Genetic Diversity and sexual-Dimorphisms are important contributors to the Inflammatory Response induced by endotoxin

Heiko Trentzsch

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Aus der Chirurgischen Klinik und Poliklinik

der Ludwig-Maximilians-Universität München

Direktor: Prof. Dr. med. K.W. Jauch

In Kooperation mit der Division of Pediatric Surgery, Department of Surgery

Johns-Hopkins-University, Baltimore, Maryland, USA

Direktor: Dr. Paul Colombani, M.D.

GENETIC DIVERSITY AND SEXUAL-DIMORPHISMS ARE IMPORTANT CONTRIBUTORS TO THE INFLAMMATORY RESPONSE INDUCED BY ENDOTOXIN

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Vorgelegt von

Heiko Trentzsch

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1. Berichterstatter:	Prof. Dr. Eugen Faist
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Meinen Eltern

"Twenty years from now you will be more disappointed by the things that you didn't do than by the ones you did do. So throw off the bowlines. Sail away from the safe harbor. Catch the trade winds in your sails. Explore. Dream. Discover."

Mark Twain

TABLE OF CONTENTS

1. INTRODUCTION

1.1. Sepsis

Injury induced by a stochastic event, such as trauma or after an operative intervention initiates an inflammatory response directed to control the initial insult. Patients, who withstand the initial injury, are still at risk to suffer serious deterioration of their health by secondary responses mounted after the initiating insult. Such secondary responses are commonly sepsis, acute respiratory distress syndrome (ARDS) and multiple organ dysfunction syndrome (MODS) (Baue 1975; Meakins 1990; Baue 1996). Morbidity and mortality associated with these conditions are a major health problem (Rangel-Frausto *et al.* 1995). A national estimate of 751,000 cases of sepsis is predicted per annum in the U.S, with an average hospital stay of 19.8 days and costs of approximately $22.100 per case (Angus *et al.* 2001).

An intriguing question that arises from clinical observations is the diversity in the outcome after severe injury. Thus, it could be hypothesized that the regulation of the response to injury is different among human beings, thus resulting in a different incidence of sepsis, ARDS and MODS.

1.2. Mediators of sepsis

Although the patho-physiological mechanism that underlines these syndromes is not exactly clear, it seems to proceed from an uncontrolled inflammatory response (Livingston *et al.* 1995). The inflammatory response is composed by the orchestrated expression of several factors directed to repair the initial insult. In addition, the inflammatory process auto-regulates itself through actions that aim at clearing components of the inflammatory cascade.

Lipopolysaccaride (LPS), or endotoxin, is a component of the outer cell-wall of gram-negative bacteria (Mayeux 1997). In patients with bacteremia, LPS can be detected in about 30% of patients (Cohen 2000). LPS is considered to play a key-role in human gram-negative septic shock (Kelly *et al.* 1997). Injection of bacterial LPS to healthy volunteers results in a hyper-dynamic metabolic state accompanied by an acute inflammatory response, which mimics several aspects of gram-negative sepsis (Bone 1992; Mayeux 1997).

LPS in circulation is recognized by a protein, named LPS-binding protein (LBP). The LPS-LBP-complex interacts with a surface receptor on monocytes and macrophages, coined CD-14 (Wright *et al.* 1990). CD-14 is a glycosyl-phospho-inositol (GPI) anchored glycoprotein, which does not possess any trans-membrane or cytosolic domains. Thus, the signal transduction triggered by LPS requires accessory membrane associated proteins. One of these accessory proteins is Toll-like receptor 4 (TLR-4). Via a complex signal transduction pathway activated by TLR-4, the transcription factor NF-κB is activated, and is translocated into the nucleus to initiate transcription of pro-inflammatory genes that encode for mediators of the inflammatory response, such as *Tumor Necrosis Factor α* (TNF- α) (Beutler *et al.* 2001).

TNF-α belongs to the family of cytokines. They can be divided into two major groups: pro-inflammatory cytokines (e.g. TNF-α, IL-1β) that initiate the inflammatory response, and anti-inflammatory cytokines (e.g. IL-10), which regulate the inflammatory process. The presence of cytokines in circulation is considered a good marker of the inflammatory response (Ertel *et al.* 1993; Volk *et al.* 1999; Hoflich *et al.* 2002). TNF- α is a pro-inflammatory cytokine that is produced by macrophages, monocytes, neutrophile granulocytes, natural killer-cells and keratinocytes. The production of TNF- α can also be induced by a vast number of stimuli, such as bacteria, fungi, tumor cells, cytokines like IL-1, IL-2, interferon γ (IFN- γ) etc. It is considered a major mediator in the physiological response to shock and sepsis with a broad range of effects on cells of the immune system. In addition, TNF- α stimulates the proliferation of T- and B-cells, the expression of *major histocompatibility complex* antigens (MHC-I and MHC-II) and modulates the expression of adhesion-molecules on endothelial cells. TNF- α also interacts with anticoagulation properties of the endothelium and promotes prostaglandin E2 formation. TNF-α is chemotactic for neutrophils and induces the production of IL-2 receptors and IFN-γ from T-cells (Aggarwal *et al.* 1996). TNF- α plasma levels peak 1.5 to 2h after LPS-injection before going back to baseline (Villa *et al.* 1995; De Maio *et al.* 1998; Remick *et al.* 2000). TNF-α has been implicated as major negative factor in the clinical outcome from sepsis in humans (Damas *et al.* 1989; Baud *et al.* 1990; Pinsky *et al.* 1993). However, studies on the effects of anti-TNF-α antibodies in humans remain controversial. Most studies could not demonstrate improved survival in septic patients (Abraham *et al.* 1995; Cohen *et al.* 1996; Reinhart *et al.* 1996; Abraham *et al.* 1998; Abraham *et al.* 2001). Interestingly, LPS-induced TNF-α levels in the supernatant of monocyte-cultures from healthy humans show inter-individual, probably HLA –associated differences (Molvig *et al.* 1988). Differences in LPS-induced TNF-α levels between A/J and B6 mice have also been observed and were referred to genetic variability within the same species (De Maio *et al.* 1998). Polymorphisms in the TNF-α gene of these two strains have been reported (Iraqi *et al.* 1997, 1999). In humans, at least two polymorphism have been evaluated for their association with increased susceptibility to severe sepsis (Stuber *et al.* 1995; Stuber *et al.* 1996; Fang *et al.* 1999; Mira *et al.* 1999; Schroeder *et al.* 1999; Schroder *et al.* 2000; Riese *et al.* 2003). These findings suggest that genetic diversity may have stronger impact on inter-individual differences than originally anticipated. Interestingly, a gender component was described in one of these studies (Schroder *et al.* 2000).

IL-10 is also produced by macrophages, T lymphocytes and epithelial cells and is one of the most important anti-inflammatory cytokines (Moore *et al.* 1993; Oberholzer *et al.* 2002). IL-10 has a direct role in the down-regulation of several pro-inflammatory cytokines, particularly TNF-α (Marchant *et al.* 1994; van der Poll *et al.* 1997). Down-regulation of TNF-α by IL-10 occurs at multiple levels including transcriptional and posttranscriptional mechanisms (Gerard *et al.* 1993; Marchant *et al.* 1994; van der Poll *et al.* 1997). The importance of IL-10 has been illustrated in mice lacking the *IL-10* gene, which develop symptoms similar to *Crohn's* disease (Kuhn *et al.* 1993). Moreover, *IL-10* knock out mice injected with bacterial lipopolysaccharide (LPS) displayed elevated levels of TNF-α as compared to wild type animals (Kuhn *et al.* 1993). Administration of exogenous IL-10 to female BALB/c mice resulted in protection from injection of LPS (Howard *et al.* 1993). Similarly, male BALB/c mice treated with IL-10 were protected from *Staphylococcal* enterotoxin-B (Bean *et al.* 1993). No protection was observed in female BALB/c mice after cecal ligation and puncture (CLP), an experimental model of sepsis (Remick *et al.* 1998). Over-expression of *IL-10* improved survival of male B6 mice after challenge with *E. coli* (Takakuwa *et al.* 2000). Early treatment with anti-IL-10 antibodies also increased survival of male A/J mice after CLP (Lyons *et al.* 1999). Interestingly, the response to an inflammatory stimulus varies between strains of inbred mice. We have previously reported that B6 mice showed higher IL-10 plasma levels in comparison with A/J mice after CLP (Stewart *et al.* 2002). Consequently, a contribution of the genetic background has to be assumed to the inflammatory response in general.

1.3. Genetic components in sepsis

A genetic component in the response to injury has recently been demonstrated. Different frequencies of mortality after injection of LPS were observed in various mouse strains (De Maio *et al.* 1998). Similar observations were made after CLP (Stewart *et al.* 2002), and mechanical, thermal and radiation injury (Radojicic *et al.* 1990). Different components of the inflammatory process, such as circulating cytokine levels, acute phase gene expression, and infiltration of polymorph-nuclear leukocytes in liver and lung have been observed to be different between various mouse strains after injection of LPS (De Maio *et al.* 1998; O'Malley *et al.* 1998) or after CLP (Stewart *et al.* 2002). Loci contributing to the LPS response have been identified in mouse (Matesic *et al.* 2000). Similarly, mutations in TLR-4 of C3H/HeJ and C57BL/10SCr mice have been identified as the responsible factor for their resistance to LPS (Qureshi *et al.* 1996; Poltorak *et al.* 1998). Studies in humans also indicate a genetic component in the response to several inflammatory conditions. The polymorphism in the promoter of the human TNF-α gene, 308(G/A), has been associated with increased risk of sepsis related mortality (Stuber *et al.* 1995; Mira *et al.* 1999). The TNF^{β}, LT^{α}-Nco1 polymorphism of the *TNF*B locus (TNFB1/2) has been correlated with increased mortality of septic patients (Stuber *et al.* 1996; Fang *et al.* 1999; Schroeder *et al.* 1999; Schroder *et al.* 2000), with an increased risk to develop severe posttraumatic sepsis (Majetschak *et al.* 1999) or with higher risk for complications after major surgery (Riese *et al.* 2003). Similarly, *Tnf* polymorphisms are associated with negative outcome from other infectious diseases, such as cerebral malaria (McGuire *et al.* 1994), leishmaniasis (Cabrera *et al.* 1995), and autoimmune diseases, such as systemic lupus erythematosus (Jacob *et al.* 1991). Consequently, genetic diversity has to be taken into account to explain the variable response observed in patients and in order to identify risk-factors that predict increased susceptibility to develop severe sepsis after a major injury.

1.4. Gender as a risk factor for the outcome from sepsis

Another confounding factor in the response to injury is gender. Male gender has been associated with a higher risk of infections after injury (Offner *et al.* 1999). After surgery, male patients require therapy in the surgical intensive care unit more frequently than females and have higher incidence of severe sepsis and septic shock as compared to females (Wichmann *et al.* 2000). Females from different species have been demonstrated to be more resistant to bacterial, viral, and parasitic infections (Klein 2000). In general, female rodents have an enhanced immunological response with respect to males, resulting in better survival after injury (Zellweger *et al.* 1997; Angele *et al.* 2000). In particular, cytokine-secretion (IL-1, IL-2, IL-3, and IL-6) from isolated peritoneal and splenic macrophages from C3H/HeN mice after hemorrhagic shock were higher in females than in males (Wichmann *et al.* 1996). Similar studies provide evidence that female C3H/HeN mice are immunologically more competent than male mice after CLP (Zellweger *et al.* 1997). Gender differences have also been demonstrated in the response after burn injury in BALB/c mice (Gregory *et al.* 2000). This lower susceptibility of females to different insults has been explained by the presence of sex steroids.

Castrated C3H/HeN male mice supplemented with 5α-dihydrotestosterone (DHT) show a decrease in the levels of pro-inflammatory cytokines after hemorrhagic shock with respect to non-castrated mice. These findings were prevented when castrated male mice were supplemented with EST (Angele *et al.* 1999). A decrease in testosterone levels by either surgical castration or pharmacological blockage has been shown to be beneficial in C3H/HeN male mice after hemorrhagic shock (Remmers *et al.* 1997; Wichmann *et al.* 1997; Remmers *et al.* 1998).

However, controversial findings have been made in studies that found no gender difference (Riche *et al.* 1996; Eachempati *et al.* 1999; Wichmann *et al.* 2000), or an even higher mortality in septic female patients (McLauchlan *et al.* 1995; Napolitano *et al.* 2001; O'Keefe *et al.* 2001). Some authors even described higher incidence of infection in female patients (Dinkel *et al.* 1994; Kollef *et al.* 1997). In summary, it seems as if there is no general consensus whether gender is a positive or negative factor in the outcome from injury and how such controversial findings may be interpreted.

Interestingly, differences in *TNFB* gene distribution of septic patients have been correlated with a better outcome in females as compared to males (Schroder *et al.* 2000). It is possible that genetic variability has major impact on gender differences in the inflammatory response or its modulation by sex-steroids and thus may explain controversial observations on gender as a potential risk-factor that determines the outcome from severe sepsis.

2. AIMS OF THE STUDY

We hypothesized that gender differences are also dependant on genetic traits. Since it is well-anticipated that sex-steroids are most likely responsible for gender differences, the effect of sex-steroids on the inflammatory process was investigated. To test this hypothesis, we designed an experimental model to answer the following question:

- Is gender a contributing factor to the LPS-induced inflammatory response?
- Can sex-steroids or hormone depletion by surgical castration modulate this response?
- Is this modulation dependent of the genetic background of the individual?
- Do sex-chromosomes carry information that is responsible for a variable response?
- Do changes in the response that were induced with sex-steroids or hormone depletion affect the outcome from endotoxic shock?

3. MATERIAL AND METHODS

3.1. Animals

Male and female A/J, AKR/J, BALB/cJ, C57BL/6J (*herein designated as B6*), and DBA/2J mice at the age of 6 weeks were obtained from Jackson Laboratory (Bar Harbor, Maine). Additional male C3H/HeN mice were purchased from Charles River Laboratory (Portage, Michigan).

B6AF1 mice (*F1-generation; herein designated as B X A*), the offspring from a B6 female and an A/J male were purchased from Jackson Laboratory (Bar Harbor, Maine). F1s bred from an A/J female and a B6 male are not commercially available. Hence, AB6F1, (*herein designated as A X B*) were bred in our laboratory animal facility.

All mice were maintained under identical environmental conditions in a pathogen-free animal facility. All procedures were carried out in accordance with the guidelines for the Care and Use of Laboratory Animals by the National Institutes of Health (NIH). The experimental protocol was approved by the Institutional Animal Care and Use Committee of Johns-Hopkins-University School of Medicine.

3.2. Castration

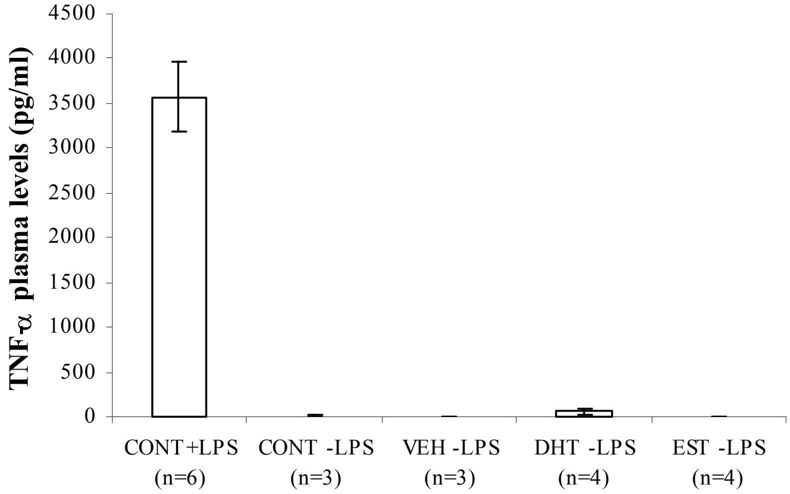
Male mice (6 weeks old) were anesthetized with an intraperitoneal injection of Avertin (400 to 500 mg/kg body weight). Avertin, a common rodent anesthetic, was composed of 0.9 mM 2,2,2 tribromoethanol (Aldrich Chemical Company, Milwaukee, Wisconsin) in an aqueous solution of 0.5 % (V/V) tert-amyl alcohol (Aldrich Chemical Company, Milwaukee, Wisconsin). After anesthesia, the abdomen was opened by low midline laparotomy, testicles were exposed from the scrotum, and were removed after ligation with a single suture (Silk 4-0; Ethicon Inc., Somerville, New Jersey). The abdominal incision was closed in two layers with absorbable suture (Polysorb 4-0; USSC, Norfalk, Connecticut). No mortalities were observed in castrated mice without LPS challenge.

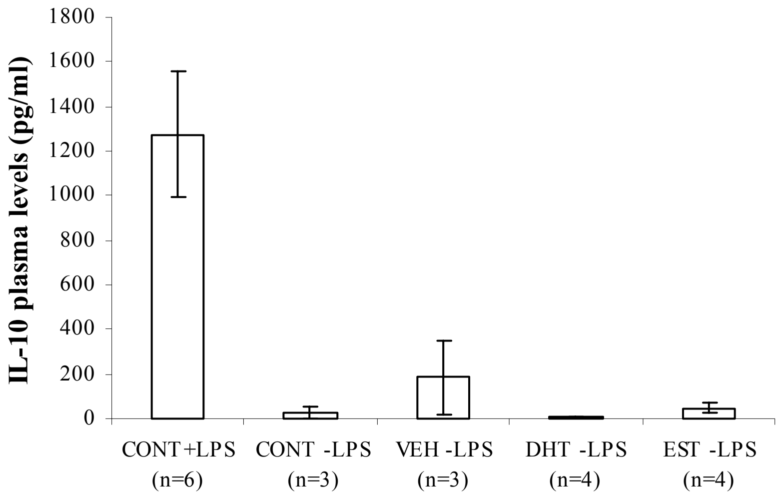
3.3. Ovariectomy

Female mice (6 weeks old) were anesthetized with an intraperitoneal injection of Avertin (400 to 500 mg/kg body weight). Each ovary was accessed via a small paravertebral incision between the lower rim of the rib cage and the upper pelvic ridge. The oviduct and adjacent blood vessels were ligated using a single suture (Silk 4-0; Ethicon Inc., Somerville, New Jersey). The ovaries were then removed and the incisions were closed in two layers with absorbable suture (Polysorb 4-0; USSC, Norfalk, Connecticut). No mortalities were observed in ovariectomized mice without LPS challenge.

3.4. Hormone supplementation

Protocol and dosage used in this experiment were followed as previously described (Angele *et al.* 1999): Following gonadectomy, hormone release pellets (Innovative Research of America, Sarasota, Florida) were implanted through a small dorsal incision into a subcutaneous pocket. These pellets release constant daily doses of hormone for up to 21 days: 0.357 mg/d of 5-α-Dihydrotestosterone (DHT), or 23.8 μg/d of 17-β-Estradiol (EST). Vehicle pellets that do not contain any active hormone served as placebo (VEH). After surgery, mice were allowed to recover for 2 weeks prior to further manipulations. Injection of saline in gonadoctomized and hormone supplemented mice did not result in any significant TNF-α or IL-10 plasma levels (**Figure 1a+b**).





<u>Figure 1 a+b:</u> TNF-α (Fig. 1*a, top*) and IL-10 (Figure 1*b, bottom*) plasma levels in male A/J mice 14 days after recovery from surgical castration. Six week old mice were castrated and received hormonal treatment with 5-α-Dihydrotestosterone (DHT) or 17-ß-Estrogen (EST) by hormone release pellets implanted during surgery. One group received pellets which do not contain any hormone (VEH). Additional, non-manipulated mice were used as control (CONT). Afterwards, all mice were maintained under identical conditions for two weeks. After fasting for 16h, mice received an intra-peritoneal injection of normal saline (-LPS). Some CONT mice received an injection of LPS (15mg/kg; CONT + LPS). After 1.5h of the injection plasma was obtained. Plasma cytokine levels were measured using an ELISA. The cytokine levels correspond to the average concentration obtained from each group ± standard error of the mean. As opposed to the high levels of cytokine induced in non-manipulated male A/J mice injected with LPS, castration and implantation of pellets (DHT, EST, VEH) did not result in any significant cytokine response after injection of normal saline.

3.5. Endotoxemia

At the age of 8 weeks, mice were subjected to endotoxic shock. By now, gonadectomized animals had recovered for 2 weeks. Mice were fasted for 16 h with access to water ad libitum. Then, they were intraperitoneally injected with E. coli 026:B6 LPS (15 mg/kg) (Diffco Laboratories, Detroit, Michigan) under aseptical conditions. After 1.5 hours of the injection, animals were anesthetized with Avertin and blood was drawn from the right ventricle after cardiac puncture, and collected into potassium-EDTA-coated Microtainer™ tubes (Becton Dickinson; Franlin Lakes, New Jersey). The same batch of LPS was used throughout all described experiments. Plasma levels of TNF-α and IL-10 were measured using a commercial ELISA-kit (BioSouce International Inc.; Camarillo, California). In the morality experiments, mice were treated as described, however survival was monitored up to 150h without any further intervention.

3.6. Vaginal smears

Smears were obtained by lavage of the vagina with approximately 18 µL of normal saline, injected through a 24G Teflon catheter on top of a 20 µL pipette. The sample was put into an Eppendorf™ tube. For better contrast and staining of nuclei, a drop of methylene-blue was added. The sample was mixed well and than spread out on a slide for microscopic evaluation.

We used modified criteria to determine the stages of estrus cycle based on Rugh (Rugh 1990): When the majority of cells in the smear were leukocytes, the sample was classified as diestrus. When clearly defined epithelial cells, some with distinct nuclei or large, squamous epithelial cells without nuclei were observed, the smear was considered estrus. Intermediate stages were not further discriminated (*For* *further detail on the murine estrus cycle see* ***APPENDIX 1: On the Estrus Cycle of the Mouse.***)

3.7. Statistical analysis

The Kruskal-Wallis One Way Analysis of Variance (ANOVA) on ranks with the 1 to k correction by Dunn was performed to determine the effect of different hormonal treatment with respect to untreated controls (CONT). Differences between two independent groups were evaluated by non-parametric comparison with Mann-Whitney-Rank Sum Test as indicated. Survival as categorical variable was analyzed using the Fisher Exact Test.

Data are expressed as mean ± standard error of the mean (SEM) unless stated otherwise. Statistical significance was accepted at $p<0.05$.

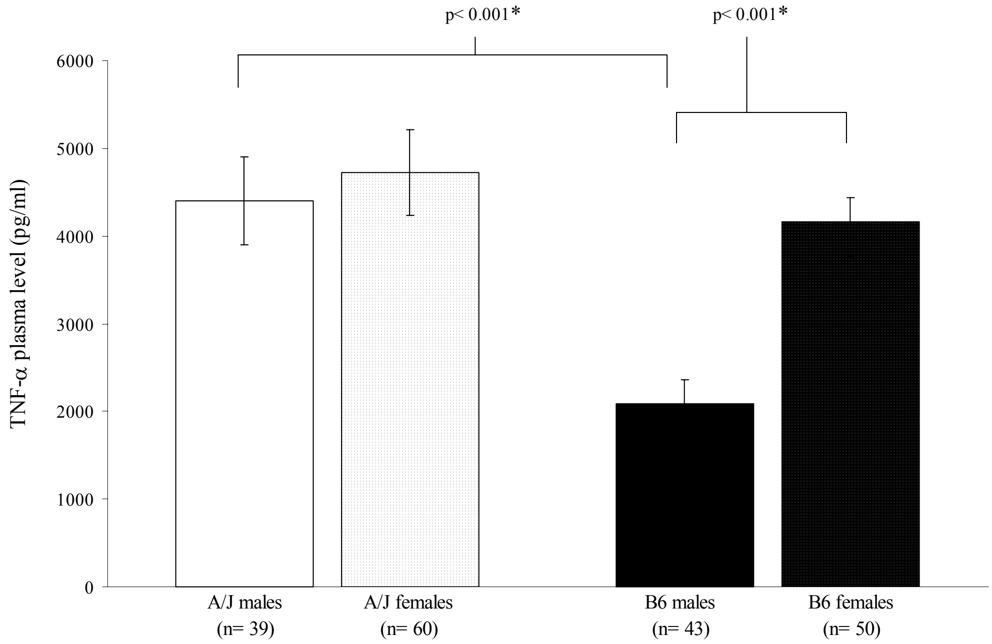
4. RESULTS

4.1. Gender differences in TNF-α plasma levels and the role of sex steroids

We compared the response to LPS between female and male B6 mice. Mice were injected with LPS (15mg/kg), which results in significant mortality within 48h, as previously reported (De Maio *et al.* 1998). Blood samples were taken 1.5 h after LPS injection for analysis of TNF-α plasma levels. This time point corresponds to the maximum detectable plasma level of this cytokine after this dose of LPS (De Maio *et al.* 1998). Female B6 mice showed TNF-α plasma levels that were two-fold higher than B6 male mice (**Figure 2**). These results reflected several independent determinations performed during 10 months to account for different stages of the estrus cycle in female mice and potential seasonal variability.

