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SEX DIFFERENCES AND ESTROGEN MODULATION OF THE CELLULAR IMMUNE RESPONSE AFTER INJURY

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Abstract

Cell-mediated immunity is extremely important for resolution of infection and for proper healing from injury. However, the cellular immune response is dysregulated following injuries such as burn and hemorrhage. Sex hormones are known to regulate immunity, and a well-documented dichotomy exists in the immune response to injury between the sexes. This disparity is caused by differences in immune cell activation, infiltration, and cytokine production during and after injury. Estrogen and testosterone can positively or negatively regulate the cellular immune response either by aiding in resolution or by compounding the morbidity and mortality. It is apparent that the hormonal dysregulation is dependent not only on the type of injury sustained but also the amount of circulating hormones. Therefore, it may be possible to design sex-specific therapies to improve immunological function and patient outcome.

Keywords

estrogen; testosterone; macrophage; neutrophil; T cell; injury; burn; trauma-hemorrhage; cytokines; immunity; inflammation

1. Introduction

There is extensive evidence in the literature for a sexual dimorphism in the immune response leading to differences in a wide array of disorders ranging from susceptibility to autoimmune disorders to mortality following injury or infection [1–8]. During the reproductive years, females have a more robust humoral and cellular immune response compared to males [2]. This is reflected in observations showing that females possess a more developed thymus, higher antibody concentrations, and a greater capability to reject tumors [9]. Physiologic levels of estrogen, like those seen during the estrus/menstrual cycle, stimulate the immune response, whereas high levels of estrogen such as those found during pregnancy are suggested to down-regulate cell-mediated immune responses [9]. Estrogen receptors are found in reproductive

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tissue, as well as certain immune cells including T cells, monocytes, and macrophages [10–16]. Direct estrogen-mediated modulation of immune cell activity includes changes in cytokine production, as well as cell activation and proliferation. Following trauma, including burn and hemorrhage, significant differences have been observed in clinical and animal studies which are thought to be due to estrogen's ability to influence the immune response following injury.

2. Changes in the immune response following trauma

Burn and hemorrhagic shock are two major insults that result in immune dysregulation, which can be affected by gonadal hormones. The immune response to injury has been described as biphasic and can result in either resolution or death. Early events result in a systemic inflammatory response (SIRS), which is mainly mediated by innate immune cells and the production of pro-inflammatory cytokines. In many patients, a compensatory anti-inflammatory response syndrome (CARS) can develop, which is associated with immune suppression and anti-inflammatory cytokine production [17,18]. This combination of responses can also be complicated with the introduction of an opportunistic infection, described as the two-hit response hypothesis or model [19,20]. The second hit can then increase the risk for multiple organ dysfunction syndrome (MODS) and multiple organ failure (MOF) (Figure 1). Clinical evidence shows that patients usually succumb to secondary infections rather than their primary injury; this infers that immune function after injury involves biological processes that are highly inefficient [21,22].

Both clinical and animal model studies demonstrate that burn injury leads to an enhanced systemic inflammatory response leading to MODS and MOF. This dysregulated immune response in humans and mice can be characterized by higher levels of interleukin-6 (IL-6), interleukin-1 β (IL-1 β), tumor necrosis factor- α (TNF- α), prostaglandin E2 (PGE-2), and lower delayed-type hypersensitivity (DTH) response and lymphocyte proliferation [23–25]. A retrospective study in burn patients demonstrated higher mortality in females when compared to males sustaining a similar sized burn injury [26–29]. Consistent with these observations, lower survival rates in female mice compared to males with 15% total body surface area (TBSA) were obtained [30]. Estrogen levels are significantly higher (10–15 fold over baseline) in females following burn injury [31], whereas concentrations of circulating testosterone are decreased [32,33]. These observations suggest that significantly higher levels of estrogen may lead to improper cell mediated immune response. This effect is also compounded with the addition of other co-morbidity factors such as alcohol use and age as discussed below.

