffer cells from proestrus females, whereas Src expression and phosphorylation were increased in Kupffer cells from males after hypoxia. Finally, our results suggest that MyD88 likely plays the central role in IL-6 release in Kupffer cells from proestrus females whereas Src is responsible for the production of IL-6 by Kupffer cells from males following hypoxia.

MATERIALS AND METHODS

Mice

C3H/HeN male and female mice, 6 to 8 weeks of age (20 to 25 g), which had been acclimated in our animal facility for at least 7 days, were used for this study. Estrus cycle in female mice was determined by daily examination of vaginal smears; only females in the proestrus cycle were used. All mouse experiments were approved by the UAB Institutional Animal Care and Use Committee and conformed to the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH publ. no. 85-23, 1996).

Model of Hypoxia

Hypoxia without soft-tissue trauma or blood loss was produced as described previously with some modification (11). Briefly, mice were placed in 2 plastic chambers (20 by 10 by 8 cm). Each chamber had an inlet and an outlet through which the hypoxic mixture (95% N_2 and 5% O_2) or compressed room air (sham group) flowed at a flow rate of 5 L/min for 1.0 h. The mice were then returned to ambient room air. No immediate or late mortality was observed with this model of hypoxia.

Previous studies have shown that arterial pO_2 decreased from baseline values of 120 mmHg to values of ~40 mmHg 20 min after the beginning of hypoxemia and remained at that level throughout the duration of hypoxemia (10).

In some experiments, male mice received a subcutaneous injection of 250 μg/kg body weight 17β-estradiol 1 d before they were subjected to hypoxia. This dose of 17β-estradiol was selected from our previous studies, which showed a salutary effect following trauma-hemorrhage (35). Additional experiments were also performed in which male mice were treated with the Src inhibitor PP1 (Biomol, Plymouth Meeting, PA, USA) intraperitoneally 1 h prior to hypoxia at a dose of 1.5 mg/kg body weight (38,39). PP1 is a potent and selective inhibitor of Src-family tyrosine kinases including Src, Fyn, Hck, and Lck (38,39). Before injection, PP1 was dissolved in dimethyl sulfoxide and diluted in phosphate-buffered saline. For the control group, the same solvent without PP1 was injected using the same procedure.

Preparation of Kupffer Cells

One hour after hypoxia, the mice were anesthetized and livers were removed for preparation of Kupffer cells. The Kupffer cells were isolated from mice as previously described with some modification (12). Briefly, the liver was immediately washed 3 to 5 times with 20 mL Hanks balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+}. This was followed by perfusion of 15 mL HBSS containing 0.02% collagenase, 0.02% trypsin inhibitor (Sigma-Aldrich Corp.), and 0.5% of 100 mM calcium chloride solution at the same perfusion rate. The liver was then removed into a 60-mm Petri dish containing HBSS with 0.02% collagenase and incubated for 20 min at 37 °C to further dissociate the cells. The cell suspension was then passed through a sterile 150-mesh stainless steel screen into cold HBSS (final volume 40 mL) and centrifuged at 50 x *g* for 3 min at 4 °C to sediment hepatocytes. The remaining cells in the supernatant were collected by centrifugation (1500 x *g* for 15 min at 4 °C) and suspended in William's E medium. The supernatant was discarded and the cell pellets were gently laid over 6 mL of 16% metrizamide (Accurate Chemical & Scientific Co., Westbury, NY, USA). The Kupffer cell layer was found at the interface of the metrizamide and the media following centrifugation (400 x *g* for 45 min at 4 °C). The cells were further washed with 25 mL William's medium. The isolated Kupffer cells were resuspended in complete William's E medium containing 10% heat-inactivated fetal calf serum and ampicillin/streptomycin and then allowed to adhere to the bottom of the plastic culture dish for 2 h; unattached cells were removed by gentle washing. This protocol provided adherent cells that were greater than 96% positive by nonspecific esterase staining and exhibited typical macrophage morphology. Adherent cell cultures were then divided into 2 groups. One was cultured for 24 h in complete William's E medium to study the cytokine production capacity; the other group was removed from the culture dish and analyzed for the purity of Kupffer cells using a rat anti-mouse macrophage monoclonal antibody F4/80 (1:10 dilution, 45 min, 4 °C; Bioproducts, Indianapolis, IN, USA) (12) followed by anti-rat IgG-FITC (1:10 dilution, 45 min, 4 °C; Sigma Chemical Co., St. Louis, MO, USA) and counterstained with 1,1′-dioct-adecyl-3,3,3′,3′-tetram-

ethyllindrocarbocyanine perchlorate (5 μg/mL, 10 min, 37 °C; Molecular Bioprobes, Eugene, OR, USA). These were 90% positive with this macrophage marker (12).