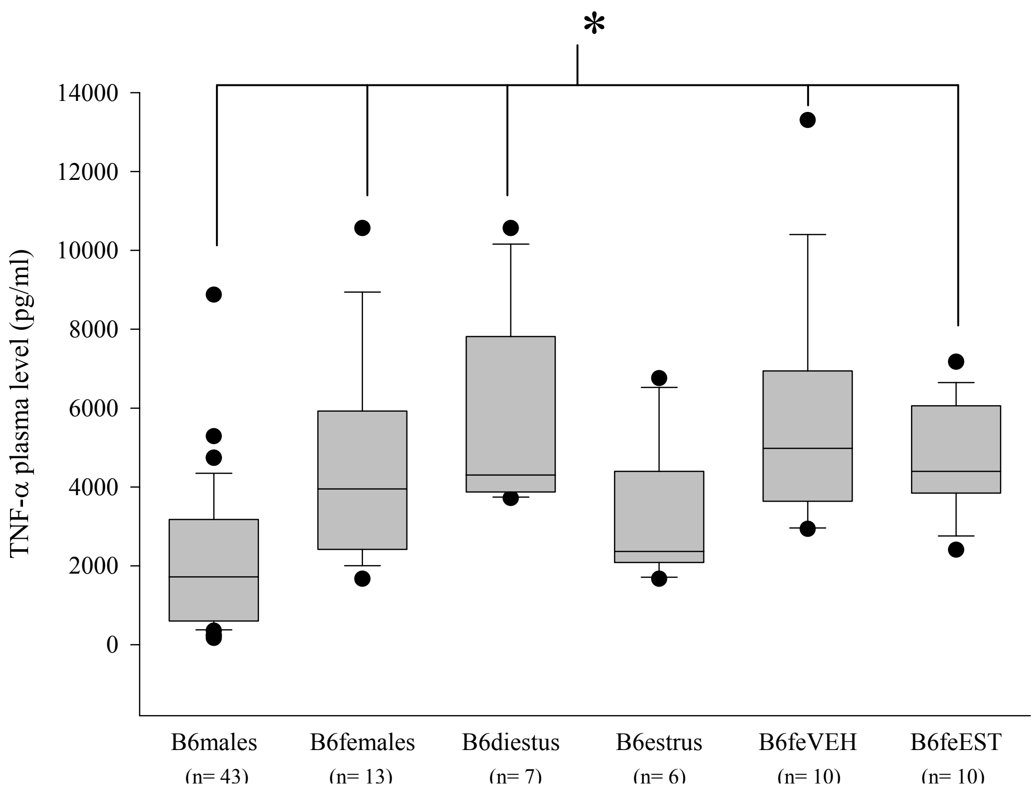
To test for possible influence of the estrus cycle, vaginal smears from an additional set of female mice was taken just before the injection of LPS. When LPS-induced TNF-α plasma levels were stratified by "estrus" and "diestrus" stage of the murine estrus cycle, no significant difference between females in estrus or diestrus could be detected (**Figure 3**). Only a trend towards lower TNF-α plasma levels of estrus (i.e. high estrogen) mice was observed. However, female B6 mice in diestrus (i.e. low estrogen) had significantly higher TNF-α levels than male B6 mice, confirming the observed gender-difference. Comparison of male B6 mice with female B6 mice in estrus did not show this difference.

Thus, the difference in LPS induced-TNF-α levels between females and males could be due to the presence of sex-steroids. To test this possibility, female and male B6 mice were gonadectomized at 6 weeks of age and supplemented with 17-β-estradiol (EST), 5-α-dihydrotestosterone (DHT) or the vehicle as placebo (VEH).



<u>Figure 2:</u> Gender differences in TNF- α levels 1.5h after injection with LPS. A/J male, A/J female, B6 male and B6 female mice at the age of 8 weeks were injected with LPS (15 mg/kg). Plasma samples were taken 1.5h after the injection. TNF- α was measured using an ELISA. The cytokine levels correspond to the average concentration obtained from all animals in the group ± standard error of the mean. Statistical significance was accepted at $*p<0.05$ obtained by pair wise comparison as indicated by lines, using Mann-Whitney-Rank Sum Test.

Two weeks after the intervention, mice were injected with LPS and plasma TNF-α levels were evaluated in plasma samples obtained 1.5 h after the injection. Castration of male mice without hormone replacement (VEH) resulted in a two-fold increase of LPS- induced TNF-α levels suggesting a suppressive effect caused by testosterone. Addition of EST to the castrated male mice resulted in a further increase of TNF-α



<u>Figure 3:</u> **Gender differences and hormonal influence on LPS-induced TNF- α levels in B6 mice after injection with LPS.** At the age of 8 weeks, B6 female and B6 female in diestrus or in estrus were injected with LPS (15mg/kg). Plasma samples were taken 1.5h after the injection. LPS-induced plasma levels of TNF-α were measured using an ELISA and were compared to those of B6 male mice after 1.5h of LPS injection. Additionally, female B6 mice at the age of 6 weeks were ovariectomized and treated with estradiol pellets (feEST) or vehicle (feVEH). After 14 days of recovery, mice were injected with LPS as described and blood sample were taken 1.5h after the injection. Plasma was obtained and TNF-α levels were measured. Cytokine levels in each female group were compared to LPS-induced TNF-α plasma levels of male B6 mice by pair wise comparison. The cytokine levels correspond to the average concentration obtained from all animals in the group ± standard error of the mean. Statistical significance was accepted at *p<0.05 obtained by pair wise comparison with respect to B6 males using Mann-Whitney-Rank Sum Test.

levels (*3-fold*) indicating an enhancing effect of this hormone. Administration of DHT resulted in LPS-induced TNF-α plasma levels similar to non-operated mice (CONT). LPS-induced TNF-α levels in EST supplemented B6 male mice were also higher (*1.5 fold*) than non-operated female mice ($p < 0.022$).

Female B6 mice showed no effect of ovariectomy or hormone replacement in the TNF-α plasma levels induced by injection of LPS (**Table 1**). Examination of vaginal smears obtained from ovariectomized female mice supplemented with EST showed the typical pattern of the estrus stage. In contrast, the pattern of female mice supplemented with DHT or placebo corresponded to a diestrus-like stage.

These results suggest a modifier role for sex-steroids in LPS-induced TNF-α response in male B6 mice. However, hormonal manipulation did not alter the response of female mice, arguing against that modifier role of sex-steroids. It is possible that other sex-related factors, such as genetic differences are involved in the response to LPS of female and male mice.

To determine if gender differences underlie variability based on genetic differences within strains of inbred mice and thus are dependant on the genetic background, we repeated the experiment in A/J mice. This strain has previously been demonstrated to show a distinctive response to LPS in comparison with B6 mice (De Maio *et al.* 1998; O'Malley *et al.* 1998; Matesic *et al.* 1999; Matesic *et al.* 2000; Stewart *et al.* 2002). Male A/J mice were found to have higher LPS-induced TNF-α plasma levels than B6 males (De Maio *et al.* 1998).

When LPS-induced TNF-α plasma levels of male A/J mice were compared with females, no gender difference was detected (**Figure 2**). Since females were not

	CONT	VEH	EST	DHT
AJ male	4400 ±503 n= 39	2926 ±374 n= 21	1743 ±261 n= 19 ↓ * x2.5	3573 ±452 n= 22
AJ female	4719 ±488 n= 60	3817 ±526 n= 18	3171 ±500 n= 21	3832 ±418 n= 17
B6 male	2086 ±279 n= 43	4158 ±763 n= 15 ↑ * x2	6298 ±844 n= 11 ↑ * x3	3130 ±377 n= 9
B6 female	4158 ±388 n= 50	5655 ±975 n= 10	4649 ±463 n= 10	3051 ±435 n= 9

Table 1: Comparison of LPS-induced TNF-α plasma levels between male and female B6 mice at the age of 8 weeks. Gonadectomized mice were operated on at the age of 6 weeks and treated with EST, DHT or VEH released from hormone pellets implanted during surgery and followed by a two-week recovery period. Non-operated mice were used as controls (CONT). All mice were maintained under identical conditions. Mice were injected with LPS (15mg/ml) and TNF-α plasma levels were measured. Data corresponds to the average TNF-α concentration obtained from each group ±standard error of the mean. Arrows indicate an increase (↑) or decrease (↓) of TNF-α plasma levels. Statistical significance was accepted at *$p<0.05$ obtained by ANOVA on Ranks and Dunn's Method as Post Hoc test with respect to CONT.

stratified by the stage of the estrus cycle, possible gender differences related to changes in hormonal levels throughout the estrus cycle may have been masked. Therefore, vaginal smears were obtained from an additional set of female A/J mice. The findings suggest a similar, albeit statistically not significant trend as observed in B6 females: stages of low estrogen levels (i.e. diestrus) are associated with high levels of LPS-induced TNF-α and stages of high estrogen (i.e. estrus) are associated with low TNF-α levels (**Figure 4**).

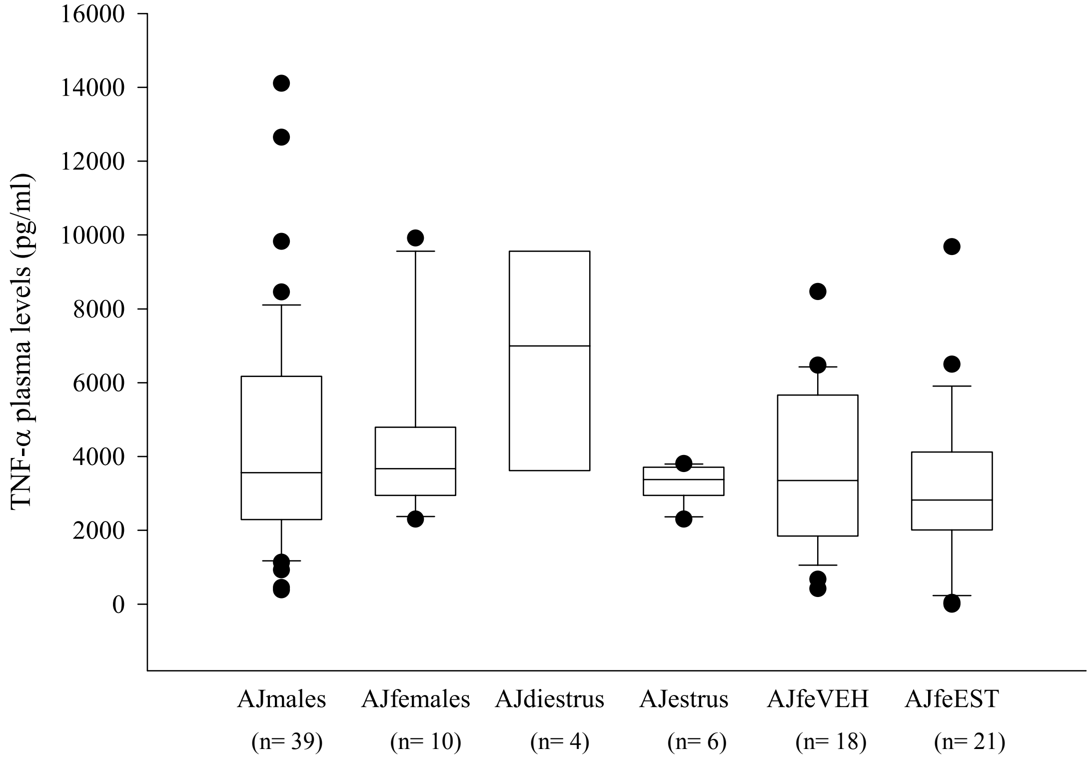


Figure 4: Gender differences and hormonal influence on LPS-induced TNF- α levels in A/J mice after injection with LPS. At the age of 8 weeks, A/J female and A/J female in diestrus or in estrus were injected with LPS (15mg/kg). Plasma samples were taken 1.5h after the injection. LPS-induced plasma levels of TNF-α were measured using an ELISA and were compared to those of A/J male mice after 1.5h of LPS injection. Additionally, female A/J mice at the age of 6 weeks were ovariectomized and treated with estradiol pellets (feEST) or vehicle (feVEH). All mice were maintained under identical conditions. After 14 days of recovery, mice were injected with LPS as described and blood sample were taken 1.5h after the injection. Plasma was obtained and TNF-α plasma levels were measured. The cytokine levels correspond to the average concentration obtained from all animals in the group ± standard error of the mean. Statistical significance was accepted at $^*p<0.05$ obtained by pair wise comparison with respect to A/J males using Mann-Whitney-Rank Sum Test

To evaluate the potential of sex-steroids to modulate TNF-α levels in this strain, A/J mice were gonadectomized and treated with hormone pellets releasing DHT or EST. An additional group was supplied with placebo pellets (VEH). In A/J male mice, there was no significant effect of castration without hormonal replacement (VEH) in LPS-induced TNF-α plasma levels. After treatment with EST, a decrease in LPS-induced TNF-α levels was observed. Ovariectomy and implantation of pellets, releasing either DHT, EST or VEH respectively, did not affect TNF-α plasma levels after injection of LPS in any female A/J group (**Table 1**). It appears that sex steroids can only modify the LPS-induced inflammatory response in male mice.

4.2. Effect of 17-β-EST on TNF-α plasma levels varies with the genetic background

We assessed whether the effect of EST on LPS-induced TNF-α plasma levels was particular to B6 mice or if it could be observed in other mouse strains. Male mice of the inbred strains AKR/J, DBA/2J, and BALB/cJ were included into the study. Statistically different levels of plasma TNF-α after LPS injection in non-manipulated mice were observed in these strains resulting in the following hierarchy: DBA/2J > AKR/J = BALB/cJ = A/J >B6 (**Table 2**, *Mann-Whitney Rank-Sum-Test, $p<0.05$*). There was no significant effect of castration without hormonal replacement (VEH) in LPS-induced TNF-α plasma levels in any of these mouse strains other than B6. All of them showed a decrease in LPS-induced TNF-α levels after treatment with EST, which is contrary to the values observed in B6 mice (**Table2**).

	A/J	AKR	DBA	BALB/cJ	B6
CONT	4400 ±503 n= 39	6070 ±1031 n= 8	12196 ±1624 n= 8	3917 ±442 n= 10	2086 ±279 n= 43
VEH	2926 ±374 n= 21	6503 ± 1148 n= 8	9916 ±781 n= 8	5476±1498 n= 10	4158 ±763 n= 15 ↑ * x2
EST	1743 ±261 n= 19 ↓ * x2.5	4110 ±306 n= 8 ↓ * x1.5	7480 ±1183 n= 8 ↓ * x1.6	2365 ±254 n= 8 ↓ * x1.7	6298 ±844 n= 11 ↑ * x3

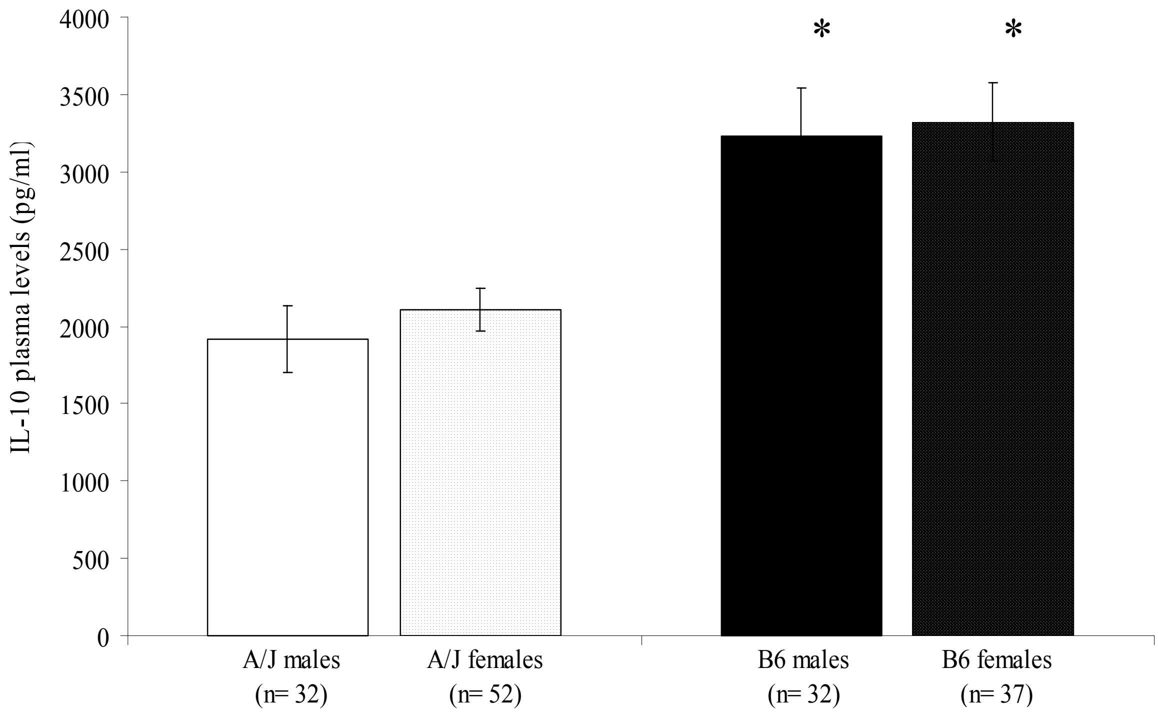
Table 2: Modulation of LPS-induced TNF-α plasma levels in male mice from different inbred strains after estradiol treatment. A/J, AKR/J, DBA/2J, BALB/cJ and B6 were castrated and supplemented with EST or VEH pellets. Non-operated mice were used as controls (CONT). Mice were injected with LPS (15mg/ml) and TNF-α plasma levels were measured. Data corresponds to the average TNF-α concentration obtained from each group ±standard error of the mean. Arrows indicate increase (↑) or decrease (↓) of TNF-α plasma levels. Statistical significance was accepted at $*p<0.05$ obtained by ANOVA on Ranks & Dunn's Method as Post Hoc test with respect to CONT.

4.3. IL-10 plasma levels in male and female B6 and A/J mice and the role of sex steroids

Male and female mice of B6 and A/J strain were injected with LPS (15 mg/kg) and blood samples were collected after 1.5 h for IL-10 detection. For this time-point, plasma IL-10 levels were observed to be maximal and with higher levels in B6 mice. However, there were no differences in LPS-induced IL-10 plasma levels between females and males of each strain (**Figure 5**). The data represents several independent experiments performed during a time period of 10 months to include possible seasonal variability. Moreover, female mice were not differentiated by the stage in the estrus cycle. Thus, this data represents the average of possible differences due to hormonal changes between males and females.

An additional set of A/J and B6 female mice was obtained and stages of the estrus cycle were determined just before the injection of LPS. While no effect of estrus or diestus was detected in B6 females **(Figure 6)**, A/J females in estrus (high estrogen) showed a trend towards increased IL-10 plasma levels (**Figure 7**). Thus a possible influence of sex-steroid on LPS-induced IL-10 plasma levels could not be excluded at least for the A/J strain. This finding implicates that hormonal effects might create differences between males and females although no relevant gender difference in LPS-induced IL-10 plasma levels was observed.

Thus, mice were gonadectomized at the age of 6 weeks and supplemented with pellets that release a daily dose of DHT or EST. These mice were maintained under hormonal replacement conditions for two weeks. As a control, gonadectomized mice were supplemented with pellets that release no hormones (VEH). After two weeks of hormone replacement, mice were injected with LPS and IL-10 plasma levels were detected 1.5 h after the injection. The three groups were compared with mice that were not operated, but maintained under the same environmental conditions as the manipulated rodents (CONT). Gonadectomy and treatment with placebo pellets (VEH) did not affect the LPS-induced IL-10 plasma levels of neither male nor female B6 mice (**Table 3**). However, EST treatment increased LPS-induced IL-10 levels in both sexes. On the contrary, DHT did not affect LPS-induced IL-10 levels in any sex. In A/J mice a comparable response was observed (**Table 3**). While DHT had no effect on IL-10 plasma levels, EST markedly increased IL-10 plasma levels in male and female mice respectively. Interestingly, ovariectomy in A/J females without hormonal replacement (VEH) resulted in an increase of LPS-induced IL-10 levels (*2.3 fold*) whereas castration did not have such an effect on male A/J mice (**Table 3**).



<u>Figure 5:</u> Gender differences in IL-10 plasma levels after injection with LPS. A/J male, A/J female, B6 males, B6 females mice at the age of 8 weeks were injected with LPS (15 mg/kg). Blood samples were taken 1.5h after injection. Plasma was obtained and IL-10 was measured by an ELISA. The cytokine levels corresponded to the average concentration obtained from each group ± standard error of the mean. The data was collected during the course of ten months to include possible seasonal variability and possible differences in the estrus cycle of female mice. LPS-induced IL-10 plasma levels were increased in both, male and female B6 mice as opposed to the A/J strain. Statistical significance was accepted at *$p<0.05$ obtained by pair wise comparison with respect to males using Mann-Whitney-Rank Sum Test.

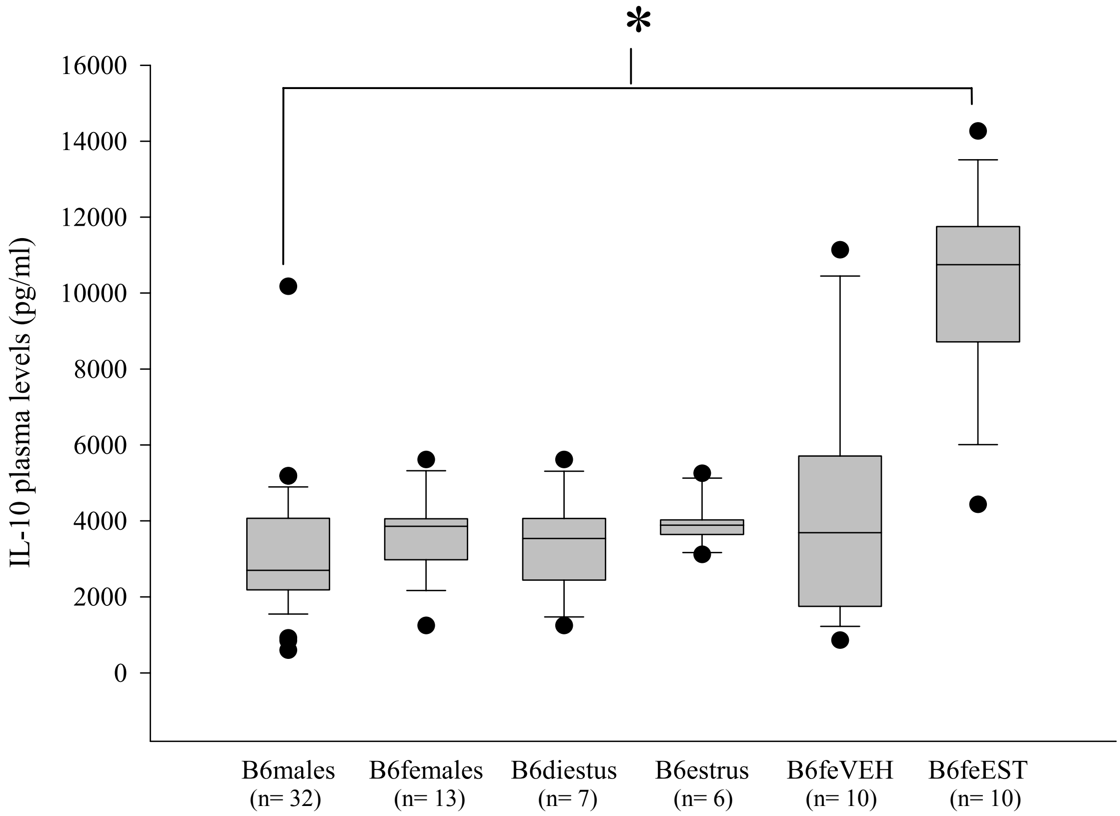


Figure 6: Gender differences and hormonal influence on LPS-induced IL-10 levels in A/J mice after injection with LPS. At the age of 8 weeks, A/J female and A/J female in diestrus or in estrus were injected with LPS (15mg/kg). Plasma samples were taken 1.5h after the injection. LPS-induced plasma levels of IL-10 were measured by the use of ELISA and were compared to those of A/J male mice after 1.5h of LPS injection. Additionally, female B6 mice at the age of 6 weeks were ovariectomized and treated with estradiol pellets (feEST) or vehicle (feVEH). After 14 days of recovery, mice were injected with LPS as described and blood sample were taken 1.5h after the injection. Plasma was obtained and TNF-α plasma levels were measured. Cytokine levels in each female group were compared to LPS-induced TNF-α plasma level of male A/J mice by pair wise comparison. The cytokine levels correspond to the average concentration obtained from all animals in the group ± standard error of the mean. Statistical significance was accepted at $^*p<0.05$ obtained by pair wise comparison with respect to B6 males using Mann-Whitney-Rank Sum Test.