Hemorrhagic shock is also characterized by an overabundant production of pro-inflammatory mediators which can lead to MODS and death [34]. Estrogen and testosterone can influence immune and cardiac function following trauma-hemorrhage and shock. Immunosuppression following trauma-hemorrhage is observed in males, however, unlike in burn injury, female mice demonstrate an enhanced immune response compared to their male counterparts [35, 36] and to ovariectomized (OVX) females, suggesting a protective role of estrogen in this particular injury [37]. Differences in immune function were observed in humans and mice in cases of trauma-hemorrhage with increased incidences of MODS and sepsis occurring in males [38]. In addition, following trauma female patients are less susceptible to sepsis and subsequent multiple organ disorder than males [39]. In a recent clinical study evaluating patients with multiple injuries, it was observed that female patients had a lower incidence of MODS and sepsis. Additionally, the females in this study cohort showed significantly lower levels of IL-6 and interleukin-8 (IL-8) in serum at early time points and lower serum levels of IL-10 at late time points than age matched male patients [40]. Consistent with these data, animal models show male mice were immunosuppressed, while female mice maintained a functional immune response following sepsis. Not surprisingly, female mice also have a significantly higher survival rate than males after sepsis [41]. Although both injuries display sex differences, the

sex with the better outcome is not the same. Interestingly, following trauma-hemorrhage, females fair better than males, while in burn the opposite is true. This disparity in outcome is most likely due to changes in circulating hormones present after injury. Consistent with these observations, treatment of injured mice with the addition or removal of sex hormones affects the morbidity and mortality after injury. These outcomes are summarized in Table 1 and discussed in more detail in the text that follows. While other hormones such as progesterone and prolactin are known to influence the immune response, the studies discussed in this review will focus mainly on estrogen and testosterone.

In females, there is a normal variation of estrogen levels during the estrous cycle. The cycle can be divided into four stages: proestrus, estrus, metaestrus, and diestrus with ovulation normally occurring during estrus. At the beginning of the cycle, estrogen secretion is very low and comparable to levels observed in males. Low levels of estrogen are found during proestrus and steadily increase reaching very high levels that are close to concentrations observed during pregnancy around day 14 in humans [42]. Additionally, the expression of gonadotropin receptors can vary across the estrous cycle. For example, in rats an increase in the expression of gonadotropin receptors occurs on the day of proestrus compared to other points in the cycle [42]. Fluctuations in gonadal hormones and their receptors may impact both the innate and adaptive immune response following injury.

3. The innate immune response following injury

Innate immunity represents the first active line of defense to infection. Innate immunity is a non-specific, but immediate response to pathogen, which is designed to 1) eliminate or control infection via phagocytosis and release of enzymes that degrade pathogens, and 2) activate the adaptive immune response via cytokine release and cell-to-cell interactions, resulting in a more specific and robust immune response. The two most studied leukocytes involved in innate immunity after burn or trauma injuries, are neutrophils and macrophages. Evidence suggests that, after injury, there is a large-scale dysregulation of the function and recruitment properties of these cells, as described below.

3.1 Neutrophils

Neutrophils play a key role in innate immunity by responding to chemokines released in specific tissues. The chemokines stimulate them to migrate from the circulatory system into tissues in order to destroy pathogens through phagocytosis and the release of soluble toxic mediators. Initial activation of neutrophils is required for proper function. Studies from Deitch et al. show that, at 3 hours after burn in rats, there is an increase in CD11b expression on phagocytes from both males and females, but the increase is more robust in males. Naïve neutrophils from male rats incubated with plasma from burned male or female rats showed similar activation of neutrophils after burn injury. Interestingly, naïve neutrophils from female rats were not able to become activated by plasma isolated from burned animals. Intestinal lymph from male burned mice also showed a greater neutrophil activating capacity compared to lymph collected from females in the proestrus stage [43]. Additionally, this study examined the activation of phagocytes after trauma and hemorrhage. Neutrophil activation, as measured by CD11b expression and respiratory burst, was increased in males after hemorrhage, but not in females. Gonadectomy decreased CD11b expression on neutrophils from males, but increased expression in females, implying that testosterone enhances and estrogen suppresses CD11b expression after injury. These trends were the paralleled when measuring respiratory burst production, or the release of reactive oxygen species from these cells. Additionally, female rats in proestrus had less of a respiratory burst production compared to males. These data were explained by showing evidence that estrogen decreases calcium influx into male neutrophils in a dose dependant manner [43]. Improper activation of neutrophils can result in

an insufficient initial response to a pathogen at the site of injury or at distal tissues such as the lungs, where secondary infections are likely to occur [44–46].

