Estrogen mediates the sex difference in post-burn immunosuppression

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Abstract

Previous studies in our laboratory have demonstrated that cell-mediated immune function was suppressed in female, but not male, mice at 10 days after burn injury and was mediated, in part, by increased production of interleukin-6 (IL-6). Because 17β-estradiol (E_2) influences immune function after trauma and the hormone is known to regulate IL-6 production, the effect of E_2 on immune function after thermal injury was examined. Increased circulating concentrations of E_2 corresponded with suppressed delayed-type hypersensitivity (DTH) and splenocyte-proliferative responses, and increased circulating concentrations of IL-6 in female mice after burn. Ovariectomy restored the suppressed DTH response and decreased IL-6 concentrations, and administration of exogenous E_2 to both ovariectomized females and intact male mice resulted in a suppressed DTH response. In addition, *in vitro* treatment with E_2 suppressed splenocyte proliferation in a macrophage-dependent manner and enhanced macrophage production of IL-6. These results strongly suggest that the sex difference in cell-mediated immunity 10 days after burn injury is mediated by altered concentrations of E_2, which in turn modulate key macrophage-derived immunoregulatory cytokines.

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Introduction

Previous studies in our laboratory have substantiated the presence of a temporal difference in the cell-mediated immune response in male and female mice (MS Gregory, DE Faunce, LA Duffner & EJ Kovacs, unpublished observations). The delayed-type hypersensitivity (DTH) and splenocyte-proliferative responses were suppressed in males at 1 and 4 days after burn injury, whereas females did not exhibit suppression until day 7. At 10 days post burn, the suppressed immune response in females corresponded with high circulating concentrations of interleukin (IL)-6, whereas the normal immune response observed in males corresponded with low circulating IL-6. Furthermore, post-burn treatment with an anti-IL-6 neutralizing antibody partially restored the DTH response in female mice. These results demonstrate that the suppressed immune function in females at 10 days post burn was mediated, in part, by increased IL-6.

Suppression of the cell-mediated immune response is one of the major complications that directly influences the outcome in burn-injury patients. After thermal injury, the macrophage has been shown to play a central role in the immune dysfunction through altered production of inflammatory mediators, such as tumor necrosis factor α (TNFα), IL-1 and -6, and prostaglandin E_2 (PGE_2) (Wood *et al.* 1987, Faist *et al.* 1988, 1996). IL-6 is a pleiotropic cytokine that was initially discovered as a regulator of B-cell maturation, but has since been found to regulate acute-phase protein induction and T-cell activation and proliferation (Akira *et al.* 1990). In addition, increases in IL-6 concentrations after trauma correlate with suppressed cellular immune responses and poor survival (Schluter *et al.* 1991, Ueyama *et al.* 1992, Drost *et al.* 1993, Bankey *et al.* 1995). Many different cell types, such as lymphocytes and fibroblasts, produce IL-6, and we and others have shown that macrophages/monocytes isolated from thermally injured animals produce high levels of IL-6 that correspond with decreased cell-mediated immune responses (Fukushima *et al.* 1994, Ogle *et al.* 1994, Faunce *et al.* 1998, MS Gregory, DE Faunce, LA Duffner & EJ Kovacs, unpublished observations).

Although we have shown that IL-6 plays a part in the sex difference in cell-mediated immune function after burn, the mechanism by which IL-6 is increased in females and not males in this circumstance remains unknown. Estrogens have been shown to alter several immune parameters, but the results of hormone action

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vary according to concentration (Grossman 1984, Schuurs & Verhuel 1990). Physiologic concentrations of estrogen stimulate humoral and cell-mediated immune responses (Paavonen *et al.* 1981, Weinstein *et al.* 1984) whereas high physiologic and superphysiologic concentrations of estrogen, such as are achieved during pregnancy, suppress both mitogenic (Purtillo *et al.* 1972, Thong *et al.* 1973) and cell-mediated immune responses (Finn *et al.* 1972, Thong *et al.* 1973). Gonadal steroid hormones are believed to alter the immune response by enhancing or impairing the responsiveness of immunocompetent cells (Paavonen 1994). Because we and others have shown that monocytes, macrophages and lymphocytes express estrogen receptors (Weusten *et al.* 1986, Frazier-Jessen & Kovacs 1995), increases in circulating 17β-estradiol (E_2) may directly alter cellular function, such as cytokine production.

Numerous studies have documented that pregnancy concentrations of E_2 suppress cell-mediated immune responses (Finn *et al.* 1972, Purtillo *et al.* 1972, Thong *et al.* 1973), possibly by altering the production of the macrophage-derived pro-inflammatory mediators, IL-1, IL-6, TNFα and PGE_2 (Cutolo *et al.* 1995, Kuckerman *et al.* 1996, Miller & Hunt 1996). Given that E_2 can regulate macrophage production of IL-6 (De *et al.* 1992, Li *et al.* 1993), and high concentrations of both E_2 and IL-6 suppress immune-cell function, we chose to investigate the relationship between E_2 and IL-6 production and immunosuppression after thermal injury in male and female mice. The studies described herein demonstrate that the sex difference in the cell-mediated immune response after thermal injury is E_2-dependent. Furthermore, these observations suggest that, in this thermal injury model, the effects of E_2 on cell-mediated immune function may involve hormonal regulation of IL-6 production by the macrophage.