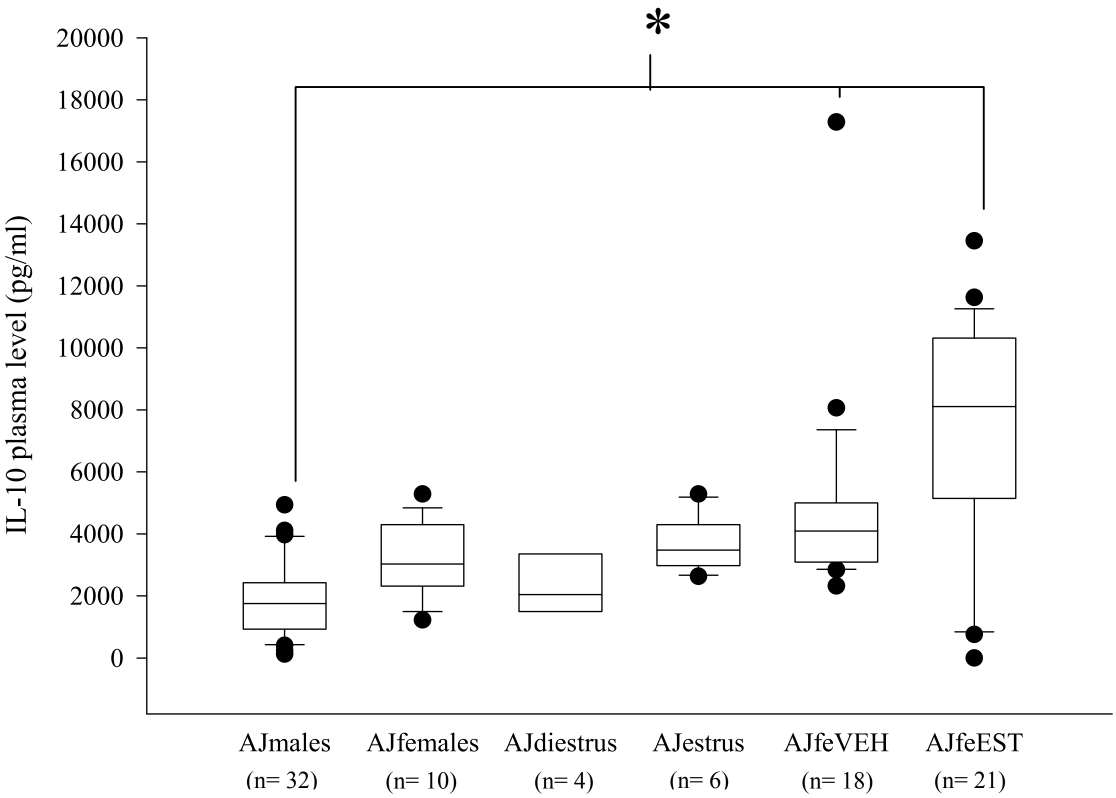


Figure 7: Gender differences and hormonal influence on LPS-induced IL-10 levels in A/J mice after injection with LPS. At the age of 8 weeks, A/J female and A/J female in diestrus or in estrus were injected with LPS (15mg/kg). Plasma samples were taken 1.5h after the injection. LPS-induced plasma levels of IL-10 were measured using an ELISA and were compared to those of A/J male mice after 1.5h of LPS injection. Additionally, female A/J mice at the age of 6 weeks were ovariectomized and treated with estradiol pellets (feEST) or vehicle (feVEH). After 14 days of recovery, mice were injected with LPS as described and blood sample were taken 1.5h after the injection. Plasma was obtained and IL-10 plasma levels were measured. This data was included into the comparison. Cytokine levels in each female group were compared to LPS-induced IL-10 plasma level of male A/J mice by pair wise comparison. The cytokine levels correspond to the average concentration obtained from all animals in the group ± standard error of the mean. Statistical significance was accepted at *$p<0.05$ obtained by pair wise comparison with respect to A/J males using Mann-Whitney-Rank Sum Test.

	CONT	VEH	EST	DHT
A/J male	1920 ±216 n= 32	3157 ±582 n= 21	5293 ±893 n= 19 ↑ * x2.8	2323 ±324 n= 22
A/J female	2110 ±139 n= 52	4846 ±800 n= 18 ↑ * x2.3	7321 ±829 n= 21 ↑ * x3.5	3053 ±446 n= 17
B6 male	3228 ±310 n= 32	3932 ±519 n= 15	6060 ±706 n= 11 ↑ * x1.9	4280 ±706 n= 9
B6 female	3322 ±252 n= 37	4558 ±1130 n= 10	10231 ±886 n= 10 ↑ * x3.1	4113 ±646 n= 9

Table 3: LPS-induced IL-10 plasma levels in eight week old male and female A/J and B6 mice treated with different sex steroids. Male mice were castrated and females were ovariectomized at the age of 6 weeks respectively. Mice were supplemented with subcutaneous hormone pellets of 17-β-estradiole (EST), 5-α-dihydroxytestosterone (DHT) or placebo (VEH) for 14 days and than injected with LPS (15mg/kg). Cytokine plasma levels were measured using an ELISA. The data correspond to the average of each group± standard error of the mean. Arrows indicate increase (↑) of plasma levels. Statistical significance was accepted at $p<0.05$ (*) obtained by ANOVA on Ranks & Dunn's Method as Post Hoc test with respect to CONT.

4.4. 17-β-Estradiole enhancement of IL-10 plasma levels during endotoxemia is determined by the genetic background

The importance of the genetic background on the response to EST was further elucidated by employing other inbred strains. Male AKR/J, DBA/2J, and BALB/cJ were castrated and supplemented with EST or VEH pellets and compared with non-operated mice. LPS-induced IL-10 plasma levels in the non-operated group were very different among the various strains. Statistical differences in IL-10 levels of these strains resulted in the following hierarchy: BALB/cJ >AKR/J =B6 > A/J =DBA/2J (**Table 4**, *Mann-Whitney Rank-Sum-Test, p<0.05*). Castration and VEH treatment did not affect LPS-induced IL-10 levels in any studied strain. Administration of EST resulted in an increase of LPS-induced IL-10 levels in AKR/J similar to A/J and B6 mice. DBA/2J and BALB/cJ did not demonstrate any further increase of LPS-induced IL-10 levels after administration of EST (**Table 4**). This data indicates that the response to EST is genetically modulated.

	A/J	**AKR**	**DBA**	**BALB/cJ**	**B6**
CONT	1920 ±216 n= 32	2918 ±568 n= 8	1348 ±124 n= 7	5117 ±484 n= 10	3228 ±310 n= 32
VEH	3157 ±582 n= 21 x1.6	4158 ±773 n= 8	1267 ±144 n= 8	6270 ±952 n= 10	3932 ±519 n= 15
EST	5293 ±893 n= 19 ↑ * x2.8	8375 ±1934 n= 8 ↑ * x2.9	1467 ±223 n= 8	4292 ±274 n= 8	6060 ±706 n= 11 ↑ * x1.9

Table 4: LPS-induced IL-10 plasma levels in male mice from different inbred strains after castration and estrogen treatment. Male A/J, AKR/J, DBA/2J, BALB/cJ and B6 mice were castrated at the age of 6 weeks, treated with subcutaneous hormone pellets of 17-β-estradiol (EST) or placebo (VEH) for 14 days and injected with LPS (15mg/kg). Blood samples were collected 1.5h after LPS injection. Cytokine plasma levels were obtained using an ELISA. The displayed cytokine levels correspond to the average concentration obtained from each group ± standard error of the mean. Arrows indicate increase (↑) or decrease (↓) of TNF-α plasma levels. Statistical significance was accepted at *$p<0.05$ obtained by ANOVA on Ranks & Dunn's Method as Post Hoc test with respect to CONT.

4.5. Investigation of sex-link of the observed phenotypes in the F1 generation

Apart from sex-steroids another possible variable that may be responsible for gender differences are the sex-chromosomes. To evaluate if sex-chromosomes are involved in the observed phenotypes we analyzed LPS-induced inflammatory response in the F1-generation bred from B6 and A/J mice. The offspring of two homozygous founder strains generates mice that are heterozygous in every single allele except from the sex-chromosomes. Since the Y-chromosome of a male F1 mouse may be contributed by an A/J or B6 father, both possible alternatives (*A X B* or *B X A*) were included. Moreover, this approach would give a rough idea on the inheritance pattern of the observed phenotypes.

Non-operated male and female F1 mice have a TNF-α phenotype similar to B6 male mice at 1.5h after injection of LPS (**Figure 8**). This suggests that this phenotype is neither sex-linked nor imprinted, and may be a B6 dominant, autosomal allele. Evaluation of the IL-10 phenotype in F1 mice clearly indicates that LPS-induced IL-10 plasma levels in B X A male and female mice follow the same pattern than B6 mice (**Figure 9**). However, IL-10 plasma levels in A X B males and females do not match the pattern in either A/J or B6. The data suggests comparable IL-10 levels between A/J males and A X B males (**Figure 9**). This might suggest involvement of the A/J X-chromosome. However, the levels of A X B females seem to be comparable to B6 mice. Moreover, IL-10 plasma levels of A X B male and female mice do not differ statistically (*p=0.35 in a pair wise comparison by Mann-Whitney-Rank Sum Test*). If IL-10 levels of A X B males and females were comparable to the B6 founder strain, no sex-link or imprinting could be postulated for this phenotype. Male F1 mice were also castrated and supplemented with sex-steroids. The levels of LPS-induced TNF-α were neither modified by castration and administration of vehicle (VEH) nor castration and hormone treatment (i.e. EST or DHT; **Table 5**). Thus, the effect of EST on LPS-induced TNF-α plasma levels in the parental generation and the effect of castration on male B6 mice disappear with the loss of homozygosity. Another explanation for the loss of these changes might be that opposing alleles neutralize each other when combined in the same individual. No effects were observed in female F1 mice (**Table 5**).

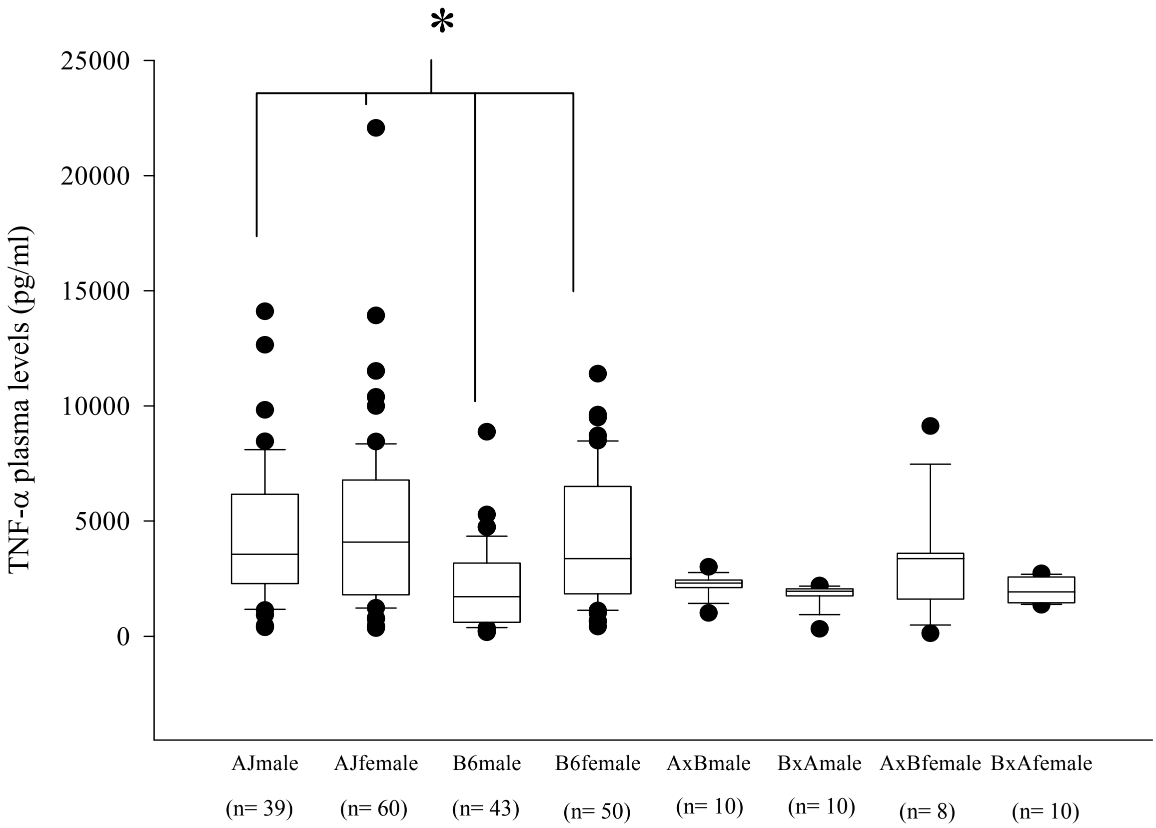


Figure 8: Comparison of LPS-induced TNF-α plasma levels in A/J, B6 and their F1-generation. Male A/J, AxB, BxA and B6 mice and female A/J, AxB, BxA and B6 mice at the age of 8 weeks were injected with LPS (15 mg/kg). Blood samples were taken 1.5h after injection. Plasma was obtained and TNF-α was measured using an ELISA. The displayed cytokine levels correspond to the average concentration obtained from each group ± standard error of the mean. Statistical significance was accepted at *$p<0.05$ obtained by pair wise comparison with respect to B6 males using Mann-Whitney-Rank Sum Test.

	CONT	VEH	EST	DHT
AxB males	2211 ±165 n= 10	2777 ± 339 n= 10	2289 ±314 n= 8	3195 ±531 n= 10
AxB females	3302 ±946 n= 8	5279 ±1605 n= 8	4030 ±1140 n= 7	2826 ±687 n= 7
BxA males	1772 ±173 n= 10	2748 ±292 n= 12	3073 ±499 n= 9	2443 ±188 n= 12
BxA females	1992 ±182 n= 10	2490 ±191 n= 12	2710 ±165 n= 12	2625 ±239 n= 12

Table 5: **LPS-induced TNF-α plasma levels in the F1-Generation of A/J and B6 mice after castration and hormonal treatment.** Male and female F1 mice were bred from A/J and B6 parental generation. Male and female AxB and BxA were gonadectomized at the age of 6 weeks respectively, and treated with subcutaneous hormone pellets of 17-β-estradiol (EST) or placebo (VEH) for 14 days. On the day of the experiment they were injected with LPS (15mg/kg). Blood samples were collected 1.5h after LPS injection. Cytokine plasma levels were obtained using an ELISA. The displayed cytokine levels correspond to the average concentration obtained from each group ± standard error of the mean. Arrows indicate increase (↑) or decrease (↓) of TNF-α plasma levels. Statistical significance was accepted at $*p<0.05$ obtained by ANOVA on Ranks & Dunn's Method as Post Hoc test with respect to CONT.

The effects of gonadectomy and implantation of hormone or VEH pellets had a similar effect on IL-10 plasma levels in F1 mice as on the parental generation: Gonadectomy with VEH-treatment or DHT-treatment respectively had no effect on LPS-induced IL-10 levels. EST-treatment clearly increased IL-10 levels in male A X B, male B X A and females B X A mice. In female A X B mice however, the increase failed statistical significance (**Table 6**).

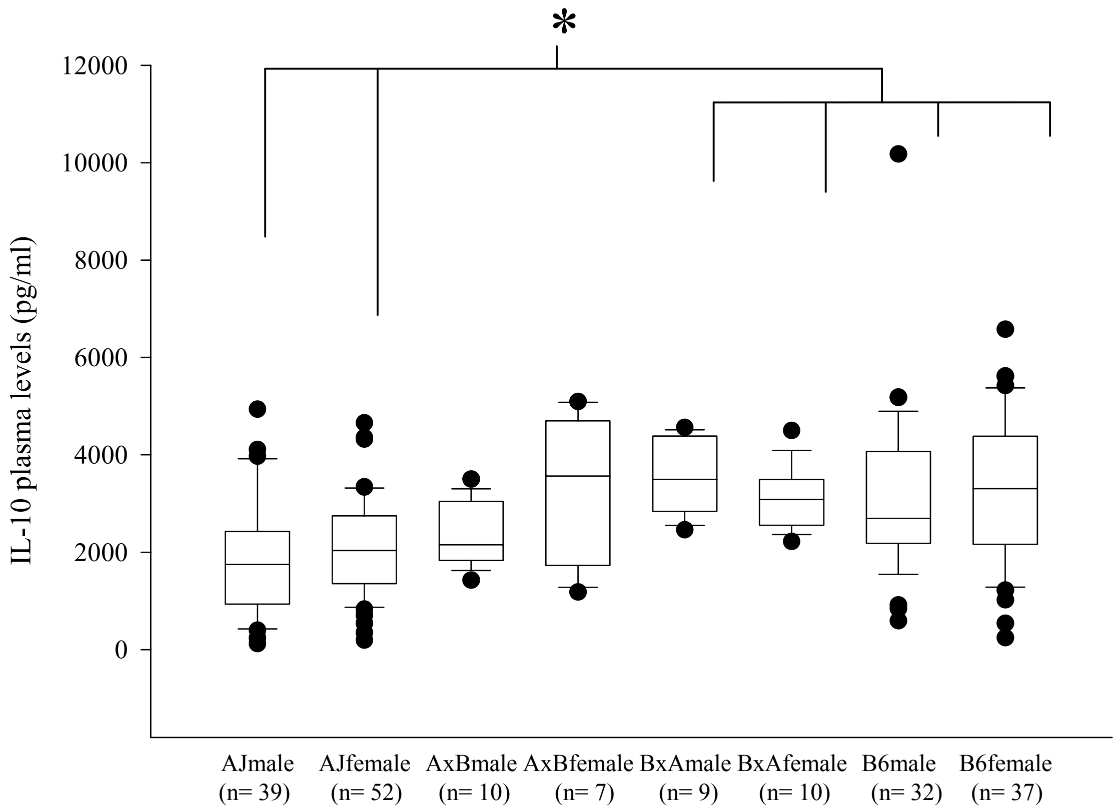


Figure 9: Comparison of LPS-induced IL-10 plasma levels in A/J, B6 and their F1-generation. Male A/J, AxB, BxA and B6 mice and female A/J, AxB, BxA and B6 mice at the age of 8 weeks were injected with LPS (15 mg/kg). Blood samples were taken 1.5h after injection. Plasma was obtained and IL-10 plasma levels were measured using an ELISA. The displayed cytokine levels correspond to the average concentration obtained from each group ± standard error of the mean. Statistical significance was accepted at *p<0.05 obtained by pair wise comparison with respect to A/J or AxB males females respectively by using Mann-Whitney-Rank Sum Test.

	CONT	VEH	EST	DHT
AxB males	2341 ±214 n= 10	3532 ±554 n= 10	6986 ±904 n= 8 ↑ * x3	3885 ±982 n= 10
AxB females	3169 ±605 n= 7	3186 ±398 n= 8	4795 ±736 n= 7 x1.5	3248 ±587 n= 6
BxA males	3516 ±270 n= 9	5012 ±510 n= 12	8736 ±1916 n= 9 ↑ * x2.5	3526 ±329 n= 12
BxA females	3128 ±216 n= 10	3796 ±305 n= 12	9254 ±665 n= 12 ↑ * x3	3506 ±178 n= 12

Table 6: **LPS-induced IL-10 plasma levels in the F1-Generation of A/J and B6 mice after castration and hormonal treatment.** Male and female F1 mice were bred from A/J and B6 parental generation. Male and female AxB and BxA were gonadectomized at the age of 6 weeks respectively, treated with subcutaneous hormone pellets of 17-ß-estradiol (EST) or placebo (VEH) for 14 days and were injected with LPS (15mg/kg). Non-operated mice were used as control (CONT). All mice were maintained under identical environmental conditions. Blood samples were collected 1.5h after LPS injection. Cytokine plasma levels were obtained using an ELISA. The displayed cytokine levels correspond to the average concentration obtained from each group ± standard error of the mean. Arrows indicate increase (↑) or decrease (↓) of IL-10 plasma levels. Statistical significance was accepted at *p<0.05 obtained by ANOVA on Ranks & Dunn's Method as Post Hoc test with respect to CONT.

4.6. Effects of EST-related changes on outcome from lethal endotoxemia

Mortality after lethal endotoxemia has previously been described to have strain specific differences (De Maio *et al.* 1998). A/J mice do not only have higher TNF-α levels and lower IL-10 plasma levels 1.5h after the injection of a lethal dose of LPS (15mg/kg), they also have a better survival rate (63% vs. 30% at 48h after the injection) (De Maio *et al.* 1998). Thus, we had to determine whether the changes in cytokine patterns observed after EST-treatment would alter the outcome. Frequency of mortality was compared between non-operated mice (CONT) and mice that were castrated and supplemented with EST or placebo (VEH). After 48h, no difference in mortality was observed between non-operated and vehicle treated B6 mice (Survival: CONT 37%, VEH 36%). In contrast, castrated mice supplemented with EST showed an increased frequency of mortality (survival EST 8%). The data was obtained from 2 independent experiments to assure reproducibility (**Figure 10**).

