SEXUAL DIMORPHISM IN SEPSIS SUSCEPTIBILITY: EFFECTS OF REPRODUCTIVE HORMONES ON MICROBIAL PATTERN RECOGNITION RECEPTOR EXPRESSION

by

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ABSTRACT

JENNIFER ANNE RETTEW. Sexual dimorphism in sepsis susceptibility: effects of reproductive hormones on microbial pattern recognition receptor expression. (Under the direction of DR. IAN MARRIOTT)

Sex-based differences in innate immune responses to bacterial infection are evident in human patients and animal models of disease. Females are less susceptible to the development of bacterial infections and subsequent bacteremia and/or sepsis while males exhibit a greater incidence of such infections and are more likely to develop fatal sequelae. While the precise effects and mechanisms of action remain to be determined, it is apparent that male and female reproductive hormones can have direct effects on the expression and function of key bacterial pattern recognition receptors on innate immune cells. Changes in the expression of these receptors are likely to have profound effects on the production of the inflammatory mediators responsible for the lethal nature of septic shock and may underlie the observed sexual dimorphism demonstrated in immune responses to bacterial endotoxins. In the present studies, we have determined the role of testosterone and estradiol on TLR4 expression from murine macrophages. Testosterone suppresses both total and cell-surface TLR4 expression from macrophages, both in vitro and in vivo, and decreases inflammatory mediator production following LPS challenge. Estradiol, however, exhibits much more complicated effects on pattern recognition receptor expression. Long-term exogenous estradiol treatment in vivo augments cell-surface but not total TLR4 expression, with a corresponding increase in inflammatory mediator production following LPS challenge. However, acute treatment of macrophages with estradiol results in an opposite effect with decreased levels of cell-surface TLR4

expression and inflammatory mediator production, and this effect was found to be mediated by the novel estrogen receptor GPR30. As such, estrogens may be able to reduce the devastating inflammation associated with acute overactive host responses such as septic shock without compromising long-term defense against infectious organisms.

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CHAPTER ONE: INTRODUCTION

While it has long been known that sex is a contributing factor in the incidence and progression of disorders associated with immune system dysregulation, it is recently come to light that sex also plays a role in susceptibility to infectious disease. Specifically, responses to bacterial infections and endotoxin differ based on sex as well as reproductive status. In discussing sex bias to bacterial infection, it is important to note that disease severity and outcome following bacterial infection are often dependant on the host inflammatory responses elicited by endotoxins produced by many bacterial species (Figure 1). Systemic inflammatory response syndrome (SIRS) describes the physiological changes associated with an overactive and systemic host response that can be due to either an infectious stimuli, such as endotoxin, but can also be caused by physiological responses to challenges such as hemorrhage. Patients with SIRS exhibit symptoms such as fever or hypothermia, tachycardia, tachypnea, and can also be associated with white blood cell count abnormalities (Martel 2002). The term SIRS encompasses sepsis, bacteremia, and endotoxemia. Sepsis occurs when organisms at a local site of infection proliferate and gain access to the blood stream via tissue damage and/or invasion mechanisms. Bacteremia and endotoxemia occur when bacteria or endotoxins such as lipopolysaccharide (LPS) are present in the blood stream, respectively. LPS is a structural component of the cell wall of Gram-negative bacteria that, while not actively secreted by these organisms, is often released into the

extracellular milieu of the host following bacterial lysis. The systemic circulation of microbes and/or endotoxin often leads to a systemic inflammatory response, and frequently sepsis (Figure 1). Severe sepsis can lead to septic shock, which is categorized by a catastrophic drop in blood pressure that results in diminished perfusion of tissues, hypoxia, and dysfunction of organs, including the kidneys, liver, lungs, and CNS. This loss of function may lead to multiple organ failure and death (Martel 2002). In fact, sepsis and the multiple organ failure associated with septic shock is the most common cause of late post-injury death in surgical intensive care units (Sauaia et al. 1995).

Sex based differences in the immune response to bacterial infection are evident at multiple levels. Both innate and adaptive immunity exhibit sexual dimorphism in human patients and mouse models of disease. While disparities between men and women in B cell activity and antibody production in response to bacterial infection and vaccination are well known and are discussed elsewhere in this volume, it is also apparent that marked differences exist between males and females in the frequency, severity, and outcome of severe sepsis and septic shock. In the following chapter, we will discuss the sex based differences in susceptibility to bacterial infection and discuss the mechanisms that may account for such sexual dimorphism.

1.1 Sex based differences exist in susceptibility to bacterial infection and sepsis with males exhibiting higher incidence and severity

Animal studies have provided evidence that males exhibit greater susceptibility to bacterial challenge than their female counterparts. Sex based differences have been observed in susceptibility of mice to *Mycobacterium marinum* infection, with males

showing higher disease severity, bacterial burden, and mortality than infected female mice (Yamamoto et al. 1991). Similarly, *Helicobacter pylori* infections in females show delayed onset of intestinal dysplasia relative to infected males, and show less intestinal inflammation and histopathology (Ohtani et al. 2007). This difference is not limited to bacterial burden as animal models of endotoxemia indicate that female mice demonstrate higher survival rates than males when subjected to severe sepsis. For example, administration of *V. vulnificus* derived LPS leads to endotoxic shock in male rats with a mortality rate of 82%. In contrast, females treated in the same manner exhibit only 21% mortality following LPS challenge (Merkel et al. 2001). Studies in mice have yielded similar results. In one study, all female mice survived LPS induced sepsis, but only 70% of their male counterparts survived a similar treatment (Laubach et al. 1998). In the cecal ligation and puncture induced model of sepsis, female mice similarly survived at a much higher rate (44%) relative to males (5%) (Kahlke et al. 2002).

Findings in human patients appear to correspond with these animal studies, with men exhibiting greater susceptibility to bacterial infection than women. A study of patients at Boston City Hospital in 1972 with bacteremia found that the incidence of infection was significantly higher in male patients than in females (McGowan et al. 1975). In addition, it has been shown that men exhibit increased mortality associated with nosocomial infection compared to their female counterparts (Dinkel and Lebok 1994), and the male sex has been identified as a major risk factor for bacterial infection following severe injury with a study showing that male patients exhibit a 58% greater risk of developing major bacterial infections following trauma (Offner et al. 1999).

Again, it is apparent that these sex differences in the incidence and/or severity of bacterial infection are mirrored by similar differences in the development of severe sepsis and septic shock. *Vibrio vulnificus* infection, which occurs following ingestion of raw or undercooked seafood, elicits endotoxic shock in humans with a fatality rate of almost 60%, but 80% of *V. vulnificus* associated mortality is observed in males (Oliver 1989). In addition, reviews of hospital cases have revealed that significantly fewer female patients were referred to the intensive care unit, and of all patients referred, men developed severe septic shock more frequently than women (Wichmann et al. 2000, Dosset et al. 2008). Women have also been found to have lower organ dysfunction scores than men following the development of severe sepsis (Adrie et al. 2007). Furthermore, the outcome following the development of sepsis and severe sepsis also differs based between the sexes, with men again exhibiting greater mortality than women (Schroder et al. 1998, Adrie et al. 2007).

As such, it appears that females are less susceptible to the development of bacterial infections and subsequent bacteremia and/or sepsis while males exhibit greater incidence and severity of bacterial infections and are far more likely to develop lethal sequelae. While these sex based differences have been appreciated for many years, the mechanisms that may account for such differences are only now becoming apparent. It is conceivable that the divergence in sepsis severity could stem from differences in circulating endotoxin levels. Indeed, one study has found that female rats exhibit significantly lower endotoxin levels following cecal ligation and puncture (Erikoglu et al. 2005). However, this has not been a consistent observation with other groups reporting contradictory results (Kono et al. 2000). A more likely mechanism for sex based

differences in sepsis severity lies in the relative production of key inflammatory cytokines that precipitate the lethal consequences of bacterial septic shock.

1.2 Sex based differences exist in the production of key inflammatory cytokines by immune sentinel cells that underlie the development of bacterial septic shock

The development of sepsis is driven by the overproduction of cytokines including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 (Blackwell and Christman 1996). The importance of these molecules in the development of septic shock is underscored by the observation that sera levels of IL-6, TNF-α, and IL-8 were significantly lower in sepsis patients that survived than those succumbing to this condition (Dosset et al. 2008, Majetschak et al. 2000). The rapid onset and progression of septic shock are testaments to the role played by innate immunity in the development of this often self destructive host response. Macrophages can perceive bacteria and endotoxins to rapidly produce large amounts of these potent inflammatory cytokines. As such, the lethal nature of septic shock is mediated, in large part, by the widespread activation of macrophages and the subsequent overproduction of inflammatory mediators.

Several studies have indicated that sexual dimorphism exists in the circulating levels of these inflammatory cytokines following infection and/or septic shock. For example, male sepsis patients have been found to have higher circulating levels of TNF-α than female patients, and this observation correlates with a worse prognosis (Schroder et al. 1998). In mice, *Escherichia coli* derived LPS has been found to elicit higher circulating levels of IL-6 in males than similarly treated female mice (Marriott et al. 2006). Interestingly, female sepsis patients have been found to exhibit higher levels of

the anti-inflammatory cytokine IL-10 than age and disease severity matched male patients (Schroder et al. 1998). While these studies appear to support the contention that the overproduction of inflammatory cytokines or diminished production of immunosuppressive cytokines correlates well with susceptibility to septic shock, it is important to note that some studies have failed to detect such sex based differences (May et al. 2008, Schroder et al. 1998), and the reason for this discrepancy is not clear.

Importantly, there is a considerable body of work to support the hypothesis that sex based differences exist at the level of inflammatory cytokine production by isolated immune sentinel cells. In vitro studies employing acutely isolated human peripheral monocytes and macrophages isolated from animals show male/female differences in the production of inflammatory cytokines and chemokines following in vitro LPS challenge. For example, peripheral monocytes from male patients produce higher levels of TNF-α than females derived cells following LPS challenge (Asai et al. 2001, Temple et al. 2008, Tiberio et al. 2004) and peripheral blood cells derived from young adult men produce significantly more TNF-α following LPS challenge than that released by similarly treated cells derived from young adult women (Moxley et al. 2002). Similarly, peritoneal macrophages isolated from male mice subjected to cecal ligation induced sepsis secrete greater amounts of TNF-α than their female derived counterparts (Kahlke et al. 2002).

These sex based differences do not appear to be limited to TNF-α. Male derived macrophages produce significantly larger amounts of the inflammatory chemokine IP-10 than macrophages from female mice (Marriott et al. 2006). This effect seems to be chemokine-specific as no sex based differences were observed in the production of MCP-1 (Marriott et al. 2006). In addition, in vitro studies using peritoneal macrophages show

that cells derived from young male mice produce higher levels of IL-1β and IL-6 following LPS challenge than similarly treated female derived cells (Kahlke et al. 2000). Furthermore, peripheral blood monocytes isolated from male patients produce higher levels of IL-6 following LPS challenge than female derived cells (Tiberio et al. 2004). However, the findings for IL-6 do not appear to be as consistent as those for TNF-α and IL-1β as several studies have reported contradictory results. For example, LPS stimulated female peripheral monocytes have been found to produce more IL-6 than male monocytes, even as these same cells produce less TNF-α (Asai et al. 2001). Furthermore, macrophages isolated from female mice after thermal injury produce higher levels of IL-6 upon LPS stimulation (Kovacs et al. 2002), and hypoxia stimulated IL-6 secretion was greater in female derived Kupffer cells, the resident macrophages of the liver (Zheng et al. 2006). While these results appear to contradict the male/female differences seen in TNF-α production, it is important to note that interpreting changes in IL-6 levels is complicated by its role in the development of TH2 T-helper cell responses.

Interestingly, sex based differences have been observed in the production of anti-inflammatory cytokines such that female derived macrophages produce significantly more than that released by male derived cells. For example, peritoneal macrophages isolated from male mice have been shown to produce significantly lower amounts of the potentially immunosuppressive prostanoid PGE_2 than female derived cells (Marriott et al. 2006). This finding would appear to agree with previous studies showing that female cells can be induced to secrete more prostanoids than males (Du et al. 1984, Leslie et al. 1987, Gregory et al. 2000a). Furthermore, in vitro studies employing female-derived splenic macrophages have shown that these cells secrete higher levels of the

immunosuppressive cytokine IL-10 than male derived cells (Kahlke et al. 2000). However, it should be noted that some studies have failed to detect such sex based differences in IL-10 secretion (Asai et al. 2001, Temple et al. 2008), while one group found greater production of mRNA encoding IL-10 following LPS stimulation of male derived mononuclear cells (Temple et al. 2008). In one study, male/female differences were observed in inflammatory cytokine production from mononuclear cells following LPS challenge, but not in IL-10 production, indicating males have proportionally less anti-inflammatory signals to balance the inflammatory cytokines (Tiberio et al. 2004). Interestingly, a study of cecal ligation and puncture induced sepsis found that exogenous IL-10 treatment increased male survival but had no effect on female survival (Kahlke et al. 2002).

Finally, it appears that the male/female differences in LPS-induced inflammatory mediator production observed in monocytes/macrophages extends to other sentinel cell types such as neutrophils (Spitzer and Zhang 1996), and may also include non-leukocytes cells. For example, LPS induced lung inflammation in male mice has been shown to be associated with higher levels of TNF-α than that seen in the airway fluid of female mice (Card et al. 2006). Gastric tissue from male mice infected with *H. pylori* demonstrates higher expression of TNF-α than that seen in infected female tissue (Ohtani et al. 2007). In addition, cardiomyocytes isolated from male mice produce greater amounts of TNF-α following LPS challenge than do female derived cells (Zhu et al. 2009).

As such, the majority of the data supports the idea that females can be protected from potentially lethal endotoxic shock in two ways: first by producing lower levels of the inflammatory mediators that precipitate the systemic effects associated with sepsis,

and second by elevating the production of immunosuppressive molecules that could serve to attenuate endotoxin-mediated systemic inflammation. Despite the consensus that male and female derived immune cells appear to differentially produce inflammatory mediators following bacterial or endotoxin exposure, the mechanisms that underlie these differences remain poorly defined.

1.3 Sex based differences exist in the expression and/or functionality of receptors for conserved bacterial motifs

The recent discovery of a family of pattern-recognition receptors with a high degree of homology to the Toll family of proteins in *Drosophila* has shed light on the means by which innate immune cells recognize a wide range of pathogens without the need for prior exposure (Wright 1999, Medzhitov and Janeway 2000). To date, at least eleven members of the Toll-like family of receptors (TLR) have been discovered in mice and humans. These receptors detect the presence of conserved microbial motifs and initiate the production of cytokines, chemokines, and co-stimulatory molecule expression (Barton and Medzhitov 2003).

As shown in Figure 2, the detection of LPS involves Toll-like receptor 4 (TLR4) and several co-receptors and binding proteins. LPS binding protein (LBP) is a type I acute phase protein that catalyses the monomerization of LPS and mediates its transfer to CD14 (Schumann et al. 1990, Wright 1999). CD14 is a co-receptor for LPS and is either expressed within the plasma membrane of innate immune sentinel cells such as macrophages or is present as a soluble molecule in the surrounding milieu. Since neither CD14 nor LBP contain a cytoplasmic component, cellular responses are initiated through

binding with TLR4 (Poltorak et al. 1998, Chow et al. 1999, Lien et al. 2000, Akira 2006). TLR4 is expressed by immune sentinel cells including monocytes/macrophages and dendritic cells (Medzhitov et al. 1997). However, TLR4 also exhibits a broader expression pattern on non-leukocytic cell types capable of inflammatory cytokine production including epithelial cells in a variety of tissues (Diamond et al. 2000; Kumar et al. 2004; Quintar et al. 2006; Hornef et al. 2002), smooth muscle cells (Quintar et al. 2006), endothelial cells (Rock et al. 1998), and resident CNS cells such as astrocytes (Lewis et al. 2008). All of these cells can therefore respond to LPS and have the potential to contribute to a systemic inflammatory response. Ligation of TLR4, facilitated by CD14 and LBP, initiates an intracellular signaling cascade that results in the activation of NF-κB, a pivotal transcription factor in the regulation of inflammatory cytokine expression. As such, widespread activation of cells via TLR4 can precipitate the overproduction of soluble immune mediators that can lead to the development of a systemic inflammatory response that underlies the lethal nature of septic shock (Palsson-McDermott and O'Neill 2004).

Importantly, male/female differences appear to exist at the level of expression of these LPS receptors, and could therefore underlie the observed differences in LPS-mediated responses in male and female-derived cells with regard to inflammatory mediator production. Male mice have been found to have significantly higher circulating levels of LBP after LPS challenge than female mice and macrophages isolated from male mice express higher levels of cell surface CD14 than female-derived cells (Marriott et al. 2006). Interestingly, this difference appears to exist at the level of protein expression rather than at the level of gene transcription, as levels of mRNA encoding CD14 were not

significantly different between male and female derived cells (Marriott et al. 2006). However, it should be noted that another group has largely failed to detect such differences, with male peritoneal macrophages demonstrating a non-significant trend to express greater amounts of cell surface CD14, while splenic macrophages showed no difference between male and female derived cells (Eisenmenger et al. 2004). Temple and coworkers also found no sex-based difference in membrane CD14 expression on human mononuclear cells; however, their use of permeabolization to examine total TLR4 protein expression on those same cells could have had confounding effects on the measurement of CD14 levels (Temple et al. 2008).

Consistent with the notion that male sentinel cells express higher levels of receptors for LPS than their female counterparts, macrophages isolated from male mice have been shown to express more TLR4 than female-derived cells (Marriott et al. 2006, Frisancho-Kiss et al. 2007). Furthermore, mononuclear cells isolated from male patients also express significantly higher levels of total TLR4 protein than do cells from female patients (Temple et al. 2008, Tiberio et al. 2004). Again, this difference appears to occur at the level of protein expression as expression of mRNA for this pattern recognition receptor was not found to significantly differ between the sexes under naive conditions (Marriott et al. 2006, Zhu et al. 2009). In contrast, at least one study has reported that hypoxia can significantly induce TLR4 mRNA expression in male Kupffer cells, the resident macrophages of the liver, over that seen in female cells (Zheng et al. 2006). Also, following LPS challenge of murine cardiomyocytes, cells derived from male animals were found to contain significantly higher levels of mRNA encoding TLR4 than cells from their female counterparts (Zhu et al. 2009), indicating that perhaps mRNA

levels can increase in male cells following challenge with infectious or endogenous "danger-signals". Finally, male peritoneal macrophages exhibit a non-significant trend to express more cell surface TLR4 following trauma hemorrhage than cells from similarly treated females (Eisenmenger et al. 2004). However, in this study there were no significant differences in such expression in naive macrophages or splenic macrophages following trauma hemorrhage.

It has been suggested that sex based differences in LPS responses may also stem from differences in the intracellular signaling pathways initiated following TLR4 ligation. Specifically, hypoxia has been shown to initiate a MyD88-dependant signaling cascade resulting in IL-6 release from female Kupffer cells. In contrast, male-derived Kupffer cells appear to rely upon a MyD88-independent signaling pathway for production of this cytokine (Zheng et al. 2006). Consistent with this finding, female Kupffer cells demonstrated greater expression levels of the adaptor protein MyD88 than males (Zheng et al. 2006). Interestingly, while Src expression levels were higher in female derived liver macrophages, hypoxia initiated a decrease in such expression in female-derived cells while eliciting an increase in Src expression in male Kuppfer cells (Zheng et al. 2006). Ligation of TLR4 initiates a MAP kinase signaling and subsequent activation of NF-κB and female-derived peritoneal macrophages have been demonstrated to shown a greater degree of p38 MAP kinase phosphorylation, and hence activation, following LPS challenge than that found in male cells (Angele et al. 2003). Furthermore, Kupffer cell NF-κB activity has been reported to be three times higher in females following alcohol-induced liver injury than similarly treated male-derived cells (Kono et al. 2000). However, at least one study found greater ERK1/2 and p38 MAP kinase

phosporylation in male derived cardiomyocytes than in female derived cells following LPS challenge (Zhu et al. 2009).

Taken together, evidence is accumulating that male/female differences exist at the level of expression of those pattern recognition receptors that can perceive bacterial components and can influence the signaling pathways that result in inflammatory cytokine production. Because activation of TLRs and their co-receptors is responsible for LPS-mediated cytokine production, sex differences in these activation pathways may underlie the sexual dimorphism observed in immune responses during sepsis and/or septic shock. While the higher expression of TLR4 and its co-receptors on male sentinel cells and the more efficient LPS-induced signaling pathways in female cells appear to be contradictory, it is important to note that TLR induces both inflammatory and immunosuppressive cytokine production. As such, it is possible that the differences seen in female signaling pathways could result in higher anti-inflammatory cytokine production rather than elevated inflammatory mediator release. Clearly, more research is required to validate such a hypothesis.

1.4 Susceptibility to bacterial infection and sepsis differ with age and reproductive status

The differences in immune responses between males and females have generally been assumed to be a consequence of reproductive hormones. Circumstantial evidence for such a hypothesis comes from the documented differences in susceptibility to bacterial infection and sepsis/septic shock observed with age and changes in reproductive status.

A study of sepsis cases in children has revealed that there are no male/female differences in susceptibility or mortality due to sepsis in pre-pubescent children over the age of one and this absence of sex based differences correlate with very low levels of reproductive hormones (Bindl et al. 2003). This study did show male/female differences in sepsis susceptibility in neonatal infants with boys exhibiting greater severity similar to that seen in adults males (Bindl et al. 2003), but it is important to note that this effect may well be due to the presence of residual sex hormones that are present at high levels during fetal development but that rapidly decrease within the first year of life. Similarly, sepsis susceptibility changes in the elderly. While these effects may be a result of the physiological changes associated with aging, changes in susceptibility to bacterial challenge appear to be at least partially attributable to alterations in circulating levels of sex hormones. In a mouse model of aging, Kahlke and co-workers (2000) found that peritoneal macrophages from aged mice release lower levels of the inflammatory cytokines IL-1β and IL-6, and greater levels of the anti-inflammatory cytokine IL-10 upon LPS stimulation compared to young mice. Importantly, the sexual dimorphism observed in cytokine production release in young mice is not apparent in aged mice (Kahlke et al. 2000). Similarly in humans, elderly sepsis patients do not display differences between the sexes in either the incidence of septic shock or shock-associated mortality seen in younger patients (Angstwurm et al. 2005). However, at least one review of hospital cases found that women had a lower risk of mortality associated with severe sepsis than men, but only in the above 50 years of age category (Adrie et al. 2007). Presumably, this would indicated that the sex difference in susceptibility to sepsis occurs post- and not pre-menopausal, however the authors note that low mortality and small

sample size in the younger than 50 category, as well as the potential of hormone replacement in post-menopausal women, could have confounded their results (Adrie et al. 2007).

Major changes in reproductive hormone production occur during female menopause and a growing body of evidence suggests that changes in immune cell populations and functions occur at this time. The ovary can function in humans from the teenage years through to the fifth decade of life. However, during the perimenopause, the first hormonal changes occur that lead to the final menstrual period. During perimenopause, circulating estrogen concentrations fluctuate greatly from low (< 120 pM) to high (2 μM) but do not become significantly different until close to the final menstrual period (Sherman and Korenman 1975, Metcalf 1988, Shideler et al. 1989). Postmenopause, plasma estrogen is undetectable and progesterone levels are consistently less than 2 nM. Ovariectomy of mice, which models surgically induced menopause, results in a decreased immune response and increased mortality following bacterial infection. Female mice that lack endogenous estrogen due to ovariectomy show an increased mortality following cecal ligation and puncture induced sepsis than age-matched intact females (Knoferl et al. 2002). Similarly, ovariectomized female mice exhibit twice the lung bacterial burden following *M. avium* infection than age-matched intact females (Tsuyuguchi et al. 2001) and mortality associated with LPS challenge is significantly elevated in ovariectomized female rats (Merkel et al. 2001). Importantly, these effects were reversed following exogenous estrogen replacement confirming that the elevated bacterial burden and susceptibility to endotoxin administration are directly

attributable to the loss of endogenous estrogen (Tsuyuguchi et al. 2001; Merkel et al. 2001).