Materials and Methods

Induction of thermal injury

Male and female BALB/c mice (10–12 weeks) from Charles River Laboratories (Portage, MI, USA) were maintained on a 12 h light : 12 h darkness cycle and had food and water available *ad libitum*. Mice were subjected to a 15% total body surface area (TBSA) dorsal scald or sham injury using a modification of the method of Walker & Mason (1968), as previously described (Faunce *et al.* 1997). Briefly, while mice were under anesthesia with Nembutal (40 mg/kg i.p.), clippers were used to remove the hair from the dorsum of each animal. Animals receiving scald injury were placed in a template allowing exposure of 15% TBSA and immersed in a 100 °C water-bath for exactly 8 s, then dried off with a towel to prevent any further scalding. Sham-treated animals were immersed in a room-temperature water-bath. Both burned and sham-injured animals were resuscitated with 1·0 ml i.p. saline and allowed to recover under a heat lamp. When animals were fully recovered from anesthesia, they were returned to their cages and maintained under standard conditions in the animal facility. The animal studies described herein were performed in accordance with the guidelines set forth by the Loyola University Chicago Institutional Animal Care and Use Committee.

Determination of DTH responses

DTH responses were induced as previously described (Faunce *et al.* 1997). Six days before thermal injury, mice were sensitized to the hapten, 2,4-dinitro-fluorobenzene (DNFB) (Acros Organics, New Jersey, NJ, USA) by applying 10 μl of a 0·5% solution of acetone–olive oil (4 : 1) directly to the skin of the abdomen. At 10 days after burn injury, mice received an eliciting dose of DNFB (20 μl 0·2% solution) applied to the pinna of the right ear. The animals used in the hormone replacement studies were sensitized to the DNFB hapten 6 days before insertion of the hormone implant, to insure that the hormone and placebo treatment did not differentially alter sensitization. The burn or sham injury was then given 5 days after the implant was inserted; thus 11 days elapsed between sensitization and burn or sham injury. The difference in sensitization timing between the unmanipulated and hormone-manipulated animals did not alter the magnitude of the DTH response in the respective sham-treated groups (see Figs 1, 3; MS Gregory & EJ Kovacs, unpublished observations). Ear thickness measurements were made just before elicitation and 24 h after elicitation. The magnitude of ear swelling was expressed as a percent change in ear thickness using the following formula: (Δ thickness/pre-elicitation thickness) × 100, where Δ thickness = post-elicitation minus pre-elicitation ear thickness. A group of naive animals received only the elicitation dose of DNFB for the determination of non-specific ear swelling attributable to the application of the hapten in vehicle. Left ear measurements were also obtained and served as internal controls for each animal. Comparisons between the mean percentage change in each group were made with analysis of variance (ANOVA) followed by Fisher's LSD *post-hoc* analysis.

In vitro *analysis of the cellular immune response*

At 10 days post burn, animals were sacrificed and the spleens were aseptically removed. Single cell suspensions of splenocytes were plated in 96-well microtiter plates at a density of 200 000 cells per well in RPMI medium supplemented with L-glutamine (2 mM), penicillin-G (100 U/ml), streptomycin (100 μg/ml), and 10% fetal bovine serum (FBS). The viability of the cells was confirmed to be >95% by trypan blue exclusion. Splenocyte cultures were incubated for 72 h at 37 °C in 5% CO_2 in the presence or absence of concanavalin A (Con A)

(2 μg/ml). Additional cultures were incubated with Con A (1 μg/ml) in the presence or absence of E_2 (300 pg/ml) (Sigma, St Louis, MO, USA). This concentration of E_2 (approximately 10^{-9} M) was used because it most closely mimicked the concentration of E_2 measured in females at 10 days post burn. Furthermore, because Con A at a concentration of 2 μg/ml induced maximal proliferation, a submaximal concentration of Con A (1 μg/ml) was used in order to examine whether E_2 modulated proliferation, to allow for detection of both stimulation and inhibition. For some studies, the splenocytes were plated for 1·5 h to remove the adherent population. Non-adherent cells were plated and cultured in the same manner as total splenocytes.

RPMI medium containing the pH indicator, phenol red, was used throughout the studies described herein, except when medium was used as a diluent for reagents in the colorimetric proliferation assay described below. Previously, we found that macrophage or lymphocyte activation and cytokine production were not sensitive to the 'estrogenic' effects of the phenol red pH indicator (Frazier-Jessen & Kovacs 1995, reported by Welshons *et al.* 1988). All studies were performed with a single lot of FBS, which was selected for low concentrations of sex steroids; a 10% solution of this lot of FBS contains 1·7 pg/ml E_2. We elected to use untreated, rather than charcoal-extracted FBS, because previous studies in our laboratory demonstrated that these low concentrations of steroid hormones do not alter leukocyte function and cytokine production (Frazier-Jessen & Kovacs 1995).