RNA Isolation

Total RNA was isolated with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Briefly, cultured macrophages or Kupffer cells (5×10^6) were lysed with 1 mL TRIzol and RNA was precipitated by isopropyl alcohol. The quality and quantity of extracted total RNA samples were examined by loading 5 μg of each sample on a 2.2% denaturing agarose gel.

Quantitative Real-Time PCR

Quantitative gene expression analysis was performed on ABI GeneAmp 7900 Sequence Detection System machine using FAM labeling probes (Applied Biosystems, Foster City, CA, USA). In 10 μL reaction mixture, 5.0 μL master mix, 4.5 mL cDNA (corresponding to 10 ng of total RNA input), and 0.5 μL gene forward and reverse primers and probe, the following were added:

IL-6 (5′-GAGAAAAGAGTTGTGCAA TGGCAAT-3′FAM),
Toll4 (5′-GGATTTATCCAGGTGTGA AATTGAA-3′FAM),
MyD88 (5′-AAGTCGCGCATCGAGGAG GACTGCC-3′FAM)

The reaction mixture was heated for 2 min at 50 °C and 10 min at 95 °C and then 40 PCR cycles (15 s at 95 °C and 60 s at 60 °C) were performed. The product accumulation was monitored by FAM fluorescence. The absence of genomic DNA contamination in the RNA samples was detected by using total RNA samples that had not been subjected to reverse transcription. 18S RNA was used as the standard housekeeping gene. The primers used for IL-6 were purchased from Applied Biosystems. The relative gene expression levels were determined from the standard curve and calculated using the software provided by the manufacturer (Applied Biosystems). The results of RQ display the calculated relative level of gene expression in the test samples (User Guide ABI Prism 7900HT).

Antibodies and Western Blot

Antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) except phospho Src (Tyr416; cat #2101) and nonphospho Src (Tyr416; cat #2102) antibodies, which were obtained from Cell Signaling Technology (Beverly, MA, USA). Phospho Src antibody detects endogenous levels of Src phosphorylated at tyrosine 416. For whole-cell lysates, Kupffer cells were washed twice with PBS and lysed in SDS lysis buffer (100 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10% glycerol, 1% SDS, 5 mM EDTA, 5 mM EGTA, 2 mM PMSF, 50 μg/mL pepstatin and leupeptin). Equivalent amounts of protein (20 μg) were separated on 10% SDS-PAGE and transferred to PVDF membranes (Millipore, Bedford, MA, USA). The membranes were blocked in TBS containing 3.5% fat dry milk and 0.1% Tween 20. Membranes were incubated with primary antibodies followed by anti-mouse or anti-rabbit horseradish peroxidase (HRP)-conjugated antibody (1:7000). Bound antibody was visualized by enhanced chemiluminescence (ECL) (Amersham Pharmacia Biotech, Arlington Heights, IL, USA) according to the manufacturer's instructions.

Cytometric Bead Array

The concentration of IL-6 in Kupffer cell supernatants was measured using commercially available CBA Mouse Inflammation Kits (BD Pharmingen, San Diego, CA, USA), according to the manufacturer's instructions. Briefly, 50 μL mixed capture beads were incubated with 50 μL supernatant and 50 μL PE detection reagent for 2 h at room temperature. The immunocomplexes were then washed and analyzed on an LSRII flow cytometer (Becton Dickinson, San Jose, CA, USA).

Flow Cytometry Analysis

The percentage of IL-6–positive Kupffer cells in the samples was measured by flow cytometry using CellQuest software (Becton Dickinson). Briefly, the cells were washed twice in FACS buffer (phosphate-buffered saline with 1% fetal bovine serum and 0.01% sodium azide) and permeabilized with perm/fix buffer on ice for 30 min. Kupffer cells ($\sim 5 \times 10^5$) were labeled for 30 min on ice with 10 mL FITC-conjugated anti-mouse F4/80 and PE-conjugated anti-mouse IL-6 monoclonal antibodies (0.6 μg mL^{-1} per 10^6 cells) (BD Biosciences). Kupffer cells were identified as F4/80-positive.

Statistical Analysis

Values are reported as mean ± SE of 4 to 6 mice in each group. Statistical analysis was carried out using 1-way ANOVA and Tukey's test, and values were considered significant at $P < 0.05$.