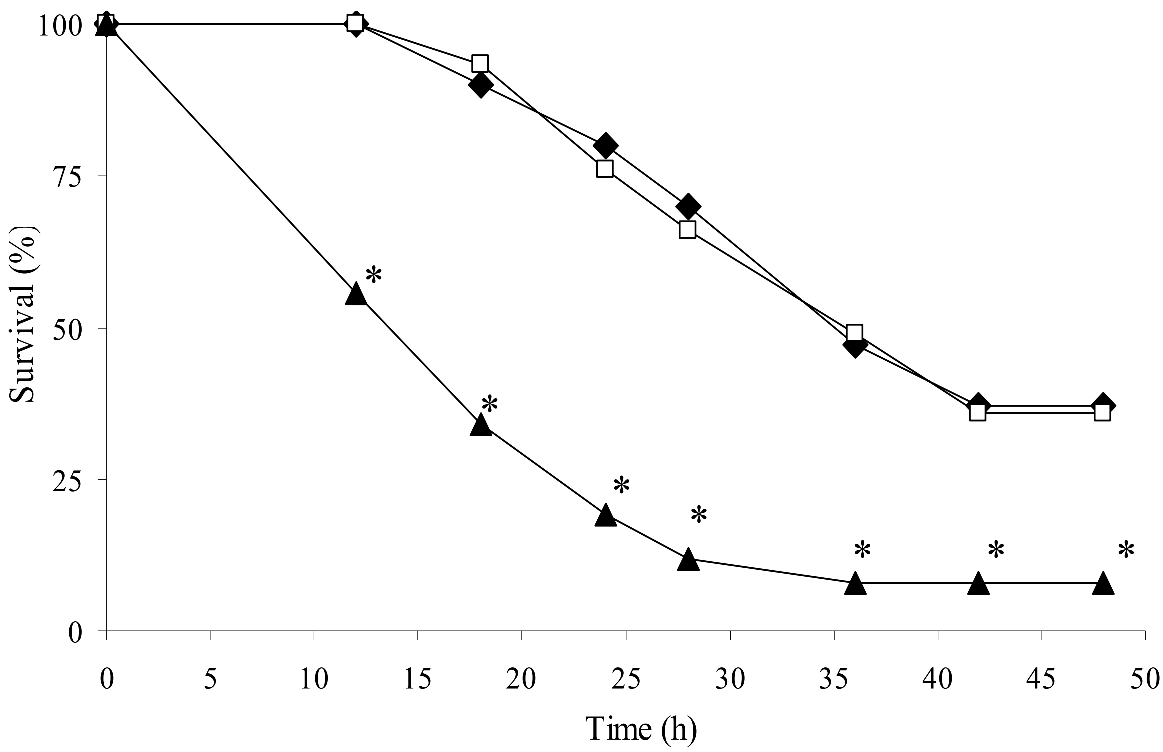
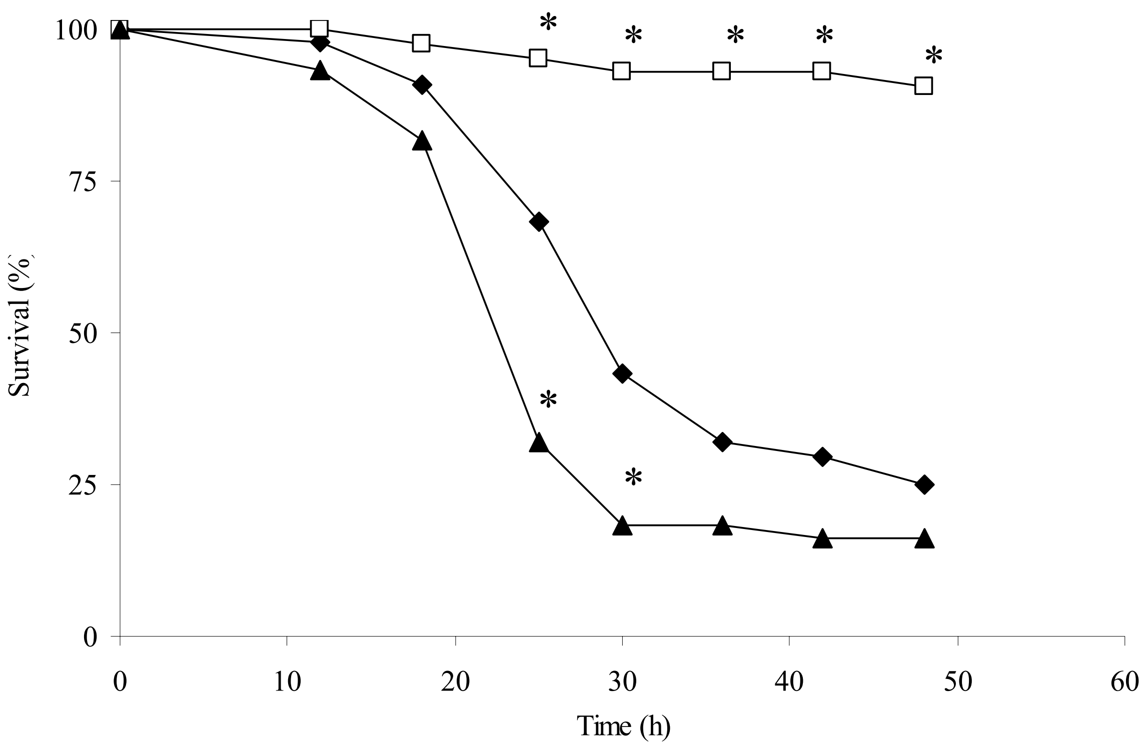


__**Figure 10:**__ **Survival of estrogen-treated B6 male mice after injection of LPS.** Male B6 mice at the age of 6 weeks were castrated and treated with subcutaneous hormone pellets of 17-β-estradiol (▲EST, *n*=27) or placebo (□VEH, *n*=30). Non-castrated mice were used as control (♦CONT, *n*=30). Two weeks after the procedure, mice were injected with LPS (15mg/ml). All mice were maintained under the same environmental conditions. Survival was monitored over 48h, at this time-point survival was 8% for EST group, 36% for VEH group and 37% for CONT. The data represents the average mortalities from 2 independent experiments to assure reproducibility. Statistical significance was accepted at *$p<0.05$ obtained with respect to CONT by using Fischer Exact Test.

Interestingly, castrated and VEH treated A/J mice had a markedly improved survival (90%) as compared to non-operated A/J males. Treatment with EST did not increase the frequency of mortality but seemed do enhance the velocity of LPS-induced mortality in this strain. The data was obtained from 3 independent experiments to assure reproducibility (**Figure 11**).

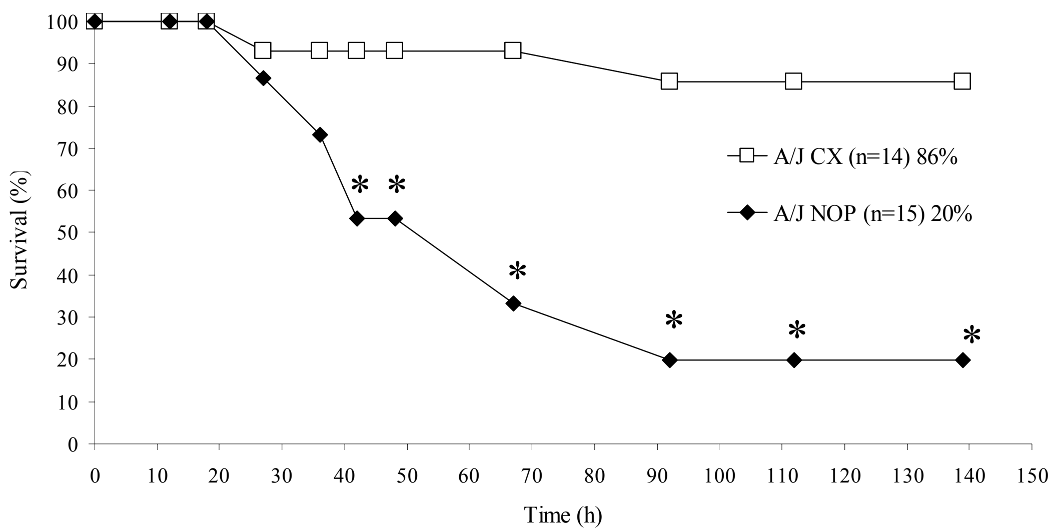


<u>Figure 11</u> Survival of estrogen-treated A/J male mice after injection of LPS. Male A/J mice at the age of 6 weeks were castrated and treated with subcutaneous hormone pellets of 17-β-estradiol (▲EST, *n*=44) or placebo (☐VEH, *n*=44). Non-castrated mice were used as control (♦CONT, *n*=44). Two weeks after the procedure, mice were injected with LPS (15mg/ml). All mice were maintained under the same environmental conditions. Survival was monitored over 48h, at this time-point survival was 16% for EST group, 90% for VEH group and 25% for CONT. The data represents the average mortalities from 3 independent experiments to assure reproducibility. Statistical significance was accepted at *$p<0.05$ obtained with respect to CONT by using Fischer Exact Test.

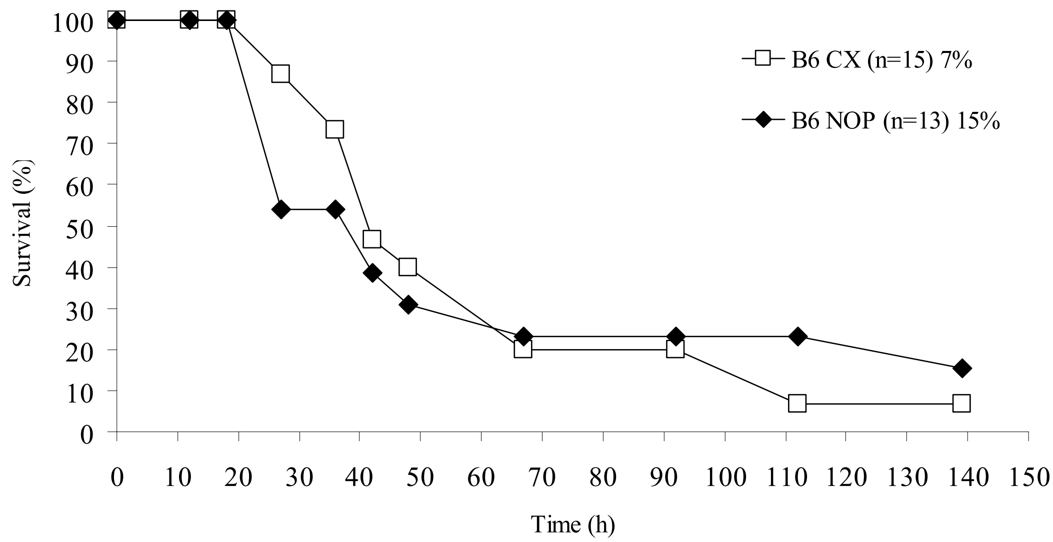
Based on this data we determined if protection by androgen depletion could be found in other strains. AKR/J, BALB/cJ and DBA/2J were included into the experiment. Mice were castrated but no VEH pellets were implanted. In order to exclude the unlike possibility that VEH pellets would affect the outcome, additional A/J and B6 mice were enrolled into this series once again. Survival was monitored for up to 150h and compared to non-manipulated mice (CONT), which were maintained in an identical environment. Although provided by a different vendor, and consequently being exposed to different environmental conditions, C3H/HeN mice were also included. This strain has previously been reported to have improved survival after pharmacological castration in a model of trauma-hemorrhage with consequent sepsis (Angele *et al.* 1997). C3H/HeN are not identical with the LPS-resistant strain C3H/HeJ provided by *Jackson Laboratory*. C3H/HeJ are hypo-responsive to LPS because of a point-mutation in the cytoplasmatic domain of TLR-4 and consequently lack signal-transduction (Poltorak *et al.* 1998). Over the time-course of 150h, we found that A/J mice were the only strain that definitely benefited from removal of the testicles (**Figure 12**). This effect turned out to be independent of the application of the placebo pellet (**Figure 11 and Figure 12a**). The overall survival from castrated A/J mice in all experiments was 89% (50 of 56) as opposed to 25% (11 of 56) in the non-operated groups. When additional castrated male A/J mice were supplemented with DHT pellets protection from LPS ceased **(Figure 13)**.

Frequency of mortality in all other strains remained unchanged, even in C3H/HeN (**Figure 12c**). For DBA/2J mice, the data may suggest attenuation of the dynamic of the clinical course, however without changing the outcome (**Figure 12d**).

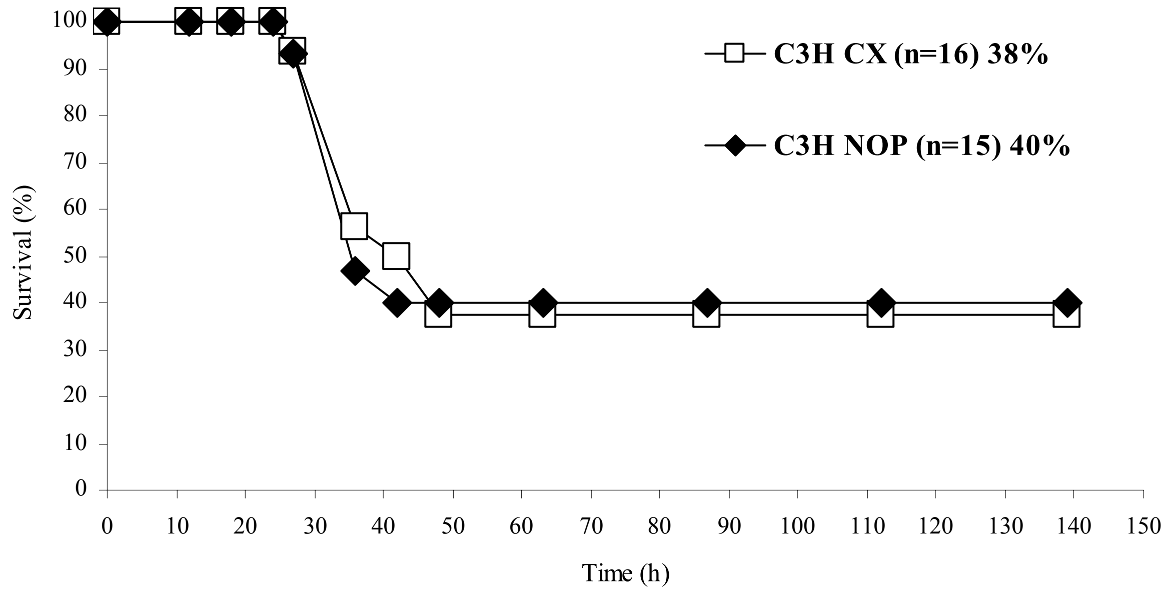
<u>Figure 12 a-f:</u> Survival of castrated mice from different inbred strains after injection of LPS. Male A/J (<u>Figure 12a</u>), B6 (<u>Figure 12b</u>), C3H/HeN (<u>Figure 12c</u>), DBA/2J (<u>Figure 12d</u>), BALB/cJ (<u>figure 12e</u>) and AKR/J (<u>Figure 12f</u>) mice were castrated at the age of 6 weeks. Non-castrated mice were used as control and maintained under identical conditions. Two weeks after the procedure, mice were injected with LPS (15mg/ml). Survival was monitored up to 150h. Statistical significance was accepted at *p<0.05 obtained by comparison with respect to CONT by using Fischer Exact Test.



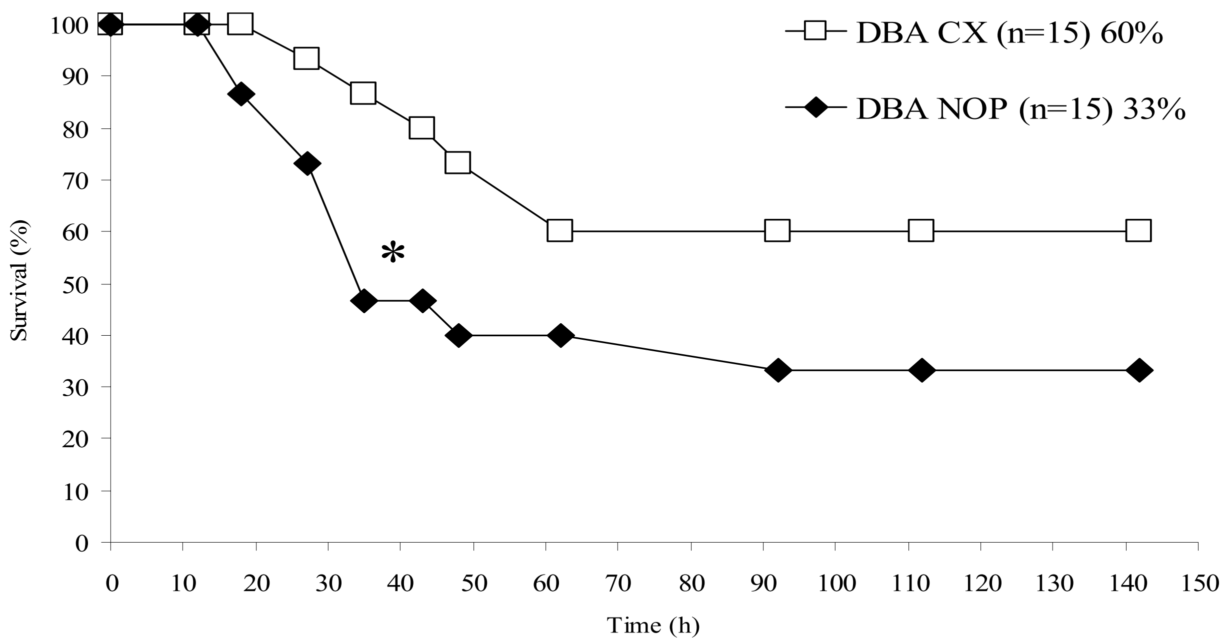
a: A/J



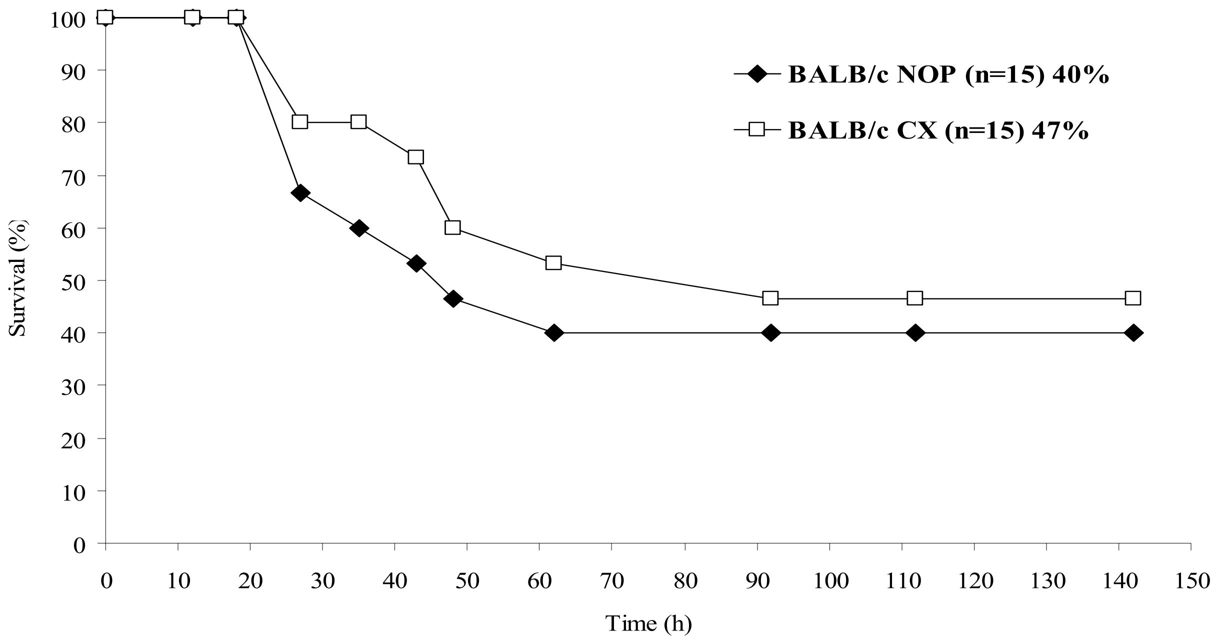
b: B6



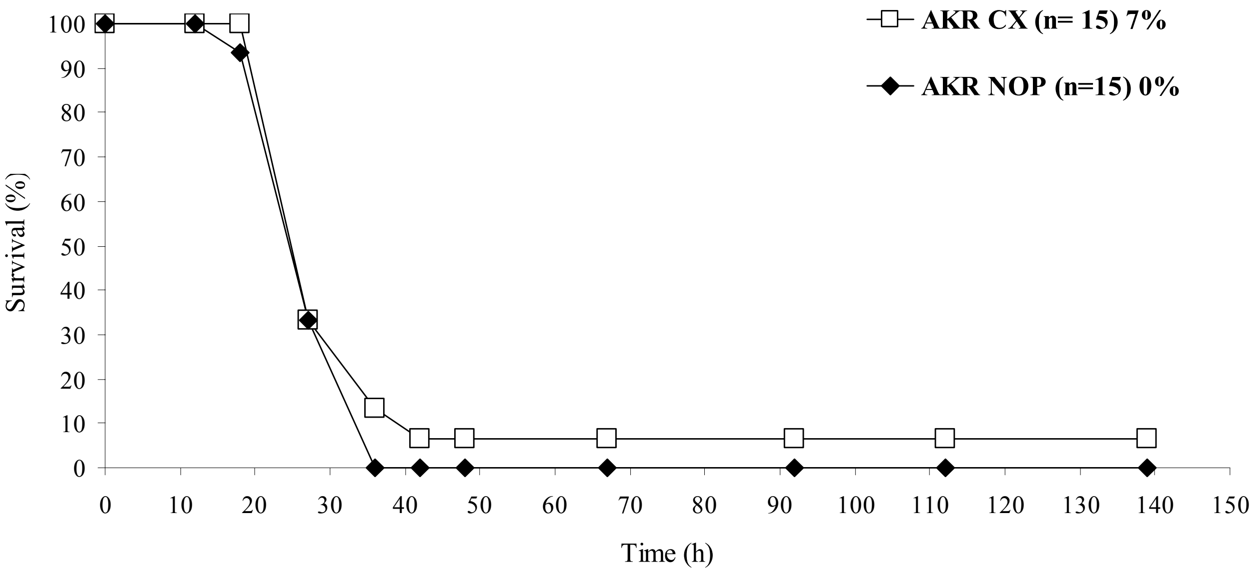
c: C3H/HeN



d: DBA/2J



e: BALB/cJ



f: AKR/J

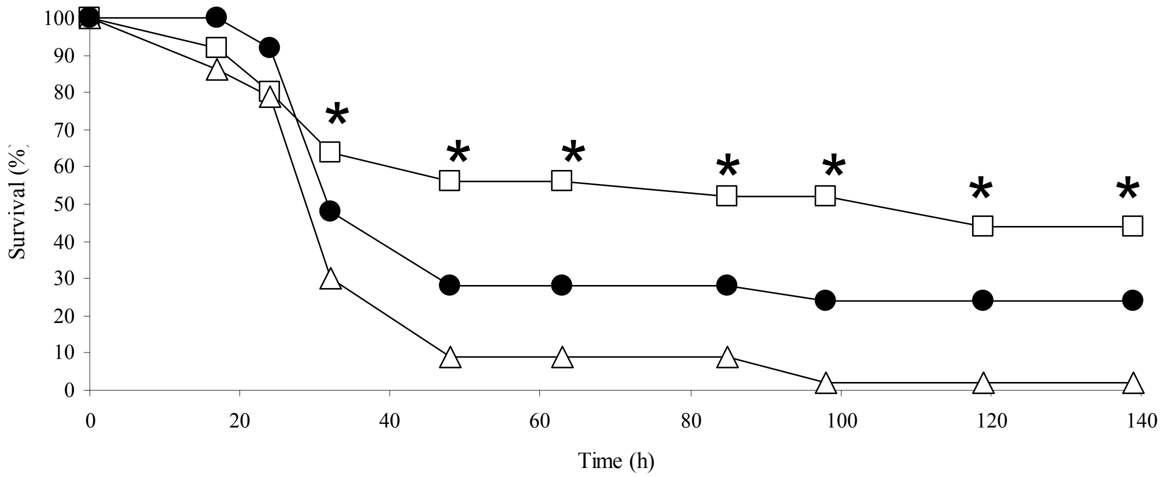


Figure 13: Survival of dihydrotestosterone-treated A/J male mice after injection of LPS. Male A/J mice were castrated (CX) at the age of 6 weeks and randomized into two groups. One group was treated with subcutaneous hormone pellets releasing 5-α-Dihydroxytestosterone (△CX+DHT, *n*=15), the other group received no further hormonal treatment (☐CX, *n*=24). Non-castrated mice (●NOP, n=25) were used as control. Two weeks after the procedure, mice were injected with LPS (15mg/ml). Survival was monitored over 140h, at this time-point survival was 42% for CX group, 7% for CX+DHT group and 24% for CONT group. Statistical significance was accepted at *$p<0.05$ obtained with respect to CONT by using Fischer Exact Test.

5. DISCUSSION

We have hypothesized that the response to injury is modified by three major factors: the type of injury, the environment and the genetic background (**Figure 14**). Prior studies in different experimental rodent models support this hypothesis (De Maio *et al.* 1998; Stewart *et al.* 2002; Trentzsch *et al.* 2003). This paradigm could explain the variability observed in the outcome of critically ill patients. In addition to these factors, gender is likely to be another component that influences the response to injury.