In humans, LPS sensitivity and endotoxin-associated mortality increase with age with postmenopausal women displaying a higher incidence of sepsis (Meyers et al. 1989, Beery 2003). Peripheral monocytes isolated from post-menopausal women produced significantly higher levels of inflammatory cytokines following LPS challenge than similarly stimulated cells from pre-menopausal women (Majetschak et al. 2000, Moxley et al. 2004). Majetschak and colleagues (2000) showed that post-menopausal female-derived monocytes secreted inflammatory cytokines at the same level as male cells. In contrast, one study showed that LPS-stimulated monocytes from post-menopausal women failed to produce comparable levels of TNF-α to those release by similarly challenge male-derived cells (Moxley et al. 2004). However, it must be noted that interpretation of this finding is complicated by the fact that half of the post-menopausal women monitored were receiving hormone replacement therapy (Moxley et al. 2004).

Finally, normal mucosal flora has been shown to vary following menopause and the subsequent fall in estrogen levels. Reductions in circulating estrogen levels correlate with loss of *lactobacilli* species in the vagina, which causes an increase in pH and coliform microorganisms, promoting growth of pathogenic bacteria and predisposing patients to infection (Gupta et al. 2006). Hormone replacement therapy appears to reverse this effect and women not receiving hormone replacement therapy have greater incidences of *E. coli* and bacteroids (Gupta et al. 2006). Such a hormone-dependent change in mucosal flora has important implications for the development of chronic inflammatory diseases and it is interesting to note that post-menopausal women receiving

hormone replacement therapy demonstrate a reduced incidence of inflammatory bowel disease (Kane and Reddy 2008).

As such, there is a considerable body of evidence that age and reproductive status can significantly influence the severity of bacterial infections and the incidence of sepsis. The lack of male/female differences in sepsis susceptibility in post-neonatal and pre-pubescent children, and the increased sepsis susceptibility of post-menopausal females that is reversed following hormone replacement, strongly indicate that reproductive hormones underlie the observed sex based differences in bacteremia and sepsis/septic shock.

1.5 Sex hormones have direct effects on susceptibility to bacterial infection

While variations in sepsis susceptibility seen with changes in age and reproductive status imply a role for reproductive hormones in the incidence and outcome of bacterial infections, more direct evidence exists that sex hormones influence acute immune responses to bacteria challenge. In general, estrogen has been considered to be "immunoprotective." However, this term fails to delineate whether estrogen acts as an immunoenhancer to combat bacterial infection, or is an immunosuppressor and protects against the overactive and damaging immune response associated with sepsis. Testosterone, on the other hand, is widely accepted to be immunosuppressive and causes an increased susceptibility of both males and females to bacterial infection. For example, exogenous testosterone administration increases female mouse susceptibility to *M. marinum* infection while castration of males, and hence removal of endogenous testosterone, attenuates such infections (Yamamoto et al. 1991).

Several studies have shown that both male and female sepsis patients exhibit abnormally low circulating levels of testosterone (Christeff et al. 1992, Fourrier et al. 1994) and death of males due to septic shock has been associated with extremely low levels of this androgen (Christeff et al. 1988). To date, the mechanisms underlying these observations are not clear. However, LPS has been reported to inhibit testosterone synthesis (Reddy et al. 2006) and so decreased testosterone levels could result in a more robust inflammatory immune response, and hence septic shock.

It should be noted that some animal studies appear to contradict the suppressive effects of testosterone on sepsis-induced mortality. In one study (Torres et al. 2005), the A/J mouse strain was reported to show less sepsis susceptibility following orchidectomy than intact males, an effect that was reversed following administration of dihydrotestosterone (DHT). Furthermore, another study has reported that castrated neonatal mice exhibited decreased sepsis susceptibility as adults (Bernhardt et al. 2007). However, a caveat of this study is that this model features the disruption of the hypothalamic-pituitary-gonadal axis and thereby preventing the formation of a normal male brain.

While a small number of studies have failed to show an effect of estrogen receptor agonists on monocyte responses to intracellular Gram-positive bacterial pathogens such as *Listeria monocytogenes* (Opal et al. 2005, Cristofaro et al. 2006), there is an overwhelming body of evidence indicating that estrogens, and in particular 17β-estradiol (E2; estradiol), are protective for bacterial infection and sepsis susceptibility. For example, estrogens have been demonstrated to increase resistance to streptococcal infections (Nicol et al. 1964). Estradiol administration has been found to increase

survival and decrease the oxidative stress along the rat gastrointestinal tract following intraperitoneal LPS challenge (Sener et al. 2005). Similarly, rats treated with intramuscular injections of estradiol and progesterone had lower endotoxemia and exhibited less liver and lung congestion following cecal ligation and puncture induced sepsis regardless of sex, while females receiving these agents had less liver inflammation and less lung edema (Erikoglu et al. 2005). Consistent with these observations, female rats treated with estrogen receptor agonists demonstrated lower circulating endotoxin levels and higher survival rates following *Pseudomonas* infection than untreated animals (Opal et al. 2005, Cristofaro et al. 2006). Furthermore, similar treatment of male and female mice increased survival following cecal ligation and puncture, while an estrogen receptor-beta agonist, but not an estrogen receptor-alpha agonist, decreased bacteremia in these animals (Opal et al. 2005, Cristofaro et al. 2006). Finally, human uterine epithelial cells treated with estradiol in culture have been found to have increased antibacterial activity following infection with *S. aureus* (Fahey et al. 2008).

In addition to experiments employing the administration of exogenous estrogen or estrogen receptor agonists, numerous studies have assessed the role played by endogenous estrogen on bacterial infection outcome and sepsis. For example, ovariectomy has been shown to markedly increase the severity of *M. avium* infection and this effect was reversed following estrogen replacement (Tsuyuguchi et al. 2001). Similarly, ovariectomy increased mortality following cecal ligation and puncture induced sepsis in female mice (Knoferl et al. 2002), and exacerbates *H. pylori* infection such that the severity of infection becomes comparable to that seen in males (Ohtani et al. 2007).

Again, this effect is reversed following exogenous estrogen treatment (Ohtani et al. 2007).

Removal of endogenous estrogens following ovariectomy also increases mortality associated with LPS challenge in rats and this effect is absent in animals that receive exogenous estrogen administration (Merkel et al. 2001). In the same study, androgenized females that have never been exposed to endogenous estrogen or progesterone, demonstrate a significantly higher mortality rate following LPS challenge than sham-androgenized females (Merkel et al. 2001). Finally, *E. coli* infections in dogs are most prevalent during diestrus, when estrogens are at their lowest levels (Surgiura et al. 2004).

Taken in concert, a large body of evidence suggests that estrogens are protective against bacterial infection and septic shock. However, it should be noted that some data suggests that high estrogen levels, such as those that might be seen in late proestrus and early estrus, have the potential to exacerbate such infections. For example, estradiol treated mice exhibit greater bacteremia and death following gonococcal infection than untreated animals (Kita et al. 1985). In addition, ovariectomized mice receiving high dose exogenous estrogen replacement demonstrate a higher incidence of *E. coli* infection in the kidney due to urinary tract infection than untreated mice (Curran et al. 2007). Furthermore, acute injection of the estrogen, estriol, was found to dramatically increase LPS-associated mortality in rats (Ikejima et al. 1998). Also, estrogen levels have been found to be higher in patients that develop an acute infection (within one day) following traumatic injury (Gee et al. 2007).

Finally, and perhaps more importantly, the increased incidence and severity of sepsis in humans correlates with sera estrone and estradiol levels. Female patients that

were admitted to the intensive care unit for sepsis had 10-20 times higher levels of these hormones than non-sepsis patients, while male sepsis patients exhibited 3-5 times higher estrogen levels (Fourrier et al. 1994). These results are in agreement with another recent study demonstrating that estradiol levels are significantly higher in non-surviving sepsis patients, regardless of sex (Dosset et al. 2008). Furthermore, the probability of death due to septic shock was found to be lowest when circulating sera estradiol levels were within the physiological range (0.01-0.37 ng/ml in females and 0.02-0.06 ng/ml in males), but increases sharply when levels were beyond this range (May et al. 2008). Interestingly, it was found that while all male sepsis patients had elevated estrone and estradiol levels in the first two days following admission to the intensive care unit, men that subsequently succumbed to septic shock were found to have maintained and even elevated estrogen levels in contrast to sepsis survivors (Christeff et al. 1992). Indeed, the correlation between sera estrone levels and sepsis severity was such that the authors of this study proposed that the levels of this hormone could be a useful predictor of outcome for sepsis patients. The increases in estrone and estradiol levels associated with sepsis do not coincide with increases in LH or FSH levels, indicating that these changes are due to increased synthesis of estrogens in the periphery (Fourrier et al. 1994). Interestingly, it has been found that the presence of LPS and/or inflammatory cytokines can increase activity of aromatase, the enzyme responsible for converting androgens into estrogens (Schmidt et al. 2000, Singh et al. 1997). Furthermore, it has been found that macrophages can actively convert androgens into the estrogen estriol (Schmidt et al. 2000). As such, high levels of circulating LPS and inflammatory cytokines in patients

with sepsis may have the ability to exacerbate symptoms by increasing production of estrogens.

Taken together, these findings indicate that while testosterone tends to increase susceptibility to bacterial infection and estrogen generally provides protection. However, it is important to note that super-physiological levels of female reproductive hormones may exert a contrary effect and negatively influence the outcome following sepsis.

1.6 Sex hormones have direct effects on immune cell function and modulate sentinel cell responses to bacterial motifs

Sexual dimorphism in acute immune responses to bacterial infections and the effects of reproductive hormones on susceptibility to such challenges indicate direct endocrine effects on the function of sentinel cells. Such a hypothesis has been supported by the presence of receptors for reproductive hormones in a variety of leukocyte types. Macrophages have been widely demonstrated to express estrogen receptors (Olsen and Kovacs 1996, Angele et al. 2000) and this sex hormone can modulate macrophage function by normal genomic or alternative non-genomic events. In vitro treatment with estradiol has been found to initiate the rapid activation of lipid secondary messengers, including phosphatidylinositol 3-kinase, in this cell type (Ghisletti et al. 2005). While classical androgen receptors do not appear to be expressed by macrophages, recent studies suggest that these sentinel cells possess non-classical cell surface receptors for testosterone (Benten et al. 2004). Treatment with this male sex hormone elicits an increase in intracellular calcium in macrophages and a resulting down-regulation of LPS-

induced c-fos promoter activity, while estrogen has the opposite effect (Benten et al. 2004).

Since testosterone appears to suppress immune responses to bacterial challenge and the overproduction of cytokines underlies the lethal nature of sepsis, one might anticipate that androgens directly alter the secretion and production of these inflammatory mediators. A considerable body of evidence has shown that male sex hormones suppress the responses of immune cells to endotoxin challenge. Removal of endogenous testosterone following castration results in an increase in LPS-induced IL-1 and IL-6 production by mouse peritoneal and splenic macrophages (Wichmann et al. 1997). In vitro treatment of murine peritoneal macrophages with testosterone decreases secretion of IL-1β following LPS challenge (Savita and Rai 1998), and causes a modest reduction in TNF-α secretion by such cells isolated from male but not female animals (Chao et al. 1995). Furthermore, it has been found that in vitro DHT treatment reduces IL-6 expression in LPS-stimulated umbilical chord endothelial cells via androgen receptors (Norata et al. 2006).

In addition to inflammatory cytokines, testosterone has been shown to attenuate the production of other immune molecules used to combat bacterial infections. Notably, macrophages isolated from castrated male mice produce more nitric oxide (NO) upon LPS stimulation indicating that endogenous testosterone suppresses NO release by these cells. Consistent with this conclusion, in vitro testosterone treatment results in decreased NO release by stimulated primary macrophages (Savita and Rai 1998) and RAW 264.7 macrophage-like cells with both pre- and post-transcriptional effects on the expression of inducible nitric oxide synthase (Friedl et al. 2000). It is clear that the immunosuppressive

effects of testosterone are not due to the peripheral aromatization and conversion of testosterone to estradiol because DHT, which is not converted to estrogen, also has suppressive effects and these actions are not affected by estrogen receptor inhibitors (Norata et al. 2006).

Finally, there is some evidence that the immunosuppressive actions of testosterone are specific to certain challenges and may be restricted based on sex. For example, it has been suggested that testosterone does not depress immune function in healthy animals but can suppress immune responses following insults such as trauma-hemorrhage (Angele et al. 1999), and female mice treated with DHT have been demonstrated to have higher in vivo TNF-α responses to LPS than untreated females and beyond that seen in male mice (Card et al. 2006). While the reasons for these discrepancies are presently unclear, it is possible that they stem from the fact that the suppressive effects of testosterone may be somewhat non-selective as androgens have also been implicated in the suppression of anti-inflammatory mediators. Orchidectomy has been shown to result in increased sera IL-10 levels after LPS challenge compared to intact males (Torres et al. 2005) and DHT can attenuate IL-10 production by LPS stimulated macrophages (Angele et al. 1999). Futhermore, orchidectomy has been shown to decrease inflammation due to infectious myocarditis. This effect is mediated by an increased proportion of anti-inflammatory regulatory M2 macrophages in the heart, indicating that testosterone inhibits anti-inflammatory populations of macrophages (Frisancho-Kiss et al. 2009).

Since the consensus is that testosterone is immunosuppressive and generally decreases inflammatory cytokine production by immune sentinel cells, one might

anticipate that estrogen would conversely increase the production of these mediators by cells such as macrophages. Indeed, several studies would seem to support this hypothesis. The production of inflammatory cytokines within the brain following intracerebral injection of LPS has been found to be attenuated in ovariectomized animals, an effect that is reversed following exogenous estrogen administration (Soucy et al. 2005). In addition, an intermediate dose of estradiol (0.1 ng/mL) results in a significant increase in TNF-α secretion by rat peritoneal macrophages stimulated with LPS (Chao et al. 1995), and acute in vivo estriol treatment has been shown to increase sera TNF-α levels and Kupffer cell TNF-α mRNA expression in rats challenged with LPS (Ikejima et al. 1998). Exposure to estradiol or estriol has been shown to increase sera TNF-α levels in female mice following in vivo LPS challenge, an effect that was antagonized by an agent that can serve as an estrogen receptor antagonist (Zuckerman et al. 1995, Zuckerman et al. 1996). Furthermore, treatment of isolated human peripheral monocytes with estradiol was found to increase TNF-α production from male derived cells (Asai et al. 2001), and peritoneal macrophages isolated after acute in vivo LPS treatment demonstrated a modest increase in the expression of mRNA encoding TNF-α after in vivo estriol exposure (Zuckerman et al. 1996). Removal of endogenous estrogens following ovariectomy has been shown to result in dramatic reductions in LPS-induced elevations of TNF-α mRNA expression in the brain, an effect that is reversed following estradiol replacement (Soucy et al. 2005). Finally, peripheral monocytes isolated from post-menopausal women with low levels of estradiol secrete significantly lower amounts of TNF-α following stimulation than cells isolated from pre-menopausal women (Verthelyi and Klinman 2000).

In addition to TNF-α, there is also evidence that estrogen can augment the production of other inflammatory cytokines. For example treatment of human peripheral monocytes with estradiol has been found to increase IL-6 production from male derived cells both before and after LPS stimulation (Asai et al. 2001). Interestingly, one study has found that while there is no difference in maximal IL-6 production in female mice following estrogen treatment, females treated with estriol produced IL-6 with faster kinetics following LPS challenge than that seen in untreated females, and this effect was inhibited by an agent that can antagonize estrogen receptors (Zuckerman et al. 1996). Peritoneal macrophages isolated from mice exposed in vivo to estradiol have been shown to exhibit increased expression of mRNA encoding for IL-6, IL-1β, IL-12, and iNOS following LPS challenge (Calippe et al. 2008). Furthermore, in vitro treatment of mouse peritoneal macrophages with estradiol has been found to increase LPS-stimulated IL-1β production (Savita and Rai 1998), while in vivo treatment of rodents with estradiol has been found to increase sera NO levels (Ikejima et al. 1998) and splenocyte production of IL-1α and IL-1β following in vitro restimulation (Dai et al. 2007). Lastly, estrogen may promote immune responses by limiting the production of the immunosuppressive cytokine IL-10, as one study has shown that in vitro estrogen treated male human peripheral monocytes produce lower amounts of this cytokine following in vitro stimulation (Asai et al. 2001).

Having made the case for estrogens enhancing the production of inflammatory mediators it might be surprising to learn that the preponderance of evidence appears to contradict this idea. For example, lower levels of IL-6 and TNF-α have been observed in the peritoneal fluid following cecal ligation and puncture in mice that were treated with

an estrogen receptor agonist (Cristofaro et al. 2006), and acute intraperitoneal treatment with estradiol attenuates LPS induced elevations in sera TNF-α levels in rats (Sener et al. 2005). Both low (less than 10^{-5} ng/mL) and high (greater than 1ng/mL) doses of estradiol have been shown to decrease secretion of TNF-α by LPS treated rat peritoneal macrophages (Chao et al. 1995). Estradiol can decrease LPS induced TNF-α expression by murine macrophages (Salem et al. 2000) and human peripheral monocytes (Vlotides et al. 2007, Asai et al. 2001). This effect does not appear to be limited to monocytes/macrophages, as gastric tissue infected by *H. pylori* produced more TNF-α following ovariectomy, an effect that was reversed by estradiol replacement (Ohtani et al. 2007). Furthermore, in vitro estradiol treatment of astrocytes decreased LPS induced TNF-α expression (Kipp et al. 2007, Lewis et al. 2008), and this decrease was inhibited by an estrogen receptor antagonist (Kipp et al. 2007). Finally, a study of trauma patients found that sera estradiol levels were negatively correlated with levels of TNF-α, though not sera levels of IL-6 or IL-8 (Gee et al. 2007).

The effects of estrogens on IL-6 levels appear to be in agreement with the findings for TNF-α production in that estrogen decreases the production of this cytokine following bacterial challenge and/or exposure to endotoxin. Acute in vivo exposure to estradiol has been found to decrease IL-6 levels following endotoxin challenge (Zuckerman et al. 1995) and macrophages elicited from estradiol treated mice expressed lower levels of mRNA encoding IL-6 than untreated mice following in vitro restimulation (Zuckerman et al. 1995). In addition to immune sentinel cells, other cell types capable of responding to LPS have been shown to produce lower levels of IL-6 following estrogen treatment. Human retinal pigment epithelial cells have been shown to have lower LPS

induced IL-6 responses following exposure to estradiol, and this effects has been demonstrated to be reversed following treatment with an estrogen receptor antagonist (Paimela et al. 2007). Acute estradiol treatment has also been found to attenuate LPS induced IL-6 production from human uterine and endometrial epithelial cells (Lesmeister et al. 2005, Fahey et al. 2008) and murine astrocytes (Cerciat et al. 2009).

In addition to TNF-α and IL-6, estrogens have been shown to decrease production of bacterial/endotoxin induced production of other inflammatory mediators, including IL-1β, inflammatory chemokines, and NO. Resident CNS cells that exert sentinel immune functions, including microglia and astrocytes, exhibit diminished LPS induced IL-1β production following exposure to estradiol or estrogen receptor agonists (Lewis et al. 2008). Similarly, gastric tissue from ovariectomized females infected by *H. pylori* has been found to contain higher levels of IL-1β than that seen in infected intact females, an effect that was reversed following estradiol replacement (Ohtani et al. 2007). Short-term in vitro exposure to estradiol has also been found to decrease murine peritoneal macrophage expression of mRNA encoding for IL-1β, IL-12, and iNOS following LPS challenge, an effect contrary to what was seen due to the presence of in vivo estradiol (Calippe et al. 2008). CXCL8 (IL-8) secretion attracts immune cells to the site of bacterial infection, and excess infiltration of leukocytes, including neutrophils, can contribute to the pathogenesis of sepsis. Treatment of female human peripheral monocytes with estradiol results in a dose dependent decrease in LPS induced mRNA expression and secretion of IL-8 (Pioli et al. 2007). Similarly, estradiol decreases expression of mRNA encoding another inflammatory chemokine, CXCL2 (MIP-2), in LPS treated macrophages (Ghisletti et al. 2005). This effect was driven by effects on NF-

κB function, as estradiol decreases p65 binding to the MIP-2 promoter (Ghisletti et al. 2005). Acute estradiol treatment has also been found to attenuate LPS induced MIP-3α production from uterine epithelial cells (Soboll et al. 2006). Murine astrocytes have been shown to have decreased levels of the inflammatory chemokine IP-10 following LPS challenge in vitro (Cerciat et al. 2009). The presence of in vivo estradiol has been reported to decrease inflammation in the brain of rats by decreasing the proportion of MHC class II immunoreactive microglia following LPS treatment. However, the dose of estradiol associated with this effect is dependant on sex, with only low doses decreasing immunoreactive microglia in male brains and only high doses of estradiol suppressing inflammation in female brains (Tapia-Gonzales et al. 2008). Finally, ovariectomy results in the elevated production of NO by macrophages upon stimulation with LPS indicating that endogenous estrogen decreases NO secretion. Consistent with this notion, in vitro treatment of macrophages (Savita and Rai 1998) and microglia (Vegato et al. 2000) with estradiol leads to diminished LPS-induced NO release, an effect reversed following exposure to an agent that can inhibit estrogen receptors (Savita and Rai 1998). Also, treatment of macrophages with endocrine disruptors results in decreased LPS-induced NO production and NF-κB activation, an effect that was found to be at least in part mediated by the classical estrogen receptors (Yoshitake et al. 2008).

The idea that estrogen limits inflammatory responses is further supported by its documented effects on the production of the immunosuppressive/anti-inflammatory cytokine IL-10. For example, estradiol treatment of orchidectomized male mice increases IL-10 secretion from LPS stimulated Kupffer cells (Angele et al. 1999). Furthermore, exogenous estradiol treatment causes a significant increase in IL-10

production in ovariectomized mice infected with *H. pylori* (Ohtani et al. 2007), and long term in vivo treatment of male mice with estradiol was found to increase splenocyte IL-10 production following in vitro concanavalin A challenge (Dai et al. 2007).

Taken together, there appears to be a consensus that testosterone limits the production of key inflammatory mediators consistent with an immunosuppressive role for this hormone. In contrast, the effects of estrogen appear to be far more controversial. While data is available to support the enhancement of immune sentinel cells responses by this hormone, a large body of work contradicts these findings and points to a suppressive action of estrogens on inflammatory cytokine and chemokine release by a number of host immune cell types. While the reasons for these disparate results are unclear, it is possible that the effects of estrogen are dependent on the particular challenges faced by the host, the kinetics of the immune response, and the effective dose of estrogen. For example, it is possible that while intermediate doses of estrogen enhance cytokine release, very low or very high doses can have the opposite effect. Alternatively, these often confusing results may again stem from the differences between immunosuppressive versus immunoprotective actions. It is important to note that in order to protect an individual during sepsis, a decreased production of potent inflammatory cytokines such as TNF-α, IL-6, and IL-1 would be desirable. Obviously, further study is necessary to clarify this issue.