A colorimetric assay was utilized to measure proliferation as previously described (Denziot & Lang 1986). Briefly, after 68 h of incubation, plates were centrifuged, the supernatant removed, and 50 μl of a 1 mg/ml solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma Chemical Co., St Louis, MO, USA) in RPMI (phenol-red-free) medium was added to each well. Phenol-red-free medium was used in the colorimetric assay as a medium in which to dilute the MTT, because the red color interferes with the colorimetric assay. Plates were then incubated for 4 h at 37 °C in 5% CO_2. After the incubation, the untransformed MTT was removed by careful inversion and blotting. The reaction product, formazan, was solubilized by adding 50 μl isopropanol. The optical density of each well was measured using an automatic plate reader at 540 nm wavelength. All data are represented as percentage of control ± S.E.M (control being the optical density (OD) obtained from Con-A-stimulated splenocytes from sham-treated animals).

Determination of serum E_2 concentration and uterine weight

Ten days post burn, animals were sacrificed by CO_2 inhalation followed by cervical dislocation. Whole blood was obtained by cardiac puncture and allowed to coagulate at room temperature for 2 h. The serum concentration of E_2 was determined by ELISA (Cayman Chemical, Ann Arbor, MI, USA) and RIA (Diagnostic Products Corp., Los Angeles, CA, USA). Uterine weight was also measured, as an index of circulating E_2 concentrations (De & Wood 1990).

Measurement of circulating and macrophage supernatant concentrations of IL-6

For assessment of circulating concentrations of IL-6, serum was collected as described above and stored at −80 °C before assay. Purified splenic macrophages were obtained from splenocyte suspensions by adherence to tissue culture plastic, as previously described (Faunce *et al.* 1997). Briefly, 200 μl total splenocyte suspension (containing $1·0 \times 10^7$ cells/ml) was plated in each well of a 96-well microtiter plate. The cells were cultured for 1·5 h at 37 °C, after which the non-adherent cells were removed by washing twice with 37 °C phosphate buffered saline (PBS). This method yields approximately 200 000 macrophages per well, which are >98% positive for Mac-3 and Di-I-acetylated low-density lipoprotein uptake (data not shown). The purified splenic macrophages were cultured in RPMI medium containing 10% FBS for 18 h in the presence or absence of lipopolysaccharide (LPS) (1 μg/ml). In addition, splenic macrophages were cultured with E_2 (300 pg/ml) and LPS (1 μg/ml). The supernatants were filtered through 0·22 μm filters and stored in aliquots at −80 °C before assay. The IL-6 content in both the serum and macrophage supernatants was determined by ELISA, using a commercially available matched-antibody-paired kit (Endogen Inc., Cambridge, MA, USA). The enzyme immunoassay was performed according to the manufacturer's specifications. Values below the level of detection of the kit (15 pg/ml) were reported as zero.

Ovariectomy and hormone administration

For hormone replacement studies, female mice were ovariectomized (OVX) to remove the primary source of endogenous estrogen. One week after surgery, OVX females and intact male mice were randomly assigned to E_2, E_2 antagonist (17α-estradiol), or placebo groups. Under methoxyflurane (Metophane; Pitman-Moore Inc., Mundelein, IL, USA) anesthesia, OVX female and intact male mice were given subcutaneous implants of slow-diffusion polymer pellets containing E_2 (0·5 mg), 17α-estradiol (5·0 mg), or placebo (0·5 mg) (Innovative Research of America, Tampa, FL, USA) as previously described (Frazier-Jessen & Kovacs 1995). The pellets were designed to deliver continuously a prescribed dose of the particular hormone for 21 days. To attempt to mimic the hormone milieu observed in female mice 10 days after burn injury, we chose to use a 0·5 mg E_2 pellet, which maintained a circulating concentration of

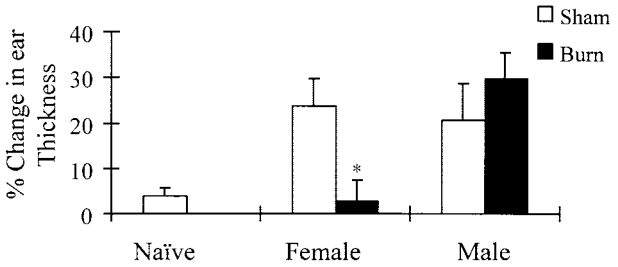

Figure 1 Effect of injury on the DTH response. Mice were sensitized to DNFB 6 days before burn or sham injury. Elicitation of DTH was performed in female and male mice at 10 days post injury. Naive animals received only the elicitation dose of DNFB for the determination of non-specific ear swelling attributable to the application of the hapten in vehicle and there was no difference between naive male and female mice. Values are means, with S.E.M. bars, $n=5$. *Significant difference from female sham ($P<0{\cdot}01$, ANOVA).

240 ± 80·7 pg/ml, as measured by RIA (Diagnostic Products Corp., Los Angeles, CA, USA). In order to block the endogenous E_2, a 5·0 mg pellet containing the 17α-estradiol was used, which would deliver a 10-fold excess of the antagonist.