RESULTS

Expression of TLR in Kupffer Cells from Male and Proestrus Female Mice following Hypoxia

Because TLR4 is a major initiator of signal transduction in Mϕ, we examined whether hypoxia affects expression of TLR4 in Kupffer cells of males and proestrus females. The expression of TLR4 mRNA in Kupffer cells from male and proestrus female mice before and after hypoxia was assessed using quantitative real-time PCR. The results were normalized using 18S mRNA levels and are presented in Figure 1. Hypoxia increased the expression of TLR4 mRNA in Kupffer cells from males and proestrus females by 3- to 4-fold (Figure 1). Because the degree of increased TLR4 was similar in male and female mice, the levels of TLR4 may not contribute to sex differences in the innate immune responses following hypoxia.

MyD88 Expression in Kupffer Cells from Male and Female Mice

Because there was no sex specificity in TLR4 expression, we explored whether the sex-specific response following hypoxia is due to differential expression of MyD88 in males and proestrus females.

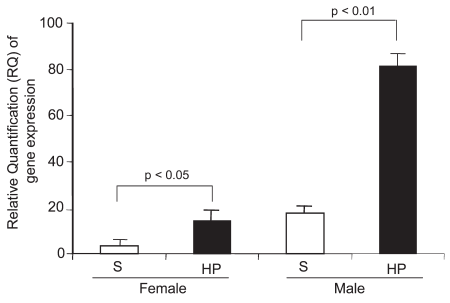

Figure 1. TLR transcripts in the Kupffer cells from male and female mice before and after hypoxia. Male and female mice were subjected to hypoxia (HP) or sham hypoxia (S) for 1.5 h and were killed 1 h after their return to room air. Their livers were removed for Kupffer cell isolation, and Kupffer cell RNA was isolated. The mRNAs of 10 ng were analyzed by real-time PCR using TLR-4–specific primer pairs. The primers of 18S mRNA were included in each reaction as an internal control to normalize relative quantities of TLR. The results are presented in RQ value, which displays the relative level of gene expression in the test samples. This represents 4 identical experiments presented as mean ± SE. *P* values are shown between 2 indicated groups.

The MyD88 adaptor molecule is the first signaling protein in the TLR pathway whose expression is critical for TLR-dependent activation of downstream signaling leading to cytokine production. The results in Figure 2A show that the expression of MyD88 adaptor protein in Kupffer cells from male mice under normoxia was very low or undetectable and this protein was not induced even after hypoxia. In contrast, the expression of MyD88 was clearly detected in Kupffer cells from proestrus female mice; however, the elevated expression was not altered by hypoxia. Quantitative analysis of the densities of MyD88 bands from 4 individual experiments further demonstrated that MyD88 expression in Kupffer cells presents a profound sex expression profile (Figure 2B). The MyD88 levels in Kupffer cells from proestrus females were significantly higher compared with the levels in Kupffer cells from males in both sham and hypoxia mice. Because proestrus females have the highest estrogen levels, it is likely that estrogen may contribute to an elevated expression of MyD88 in females.

To study the potential effects of estrogen, a group of C3H/HeN male mice was injected subcutaneously with 5 μg 17β-estradiol (E2) 1 d before hypoxia. Kupffer cells were isolated and analyzed for MyD88 expression. The results in Figure 2A and B suggest that E2 administration in male mice profoundly increased the expression of MyD88 protein in Kupffer cells from males treated with E2 (male + E2 lanes). Hypoxia, however, did not further influence the expression of MyD88 in Kupffer cells from E2-treated male mice. This increased MyD88 expression in Kupffer cells from E2-treated male mice further supports the hypothesis that E2 may play a critical role in the regulation of MyD88 expression.

The levels of MyD88 transcript in Kupffer cells from males and proestrus females were also determined by real-time PCR. Consistent with immunoblot analysis, the results shown in Figure 2C further confirm that the levels of MyD88 transcript in Kupffer cells from sham and hypoxia male mice were markedly lower than those seen in Kupffer cells from females. Treatment of male mice with E2 enhanced the expression of MyD88; however, MyD88 expression in E2-treated mice was not further influenced by hypoxia (Figure 2C). The expression of MyD88 transcript in Kupffer cells from proestrus females was high under normoxia and decreased significantly following hypoxia.