Other studies have shown that estrogen reduces neutrophil invasion into tissues, while testosterone has an opposite effect [47–49]. MPO and elastase are two of the soluble toxic mediators in a neutrophil's arsenal used to kill bacterial and fungal pathogens. MPO uses hydrogen peroxide to form hypochlorous acid, which is bactericidal. This enzyme is often used as an index of neutrophil activation or content. MPO activity induced by burn injury was significantly reduced following castration of male mice [47]. In addition to a decrease in MPO, estrogen treatment significantly reduced burn-induced innate cell infiltration in the liver and lung. The androgen receptor blocker, cyproterone acetate, reduced MPO levels but not as effectively as estrogen treatment but this may be due to a suboptimal blockade of the androgen receptor [47]. These and other studies show that both testosterone and estrogen play a role in leukocyte recruitment and effector function. Where testosterone promotes recruitment of neutrophils, estrogen inhibits their recruitment and activation. Additional evidence shows that circulating numbers of these cells are more robust in males as compared to females 7 days after burn injury with prior exposure to ethanol (J. Karavitis and E.J. Kovacs, unpublished observations), supporting the idea that estrogen may down-regulate neutrophil infiltration into a site of injury or infection leading to a worse prognosis in females following burn injury.

Other interesting results have been seen in neutrophil recruitment after trauma and hemorrhage in murine models of injury. A recent study by Frink et al. examined whether male mice subjected to trauma-hemorrhage treated with 17- β -estradiol (estrogen) immediately after injury, produce KC, a neutrophil chemoattractant cytokine, and have MPO levels similar to saline treated mice two hours after initial injury. Their studies show that at two hours after injury, estrogen treatment decreased circulating levels and Kupffer cell production of KC, TNF- α and IL-6, as well as KC gene expression and protein in the lung. This was accompanied by a decrease in inflammatory cell infiltration and edema formation in the lung [50]. These results suggest that estrogen prevents lung neutrophil infiltration in part by decreasing KC during the post-trauma immune response. Also, increases in intercellular adhesion molecule-1 (ICAM-1) and cytokine-induced neutrophil chemoattractant (CINC)-1 and -3, typically seen in liver after trauma-hemorrhage are attenuated with flutamide, an androgen-receptor blocker, [51] indicating that blocking testosterone may decrease recruitment of phagocytes in distal organs following injury. After an intratracheal instillation of bacterial lipopolysaccharide (LPS), as a model used for bacterial infection, male and OVX female mice showed increased levels of IL-1 β in bronchial alveolar lavage (BAL) fluid, serum levels of IL-6, and lung homogenate levels of intercellular adhesion molecule-1 (ICAM-1) [52]. The up-regulated levels of IL-1 β , IL-6, and ICAM-1 were abrogated in the OVX mice that have been administered estrogen, resulting in albumin and neutrophil content in the lungs at the level of intact female mice [52].

Similar effects of estrogen on neutrophil recruitment were observed following other types of injury. In a model of acute vascular injury, a balloon injury is induced in the common carotid artery. This injury to the carotid artery is associated with infiltration of leukocytes including phagocytes, granulocytes, and lymphocytes in the adventitia surrounding the site of injury [53,54]. Ovariectomized rats have a significant leukocyte infiltrate following arterial injury [55]. Consistent with this observation, estrogen treatment of OVX rats yielded a decrease in migration of neutrophils and monocytes into the damaged artery, suggesting a protective effect [55]. In the rat model of acute vascular injury, expression of mRNA in the common carotid artery for adhesion molecules P-selectin, vascular cell adhesion molecule-1, and ICAM-1, as well as chemoattractants CINC-2 β and monocyte chemoattractant protein-1 (MCP-1), are increased in injured arteries of OVX rats at 2 hours. Administration of estrogen (20 μ g/kg) after injury attenuated expression of the adhesion molecules and chemoattractants [56]. P-selectin,

CINC-2 β , and MCP-1 stayed lowered in the estrogen treated rats 24 hours after injury. The decrease in adhesion molecules and chemokines is modulated by estrogen and could be a result of a reduction of cell-surface integrin expression [57], or reductions of oxidative stress [58–60].