1.7 Sex hormones have direct effects on the expression of pattern recognition receptors and their signal transduction pathways in immune sentinel cells

As discussed previously, it has now become apparent that acute immune responses of key sentinel cells to bacterial pathogens are initiated via the recognition of conserved microbial motifs including LPS and bacterial lipoproteins by receptors, TLR4 and TLR2, respectively. In addition, the recognition of LPS is facilitated by co-receptors such as CD14 and molecules including LBP. Recognition of bacterial components by these receptors initiate MAP kinase cascades and NF-κB activation via adaptor molecules including MyD88. The activation of NF-κB will then alter gene transcription of pro- and anti-inflammatory cytokines precipitating the "cytokine storm" that underlies bacterial septic shock.

In accordance with the inhibitory effects of androgens on cytokine production, testosterone has been shown to attenuate LPS-induced activation of p38 MAP kinase but not ERK1/2 or JNK/SAPK activation in macrophages (Benten et al. 2004), while DHT appears to interfere with the ability of NF-κB to bind to DNA promoters in umbilical cord endothelial cells (Norata et al. 2006). Similarly, and in agreement with the documented ability of estrogen to limit inflammatory cytokine production, female sex hormones have been shown to inhibit NF-κB activity. For example, treatment with an estrogen receptor agonist can cause a decrease in cellular NF-κB reporter activity (Opal et al. 2005) and acute estradiol treatment has been found to decrease DNA binding of NF-κB (Paimela et al. 2007, Ghisletti et al. 2005). It appears that estradiol can regulate NF-κB activity at the level of nuclear translocation. Estradiol retains p65/Rel-A and c-Rel in the cytoplasm of immune cells following stimulation (Ghisletti et al. 2005, Dai et al. 2007), regardless of IκBα synthesis, phosphorylation, or degradation (Ghisletti et al. 2005), and appears to target the microtubule-associated transport system used by NF-κB

subunits (Ghisletti et al. 2005). Interestingly, estradiol was not found to inhibit the nuclear translocation of the p50 subunit and increases the expression of Bcl-3, a protein that binds to p50 homodimers to permit transcription of NF-κB responsive genes (Dai et al. 2007). Furthermore, estradiol has been found to affect the activation of the PI3K/Akt pathway, which in turn inhibits NF-κB activity and resulting inflammatory cytokine secretion. While short-term in vitro treatment with estradiol enhances murine macrophage phosphorylation of Akt, chronic in vivo estradiol exposure results in the opposite phenomenon, as well as inhibition of PI3K activity, following LPS challenge of these cells (Calippe et al. 2008). As such, these effects might provide several mechanisms underlying the seemingly paradoxical ability of estrogen to both inhibit and promote inflammatory gene expression.

While reproductive hormones may have specific effects on the amount or function of signal transduction components, it is clear that endocrine-induced changes in the level of expression of pattern recognition receptors, their co-receptors, or adaptor molecules would have a profound influence on downstream cellular events including NF-κB activation and cytokine production. Importantly, one recent study indicated that sex hormones can directly alter the expression of innate receptors for bacterial components as summarized in Figure 2. It has been shown that rat prostate cells from orchidectomized animals express higher levels of TLR4 following *E. coli* infection compared to intact males but were restored to normal levels following testosterone replacement (Quintar et al. 2006).

Once again, while the effects of testosterone appear to be consistent, the effects of estrogens on pattern recognition receptor expression are more complicated as acute

administration of estrogen fails to alter such expression in vitro. For example, acute estrogen treatment of macrophage-like cell lines (Vegato et al. 2004, Vlotides et al. 2007), and Kupffer cells (Ikejima et al. 1998), or LPS challenged human monocytes (Pioli et al. 2007), fails to significantly alter TLR4 or CD14 expression. In contrast, a number of studies show that estrogen can have significant effects on pattern recognition receptor expression in vivo. For example, in vivo administration of estriol can elevate expression of mRNA encoding CD14 and LBP in Kupffer cells (Ikejima et al. 1998), and ovariectomy results in markedly lower TLR2 transcription in the brain in response to LPS compared to that seen in intact females, an effect that can be reversed following exogenous estrogen replacement (Soucy et al. 2005). One study with low sample size found that macrophages from estradiol treated ovariectomized mice expressed slightly, but not significantly, higher levels of cell surface CD14 (Calippe et al. 2008). Finally, expression of MyD88, an important adaptor protein in TLR signaling, was found to be increased following in vivo treatment of male mice with estradiol (Zheng et al. 2006). However, it has been found that chronic in vivo treatment of ovariectomized mice with estradiol fails to alter total cellular TLR4 protein content in peritoneal macrophages (Calippe et al. 2008). While hormone replacement therapy has not been reported to alter the levels of TLR4 expression in circulating monocytes isolated from elderly post-menopausal women undergoing exercise training (Flynn et al. 2003), it must be noted that the results of this study are difficult to interpret due to the physiological effects of aging and exercise.

That being said, at least one study has shown contrary effects of estradiol on TLR expression. Levels of mRNA encoding for several TLRs have been found to vary with

the estrous cycle in the human uterine endometrium. Higher levels of mRNA encoding for TLR2, TLR3, TLR4, and TLR9 correlate with low levels of estradiol. Furthermore, endometrial cells treated in vitro with estradiol for 72 hours, but not 24 or 48 hours, exhibited reduced expression of mRNA encoding for TLR4, but not TLR2, TLR3, or TLR9 (Hirata et al. 2007). Interestingly, progesterone treatment of endometrial cells for 72 hours yield higher levels of mRNA encoding for TLR4, and normalized levels when co-treated with estradiol (Hirata et al. 2007).

1.8 The novel G protein coupled receptor GPR30 binds to estradiol and elicits rapid cellular responses

One explanation for the apparently contradictory actions of estrogens could be due to the existence of multiple types of receptors for this hormone. Rapid signaling events and genomic actions of estrogen have previously been exclusively attributed to the actions of the classical nuclear hormone estrogen receptors, ERα and ERβ. These receptors bind to estrogen intracellularly and typically translocate to the nucleus where they bind to estrogen response elements on DNA to activate or repress transcription (reviewed in Beato 1989). However, the recent discovery of a non-classical membrane estrogen receptor that also mediates rapid non-genomic events has shed light on different actions of estrogen. The G protein coupled receptor GPR30 is a seven transmembrane receptor that has very high affinity for 17β-estradiol and not other estrogens, progesterone, or testosterone (Thomas et al. 2008, Revankar et al. 2005). While structurally unrelated to the classical estrogen receptors, overwhelming evidence has shown that GPR30 binds to estradiol and mediates rapid actions of this hormone.

Estrogen bound GPR30 has been found to initiate several rapid cellular effects that are not mediated by the traditional estrogen receptors. GPR30 promotes estradiol activation of the MAP kinase signaling molecules ERK1 and ERK2 via the Gβγ protein subunit (Filardo et al. 2000). The Gα subunit has been found to cause activation of adenylyl cyclase and an increase in cAMP after GPR30 binding to estradiol (Filardo et al. 2002, Hsieh et al. 2007a). Importantly, both of these effects were found to be estrogen receptor independent. The actions of estradiol binding to GPR30 are very rapid, with an increase in activation of adenylyl cyclase and cAMP found within 10 to 15 minutes of binding (Thomas et al. 2008, Filardo et al. 2002, Filardo et al. 2007). GPR30 has also been found to initiate an intracellular calcium influx (Revankar et al. 2005, Filardo et al. 2002, Funakoshi et al. 2006, Wang et al. 2008). Intracellular calcium is elevated by 10 seconds after estrogen treatment, and calcium stays elevated for over 5 minutes (Funakoshi et al. 2008).

Importantly, the selective estrogen receptor modulator 4-hydroxytamoxifen and the classical estrogen receptors ERα and ERβ antagonist ICI 182780, are both agonists for GPR30 (Thomas et al. 2008, Pang et al. 2008). ICI 182780 has previously been considered a pure anti-estrogen because of its inhibitory effects on the nuclear hormone estrogen receptors. However, recent evidence has shown that this compound is in fact an agonist for GPR30, mimicking the effects of estradiol (Filardo et al. 2000, Pang et al. 2008, Teng et al. 2008, Kamanga-Sollo et al. 2008). ICI 182780 and tamoxifen have competitive affinity for GPR30, at about 10% of the affinity of estradiol (Thomas et al. 2008). It has been found that the molecule G1 is a specific agonist for GPR30, and initiates activity in this receptor similar to estradiol (Pang et al. 2008, Teng et al. 2008,

Kamanga-Sollo et al. 2008, Kuhn et al. 2008). G1 has no activity for the classical estrogen receptors, nor does it cause effects in cells lacking GPR30 (Bologa et al. 2006). GPR30 alone, and not the classical estrogen receptors, has been found to mediate important effects of estradiol in several cell types (Wang et al. 2008, Kuhn et al. 2008). In some cases, GPR30 has been found to work in conjunction with ERα to induce proliferation (Kamanga-Sollo et al. 2008, Sirianni et al. 2008). Crosstalk between these two receptors can be important for cellular function. Whereas G1 and PPT (GPR30 and ERα agonists, respectively) initiate estrogenic effects, ICI 182780 blocks the effects of estrogen because of inhibition of the ERα component of signaling (Sirianni et al. 2008). As such, estradiol has the potential to initiate several different cellular responses, some of which could be contradictory, depending on the type of receptor to which it binds.

1.9 Summary

Male/female differences in innate immune responses to bacterial infection are evident in human patients and rodent models of disease. Females are less susceptible to the development of bacterial infections and subsequent bacteremia and sepsis, whereas males exhibit a greater incidence of such infections and are more likely to develop fatal sequelae. Females are protected from septic shock in two ways: 1) by producing lower levels of the proinflammatory cytokines responsible for the lethal nature of septic shock; 2) by elevating the production of anti-inflammatory molecules. Although evidence is accumulating that sex differences exist at the level of expression of PRRs for bacterial motifs and downstream signaling pathways, the mechanism of action is controversial as cells from males express higher levels of TLR4 and its co-receptors, whereas LPS-

induced signaling pathways in female cells are more efficient at recognizing bacterial pathogens than are male cells.

The observed differences in susceptibility to bacteria and endotoxin between the sexes may be caused by the actions of sex steroids. Both age and reproductive status significantly influence the severity of bacterial infections and the incidence of sepsis. Testosterone also increases susceptibility to infection in rodent models, whereas estrogen generally reduces susceptibility. Androgens, however, limit the production of key inflammatory mediators by immune cells following bacterial endotoxin exposure. Conversely, the role played by estrogens is debated, with evidence for both enhancement and suppression of immune responses. A possible mechanism for the effects of sex hormones on immune responses to bacterial pathogens may be that testosterone and estrogen exert direct effects on the expression of PRRs, including TLR4 and its co-receptors, and can modulate the signal transduction pathways employed by these receptors to initiate cytokine expression. Consequently, sex hormones affect proinflammatory cytokine expression. As such, it is the goal of this dissertation to elucidate some of the controversy surrounding the roles of reproductive hormones in susceptibility to sepsis.

1.10 Figures

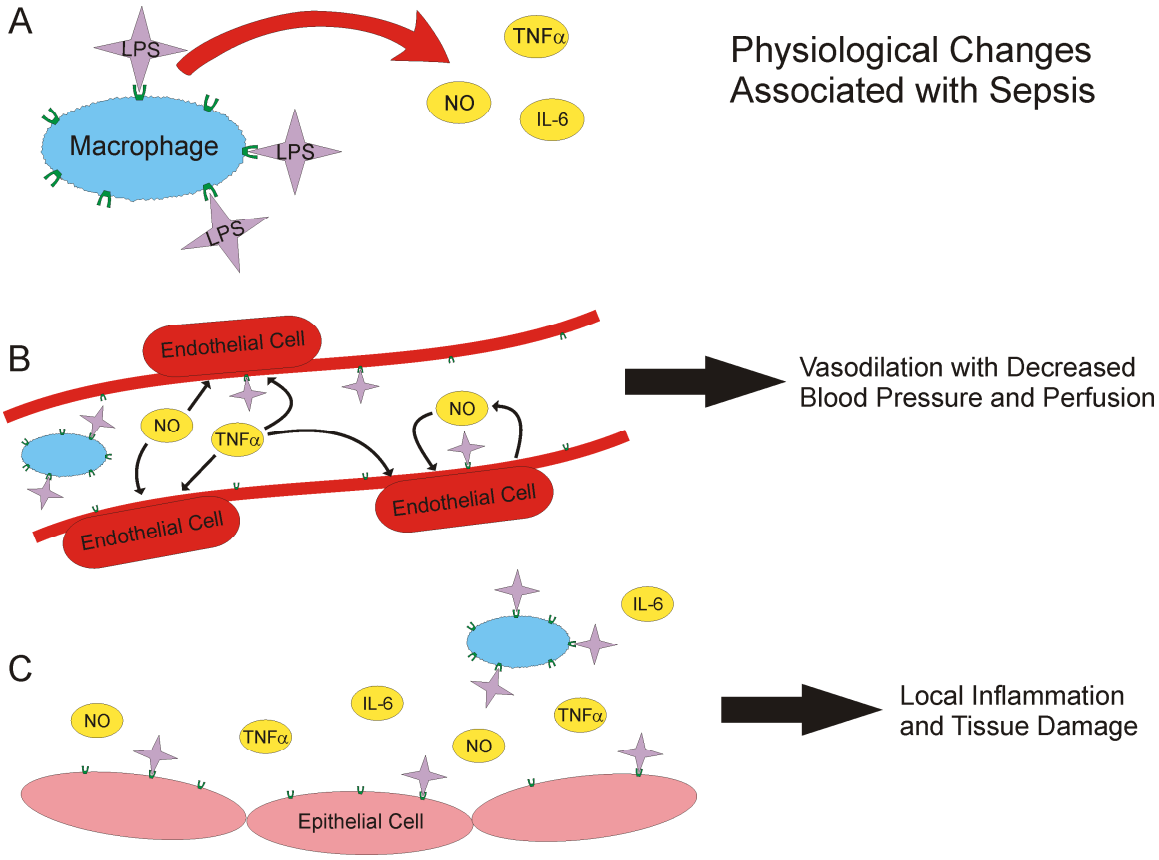

FIGURE 1: Physiological changes associated with sepsis. Immune sentinel cells responding to LPS release inflammatory mediators (panel A) that in turn act on target tissues to cause vasodilation, decreased perfusion, inflammation, and tissue damage (panels B and C).

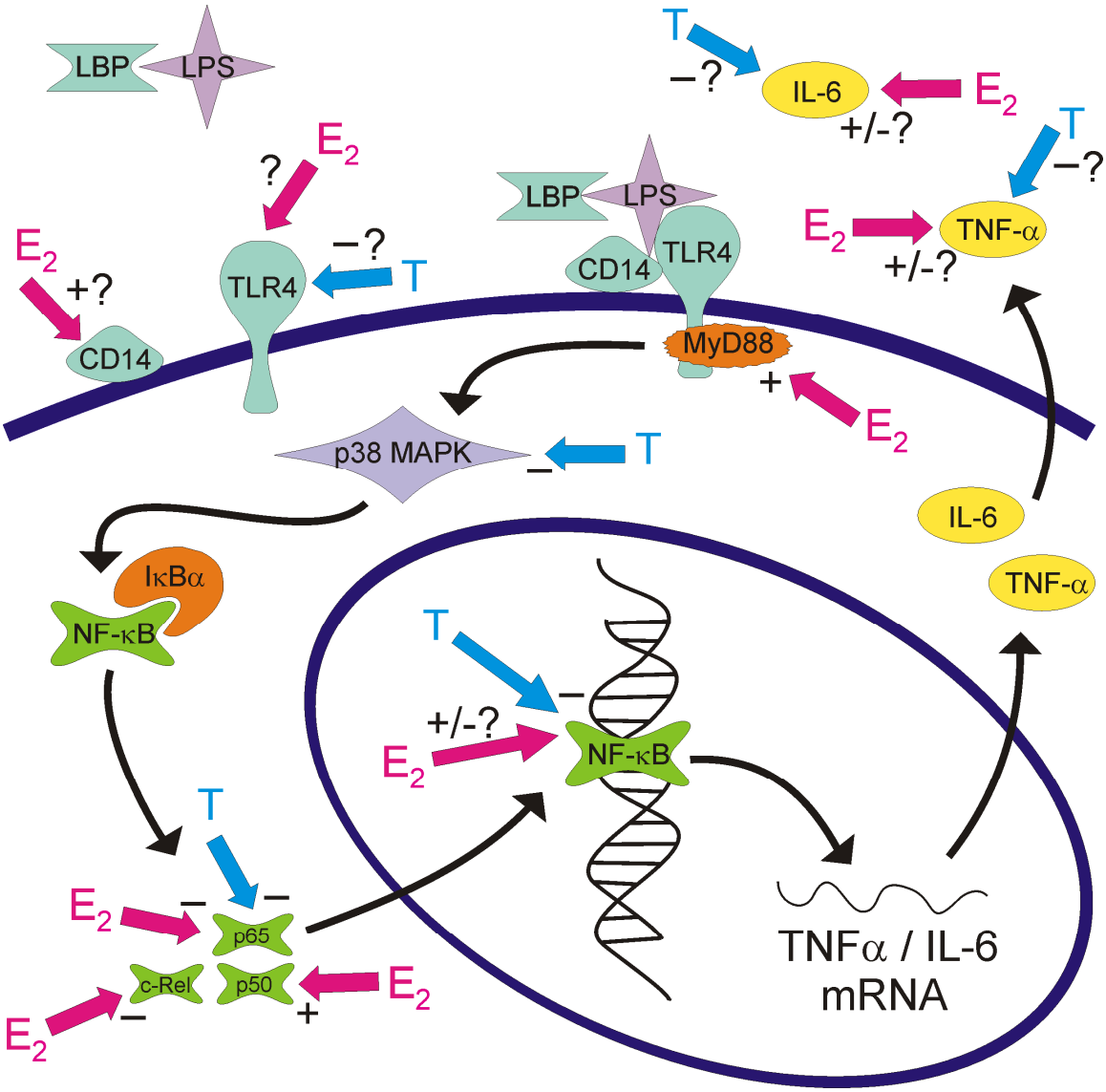

FIGURE 2: Mechanisms underlying LPS-induced inflammatory cytokine production by host sentinel cells and possible points of regulation by reproductive hormones. LBP: lipopolysaccharide binding protein, LPS: lipopolysaccharide, TLR4: Toll-like receptor 4, p38 MAPK: mitogen-activated protein kinase p38, NF-κB: nuclear factor kappa B, E_2: estrogens (and pink arrow), T: testosterone (and blue arrow). Plus sign indicates a stimulatory effect while negative sign indicates an inhibitory effect, and question mark indicates unknown or unclear.

CHAPTER TWO: MATERIALS AND METHODS

2.1 Macrophage-like cell line culture

RAW 264.7 macrophage-like cells originally derived from male cells (CRL-2278; ATCC, Manassas, VA, USA) were grown on Cellstar culture plates (Greiner Bio-one, Monroe, NC) in RPMI 1640 (Cellgro, Washington, DC) containing 2% NuSerum (BD, 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 2.6 X 10^{-10}M testosterone, less than 2.5 X 10^{-10}M 17β-estradiol, and less than 3.1 x 10^{-10}M progesterone. Some cells were co-cultured with varying doses of estradiol (Sigma Aldrich Co., St. Louis, MO), testosterone (Sigma Aldrich Co., St. Louis, MO), ICI 182780 (Tocris Bioscience, Ellisville, MO), or G1 (Cayman Chemical, Ann Arbor, MI).

2.2 Surgical orchidectomy and ovariectomy, and hormone replacement

Male and female C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME, USA), 8 weeks of age, underwent bilateral gonadectomy under inhalant isoflurane anesthesia. Male mice underwent orchidectomy via scrotal incision to remove the main endogenous source of testosterone. Female mice underwent bilateral ovariectomy under inhalant isoflurane anesthesia to remove the main endogenous source of estrogens. Groups of adult mice were bilaterally gonadectomized or sham gonadectomized and allowed to

recover for five weeks. This time period not only ensures that androgens produced by the testis and estrogens produced by the ovary have been metabolized and are no longer present in the blood (Merkel et al. 2001), it is also sufficient to allow the turnover of immune cells generated under the influence of these reproductive hormones. Mice in the sham-operated group underwent the same anesthesia and incision procedure but the gonads were not excised.

Hormone replacement was achieved as follows. Male orchidectomized mice received subcutaneous injections of testosterone proprionate in corn oil (5 mg/kg) every three days for five weeks. Female hormone replacement was achieved essentially as described previously by Cohen and collegues (1993). Immediately following ovariectomy, silastic 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 subcutaneously to deliver exogenous hormone throughout the five week experimental period. Hormone packed in silastic tubing implants have previously been shown to provide elevated levels of hormone for this time period (Cohen et al. 1993). 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 estradiol implants ($OVX+E_2$), and ovariectomized animals receiving both progesterone and 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.

2.3 *In vivo* endotoxin treatment

Gonadectomized, sham operated, and gonadectomized animals receiving hormone replacement were given intraperitoneal (i.p.) injections of lipopolysaccharide (LPS) (5 mg/kg) isolated from *E. coli* (> 500,000 EU/mg; Sigma Chemical Co., St. Louis, MO). At varying time points post-treatment, animals were studied for behavior and appearance prior to euthanasia and analysis of sera content. The severity of endotoxic shock was scored according to a system modified from that previously employed by Liu et al. (2005), in which a score of 1 was given to mice with ruffled fur but no detectable behavioral differences compared to untreated mice, a score of 2 was given to mice with percolated fur and a huddle reflex but that were still active, a score of 3 was given to mice that were less active and were relatively passive when handled, a score of 4 was assigned to inactive mice that exhibited only limited response when handled, and a score of 5 was given to moribund mice.

2.4 Isolation of murine peritoneal macrophages

Elicited peritoneal macrophages were isolated as previously described by our laboratory (Marriott et al. 1998, Marriott et al. 2000, Marriott et al. 2006). Briefly, mice from each treatment group received intraperitoneal injections of 200 µl incomplete Freunds adjuvant (Sigma-Aldrich, St. Louis, MO). Three days later, the peritoneal cavities were lavaged with RPMI 1640 (7 x 1.5 ml per animal) containing 10% FBS (Atlanta Biologics, Norcross, GA) to remove the peritoneal macrophages. After washing twice in RPMI 1640, adherent macrophages were cultured in RPMI 1640 containing 2% FBS and gentamycin. Some cells were co-cultured with varying doses of testosterone and estradiol.

2.5 Isolation and cell culture of bone marrow-derived macrophages

Mice were euthanized and marrow was flushed from isolated femurs with RPMI 1640 (Cellgro, Washington, DC) containing 10% FBS (Atlanta Biologics, Norcross, GA) to isolate bone marrow cells. After washing twice in RPMI 1640, adherent cells were cultured in RPMI 1640 containing gentomycin, 2% NuSerum (BD, Franklin Lakes, NJ, USA) to minimize exposure to reproductive hormones, and 25% LADMAC media as a source of macrophage colony stimulating factor (M-CSF) to elicit macrophage development. Cells were cultured for up to two weeks, and some groups were co-cultured with varying doses of estradiol (Sigma Aldrich Co., St. Louis, MO).

