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Sex-Based Differences In Cytokine Production after Burn Injury: A Role of IL-6

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Abstract

Background—Sex-specific differences have been found in the survival of patients after a burn injury. Using a murine model of burn injury, suppression of cell-mediated immunity occurs in a sex-specific manner. Immunosuppression correlated with elevated circulating interleukin-6. This study examines sex differences in cytokine production after injury and the role of interleukin-6.

Study Design—A murine model of dorsal scald injury was employed to examine differences in splenocyte production of interleukin-2, interleukin-4, and interferon- γ in male versus female mice, as well cytokine production in interleukin-6 deficient males.

Results—At 24 hours after burn injury, there was significantly greater suppression of TH1 cytokine production in males than in females. However, interleukin-6 deficient male mice had improved cytokine production relative to wild type mice.

Conclusions—The suppression of the cell-mediated immune response in males correlated with lower T-helper 1 cytokine production, compared to females. Additionally, the immune response and cytokine production is improved by interleukin-6 deficiency.

Introduction

Sex is a significant factor in a patient's survival following burn injury, with females experiencing a higher level of mortality than males.¹⁻³ In addition, differences in infections and immune responses have also been reported between the sexes after a burn injury.⁴⁻⁶ Similar sex-based variations have been mimicked in a murine model of thermal injury.^{7,8}

Prior studies have indicated the presence of temporal differences in the immune responses of males and females after burn injury.⁷ In these studies, males had a greater inhibition of cell-mediated immune responses 24 hours after injury than females, whereas females were more inhibited than males 10 days after injury. Moreover, the sex-based differences can be reversed by administering 17 β estradiol to male mice⁸ and either ovariectomy or administration of an

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estrogen receptor antagonist to female mice.⁸ Interestingly, a lower delay-type hypersensitivity (DTH) and splenocyte proliferation occurred when serum interleukin-6 levels (IL-6) were significantly elevated above sham levels. This immune suppression could be partially restored by administration of an IL-6 neutralizing antibody⁹ or by blocking estrogen.⁸ However, sex differences in the production of cytokines after burn injury, aside from IL-6, have not been fully studied.

The present studies examine the differences in cytokine production by males and female mice after thermal injury, and whether the heightened suppression seen in males is mediated by IL-6.

Methods

Animals

Male and female BALB/C mice, aged 2-3 months were obtained from Harlan Sprague-Dawley (Indianapolis, IN). Interleukin-6 knockout (KO) mice were created as previously described,¹⁰ backcrossed onto the BALB/C background, and a colony was maintained at the Taconic Laboratories (Germantown, NY) for exclusive use by our laboratory. The animals were maintained on a 12-hour light-dark cycle and were provided with food and water ad libitum. All animal experimentation was performed in accordance with the guidelines established by the Loyola University Chicago Institutional Animal Care and Use Committee.

Induction of Burn Injury

Mice were subjected to a 15% total body surface area (TBSA) dorsal scald injury as previously described.¹¹ Briefly, mice were anesthetized (40 mg/kg sodium pentobarbital, i.p.) and their dorsal surfaces were shaved with clippers to remove the hair. The animals were then placed in a plastic template that exposed 15% TBSA and immersed in either a 100°C (burn injury) or a room temperature (sham injury) water bath for 8 seconds. Following injury, the mice were immediately dried off and allowed to recover under a heating lamp. Both sham and burn injured mice received fluid resuscitation. Because of concerns about the circadian rhythms of hormones, like corticosterone, and the effects of the hormones on immunity, all animal procedures were carried out between 8 and 10 in the morning.

Splenic Supernatant Preparation

Twenty-four hours after injury, the animals were sacrificed by CO₂ inhalation, followed by cervical dislocation. Immediately after death, spleens were aseptically removed and splenocyte cultures were established as previously described.¹² Briefly, splenocytes were suspended in RPMI supplemented with L-glutamine (2 mM), penicillin-G (100 U/ml), streptomycin (100 ug/ml), 2-mercaptoethanol (5×10^{-5}), HEPES (50 mM), and 10% fetal bovine serum (Gibco, Grand Island, NY). The viability of the cells was confirmed to be >98% by trypan blue exclusion. Single cell suspension were plated at 2.5×10^5 cells/well in the presence or absence of Concanavalin A (Con A; 2 µg/ml; Sigma Chemical, St. Louis, MO) and incubated at 37°C. After 18 hours incubation, the supernatants were collected and stored for later analysis.