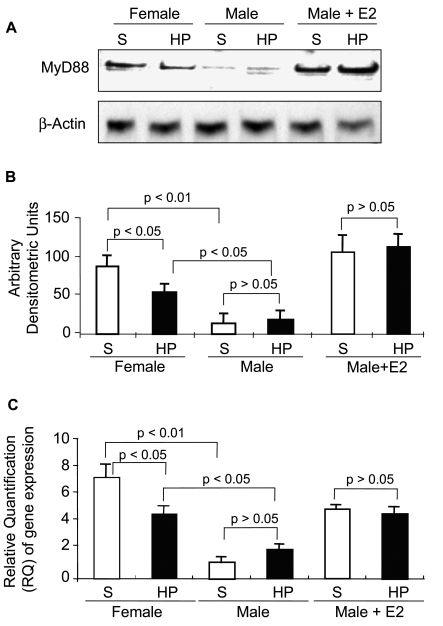

Figure 2. MyD88 expression in the Kupffer cells from female and male mice. Three groups, untreated female and male mice and male mice treated with 17β-estradiol for 1 d (Male + E2), were subjected to hypoxia (HP) or normoxia (S), and then Kupffer cells were prepared. (A) A representative immunoblot showing MyD88 protein (34 kDa) levels in Kupffer cells of various groups. β-Actin was used as a protein loading control. (B) The expression of MyD88 protein was quantitatively analyzed densitometrically, and the densitometric values from 4 individual experiments were pooled and are presented as mean ± SE. (C) Real-time PCR for MyD88 transcript. The experiment was repeated 3 times and the results are presented as mean ± SE. *P* values are shown between 2 indicated groups.

Src Expression in Kupffer Cells from Male and Female Mice

In contrast to MyD88, the results shown in Figure 3A and B indicated an increased expression of Src in Kupffer cells of sham female mice compared with male sham control. Hypoxia resulted in a significant decrease in Src expression in Kupffer cells of proestrus females, in contrast to males, in which hypoxia significantly increased Src expression (S vs. HP male, Figure 3A). Furthermore, administration of E2 in male mice did increase the expression levels of Src in Kupffer cells, but the levels of Src in E2-treated male Kupffer cells were not further affected following hypoxia (Figure 3A and B).

Assessment of IL-6 Transcripts in the Kupffer Cells

We determined IL-6 levels in the Kupffer cells before and after hypoxia in male and proestrus female mice. The

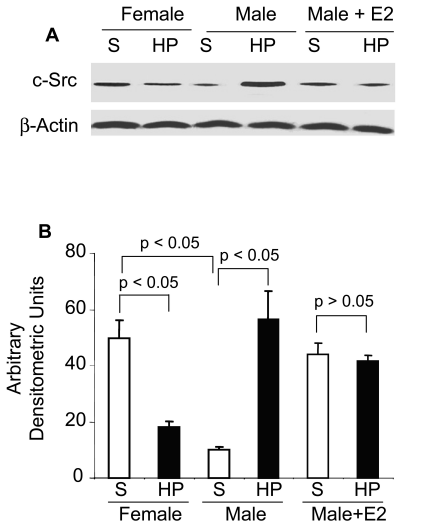

Figure 3. Src expression in the Kupffer cells from female and male mice. Untreated female and male mice, plus a separate group of male mice treated with 17β-estradiol for 1 d (Male + E2), were subjected to hypoxia (HP) or normoxia (S), and Kupffer cells were isolated. Kupffer cells were lysed, and the lysates were analyzed for Src protein contents. (A) A representative immunoblot of Src proteins in the Kupffer cells and β-actin. (B) The expressions of Src protein were quantitatively analyzed densitometrically, and the densitometric values from 4 individual experiments were pooled and are presented as mean ± SE.

IL-6 mRNA in the Kupffer cells from sham males was markedly lower than that in sham females (Figure 4). However, hypoxia stimulated IL-6 mRNA only in males. In contrast, the levels of IL-6 mRNA were high in sham females, and the levels were significantly decreased by hypoxia. Furthermore, the results in Figure 4 show that the treatment of mice with E2 enhances IL-6 mRNA expression in male mice to levels seen in proestrus female shams. Hypoxia appeared to decrease the cytokine mRNA in E2-treated male mice when compared with sham controls, but the decrease was statistically insignificant ($P > 0.05$).

To further confirm that E2 downregulates IL-6 expression, we determined the expression of IL-6 in Kupffer cells in ex vivo studies. For these studies, Kupffer cells were isolated from male mice and incubated with E2 at indicated concentrations for 5 h (Figure 5). The intracellular IL-6 levels were determined by flow cytometry. Figure 5A shows a representative FACS analysis. The IL-6–positive Kupffer cells (F4/80-positive) decreased from an initial 33% to 17% following E2 treatment. Furthermore, the results summarized in Figure 5B show that IL-6 expression can be lowered to approximately 40% in Kupffer cells by the addition of 10^{-9} M E2. These results thus suggest that E2-mediated downregulation of MyD88 is likely to contribute to the decreased IL-6 mRNA and intracellular levels following hypoxia in the proestrus females. In contrast, in males Src, rather than MyD88, is likely the protein kinase that regulates IL-6 because the increase in IL-6 expression in male Kupffer cells was observed in the absence of MyD88. To clarify the mechanism for increased IL-6 expression in males by Src, additional experiments as described below were carried out.