Results

Sex differences in cell-mediated immune responses 10 days after burn injury

The DTH and splenocyte proliferative assays were used as indices of cell-mediated immunity, to examine the immune response of male and female mice to burn injury. In the absence of burn injury, the immune responses of sham-treated males and females were indistinguishable, but at 10 days after burn injury, females exhibited significant suppression of both the DTH and splenocyte-proliferative responses (Figs 1, 2). In contrast, neither the DTH nor the splenocyte-proliferative responses were suppressed in males and, in most studies, male mice exhibited responses that were enhanced above those in sham-treated animals. These results support previous findings (MS Gregory, DE Faunce, LA Duffner & EJ Kovacs, unpublished observations) that a sex difference exists in both the DTH and splenocyte-proliferative responses after thermal injury.

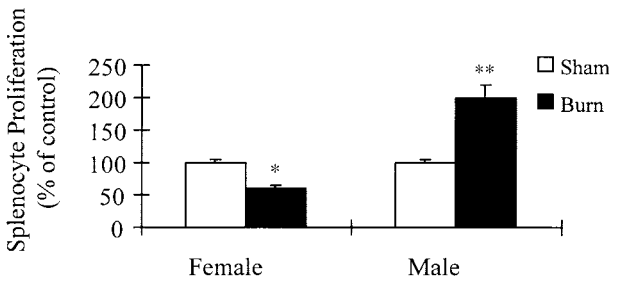

Figure 2 Effect of thermal injury on the splenocyte-proliferation responses. Splenocytes from female and male were mice harvested at 10 days post injury and cultured for 72 h in the presence of Con-A (2 µg/ml). Values are means, with S.E.M. bars, $n=8$. Control was the OD obtained from Con-A-stimulated splenocytes from sham-treated animals. Significant differences: *$P<0{\cdot}05$ compared with female sham, **$P<0{\cdot}01$ compared with male sham (ANOVA).

Table 1 Serum E_2 concentrations and uterine weight 10 days after burn injury. Values are means ± S.E.M.; $n=7$–8, for serum E_2 and $n=4$–7 for uterine weight

	Serum E_2 (pg/ml)	**Uterine weight** (mg)
Group		
Female sham	51·9 ± 14·8	77·8 ± 10·7
Female burn	114·5 ± 26·1*	121 ± 11·2*
Male sham	27·3 ± 14·8	NA
Male burn	46·5 ± 16·2	NA

NA=not applicable. *Significantly different from sham-treated controls ($P<0{\cdot}05$, ANOVA).

Effects of thermal injury on circulating concentrations of serum E_2 and uterine weight

To determine whether the circulating concentration of E_2 was altered in mice after burn injury, as is observed in burn patients (Dolecek 1985, 1989), serum E_2 concentrations were measured. Ten days after burn injury, circulating concentrations of E_2 in female mice were significantly increased in comparison with those in sham-treated controls ($P<0{\cdot}05$; Table 1). In contrast, there was a non-significant increase in E_2 in male mice at 10 days post burn. Although E_2 was increased in both thermally injured female and male mice, it is possible that a threshold concentration of E_2 must be achieved in order to observe suppression. Furthermore, the increased E_2 concentrations in the serum of female mice corresponded with a significant increase in uterine weight ($P<0{\cdot}01$).

Effect of E_2 treatment on the DTH response in female mice at 10 days post burn

To elucidate further the involvement of E_2 in the suppressed DTH response in female mice after burn injury, OVX mice were used to examine the role of exogenous E_2 and the E_2 antagonist, 17α-estradiol, on the DTH response after burn and sham injury. Both cycling and OVX+placebo sham-treated female mice were able to mount adequate DTH responses, as indicated by the 20–30% increase in ear thickness (Fig. 3). Whereas cycling females exhibited a suppressed DTH response 10 days after burn injury, OVX females given placebo hormone exhibited a DTH response equal to that of OVX sham-treated controls. The administration of exogenous E_2 to

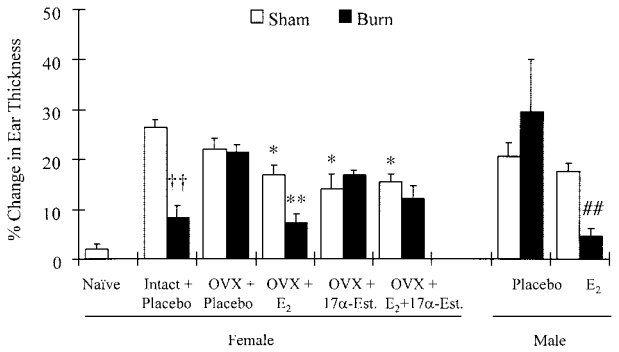

Figure 3 Effect of E_2 on the DTH response at 10 days post burn injury. Mice were sensitized to DNFB 6 days before treatment with hormones (E_2: 0·5 mg 21-day slow-release polymer pellet; 17α-estradiol (17α-Est.) 5 mg 21-day slow-release polymer pellet; both E_2+17α-estradiol; or placebo). Thermal or sham injury was performed 5 days after hormone treatment. Elicitation of DTH was examined 10 days after injury. Values are means, with S.E.M. bars, $n=5$ per group. Significant differences: *$P<0·05$ compared with female sham (OVX+placebo); $P<0·01$ compared with ††female sham (intact placebo), **female sham (OVX E_2), ##male sham E_2 (ANOVA).