In order to determine which estrogen receptors mediate the effects, ER- α or ER- β agonists were administered after injury [61]. Administration of the ER- α agonist propyl pyrazole triol (PPT), but not the ER- β , agonist diarylpropionitrile (DPN), lowered the chemokines, CINC-1 and CINC-3, and the adhesion molecule ICAM-1 levels in the liver. In contrast, DPN but not PPT significantly decreased CINC-1 and ICAM-1 expression in the lung. Neither ER subtype requirement was observed in the intestine, showing that either PPT or DPN was able to lower CINC-1, CINC-3 and ICAM-1 levels [61]. Interestingly, the effects of estrogen administration on neutrophil sequestration into tissues following trauma-hemorrhage are receptor subtype and tissue-specific. These data provide additional evidence to help explain how estrogen reduces tissue damage after trauma/hemorrhage.

3.2 Macrophages

Similar to neutrophils, sex differences are observed in macrophage function following injury. At early time points (1 day post-burn) after burn injury, splenic macrophages isolated from burned male mice showed increased levels of IL-6 production in response to LPS that returned to sham levels by day four [30]. This is in contrast to splenic macrophages isolated from burned females, where there is a significant increase in IL-6 production in response to LPS that stays elevated out to 10 days after burn injury [30]. The macrophage dysfunction observed in burn-injured females may have effects on other cell types as well. Suppression of splenocyte proliferation reported for cells from female, but not male mice at 10 days after burn injury is abrogated upon removal of macrophages, implying that the aberrant responses are mediated by these activated macrophages [30].

To further validate the role of estrogen on macrophage function after injury, splenic macrophages from sham and burn animals were incubated in vitro with LPS plus estrogen. Estrogen treatment (300 pg/ml) resulted in significantly higher levels of IL-6 production in macrophages from sham-injured mice, similar to levels of production by macrophages from burned animals. Interestingly, further elevations of IL-6 were not observed in estrogen plus LPS treated macrophages isolated from burned mice, suggesting a saturation level of IL-6 production by macrophages in response to LPS treatment after burn in culture [30]. Conversely, splenic macrophages isolated 24 hours after male mice that were exposed to ethanol followed by burn injury showed a decrease in IL-6 production when treated with LPS and estrogen [33]. This evidence shows that the hyper-inflammatory response expressed after burn injury can be mitigated with exposure to low levels of estrogen.

In a mouse model of trauma and hemorrhagic shock, Angele et al treated castrated male mice with either vehicle, 5 α -dihydrotestosterone (DHT), estrogen, or a combination of both steroid hormones for 14 days prior to injury. The advantage of using DHT is that because it is the active form of testosterone, it cannot be converted back to testosterone and further metabolized into estrogen by aromatase. Twenty-four hours after injury, immune function was measured by assaying plasma cytokine levels, as well as cytokine levels in Kupffer cell, splenic macrophages, and peritoneal macrophage cultures supernatants. Regardless of the source of macrophages, in DHT-treated mice, there was a decrease in the production of the proinflammatory cytokines, IL-1 β and IL-6, after trauma-hemorrhage when compared with the levels of cytokines produced by cells from sham-injured mice. Estrogen treatment also lowered pro-inflammatory cytokines by macrophages. Interestingly, the release of IL-10 from Kupffer cells from DHT-treated injured animals was increased compared with that in sham-operated animals, but was decreased in estrogen-treated mice under such conditions [62]. These

results suggest that both male and female sex steroid hormones exhibit immunomodulatory properties with respect to macrophage responses after trauma-hemorrhage, and implicate the potential difference in outcome after injury between sexes.