Cytokine Analysis

IL-2, IL-4, and IFN γ levels were measured using commercially available ELISA kits (Endogen, Woburn, MA). The detectable limit for each ELISA is 15 pg/ml (IL-2), 10 pg/ml (IL-4), and 100 pg/ml (IFN γ). All procedures were carried out according to the manufacturer's instructions.

Statistical Analysis

Data are presented as the mean $\pm$ SEM, unless otherwise noted. Percent control was calculated by using the following formula: $[1 - (\text{mean sex-matched sham-injured value} - \text{measured value})]$

(mean sex-matched sham-injured value)] $\times 100\%$. Differences between groups were determined by ANOVA and Newman-Keuls post hoc tests using GB-Stat School Pack software (Dynamic Microsystems, Inc., Silver Springs, MD). A difference of $p < 0.05$ was considered significant.

Results

Serum IL-6

Based upon previous studies, which indicated that elevated IL-6 correlates with increased mortality and immunosuppression after burn injury, serum levels of IL-6 were assayed.⁷ Male and female sham-injured wild type mice showed no significant difference in circulating IL-6 levels (58 ± 4 pg/ml and 154 ± 54 pg/ml, respectively). After injury, males had significantly higher IL-6 levels than burn-injured females ($p < 0.05$), with the males having nearly 40 times higher IL-6 than their sham-injured counterparts (Fig. 1).

Splenocyte Cytokine Production

Previous studies demonstrated that suppression of Con A-induced splenocyte proliferation was greater in burn-injured male mice than in females.⁷ As a corollary to splenocyte proliferation, levels of interleukin-2 (IL-2) production, a TH1 cytokine, were assayed. In the absence of injury, Con A-stimulated splenocytes from sham-injured male mice produced $15,410 \pm 1,878$ pg/ml of IL-2, while activated cells from females produced $13,077 \pm 583$ pg/ml of IL-2 (Fig. 2). In vitro stimulation of cells from burn-injured male mice had an 82.3 % reduction in the level of production of IL-2 relative to cells from sham-injured males ($p < 0.01$). In comparison, the production of IL-2 by cells from burn-injured females decreased by only 33.6 % ($p < 0.01$). Thus, the relative suppression was significantly greater in cells from burn-injured male mice than in female mice ($p < 0.01$).

To further examine changes in TH1 cytokine production, Con A-induced splenocyte production of interferon- γ (IFN γ) was also examined at 24 hours after injury. As with IL-2, splenocytes from male burn-injured mice had a greater decrease in IFN γ levels than cells from burn-injured females (Fig. 3). Spleen cells from sham-injured males produced $97,517 \pm 24,015$ pg/ml of IFN γ , whereas production by cells from burn-injured male mice was reduced by 86.6% ($p < 0.01$). Splenocytes from sham-injured females had $19,519 \pm 849$ pg/ml of IFN γ , while cells from burn-injured female production of IFN γ decrease by 23.1 % ($p < 0.01$). As with IL-2, the relative suppression in splenocyte production of IFN γ was significantly greater in cells from burn-injured male mice than cells from female mice ($p < 0.01$). The results of the changes in the levels of IFN γ and IL-2 suggest that after injury there is a greater impairment of TH1 cytokine production in male mice than in females at this early post-injury time point.

Interleukin-4 (IL-4) production by splenocytes was assayed in order to examine TH2 cytokine production. The level of IL-4 from injured male mice was significantly lower, when compared to sham injury (222 ± 35 pg/ml versus 54 ± 6 pg/ml), decreasing by 75.7 % ($p < 0.01$) (Fig. 4). Interestingly, IL-4 production was equally suppressed in female mice after injury relative to sham (398 ± 83 pg/ml versus 167 ± 51 pg/ml), a reduction of 58.1 % ($p < 0.01$). Thus, while IL-4 levels decrease for both sexes after injury, the decreases are of equal magnitude.