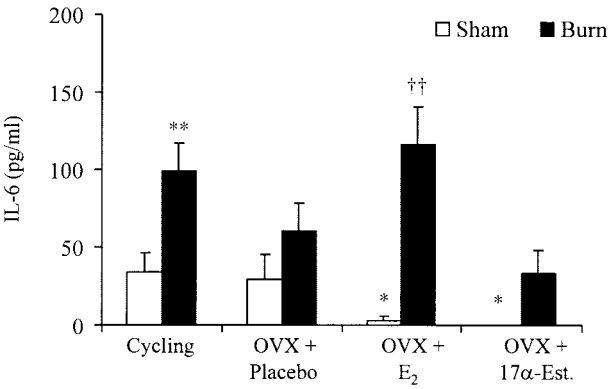

Figure 4 Effect of E_2 on circulating IL-6 concentrations in burned female mice. Blood was obtained by cardiac puncture from female mice 10 days post burn injury. Serum IL-6 content was measured by ELISA. Values are means, with S.E.M. bars, $n=6$ per group. 17α-Est., 17α-estradiol. Significant differences: *$P<0·05$ compared with OVX+placebo sham; $P<0·01$ compared with **cycling sham, ††OVX+E_2 sham (ANOVA).

OVX females before burn injury resulted in a significant suppression of the DTH response at 10 days post burn, compared with responses in OVX+placebo and OVX+E_2 sham-treated controls ($P<0·01$). Furthermore, OVX female mice treated with 17α-estradiol did not exhibit a suppressed DTH response at 10 days post burn compared with OVX+17α-estradiol sham-treated controls. However, the administration of both E_2 and 17α-estradiol to OVX females partially restored the suppressed DTH response observed in thermally injured OVX females given E_2 alone.

Effect of E_2 treatment on the DTH response after burn injury in male mice

High concentrations of E_2 corresponded with a suppressed DTH response in females 10 days after burn injury. Therefore, parallel studies were performed to determine whether administration of exogenous E_2 could suppress the DTH response of male mice at this time. Intact male mice were given subcutaneous implants of E_2 (0·5 mg) 5 days before burn or sham injury. Sham-treated male mice given placebo hormone (Fig. 3) exhibited a normal DTH response comparable to that of sham-treated controls not given hormone pellets (Fig. 1). Furthermore, treatment with placebo hormone pellets did not alter the DTH response after burn injury. The DTH response of sham-treated male mice given E_2 did not differ from that of placebo treated sham controls, but there was a suppressed DTH response in burned males given E_2.

Effect of thermal injury on circulating IL-6 concentrations

IL-6 is a pleiotropic cytokine that, in high concentrations, can inhibit immune function (Zhou *et al.* 1991, 1992). Previous studies in our laboratory have shown that immune dysfunction after thermal injury is mediated, in part, by increased concentrations of IL-6 (Faunce *et al.* 1998, MS Gregory, DE Faunce, LA Duffner & EJ Kovacs, unpublished observations). At 10 days post burn, the circulating concentration of IL-6 in male mice (116 ± 25·8 pg/ml) was not significantly increased compared with that in the sham-treated group (66·7 ± 20·8 pg/ml). In contrast, the circulating concentration of IL-6 in thermally injured female mice (139·5 ± 18·2 pg/ml) was markedly increased when compared with that in sham-treated controls (61·5 ± 10·6 pg/ml; $P<0·01$).

Effect of E_2 treatment on circulating IL-6 10 days after burn injury

OVX females given placebo hormone replacement before burn injury exhibited a normal DTH response, and the administration of E_2 to OVX females before burn injury resulted in significant suppression of the DTH response. Furthermore, high circulating IL-6 concentrations corresponded with both suppressed DTH responses and increased E_2 concentrations in females 10 days post burn. Therefore, circulating concentrations of IL-6 after OVX and hormone manipulation in female mice were measured. The administration of E_2 to sham-treated OVX females resulted in a suppression of circulating IL-6, which agrees with other studies showing that, under normal conditions, E_2 downregulates IL-6 production (Fig. 4) (Stein & Yang 1995, Depshpande *et al.* 1997). At 10 days post burn, intact cycling females exhibited a three-fold

increase in circulating IL-6 concentrations, whereas OVX females given placebo hormone exhibited IL-6 concentrations equal to those of sham-treated controls. In addition, the administration of E_2 before burn injury resulted in a four-fold increase in circulating IL-6 concentrations over those in sham-treated controls, and these concentrations were similar to those observed in cycling females subjected to thermal injury. Furthermore, treatment of female mice with 17α-estradiol resulted in IL-6 concentrations comparable to those of sham-treated intact females. These data demonstrate that, whereas *in vivo* treatment with E_2 decreased circulating concentrations of IL-6 production in sham-treated animals, E_2 replacement increased the circulating concentrations of IL-6 in thermally injured female mice. Taken together, these results suggest that the normal estrogen regulation of IL-6 production is altered after thermal injury in female mice.