To complement these studies, work by Dr. Knoferl investigated the effects of estrogen on immune responses after trauma and hemorrhagic injury by using a model, in which male mice received subcutaneous estrogen or vehicle at the beginning of resuscitation [63]. The depressed proinflammatory cytokine (IL-1 and IL-6) production by splenic and peritoneal macrophages from mice receiving vehicle treatment was restored in mice receiving estrogen-treatment. In contrast, the sustained release of the anti-inflammatory cytokine IL-10 by splenocytes and splenic and peritoneal macrophages obtained from vehicle-treated mice after hemorrhage was reduced in estrogen-treated mice. To confirm that gonadal hormones were responsible for altering immune function, OVX female mice (and controls given laparotomy) were subjected to hemorrhagic shock. One day post-injury, peritoneal macrophage production of IL-1 β and IL-6 was significantly depressed in OVX mice compared to controls. Production of IL-6 and IL-12 levels by splenic macrophages was also significantly depressed in these mice. The levels of these cytokines were restored by estrogen replacement treatment [64]. Interestingly, earlier time points also showed differences due to estrogen. At two hours after injury, the release of IL-1 β and IL-6 by splenic and peritoneal macrophages from proestrus mice was maintained [65] or increased [37] after injury, whereas the release of IL-1 β and IL-6 by macrophages from OVX mice was depressed by approximately 50% compared to proestrus female mice. In contrast, trauma/hemorrhage resulted in a 4-fold increase in production of TNF- α by Kupffer cells from OVX females and a 5-fold increase in plasma concentrations of TNF- α in comparison to cells isolated from intact females. TNF- α levels in the circulation did not differ in proestrus mice under such conditions [36]. These studies validate that estrogen, after traumatic injury can result in a more robust immune response.

Prostaglandin E₂ (PGE₂) may also be contributing to the immune suppression observed in female mice following burn injury. Prostaglandins have long been shown to play a pivotal role in mediating the cellular immune response following trauma and burn injury. Increased circulating levels of PGE₂ inhibit T-cell mitogenesis, macrophage antigen presentation, and phagocytosis [66–68] that may ultimately lead to a poor clinical outcome in burned mice and humans. In the mouse model of burn injury, there was a 4-fold increase in PGE₂ production by LPS-stimulated macrophages from burn-injured females compared to sham-treated controls at 10 days post injury, as shown by Gregory et al [69]. In contrast, there were no significant differences in PGE₂ production in LPS-stimulated macrophages from burn-injured males compared to that in sham-treated controls [69]. Recent studies by Schneider et al [70] show PGE₂ increases in male mice after trauma-hemorrhage. This was not observed in female mice after injury [70]. Other trauma-hemorrhage studies show that macrophage production of PGE₂, TNF- α , and IL-6 was significantly increased in injured female mice compared with female controls, but there were no differences in injured male mice compared with sex-matched controls. PGE₂ and TNF- α production by male mice following traumatic injury was significantly less than that produced by injured proestrus females [71]. Due to the dichotomy in the literature, further studies need to be performed to further understand the role of PGE₂ in the immune response following trauma.

In addition to injury, wound healing is also affected by sex hormones. Ashcroft et al. [72] reported on dermal wound healing in aged males and females. Subjects had two upper inner arm punch biopsies and received either estrogen treatment or placebo for 24 hours. The wounds were excised at either day 7 or day 80 post-wounding and the degree of wound healing was measured. Compared to placebo, estrogen treatment was beneficial, decreasing wound size at day 7 in both males and females. Hormone treatment increased both collagen and fibronectin at the earlier time point. In addition, estrogen enhanced the strength of the wound at day 80

suggesting a role for the hormone in possible therapeutics to promote faster wound healing in the aged. Estrogen has also been shown to induce angiogenesis, a process necessary for proper wound healing [73]. Using an *in vitro* model, estrogen (10^{-8} mol/L) given to a scraped monolayer of endothelial cells showed increased cell migration, cell proliferation, and organization into tubular networks [73]. These data demonstrate that estrogen promotes wound healing in a population controlled for circulating estrogen levels and suggest that the hormone is capable of exhibiting direct effects on multiple cell types involved in the healing process. Moreover, they may help explain why females have a better prognosis after trauma than males.

In contrast to these observations, the opposite response has been found for oral wound healing. A recent clinical study by Engeland and colleagues found that similar size wounds in the oral mucosa heal faster in men than in women [74]. Thus, in the oral mucosa, estrogen and testosterone can affect the inflammatory cell infiltrate and therefore influence the rate of healing. Testosterone is known to down-regulate inflammation, possibly leading to improved healing rate observed in men. The exact role of estrogen on oral mucosal wound healing is unknown at this time, but the pro-inflammatory properties of estrogen may in fact cause a delay in healing since mucosal wound closure is associated with lower levels of inflammatory cells than in dermal wound closure [74].