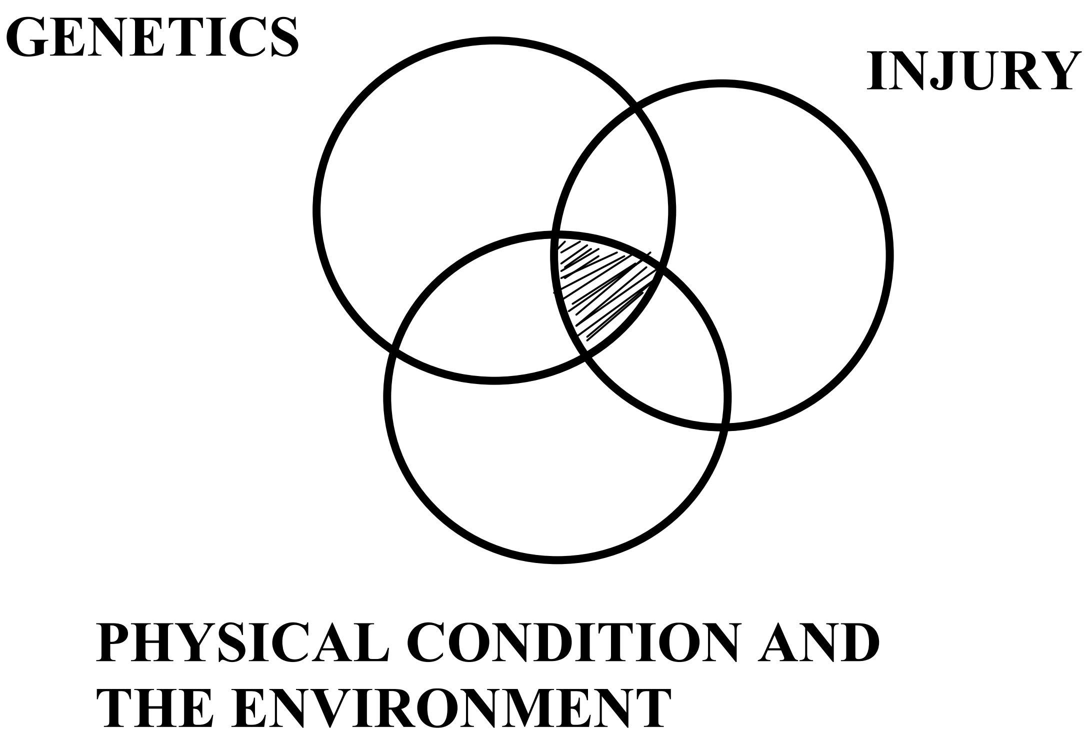


Figure 14: The "Three-Circle-Theory". This model considers determination of the inflammatory response as an intersection of three major components: The type of injury, the physical condition of the individual and its environment and the genetic make-up. As depicted here, the inflammatory response that can be measured after a certain injury such as injection of LPS can be understood as the hatched intersection of the three circles.

Clinical studies have shown that male septic patients have a higher risk of mortality with respect to the female counterpart (Schroder *et al.* 1998; Bauerle *et al.* 2000; Schroder *et al.* 2000). However, these observations have been challenged by other studies that found no gender difference (Riche *et al.* 1996; Eachempati *et al.* 1999; Wichmann *et al.* 2000), or an even higher mortality in septic female patients (McLauchlan *et al.* 1995; Napolitano *et al.* 2001; O'Keefe *et al.* 2001). A higher incidence of infection (Offner *et al.* 1999) and sepsis (McGowan *et al.* 1975) has also been associated with male patients. In contrast, two other studies have shown a higher incidence of infection in female patients (Dinkel *et al.* 1994; Kollef *et al.* 1997). In summary, there is no general consensus whether gender is a positive or negative factor in the outcome from injury. Genetic diversity may contribute to such contradicting results. However, genetic diversity has never really been considered in such studies. Only one study has so far suggested a gender-contribution on increased risk for mortality from sepsis associated with TNF-α polymorphisms (Schroder *et al.* 2000). The contribution genetic variability of the inflammatory response in regard to sex-steroids however remains unconsidered so far.

The importance of genetics in the response to injury has recently been demonstrated in animal models of endotoxemia (De Maio *et al.* 1998) and sepsis (Stewart *et al.* 2002). This genetic contribution has been evaluated at the level of mortality as well as at different components of the inflammatory process, such as cytokines (De Maio *et al.* 1998; Stewart *et al.* 2002), end-organ damage (O'Malley *et al.* 1998), and spleenocyte proliferation (Matesic *et al.* 1999). Clinical studies have been initiated to evaluate genetic components in sepsis research (Schroder *et al.* 2000; Tabrizi *et al.* 2001; Riese *et al.* 2003).The potential contribution of genetics to the inflammatory response became more important with completion of the Human

Genome Project. The sequencing data of the human genome revealed 1.42 million single nucleotide polymorphisms (SNP) within the 3 billion base pairs that built the whole human genome. This one tenth percent of differences implies that there are 3 million possible differences between 2 individuals (Sachidanandam *et al.* 2001). Such genetic diversity provides solid bases for differences in the response to many pathological situations including sepsis. To evaluate the linkage between gender differences and their variability based on genetic differences, we have investigated this relationship in a model of endotoxemia in mice with dissimilar genetic background. Since these mice were subjected to the same insult and were maintained in an identical environment (**Figure 14**), the only variable that may account for such differences is the genetic background.

5.1. Gender and genetics determine LPS-induced cytokine plasma levels through sex-steroidal modulation

LPS-induced TNF-α plasma levels were different between male and female B6 mice indicating a gender-significant difference (**Figure 2**). By evaluating different stages of the estrus cycle in female B6 mice, our data suggests a hormonal bases for the observed gender difference. Surprisingly, we found that the estrus stage (high estrogen) was associated with rather depressed LPS-induced TNF-α levels, voiding the difference between male and female B6 mice, while females in di-estrus (low estrogen) displayed high TNF-α plasma levels that characterize this gender difference. With TNF-α playing a key role in induction of the inflammatory response, this observation might object the established dogma of a more active immune system in females. And moreover, estrogen has been described to increase TNF-α plasma levels after LPS challenge in female BALB/c mice (Zuckerman *et al.* 1995;

Zuckerman *et al.* 1996). However, decreased TNF-α expression and plasma levels after estrogen-treatment of female C3H/He mice has been found after inflammatory stimuli with protease peptone (Salem *et al.* 2000). These observations raise concern what effect estrogen actually has on TNF-α production and what differences between exogenous hormone treatment and physiological plasma levels exist. Actually, few data is available on estrogen-related effects on this cytokine throughout the estrus cycle and studies in humans remain controversial (Angstwurm *et al.* 1997; Schwarz *et al.* 2000; Bouman *et al.* 2001).

The data on B6 mice clearly provides evidence for the hormone dependent modulation of the inflammatory response and thus implies gender-specific differences herein. Moreover, comparison with another strain of inbred mice (*i.e.* A/J) revealed that genetic diversity may have important impact on gender-differences in the inflammatory response and their modulation by sex-steroids: Although female A/J mice showed similar trends in the modulation of LPS-induced TNF-α plasma levels depending on the stage of the estrus cycle, there was no gender-difference detectable between male and female A/J mice (**Figure 4**), and androgen-depletion of male A/J mice did not change TNF-α plasma levels in the response to LPS injection (**Table 1**). Differences in hormone secretion, plasma protein binding, receptor affinity and density or hormone clearance and degradation may provide possible explanation for the variable qualitative and quantitative inflammatory response observed between these two strains. A number of such differences including plasma sex-steroid concentrations between various strains of inbred mice have been reported (Crispens 1975). They are likely to be result of differences in the genetic composition of each strain.

To compensate for strain-differences in hormonal levels or cycle-dependant variability, mice were gonadectomized and supplemented with subcutaneous, constant release pellets of DHT or EST to deliver equal amounts of hormone into the mice. This model has previously been reported to result in physiologic hormone plasma levels in mice (Angele *et al.* 1999). Interestingly, no effect on TNF- α levels after hormone depletion by ovariectomy and VEH or ovariectomy with supplementation with EST or DHT was observed in either B6 or A/J female mice (**Table 1**). A similar observation has previously been made in humans, too. LPS-induced TNF-α levels were increased by EST treatment of ex-vivo white blood cells (PBMC) from male volunteers while there was no effect in female PBMC (Asai *et al.* 2001). It remains unclear, why females show this poor response to hormone manipulation. It may be the result of greater tolerance towards changes in sex-hormone plasma levels that comes with cycle dependant fluctuation. Females may have differences in control and regulation of receptor-density as compared to males. It has been demonstrated that the endocrine modulation of the inflammatory response in rats is most likely to be controlled by the adrenal gland and may be dependant on the immune-reactivity of estrogen receptor α in the medullar cells (Green *et al.* 1999).

Interestingly, administration of estriol (*an estrogen agonist*) to female BALB/c mice resulted in a dramatic increase in LPS-induced TNF-α levels approximately 1 hour after the injection. This increase was suppressed by administration of tamoxifen, an estrogen antagonist, (Zuckerman *et al.* 1996). Thus, females like BALB/c and by implication all other females with a genetic background other than B6 or A/J may be better responders to EST-treatment.

The comparison of castrated and VEH treated or castrated and hormone treated male A/J and B6 mice revealed two important observations that clearly support our hypothesis, that the genetic background may exert different effects on sex-steroidal modulation of the inflammatory response: First, depressive effects of androgens on LPS-induced TNF- α plasma levels were observed in B6 males only. Second, EST-treatment showed marked effects in males of either strain, however with opposite effects (**Table 1**). When other inbred strains (AKR/J, DBA/2J, and BALB/cJ) were included, it became apparent, that both observations are unique for B6 males (**Table 2**). Depressive effects of androgens on the inflammatory response have previously been described (Angele *et al.* 1997; Angele *et al.* 1998; Angele *et al.* 1999). Androgen depletion by castration prevents this depression and can be reverted by pharmacological blockade of testosterone receptors with Flutamide (Angele *et al.* 1997; Wichmann *et al.* 1997). Our studies showed that DHT-treatment was capable to reverse the effects of androgen depletion in castrated male B6 mice (**Figure 13**).

After treatment with EST reduced TNF- α plasma levels after LPS injection were observed in male mice except from B6 males (**Table 2**). Data from female A/J and B6 mice in the estrus stage (high estrogen) of the cycle suggest similar tendencies in the effect of EST on LPS-induced TNF-α plasma levels (**Figure 3 and Figure 4**). Depression of LPS-induced TNF-α levels under the influence of EST has previously been observed. For example, peritoneal macrophages of 17-β-estradiol-treated C3H/He mice infected with Listeria monocytogenes show decreased gene expression and production of TNF-α (Salem *et al.* 1999). After treatment with estrogen, LPS-induced TNF-α production of murine macrophages isolated from female BALB/c mice was reduced, possibly by interaction with NF-κB (Deshpande *et al.* 1997). However, data from the F1 generations bred from A/J and B6 mice suggests that the responsible alleles need to be homozygous (**Table 5**).

Our data suggests that the gender difference in TNF- α plasma levels after injection of LPS between B6 males and B6 females may be a result of differences in estrogen concentrations. EST treatment of males decreased LPS-induced TNF- α levels and plasma levels in estrus B6 females are comparable to males. Thus times of high estrogen blood levels seem to be responsible for the gender difference. Surprisingly, B6 females supplemented with EST after ovariectomie showed LPS-induced TNF-α plasma levels comparable to those found in di-estrus B6 females and ovariectomized female B6 mice with VEH treatment (*Mann-Whitney Rank Sum Test,* $p=0.807$). Moreover, they also have higher TNF- α levels than male B6 mice (*ANOVA on Ranks with Dunn's correction,* $p<0.05$). The amount of EST in the pellets may be very different from physiological 17-β-estradiol levels of naïve B6 females in estrus. Sex-steroids are known to function within a broad range of concentrations but may have functional optimum at a specific dosage (Goretzlehner 1991; Asai *et al.* 2001). Consequently, there may be dose-dependant variability in the capacity of sex-steroids to modulate the inflammatory response. By implication this includes strain-specific differences that are based on the genetic background. Thus, variable effects of sex steroids among inbred mice of different strains may also be a result of physiologic hormone plasma levels characteristic for each strain.

Despite the lack of gender-differences in LPS-induced IL-10 plasma levels in B6 or A/J mice (**Figure 5**) hormonal modulation of the LPS-induced IL-10 response was suggested by stratification for different stages of the estrus cycle: In female A/J mice we observed a trend towards higher LPS-induced IL-10 plasma levels during estrus (high estrogen) as compared to diestrus (**Figure 7**). No such effect was observed in B6 females (**Figure 6**). Again, such differences may be explained by inter-strain variability. Further experiments revealed that LPS-induced IL-10 plasma levels can be enhanced by EST-treatment in both sexes of either A/J or B6 mice (**Table 3**). While the TNF- α phenotype could not be reproduced in the F1-Generations (**Table 5**), the effect of EST on LPS-induced IL-10 plasma levels could be observed in the F1-generations bred from A/J and B6 mice (**Table 6**). Castrated male AKR mice treated with EST also experienced an increase in LPS-induced IL-10 levels (**Table 4)**. The presence of steroid responsive elements in the promoter region of the IL-10 gene may provide a possible explanation for this finding (Kim *et al.* 1992; Kube *et al.* 2001). However, the identified elements are not typical and functional assays have not been performed yet. Interestingly, two of the evaluated strains, *i.e.* DBA/2J and BALB/cJ, were identified as non-responders to EST treatment (**Table 4).** Estrogen responsive elements in the IL-10 promoter might be missing, be defective, or require a different dosage of EST for optimal function. Dose-dependency of hormonal effects has been described in humans (Goretzlehner 1991; Asai *et al.* 2001) and may explain our finding in the LPS-induce TNF-α plasma levels of B6 mice.

Regulatory pathways other than interaction of EST with the promoter of the IL-10 gene may be possible. For example, estrogen has also been reported to regulate IL-6 expression (Girasole *et al.* 1992; Deshpande *et al.* 1997), but to date, no estrogen responsive element has been detected in the IL-6 promoter (Ray *et al.* 1994; Deshpande *et al.* 1997). Indeed, the ability to decrease IL-6 levels has been related to direct interaction of estrogen with NF-κB (Ray *et al.* 1994; Stein *et al.* 1995). In conclusion, it is possible that sex-steroids regulated the inflammatory process at different levels.

With respect to the effects of EST on LPS-induced IL-10 plasma levels, an alternative explanation for the TNF-α data come into view: Interleukin 10 down-regulates TNF-α expression and thus is responsible for a balancing effect on the proinflammatory response (Gerard *et al.* 1993; Marchant *et al.* 1994; van der Poll *et al.* 1997). However, there is evidence in our data that makes this scenario seem less likely: First, IL-10 plasma levels after LPS challenge are increased in castrated B6 mice supplemented with EST. Consequently, a result of an inhibitory effect of IL-10 should cause depression of TNF-α of these mice (**Table 1**). However, this observation may be explained by traits in the genetic background of the B6 strain. Actually, A/J and AKR/J do show decreased TNF-α levels along with increased IL-10 levels after EST treatment.

Second, DBA/2J and BALB/cJ mice are unresponsive to EST-treatment and maintain the level of LPS-induced IL-10 levels (**Table 4**). However, LPS-induced TNF- α plasma levels also decrease with EST treatment in these two strains (**Table 2**). There may be another regulatory mechanism involved in the down-regulation of TNF- α. It is also possible, that the unresponsiveness of DBA/2J and BALB/c mice actually is an artifact from sub-optimal dosage.

Ovariectomized female A/J mice show an increase in LPS-induced IL-10 plasma levels (**Table 3**). Apparently this does not match with the concept of IL-10 modulation thru EST. It is however possible, that this finding is a consequence of regulation at an endocrine location other than the ovaries. Studies in rats suggest medullar cells in the adrenal gland to be responsible for gender differences in the inflammatory response (Green *et al.* 1999).

5.2. F1-generation: contribution of sex-chromosomes to TNF-α and IL-10 phenotypes

There are two possible mechanisms behind gender differences of any kind. One is a difference in hormonal activity, i.e. sex-steroids; the other is availability of alternative genetic information. In a genetic sense, sex-chromosomes are the only thing that make the difference between male and female (Passarge 1994): By looking at meiosis, it becomes clear, that apparently every single autosome is exchangeable between male and female without changing the genetic sex. However, the presence of an intact Y-chromosome will determine male sex of the embryo. Patients with two X chromosomes and an accessory Y-chromosome will clinically present with a male phenotype (*Klinefelter-Syndrome*), and individuals with only one X-chromosome will develop a female phenotype (*Turner-Syndrome*) as long as no Y-chromosome is present.

Interestingly, the sex-determination is not dependant on the complete Y-chromosome. The critical region that determines biologic sex lies on the distal short arm of the Y-chromosome. The physical map of this region shows the following subsets: The most distal part of the Y-chromosome is considered the pseudoautosomal region (PAR). Because of the homology with the distal part of the short arm of the X-chromosome homologue pairing and crossing-over may occur in this region of the Y-chromosome during meiosis - just like on any other autosome – without changing the sex.

The adjacent region to PAR is segment 1. The total to a length of these two segments is 2500kb. The following segments 2 to 7 do not contain any relevant gene for male sex-determination. Located adjacent to PAR is the proximal part of segment 1 (1 A1), were the sex-determining region Y (SRY) lays (Wolf *et al.* 1992). This rather short sequence of just 35kb is most likely to be identical with the testis-determinating factor (TDF). After transfection with the Sry-Region (the murine 14kb equivalent of the human SRY), transgenic mice with female genotype (X/X) will develop as males (Koopman *et al.* 1991). Similar sex-reversal as the result of an SRY-exchange by crossing-over during meiosis (a risk that comes with the close proximity of SRY to PAR) or secondary to a defective SRY through single nucleotide mutations have been described in humans (McElreavy *et al.* 1992; Wolf *et al.* 1992; Affara *et al.* 1993).

Once the sex is determined genetically by sex-chromosomes, the development of each sex (*sex-differentiation*) can evolve. It is a complex, time-dependant process that requires the expression of a multitude of genes in different tissues. This gene-expression is controlled by sex-hormones, like estrogen or testosterone. These hormones are primarily produced in the gonads. The presence of SYR initiates the development of testicles from the so far undifferentiated gonads. This step grants the production of high levels of androgens and thus causes differentiation of a male phenotype determined by a male genotype. In the absence of SRY the undifferentiated gonads will develop as ovaries (Ganong 1993).

During sex-differentiation, sex-steroids are required as mediators to develop gender-specific features. The significance of the hormonal gene-regulation becomes apparent when the normal function is disturbed. For example, a defective testosterone receptor can cause testosterone-resistance. Despite a male genotype (X/Y), a female phenotype will evolve. This disorder is known as testicular feminism. The importance of estrogen in the female sex-differentiation is also illustrated in the adreno-genital syndrome (AGS). This disease is based on an estrogen-deficit caused by a defective 21-hydroxilase. As a result metabolites of the estrogen-biosynthesis are shifted into pathways of the androgen-production. The surplus in androgen hormones results in male features despite a female genotype (X/X) (Ganong 1993).

Although the amount of DNA that actually makes the difference between male and female is very small, elucidation of the role of sex chromosomes in a study on genetic impact on gender differences of the inflammatory response is mandatory.

Data on the TNF-α phenotype in F1 mice indicate that neither the gender difference in B6 mice nor the response to sex-steroids, in particular the modulation of the TNF-α by EST is linked to the sex-chromosomes or is genetically imprinted and requires homozygosity of the underling allele. Additionally, the opposite effects of EST on LPS-induced TNF-α levels may be the result of opposing alleles that may neutralize the parental phenotype in the F1-generation (**Table 5**).

Data obtained from the F1 generation on the IL-10 phenotype may allow exclusion of any linkage to sex-chromosomes or imprinting, too. However, B X A respond like B6 only. Statistically there is no difference between A/J males and A x B or B6 males and A X B, respectively. A X B females follow the pattern of B6 mice, though. This may suggest a possible linkage to the A/J X-chromosome or maternal imprinting (**Figure 9**). The problem with this experiment however is that A X B mice are not commercially available and thus had to be bred in the institutional animal facility. Thus, a larger number of batches with a smaller number of animals per group were used per experiment. Consequently, environmental factors may differ from those for mice that were obtained from commercial sources, which may disturb the accuracy of this experiment. Moreover, female F1 mice were not stratified by stage of the estrus cycle, which may increase the variability in the control groups.

A mapping approach currently under way suggests candidate genes located on chromosome 13 for the IL-10 phenotype in non-manipulated male mice after injection of LPS and chromosome 9 for the TNF-α phenotype (*unpublished data*). However, the IL-10 gene is located on murine chromosome 1, the TNF-α gene is located on murine chromosome 17. In summary, the observed phenotypes in this study are very likely to follow a multi-factorial pattern of inheritance, involving a number of loci on different autosomes rather than to be linked to sex-chromosomes.

Which loci significantly contribute to the observed effects of sex-steroids and gender-differences will have to be elucidated in future mapping approaches. Distribution of the TNF-α plasma levels after EST treatment in A/J and B6 males suggest that this phenotype may be suitable for comparative mapping strategies (**Figure 15**). Such concepts have previously been described (Matesic *et al.* 1999; Matesic *et al.* 2000).

Besides sex steroids and sex chromosomes, other, yet unknown factors related to gender may be involved. Such factors have been suggested by the fact, that mortality due to infection is higher in newborn boys than in girls (Wells 2000). Additionally, a clinical study in burned children at an average age of 5 years reports a higher mortality rate in boys with respect to girls (Barrow *et al.* 1990). Sex-steroids obviously play a secondary role in these two populations. A comparable study on burns, in sexually mature adults, female sex was found as an independent risk-factor for adverse outcome (O'Keefe *et al.* 2001). Such contradicting results may correlate with gender differences independently of sex-steroids.

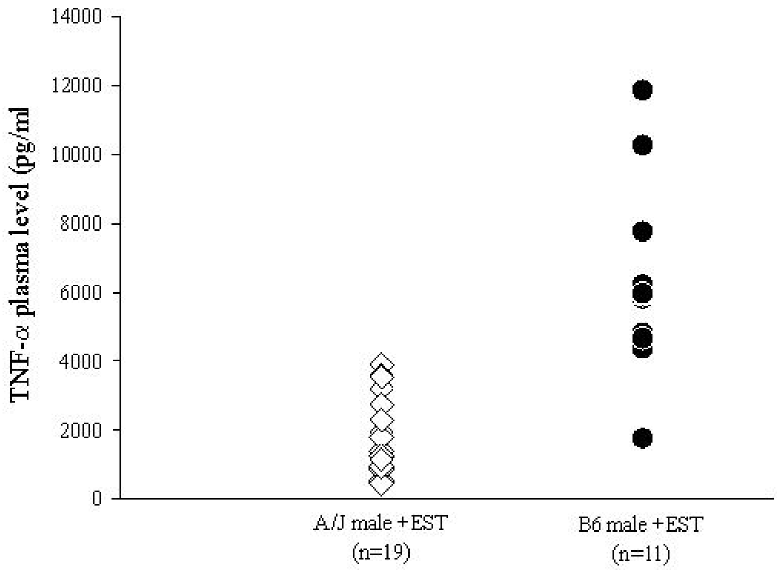


Figure 15: LPS-induced TNF-α plasma levels after estrogen-treatment in castrated A/J and B6 male mice. Mice underwent surgical castration by removal of their testicles at the age of 6 weeks. Subcutaneous constant release pellets containing 17-Estradiol (EST) were implanted. After 14 days of recovery, mice were injected with LPS (15mg/kg). TNF-α plasma levels (pg/ml) were measured 1.5h after the injection using an ELISA. Each dot represents the TNF-α plasma levels in each individual mouse in the respective group (A/J or B6).

5.3. Protection from lethal endotoxic shock by castration depends on genetic background

Androgen-depletion by surgical or pharmacological castration has been proposed to modulate the inflammatory response after hemorrhagic shock and improve outcome from subsequent sepsis (Wichmann *et al.* 1996; Angele *et al.* 1997; Angele *et al.* 1999). In analogy, female sex-steroids may have salutary effects to improved outcome (Zellweger *et al.* 1997; Angele *et al.* 2000; Knoferl *et al.* 2002).

Indeed, castrated A/J male mice were protected against lethal endotoxemic shock (**Figure 12a**). Survival was markedly improved over the evaluated time-course and restoration of the androgen hormonal environment by implantation of DHT pellets reversed this protection (**Figure 13**). In conclusion, androgen depletion results in protection from LPS. The most striking finding of our mortality-studies, however is that the benefit is restricted exclusively to the A/J genetic background. Castration may have decelerated the frequency of mortalities in DBA/2J mice though (**Figure 12d**). All other examined strains had an unchanged outcome after surgical castration. C3H/HeN mice (**Figure 12c**) have been reported to have survival-benefits after androgen depletion in a model of abdominal sepsis following hemorrhagic shock (Angele *et al.* 1997). In our model, they showed no improved outcome. Consequently, protection by androgen depletion may not only depend on the genetic background but moreover may change with the type of injury. Qualitative and quantitative divergence in the response to different types of injury can be observed in animal models: CLP produces a quite different cytokine response as observed during endotoxemia (Villa *et al.* 1995; De Maio *et al.* 1998; Remick *et al.* 2000; Stewart *et al.* 2002). Variable extend of an injury influences the inflammatory response as demonstrated in a model of combined insult using CLP and endotoxic shock (Trentzsch *et al.* 2003).