Effect of E_2 on total and non-adherent splenocyte proliferation

We have previously demonstrated that supernatants from splenic macrophages obtained from female mice 10 days post burn suppressed normal splenocyte proliferation, and that increased concentrations of macrophage-derived IL-6 corresponded with suppressed splenocyte proliferation (MS Gregory, DE Faunce, LA Duffner & EJ Kovacs, unpublished observations). To determine whether estrogen suppresses splenocyte proliferation through interaction with the macrophage or through interaction with the non-adherent T-cell or B-cell populations, total and non-adherent splenocytes were cultured in parallel. *In vitro* treatment with E_2 (300 pg/ml) suppressed total splenocyte proliferation by nearly 30% in cultures prepared from sham-treated females compared with Con A stimulation alone ($P<0{\cdot}01$; Fig. 5). In contrast, induction of proliferation in non-adherent splenocytes exposed to E_2 was not altered, suggesting that E_2 is acting directly on the macrophage and not the non-adherent population. Furthermore, E_2 treatment failed to suppress further the proliferation of splenocytes prepared from females 10 days post burn, possibly because these cells had already been exposed to significantly increased concentrations of E_2 *in vivo*.

Effect of in vitro *E_2 on macrophage production of IL-6*

E_2 did not alter IL-6 production by unstimulated macrophages, but the hormone significantly enhanced IL-6 production by LPS-stimulated macrophages prepared from sham-treated females compared with the effect of LPS alone (Fig. 6; $P<0{\cdot}05$). Furthermore, these concentrations of IL-6 were similar to those measured in LPS-stimulated macrophage cultures prepared from thermally injured female mice. Interestingly, no further increase in IL-6 production was induced by E_2 in LPS-treated macrophages from thermally injured female mice. This lack of

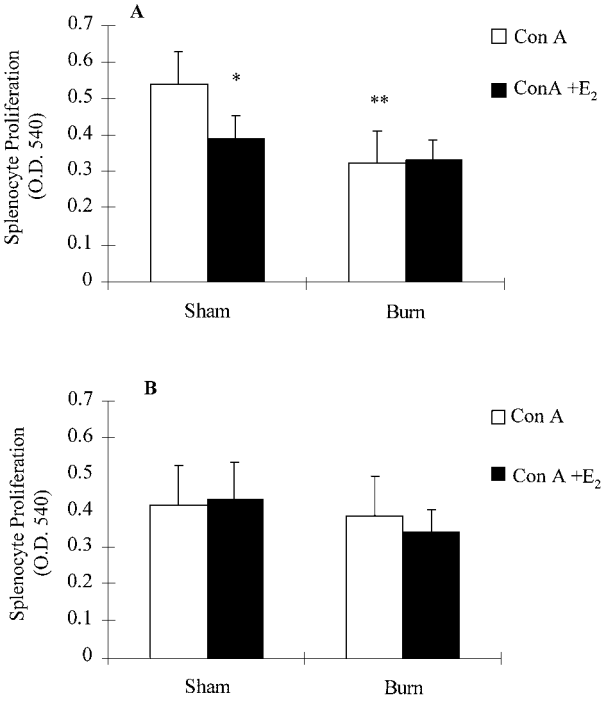

Figure 5 Effect of E_2 on total and non-adherent splenocyte proliferation in female mice. Splenocytes were harvested from female mice at 10 days post sham or burn injury. Total (A) and non-adherent (B) splenocytes were prepared as described in Methods and cultured in the presence of Con A (1 μg/ml) alone or in conjunction with E_2 (300 pg/ml). Proliferation was measured using the MTT assay. Values are means, with S.E.M. bars, $n=8$. Significant differences: *$P<0{\cdot}05$, **$P<0{\cdot}01$, compared with sham stimulated with Con A alone (ANOVA).

E_2-mediated increase in IL-6 production corresponded with the inability of E_2 to alter proliferation in cultures prepared from these female mice at 10 days post burn (Fig. 5). Again, this may be due to the *in vivo* exposure of

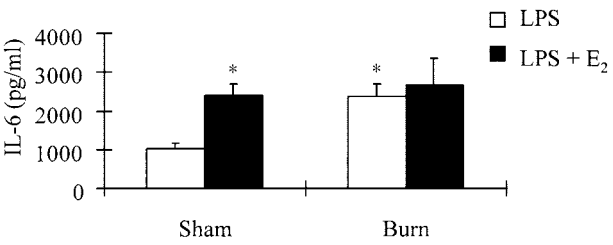

Figure 6 Effect of E_2 on macrophage production of IL-6. Splenic macrophages were purified from female mice at 10 days post burn and cultured at a density of 200 000 cells/well in the presence of LPS (1 μg/ml) alone or in conjunction with E_2 (300 pg/ml) for 18 h. Values are mean supernatant IL-6 concentrations, with S.E.M. bars, $n=4$–6. *Significantly different from sham-treated female controls stimulated with LPS alone ($P<0{\cdot}05$, ANOVA).

these macrophages to the high circulating concentrations of E_2 that were measured after thermal injury.