4. Adaptive Immune Response following Injury

As with infection, the adaptive arm of the immune response is critical for recovery from injury. Following burn, there is an increase in circulating estrogen levels in mice, reaching levels of the hormone capable of attenuating DTH responses and Concanavalin-A (Con-A)-stimulated splenocyte proliferation [75]. At late time points following burn injury (10 days), suppression of the immune response in females was observed. Similarly, intact male mice given burn and estrogen had significantly decreased DTH responses as did OVX female mice given estrogen at 10 days post burn. In contrast, treatment with an estrogen antagonist (17- α estradiol) restored this memory response, as well as IL-6 production, in intact female mice given burn injury to that of sham levels [75]. Cellular proliferation following stimulation with Con-A was measured *ex vivo* in mice subjected to a 15% total body surface area (TBSA) burn injury. Temporal differences were observed at several time points in male and female mice following burn, in which cell proliferation in male mice was significantly suppressed 1 day after burn and then rebounded to levels similar to sham treated animals by 7 days post injury. In contrast, proliferation of spleen cells in female mice was observed to be higher on day 4 post burn followed by a 50% suppression at later time points [30]. The composition of these cells was analyzed by flow cytometry and differences in the types of cells responding were observed in male and female mice. Female mice receiving burn injury had a 40% decrease in CD4⁺ T cells compared to sham treated females. There was only a 16% decrease in CD4⁺ T cells in burned male mice compared to sham treated males [30].

T lymphocytes have been demonstrated to play an important role in the immune response to burn injury. However, there are conflicting reports in the literature as to the exact role that the subsets of T cells play. One paradigm of T cell response to burn injury suggests that CD4⁺ T cells are pro-inflammatory and produce Th1 cytokines in response to the injury, while CD8⁺ T cells are counter-inflammatory and regulatory in nature [76]. Consistent with this paradigm, some studies demonstrate a Th2 (Tc2) phenotype cytokine secretion by CD8⁺ T cells with a decreased antigen-specific proliferative and cytolytic response [77,78]. However, other groups have evidence of CD8⁺ T cells producing Th1 (Tc1) cytokines similar to their CD4⁺ T cell counterparts following burn [79]. To add confusion to the story, other laboratories suggest it is CD4⁺ CD25⁺ T regulatory cells that are the main players in down-regulating the aberrant inflammatory response following burn [80]. Rag1^{-/-} mice, which genetically lack B and T

cells, produced a heightened cytokine response to LPS stimulation following burn injury, which was then dampened with the adoptive transfer of CD4⁺ CD25⁺ T cells [80].

In trauma-hemorrhage, there is a switch in cytokine production by T cells to a Th2 profile instead of Th1. Untreated male mice, castrated male mice given DHT, and DHT-treated female mice displayed elevated levels of IL-10 with lower levels of IL-2 and IFN- γ following trauma-hemorrhage compared to sham treatment [81]. Consistent with this, both male mice and OVX females can have their immune function restored or “rescued” by administration of estrogen, as demonstrated by significantly lower levels of IL-10 following trauma-hemorrhage [82]. The divergent pattern of cytokine secretion was linked to the differing expression of estrogen receptors in these mice following trauma. In studies using EM-800, an estrogen receptor antagonist, a decrease in splenocyte proliferation and Th1 cytokine production in mice given EM-800 was observed [82] suggesting that the immune protection in proestrus female mice following hemorrhage was dependent on estrogen and not another hormone. The exact mechanism for estrogen’s protective effect and promotion of a Th1 immune response is currently being studied.