2.6 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 (Marriott et al. 2006). Cells were isolated and a phycoerythrin-conjugated antibody directed against TLR4/MD-2 (Clone MTS510, eBioscience) or a FITC-conjugated antibody directed against CD14 (Clone rmC5-3) was added for 45 min at 4ºC. Cells were then washed and assayed by FACS analysis for the proportion of CD14 or TLR4/MD-2 positive cells relative to fluorescence obtained in cells stained with an FITC- or PE-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, San Diego, CA). In some experiments, 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 co-cultured with 400 ng/ml labeled LPS for 45 minutes prior to 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.

2.7 Quantification of TNF-α, IL-6, IL-10, LBP, and PGE_2 production

Capture ELISAs were performed to quantify interleukin 6 (IL-6) levels using a commercially available capture antibody against IL-6 (clones MP5-20F3; BD PharMingen), a biotinylated anti-mouse 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). Tumor necrosis factor alpha (TNF-α), interleukin 10 (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. Prostaglandin E_2 (PGE_2) levels were quantified using a commercially available EIA kit according to the directions provided by the manufacturer (Assay Designs, Ann Arbor, MI), and the minimum detectable level in this assay was 39.1 pg/ml for PGE_2. All determinations were made in duplicate from the indicted number of sera samples.

2.8 Quantification of sera hormone levels

Sera estradiol and progesterone levels were quantified at the time of sacrifice using commercially available EIA assays according to the directions provided by the manufacturer (Oxford Biomedical Research, Oxford, MI).

2.9 Isolation of RNA and semi-quantitative reverse transcribed PCR

Total RNA was isolated from macrophages using TRIzol reagent (Invitrogen, Carlsbad, CA) and was reverse transcribed in the presence of random hexomers using 200 U RNase H^-, Moloney leukemia virus reverse transcriptase (RT) (Promega, Madison, WI) in the buffer supplied by the manufacturer. Polymerase chain reactions (PCR) were performed to determine the expression of mRNA encoding GPR30. Positive and negative strand PCR primers used, respectively, were 5'-AAGCCATGGATGCGACTACT-3' and 5'-CGCCAGGTTGATGAACA-3' to amplify mRNA encoding murine GPR30 (311 bp fragment). Primers were designed by using Oligo 4.0 primer analysis software (National Biosciences Inc., Plymouth, MA) based on their location in different exons of the genomic sequences for GPR30 in addition to their lack of significant homology to sequences present in GenBank (MacVector Sequence analysis software, IBI, New Haven CT). The identity of the PCR amplified fragments was verified by size comparison with DNA standards (Promega). The input RNA was normalized to the expression of the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (G3PDH).

2.10 Western blot analysis for GPR30

Protein samples were collected from macrophages using a SDS protein buffer containing 125 mm Tris Base, 20% glycerol, 2% lauryl sulfate, 0.01% BPB, and 2% 2-mercaptoethanol. Protein samples were electrophoresed on SDS-polyacrylamide gels and transferred to Immobilon-P Transfer Membranes (Milipore, Bedford, MA). A rabbit anti-human polyclonal primary antibody (which cross reacts with mouse) as used to detect GPR30 (MBL International, Woburn, MA) at a 1:500 dilution. After reacting with the primary antibody, blots were washed and incubated in the presence of an anti-rabbit IgG antibody conjugated to horseradish peroxidase (Cell Signaling Technology, Beverly, MA). Bound enzyme was detected with the enhanced chemiluminescent (ECL) system (Amersham Pharmacia Biotech, Arlington Heights, IL). Blots were exposed to Pierce CL-X Posure Film (Pierce, Rockford, IL) for one hour and developed.

2.11 Silencing RNA-mediated GPR30 knockdown

Three validated Stealth RNAi™ siRNA duplexes targeting murine GPR30, as well as universal negative control siRNA not homologous to anything in the vertebrate transcriptome, were purchased from Invitrogen (Carlsbad, CA). RAW 264.7 cells were transfected with siRNAs using Tfx-20 transfection reagent (Promega, Madison, WI) according to the directions provided by the manufacturer. GPR30 siRNA duplex Oligo ID: MSS233774 was chosen based on optimization experiments using RAW 264.7 cells and the Tfx-20 transfection reagent. Serum-free media was replaced with complete media following one hour transfection. At 48 hours after transfection, cells were assayed

for efficiency of knockdown, as assessed by PCR, and TLR4 levels, as assessed by flow cytometry.

2.12 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 a Student's t test, a paired Student's *t* test, or a one way ANOVA as appropriate using commercially available software (SAS v9.1.3, SAS Institute Inc., Cary, NC). In all experiments, results were considered statistically significant when a P-value of less than 0.05 was obtained. Results are presented as the mean or the geometric mean of fluorescence intensity +/- SEM.

CHAPTER THREE: TESTOSTERONE REDUCES MURINE MACROPHAGE EXPRESSION OF TOLL-LIKE RECEPTOR 4

3.1 Rationale

Sex differences have long been known to be a contributory factor in the incidence and progression of disorders associated with immune system dysregulation (as reviewed in Beery et al. 2003). More recently, evidence has accumulated that sex may also play an important role in infectious disease susceptibility (as reviewed in Marriott and Huet-Hudson 2006). In general, females generate more robust and potentially protective humoral and cell-mediated immune responses following antigenic challenge than their male counterparts. For example, influenza infection elicits greater severity and hospitalization in male patients (Quach et al. 2003), and females generate greater humoral and cell mediated responses to herpes simplex viruses (Knoblich et al. 1983) and cytomegalovirus (Villacres et al. 2004). Women have been found to have higher circulating levels of IgM than men (Butterworth et al. 1967) and this difference is most apparent at puberty (Lichtman et al. 1967, Grundbacher 1972) suggesting a role for reproductive hormones in the development of this gender bias. Furthermore, cell-mediated host responses have also been suggested to exhibit sexual dimorphism and female mice have been shown to mount more vigorous T-cell responses to exogenous and allogeneic antigens than males (Weinstein et al. 1984). This notion is further supported by the observation that early cell-mediated immune responses to thermal injury are more robust in females than their male counterparts (Gregory et al. 2000b).

Differences in immune responses between males and females have generally been assumed to be a consequence of the actions of reproductive hormones. Androgens have been shown to have suppressive effects on immune functions following trauma or trauma-hemorrhage and subsequent sepsis (Wichmann et al. 1996, Angele et al. 1999, Angele et al. 2000). Furthermore, testosterone and other androgens such as dihydrotestosterone, have been shown to decrease immunoglobulin and cytokine production, and to limit lymphocyte proliferation (as reviewed in Angele et al. 2000 and Olsen and Kovacs 1996). These observations are consistent with the demonstration that exogenous testosterone administration increases female susceptibility to *M. marinum* infection, and castration attenuates such infections in male mice (Yamamoto et al. 1991). While classical androgen receptors do not appear to be expressed by macrophages, recent studies suggest that these important sentinel cells possess non-classical cell surface receptors for male sex hormones (Liu et al. 2005). The presence of such receptors could explain the documented ability of testosterone to down-regulate LPS-induced activation of the pro-inflammatory transcriptional regulators and immune responses in isolated macrophages (Benten et al 2004). To date, it is unclear how testosterone alters the immune responsiveness of macrophages.

In the present study we demonstrate that testosterone reduces expression of TLR4 on a macrophage cell-line and cultured primary macrophages. Furthermore, we have extended these in vitro findings to an in vivo analysis of TLR4 expression on monocytes/macrophages and report an increase in TLR4 levels on these cells in the absence of endogenous testosterone. Taken together, these data are in agreement with the recent observation that TLR4 expression is increased in the prostate following castration

(Quintar et al. 2006) and provides a potential mechanism underlying the immunosuppressive effects of testosterone.

3.2 Results

To begin to determine the in vitro effects of testosterone on cell surface TLR4 expression we have utilized a murine macrophage-like cell line, RAW 264.7. This cell line was cultured in media containing 2% NuSerum, conditions designed to minimize exposure to reproductive hormones. These cells were exposed to varying concentrations of testosterone propionate (1-1000 nM) for various time periods prior to analysis of cell surface TLR4 by flow cytometry. As shown in Figure 3A and 3B, testosterone elicited an approximately 10% reduction in fluorescence attributable to cell surface TLR4 at doses as low as 100 nM (final ethanol concentration of 0.0002%) and this effect was significant ($n = 16$ per group, $p < 0.05$) at a dose of 1 μM (final ethanol concentration of 0.002%). This effect was most apparent at 24 hours following testosterone administration (Figure 3C).

To determine whether the reduction in cell surface TLR4 expression was due to receptor internalization, we have assessed the effect of testosterone on relative TLR4 protein content in permeabilized cells by flow cytometry. In these studies, 24 hour treatment of RAW 264.7 cells with 1 μM testosterone elicited a statistically significant 7.3% decrease in cellular TLR4-asssociated fluorescence (fluorescence geometric means of 7.37 +/- 0.14 versus 6.83 +/- 0.13 in untreated and testosterone treated cells, respectively; $n = 12$ per group, $p < 0.05$) indicating that receptor internalization does not account for the apparent decrease in cell surface TLR4 expression in this cell line.

To begin to assess the functional significance of testosterone-mediated decreases in TLR4 expression in this macrophage-like cell line, we have determined whether testosterone alters the sensitivity of these cells to the TLR4 ligand, LPS. RAW 264.7 cells were exposed to testosterone (1 μM) for 24 hours prior to challenge with LPS (250 ng/ml) and the production of the inflammatory cytokine, TNF-α, was assessed after 12 hours by specific capture ELISA. We report that testosterone significantly decreased LPS-induced TNF-α production from 703 +/- 15 pg/ml to 620 +/- 20 pg/ml ($n = 12$ per group, $p < 0.05$) consistent with a decreased functional responsiveness of these cells to this TLR4 ligand.

Having determined optimal doses and kinetics using a cell line, we have confirmed the effect of testosterone on TLR4 expression in primary murine peritoneal macrophages. Macrophages were elicited from orchidectomized animals that are largely devoid of endogenous androgens, sham orchidectomized mice, and orchidectomized mice with testosterone replacement. Cells were cultured either in the presence or absence of 1 μM testosterone and TLR4 cell surface expression was assessed at 24 hours post testosterone treatment by flow cytometry. As shown in Figure 4, testosterone treatment again elicited a modest but significant decrease in cell surface expression of TLR4 on macrophages derived from orchidectomized mice ($n = 16$ per group, $p < 0.05$, paired Student's t test). In contrast, cells derived from orchidectomized mice that received in vivo testosterone replacement or sham orchidectomized mice failed to show sensitivity to in vitro testosterone addition, suggesting that the effects of this hormone are long lasting and maximal at even endogenous levels.

To determine whether the reduction in cell surface TLR4 expression was due to receptor internalization or decreased TLR4 protein levels, we have assessed the effect of testosterone on relative TLR4 protein content in permeabilized cells by flow cytometry. In these studies, 24 hour treatment of primary macrophages with 1 μM testosterone decreased TLR4-associated fluorescence in cells from 5 of 7 orchidectomized animals with an average decrease of 13.8% (fluorescence geometric means of 7.60 +/- 0.62 versus 6.55 +/- 0.58 in untreated and testosterone treated cells, respectively; $n = 5$, $p < 0.05$) indicating that testosterone induced decreases in cell surface TLR4 expression cannot be explained on the basis of receptor internalization.

To begin to assess the functional significance of testosterone-mediated decreases in TLR4 expression in primary macrophages, we have determined whether testosterone alters the sensitivity of these cells to LPS. Peritoneal macrophages were again isolated from orchidectomized and sham orchidectomized animals, and orchidectomized mice with testosterone replacement, and cells were cultured either in the presence or absence of 1 μM testosterone. At 24 hours post testosterone treatment, cells were challenged with LPS (250 ng/ml) and the production of the inflammatory cytokine, TNF-α, was assessed by specific capture ELISA. As shown in Figure 5, testosterone significantly decreased LPS-induced TNF-α production by cells derived from orchidectomized mice ($n = 16$ per group, $p < 0.05$, paired Student's t test) consistent with a decreased functional responsiveness of these cells to this TLR4 ligand. In agreement with the inability of testosterone to reduce TLR4 expression on cells derived from sham orchidectomized mice or orchidectomized mice that received testosterone replacement (Figure 4),

testosterone failed to elicit significant decreases in LPS sensitivity in these cells (Figure 5).

To confirm that testosterone suppresses cell surface TLR4 expression on these immune cells in vivo, we have examined TLR4 expression on monocytes/macrophages acutely isolated from orchidectomized, sham orchidectomized, and orchidectomized mice that received testosterone replacement. As shown in Figure 6A, orchidectomy elicits a significant decrease ($n = 16$ animals per group, $p < 0.05$) in animal body weight that is prevented by exogenous testosterone replacement as previously reported (Hooper et al. 1986). Peritoneal monocytes/macrophages from sham orchidectomized animals express very low levels of cell surface TLR4 (Figures 6B and C). Importantly, cells from orchidectomized mice express approximately 25% greater TLR4 expression ($n = 16$ animals per group, $p < 0.05$) than sham operated animals. This effect was largely abolished in cells derived from animals that received exogenous testosterone replacement (Figures 6B and C). These data indicate removal of endogenous testosterone, and hence elimination of its suppressive effects, elevates in vivo TLR4 expression on this key sentinel immune cell type.

To begin to test the physiological relevance of these changes in TLR4 expression, we have investigated the susceptibility of male mice to endotoxic shock following removal of endogenous testosterone. Orchidectomized, sham orchidectomized, and orchidectomized mice that received testosterone replacement, were challenged with a sub-lethal dose of LPS (5 mg/kg, i.p.). At 24 hours post-treatment the severity of endotoxic shock was assessed according to appearance and behavior using a scoring system modified from that employed by Liu and colleagues (2006). Animals were then

euthanized and sera isolated for inflammatory cytokine content. As shown in Figure 7, orchidectomized mice exhibited a markedly elevated susceptibility to endotoxin to that seen in sham treated animals with a close correlation between severity scores and sera levels of the inflammatory cytokine, IL-6 ($n = 6\text{-}7$ animals per group, $p < 0.05$). Importantly, this effect was abolished in animals that received exogenous testosterone replacement (Figures 7A and 7B). These data indicate removal of endogenous testosterone elevates susceptibility to endotoxic shock and is consistent with the observed in vivo increase in TLR4 expression on immune cells.

3.3 Conclusions

It has recently been recognized that sex based differences may influence host responses to infectious organisms. Examples include the observation that female deer have lower parasite loads than males and the finding that helminth infections are generally more severe in males than females (Zuk and McKean 1996, Poulin 1996). These phenomena appear to correlate with clinical and laboratory studies demonstrating that females generally exhibit greater adaptive immune responses following antigenic challenge than males (as reviewed in Marriott and Huet-Hudson 2006). Importantly, many of these differences become apparent at puberty (Lichtman et al. 1967, Grundbacher 1972) 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 Angele et al. 2000). Androgens have been shown to have suppressive effects on immune functions following trauma or trauma-hemorrhage and subsequent sepsis (Wichmann et al. 1996,

Angele et al. 1999, Angele et al 2000). Furthermore, male reproductive hormones have been shown to decrease immunoglobulin and cytokine production, and to limit lymphocyte proliferation (as reviewed in Angele et al. 2000 and Olsen and Kovacs 1996). These observations are consistent with the demonstration that exogenous testosterone administration increases female susceptibility to *M. marinum* infection, and castration attenuates such infections in male mice (Yamamoto et al. 1991). Recent studies suggest that macrophages, a key sentinel immune cell type, possess non-classical cell surface receptors for androgens (Liu et al. 1005) and that testosterone can decrease LPS-induced activation of transcription factors that regulate inflammatory responses (Benten et al. 2004). However, it is currently unclear how testosterone alters the immune responsiveness of macrophages.

In the present study we demonstrate that in vitro exposure to testosterone elicits significant decreases in the expression of cell surface TLR4 in a macrophage-like cell line and we have confirmed this effect in primary murine macrophages. Interestingly, we show that the ability of testosterone to decrease TLR4 expression on primary macrophages is only apparent on cells derived in the absence of endogenous gonadal androgens as cells isolated from sham orchidectomized animals or orchidectomized animals that receive testosterone replacement fail to demonstrate such sensitivity. This finding suggests that endogenous testosterone exerts sustained effects on cell surface TLR4 molecule expression and is characteristic of the long lasting actions of reproductive steroid hormones on cellular machinery. The testosterone-mediated decrease in cell surface TLR4 expression does not appear to be due to receptor internalization as significant decreases in TLR4 expression were also observed in permeabilized cells.

Furthermore, we have demonstrated the ability of endogenous testosterone to decrease TLR4 expression on immune cells in vivo by showing that levels of this microbial pattern recognition receptor on acutely isolated peritoneal monocytes/macrophages derived from orchidectomized mice are significantly higher than those seen on cells obtained from sham orchidectomized animals or orchidectomized animals that received testosterone replacement. Importantly, this elevation in TLR4 expression in vivo correlates with a dramatic increase in endotoxin susceptibility in orchidectomized animals. Taken together, the present findings demonstrate that the presence of testosterone in vitro or in vivo significantly decreases the cell surface expression of a critical receptor for microbial components and inflammatory signals liberated from injured tissues on an important sentinel immune cell type.

In our in vitro studies, acute administration of exogenous testosterone evoked maximal reductions of 7-10% in TLR4 expression on macrophages and one might be tempted to question the functional significance of such an effect. However, it is important to note that the number of these receptor molecules on the surface of immune cells is relatively low and so even modest changes in the level of expression may have marked effects on cellular responsiveness. In this study, we have begun to assess the functional relevancy of testosterone-mediated reductions in TLR4 expression by measuring inflammatory cytokine production elicited by a TLR4-specific ligand. We show that testosterone significantly decreases LPS-induced TNF-α production by primary macrophages generated in the absence of endogenous androgens and a macrophage-like cell line and, while we cannot rule out possible effects of testosterone on the signal transduction pathway, its effects on inflammatory cytokine production

correlate well with the observed changes in TLR4 expression. If these in vitro findings were reproduced in vivo, then a 10% difference in inflammatory mediator production would be anticipated to have profound effects on disease outcome. However, it is quite possible that our in vitro studies underestimate the chronic influence of testosterone on immune cell function. This notion is supported by our in vivo studies showing that the removal of endogenous testosterone elicits a more marked effect on TLR4 expression on in situ monocytes/macrophages that that seen in vitro. Importantly, this effect correlates with a profound increase in susceptibility to in vivo endotoxin challenge.

Our results are in agreement with a recent study demonstrating that the androgen, dihydrotestosterone can inhibit the expression of mRNA encoding TLR4 in human endothelial cells derived from neonatal tissue and can reduce LPS-mediated inflammatory mediator production by this cell type (Norata et al. 2006). In addition, the present in vivo studies also support the findings of Quintar and colleagues (2006) showing that prostate TLR4 protein expression is elevated in castrated rats. Furthermore, the present study could provide a mechanism underlying the ability of testosterone to down-regulate LPS-induced activation of the pro-inflammatory transcriptional regulator, p38 MAP kinase, and reduce nitric oxide production in macrophage-like cell lines (Benten et al. 2004). However, it is important to note that the cellular effects of reproductive hormones are complex and so ascribing specific roles to each hormone is fraught with peril. For example, while a number of studies indicate that testosterone alters inflammatory cytokine release by macrophages following bacterial endotoxin exposure (Angele et al. 1998a and 1998b), others have provided contrary evidence and this has led to the suggestion that male reproductive hormones only exert such effects in

immunocompromised hosts (Angele et al. 1999). As such, while it is apparent that the precise role of testosterone in the regulation of immune function remains contentious, the present demonstration that testosterone can modulate the expression of a key receptor for "danger signals" in vitro and in immune competent animals may represent an important mechanism underlying the immunosuppressive effects of this androgen.

3.4 Figures

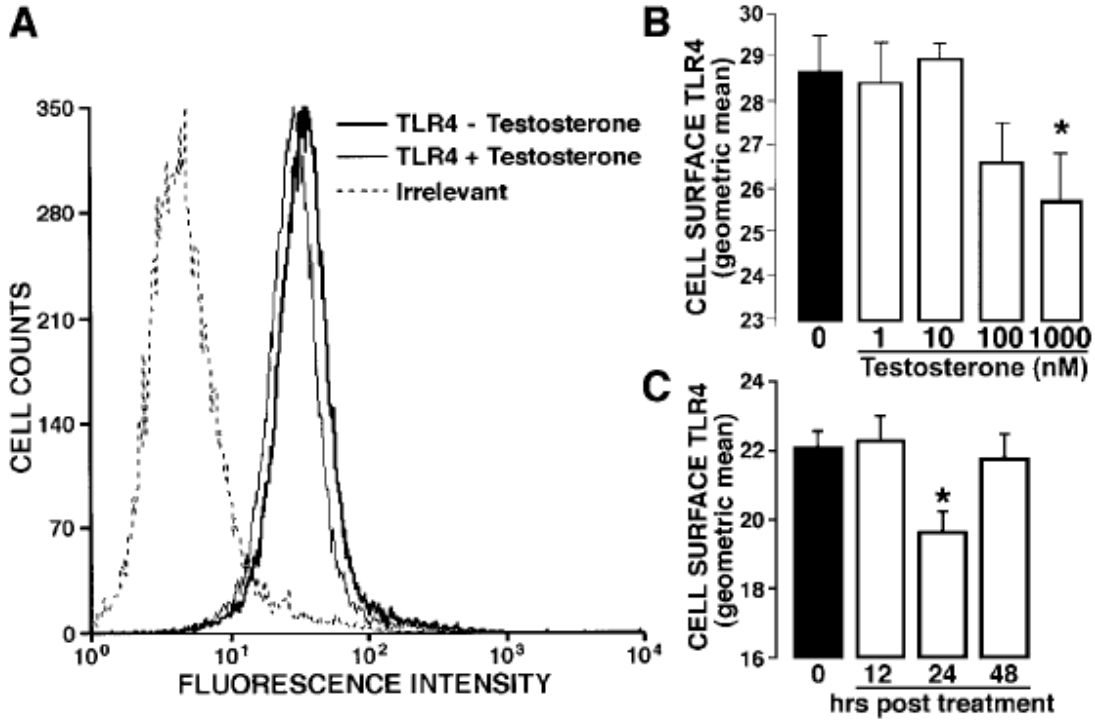

FIGURE 3: Testosterone decreases cell surface TLR4 expression on a macrophage-like cell line in a dose and time dependant manner as determined by flow cytometry. RAW 264.7 cells were cultured in 2% NuSerum-containing media to minimize exposure to reproductive hormones and were untreated (0) or exposed to varying concentration of testosterone for indicated times and analyzed for cell surface TLR4 by flow cytometry. Panel A is a representative experiment showing changes in TLR4 immunofluorescence following 24 hr exposure to 1 μM testosterone. Trace labeled Irrelevant indicates fluorescence obtained with a control antibody directed against an irrelevant antigen. Panel B shows the average immunofluorescence intensity (as geometric means) following exposure to increasing doses of testosterone (1-1000 nM). N = 10 per group, asterisk indicates significant difference from untreated cells. Panel C shows average immunofluorescence intensity (as geometric means) following exposed to testosterone (1 μM) for 12, 24 or 48 hrs. N = 16, asterisk indicates significant difference from untreated cells.