Src Expression and the Production of IL-6

To assess the role of Src in IL-6 production by Kupffer cells from male mice, we examined whether the increase in protein expression was accompanied by an increase in its phosphorylation (i.e., activation) and whether inhibition of Src activation prevents the increase in IL-6 production following hypoxia. Results from these experiments, summarized in Figure 6, show that hypoxia, similar to protein expression, significantly increased Src phosphorylation in Kupffer cells of male mice. Treatment of mice with the Src inhibitor PP-1 prevented the increase in both Src phosphorylation and protein expression following hypoxia. We then examined whether similar treatment of mice also prevented the increase in IL-6 production. The results indicate that Kupffer cells isolated from male mice subjected to hypoxia show a significantly increased IL-6 production compared with sham controls (Figure 7). Treatment of mice with Src inhibitor PP1 prevented the increase in Kupffer cell

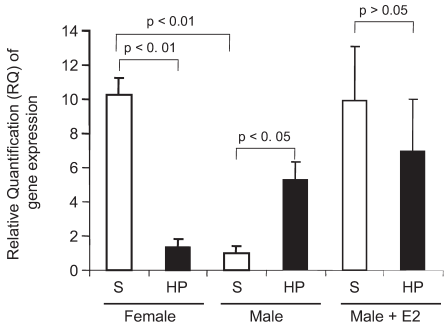

Figure 4. IL-6 mRNA expressions in Kupffer cells from male and female mice by real-time RT-PCR. Three groups, untreated female and male mice and male mice treated with 17β-estradiol for 1 d (Male + E2), were subjected to hypoxia (HP) or normoxia (S), and Kupffer cells were prepared. Kupffer cell RNA was isolated and real-time PCR was performed for IL-6 mRNAs. The experiment was repeated 3 times and the results are presented as mean ± SE. *P* values are shown between 2 indicated groups.

IL-6 production. These observations further suggest that increased IL-6 in males induced by hypoxia is regulated via the Src pathway.

DISCUSSION

Our previous studies have demonstrated that hypoxia produces alterations in immune cell functions (10,11), similar to trauma-hemorrhage (13), in males but not in proestrus females. Because alterations in Mϕ functions, including the increased production of IL-6, are a good predictor of mortality (18), the present study was conducted to determine the mechanism of IL-6 release by Kupffer cells under hypoxia in male and proestrus female mice. Because studies have shown the involvement of TLR4 in the release of IL-6 (6,7), we examined the effect of hypoxia on the expression of TLR gene and the downstream signaling components in the TLR-mediated pathway for IL-6 release by Kupffer cells from male and proestrus female mice. Others have reported that hypoxia diminished TLR4 expression on endothelial cells and that this change is mediated by mitochondrial ROS leading to attenu-