4.1 Burn and co-morbidity factors

The immune suppression observed in burn is exaggerated with the addition of alcohol. Patients with alcohol on board at the time of injury suffer from increased infectious and/or surgical complications and mortality [83–85]. In animal studies, female mice were observed to have a higher morbidity than males following ethanol and burn in both survival and immune function (DE Faunce, J Karavitis, and EJ Kovacs, unpublished observations). Numerous studies have demonstrated the detrimental effect of ethanol on immune function including decreased lymphocyte activation following antigen-stimulation, decreased neutrophil infiltration and phagocytic capability, and altered cytokine production by both T cells and macrophages [86–91]. The immunosuppression caused by ethanol is exaggerated when in association with burn injury and can result in increased susceptibility to bacterial infection [92–95]. Consistent with immune suppression in males following burn plus alcohol, flutamide had no effect on immune responses in sham animals but restored cellular proliferation and the DTH response in burned male mice [33]. Flutamide did not increase circulating levels of testosterone [33], but it did increase estrogen levels (KAN Messingham, MA Emanuele, and EJ Kovacs unpublished observations) as well as estrogen receptor expression following burn [96]. Additionally, as mentioned earlier, burn causes a rise in estrogen levels in female mice to those resembling pregnancy (immunosuppressive) where levels of estrogen in burned male mice reach low levels such as that of sham females (immunostimulatory) [75]. These data suggest that specific levels of gonadal steroids are a key component to a positive outcome following injury.

Interestingly, treatment of male mice with low levels of estrogen (proestrus levels) was beneficial and restored cell-mediated immune response following ethanol and burn. The treatment attenuated the increased production of pro-inflammatory cytokines such as IL-6 and improved the survival of these mice after challenge with *Pseudomonas aeruginosa*, a gram-negative bacteria commonly affecting burn patients [33]. Other treatments, including DHT, did not improve immune responses. Whereas flutamide was shown to improve the cellular response post burn injury, DHT did not improve the immune response following burn or burn plus alcohol [97]. These data are consistent with previous work by other groups showing impaired mitogenic B and T lymphocyte responses as well as antibody production in mice subjected to a 25% TBSA burn were not restored with treatment of DHT [98]. In contrast, using a precursor of testosterone and estrogen, dehydroepiandrosterone (DHEA), to treat female mice slightly enhanced immune response compared to vehicle-treated females following trauma-hemorrhage [82]. Furthermore, blocking testosterone through castration resulted in immune recovery and increased survival in male mice subjected to traumatic injury

[37]. Conversely, administration of testosterone to female mice led to a significant depression of splenocyte proliferation and cytokine secretion after traumatic injury comparable to that seen in intact male mice [99].

Chronic alcohol exposure in females caused increased levels of estrogen initially, and then significantly decreased them [100,101]. Additionally, the increased immunity observed in females is negated following introduction of alcohol immunity [102–104]. Some recent *in vivo* work in our laboratory evaluating differences between male and female mice following acute ethanol exposure and burn injury found significant differences between male and female mice in T cell function. At seven days post burn, female mice had lower levels of mitogen-induced splenocyte proliferation after burn injury with or without ethanol compared to their male counterparts. Additionally, female mice given burn or burn plus ethanol demonstrated lower levels of IFN- γ . This decrease in lymphocyte response was not due to a lack of stimulatory cytokines, such as IL-2, production since IL-2 levels were elevated in female mice in all treatment groups (J. Karavitis and E.J. Kovacs, unpublished observations).

Another confounding factor in burn injury is age. Elderly burn patients suffer from a significantly higher rate of mortality compared to young patients with a similar injury [105]. This poor outcome is suggested to be related to the decline of gonadal steroid hormones in elderly female patients following menopause [4]. Pre-menopausal levels of estrogen and hormone-replacement therapy correlate with improved immune parameters in many patients [106,107]. In animal models, aged mice have a higher mortality rate than young adult mice following 15% TBSA [108]. Aged mice also demonstrate significant decreases in DTH responses, Con A-stimulated splenocyte proliferation, and increases in IL-4 and IL-10 production suggesting a shift to a Th2 immune response [109]. Several studies were performed looking at the immune response following burn injury in young adult and aged mice. In aged mice, lower DTH responses and higher IL-6 levels were observed. However, in this study immunity was restored in aged female mice given estrogen replacement therapy. Hormone-treated mice had higher rates of survival, DTH responses, and lower IL-6 production than placebo-treated animals [110]. Similarly, aged mice given estrogen therapy demonstrated increases in DTH responses and IFN- γ [111]. Therefore, a restoration in estrogen levels boosts the immune system in aged female mice following injury.