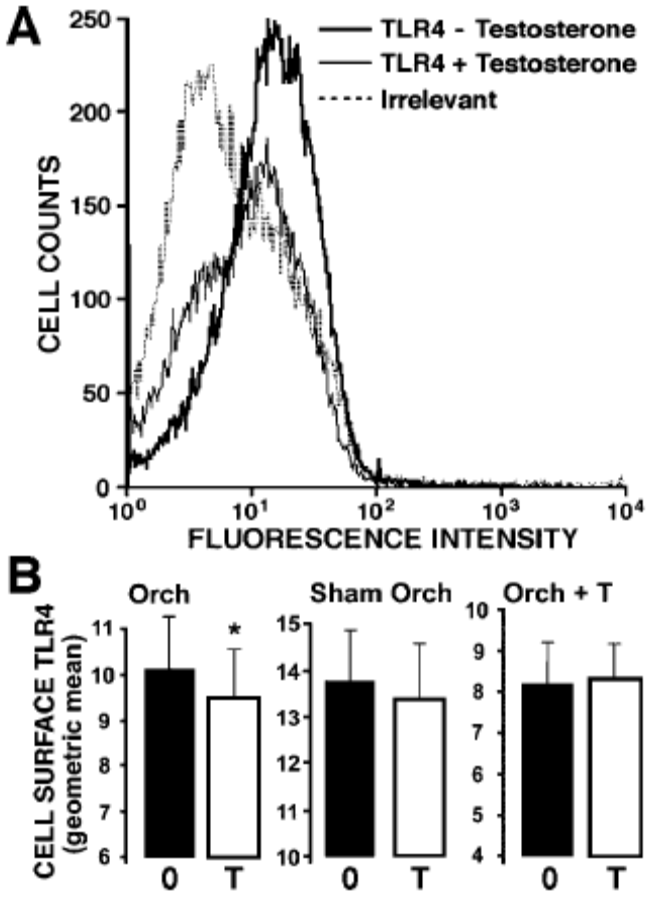

FIGURE 4: Testosterone decreases cell surface TLR4 expression on cultured primary macrophages derived from animals largely devoid of endogenous androgens. Macrophages were isolated from orchidectomized (Orch) or sham orchidectomized (Sham Orch) mice or animals that were orchidectomized and received s.c. injections of testosterone (Orch + T). Cells were untreated or exposed to testosterone (1 μM) for 24 hrs and analyzed for cell surface TLR4 by flow cytometry. Panel A is a representative experiment showing changes in TLR4 immunofluorescence on macrophages from an orchidectomized mouse following exposure to 1 μM testosterone. Trace labeled Irrelevant indicates fluorescence obtained with a control antibody directed against an irrelevant antigen. Panel B shows average TLR4 immunofluorescence intensities (as geometric means) following exposure to testosterone (T). N = 16 animals per group, asterisk indicates significant difference from untreated cells as determined by paired Student's t test.

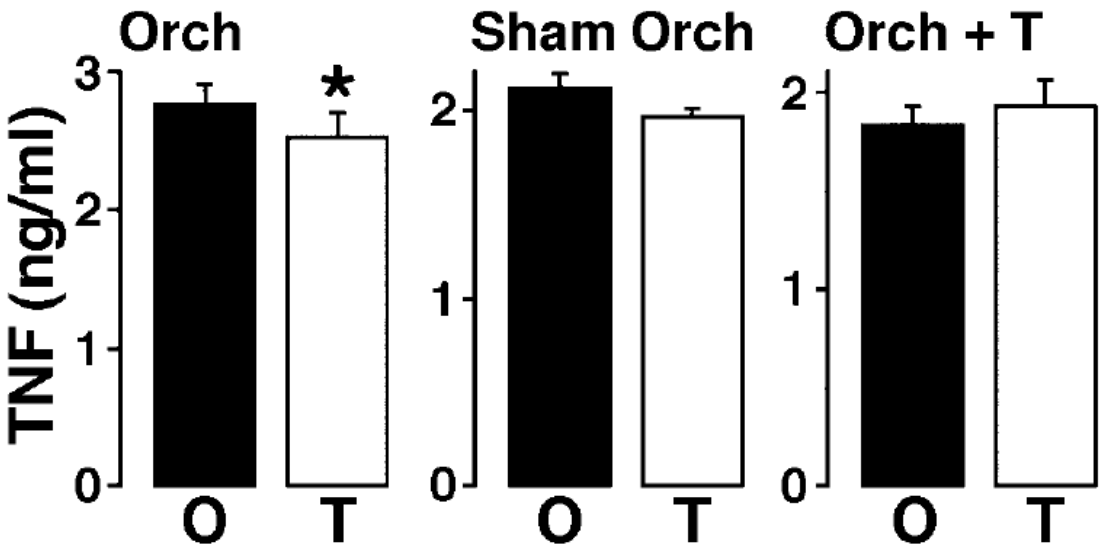

FIGURE 5: Testosterone decreases TLR4-mediated inflammatory cytokine production by cultured primary macrophages derived from animals largely devoid of endogenous androgens. Macrophages were isolated from orchidectomized (Orch) or sham orchidectomized (Sham Orch) mice or animals that were orchidectomized and received s.c. injections of testosterone (Orch + T). Cells were untreated (0) or exposed to testosterone (T; 1 μM) for 24 hrs. These cells were then challenged with LPS (250 ng/ml) for 12 hrs and culture supernatants were assayed for the presence of TNF-α by specific capture ELISA. N = 16 animals per group, asterisk indicates statistically significant difference from cells that were not exposed to testosterone in vitro as determined by paired Student's t test.

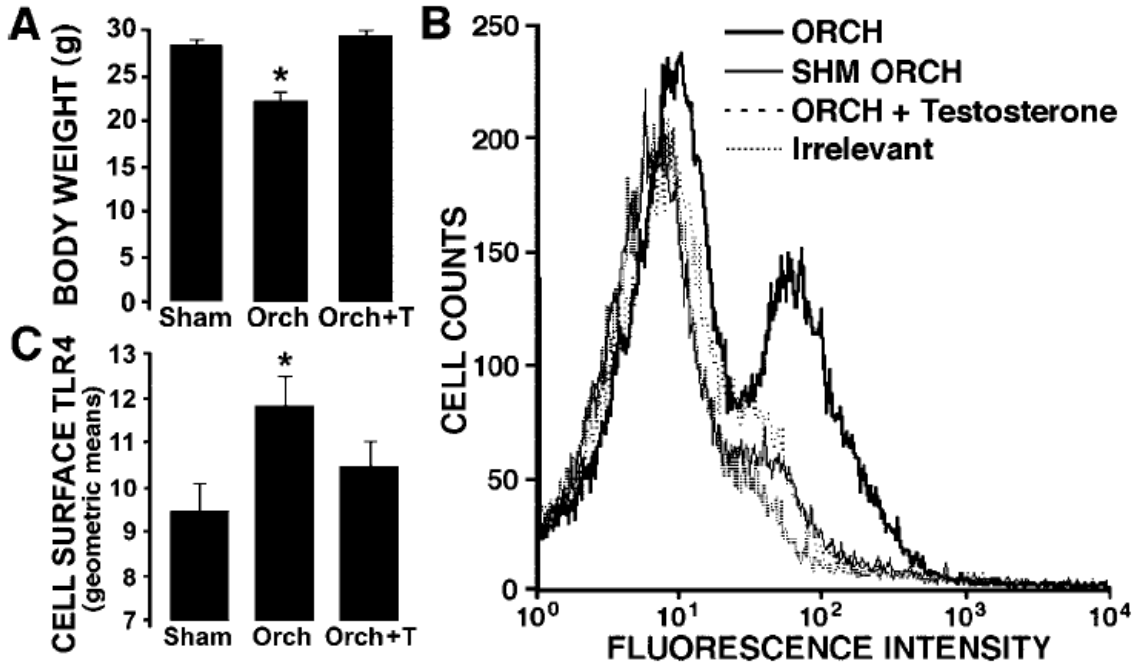

FIGURE 6: Endogenous testosterone decreases cell surface TLR4 expression on monocytes/macrophages in vivo. Elicited peritoneal monocytes/macrophages were acutely isolated from orchidectomized (Orch) or sham orchidectomized (Sham Orch) mice or animals that were orchidectomized and received s.c. injections of testosterone (Orch + Testosterone) and analyzed for cell surface TLR4 expression by flow cytometry. Panel A shows changes in body weight with each treatment regimen and indicates that testosterone replacement reverses the weight loss observed following orchidectomy. Panel B is a representative experiment showing changes in TLR4 immunofluorescence on monocytes/macrophages derived from each treatment group. Trace labeled Irrelevant indicates fluorescence obtained with a control antibody directed against an irrelevant antigen. Panel C shows the average immunofluorescence intensities (as geometric means) of TLR4 expression on cells from each treatment group. N = 16 animals per group, asterisk indicates significant difference from cells derived from sham orchectomized animals.

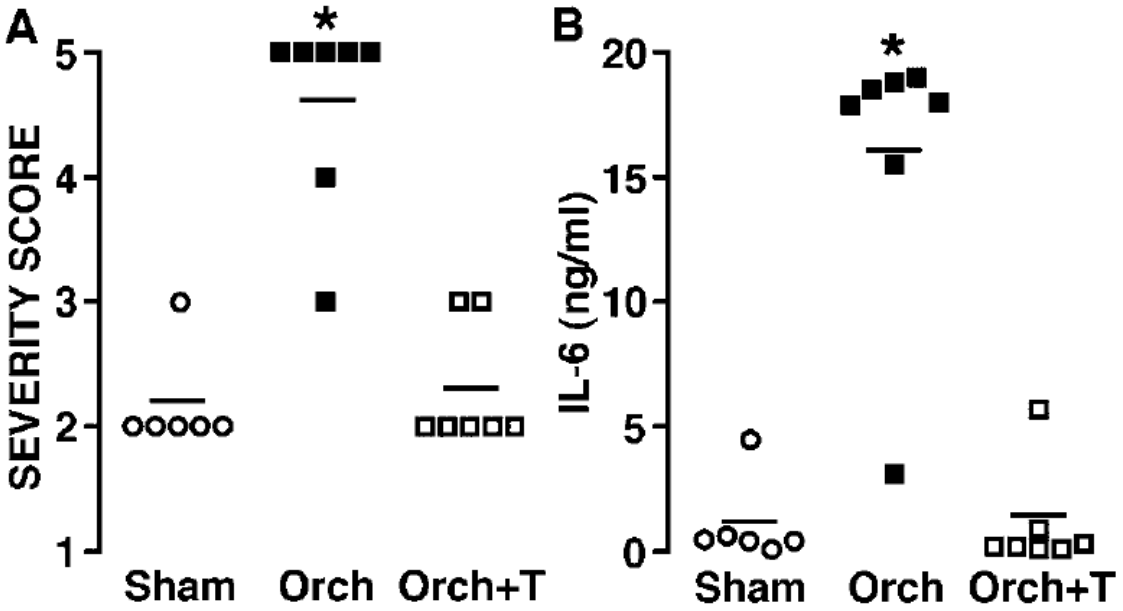

FIGURE 7: Removal of endogenous testosterone elevates susceptibility to endotoxic shock in vivo. Orchidectomized (Orch), sham orchidectomized (Sham), and orchidectomized mice that received testosterone replacement (Orch+T), were challenged with a sub-lethal dose of LPS (5 mg/kg, i.p.) for 24 hours. Panel A: severity of endotoxic shock assessed according to appearance and behavior and 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). Panel B: sera IL-6 content for each animal in the three treatment groups. Bars indicate group averages and asterisks indicate significant differences from sham orchectomized animals.

CHAPTER FOUR: ESTROGEN AUGMENTS CELL SURFACE TLR4 EXPRESSION ON MURINE MACROPHAGES AND REGULATES SEPSIS SUSCEPTIBILITY IN VIVO

4.1 Rationale

It has become increasingly apparent that sexual dimorphism exists in infectious disease susceptibility (as reviewed in Marriott and Huet-Hudson 2006). In general, females generate more robust and potentially protective humoral and cell-mediated immune responses following 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 (Zuk and McKean 1996, Poulin 1996). In addition, influenza infection elicits greater severity and hospitalization in male patients (Quach et al. 2003). Furthermore, this sexual dimorphism in susceptibility to infectious disease extends to cases of sepsis, where male patients exhibit a 70% mortality associated with sepsis, but females only show 26% associated mortality (Schroder et al. 1998). Interestingly, women have been found to have higher circulating levels of IgM than men (Butterworth et al. 1967) and this difference is most apparent at puberty (Lichtman et al. 1967, Grundbacher 1972) suggesting a role for reproductive hormones in the development of this male/female bias.

Estrogens have been found to promote increased resistance to streptococcal infections (Nicol et al. 1964) while androgens have been shown to suppress host immune responses (as reviewed in Angele et al. 2000 and Olsen and Kovacs 1996). Indeed, high

levels of estrogens can elevate immune responses to a point at which they are detrimental to the host. For example, estradiol or estriol treated rodents exhibit greater mortality following gonococcal infection (Kita et al. 1985) or endotoxin challenge (Ikejima et al. 1998), respectively, than untreated animals. Furthermore, it has been found that sera levels of 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 sex (Schroder et al. 1998, Fourrier et al. 1994, Dossett et al. 2008, May et al. 2008).

The mechanisms underlying innate immune responses to bacterial endotoxins are only now becoming apparent. As such, it is feasible that reproductive hormone-based differences in the level of expression of molecules that perceive bacterial motifs could lead to significant differences in the magnitude of inflammatory host responses following endotoxin exposure. In this study, we show that removal of endogenous estrogens decreases circulating levels of key inflammatory mediators following 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, we show that acute in vitro exposure to exogenous 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 supra-physiological levels of estradiol result 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.

4.2 Results

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 sub-lethal dose of LPS (5 mg/kg, i.p.). Sera levels of estradiol were decreased by 61.4% in ovariectomized animals. At 20 hrs after treatment the severity of endotoxic shock was assessed according to appearance and behavior using a scoring system modified from that employed by Liu et al. (2005). Animals were then euthanized and sera isolated for cytokine content by specific capture ELISAs. As shown in Figure 8, ovariectomized animals exhibited markedly lower LPS-induced sera levels of IL-6 (Figure 8A) and TNF-α (Figure 8B) than age matched intact females ($n = 6\text{-}7$ animals per group; $P < 0.05$). It is important to note that we did not observe significant differences in endotoxic shock severity scores between these groups (Figure 8C). However, this observation could be explained by our finding that sera levels of the anti-inflammatory cytokine, IL-10, were also significantly higher ($P < 0.05$) in sham ovariectomized animals than in gonadectomized mice (Figure 8D). Together, these data show that removal of endogenous estrogens results in reduced host cytokine responses to endotoxin challenge.

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 have determined the circulating levels of LBP in sera isolated from ovariectomized and sham gonadectomized animals. As shown in Figure 9A, circulating

levels of LBP were significantly lower in female mice following 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 have investigated the expression of pattern recognition receptors on acutely isolated macrophages from intact and gonadectomized female animals. As shown in Figure 9B, 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 (Figure 9C). Interestingly, this effect appears to be limited to TLR4, as the LPS co-receptor CD14 failed to demonstrate detectable differences in expression in the absence or presence of endogenous estrogen (Figure 9D). 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 if acute treatment of macrophages with estradiol can similarly elevate TLR4 expression on macrophages, we investigated the effects of exogenous estradiol on the macrophage-like cell line, RAW 264.7, and cultured primary macrophages derived from ovariectomized and sham ovariectomized animals. As shown in Figure 10A, 24 hr treatment with estradiol (1×10^{-8} M 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 to minimize exposure to reproductive hormones. Similarly, TLR4 expression was not altered on primary macrophages derived from either

ovariectomized or sham ovariectomized animals (Figure 10B). Consistent with the data presented in Figure 10C, 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 versus 9.06 +/- 0.17, respectively) ($P < 0.05$). Together, these data indicate that acute in vitro exposure to physiological levels of estradiol is not sufficient to alter cell surface TLR4 expression on macrophages.

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 following long term high level estradiol and/or progesterone replacement. Mice were ovariectomized or sham ovariectomized and a group of the gonadectomized animals received silastic implants containing either estradiol, progesterone, or one implant of each estradiol and progesterone. Such implants have been previously been shown to continually release hormone for at least 35 days (Cohen and Milligan 1993). In the present study, the implants were not depleted at 5 weeks following ovariectomy. After sacrifice, implants were removed and weighed, and the presence of hormone contents confirmed visually. On average, estradiol implant content weight decreased from 3.97 +/- 0.18 mg to 2.83 +/- 0.20 mg, and progesterone implant content weight decreased from 4.74 +/- 0.18 mg to 1.99 +/- 0.08 mg. Sera levels of estradiol were 390 +/- 42 pg/ml at the time of sacrifice in animals receiving estradiol implants, far in excess of the sera levels of estradiol in intact females (56 +/- 38 pg/ml). At sacrifice, 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 estradiol implants showed a significant increase in susceptibility to LPS challenge (5 mg/kg, i.p.) over that seen in either intact mice or ovariectomized animals that did not receive estradiol replacement. As shown in Figure 11A, ovariectomized animals receiving exogenous estradiol showed significantly higher inflammatory cytokine levels at 8 hrs following 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 estradiol and progesterone showed similar elevations in inflammatory cytokine levels ($n = 7\text{-}15$, $P<0.05$). 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 estradiol treatment group, as well as animals receiving both estradiol and progesterone, becoming moribund as rapidly as 8 hrs post-challenge (Figure 11B). While there was a trend for increased sera levels of the anti-inflammatory cytokine IL-10 in animals receiving estradiol treatment, this difference was not statistically significant.

Importantly, these differences cannot be attributed to treatment-induced changes in body weight. Prior to 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). While ovariectomized animals weighed significantly more than sham ovariectomized animals (20.6 +/- 0.2 versus 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 estradiol demonstrated a decrease in body weight following long-term ovariectomy. While 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 (Bryson and Bischoff 1979, Game et al. 2008).

To investigate the mechanisms underlying the increased susceptibility of mice receiving high levels of exogenous estradiol to endotoxin challenge, we have assessed the relative expression of critical innate immune receptors for LPS in these animals. Mice were ovariectomized, sham ovariectomized, or ovariectomized and given silastic implants containing estradiol, progesterone, or both estradiol and progesterone prior to isolation of sera and peritoneal macrophages. As shown in Figure 12A, circulating levels of LBP were significantly higher in gonadectomized mice receiving exogenous 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 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 (Figure 12B). Importantly, the increased ability of macrophages to bind LPS was mirrored by significant increases in the cell surface expression of CD14 (Figure 12C) and TLR4 (Figure 12D) 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 (Figure 12E). Rather, these differences were due to a reduction in the relative proportion of these receptors on the cell surface.

An ability of exogenous 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 Figure 12, the presence of progesterone-containing implants failed to augment sera levels of LBP (Figure 12A), levels of LPS binding to macrophages (Figure 12B), cell surface levels of CD14 on these cells (Figure 12C), or either cell surface TLR4 expression or total TLR4 content in macrophages (Figures 12D and 12E, respectively). Similarly, co-administration of progesterone with estradiol failed to significantly effect expression of most of these parameters compared to estradiol treatment alone (Figure 12). While co-administration of progesterone with estradiol did significantly increase LPS binding to acutely isolated macrophages (Figure 12B), it is important to note that no significant changes in cellular CD14 or TLR4 expression were observed and so the mechanisms underlying this effect are unclear.

4.3 Conclusions

It has recently been recognized that sex based differences may influence host responses to infectious organisms. In general, females generate more robust and

potentially protective humoral and cell-mediated immune responses following antigenic challenge than their male counterparts (as reviewed in Marriott and Huet-Hudson 2006). Studies have found that females generate greater immune responses and exhibit less hospitalization associated with viral infections (Quach et al. 2003, Knoblich et al. 1983, Villacres et al. 2004). Furthermore, males have been found to have more severe parasitic infections than their female counterparts (Zuk and McKean 1996, Poulin 1996). Importantly, many of these differences become apparent at puberty (Lichtman et al. 1967, Grundbacher 1972) 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 Angele et al. 2000). Estrogens have been demonstrated to increase resistance to streptococcal infections (Nicol et al. 1964) while the removal of endogenous estrogens have been shown to markedly increase the severity of M. avium infections, an effect that can be reversed following estradiol replacement (Tsuyuguchi et al. 2001). 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 following endotoxin challenge. While circulating levels of the inflammatory cytokines, IL-6 and TNF-α, are decreased following estrogen removal, levels of the anti-inflammatory 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 sequelae associated with excessive

pro-inflammatory cytokine production and protect against endotoxemia (Londono et al. 2008, Emmanuilidis et al. 2001, van der Poll et al. 1995). IL-10 deficient mice exhibit increased bacteriemia, increased inflammatory TNF-α secretion, and increased mortality associated with bacterial infection (Londono et al. 2008).

Importantly, this decreased cytokine response to endotoxin administration following 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 following LPS challenge as compared to intact females (Soucy et al. 2005). As such, the reduced expression of critical molecules used in the recognition of LPS, provides a potential mechanism underlying diminished in vivo cytokine responses following 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 have previously 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. In this study, we have assessed the acute in vitro effects of 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 (Ikejima et al. 1998), or estradiol treatment of macrophage-like cell lines (Vegato et al. 2004, Vlotides et al. 2007) or LPS challenged human monocytes (Pioli et al. 2007), does not significantly alter TLR4 or CD14 expression. The actions of endogenous in vivo 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.

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 have assessed the effects of exogenous estradiol replacement on the level of expression of innate receptors for LPS. We demonstrated that supra-physiological levels of estradiol following 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 (Ikejima et al. 1998).

While estradiol treatment resulted in increased TNF-α secretion and greater susceptibility to endotoxin, it did not result in significantly higher sera levels of the anti-

inflammatory cytokine, IL-10. Since IL-10 provides protection against sepsis (Londono et al. 2008, Emmanuilidis et al. 2001, van der Poll et al. 1995), lower levels of IL-10 secretion would be expected to correlate with increased susceptibility to endotoxin challenge. Consistent with this notion, pregnant mice which have high circulating estrogen levels demonstrate increased TNF-α expression, suppressed IL-10 levels, and increased mortality following LPS challenge (Vizi et al. 2001).

Our findings indicate that 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 estradiol treatment (Calippe et al. 2008). As such, it appears that 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.

It is interesting to note that macrophages isolated from animals that received replacement of both estradiol and progesterone demonstrated higher total LPS binding capacity than those that received estradiol alone without significantly different levels of TLR4 or CD14 expression. As such, we suggest that progesterone in concert with high levels of 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 to their ovariectomized counterparts. However, such immune responses do not render these mice more susceptible to endotoxic shock, perhaps due to a concurrent increase in anti-inflammatory cytokine levels. In contrast, animals that have supra-physiological levels of estrogens demonstrate sensitized innate immune cells resulting in a more rapid and elevated inflammatory response following 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 non-surviving sepsis patients, regardless of sex (Dossett et al. 2009, May et al. 2008).