Discussion

A number of studies have documented the significant influence that gonadal steroid hormones have on normal immune function, and have concluded that physiologic concentrations of the female hormone, estrogen, stimulates, whereas the male hormone, testosterone, suppresses, cell-mediated immunity (Paavonen *et al.* 1981, Grossman 1984). Under normal conditions, females have a stronger cellular and humoral immune response compared with males, leading to increased susceptibility to autoimmune diseases (Ansar-Ahmed *et al.* 1985). However, high physiologic concentrations of estrogen, such as those achieved during pregnancy, have been shown to suppress both mitogenic (Thong *et al.* 1973) and cell-mediated immune responses (Finn *et al.* 1972). Although there is considerable scientific evidence demonstrating how estrogen alters immune function, the reports are somewhat inconsistent. These inconsistencies are probably attributable to the use of different concentrations of estrogen, different types of estrogen (estriol, estrone and artificial estrogens), and different cell types (Ansar-Ahmed *et al.* 1985, Schuurs & Verheul 1990, Frazier-Jessen &Kovacs 1995, Miller & Hunt 1996, Depshpande *et al.* 1997).

The studies presented herein demonstrate that increased serum concentrations of E_2 in female mice play a significant role in the suppressed DTH and splenocyte-proliferative responses observed 10 days after burn injury. Although several studies have examined cell-mediated immune function in either male or female mice after thermal injury, there are no reports in which the immune response of both sexes were directly compared at 10 days post burn. Others have investigated the sex difference in immune function and survival after hemorrhagic shock and sepsis, and reported increased survival and immune function in females compared with those in males 24 h after injury (Wichmann *et al.* 1996, Angele *et al.* 1997, Zellweger *et al.* 1997). Thus, at this early time after injury, female mice tolerate both sepsis and hemorrhagic shock better than males. This observation is in agreement with our data demonstrating that, at 24 h after scald injury, male mice, but not females, exhibit suppressed cell-mediated immune function (MS Gregory, DE Faunce, LA Duffner & EJ Kovacs, unpublished observations).

In support of the findings within the present report, several other clinical studies have demonstrated that immune function after trauma is influenced by altered concentrations of gonadal steroid hormones such as E_2 (Dolecek 1985, 1989, Plymate *et al.* 1987, Christeff *et al.* 1988, Fourrier *et al.* 1994). Similar to the fluctuations in the concentrations of gonadal steroid hormones observed during the estrous cycle and pregnancy, the onset of critical illness has also been shown to alter the production of these hormones. Circulating concentrations of E_2 are enhanced, whereas testosterone concentrations are depressed in male patients within the first 48–72 h of myocardial infarction, sepsis or shock, and after thermal injury (Klaiber *et al.* 1982, Woolf *et al.* 1985, Christeff *et al.* 1988, 1992, Fourrier *et al.* 1994). Studies have also shown that circulating estrogen is increased to a greater concentration (Fourrier *et al.* 1994) in septic females than in males, and remains increased for an extended period of time; thus more dramatic consequences of estrogen would be observed in females.

In addition to the direct control that estrogen has on lymphocyte and macrophage function, the hormone has also been shown to alter the hypothalamic–pituitary–adrenal (HPA) axis, through modulating the release of both hypothalamic and pituitary peptides (Grossman 1991, Shupnik 1996), which could in turn mediate the actions of estrogen on the immune system. Under normal conditions, increased concentrations of E_2 inhibit the secretion of gonadotropin-releasing hormone, which would feed back and inhibit gonadal function, thus decreasing the production of gonadal steroids (Grossman 1991). Interestingly, after thermal injury, gonadal function is inhibited, but circulating concentrations of E_2 remain increased (Dolecek 1989); therefore, the source of the increased estrogen has been under investigation. The findings of some studies have suggested that the significant increase in estrogen production that occurs during sepsis may occur through cortisol-stimulated aromatization of androgens in adipose tissues (Simpson *et al.* 1981, Lephart *et al.* 1987). Alternatively, this increase in the circulating concentration of estrogen may be due, in part, to a decrease in estrogen catabolism, which can occur during liver failure (Kley *et al.* 1976, Demelia *et al.* 1987).

The source of increased estrogen after burn injury remains under debate, but the effect of the hormone on immune function also has not been resolved. Under normal physiologic conditions, estrogen has been shown to enhance the production of other hormones, including prolactin and growth hormone which, in turn, stimulate the immune response (Lieberman *et al.* 1981, Grossman 1991). Therefore, it is possible that the increased concentrations of E_2 measured in females at 10 days post burn (Table 1) could alter immune function indirectly through the upregulation of prolactin production. However, the E_2-enhanced production of pituitary-derived prolactin is maximal at physiologic concentrations of E_2 (Lieberman *et al.* 1981). Although circulating concentrations of prolactin were not measured in the present study, it is unlikely that the consequences of E_2 function were mediated by prolactin. The concentrations of E_2 were greater than those that stimulate prolactin production. Furthermore, prolactin stimulates immune function, whereas thermally injured mice with high E_2 concentrations were immunosuppressed.