5. Mechanisms Section

It is clear that sex hormones have a direct influence on immune function following injury, although the exact mechanisms involved are not clearly understood. The major effects of estrogen are mediated through two different receptors, ER- α and ER- β , which are expressed in immune cells [96]. Interestingly, no differences in the expression of androgen and estrogen receptors in male and female mice following trauma or following burn [96]. However, pre-treatment of flutamide increased the expression of ER in T cells in animals following both sham and trauma-hemorrhage [96]. IL-6 is lower in normal male mice following trauma-hemorrhage, but treatment with flutamide increased production of IL-6 levels in male mice after trauma-hemorrhage to that of sham levels suggesting increases in ER expression may be protective in males in certain injuries. Following trauma, the expression of ER- β was significantly different in OVX mice; specifically it was decreased in proestrus mice but increased in OVX mice. Therefore, down-regulation of ER- β may then change expression of cytokines by T cells in these mice [112]. Other studies have suggested a spatial difference in immune-regulation by the two receptors with respect to tissue. Data examining cytokine levels in plasma and spleen suggest that systemic cytokine levels are mediated through ER- α , while production of cytokines by splenocytes may be more dependent on ER- β [113]. In addition to cytokine expression, estrogen is capable of regulating intracellular Ca²⁺ mobilization and release of inducible nitric oxide synthase within leukocytes [114,115].

One mechanism for estrogen in modulation of the immune response is regulation of inflammatory gene expression, which may be a direct or indirect effect. As mentioned above, exposure of macrophages *in vitro* to estrogen causes an increase in production of TNF- α and IL-1 β [116,117]. Expression of other cytokines, like IL-6, are more dose-dependent in that proestrus levels of estrogen inhibit IL-6, whereas pregnancy levels of estrogen increases expression of IL-6 [30,118,119]. Cytokine regulation, by steroid hormones, including estrogen, occurs in two major fashions based on the presence of estrogen response elements (ERE) in the promoter region of the gene. The IFN- γ gene contains an ERE in its promoter allowing for direct regulation of expression by estrogen. This regulation by estrogen is demonstrated by the dramatic increase in expression of IFN- γ mRNA following stimulation of cells with estrogen [120]. All other cytokines, such as IL-6, lack an ERE [121,122], but can be tightly regulated by estrogen [119]. IL-6 expression is regulated indirectly through the binding of estrogen to its receptor. The receptor homo- or hetero-dimers bind to fos or jun thereby blocking their ability to bind to AP-1 sites. Additionally, the receptor dimers can bind to NF- κ B or CEBP- β [119,123]. NF- κ B has been observed to be up-regulated in patients with SIRS along with the cytokines that it is known to regulate, indicating that NF- κ B may be an important mediator for estrogen's effect on the immune response after injury [124].

6. Conclusion

While estrogen may use different mechanisms to control cytokine expression, it is still an important regulatory molecule whose levels of expression may influence the outcome following injury. Design of sex-specific treatments could be employed to influence morbidity and mortality in patients receiving severe injuries. For males, administration of low doses of estrogen or blockade of androgen receptor with flutamide may ameliorate the immunosuppression observed early on following burn or hemorrhage and shock. For females, administration of testosterone or blockade of estrogen may help to decrease the effect of high levels of estrogen on post-injury immunity and therefore hopefully improve the outcome. Additionally, NF- κ B may also be a target for treatment with anti-oxidants to prevent it from binding estrogen-receptor dimers and subsequently inhibiting expression pro-inflammatory cytokines such as IL-6.

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Figure 1.

Schematic of events following injury, with the circles representing innate (filled) and adaptive (open) immune responses. SIRS = systemic inflammatory response syndrome; CARS = compensatory anti-inflammatory response syndrome; MODS = multiple organ dysfunction syndrome; MOF = multiple organ failure.

Table 1

Effects on Morbidity and Mortality in Different Hormonal Treatment Groups

Male	Treatment Intact @ GDX *# Estrogen	Burn Injury Beneficial Detrimental Beneficial	Trauma Hemorrhage Detrimental Beneficial Beneficial
Female	Intact @ OVX # OVX + Estrogen	Detrimental Beneficial Detrimental	Beneficial Detrimental Beneficial

Effects are listed as beneficial or detrimental when compared to the outcome in the opposite sex (@) or compared to the outcome in the intact same sex (#). Male mice depleted of testosterone either by gonadectomy (GDX) or treatment with flutamide are marked with *. Ovariectomized female mice are listed as OVX. Estrogen treatment effects were compared to similarly treated mice given vehicle.