4.4 Figures

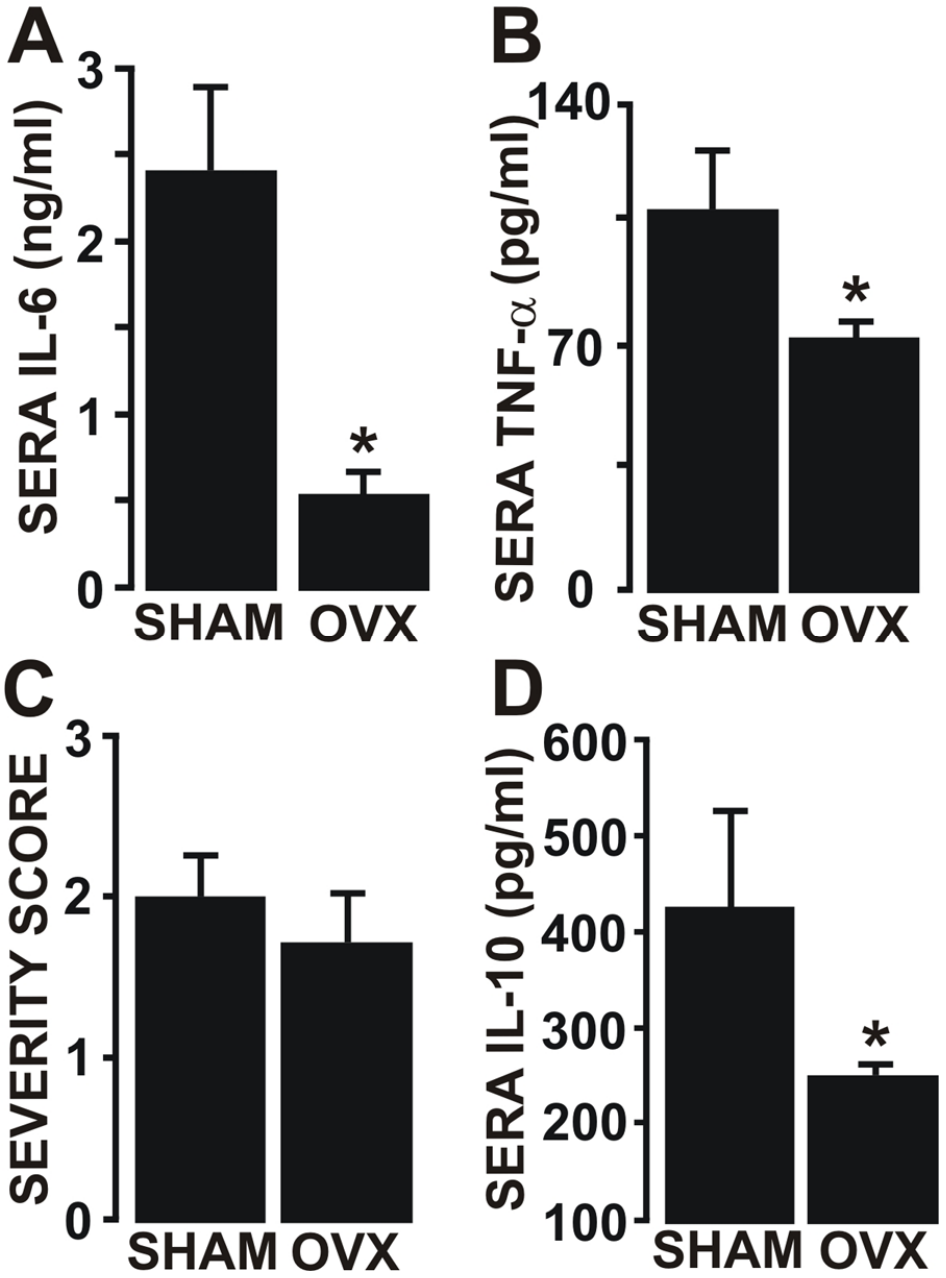

FIGURE 8: Removal of endogenous estrogen reduces circulating levels of cytokines resulting from in vivo endotoxin challenge. Mice were sham ovariectomized (SHAM: n = 6) or ovariectomized (OVX: n = 7) and received an i.p. challenge with LPS (5 mg/kg). At 20 hrs post challenge, sera was isolated and assayed for the presence of IL-6 (Panel A), TNF-α (Panel B), or IL-10 (Panel D) by specific capture ELISA. Panel C shows severity of endotoxic shock assessed according to appearance and behavior 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). Asterisk indicates significant difference from sham ovariectomized animals.

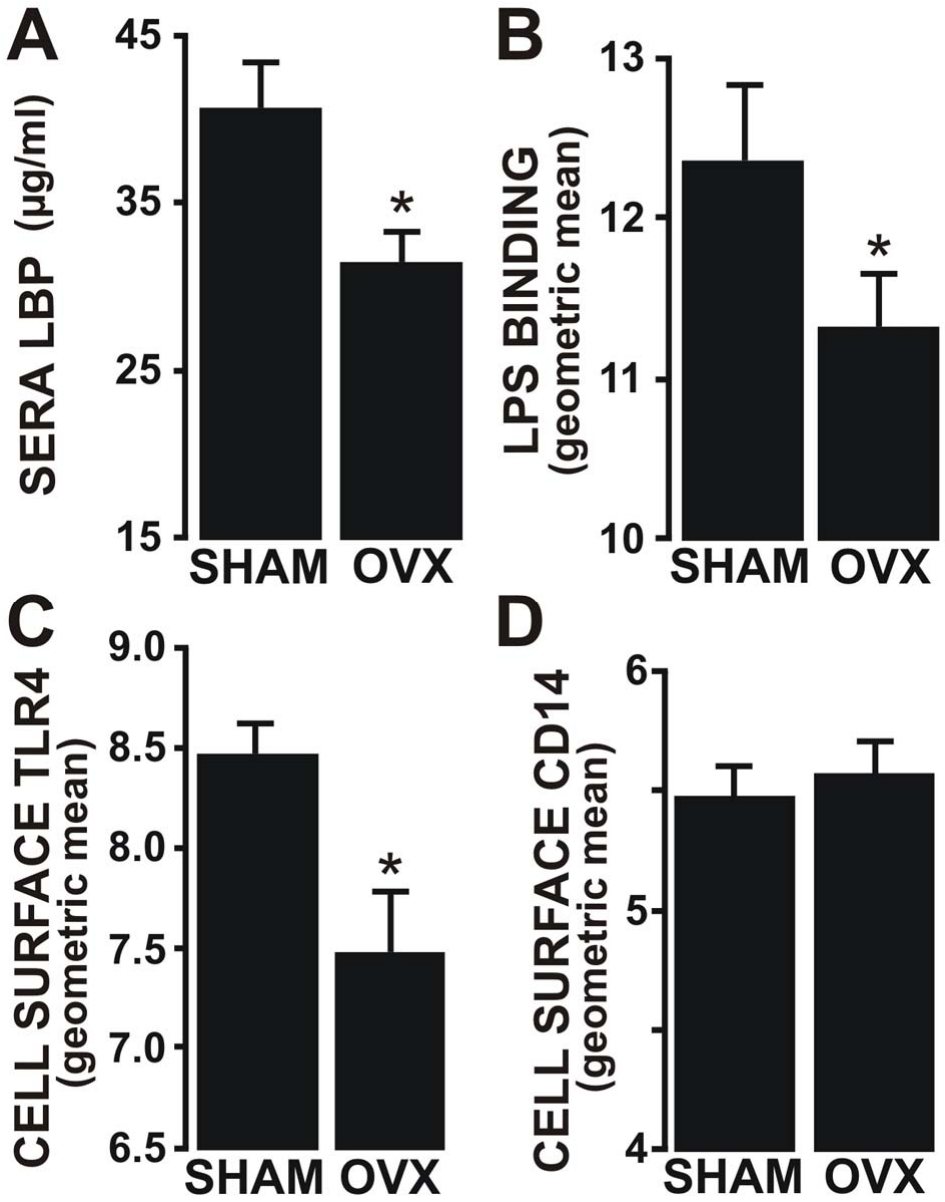

FIGURE 9: Removal of endogenous estrogen decreases expression of key molecules necessary for the innate immune recognition of LPS. Mice were sham ovariectomized (SHAM) or ovariectomized (OVX) five weeks prior to sera collection and isolation of peritoneal monocytes/macrophages. Sera levels of LBP were assessed by specific capture ELISA (Panel A: SHAM n = 8, OVX n = 7). Total LPS binding (Panel B: SHAM n = 7, OVX n = 8), and cell surface TLR4 (Panel C: SHAM n = 11, OVX n = 12) and CD14 (Panel D: SHAM n = 7, OVX n = 8) expression on isolated macrophages was determined by flow cytometry. Data is shown as the average fluorescence intensity (as geometric means) for each group. Asterisk indicates significant difference from sham ovariectomized animals.

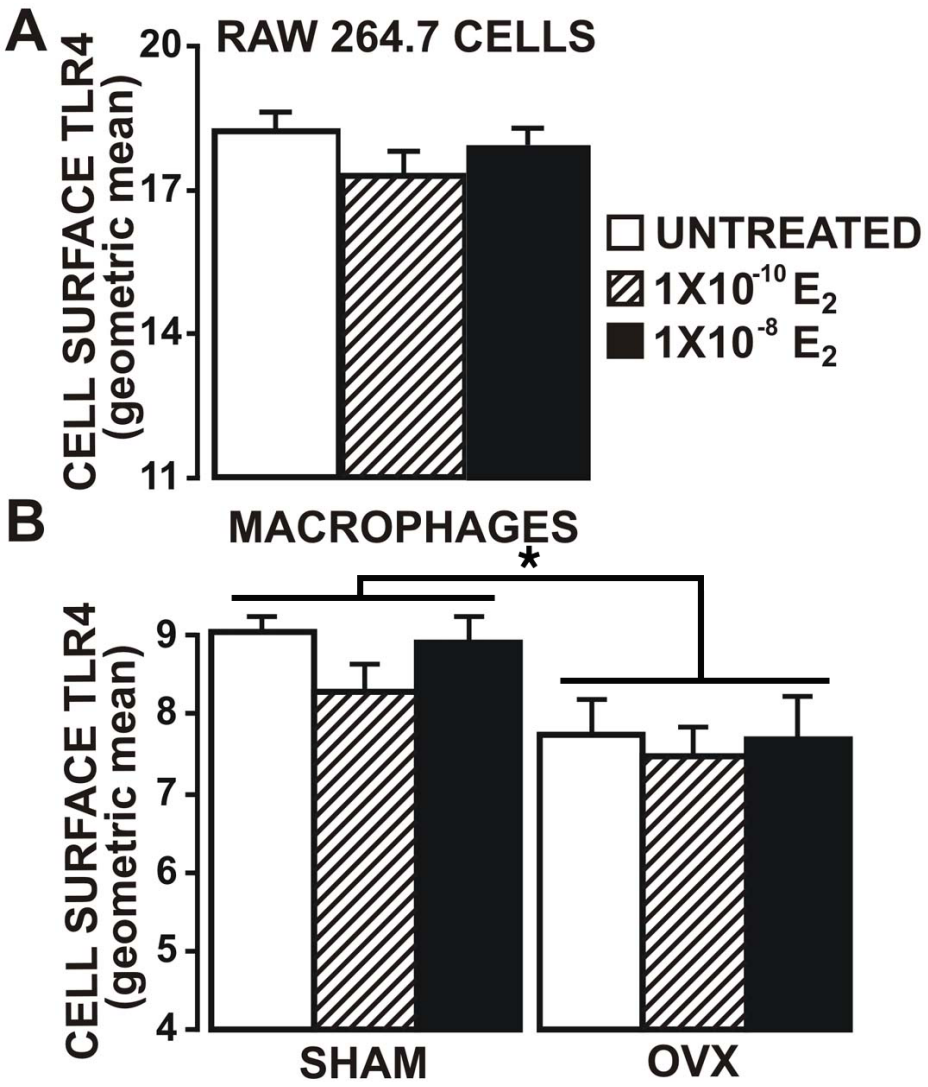

FIGURE 10: Acute in vitro exposure to exogenous estradiol fails to augment cell surface TLR4 expression on a macrophage-like cell line and primary macrophages. Panel A: RAW 264.7 cells were untreated or exposed to estradiol (E_2: 1 X 10^{-10} M or 1 X 10^{-8} M) for 24 hours prior to analysis of cell surface TLR4 expression by flow cytometry (n = 18). Panel B: peritoneal macrophages derived from sham ovariectomized (SHAM) or ovariectomized (OVX) mice (n = 8 animals in each group) were untreated or exposed to estradiol (E_2: 1 X 10^{-10} M or 1 X 10^{-8} M) for 24 hours prior to analysis of cell surface TLR4 expression by flow cytometry. Data is shown as the average fluorescence intensity (as geometric means) for each group. Asterisk indicates a significant difference between the average of all ovariectomized mice and the average of all intact animals.

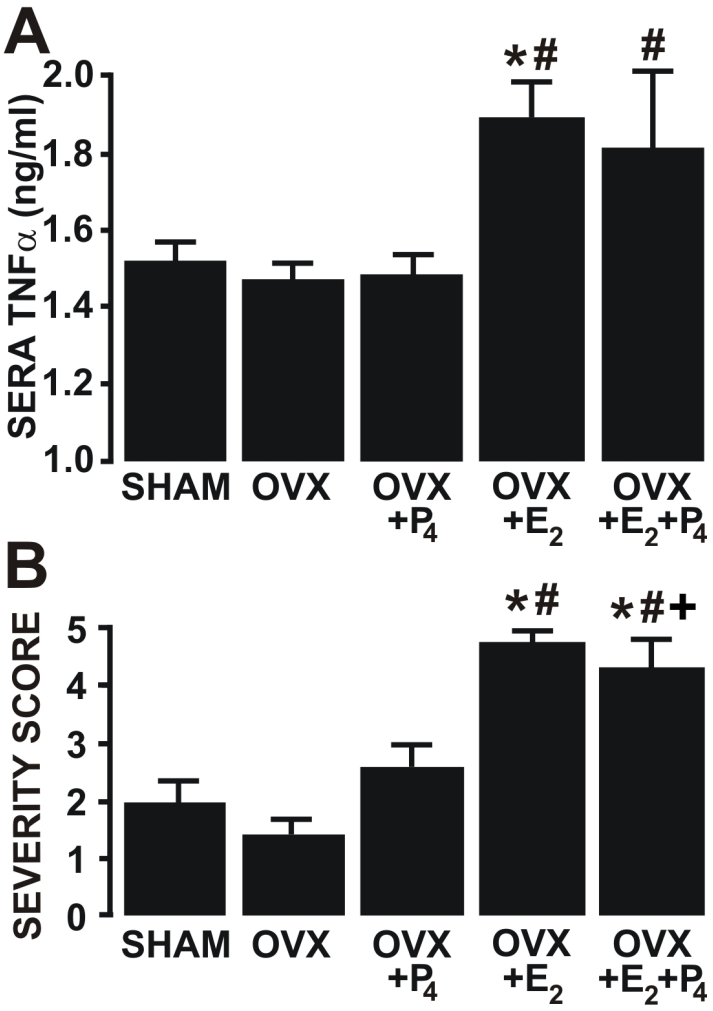

FIGURE 11: Administration of high levels of exogenous estradiol renders females more susceptible to endotoxic shock. Mice were sham ovariectomized (SHAM: n = 15), ovariectomized (OVX: n = 15), ovariectomized and received exogenous progesterone replacement (OVX+P_4: n = 7), ovariectomized and received exogenous estradiol replacement (OVX+E_2: n = 15), and ovariectomized and received exogenous estradiol and progesterone replacement (OVX+E_2+P_4: n = 7), for five weeks prior to LPS challenge (5 mg/kg i.p.). At 8 hrs post-challenge, sera was isolated and assayed for the presence of TNF-α (Panel 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 (Panel B). Severity was scored from 1 (no detectable behavioral differences) to 5 (moribund). Asterisk indicates significant difference from sham ovariectomized animals, pound symbol indicates significant difference from ovariectomized animals that did not receive estrogen replacement, and plus symbol indicates significant difference between progesterone treated animals in the absence or presence of estradiol co-treatment. All data was analyzed statistically by one-way ANOVA with Tukey's post-hoc analysis.

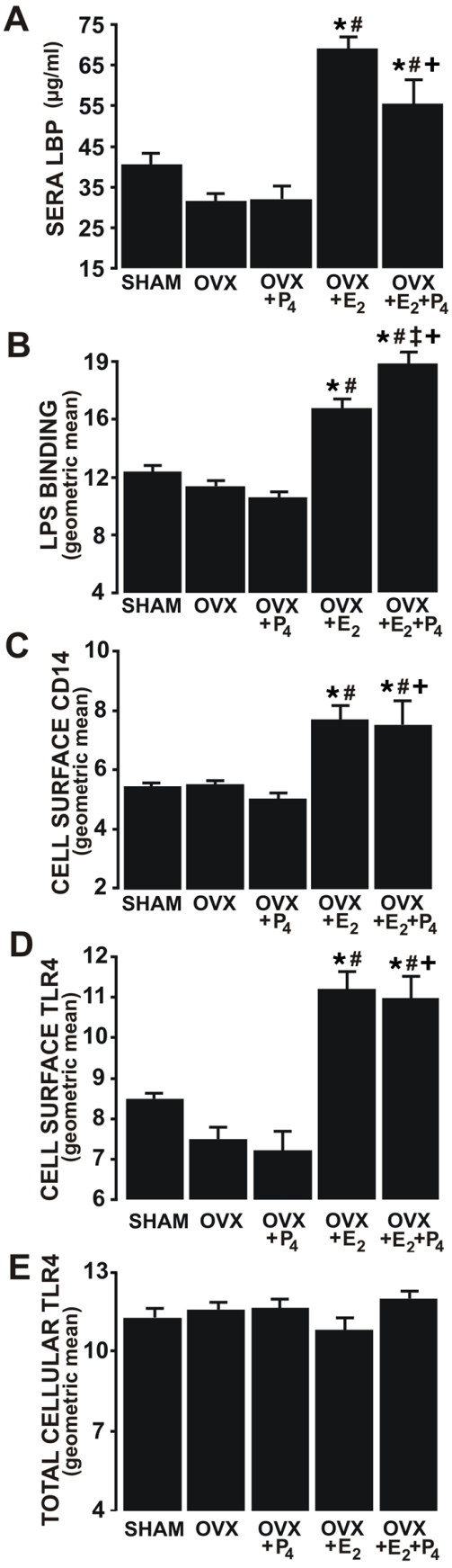

FIGURE 12: Administration of exogenous estradiol elevates cell surface expression of pattern recognition receptors for LPS on murine macrophages. Mice were sham ovariectomized (SHAM), ovariectomized (OVX), or ovariectomized with estradiol (OVX+E_2) or progesterone (OVX+P_4) replacement, or received replacement of both hormones (OVX+E_2+P_4), prior to sera collection and isolation of peritoneal monocytes/macrophages. Sera levels of LBP were assessed by specific capture ELISA (Panel A; n = 3-8). Total LPS binding (Panel B; n = 4-8), and cell surface CD14 (Panel C; n = 4-8) and TLR4 (Panel D; n = 7-12) expression on isolated macrophages was determined by flow cytometry. In Panel E, peritoneal macrophages were permeabilized and total cellular content of TLR4 was assessed by flow cytometry (n = 4-8). Data is shown as the average fluorescence intensity (as geometric means) for each group. Asterisk indicates significant difference from sham ovariectomized animals, pound symbol indicates significant difference from ovariectomized animals, double dagger indicates significant difference between estradiol treated animals in the absence or presence of progesterone co-treatment, and plus symbol indicates significant difference between progesterone treated animals in the absence or presence of estradiol co-treatment. All data was analyzed statistically by one-way ANOVA with Tukey's post-hoc analysis.

CHAPTER FIVE: LONG-TERM BIPHASIC EFFECTS OF ESTROGEN EXPOSURE ON MURINE BONE MARROW-DERIVED MACROPHAGE TLR4 EXPRESSION

5.1 Rationale

Evidence is accumulating that a sexual dimorphism exists in susceptibility to infectious disease. In general, females generate more robust and potentially protective humoral and cell-mediated immune responses following antigenic challenge than their male counterparts. This difference in immune responses has generally been assumed to be a consequence of the actions of reproductive hormones. Androgens have been shown to have suppressive effects on immune functions following trauma or trauma-hemorrhage and subsequent sepsis (as reviewed in Marriott and Huet-Hudson 2006). The role of estrogens, however, appears to be more complex. Numerous studies have shown that estrogens augment immune responses and have been demonstrated to increase resistance to infection. For example, estrogens have been found to be protective with treatment resulting in increased resistance to streptococcal infections (Nicol et al. 1964). However, some groups have shown that estrogens can exacerbate the symptoms of microbial challenge. Estrogen treated rodents exhibit greater mortality following gonococcal infection (Kita et al. 1985) or endotoxin challenge (Ikejima et al. 1998) than untreated animals. As such, the mechanisms by which estrogens can exert immunoprotective effects continue to be debated.

Reproductive hormones have been found to modulate the immune responsiveness of macrophages. Our lab has shown that testosterone suppresses the expression of a key

microbial pattern recognition receptor TLR4, consistent with the immunosuppressive actions of testosterone. Furthermore, we have recently discovered that the presence of high levels of estradiol in vivo increases cell-surface TLR4 expression on macrophages, while 24 hours in vitro exposure to this female reproductive hormone fails to elicit such effects. However, the mechanism by which estradiol augments TLR4 expression remains unclear.

In this study, we have addressed the hypothesis that estradiol increases TLR4 expression during macrophage development and maturation. We show that bone marrow-derived macrophages isolated from ovariectomized animals that received high dose estradiol treatment in vivo have significantly less cell surface TLR4 expression than those derived from sham treated mice or untreated gonadectomized animals following short-term culture. However, longer-term culture of these cells resulted in an apparent reversal of this pattern with restoration of TLR4 expression on bone marrow-derived macrophages isolated from estradiol treated animals and reduction in the expression of this pattern recognition receptor on cells from untreated gonadectomized mice in a manner that is consistent with our previous observations in acutely isolated murine peritoneal macrophages.

5.2 Results

To begin to examine the effects of estrogen on bone marrow-derived macrophages, myeloid precursors were isolated from the bone marrow of ovariectomized or sham ovariectomized mice or animals that were ovariectomized and received implants of estradiol. Adherent myeloid precursors were cultured and induced to differentiate into

CD11b+ cells as confirmed by staining with a fluorescent antibody against CD11b and analysis by flow cytometry. Bone marrow-derived macrophages were stained for TLR4 and examined via flow cytometry. As shown in Figure 13A, bone marrow-derived macrophages isolated from ovariectomized animals receiving high levels of estrogen replacement had significantly lower expression of cell-surface TLR4 expression following eight days in culture ($n = 10\text{-}23$ per group, $p < 0.05$, ANOVA) than either macrophages obtained from intact females or from ovariectomized mice. To determine whether the reduction in cell-surface TLR4 expression was due to decreased total protein, we have assessed the relative TLR4 protein content in permeabilized cells by flow cytometry. As shown in Figure 13B, total TLR4 expression is not significantly altered due to the presence or absence of in vivo estrogen during culture of bone marrow-derived macrophages ($n = 10\text{-}15$).

To determine the long term effects of estrogen on myeloid precursors, bone marrow-derived macrophages were co-cultured with varying doses of estradiol and assayed for cell surface TLR4 expression following long-term (sixteen days) exposure. As seen in Figure 14, neither low physiological dose (10^{-10}M estradiol) nor high physiological dose (10^{-8}M estradiol) altered cell surface TLR4 expression on bone marrow-derived macrophages from ovariectomized females, intact females, or ovariectomized mice that received estradiol implants ($n = 4$). However, following sixteen days in culture, bone marrow-derived macrophages from ovariectomized mice express significantly lower levels of cell surface TLR4 than cells from their sham operated counterparts (Figure 15). Furthermore, we found that bone marrow-derived macrophages from ovariectomized mice receiving high doses of in vivo estradiol express significantly

higher levels of cell surface TLR4 than cells from either of the other two groups (Figure 15), contrary to what is seen following short-term (eight days) culture.