A mechanistic explanation for protection through androgen depletion has not yet been provided. The evaluated cytokines may suggest reduced TNF-α plasma levels and increased IL-10 plasma levels in A/J male mice after castration as a possible explanation. This observation may suggest a shift in the ratio of pro- vs. anti-inflammatory activity. Although, the cytokine levels lack statistical significance, the analysis of the TNF/IL-10 ratio of these mice indicates a significant drop (**Table 7a**) suggesting attenuation of the predominantly pro-inflammatory state towards an anti-inflammatory state. EST-treatment of A/J males after castration also results in a decrease of TNF-α and an increase of IL-10 plasma levels. Indeed, the TNF/IL-10 ratio is diminished even further and thus the inflammatory response is changed from a predominantly pro-inflammatory response towards an anti-inflammatory state (**Table 7b).** Interestingly, this was not associated with improved survival and actually accelerated the clinical course, although the outcome after all was unchanged (**Figure 11).**

Improved survival may depend on the ideal equilibrium of pro- and anti-inflammatory components of the inflammatory response. Pharmacological approaches to attenuated sepsis in order to improve survival of septic patients aim at anti-inflammatory strategies. The latest promising candidates for "magic bullets"[1] are Afelimomab, a new F(ab`)2 antibody fragment against TNF-α; and activated protein C (*Xigirs™*), which actually possesses anti-inflammatory properties by inhibiting the LPS-induced liberation of TNF-α (Bloos *et al.* 2002; Hotchkiss *et al.* 2003). However, these adjuncts are restricted to the early phase of sepsis and thus illustrate the delicacy of the pro-/anti-inflammatory equilibrium that is mandatory for survival from sepsis. Overwhelming anti-inflammatory predominance may cause depression of the cellular immune function and consequently predispose to increased risk of infectious complication, most likely to result in a fatal course (Faist *et al.* 1996; Oberholzer *et al.* 2002).

Testosterone blockage with Flutamide protected male C3H/HeN mice from septic insults after hemorrhagic shock (Angele *et al.* 1997). In a number of clinical

[1] A fundamental concept of modern pharmacology is that each pathologic condition can be corrected or reversed by a single pharmacological compound. Paul Ehrlich, who first enunciated this concept, called such compounds "*magic bullets*".

studies, a decrease of testosterone levels has been described in male patients after burns (Lephart *et al.* 1987), trauma (Majetschak *et al.* 2000), and sepsis or septic shock (Christeff *et al.* 1988; Christeff *et al.* 1992; Fourrier *et al.* 1994; Schroder *et al.* 1998). Assuming that low testosterone may offer protection from injury, this response may be interpreted as a possible strategy of the organism to adapt to stressful conditions. Surprisingly, this decrease has been reported to be accompanied by an increase in estrogen levels, (Benassayag *et al.* 1984; Christeff *et al.* 1988; Christeff *et al.* 1992; Fourrier *et al.* 1994; Schroder *et al.* 1998; Majetschak *et al.* 2000) and two clinical studies report increased mortality among male patients with such hormonal changes (Schroder *et al.* 1998; Majetschak *et al.* 2000). However, testosterone concentrations in severely ill male patients inversely correlated with APACHE scores (Luppa *et al.* 1991), implying that severely ill patients with high testosterone levels have low APACHE score and thus have a better likely-hood to survive. Taking our mortality studies in castrated male mice into consideration, such contradictive findings may be explained by genetic differences in the studied cohort's gene-pool**.**

Table 7a	**AKR/J**	**A/J**	**B6**	**BALB/cJ**	**DBA/2J**
CONT	2.3 ±0.4 n= 8	3.2 ±0.4 n= 31	0.8 ±0.1 n= 32	0.9 ±0.2 n= 10	9.4 ±1.6 n= 7
CX (+VEH)	1.6 ±0.1 n= 7 p= 0.173	1.7 ±0.4 n= 21 **p= 0.004***	1.2 ±0.2 n= 15 p= 0.182	1.0 ±0.3 n= 9 p= 0.967	8.8 ±1.5 n= 8 p= 0.397

Table 7b	**CONT**	**CX +VEH**	**CX +DHT**	**CX +EST**
A/J	3.2 ±0.4 n= 31	1.7 ±0.4 n= 21 **p= 0.004***	4.2 ±1.5 n= 22 p= 0.179	0.5 ±0.1 n= 19 **p< 0.001*#**

Table 7a+b : Effects of castration on the TNF-α/IL-10 ratio in male mice. Male mice were castrated (CX) and supplemented with vehicle pellets (VEH) at the age of 6 weeks. After recovery for 14 days, LPS was injected (15mg/kg). Blood samples were obtained upon sacrifice 1.5h after the injection and TNF-α and IL-10 plasma levels were measured using an ELISA. Non-operated mice of each strain were maintained under identical conditions and used as control (CONT). **Table 7a** shows baseline ratios of the "inflammatory coefficient" (*i.e.* ratio of TNF-α/IL-10) of mice from various inbred strains (AKR/J n=8; A/J n=31; B6 n=32; BALB/cJ n=10; and DBA/2J n=7) in comparison with ratios of mice after surgical castration and implantation of vehicle pellets (CX+VEH: AKR/J n=7; A/J n=21; B6 n=15; BALB/cJ n=9; and DBA/2J n=8). Data corresponds to the average ratio obtained from all animals in the group ± standard error of the mean. Statistical significance was accepted at *p< 0.05 obtained by Mann-Whitney-Rank Sum Test. **Table 7 b** shows changes in TNF-α/IL-10 ratio in male A/J mice after castration and hormonal treatment. Mice were treated as described. Additional males were castrated (CX) and supplemented with hormone pellets (DHT, n=22; EST, n=19). Data corresponds to the average TNF-α/IL-10 ratio obtained from all animals in the group ± standard error of the mean. Statistical significance was accepted at *p< 0.05 obtained by Mann-Whitney Rank Sum Test for pair-wise comparison VEH vs. CONT and #p<0.05 for pair-wise comparison EST vs. VEH.

5.4. Critical effects of EST on LPS-induced mortality

Modulation of the inflammatory response during endotoxemia in EST-treated male B6 mice increased mortality significantly (**Figure 10**). This finding contradicts with the idea, as concluded from many studies, that female rodents have better survival than males after injury as a result of sex-steroids (Zellweger *et al.* 1997; Angele *et al.* 2000; Diodato *et al.* 2001; Knoferl *et al.* 2002). Interestingly, no differences in mortality between female and male B6 mice after injection of LPS have been observed (Laubach *et al.* 1998). Since the genetic background seems responsible for gender differences and sex-steroidal modulation of the inflammatory response, B6 may not have a suitable make-up for showing gender differences in outcome and inflammatory response in a model of endotoxemia. This strain may also be more susceptible to adverse effects of EST. Activated CD4+ T-cells (*helper T-cells*) secrete cytokines with either one of two distinct and antagonistic profiles. They secrete either cytokines with inflammatory properties (*type 1 helper T-cell*; Th-1) including TNF-α, IFN- γ, and interleukin 2, or cytokines with anti-inflammatory properties (*type 2 helper T-cells* ; Th-2), such as interleukin 4 and IL-10 (Abbas *et al.* 1996; Opal *et al.* 2000). The Th-1 response plays a role in activation and recruitment of other T-cells and macrophages; the Th-2 response regulates antibody secretion from B-cells and exerts anti-inflammatory properties via IL-10. Th-1 and Th-2 clones are reciprocally regulated by their secreted cytokines: INF- γ inhibits the proliferation of Th-2 clones whereas IL-10 inhibits that of Th1 clones (Giron-Gonzalez *et al.* 2000). A shift in the T-cell function towards Th-2 is believed to play a key role in cell-mediated immune dysfunction after trauma and critical illness, which is associated with an increased risk of septic complications (Faist *et al.* 1996; Hotchkiss *et al.* 2003). Such changes in T-cell function have been demonstrated in humans after burns (Zedler *et al.* 1999) and abdominal sepsis (Heidecke *et al.* 2000). A similar shift from Th-1 to Th-2 type response has also been observed in animal models of trauma hemorrhage (Ayala *et al.* 1996; Schneider *et al.* 2000). Interestingly, estrogen enhances phospholipase A2 activity and consequently increases prostaglandin production (Dey *et al.* 1982). Production of prostaglandin E2 from monocytes promotes the described shift in T-cell function from Th-1 to Th-2 (Faist *et al.* 1996). Based on this concept, estrogen may be rather fatal than beneficial in septic patients. Indeed, an increase in estrogen levels can be observed in such patients (Benassayag *et al.* 1984; Christeff *et al.* 1988; Christeff *et al.* 1992; Fourrier *et al.* 1994; Schroder *et al.* 1998; Majetschak *et al.* 2000). The increase in plasma estrogen levels is accompanied by decreasing testosterone levels and as observed in two clinical studies (Schroder *et al.* 1998; Majetschak *et al.* 2000) this finding correlates with increased mortality associated with male gender. A state of high estrogen and low testosterone reflects conditions similar to those in our mortality study and thus may explain the severe effects of EST-treatment on castrated B6 males (**Figure 10**). High estrogen plasma levels may contribute to the shift in T-cell function that is associated with posttraumatic immune changes. As studies in humans may suggest, the major sex-dimorphic difference in the inflammatory response is a predominantly Th-2 biased response in females as opposed to a predominantly Th-1 response in males (Giron-Gonzalez *et al.* 2000). This situation is likely to be determined by estrogen-action. Estrogen-treatment promotes suppression of the Th-1 type response in the delayed-type hypersensitivity response to purified protein derivatives in mice (Salem *et al.* 2000). We have observed markedly increased IL-10 plasma levels after EST-treatment in several strains of inbred mice (**Table 3 and Table 4).** Interleukin 10 is a defining cytokine of the Th-2 response (Faist *et al.* 1996) and has been shown to down-regulate the production of several pro-inflammatory cytokines, including TNF-α, IL-1, IL-6, IL-12 and IL-18 (Moore *et al.* 1993). However, the beneficial effect of IL-10 in the response to injury is controversial. Exogenously administration of IL-10 has been shown to reduce several aspects of the inflammatory process and improve survival in experimental models of endotoxemia (Howard *et al.* 1993) and against staphylococcal enterotoxin B (Bean *et al.* 1993). On the other hand, similar experiments in animal models of sepsis and thermal injury have shown no effect on inflammatory mediators, increased T-cell dysfunction, and elevated mortality (Oberholzer *et al.* 2002). Further studies will have to determine whether EST can be helpful to modulate the inflammatory response in order to protect from adverse outcome after injury. Correct dosage might be of critical importance (Goretzlehner 1991) and so may be the right equilibrium between androgen and estrogens (Angele *et al.* 1998). However, the genetic background and type of injury may restrict such approach to an exclusive group of patients. In our model, we were not able to identify such a group within the evaluated gene-pool.

5.5. Clinical and scientific relevance of the findings

Our findings illustrate, that the modulation of the inflammatory response by sex-steroids depends on the genetic make-up of the subject. During sex-differentiation, sex-steroids control the expression of genes that are responsible for the formation of gender-specific features. Studies suggest that genes encoding mediators of the inflammatory response are under sex-steroidal control: Estrogen receptors (ERs) have been identified in the nuclei of various human immune cells, such as monocytes (Wada *et al.* 1992; Ben-Hur *et al.* 1995), macrophages (Gulshan *et al.* 1990), and T cells(Cohen *et al.* 1983; Cutolo *et al.* 1993). There is evidence of membrane-bound ERs on the surface of monocytes (Stefano *et al.* 1999). Steroid responsive elements are located in the promoter region of the IL-10 gene (Kim *et al.* 1992; Kube *et al.* 2001). Estrogen may possibly control cytokine production by direct interaction with NF-κB (Ray *et al.* 1994; Stein *et al.* 1995). Apparently, sex-steroids regulate the inflammatory process at different levels and all of these mechanisms offer a vast number of possible genetic differences.

However, the effect of sex-steroids on the inflammatory response is not heterogeneous: A decrease in LPS-induced production of TNF-α was observed in murine splenic macrophages from BALB/c mice treated with EST (Deshpande *et al.* 1997). In contrast, an increase in this cytokine was observed in peritoneal macrophages derived from female BALB/c mice (Zuckerman *et al.* 1996), or male rats (Chao *et al.* 1994) both treated with LPS and EST. A reduction in IL-6 levels was found in BALB/c splenic macrophages treated with LPS and EST (Deshpande *et al.* 1997), which is the opposite observation as in peritoneal macrophages from female BALB/c mice under similar conditions (Zuckerman *et al.* 1996). While these findings may indicate different responses in distinct compartments of the inflammatory cascade and thus may contribute to the contradicting results, our data clearly demonstrates that the genetic background determines variable modulation of the inflammatory response by sex-steroids. This genetic component can be easily visualized by analyzing the ratio between IL-10 and TNF-α of each strain (**Figure 16 a + b**). Changes in this ratio by administration of EST are obviously very different and predicting the effect seems impossible.

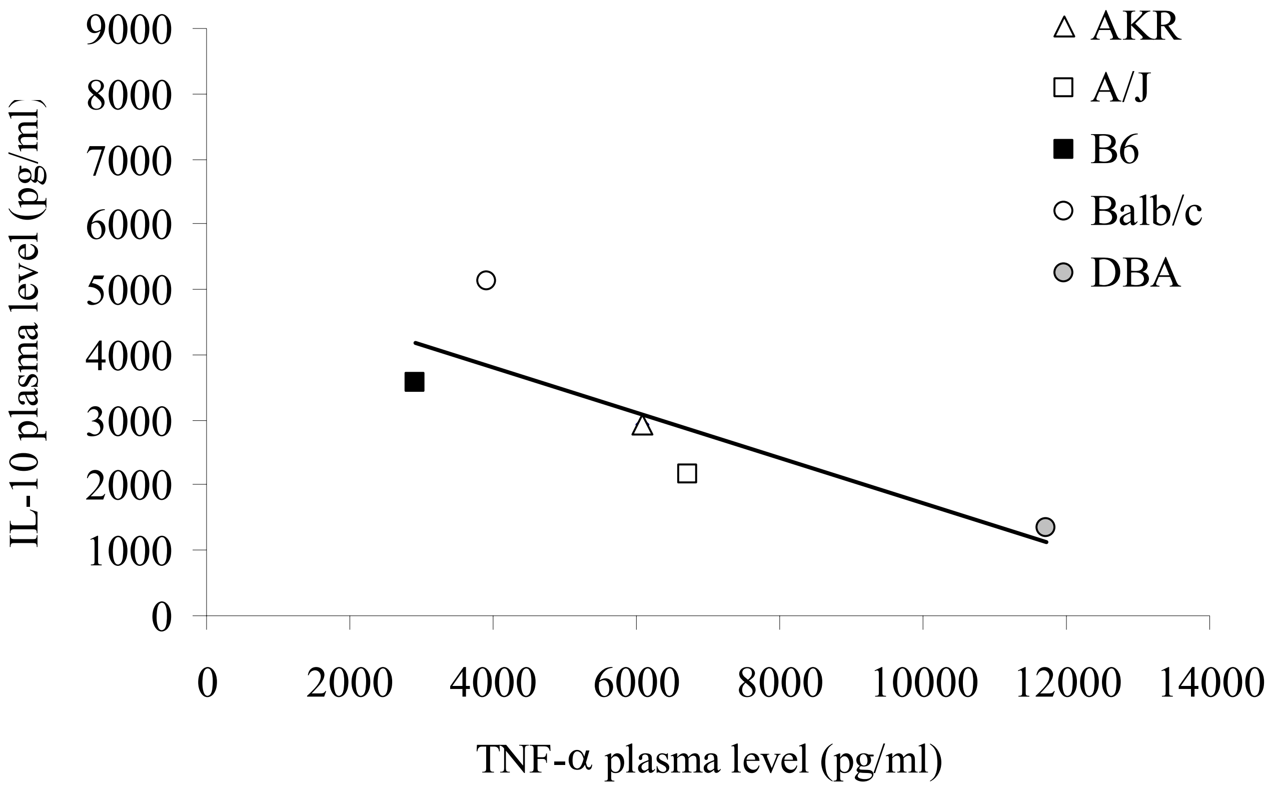


Fig. 16 a: Ratio in CONT (no EST)

Figure 16 a+b: Strain-specific variability in TNF/IL-10 ratio of male mice 1.5h after injection of LPS. **Figure 16a** shows the distribution of average TNF/IL-10 ratios of non-manipulated, 8 week old male mice after injection of LPS (15mg/kg). AKR n= 8, A/J n=10, B6 n=6, BALB/c n=10, DBA/2J n=7**.** **Figure 16b** additionally shows the distribution of TNF/Il-10 ratio in male mice that were castrated at the age of 6 weeks and received EST-treatment for 2 weeks. Arrows indicate the shift in the ratio that resulted from the EST-treatment. AKR (n=8), A/J (n=7), B6 (n=6), BALB/c (n=8), DBA/2J (n=8). Each dot represents the average TNF/IL-10 ratio of each group. Arrows indicate the shift in the ratio when compared with non-manipulated CONT.

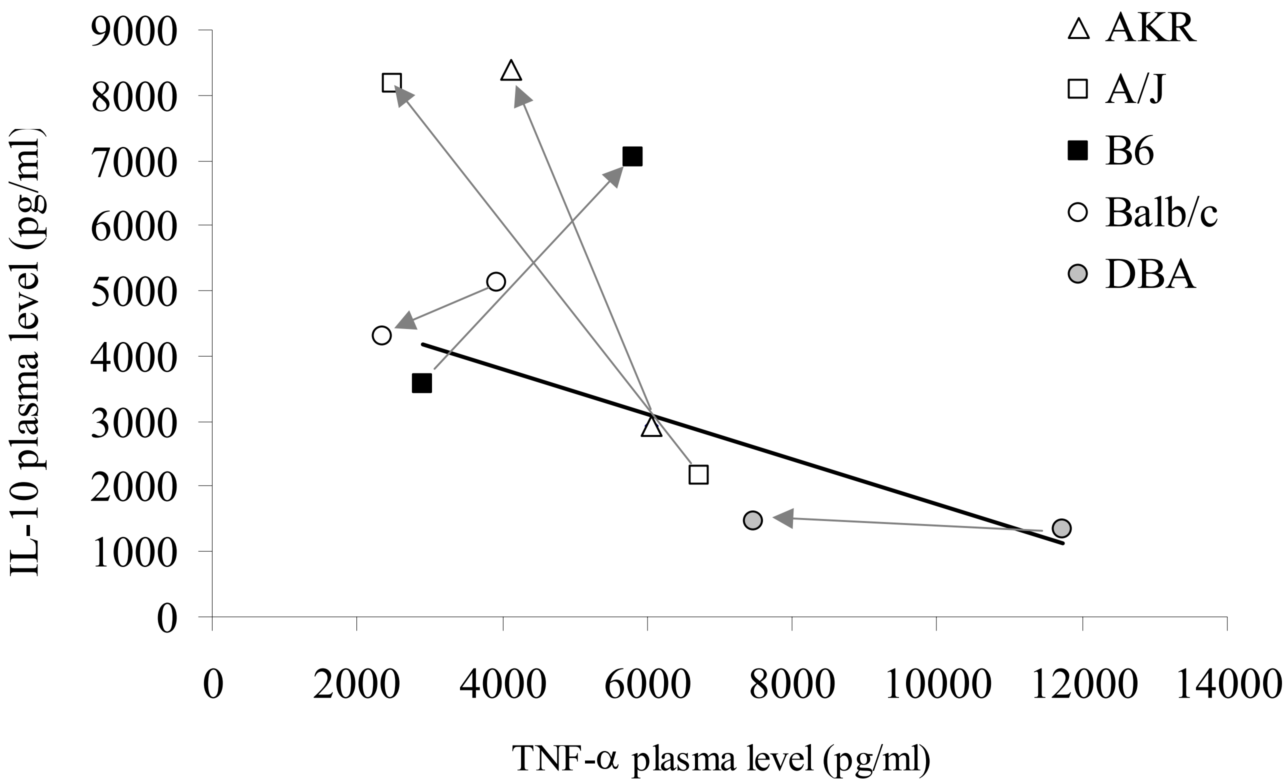


Fig. 16 b: Changes in Ratio with EST

We are aware, that findings in mice are difficult to be extrapolated to men. However, our observations suggest a critical role of hormones and hormonal treatment in critically ill patients. Sex steroids are routinely employed in the clinical field, for example in prostate cancer therapy and hormone replacement therapy (HRT). Consequently, information on how hormones influence the body's homeostasis is of importance. This became illustrated most recently by the confusion caused by the halt of the estrogen plus progestin (*Prempro™*) component in the Women's Health Initiative (WHI), a randomized, placebo-controlled and blinded, multi-center trail enrolling 27.348 patients for evaluation of HRT. As a matter of fact, 22.3 million prescriptions make Prempro™ the second most frequently prescribed hormone replacement medication throughout the US in 2000 (Kreling *et al.* 2001). The data and safety monitoring board (DSMB) of the WHI made their recommendation to halt the Prempro™ treatment-arm, after Prempro™ medication was associated with an increased risk of invasive breast cancer, stroke, coronary heart disease, and pulmonary embolism. An overall measurement suggested that HRT with estrogen plus progestin would do more harm than good (*i.e. reduced risk of colorectal cancer and reduced hip-fractures*) (Fletcher *et al.* 2002; Rossouw *et al.* 2002). As a result of the DSMB-recommendation, physicians worldwide had to wonder if HRT could be considered safe. Epidemiologic data of the WHI indicates that the majority of patients enrolled in this trail were Caucasians. One may speculate if the increased risk would have been reduced, if patients with potential genetic risk-factors would have been excluded.

In another study on men after coronary artery bypass found reduced leukocyte activation after estrogen treatment which may contribute to improved graft survival (Wei *et al.* 2001). However, the sample size was too small to detect improved outcome and there were no significant changes in cytokine profiles. If they successfully prevented sepsis and multiple organ failure was not evaluated. Apparently, EST-treatment was tolerated better than in our model. This may be a result of the type of injury, differences in environmental factors, or genetics (*including genetic differences between species!*).

Our data supports the hypothesis that clinical application of sex-steroids or alteration of sex-hormone profiles in critically ill patients may be difficult to asses without consideration of the genetic background. Quantitative trait loci that are responsible for the phenotypes observed in our mouse model may have homologies in the human genome and thus one day may allow identification of patients at risk to develop sepsis or to adjust their therapeutic regiments.

Preliminary sequencing data from the human genome project reveals that the human genome consists of possibly 25,000 to 40,000 genes. Only twice as many as in fruit fly, worm, or plant and about the same number of genes that built the murine genome (Waterston *et al.* 2002).

Genomics, the science of determining the functions of individual gene segments, facilitates structural homologies between evolutionarily, closely related genomes. Its success is based on a simple concept: Functionally important sequences are more likely to retain their sequence during evolution than non-functional sequences. So DNA sequences that are conserved between species are likely to have important function. Comparison of genomes of closely related species may also help to identify gene-control regions (Rubin 2001). We may assume that homologies of loci that control the inflammatory response between mice and men exist. An assumption that has been fueled by disclosure of sequencing data of the murine genome (Waterston *et al.* 2002).

5.6. Comments on methodology

We have based our experimental approach to the role genetic contribution to sex-steroidal modulation of the inflammatory response on the premises that the inflammatory response is the intersection of three major components: type of injury, environmental factors and the genetic background (**Figure 14**). To disclose differences that are determined by either one of these three factors, uncompromised experimental control of the other factors is mandatory. In our model, we studied the inflammatory response induced by injection of LPS to inbred mice. The use of small rodents provides a lot of advantages. They are inexpensive, widely available in large numbers, at the same age and sex, genetically identical, free of specific pathogens, and on the same diet, which minimize biological variability.

LPS-models are well-know for their reliable and reproducible results (Chaudry 1999). TLR4 seems to be the sole gateway to the LPS-induce inflammatory response in mice, by implication in all mammals. LPS itself is not capable to evoke shock, tissue injury, and other effects through non-specific interaction. Instead it can be anticipated that these effects are a product of the pro-inflammatory cascade activated by this single pathway (Beutler *et al.* 2001). Highly reproducible phenotypes are the fundamental bases for comparative mapping-strategies. LPS-injections require only minimal animal manipulation and thus warrant little alteration of the organism's homeostasis after the inflammatory challenge. Our experience with the LPS-model reassures us that observations in this model are highly reproducible (De Maio *et al.* 1998).

Apparently, gram-negative sepsis can not be restricted to LPS alone. Consequently, experimental endotoxemia is probably a better model of inflammation and acute phase response than of authentic sepsis. However, we think that the early events of the acute phase and inflammatory response are key-factors in the pathogenesis of sepsis and the resulting clinical course. Endotoxic shock provides an excellent model to study the impact of the genetic background on the inflammatory response because it reduces the risk of other pathways creating too much background, which actually may mask genetic differences.