In addition to the actions of estrogen on the HPA axis, the hormone can also directly modulate the production of macrophage-derived cytokines. High or pregnancy concentrations of estrogen enhance macrophage production of TNFα, IL-1, IL-6, and PGE_2 (Hu *et al.* 1988, Li *et al.* 1993, Chao *et al.* 1995, Frazier-Jessen & Kovacs 1995). These same mediators are increased in the serum and tissues of patients with sepsis and in animal models of thermal injury (Waage *et al.* 1989, Ogle *et al.* 1994). Further studies reveal that IL-6 is the best predictor of poor prognosis after burn and trauma (Schluter *et al.* 1991, Ueyama *et al.* 1992, Drost *et al.* 1993, Bankey *et al.* 1995).

IL-6 has a broad spectrum of activities and, under normal physiologic conditions, has a key stimulatory role in maintaining normal cellular immune function (Liu *et al.* 1994). Furthermore, Kopf and colleagues (1994) have demonstrated, through the development of an IL-6 knockout mouse, the importance of IL-6 in maintaining cellular homeostasis. In particular, both the T- and B-cell-mediated immune responses are impaired in the IL-6 knockout mice (Kopf *et al.* 1994).

Although IL-6 is typically thought of as immunostimulatory under normal circumstances, several clinical conditions, such as burn, trauma and infection, have been associated with abnormally high concentrations of IL-6 and are characterized by abnormal immune function (Zhou *et al.* 1992). Increased circulating concentrations of IL-6 correlate with impaired T-cell responses after trauma (Zhou *et al.* 1991). In addition, the outcome after burn and sepsis can be improved by blocking the actions of IL-6 with an anti-IL-6 neutralizing antibody (Starnes *et al.* 1990, Genarri *et al.* 1994). Furthermore, we have demonstrated that the sex difference in cell-mediated immune function is mediated in part through alterations in macrophage production of IL-6, and post-burn treatment with an anti-IL-6 neutralizing antibody partially restores immune function (MS Gregory, DE Faunce, LA Duffner & EJ Kovacs, unpublished observations).

The data presented in this paper demonstrate that the suppressed cell-mediated immune response observed in females 10 days after burn injury can be restored upon removal of the ovaries or administration of an estrogen antagonist (Fig. 3). Furthermore, the suppressed DTH response corresponded with increased concentrations of circulating IL-6 (Fig. 4). Surprisingly, though, the sham-treated OVX females given high, pregnancy, concentrations of E_2 exhibited a suppressed DTH response that did not correspond with increased concentrations of IL-6. In fact, the circulating concentrations of IL-6 were decreased compared with those in animals given OVX+placebo. These observations demonstrate that estrogen may differentially alter IL-6 production, depending on the environment. Under normal conditions, E_2 downregulates IL-6 production whereas, in the post-burn period, E_2 may upregulate it. Prolonged exposure to increased concentrations of E_2 *in vivo* may induce alterations in estrogen receptor levels or sensitivity, or both, which could lead to differences in E_2 regulation of IL-6 production. We have demonstrated that splenic macrophages express estrogen receptors α and β (MS Gregory & EJ Kovacs, unpublished observation), but further analysis is necessary to determine whether the concentrations of these receptors are altered after thermal injury.

Alternatively, because treatment with an anti-IL-6-neutralizing antibody only partially restored the DTH response in females at 10 days post burn, regulation of the cell-mediated immune response by estrogen may be controlled by other mediators in addition to IL-6 (MS Gregory, DE Faunce, LA Duffner & EJ Kovacs, unpublished observations). Our data indicate that E_2 suppresses splenocyte proliferation and that this suppression is macrophage dependent. Therefore, other macrophage-derived mediators may be involved. Many studies have demonstrated that IL-6 and PGE_2 are simultaneously increased after burn injury, but it is not known whether these mediators regulate each other. After trauma, macrophages rapidly produce and release PGE_2 (Faist *et al.* 1988, 1996). This arachidonic acid metabolite is a very powerful immune suppressant that suppresses T-cell mitogenesis, IL-2 production, and IL-2 receptor expression (Faist *et al.* 1996). Numerous studies have also demonstrated that estrogen upregulates macrophage-derived PGE_2 production (Du *et al.* 1984, Chang 1989) and that PGE_2, in turn, can enhance IL-6 production (Miller & Baker 1979, Fukushima *et al.* 1994). Therefore, E_2 may be suppressing cell-mediated immune function in female mice at 10 days post burn through increasing circulating concentrations of both PGE_2 and IL-6 during the post-burn period.

In conclusion, this study demonstrates that there is a sex difference in the cell-mediated response 10 days after burn injury. Furthermore, these data strongly suggest that E_2 is an important regulator of this sex difference in immune function, and that the hormone functions, in part, through the control of IL-6 production by macrophages. Therefore, manipulation of the hormone milieu may improve the immune status of the critically burned female patient.

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