5.3 Conclusions

Numerous studies have shown that estrogens augment immune responses and have been demonstrated to increase resistance to infection. For example, estrogens have been found to be protective with exposure resulting in increased resistance to streptococcal infections (Nicol et al. 1964). However, the mechanisms by which estrogens can exert immunoprotective effects continue to be debated. Previous studies in our lab have shown that the absence of endogenous estrogens results in a significant decrease in cell surface, but not total, TLR4 expression on peritoneal macrophages, while supra-physiological levels of exogenous estradiol replacement elicits a significant increase in cell surface TLR4 on these cells above what is seen in cells derived from normal cycling females. As these effects are seen only in vivo and not when macrophages are treated with estradiol in vitro for 24 hours, one hypothesis is that estradiol requires long-term exposure to exert its effects on cell surface TLR4 expression, as might be envisaged to occur during macrophage development.

In this present study, we demonstrate that CD11b+ bone marrow-derived macrophages from ovariectomized animals receiving estradiol replacement exhibit decreased expression of cell-surface TLR4 following 8 days in culture. This reduction is approximately 20% lower than TLR4 levels on cells derived from intact and ovariectomized animals. These results are not consistent with our previous studies showing in vivo estrogen exposure elevates cell-surface expression of TLR4 on

peritoneal macrophages. Importantly, bone marrow-derived macrophages from ovariectomized animals receiving estradiol replacement did not exhibit changes in total protein levels of TLR4 as compared cells derived from their ovariectomized or intact counterparts. As our previous studies also found no difference in total macrophage TLR4 protein expression, this indicates that, despite conflicting effects of estradiol on cell surface TLR4 expression, in vivo estradiol replacement does not affect the total cellular production of TLR4 protein. Other groups have also failed to detect differences in total cellular TLR4 protein levels following in vivo exposure to estrogens (Ikejima et al. 1998, Calippe et al. 2008), indicating that estradiol exposure exerts its effects only on the proportion of TLR4 on the surface of macrophages by an as of yet undetermined manner.

We show that long-term two week culture of myeloid precursors resulted in an apparent reversal of the pattern shown following one week of culture. Bone marrow-derived macrophages isolated from estradiol treated animals exhibit restored levels of cell-surface TLR4 that are significantly higher than levels observed from intact females. Furthermore, cells isolated from ovariectomized mice exhibited significantly lower levels of cell-surface TLR4 than cells isolated from either intact of estradiol treated animals. This finding is consistent with our previous observations of cell-surface TLR4 expression from peritoneal macrophages. Interestingly, in vitro treatment of bone-marrow derived cells with varying doses of estradiol during this two week period failed to mimic these effects. The temporal pattern shift of TLR4 expression of bone marrow-derived macrophage occurred in the absence of any apparent stimulus other than long-term culture and development of cells. These findings suggest that estradiol may exert effects in vivo prior to macrophage maturation.

Taken together, our previous studies have shown that in vivo exposure to estradiol elevates cell surface expression of a critical microbial pattern recognition receptor on a key sentinel immune cell type. However, these studies demonstrate that long term in vitro treatment of estradiol during macrophage development fails to mimic such effects. Importantly, the present study shows that while myeloid precursors derived from animals with supra-physiological levels of estradiol initially have lower cell-surface TLR4 expression, bone marrow-derived macrophages from these mice exhibit significantly higher levels of TLR4 following two week culture, similar to that seen in mature isolated peritoneal macrophages. As such, these results suggest that the in vivo effects of long-term estradiol on TLR4 expression occur prior to macrophage development. Supra-physiological levels of estradiol in vivo can exert biphasic and sustained effects on cell-surface TLR4 expression during maturation of this key immune sentinel cell.

5.4 Figures

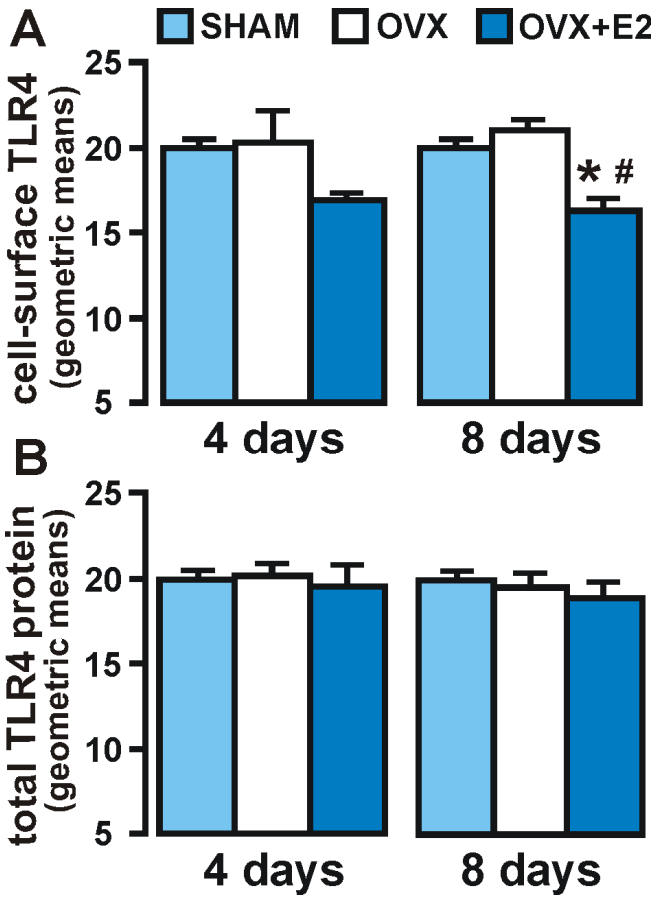

FIGURE 13: Supra-physiological levels of in vivo estradiol decreases cell surface TLR4 expression but not total TLR4 protein in bone marrow-derived macrophages after 8 days in culture. Bone marrow cells were isolated from ovariectomized (OVX) or sham ovariectomized (SHAM) mice or animals that were ovariectomized and received implants of estradiol (OVX+E2) and induced to differentiate into CD11b+ cells. Cells were analyzed for TLR4 expression by flow cytometry. Cell surface TLR4 expression (panel A) and total TLR4 protein (panel B) are shown for bone marrow-derived macrophages after 4-8 days in culture (N = 10-23). * indicates significant difference from cells derived from sham ovariectomized animals, and # indicates significant difference from cells derived from ovariectomized animals.

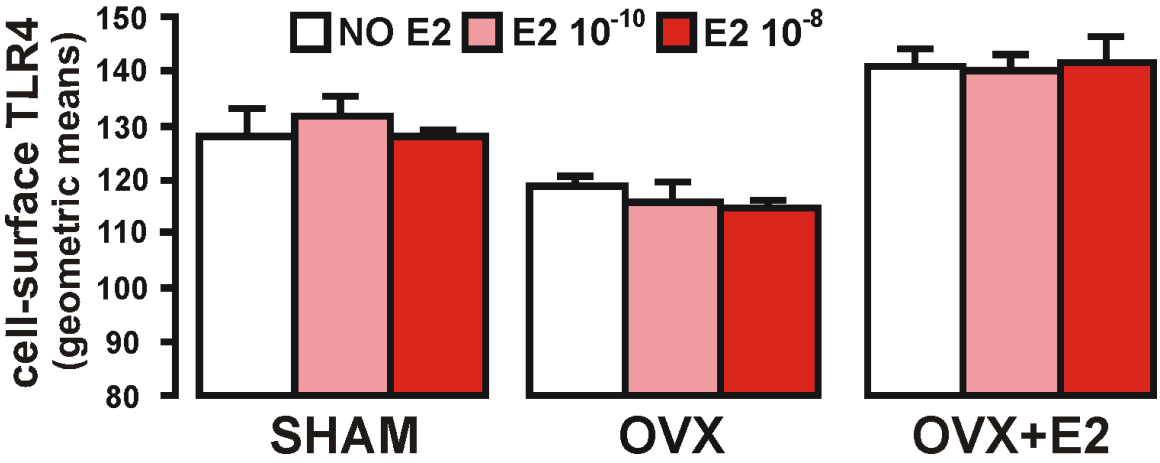

FIGURE 14: Long-term (16 day) in vitro estradiol treatment fails to elicit changes in TLR4 expression on bone marrow-derived macrophages. Bone marrow cells were isolated from sham ovariectomized (SHAM), ovariectomized (OVX), or ovariectomized with estrogen replacement (OVX+E2) and induced to differentiate into CD11b+ cells. Cells were exposed to estradiol (10^{-10}M E2 and 10^{-8}M E2) for two weeks, and then analyzed for TLR4 expression by flow cytometry (N = 4 animals).

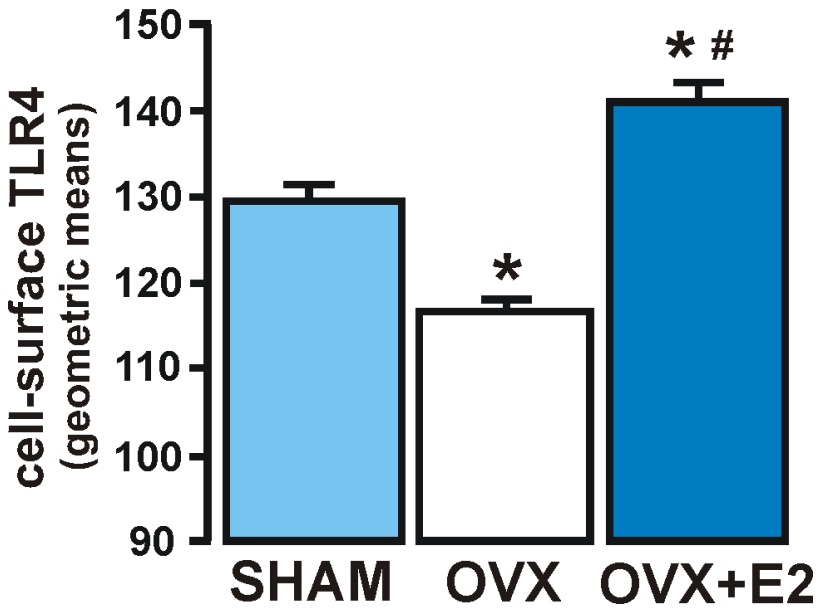

FIGURE 15: Supra-physiological levels of in vivo estradiol exert sustained effects on cell-surface TLR4 expression of macrophages during 16 days of culture. Bone marrow cells were isolated from sham ovariectomized (SHAM), ovariectomized (OVX), or ovariectomized with estrogen replacement (OVX+E2) and induced to differentiate into CD11b+ cells. Cells were analyzed for TLR4 expression by flow cytometry after 16 days in culture (data pooled from Figure 14). * indicates significant difference from cells derived from sham ovariectomized animals, and # indicates significant difference from cells derived from ovariectomized animals.

CHAPTER SIX: GPR30/GPER-1 MEDIATES RAPID DECREASES IN TLR4 EXPRESSION ON MURINE MACROPHAGES

6.1 Rational

It has become increasingly apparent that sexual dimorphism exists in susceptibility to infectious diseases (as reviewed in Marriott and Huet-Hudson 2006). In general, females generate more robust and potentially protective immune responses following antigenic challenge than their male counterparts. Furthermore, this sexual dimorphism extends to bacterial sepsis, where male patients exhibit 70% mortality while it is lethal in only 26% of females (Schroder et al. 1998). Such differences in immunity have generally been assumed to be a consequence of the actions of reproductive hormones. Consistent with such a hypothesis, estrogens have been found to promote increased resistance to streptococcal infections (Nicol et al. 1964) while we have shown that androgens suppress acute host immune responses to bacterial endotoxin challenge. However, ascribing precise roles in immune responses to estrogens has proven challenging as the literature is rife with apparently contradictory observations demonstrating that estrogens can both augment and limit host immunity.

While the ability of estrogens to evoke opposite effects on immune function may depend on the level of these sex hormones and/or the acute or chronic nature of the host response, an alternative explanation may lie in the expression of both classical and non-classical estrogen receptors by immune cell types. G protein-coupled receptor 30 (GPR30; also known as G protein-linked estrogen receptor 1) is a membrane-bound

molecule that has been found to mediate several rapid cellular effects of estrogen including the activation of the MAP kinase signaling cascade, cAMP activation, and intracellular calcium mobilization (Filardo et al. 2002, Filardo et al. 2007). As such, it has been suggested that GPR30 is a non-classical receptor for estrogen although this notion remains controversial (as discussed in Langer et al. 2010). GPR30 has been found to be expressed by a wide variety of tissues and cell types (as reviewed in Prossnitz et al. 2008) including those that exhibit immune functions (Kanda and Wantanabe 2003, Blasko et al. 2009). It is therefore conceivable that estrogen could elicit rapid changes in the immune responses of these cells via this membrane-bound receptor.

Cellular responses to gram-negative bacteria-derived lipopolysaccharide (LPS) are mediated by Toll-like receptor 4 (TLR4). Ligation of TLR4 activates transcription factors that initiate the production of inflammatory cytokines and chemokines. These molecules promote protective immune responses but their over-production leads to systemic inflammatory responses and the catastrophic drop in blood pressure associated with bacterial septic shock (as reviewed in Akira 2006, Palsson-McDermott and O'Neill 2004). We have previously demonstrated the ability of super-physiological levels of 17β-estradiol to significantly elevate TLR4 expression on macrophages and to increase susceptibility of mice to in vivo LPS challenge. In agreement with this finding, other groups have shown that long term treatment of mice with estrogens increases inflammatory mediator production following challenge with bacteria or their products, and that this effect is mediated by the classical nuclear estradiol receptor, estrogen receptor alpha (ERα) (Soucy et al. 2005, Calippe et al. 2008). In the present study, we

demonstrate that 17β-estradiol can conversely elicit rapid decreases in cell surface TLR4 expression on macrophages and show that such effects are mediated by GPR30.

6.2 Results

To begin to determine the acute direct effects of estrogens on macrophages, we have exposed RAW 264.7 macrophage-like cells cultured in charcoal-stripped FBS to minimize the effects of reproductive hormones to 17β-estradiol for various periods prior to analysis of cell-surface TLR4 by flow cytometry. As shown in Figure 16A, 17β-estradiol treatment failed to elicit significant changes in cell-surface TLR4 expression at 24 to 72 hours following exposure. However, cell-surface TLR4 expression on RAW 264.7 cells was significantly decreased by approximately 20% ($p < 0.05$) one hour after 17β-estradiol addition (Figure 16B).

To begin to determine whether estrogen elicits rapid decreases in TLR4 levels on macrophages via a non-classical membrane receptor, we confirmed the expression of GPR30 in these cells. As shown in Figure 17A, both RAW 264.7 cells and primary murine peritoneal macrophages constitutively express mRNA encoding GPR30 as determined by semi-quantitative PCR. Importantly, we have confirmed that resting macrophage-like cell line and primary macrophages derived from male and female mice express GPR30 protein as determined by immunoblot analysis (Figure 17B).

To assess the role of GPR30 in acute estrogen-mediated reductions in TLR4 expression, we have used silencing RNA to knockdown expression of this receptor in RAW 264.7 cells. Cells were transfected with either siRNA directed against GPR30 or a negative control duplex 48 hours prior to experimentation, and we have confirmed that

the GPR30 specific siRNA duplex markedly attenuates GPR30 mRNA levels in RAW 264.7 cells (Figure 18A). We then assessed the effects of estrogen on TLR4 expression by RAW 264.7 cells following transfection. As shown in Figure 18B, 17β-estradiol elicits a significant decrease in cell-surface TLR4 expression on cells transfected with a negative control duplex at 60 to 120 minutes following treatment. Importantly, such treatment failed to elicit significant changes in cell-surface TLR4 expression following transfection with siRNA directed against GPR30 (Figure 18C).

To further confirm that the ability of estrogens to elicit rapid decreases in TLR4 expression on macrophages is mediated by GPR30 we have examined the effects of GPR30 specific agonists. RAW 264.7 cells were acutely treated with ICI 182780, a compound that has been widely employed as an inhibitor of the classical nuclear estrogen receptors but is also thought to act as a GPR30 agonist (Thomas et al. 2005). As shown in Figure 19A, ICI 182780 elicited a significant decrease cell-surface TLR4 expression at 60 and 120 minutes following treatment, with maximal reduction of approximately 18%. We have also employed a second GPR30 agonist, G1, which does not bind to ERα or ERβ (Bologa et al. 2006). As shown in Figure 19B, treatment of cells with G1 results in a significant decrease in cell-surface TLR4 expression as rapidly as 10 minutes post exposure with a maximal reduction at 120 minutes of approximately 27% (Figure 19B).

We have extended our findings with this macrophage-like cell line to primary cells in experiments employing peritoneal macrophages isolated from male C57BL6 mice and we show that G1 similarly elicits a significant decrease in cell-surface TLR4 expression on primary peritoneal macrophages within 10 minutes following treatment (Figure 19C). Furthermore, we have confirmed that the ability of G1 to down-regulate

TLR4 expression is mediated via GPR30 using silencing RNA directed against this receptor. As shown in Figure 19D, G1 induced a reduction in TLR4 expression in RAW 264.7 cells transfected with a negative control duplex. Importantly, this effect was absent in cells transfected with siRNA directed against GPR30 (Figure 19E).

To determine the biological significance of GPR30-mediated reductions in macrophage cell-surface TLR4 expression, we have assessed the effects of GPR30 agonists on the LPS-induced immune responses of these sentinel cells. As shown in Figure 20A, G1 treated RAW 264.7 cells demonstrated reduced ability to bind fluorescently labeled LPS as determined by flow cytometry. Importantly, RAW 264.7 cells acutely treated with this GPR30 agonist produced significantly lower levels PGE_2 (Figure 20B; approximately 46% less) at 2 hours following LPS challenge, and the reduced production of the key inflammatory cytokines IL-6 (Figure 20C; approximately 34% lower) and TNF-α (Figure 20D; approximately 12% lower) at 24 hours after exposure to LPS.

6.3 Conclusions

Sex-based differences in host responses to microbial pathogens have been proposed to result from the direct effect of reproductive hormones on immune cells that bear receptors for these sex steroids. Such a hypothesis is supported by findings that the greatest sex dimorphisms in immune responses is seen in adults of reproductive age. Furthermore, recent research has shown that reproductive hormones, in particular testosterone and estrogen, have significant effects on immune responses. Testosterone is widely accepted to be immunosuppressive and can increase susceptibility of both males

and females to bacterial infection. In contrast, estrogens are suggested to be immunoprotective and have been found to increase resistance to both bacterial infection (Ohtani et al. 2007, Nicol et al. 1964, Tsuyuguchi et al. 2001) and endotoxin challenge (Sener et al. 2005, Erikoglu et al. 2005). However, studies employing exogenous estrogen treatment have yielded conflicting results that may result in differences in concentrations of estrogens used or the treatment regimen. Indeed, recent in vivo studies in our laboratory have shown that endogenous levels of estrogens promote robust immune response to LPS in mice without increasing susceptibility to endotoxin challenge, but long-term super-physiological doses of 17β-estradiol elevates LPS-induced inflammatory cytokine production and dramatically increases sepsis severity. Finally, it has been reported that short term in vitro treatment with 17β-estradiol attenuates macrophage responses to LPS, while chronic in vivo estrogen exposure dramatically increases LPS-induced inflammatory cytokine expression, an effect that was mediated by ERα (Calippe et al. 2008). To date, the mechanisms that underlie this apparent paradox have not been identified.

GPR30 is a membrane bound molecule that has been shown to mediate rapid cellular events in a variety of cell types and has been proposed to serve as a non-classical estrogen receptor (as reviewed in Prossnitz et al. 2008). It should be noted that while several groups have provided experimental evidence that GPR30 serves as a novel estrogen receptor, others suggest that this molecule functions to modulate classical estrogen receptor-mediated effects (Levin 2009). Defining the role of GPR30 has been further complicated by the current absence of reliable GPR30 deficient mouse models (as discussed in Langer et al. 2010). This putative membrane-bound estrogen receptor is

expressed by a variety of cell types including those that have immune functions. GPR30 has been reported in myeloid cells including the monocytic cell line THP-1, human peripheral blood monocytes, and rat microglia (Kanda and Wantanabe 2003, Blasko et al. 2009). In the present study, we have demonstrated that both primary murine peritoneal macrophages and a macrophage-like cell line constitutively express this molecule at the level of both mRNA and protein expression. As such, the presence of this molecule in macrophages could represent a means by which female sex steroids can rapidly alter immune cell function.

We have demonstrated that short term in vitro treatment with 17β-estradiol elicits rapid decreases in TLR4 expression on macrophages and this could explain the finding by Calippe and co-workers (2008) that such treatment attenuates macrophage responses to LPS. Importantly, we show that two GPR30 specific agonists that do not employ classical nuclear estrogen receptors can mimic such effects in both RAW 264.7 cells and primary macrophages. Furthermore, we have established that the rapid effects of 17β-estradiol on TLR4 expression are due to the presence of GPR30 and confirmed that the actions of G1 are similarly mediated by this putative estrogen receptor by employing siRNA techniques to knockdown GPR30 expression in a transfectable macrophage-like cell line. Taken together, these experiments clearly demonstrate that GPR30 mediates the ability of estrogens to rapidly down-regulate the expression of a critical microbial pattern recognition receptor on this sentinel immune cell.

Finally, we have established the functional significance of GPR30-mediated reductions in cell surface TLR4 expression on macrophages by demonstrating that these cells have a decreased ability to respond to bacterial endotoxin challenge. We show that

pre-treatment of macrophage-like cells with a GPR30 specific agonist significantly reduces LPS binding to these cells and inhibits LPS-induced production of an inflammatory prostanoid and key inflammatory cytokines. Again, these findings could help to explain the previous report that two-hour 17β-estradiol treatment significantly reduces LPS-mediated increases in inflammatory mediator mRNA expression in murine peritoneal macrophages (Calippe et al. 2008). Furthermore, the present demonstration that GPR30 can mediate rapid changes in innate immune receptor expression by myeloid cells appears to be consistent with the recent demonstration that exposure of human blood mononuclear cells to G1 for one hour is sufficient to reduce TNF-α and IL-6 production initiated by a ligand for the pattern recognition receptor(s) TLR3 and/or MDA5 (Blasko et al. 2009).

While estrogens are generally considered to be “immunoprotective", this term fails to distinguish between their ability enhance host immune defenses to combat infection and their ability to suppress such responses to protect against potentially lethal inflammation. The balance between the immunosuppressive and immunoenhancing activities of reproductive sex steroids appears to be influenced by both the duration and level of exposure of immune cells to them. The present study may shed light on this issue by showing the functional presence of a membrane-bound molecule on macrophages that has been suggested to function as a non-classical estrogen receptor, and the demonstration that this molecule can mediate rapid cellular effects that modulate this cell's sensitivity to microbial motifs. As such, estrogens may be able to reduce the devastating inflammation associated with acute overactive host responses such as septic shock without compromising long-term defense against infectious organisms. Finally,

the present demonstration that G1 is effective in limiting inflammatory responses, in concert with the previous finding that this GPR30 ligand can reduce disease severity of a mouse model of CNS inflammation (Blasko et al. 2009), raises the exciting possibility that such agonists may have therapeutic potential.