Many LPS preparations are contaminated with other bacterial products reacting with different Toll receptors, which may explain some of the discrepancies in the literature with regard to LPS (Hirschfeld *et al.* 2000). Although a non-extracted mixture might represent real biology, it complicates experimentation. Only when LPS is re-extracted it acts as a pure TLR4 activator. To keep the experimental conditions in this study constant, we utilized LPS from a single batch only.

LPS produces a cytokine-rich inflammatory response. To quantify the inflammatory response and to detect changes in the response after hormone manipulation, we selected two of the possible markers involved, TNF-α and IL-10. These two markers have been considered as major players of the LPS-induced inflammatory response. A large body of evidence on these two markers has been accumulated, providing a broad basis for discussion of the findings. Detection by the use of ELISA-Kits is state of the art (Ertel *et al.* 1993).

Studying hormonal effects is a big experimental challenge. Endocrine activity underlies dynamic changes that lie beyond experimental control or simply exceed a practical approach. Obviously the biggest challenge in studies on sex-hormones is the reproductive cycle of females that is accompanied with a load of hormonal changes over a short period of time. As outlined in **Appendix 1**, the window of a certain condition may close over the time-course of an experiment and thus complicates experimental conditions (*e.g. mortality-studies*). Moreover, misinterpretation of smears may occur (*e.g. anestrus or pseudopregnancy*). For example, cycle-related changes on cytokine, such as TNF-α in humans remains controversial (Angstwurm *et al.* 1997; Schwarz *et al.* 2000; Bouman *et al.* 2001). The evaluation of cycle-determined differences is complicated and difficult to perform even in the controlled environment of an animal model. For this reason and to control for strain-specific differences in hormonal features, we decided to control hormonal activities by applying defined doses of hormone into gonadectomized animals. This model has previously been reported to result in physiologic hormone plasma levels in mice (Angele *et al.* 1999).

Administration of LPS and estriol (*an estrogen agonist*) to female BALB/c mice did not affect IL-6 serum levels, but did change the kinetics of its appearance in the circulation (Zuckerman *et al.* 1996). We did not check for changes in the kinetics of our model. It is possible that IL-10 plasma levels in DBA/2J and BALB/cJ mice after EST treatment reached peaks either before or after our designated time-point that we may have missed. This may explain why we did not see any response to DHT and why female mice seemed to be unresponsive to hormonal manipulation. However, we feel comfortable with this decision, since changes in the kinetics may be result of strain-specific traits and thus would be referred to as a result of different genetics. However such changes would not be suitable as map-able phenotypes and therefore are of minor interest.

6. SUMMARY

We have shown that gender is a contributing factor in the LPS-induced inflammatory response of B6 mice. Gender-dimorphisms of the inflammatory response appear to be associated with hormonal differences. However, this contribution is dependent on the genetic background, as demonstrated in comparison with A/J mice. Additionally, we found that treatment with sex-steroid modulates LPS-induced mediators of the inflammatory response, such as IL-10 and TNF-α. However, male mice seem to be better responders to such manipulation. Moreover, the effects were dependent on genetic differences. Some mouse strains revealed to be non-responders to changes in the hormonal environment, *i.e.* IL-10 levels after EST-treatment in DBA2/J and BALBc/J mice, while others showed opposing response, *i.e.* TNF-α levels of EST-treated A/J and B6 mice. Thus, our data suggests that gender and genetic diversity combine to modulate the response to a particular injury. However, the effects of sex-steroids and the observed gender-differences seem to be independent of sex-chromosomes. We also evaluated effects of hormonal manipulation at the levels of mortality. Androgen depletion is considered a mainstay of gender-related differences and may improve survival. Interestingly, castration protected only A/J mice against LPS. Such protective effects may be secondary to sex-steroid controlled changes in the ratio of pro- vs. anti-inflammatory components of the inflammatory cascade. However, we concluded that protection is dependent on the type of injury and the genetic background. EST-treatment of A/J and B6 males did not improve outcome from endotoxic shock.

If our findings in mice could be extrapolated to humans, they might explain contradictory observations of clinical studies on gender differences. Genetic markers might better delineate the contribution of gender in the response to injury in the

clinical setting. Thus, research on such markers needs to be intensified in the future. Consequently, this information is of importance for planning basic science experiments, clinical trials and for the development of therapies that ameliorate the secondary effects of injury.

7. ZUSAMMENFASSUNG

Die hier vorliegenden Daten zeigen deutliche geschlechtsspezifische Unterschiede in der LPS-induzierten entzündlichen Antwort von B6-Mäusen. Geschlechtsspezifische Dimorphismen der entzündlichen Antwort scheinen mit hormonell bedingten Unterschieden assoziiert zu sein. Der Vergleich mit A/J-Mäusen weist hierbei jedoch eine unterschiedliche Ausprägung in Abhängigkeit von genetischen Faktoren auf. Darüber hinaus wurde festgestellt, dass die hier untersuchten Marker TNF-α und IL-10 durch Behandlung mit Sexual-Steroiden moduliert werden können. Männliche Tiere reagieren dabei allerdings deutlich besser auf diese Beeinflussung von außen. Auch hier wurde eine Abhängigkeit von genetischen Faktoren gezeigt: Einige der untersuchten Mausstämme erwiesen sich von den Veränderungen der hormonellen Bedingungen unbeeinflusst, wie an den IL-10 Plasmaspiegel von DBA2/J und BALBc/J Mäusen nach EST-Behandlung zu sehen ist, während andere Stämme gar gegensätzliche Antworten zeigten, wie an den TNF-α Plasmaspiegeln nach EST-Behandlung von A/J und B6 Mäusen erkennbar wird.

Somit erlauben die vorgelegten Daten die Schlussfolgerung, dass biologisches Geschlecht und individuelle genetische Ausstattung gemeinsam einen messbaren Einfluss auf die entzündliche Antwort nach einer bestimmten Verletzung haben. Die Effekte von Sexualsteroiden und Geschlechtsunterschieden sind dabei unabhängig von den Geschlechtschromosomen. Da Androgene allgemein als hauptsächlich verantwortlich für die geschlechtsspezifischen Unterschiede der entzündlichen Antwort eingeschätzt werden, haben wir von der Verringerung der systemischen Androgenspiegel einen Überlebensvorteil erwartet. Um so interessanter war die Beobachtung, dass diesbezüglich lediglich A/J Mäuse nach chirurgischer Kastration vor den Auswirkungen der LPS-Gabe geschützt waren. Dieser protektive Effekt könnte die Folge von sexual-steroid-abhängigen Änderungen in der Relation von pro- und antiinflammatorischer Komponente der entzündlichen Antwort sein. Es ist anzunehmen, dass dieser Schutz nur bei entsprechender genetischer Konstellation und wahrscheinlich in Abhängigkeit vom Verletzungsmechanismus zustande kommt. EST-Behandlung von männlichen A/J and B6 Mäusen brachte kein verbessertes Überleben nach Endotoxinschock.

Könnte man diese Ergebnisse auf Menschen übertragen, so ließe sich hieraus eine Erklärung für gegensätzliche Beobachtungen bei geschlechtsspezifischen Unterschieden in klinischen Studien ableiten. Genetische Marker könnten helfen, die Einflüsse des biologischen Geschlechts auf die entzündliche Antwort klinisch besser untersuchen zu können. Die Suche nach solchen Markern sollte in Zukunft intensiviert werden, da ihnen auch eine große Bedeutung für das Design von Laborexperimenten und klinischen Studien zukommt, aus denen sich dann eventuell sogar therapeutische Ansätzen zur Milderung der sekundären Effekte von Verletzungen ableiten lassen.

APPENDIX 1: On the Estrus Cycle of the Mouse.

Female mice have a poly-estrus cycle that persists throughout the whole year. Central endocrine regulation involves Gonadotrophine Releasing Hormone (GnRH), Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH) that are derived from hypothalamus and piturity gland and control levels of estradiol and progesterone, which themselves contribute to the regulation of the estrus cycle through positive feed-back mechanism on the central regulation. The ovaries are the major source of estradiol and progesterone. The growing oocyte produces estradiol.

Throughout the cycle, phases of predominant estradiol-levels or predominant progesterone-levels create four stage of the estrus cycle: diestrus, proestrus, estrus and metestrus (also know as postestrus or metaestrus; subdivision into phase I and II may occur). The estradiol/progesterone-ratio changes over these stages (Nelson *et al.* 1981). Consequently, metabolic changes occur along the cycle: Proestrus and estrus are anabolic stages with active growth, whereas metestrus is a catabolic stage of degenerative changes. Diestrus is a quiescent stage with slow growth (Schwacha *et al.* 2001). Morphologic changes in the cells reflect these metabolic changes. There are three types of cells commonly found in smears obtained from the vagina: Polymorphonuclear cells (*i.e.* leucocytes), nucleated epithelium cells and cornified epithelium cells. The morphology and number of these three types reflects the phase of the cycle and has been well described (Barkley *et al.* 1981; Rugh 1990; Schwacha *et al.* 2001):

Peak levels of estrogen characterize **proestrus** (20-25 pg/ml in CD-1 mice (Campbell *et al.* 1976) and CBA/J NIA mice (Kahlke *et al.* 2000); up to 28 pg/ml in B6 (Nelson *et al.* 1981)). Smears show approximately even numbers of leucocytes and primarily nucleated epithelium cells. Only few cornified cells are present. This phase takes 24 to 36h. While estradiol-levels start to decline with the onset of **estrus**, progesterone starts to increase. The epithelium cells of the vagina show an accelerated turnover. A lot of huge, squamous, cornified cells without nuclei are obtained in the smear. Leukocytes are missing completely. In the early phase of estrus, clearly defined epithelial cells with distinct nuclei can also be found. Estrus, (from Greek o*estrum* for "heat") is the beginning of the reproductive state in the cycle. Ovulation starts 2 to 3 hours after the onset of estrus (Crispens 1975). However, it is the shortest phase of all and takes only 12 to 14h (Crispens 1975). Extended duration of estrus up to 72h has been described (Schwacha *et al.* 2001). Throughout **metestrus**, estrogen-levels further decline (as low as 5 pg/ml in CD-1 mice (Campbell *et al.* 1976)) and progesterone-levels remain high (9.9 ng/ml have been described on day 0 of pregnancy (Crispens 1975), which is comparable to maximum progesterone levels during metestrus in CD-1 mice (Campbell *et al.* 1976), slightly lower levels of 4 ng/ml were found in B6 (Nelson *et al.* 1981)). Now, the epithelium cells are large, folded and with translucent nuclei. They lie in even numbers with leucocytes. This phase takes 24 to 48h (Crispens 1975) but can extend up to 120h (Schwacha *et al.* 2001). **Diestrus** starts as progesterone-levels go back to baseline (~2 ng/ml progesterone during proestrus (Campbell *et al.* 1976; Nelson *et al.* 1981)). Estradiol remains low. The smear will now produce almost exclusively leukocytes. This phase takes 36 to 72h before rising estradiol levels lead into a new onset of proestrus. Under certain conditions, diestrus is prolonged for several days.

An easy way to determine the stage of the estrus cyle is to obtain smears through lavage with isotonic saline solution as described by Rugh (Rugh 1990). An alternative technique is inspection of the typical changes found in the genital by Champlin (Champlin *et al.* 1973). While this technique is less invasive than a lavage, which is considered a low-stress procedure anyhow, inspection demands a lot of experience in order to make valid predictions on the stage of the cycle. However, it may be preferable for repeated sampling. Repeated smearing, especially with cotton swabs is likely to result in vaginal cornification or the induction of **pseudopregnancy**.

The duration of the cycle has an average duration of 4 to 5 days, however most authors report variable length of the different phases. The total of days may exceed this period and thus cycle length varies between 3 to 9 days. In fact, regularly recurring cycles are rare in the mouse other than in rats (Barkley *et al.* 1981). Several factors have impact on the estrus cycle: day-night-phases (Campbell *et al.* 1976), age (Nelson *et al.* 1981) and even genetic background (Barkley *et al.* 1981) influence its delicate dynamics. CF-1 female mice subjected to short-term food deprivation (24h or 48h) showed delay of ovulation by a week or more when 48 h of food deprivation was initiated in diestrus. Lesser delays occurred when food deprivation began in estrus (Bronson *et al.* 1985).

The absence of males or housing large groups of females together in the same cage may result in irregular cycling (i.e. **prolonged diestrus)**. Two different types can be distinguished: anestrus or pseudopregnancy. **Anestrus** is characterized by prolonged diestrus predominantly when large groups of females are housed together and rapidly changes to estrus upon pairing with males. **Pseudopregnancy** shows formation of deciduomata and occurs preferablely in small groups of females without presence of males. For both phenomena, a genetic contribution has been suggested. Olfactory stimuli *i.e.* presence of males or male urine exposure are capable to override prolonged phases of diestrus and synchronize the onset of estrus (McKinney 1972; Barkley *et al.* 1981).

ABBREVIATIONS

$	U.S. Dollar
%	percent
μg	microgram ($1x10^{-9}$ kg)
/d	per day
μL	micro liter ($1x10^{-9}$ L)
A X B	AB6F1 mice (F1-generation; the offspring from an A/J female and a B6 male)
AGS	adreno-genital syndrome
ANOVA	Analysis of Variance
APACHE	acute physiology and chronic health evaluation (scoring system)
ARDS	Acute Respiratory Distress Syndrome
B X A	B6AF1 mice (F1-generation; the offspring from a B6 female and an A/J male)
B6	inbred mouse strain C57BL/6J
CD-14	Cluster of Differentiation 14
CD-4	Cluster of Differentiation 4
CLP	cecal ligation and puncture
CONT	control
CX	castrated
DHT	5α-dihydrotestosterone
DNA	deoxyribonucleic acid
DSMB	data and safety monitoring board
E. coli	Escherichia coli
EDTA	ethylene diamine tetraacetic acid
ELISA	enzyme-linked immuno sorbant assay
ERs	estrogen receptors
EST	17-β-Estradiol
F1	1st filial generation
FSH	follicle stimulating hormone
G	gauge, unit for outer diameter of a catheter
g	gram ($1x10^{-3}$ kg)

GnRH	gonadotrophine releasing hormone
h	hours
HLA	human leukocyte antigens
HRT	hormone replacement therapy
IFN-γ	interferon γ
IL-10	interleukin 10
IL-1ß	interleukin 1ß
IL-2	interleukin 2
IL-3	interleukin 3
IL-6	interleukin 6
kb	kilo base-pairs
kg	kilogram
L	liter
LBP	LPS-binding protein
LH	luteinizing hormone
LPS	lipopolysaccaride
mg	milligram ($1x10^{-6}$ kg)
MHC-I	major histocompatibility complex class 1
MHC-II	major histocompatibility complex class 2
ml	milliliter ($1x10^{-6}$ L)
MODS	Multiple Organ Dysfunction Syndrome
n	number of mice in the designated experimental group
NF-κB	nuclear factor κB
ng	nanogram ($1x10^{-12}$ kg)
NIH	National Institutes of Health
p	p-value, probability of a statistic occurring by chance
PAR	pseudoautosomal region
pg	pictogram ($1x10^{-15}$ kg)
SRY	sex-determining region Y
TDF	testis-determinating factor

Th1	type 1 helper T-cell
Th2	type 2 helper T-cell
TLR-4	Toll-like receptor 4
TNF-α	Tumor Necrosis Factor α
U.S.	United States of America
V/V	volume per volume
VEH	vehicle, i.e. placebo-pellet without hormone
WHI	Women's Health Initiative
X/X	female genotype as indicated by two x chromosomes
X/Y	male genotype as indicated by x and y chromosome

REFERENCES

Abbas, A. K., K. M. Murphy and A. Sher: "Functional diversity of helper T lymphocytes." Nature 383(6603): 787-93; 1996

Abraham, E., A. Anzueto, G. Gutierrez, *et al.*: "Double-blind randomised controlled trial of monoclonal antibody to human tumour necrosis factor in treatment of septic shock. NORASEPT II Study Group." Lancet 351(9107): 929-33.; 1998

Abraham, E., P. F. Laterre, J. Garbino, *et al.*: "Lenercept (p55 tumor necrosis factor receptor fusion protein) in severe sepsis and early septic shock: a randomized, double-blind, placebo-controlled, multicenter phase III trial with 1,342 patients." Crit Care Med 29(3): 503-10.; 2001

Abraham, E., R. Wunderink, H. Silverman, *et al.*: "Efficacy and safety of monoclonal antibody to human tumor necrosis factor alpha in patients with sepsis syndrome. A randomized, controlled, double-blind, multicenter clinical trial. TNF-alpha MAb Sepsis Study Group." Jama 273(12): 934-41.; 1995

Affara, N. A., I. J. Chalmers and M. A. Ferguson-Smith: "Analysis of the SRY gene in 22 sex-reversed XY females identifies four new point mutations in the conserved DNA binding domain." Hum Mol Genet 2(6): 785-9; 1993

Aggarwal, B. B. and K. Natarajan: "Tumor necrosis factors: developments during the last decade." Eur Cytokine Netw 7(2): 93-124; 1996

Angele, M. K., A. Ayala, B. A. Monfils, *et al.*: "Testosterone and/or low estradiol: normally required but harmful immunologically for males after trauma-hemorrhage." J Trauma 44(1): 78-85; 1998

Angele, M. K., M. W. Knoferl, M. G. Schwacha, *et al.*: "Sex steroids regulate pro- and anti-inflammatory cytokine release by macrophages after trauma-hemorrhage." Am J Physiol 277(1 Pt 1): C35-42.; 1999

Angele, M. K., M. G. Schwacha, A. Ayala, *et al.*: "Effect of gender and sex hormones on immune responses following shock." Shock 14(2): 81-90.; 2000

Angele, M. K., M. W. Wichmann, A. Ayala, *et al.*: "Testosterone receptor blockade after hemorrhage in males. Restoration of the depressed immune functions and improved survival following subsequent sepsis." Arch Surg 132(11): 1207-14.; 1997

Angstwurm, M. W., R. Gartner and H. W. Ziegler-Heitbrock: "Cyclic plasma IL-6 levels during normal menstrual cycle." Cytokine 9(5): 370-4; 1997

Angus, D. C., W. T. Linde-Zwirble, J. Lidicker*, et al.*: "Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care." Crit Care Med 29(7): 1303-10; 2001

Asai, K., N. Hiki, Y. Mimura*, et al.*: "Gender differences in cytokine secretion by human peripheral blood mononuclear cells: role of estrogen in modulating LPS-induced cytokine secretion in an ex vivo septic model." Shock 16(5): 340-3; 2001

Ayala, A., W. Ertel and I. H. Chaudry: "Trauma-induced suppression of antigen presentation and expression of major histocompatibility class II antigen complex in leukocytes." Shock 5(2): 79-90; 1996

Barkley, M. S. and G. E. Bradford: "Estrous cycle dynamics in different strains of mice." Proc Soc Exp Biol Med 167(1): 70-7.; 1981

Barrow, R. E. and D. N. Herndon: "Incidence of mortality in boys and girls after severe thermal burns." Surg Gynecol Obstet 170(4): 295-8.; 1990

Baud, L., J. Cadranel, G. Offenstadt*, et al.*: "Tumor necrosis factor and septic shock." Crit Care Med 18(3): 349-50; 1990

Baue, A. E.: "Multiple, progressive, or sequential systems failure. A syndrome of the 1970s." Arch Surg 110(7): 779-81.; 1975

Baue, A. E.: "MOF/MODS, SIRS: an update." Shock 6(Suppl 1): S1-5.; 1996

Bauerle, R., A. Rucker, T. C. Schmandra*, et al.*: "Markov cohort simulation study reveals evidence for sex-based risk difference in intensive care unit patients." Am J Surg 179(3): 207-11.; 2000

Bean, A. G., R. A. Freiberg, S. Andrade*, et al.*: "Interleukin 10 protects mice against staphylococcal enterotoxin B-induced lethal shock." Infect Immun 61(11): 4937-9; 1993

Benassayag, C., N. Christeff, M. C. Auclair*, et al.*: "Early released lipid-soluble cardiodepressant factor and elevated oestrogenic substances in human septic shock." Eur J Clin Invest 14(4): 288-94; 1984

Ben-Hur, H., G. Mor, V. Insler*, et al.*: "Menopause is associated with a significant increase in blood monocyte number and a relative decrease in the expression of estrogen receptors in human peripheral monocytes." Am J Reprod Immunol 34(6): 363-9; 1995

Beutler, B. and A. Poltorak: "Sepsis and evolution of the innate immune response." Crit Care Med 29(7 Suppl): S2-6; discussion S6-7; 2001

Bloos, F. and K. Reinhart: "[Anti-inflammatory treatment in sepsis]." Chirurg 73(11): 1087-92; 2002

Bone, R. C.: "Toward an epidemiology and natural history of SIRS (systemic inflammatory response syndrome) [see comments]." Jama 268(24): 3452-5; 1992

Bouman, A., H. Moes, M. J. Heineman, *et al.*: "The immune response during the luteal phase of the ovarian cycle: increasing sensitivity of human monocytes to endotoxin." Fertil Steril 76(3): 555-9; 2001

Bronson, F. H. and F. A. Marsteller: "Effect of short-term food deprivation on reproduction in female mice." Biol Reprod 33(3): 660-7.; 1985

Cabrera, M., M. A. Shaw, C. Sharples, *et al.*: "Polymorphism in tumor necrosis factor genes associated with mucocutaneous leishmaniasis." J Exp Med 182(5): 1259-64; 1995

Campbell, C. S., K. D. Ryan and N. B. Schwartz: "Estrous cycles in the mouse: relative influence of continuous light and the presence of a male." Biol Reprod 14(3): 292-9.; 1976

Champlin, A. K., D. L. Dorr and A. H. Gates: "Determining the stage of the estrous cycle in the mouse by the appearance of the vagina." Biol Reprod 8(4): 491-4.; 1973

Chao, T. C., P. J. Van Alten and R. J. Walter: "Steroid sex hormones and macrophage function: modulation of reactive oxygen intermediates and nitrite release." Am J Reprod Immunol 32(1): 43-52.; 1994

Chaudry, I. H.: "Sepsis: lessons learned in the last century and future directions." Arch Surg 134(9): 922-9; 1999

Christeff, N., C. Benassayag, C. Carli-Vielle, *et al.*: "Elevated oestrogen and reduced testosterone levels in the serum of male septic shock patients." J Steroid Biochem 29(4): 435-40; 1988

Christeff, N., A. Carli, C. Benassayag, *et al.*: "Relationship between changes in serum estrone levels and outcome in human males with septic shock." Circ Shock 36(4): 249-55; 1992

Cohen, J.: "The detection and interpretation of endotoxaemia." Intensive Care Med 26(Suppl 1): S51-6.; 2000

Cohen, J. and J. Carlet: "INTERSEPT: an international, multicenter, placebo-controlled trial of monoclonal antibody to human tumor necrosis factor-alpha in patients with sepsis. International Sepsis Trial Study Group." Crit Care Med 24(9): 1431-40.; 1996

Cohen, J. H., L. Danel, G. Cordier*, et al.*: "Sex steroid receptors in peripheral T cells: absence of androgen receptors and restriction of estrogen receptors to OKT8-positive cells." J Immunol 131(6): 2767-71; 1983

Crispens, C. G.: Handbook on the Laboratory Mouse, Charles C Thomas Springfield Illinois; Pages; 1975

Cutolo, M., S. Accardo, B. Villaggio*, et al.*: "Presence of estrogen-binding sites on macrophage-like synoviocytes and CD8+, CD29+, CD45RO+ T lymphocytes in normal and rheumatoid synovium." Arthritis Rheum 36(8): 1087-97; 1993

Damas, P., A. Reuter, P. Gysen*, et al.*: "Tumor necrosis factor and interleukin-1 serum levels during severe sepsis in humans." Crit Care Med 17(10): 975-8.; 1989

De Maio, A., M. L. Mooney, L. E. Matesic*, et al.*: "Genetic component in the inflammatory response induced by bacterial lipopolysaccharide." Shock 10(5): 319-23.; 1998

Deshpande, R., H. Khalili, R. G. Pergolizzi*, et al.*: "Estradiol down-regulates LPS-induced cytokine production and NFkB activation in murine macrophages." Am J Reprod Immunol 38(1): 46-54.; 1997

Dey, S. K., R. C. Hoversland and D. C. Johnson: "Phospholipase A2 activity in the rat uterus: modulation by steroid hormones." Prostaglandins 23(5): 619-30; 1982

Dinkel, R. H. and U. Lebok: "A survey of nosocomial infections and their influence on hospital mortality rates." J Hosp Infect 28(4): 297-304; 1994

Diodato, M. D., M. W. Knoferl, M. G. Schwacha*, et al.*: "Gender differences in the inflammatory response and survival following haemorrhage and subsequent sepsis." Cytokine 14(3): 162-9; 2001

Eachempati, S. R., L. Hydo and P. S. Barie: "Gender-based differences in outcome in patients with sepsis." Arch Surg 134(12): 1342-7.; 1999

Ertel, W. and E. Faist: "[Immunologic monitoring after severe trauma]." Unfallchirurg 96(4): 200-12; 1993

Faist, E., C. Schinkel and S. Zimmer: "Update on the mechanisms of immune suppression of injury and immune modulation." World J Surg 20(4): 454-9; 1996

Fang, X. M., S. Schroder, A. Hoeft*, et al.*: "Comparison of two polymorphisms of the interleukin-1 gene family: interleukin-1 receptor antagonist polymorphism contributes to susceptibility to severe sepsis." Crit Care Med 27(7): 1330-4; 1999

Fletcher, S. W. and G. A. Colditz: "Failure of estrogen plus progestin therapy for prevention." Jama 288(3): 366-8; 2002

Fourrier, F., A. Jallot, L. Leclerc*, et al.*: "Sex steroid hormones in circulatory shock, sepsis syndrome, and septic shock." Circ Shock 43(4): 171-8; 1994

Ganong, W. F.: The Gonades: Development & Function of the Reproductive System. Review of Medical Physiology. Conneticut, Appleton & Lange: 375-413; 1993.