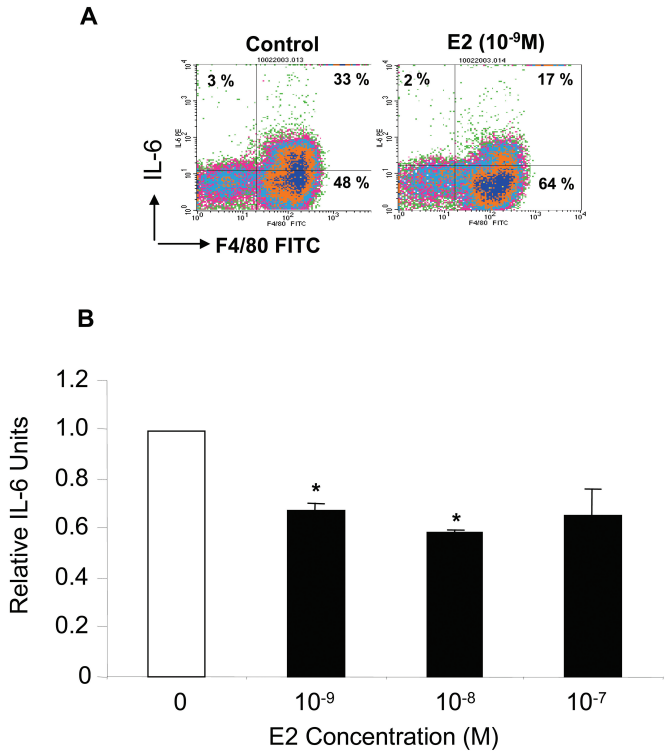

Figure 5. Effect of estrogen on intracellular levels of IL-6. Kupffer cells isolated from male mice were incubated with indicated concentrations of E2 for 5 h. The intracellular IL-6 levels in cells were analyzed using flow cytometry. (A) A representative FACS result of IL-6 in Kupffer cells with (E2) and without E2 (Control) treatment showing the percentages of IL-6–positive (upper right) and IL-6–negative (lower right) in F4/80-positive Kupffer cells (right). (B) Relative IL-6, which was calculated by the ratio of IL-6-FITC–positive cells in E2-treated samples to IL-6-FITC–positive cells in E2-untreated control sample. These values are presented as mean ± SE of 3 identical experiments. **P* < 0.05 vs. untreated cells.

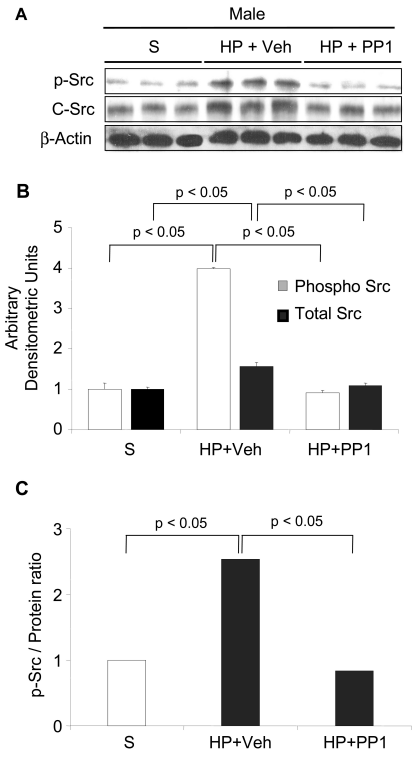

Figure 6. Effect of PP1 on Src phosphorylation and protein expression in the Kupffer cells from male mice following hypoxia. Male mice were treated intraperitoneally with Src inhibitor PP1 at a dose of 1.5 mg/kg body weight 1 h before hypoxia. Kupffer cells were isolated, lysed, and immunoblotted for Src phosphorylation and protein levels. (A) A representative immunoblot analysis of Src phosphorylation and protein levels in Kupffer cells. (B) The membranes were stripped and reprobed for β-actin for protein loading control. The blots were quantitatively analyzed by densitometry and the densitometric units from more than 4 animals in each group were pooled and are shown as mean ± SE. In addition, densitometric values for phosphorylation were normalized to the total protein. (C) Normalized phosphorylation is shown as mean ± SEM. *P* values are shown between 2 indicated groups.

ation of AP-1 transcriptional activity (40,41). However, our results indicated that hypoxia produced a 3- to 4-fold increase in TLR4 expression in Kupffer cells of both males and proestrus females. Although the maximal increase in TLR4 expression induced by hypoxia appears profound in males when compared with the maximal increase in proestrus females, the percent hypoxia-induced increase in expression was approximately the same in both males and proestrus females (Figure 1). These results imply that the increase in TLR4 expression occurs in both males and females following hypoxia and thus sex does not play a role in TLR4 expression. The effect of TLR4 upregulation in Kupffer cells following hypoxia is not yet clear. This study reveals that increased TLR4 expression following hypoxia appears to differentially regulate the production of IL-6 in males and proestrus females. It has been recently reported that early increases in TLR2/4 gene and TLR4 protein expression correlate with mortality, whereas blunting TLR gene and protein expression correlates with improved long-term survival, implying an important role of increased TLR2/4 in the proinflammatory response and pathophysiology of polymicrobial sepsis (42).

Because TLR4 expression increased in Kupffer cells from males and females following hypoxia independent of sex, we examined whether hypoxia modulates down-signaling components in the TLR pathway for the release of IL-6. Our results indicate that the expression of adaptor protein MyD88 in Kupffer cells from males is barely detectable. The suppressed expression of MyD88 in Kupffer cells from males leads to constitutive decrease of active signaling components in the major MyD88-dependent pathway following hypoxia. This suppression in MyD88-dependent pathway in Kupffer