6.4 Figures

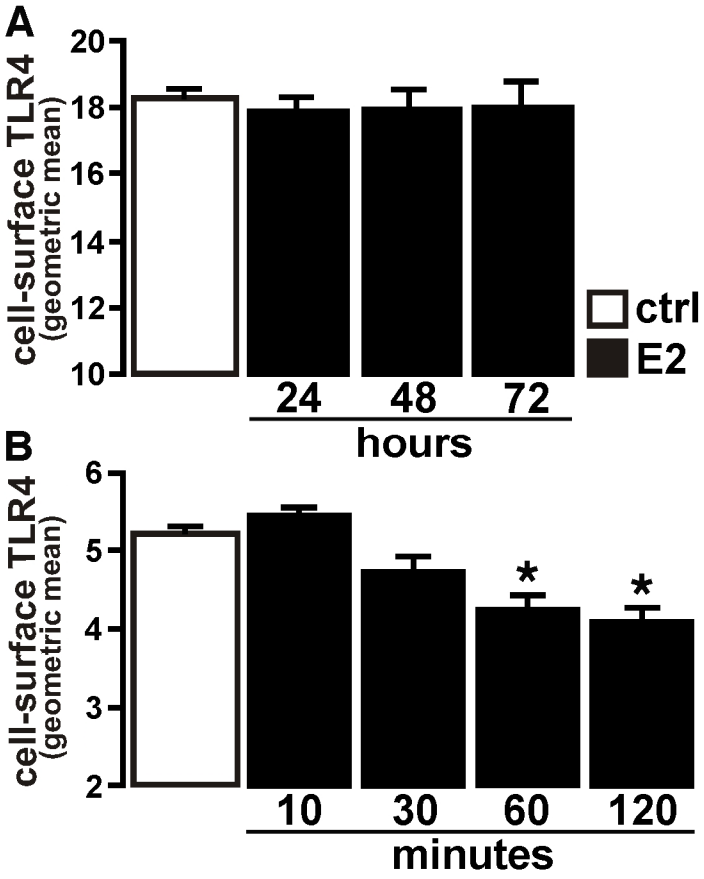

FIGURE 16: Estrogens decrease macrophage cell-surface TLR4 expression following short but not long term in vitro exposure. RAW 264.7 cells were untreated (ctrl) or exposed to long-term (1-3 days; Panel A, n = 6-18) or short-term (10-120 minutes; Panel B, n = 12) 17β-estradiol (E2; 1 x 10^{-6} M) treatment and then assayed for cell-surface TLR4 expression. Asterisk indicates a statistically significant difference from untreated cells as determined by ANOVA.

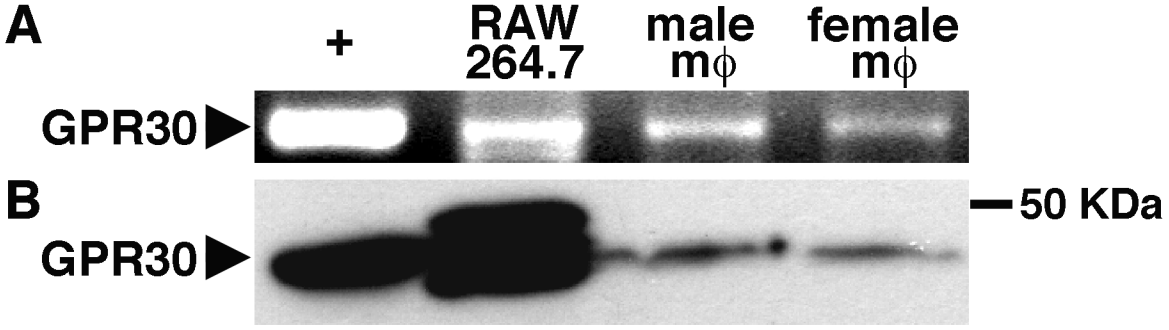

FIGURE 17: GPR30, a putative membrane-bound estrogen receptor, is expressed by macrophages. Panel A: primary murine macrophages and the RAW 264.7 macrophage-like cell line express mRNA encoding GPR30 as assessed by semi-quantitative RT-PCR. Panel B: primary murine macrophages and RAW 264.7 cells express protein for GPR30 as assessed by Western blot analysis. Positive controls for mRNA encoding GPR30 (uterus) and GPR30 protein expression (brain) are shown (+). Results shown are representative of 4 separate experiments.

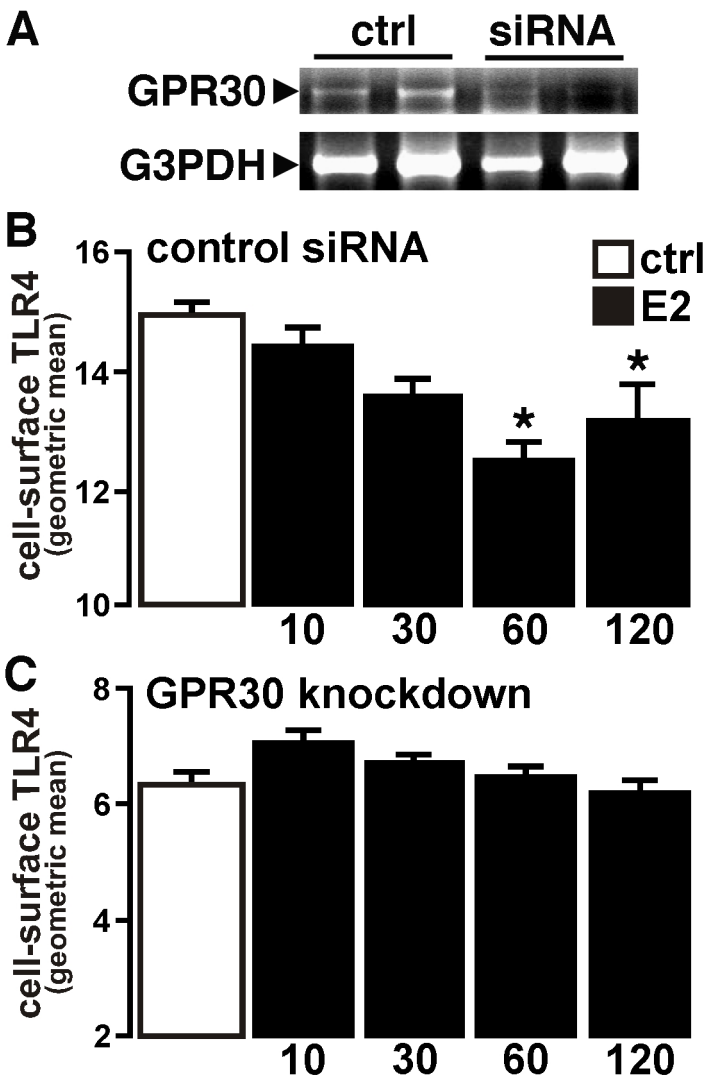

FIGURE 18: Estrogen-mediated reductions in TLR4 expression are abolished following GPR30 knockdown. RAW 264.7 cells were transfected with either siRNA directed against GPR30 or a negative control duplex. Panel A: GPR30 mRNA expression was assessed by RT-PCR in cells transfected with the negative control duplex (ctrl) and cells in which GPR30 was knocked down (siRNA). Panel B shows cell-surface TLR4 expression of untreated cells (ctrl), or following 17β-estradiol (E2; 1 x 10^{-6} M) treatment of cells transfected with a negative control duplex (n = 6). Panel C shows cell-surface TLR4 expression of untreated cells (ctrl), or following 17β-estradiol (E2; 1 x 10^{-6} M) treatment of cells transfected with siRNA directed against GPR30 (n = 6). Asterisk indicates statistically significant difference from untreated cells as determined by ANOVA.

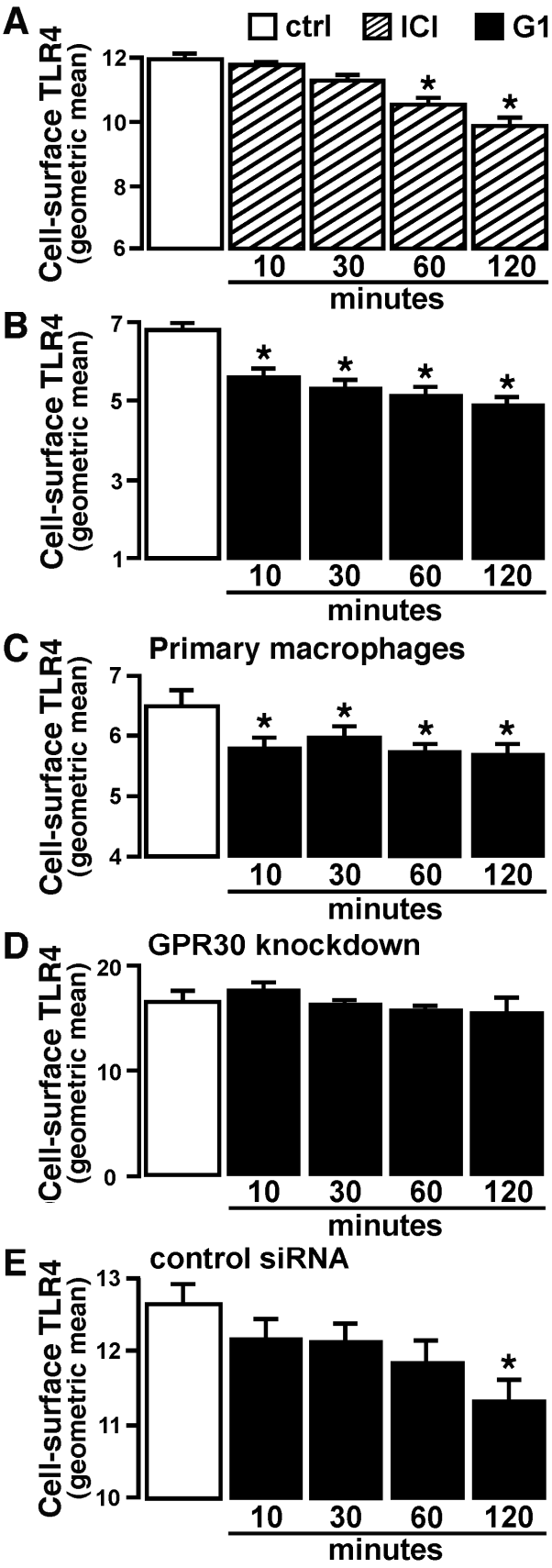

FIGURE 19: GPR30 agonists decrease cell-surface TLR4 expression on macrophages. Panel A: RAW 264.7 cells were untreated (ctrl) or treated with ICI 182780 (ICI; 1 x 10^{-5} M) and then assayed for cell-surface TLR4 expression (n = 6). Panel B: RAW 264.7 cells were treated with G1 (1 x 10^{-6} M) and then assayed for cell-surface TLR4 expression (n = 12). Panel C: primary peritoneal macrophages were treated with G1 (1 x 10^{-7} M) and then assayed for cell-surface TLR4 expression (n = 6). Panel D shows the effect of transfection of RAW 264.7 cells with siRNA directed against GPR30 on G1-mediated reductions in TLR4 expression (n = 4). Panel E shows the absence of effects of transfection of RAW 264.7 cells with negative control duplex on G1-mediated reductions in TLR4 expression (n = 6). Asterisk indicates significant difference from untreated cells as determined by ANOVA.

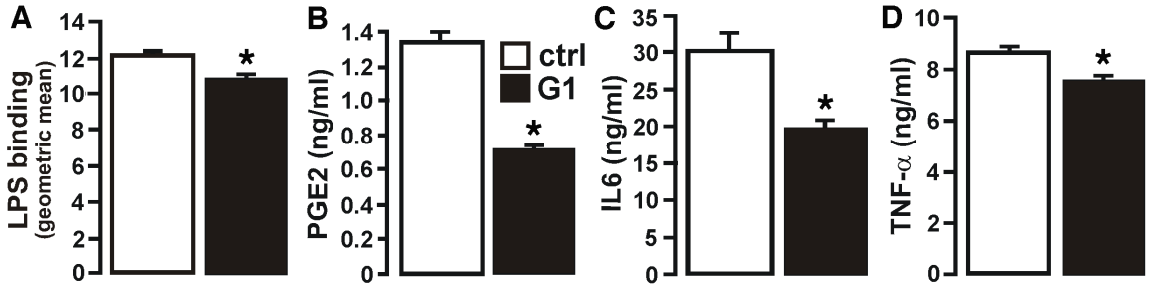

FIGURE 20: GPR30 ligation attenuates endotoxin-induced macrophage immune responses. Panel A: RAW 264.7 cells were untreated (ctrl) or treated with G1 for 30 minutes and then assayed for their ability to bind fluorescently labeled LPS by flow cytometry (n = 4). Panel B: RAW 264.7 cells were treated with G1 for 2 hours prior to the addition of LPS and PGE2 production was assessed at 2 hours following endotoxin challenge (n = 5). RAW 264.7 cells were treated with G1 for 2 hours prior to exposure to LPS and IL-6 (Panel C) and TNF-α (Panel D) content was assessed at 24 hours following endotoxin challenge (n = 12). Asterisk indicates significant difference from control cells as determined by Student's *t*-test.

CHAPTER SEVEN: SUMMARY AND CONCLUSIONS

7.1 Reproductive hormones alter pattern recognition receptor expression on macrophages

Sex-based differences in innate immune responses to bacterial infection are evident in human patients and animal models of disease. Females are less susceptible to the development of bacterial infections and subsequent bacteremia and/or sepsis while males exhibit a greater incidence of such infections and are more likely to develop fatal sequelae. While the precise effects and mechanisms of action remain to be determined, it is apparent that male and female reproductive hormones can have direct effects on the expression and function of key bacterial pattern recognition receptors on innate immune cells. Changes in the expression of these receptors are likely to have profound effects on the production of the inflammatory mediators responsible for the lethal nature of septic shock (as shown in Figure 1) and may underlie the observed sexual dimorphism demonstrated in immune responses to bacterial endotoxins.

Androgens, such as testosterone, are widely accepted as immunosuppressive. We have shown that testosterone decreases TLR4 expression on macrophages, both in vitro and in vivo. This implies a direct effect of testosterone to suppress the ability of macrophages to recognize and respond to bacterial LPS. Indeed, testosterone decreases both total and cell-surface TLR4 expression, indicating that this reproductive hormone either alters recycling and/or gene expression of TLR4, and that this decrease is not

simply due to receptor internalization. Importantly, testosterone also suppresses inflammatory mediator production from macrophages following LPS challenge. As such, the evidence that testosterone can modulate the expression of a key receptor for LPS may represent an important mechanism underlying the immunosuppressive effects of this androgen.

Estrogens, such as estradiol, are generally considered to be immunoprotective. However, this term fails to distinguish between their ability enhance host immune defenses to combat infection and their ability to suppress such responses to protect against the overactive and damaging inflammatory immune response associated with sepsis. The balance between the immunosuppressive and immunoenhancing activities of estrogens appears to be influenced by both the duration and level of exposure to immune cells.

We have shown that estradiol significantly increases cell-surface TLR4 expression on macrophages, enhancing the ability of these cells to recognize and respond to LPS. 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. However, such immune responses do not render intact female mice more susceptible to endotoxic shock, perhaps due to a concurrent increase in immunosuppressive responses. In contrast, animals that have supra-physiological levels of estrogens demonstrate sensitized innate immune cells resulting in a more rapid and elevated inflammatory response following endotoxin challenge and hence greater sepsis severity. This notion is supported by a study of peripheral monocytes from pre-menopausal women with normal menstrual cycles. Interestingly, the activity of

monocytes correlated with the menstrual cycle. As sera estradiol levels increased, cytokine production from monocytes also increased. Likewise, when sera estradiol levels decreased over time during a normal cycle, so did cytokine levels (Verthelyi and Klinman 2000).

Estradiol treatment in vivo increased cell-surface TLR4 expression, but not total protein levels, indicating that estradiol is somehow involved in trafficking of this receptor to the cell surface. As we found that estradiol only augmented TLR4 expression in vivo and not following short-term in vitro treatment, we then analyzed the effects of long-term culture of bone marrow-derived macrophages with estradiol. We have shown that culture with estradiol for two weeks fails to alter cell-surface TLR4 expression. However, macrophages derived from animals receiving long-term supra-physiological levels of exogenous estradiol over time develop higher levels of TLR4 compared to cells from intact females, in the absence of any other known stimulus. This indicates that the long-term effects of estradiol that increase cell-surface TLR4 expression may be indirect. Furthermore, these effects occur prior to macrophage development and exert sustained effects during maturation of this key immune sentinel cell. The long lasting sustained effects of estradiol could be due to the well known epigenetic effects of this reproductive hormone and environmental estrogens (Prins et al. 2008, McLachlan et al. 2006, Guerrero-Bosangna et al. 2005). Verification of such a hypothesis would require further study.

Finally, we have shown that acute in vitro treatment of macrophages with estradiol reduces TLR4 expression, an opposite effect to what is seen due to long-term exposure to estradiol in vivo. The roles of estrogens in modulating immune responses are

clearly complex, but we have shed some light on this issue by showing the functional presence of the non-classical estrogen receptor GPR30, and the demonstration that this molecule can mediate rapid cellular effects that modulate macrophages' sensitivity to microbial motifs. The rapid decrease in sensitivity to LPS would be especially important during the onset of sepsis. While chronic infections may last for months or even years, the development of sepsis occurs much more quickly, within one to a few days post exposure to endotoxin. The potential for estradiol to limit overactive host immune responses to LPS could provide a mechanism by which pre-menopausal women are less susceptible to the development of sepsis than men. Furthermore, the demonstration that the GPR30 specific agonist G1 is effective in limiting inflammatory responses raises the possibility that such agonists may have therapeutic potential.

In summary, we have shown the immunosuppressive effects of testosterone are fairly simple compared to the dichotomous effects of estradiol. Testosterone decreases TLR4 expression, and suppresses subsequent inflammatory responses and susceptibility to sepsis. The effects of estrogen, however, are contingent on the dosage, timing, and manner of treatment. As such, estrogens may be able to reduce the devastating inflammation associated with acute overactive host responses such as septic shock without compromising long-term defense against infectious organisms. In this way, the effects of estradiol resemble a balancing seesaw, in which two extremes can push the host immune response off balance. Supra-physiological long-term estradiol can exacerbate the symptoms of sepsis by increasing damaging inflammatory responses. However, too little estradiol can limit immune responses and compromise the ability of the host to respond to pathogens. 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 non-surviving sepsis patients, regardless of sex (Dossett et al. 2009, May et al. 2008). We suggest that the perfect balance of physiological levels of estradiol, as seen in normal cycling females, balancing the seesaw at the point where females exhibit greater resistance to both infectious challenge and the development of sepsis. As such, cycling levels of estradiol in the physiological range has earned the term "immunoprotective."

7.2 Potential for future studies

Sex steroid hormones can elicit demonstrable changes in the expression and function of microbial pattern recognition receptors and can significantly alter the production of soluble immune mediators responsible for lethal septic shock. However, linking these effects to the sex differences in susceptibility to bacterial infection and sepsis severity remains challenging as the two ideas are in some cases seemingly paradoxical. For example, testosterone suppresses the production of inflammatory mediators and decreases susceptibility to sepsis, yet men exhibit greater severity and higher mortality associated with sepsis than do women. While the precise mechanisms remain to be determined, we propose a scenario, summarized in Figure 21, in which male sepsis patients exhibit reduced levels of immunosuppressive testosterone due to inhibition of testosterone production by LPS (Reddy et al. 2006), and highly elevated levels of estrogen, perhaps due to increased activity of aromatase from LPS and/or inflammatory cytokines (Schmidt et al. 2000, Singh et al. 1997). This, at least in males, leads to an excessive and damaging systemic immune response. Together, reduced testosterone and

elevated estrogen levels in males would facilitate expression of TLR4, CD14, and LBP rendering sentinel immune cells more sensitive to bacterial LPS and leading to markedly elevated levels of soluble inflammatory mediators thereby precipitating septic shock. These effects are on top of already increased levels of TLR4, CD14, and LBP found in males compared to females (Marriott et al. 2006). Further research is required to validate such a hypothesis and to develop a therapeutic intervention during the development of sepsis to counter these effects.

Estradiol has been proposed by a wide variety of investigators as a therapeutic treatment for patients suffering from trauma induced septic shock (Angele et al. 1999, Ghisletti et al. 2005, Hsieh et al. 2007b, Knoferl et al. 2002, Ohtani et al. 2007, Sener et al. 2005, Raju et al. 2008). However, estradiol has complicating effects if dosage is too high or treatment too prolonged. Also, estradiol has multiple receptors to which it can bind and activate a variety of cellular responses. The recent discovery of GPR30 and its specific ligand G1, which does not bind to the classical nuclear estrogen receptors, has opened the door to specifically targeting certain estrogenic effects without initiating responses from all estrogen receptors. We demonstrated that G1 significantly decreases TLR4 expression within 10 minutes, and it effectively limits inflammatory responses 24 hours after treatment, indicating it exerts sustained effects. This is in concert with the previous finding that this GPR30 ligand can reduce disease severity of a mouse model of CNS inflammation (Blasko et al. 2009), which raises the exciting possibility that such agonists may have therapeutic potential. As such, further research is warranted to investigate the ability of G1 or other GPR30 specific targets to limit immune responses in patients exhibiting symptoms of severe sepsis and septic shock.

7.3 Figures

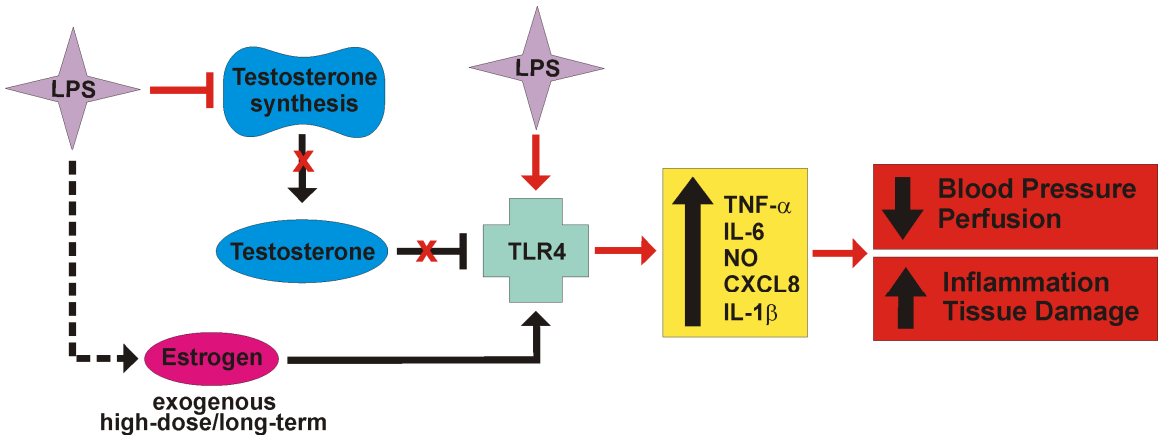

Figure 21: Putative mechanism by which testosterone and high levels of exogenous estrogen can exacerbate the symptoms of sepsis by affecting levels of TLR4. LPS inhibits testosterone synthesis thereby removing the inhibitory effect of testosterone on TLR4 expression on sentinel cells. Elevation of estrogen levels (perhaps via a direct action of LPS) increases TLR4 expression. LPS is recognized via TLR4 on these sensitized cells resulting in a massive release of inflammatory mediators precipitating septic shock.

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APPENDIX

PUBLICATIONS

Rettew JA, YM Huet-Hudson, I Marriott. 2008. Testosterone reduces macrophage expression in the mouse of toll-like receptor 4, a trigger for inflammation and innate immunity. Biology of Reproduction 78: 432-437

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Rettew JA, SH McCall, I Marriott. GPR30/GPER-1 mediates rapid decreases in TLR4 expression on murine macrophages. Submitted for publication.