IL-6 Deficient Males

Given that IL-6 has previously been shown to be partially responsible for the sex-specific changes in splenocyte proliferation and DTH response,⁷ IL-6 deficient male mice were assayed for their production of cytokines after injury. There was no difference in the level of production of IL-2 from con A-induced splenocytes from sham-injured and burn-injured IL-6 deficient male mice, $6,907 \pm 808$ pg/ml and $4,372 \pm 1504$ pg/ml, respectively. IL-6 deficiency in males

diminished the fluctuation in IL-2 production after injury, compared to wild type males ($p < 0.05$) (Table 1). Likewise, changes in the levels of IFN γ production by splenocytes was also not as great. Cells from IL-6 deficient males produced $43,294 \pm 12,047$ pg/ml of IFN γ after sham injury, versus $25,949 \pm 17,064$ pg/ml of IFN γ after burn injury. Finally, IL-4 production was also not as suppressed in IL-6 deficient males, as it was in wild type males. Splenocytes from IL-6 deficient mice produced 189 ± 29 pg/ml of IL-4 after sham injury, whereas after injury, cells from sham-injured mice produced 163 ± 65 pg/ml of IL-4 ($p < 0.05$).

Discussion

Burn injury and other forms of trauma lead to alterations in immune function, leaving patients more susceptible to infections and sepsis.^{2,13,14} Furthermore, the degree of sepsis correlates with the risk of mortality.¹⁵

Clinical and epidemiological studies have demonstrated that the risk of mortality after burn injury is influenced by the sex of the subject.^{1,16} In addition, sex-based differences in the immune response after traumatic injury have also been extensively documented.^{7,17-20} A recent report from the multicenter Inflammation and the Host Response to Injury Large Scale Collaborative Program reaffirmed the sex dimorphism after traumatic injury and further associated this with an elevation of IL-6 expression in males.²¹

Prior studies by this laboratory have demonstrated that a 15% TBSA burn injury in mice correlates with a sex-specific immune response.⁷ In those studies, males had a suppressed cell-mediated immune response, as demonstrated by the lower DTH response and splenocyte proliferation and increased IL-6, 24 hours after injury. At the same time point, females had a near normal immune response. Additionally, many of the sex-based effects on immunity after burn can be reverse by altering the sex-hormone milieu⁸ as was seen for traumatic injury.^{20, 22, 23} The present study expands upon those findings to demonstrate sex-specific changes in the production of TH1 cytokines.

Attenuated T cell proliferation is accompanied by diminished IL-2 production, as it serves as a potent stimulator of T cell proliferation.²⁴⁻²⁶ Human studies have shown lower IL-2 production in stimulated peripheral blood mononuclear cells of burn patients,²⁷ with similar results having been reported in animal models.²⁸ The present studies demonstrate that at 24 hours post-injury, production of IL-2 by splenocytes from wild type male mice is more markedly decreased relative to comparably treated cells from females. This may contribute to the previously reported attenuated proliferation of splenic cells obtained injured males that is seen at this time point.⁷ The greater suppression of IL-2 production cells from wild type male mice subjected to burn injury was much less dramatic in the IL-6 KO mice. This observation is consistent with our earlier work demonstrating that *in vivo* and *in vitro* administration of an IL-6 neutralizing antibody abrogates the post-burn suppression of splenocyte proliferation.^{7,9}