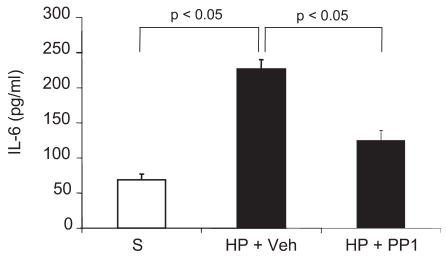

Figure 7. Effect of PP1 on cytokine production capacity of Kupffer cells in male mice following hypoxia. Male mice were treated intraperitoneally with Src inhibitor PP1 at a dose of 1.5 mg/kg body weight 1 h prior to hypoxia. Kupffer cells were isolated and cultured for 24 h at 37 °C, and the release of IL-6 in the supernatant was measured using a cytometric bead array. The results as shown are mean ± SEM. *P* values are shown between 2 indicated groups.

cells from males but not in Kupffer cells from proestrus females may be responsible for the depression of innate immune functions induced by hypoxia or trauma-hemorrhage in males. Studies have also elucidated the role of MyD88 in mediating TLR signaling (19,24). Following activation, the adapter protein MyD88 is recruited to the cytoplasmic domain of TLRs and activates a downstream kinase cascade involving IKK and IκB, leading NF-κB to translocate to the nucleus where it can promote new gene expression (19,24). Recently it has been reported that the tolerance for endotoxin seen in normally responsive cells may be associated with the failure of MyD88 to recruit the TLRs (43). Thus, our study provides a novel insight into the mechanism responsible for the sex dimorphic difference in the innate immune response induced by hypoxia in males and proestrus females.

MyD88 adaptor molecule is an essential element for the signaling of IL-1R/TLR family and plays a crucial role in host Mϕ against extracellular gram-positive bacteria. Studies have shown that MyD88-deficient mice are more susceptible to infection, suggesting a defective response to many bacterial components and cytokines such as IL-1 and IL-18 (21,44). Others have analyzed the alterations in signaling cascades in the genetically deficient MyD88 macrophages and found that MyD88 is directly associated with kinase activity and tyrosine phosphorylation such as IRAK and Pyk2 (45). Upon activation of the TLR pathway, the adaptor protein MyD88, the protein kinase IRAK, and the adaptor molecule TRAF-6 aggregate with TLR, forming the receptor complex that results in the interaction of downstream molecules and the subsequent activation of NF-κB (46). Furthermore, MyD88-induced NF-κB activation is inhibited by the dominant negative versions of TRAF6 and IRAK, which also inhibit IL-1–induced NF-κB activation (21). It is therefore not surprising that the extremely low expression of MyD88 in Kupffer cells of males may result in both severely impaired bacterial recognition and failure of TLR-dependent signaling pathway, which may contribute to the increased susceptibility of male mice to infection.

Recent studies using MyD88-mutant mice indicated that TLRs use an alternative adaptor molecule, TIR-containing adaptor molecule 1 (TICAM-1), to activate cellular responses (47,48). In MyD88-deficient mice, although LPS (TLR4 agonist) or dsRNA (TLR3 agonist) activate NF-κB induction, the response is delayed. Recent studies suggest that Src family members play a regulatory role in TLR4-mediated iNOS expression in a MyD88-independent pathway (49). Src-family tyrosine kinases have been implicated in playing roles in many signaling pathways in immune cells. It is also known that LPS can activate Src family members including Lyn, Fgr, and Hck (50,51). LPS induces autophosphorylation of Lyn kinase in wild-type, but not in TLR4-mutant (C3H/HeJ), Mϕ (52). Overexpression of a constitutively active Hck enhanced the production of TNF in response to LPS in Mϕ, whereas antisense oligonucleotides to Hck interfere with LPS-induced TNF production (52). We examined Src levels in Kupffer cells from male and female mice and the results showed that hypoxia profoundly diminished the levels of Src in Kupffer cells from proestrus female mice, whereas it stimulated the levels of Src in Kupffer cells from males compared with their sham controls. Furthermore, the increase in Src expression was accompanied by an increase in its phosphorylation, suggesting that hypoxia increases Src kinase activity. Treatment of males with Src inhibitor PP1 prevented the increase in IL-6 production following hypoxia. Thus, it is likely that MyD88 and Src tyrosine kinase are differently regulated following hypoxia in males and females. While MyD88 may be critical to IL-6 production in proestrus females, the increase in Src protein and phosphorylation likely plays the predominant role in increased IL-6 production in male Kupffer cells. We recognize that Src is one of the several members of Src family tyrosine kinases, and the antibodies to Src protein that we have used may cross react with other Src family members. In view of this, it is possible that other Src family members may also be involved in the regulation of IL-6 in male Kupffer cells following hypoxia.