While there was also a decrease in the production of IFN γ by splenocytes from both male and female mice after injury, the percentage of depression was greater in male mice. Others reported similar finding in the trauma-hemorrhage model, where IFN γ suppression was greater in cells from males at 24 hours after injury.¹⁹ Additionally, Puyana²⁹ described a decrease in IFN γ in α CD3/ α CD4-stimulated peripheral T cells in a subset of trauma patients, which correlated with increased risk of multiple organ dysfunction. The reduction in IFN γ levels was not seen in cells from IL-6 deficient male mice, suggesting that the significantly elevated IL-6 levels of males after injury is responsible for the further excessive reduction in IFN γ in cells from males, relative to females. The ability of IL-6 to regulate IFN γ production has been shown in *in vitro* studies, which have described IL-6 as upregulating the production of the suppressors of cytokine signaling (SOCS), including SOCS1.³⁰ The increase in SOCS1 blocks an IFN γ

feedback loop, which under normal conditions further increases IFN γ production. This pathway is further supported by evidence that burn injury alters the expression of members of the SOCS family.³¹

In contrast to the Th1 cytokines IL-2 and IFN γ , the Th2 cytokine IL-4 does not show a sex-based dimorphism after traumatic injury. In the trauma-hemorrhage model, splenocyte and peripheral blood mononuclear cell production of the Th2 cytokines IL-4 and IL-10 were similar in male and female mice.³² This lack of dimorphism in Th2 cytokine production is similar to that reported herein. However, in contrast, trauma-hemorrhage resulted in increased cytokine production. This difference may be related to differences in the models. Despite the absence of a sex difference, the lack of IL-6, in IL-6 deficient mice, resulted in improvement in IL-4 production by splenocytes from male mice. The mechanisms responsible for mediating this IL-6 dependent response remains to be elucidated, however, this phenomena has been seen in various other models of inflammation, infection, and after *in vitro* immune stimulation.^{33,34} Further work is needed to better determine the pathway by which IL-6 can influence IL-4 levels following trauma.

The data presented herein confirm previously published reports showing that cytokine production after injury differs between males and females at early time points, and that males have a heightened suppression of the TH1 cytokine production. Furthermore, the suppression of TH1 and TH2 cytokines is mediated by IL-6. This animal model begins to offer some insight into immune mechanisms by which elevated IL-6 in males is associated with an increased risk of infection, sepsis, and death.

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Abbreviations

Con A	concanavalin A
DTH	delay-type hypersensitivity
IFN γ	interferon-gamma
IL-2	interleukin-2
IL-4	interleukin-4
IL-6	interleukin-6
KO	knockout
SOCS	Suppressors of cytokine signaling
TBSA	total body surface area

Fig. 1.

Splenocyte production of IL-6 after stimulation with Con A. Data were combined from 3 individual experiments and are shown as mean $\pm$ SEM. n=4-8 mice per group. *p<0.01 from male sham-injured mice, #p<0.01 from female sham-injured mice.

Fig. 2. Splenocyte production of IL-2 after stimulation with Con A. Data were combined from 5 individual experiments and are shown as mean $\pm$ SEM. n=6-14 mice per group. *p<0.01 from male and female sham-injured mice, #p<0.01 from female burn-injured mice.

Fig. 3.

Splenocyte production of IFN γ after stimulation with Con A. Data were combined from 5 individual experiments and are shown as mean $\pm$ SEM. n=4-18 mice per group. *P<0.01 from male sham-injured mice, ^P<0.05 from male sham-injured mice, #p<0.01 from female sham-injured mice @p<0.05 from female burn-injured mice.

Fig. 4. Splenocyte production of IL-4 after stimulation with Con A. Data were combined from 5 individual experiments and are shown as mean $\pm$ SEM. $n=7-18$ mice per group. * $p<0.01$ from male and female sham-injured mice.

Table 1

Splenocyte cytokine production in wild type and IL-6 KO mice. Data are expressed as % control, were combined from 3 individual experiments, and are shown as mean burn-injured result $\pm$ SEM. n=4-9 male mice per group. *p<0.01 from burn-injured wild type mice.

	Wild-type, %	IL-6 KO, %
IL-2	17.28 $\pm$ 2.40	63.31 $\pm$ 21.78*
IFN γ	24.17 $\pm$ 15.34	48.28 $\pm$ 35.11
IL-4	24.24 $\pm$ 2.72	86.24 $\pm$ 34.32*