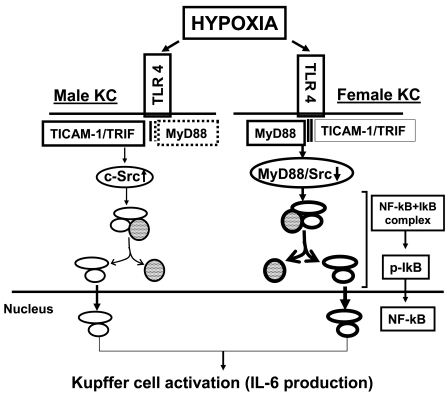

Figure 8. A schematic model for sex difference in TLR signaling pathways. Hypoxia induced an increase in Kupffer cell TLR4 expression in both males and females. In female mice (right side) both MyD88 and Src are downregulated; independent pathways are stimulated. In males while MyD88 is not expressed, the expression and phosphorylation of Src, on the other hand, is significantly increased following hypoxia. The differential regulation of MyD88 and Src in males and females following hypoxia is likely to contribute differential expression of IL-6 in Kupffer cells from males and females.

We recognize that our studies suggest significant differences in the production of IL-6 by Kupffer cells even without in vitro stimulation with a known TLR4 ligand. Although the actual mechanism by which hypoxia induces IL-6 release remains unclear, there is evidence that TLR4 activation is not exclusively facilitated by exogenous LPS or its domain lipid A but also via endogenous factors such as heat shock proteins (HSP). Although the precise mechanism by which hypoxia induces TLR-4–mediated IL-6 release remains unclear, we speculate that hypoxia might activate TLR4 via the well-established nitric oxide (NO) pathway that in turn triggers HSP. Studies have shown that the immunostimulatory activity of HSP60, for example, is mediated by TLR4 (53), and thus TLR4-dependent signaling cascades may be initiated under hypoxic stress even in the absence of LPS. However, more studies are needed to confirm this paradigm.

We also recognize that our study did not use MyD88-knockout mice, and thus we have not completely ruled out the role of MyD88 in IL-6 production. However, because the activation of Src kinases in males occurs in the absence of an increase in MyD88, it indicates that MyD88 is not critical to the activation of Src kinase. Nonetheless, the specific mechanism by which hypoxia increases Src kinases remains to be established. Moreover, additional studies using MyD88-knockout animals will shed further light on the role of MyD88-dependent and -independent pathways in Kupffer cell production of IL-6 following hypoxia in males and proestrus females. It can also be argued that to rule out a role of Src in female Kupffer cell production of IL-6, we should have treated proestrus females with Src inhibitor. However, because we found a decrease in Src protein expression in female Kupffer cells following hypoxia, we did not believe that further inhibition of Src by treating with Src inhibitor would add further information to the data in proestrus females. Although it is likely that a decrease in protein may not necessarily decrease its kinase activity, data shown in Figure 6 clearly suggest that both Src protein expression and phosphorylation were increased simultaneously in Kupffer cells from males following hypoxia. Because there was an increase in Src kinase activation in males, male animals were treated with Src inhibitor to determine whether the increase in Src has a role in increased IL-6 production in males following hypoxia.

The results from this study, summarized in Figure 8, suggest that hypoxia leads to a differential regulation of MyD88 and Src proteins in Kupffer cells of male and proestrus female mice. We found that both MyD88 and Src in Kupffer cells are overexpressed in normal proestrus females compared with normal males. As a result of hypoxia, the expression of MyD88 and Src are reduced in proestrus females. On the other hand, the expression of MyD88 in males remained barely detectable following hypoxia; however, the expression of Src in Kupffer cells was increased significantly under those conditions. The production of IL-6 was decreased in proestrus females and increased in males following hypoxia. Following administration of 17β-estradiol, MyD88 and Src expression, as well as IL-6 production, in male Kupffer cells were increased to levels comparable to female Kupffer cells. Thus, the decreased MyD88 expression in females after hypoxia is likely the cause for the decreased Kupffer cell IL-6 production. In males, however, hypoxia increased Src kinase activity. This, along with the finding that treatment of mice with Src inhibitor PP1 prevented IL-6 increase, suggests that in male Kupffer cells, TLR4 mediates its action predominantly via Src and not MyD88. Additional studies using the available appropriate knockout mice are needed to further elaborate the role of MyD88 and Src in sex-specific innate immune responses.

In conclusion, this study reveals TLR4-dependent activation of innate immune responses in mice following hypoxia. Nonetheless, regulation of MyD88 and Src expressions in Kupffer cells appear to be different in males and proestrus females and correlate with sex dimorphic IL-6 release, which is a characteristic feature of the suppression of immune functions following hypoxic injury.

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