Gerard, C., C. Bruyns, A. Marchant*, et al.*: "Interleukin 10 reduces the release of tumor necrosis factor and prevents lethality in experimental endotoxemia." J Exp Med 177(2): 547-50; 1993

Girasole, G., R. L. Jilka, G. Passeri*, et al.*: "17 beta-estradiol inhibits interleukin-6 production by bone marrow-derived stromal cells and osteoblasts in vitro: a potential mechanism for the antiosteoporotic effect of estrogens." J Clin Invest 89(3): 883-91; 1992

Giron-Gonzalez, J. A., F. J. Moral, J. Elvira*, et al.*: "Consistent production of a higher TH1:TH2 cytokine ratio by stimulated T cells in men compared with women." Eur J Endocrinol 143(1): 31-6; 2000

Goretzlehner, G.: "[Pharmacokinetics of synthetic sex steroids in hormonal contraceptives]." Zentralbl Gynakol 113(7): 403-10; 1991

Green, P. G., S. R. Dahlqvist, W. M. Isenberg*, et al.*: "Sex steroid regulation of the inflammatory response: sympathoadrenal dependence in the female rat." J Neurosci 19(10): 4082-9; 1999

Gregory, M. S., D. E. Faunce, L. A. Duffner*, et al.*: "Gender difference in cell-mediated immunity after thermal injury is mediated, in part, by elevated levels of interleukin-6." J Leukoc Biol 67(3): 319-26; 2000

Gulshan, S., A. B. McCruden and W. H. Stimson: "Oestrogen receptors in macrophages." Scand J Immunol 31(6): 691-7; 1990

Heidecke, C. D., H. Weighardt, T. Hensler*, et al.*: "[Immune paralysis of T-lymphocytes and monocytes in postoperative abdominal sepsis. Correlation of immune function with survival]." Chirurg 71(2): 159-65; 2000

Hirschfeld, M., Y. Ma, J. H. Weis*, et al.*: "Cutting edge: repurification of lipopolysaccharide eliminates signaling through both human and murine toll-like receptor 2." J Immunol 165(2): 618-22.; 2000

Hoflich, C. and H. D. Volk: "[Immunomodulation in sepsis]." Chirurg 73(11): 1100-4; 2002

Hotchkiss, R. S. and I. E. Karl: "The pathophysiology and treatment of sepsis." N Engl J Med 348(2): 138-50; 2003

Howard, M., T. Muchamuel, S. Andrade, *et al.*: "Interleukin 10 protects mice from lethal endotoxemia." J Exp Med 177(4): 1205-8; 1993

Iraqi, F. and A. Teale: "Cloning and sequencing of the tnfa genes of three inbred mouse strains." Immunogenetics 45(6): 459-61; 1997

Iraqi, F. and A. Teale: "Polymorphisms in the Tnfa gene of different inbred mouse strains." Immunogenetics 49(3): 242-5; 1999

Jacob, C. O., F. Hwang, G. D. Lewis, *et al.*: "Tumor necrosis factor alpha in murine systemic lupus erythematosus disease models: implications for genetic predisposition and immune regulation." Cytokine 3(6): 551-61; 1991

Kahlke, V., M. K. Angele, A. Ayala, *et al.*: "Immune dysfunction following trauma-haemorrhage: influence of gender and age." Cytokine 12(1): 69-77.; 2000

Kelly, J. L., C. O'Sullivan, M. O'Riordain, *et al.*: "Is circulating endotoxin the trigger for the systemic inflammatory response syndrome seen after injury?" Ann Surg 225(5): 530-41; discussion 541-3; 1997

Kim, J. M., C. I. Brannan, N. G. Copeland, *et al.*: "Structure of the mouse IL-10 gene and chromosomal localization of the mouse and human genes." J Immunol 148(11): 3618-23; 1992

Klein, S. L.: "The effects of hormones on sex differences in infection: from genes to behavior." Neurosci Biobehav Rev 24(6): 627-38.; 2000

Knoferl, M. W., M. K. Angele, M. D. Diodato, *et al.*: "Female sex hormones regulate macrophage function after trauma-hemorrhage and prevent increased death rate from subsequent sepsis." Ann Surg 235(1): 105-12; 2002

Kollef, M. H., L. Sharpless, J. Vlasnik, *et al.*: "The impact of nosocomial infections on patient outcomes following cardiac surgery." Chest 112(3): 666-75; 1997

Koopman, P., J. Gubbay, N. Vivian, *et al.*: "Male development of chromosomally female mice transgenic for Sry." Nature 351(6322): 117-21; 1991

Kreling, D. H., D. A. Mott and J. B. Wiederholt: Prescription Drug Trends - a chartbook update, The Henry J. Kaiser Family Foundation: 35; 2001.

Kube, D., H. Rieth, J. Eskdale*, et al.*: "Structural characterisation of the distal 5' flanking region of the human interleukin-10 gene." Genes Immun 2(4): 181-90; 2001

Kuhn, R., J. Lohler, D. Rennick*, et al.*: "Interleukin-10-deficient mice develop chronic enterocolitis." Cell 75(2): 263-74; 1993

Laubach, V. E., P. L. Foley, K. S. Shockey*, et al.*: "Protective roles of nitric oxide and testosterone in endotoxemia: evidence from NOS-2-deficient mice." Am J Physiol 275(6 Pt 2): H2211-8.; 1998

Lephart, E. D., C. R. Baxter and C. R. Parker, Jr.: "Effect of burn trauma on adrenal and testicular steroid hormone production." J Clin Endocrinol Metab 64(4): 842-8; 1987

Livingston, D. H., A. C. Mosenthal and E. A. Deitch: "Sepsis and multiple organ dysfunction syndrome: a clinical-mechanistic overview." New Horiz 3(2): 257-66.; 1995

Luppa, P., R. Munker, D. Nagel*, et al.*: "Serum androgens in intensive-care patients: correlations with clinical findings." Clin Endocrinol (Oxf) 34(4): 305-10; 1991

Lyons, A., A. Goebel, J. A. Mannick*, et al.*: "Protective effects of early interleukin 10 antagonism on injury-induced immune dysfunction." Arch Surg 134(12): 1317-23; discussion 1324; 1999

Majetschak, M., B. Christensen, U. Obertacke*, et al.*: "Sex differences in posttraumatic cytokine release of endotoxin-stimulated whole blood: relationship to the development of severe sepsis." J Trauma 48(5): 832-9; discussion 839-40.; 2000

Majetschak, M., S. Flohe, U. Obertacke*, et al.*: "Relation of a TNF gene polymorphism to severe sepsis in trauma patients." Ann Surg 230(2): 207-14; 1999

Marchant, A., C. Bruyns, P. Vandenabeele*, et al.*: "Interleukin-10 controls interferon-gamma and tumor necrosis factor production during experimental endotoxemia." Eur J Immunol 24(5): 1167-71; 1994

Matesic, L. E., A. De Maio and R. H. Reeves: "Mapping lipopolysaccharide response loci in mice using recombinant inbred and congenic strains." Genomics 62(1): 34-41; 1999

Matesic, L. E., E. L. Niemitz, A. De Maio*, et al.*: "Quantitative trait loci modulate neutrophil infiltration in the liver during LPS-induced inflammation." Faseb J 14(14): 2247-54.; 2000

Mayeux, P. R.: "Pathobiology of lipopolysaccharide." J Toxicol Environ Health 51(5): 415-35.; 1997

McElreavy, K., E. Vilain, N. Abbas*, et al.*: "XY sex reversal associated with a deletion 5' to the SRY "HMG box" in the testis-determining region." Proc Natl Acad Sci U S A 89(22): 11016-20; 1992

McGowan, J. E., Jr., M. W. Barnes and M. Finland: "Bacteremia at Boston City Hospital: Occurrence and mortality during 12 selected years (1935-1972), with special reference to hospital-acquired cases." J Infect Dis 132(3): 316-35; 1975

McGuire, W., A. V. Hill, C. E. Allsopp*, et al.*: "Variation in the TNF-alpha promoter region associated with susceptibility to cerebral malaria." Nature 371(6497): 508-10; 1994

McKinney, T. D.: "Estrous cycle in house mice: effects of grouping, preputial gland odors, and handling." J Mammal 53(2): 391-3.; 1972

McLauchlan, G. J., I. D. Anderson, I. S. Grant*, et al.*: "Outcome of patients with abdominal sepsis treated in an intensive care unit." Br J Surg 82(4): 524-9.; 1995

Meakins, J. L.: "Etiology of multiple organ failure." J Trauma 30(12 Suppl): S165-8.; 1990

Mira, J. P., A. Cariou, F. Grall*, et al.*: "Association of TNF2, a TNF-alpha promoter polymorphism, with septic shock susceptibility and mortality: a multicenter study." Jama 282(6): 561-8; 1999

Molvig, J., L. Baek, P. Christensen*, et al.*: "Endotoxin-stimulated human monocyte secretion of interleukin 1, tumour necrosis factor alpha, and prostaglandin E2 shows stable interindividual differences." Scand J Immunol 27(6): 705-16.; 1988

Moore, K. W., A. O'Garra, R. de Waal Malefyt*, et al.*: "Interleukin-10." Annu Rev Immunol 11: 165-90; 1993

Napolitano, L. M., M. E. Greco, A. Rodriguez*, et al.*: "Gender differences in adverse outcomes after blunt trauma." J Trauma 50(2): 274-80; 2001

Nelson, J. F., L. S. Felicio, H. H. Osterburg*, et al.*: "Altered profiles of estradiol and progesterone associated with prolonged estrous cycles and persistent vaginal cornification in aging C57BL/6J mice." Biol Reprod 24(4): 784-94.; 1981

Oberholzer, A., C. Oberholzer and L. L. Moldawer: "Interleukin-10: A complex role in the pathogenesis of sepsis syndromes and its potential as an anti-inflammatory drug." Crit Care Med 30(1 Supp): S58-S63; 2002

Offner, P. J., E. E. Moore and W. L. Biffl: "Male gender is a risk factor for major infections after surgery." Arch Surg 134(9): 935-8; discussion 938-40.; 1999

O'Keefe, G. E., J. L. Hunt and G. F. Purdue: "An evaluation of risk factors for mortality after burn trauma and the identification of gender-dependent differences in outcomes." J Am Coll Surg 192(2): 153-60; 2001

O'Malley, J., L. E. Matesic, M. C. Zink*, et al.*: "Comparison of acute endotoxin-induced lesions in A/J and C57BL/6J mice." J Hered 89(6): 525-30; 1998

Opal, S. M. and V. A. DePalo: "Anti-inflammatory cytokines." Chest 117(4): 1162-72; 2000

Passarge, E.: Taschenatlas der Genetik. Stuttgart; New York, Thieme; Pages; 1994

Pinsky, M. R., J. L. Vincent, J. Deviere*, et al.*: "Serum cytokine levels in human septic shock. Relation to multiple-system organ failure and mortality." Chest 103(2): 565-75.; 1993

Poltorak, A., X. He, I. Smirnova*, et al.*: "Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene." Science 282(5396): 2085-8.; 1998

Qureshi, S. T., L. Lariviere, G. Sebastiani*, et al.*: "A high-resolution map in the chromosomal region surrounding the Lps locus." Genomics 31(3): 283-94; 1996

Radojicic, C., B. Andric, M. Simovic*, et al.*: "Genetic basis of resistance to trauma in inbred strains of mice." J Trauma 30(2): 211-3; 1990

Rangel-Frausto, M. S., D. Pittet, M. Costigan*, et al.*: "The natural history of the systemic inflammatory response syndrome (SIRS). A prospective study." Jama 273(2): 117-23.; 1995

Ray, A., K. E. Prefontaine and P. Ray: "Down-modulation of interleukin-6 gene expression by 17 beta-estradiol in the absence of high affinity DNA binding by the estrogen receptor." J Biol Chem 269(17): 12940-6; 1994

Reinhart, K., C. Wiegand-Lohnert, F. Grimminger*, et al.*: "Assessment of the safety and efficacy of the monoclonal anti-tumor necrosis factor antibody-fragment, MAK 195F, in patients with sepsis and septic shock: a multicenter, randomized, placebo-controlled, dose-ranging study." Crit Care Med 24(5): 733-42.; 1996

Remick, D. G., S. J. Garg, D. E. Newcomb*, et al.*: "Exogenous interleukin-10 fails to decrease the mortality or morbidity of sepsis." Crit Care Med 26(5): 895-904; 1998

Remick, D. G., D. E. Newcomb, G. L. Bolgos*, et al.*: "Comparison of the mortality and inflammatory response of two models of sepsis: lipopolysaccharide vs. cecal ligation and puncture." Shock 13(2): 110-6; 2000

Remmers, D. E., W. G. Cioffi, K. I. Bland*, et al.*: "Testosterone: the crucial hormone responsible for depressing myocardial function in males after trauma-hemorrhage." Ann Surg 227(6): 790-9.; 1998

Remmers, D. E., P. Wang, W. G. Cioffi*, et al.*: "Testosterone receptor blockade after trauma-hemorrhage improves cardiac and hepatic functions in males." Am J Physiol 273(6 Pt 2): H2919-25.; 1997

Riche, F., Y. Panis, M. J. Laisne*, et al.*: "High tumor necrosis factor serum level is associated with increased survival in patients with abdominal septic shock: a prospective study in 59 patients." Surgery 120(5): 801-7.; 1996

Riese, J., K. Woerner, P. Zimmermann*, et al.*: "Association of a TNFbeta gene polymorphism with complications after major abdominal operations." Shock 19(1): 1-4; 2003

Rossouw, J. E., G. L. Anderson, R. L. Prentice*, et al.*: "Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial." Jama 288(3): 321-33; 2002

Rubin, G. M.: "The draft sequences. Comparing species." Nature 409(6822): 820-1; 2001

Rugh, R.: The mouse - Its Reproduction and Development, Oxford University Press; Pages; 1990

Sachidanandam, R., D. Weissman, S. C. Schmidt*, et al.*: "A map of human genome sequence variation containing 1.42 million single nucleotide polymorphisms." Nature 409(6822): 928-33.; 2001

Salem, M. L., M. S. Hossain and K. Nomoto: "Mediation of the immunomodulatory effect of beta-estradiol on inflammatory responses by inhibition of recruitment and activation of inflammatory cells and their gene expression of TNF-alpha and IFN-gamma." Int Arch Allergy Immunol 121(3): 235-45; 2000

Salem, M. L., G. Matsuzaki, K. Kishihara*, et al.*: "beta-estradiol suppresses T cell-mediated delayed-type hypersensitivity through suppression of antigen-presenting cell function and Th1 induction." Int Arch Allergy Immunol 121(2): 161-9; 2000

Salem, M. L., G. Matsuzaki, G. A. Madkour*, et al.*: "Beta-estradiol-induced decrease in IL-12 and TNF-alpha expression suppresses macrophage functions in the course of Listeria monocytogenes infection in mice." Int J Immunopharmacol 21(8): 481-97; 1999

Schneider, C. P., E. A. Nickel, T. S. Samy*, et al.*: "The aromatase inhibitor, 4-hydroxyandrostenedione, restores immune responses following trauma-hemorrhage in males and decreases mortality from subsequent sepsis." Shock 14(3): 347-53; 2000

Schroder, J., V. Kahlke, M. Book*, et al.*: "Gender differences in sepsis: genetically determined?" Shock 14(3): 307-10; discussion 310-3.; 2000

Schroder, J., V. Kahlke, K. H. Staubach*, et al.*: "Gender differences in human sepsis." Arch Surg 133(11): 1200-5.; 1998

Schroeder, S., M. Reck, A. Hoeft*, et al.*: "Analysis of two human leukocyte antigen-linked polymorphic heat shock protein 70 genes in patients with severe sepsis." Crit Care Med 27(7): 1265-70; 1999

Schwacha, M. G., P. Wang and I. H. Chaudry: "Trauma Models for Studying the Influence of Gender and Aging." Surgical Research: 357-366; 2001

Schwarz, E., C. Schafer, J. C. Bode*, et al.*: "Influence of the menstrual cycle on the LPS-induced cytokine response of monocytes." Cytokine 12(4): 413-6; 2000

Stefano, G. B., V. Prevot, J. C. Beauvillain*, et al.*: "Estradiol coupling to human monocyte nitric oxide release is dependent on intracellular calcium transients: evidence for an estrogen surface receptor." J Immunol 163(7): 3758-63; 1999

Stein, B. and M. X. Yang: "Repression of the interleukin-6 promoter by estrogen receptor is mediated by NF-kappa B and C/EBP beta." Mol Cell Biol 15(9): 4971-9; 1995

Stewart, D., W. B. Fulton, C. Wilson*, et al.*: "Genetic contribution to the septic response in a mouse model." Shock 18(4): 342-7; 2002

Stuber, F., M. Petersen, F. Bokelmann*, et al.*: "A genomic polymorphism within the tumor necrosis factor locus influences plasma tumor necrosis factor-alpha concentrations and outcome of patients with severe sepsis." Crit Care Med 24(3): 381-4; 1996

Stuber, F., I. A. Udalova, M. Book*, et al.*: "-308 tumor necrosis factor (TNF) polymorphism is not associated with survival in severe sepsis and is unrelated to lipopolysaccharide inducibility of the human TNF promoter." J Inflamm 46(1): 42-50; 1995

Tabrizi, A. R., B. A. Zehnbauer, B. D. Freeman*, et al.*: "Genetic markers in sepsis." J Am Coll Surg 192(1): 106-17; quiz 145-6; 2001

Takakuwa, T., S. Endo, Y. Shirakura*, et al.*: "Interleukin-10 gene transfer improves the survival rate of mice inoculated with Escherichia coli." Crit Care Med 28(8): 2685-9; 2000

Trentzsch, H., D. Stewart, C. N. Paidas, *et al.*: "The combination of polymicrobial sepsis and endotoxin results in an inflammatory process that could not be predicted from the independent insults." J Surg Res 111(2): 203-8; 2003

van der Poll, T., P. M. Jansen, W. J. Montegut, *et al.*: "Effects of IL-10 on systemic inflammatory responses during sublethal primate endotoxemia." J Immunol 158(4): 1971-5; 1997

Villa, P., G. Sartor, M. Angelini, *et al.*: "Pattern of cytokines and pharmacomodulation in sepsis induced by cecal ligation and puncture compared with that induced by endotoxin." Clin Diagn Lab Immunol 2(5): 549-53; 1995

Volk, H. D., P. Reinke and W. D. Docke: "Immunological monitoring of the inflammatory process: Which variables? When to assess?" Eur J Surg Suppl(584): 70-2; 1999

Wada, K., T. Itoh, M. Nakagawa, *et al.*: "Estrogen binding sites in peripheral blood monocytes and effects of danazol on their sites in vitro." Gen Pharmacol 23(4): 693-700; 1992

Waterston, R. H., K. Lindblad-Toh, E. Birney, *et al.*: "Initial sequencing and comparative analysis of the mouse genome." Nature 420(6915): 520-62; 2002

Wei, M., P. Kuukasjarvi, S. Kaukinen, *et al.*: "Anti-inflammatory effects of 17beta-estradiol pretreatment in men after coronary artery surgery." J Cardiothorac Vasc Anesth 15(4): 455-9; 2001

Wells, J. C.: "Natural selection and sex differences in morbidity and mortality in early life." J Theor Biol 202(1): 65-76.; 2000

Wichmann, M. W., M. K. Angele, A. Ayala, *et al.*: "Flutamide: a novel agent for restoring the depressed cell-mediated immunity following soft-tissue trauma and hemorrhagic shock." Shock 8(4): 242-8.; 1997

Wichmann, M. W., D. Inthorn, H. J. Andress, *et al.*: "Incidence and mortality of severe sepsis in surgical intensive care patients: the influence of patient gender on disease process and outcome." Intensive Care Med 26(2): 167-72.; 2000

Wichmann, M. W., R. Zellweger, C. M. DeMaso, *et al.*: "Enhanced immune responses in females, as opposed to decreased responses in males following haemorrhagic shock and resuscitation." Cytokine 8(11): 853-63; 1996

Wichmann, M. W., R. Zellweger, C. M. DeMaso, *et al.*: "Mechanism of immunosuppression in males following trauma-hemorrhage. Critical role of testosterone." Arch Surg 131(11): 1186-91; discussion 1191-2; 1996

Wolf, U., W. Schempp and G. Scherer: "Molecular biology of the human Y chromosome." Rev Physiol Biochem Pharmacol 121: 147-213; 1992

Wright, S. D., R. A. Ramos, P. S. Tobias, *et al.*: "CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein." Science 249(4975): 1431-3; 1990

Zedler, S., R. C. Bone, A. E. Baue, *et al.*: "T-cell reactivity and its predictive role in immunosuppression after burns." Crit Care Med 27(1): 66-72; 1999

Zellweger, R., M. W. Wichmann, A. Ayala, *et al.*: "Females in proestrus state maintain splenic immune functions and tolerate sepsis better than males." Crit Care Med 25(1): 106-10.; 1997

Zuckerman, S. H., S. E. Ahmari, N. Bryan-Poole, *et al.*: "Estriol: a potent regulator of TNF and IL-6 expression in a murine model of endotoxemia." Inflammation 20(6): 581-97.; 1996

Zuckerman, S. H., N. Bryan-Poole, G. F. Evans, *et al.*: "In vivo modulation of murine serum tumour necrosis factor and interleukin-6 levels during endotoxemia by oestrogen agonists and antagonists." Immunology 86(1): 18-24; 1995

CURRICULUM VITAE

20. November 1971	born in Hannover/ Germany
25. May 1992	Graduation from Bismarckgymnasium, Hannover (Abitur)
June 1992 - October 1993	Civilian Service ("*Zivildienst*") at Johanniter-Unfall-Hilfe (JUH) e.V. - KV Hannover and Certification as Emergency Medical Technician (EMT). JUH Landesschule, Wennigsen.
November 1993	Starting Medical School, Ludwig-Maximilians-University (LMU) München
14. September 1995	**Preclinical Exam („*Ärztliche Vorprüfung*")**
April 1995	Clinical Internship ("*Famulatur*"), Dept.of Orthopedics, Krankenhaus Am Urban, Berlin.
23. September 1996	**States Examination Part 1 ("*1. Staatsexamen*")**
October 1996	Practitioner Internship ("*Praxis-Famulatur*"), Dr. H. Fleischer, Cardiology, Würzburg.
1997-2000	Research Scholar, Div. of Clinical Research, Dept. of Surgery, Klinikum Grosshadern, LMU München. Mentor: Prof. Dr. med. Eugen Faist.
August 1997 -September 1997	*Famulatur*, Dept. of Surgery, General Surgery and outpatient-center, Klinikum Grosshadern, LMU München.
October 1998	*Famulatur*, Dept of Surgery, Surgical Endoscopie, Klinikum Grosshadern, LMU München
12. April 1999	**States Examination Part 2 ("*2. Staatsexamen*")**
1999 – 2000	School of Medicine Final Year Rotations ("*Praktisches Jahr*") Dept. of Anesthesia and Critical Care Medicine, Klinikum Grosshadern, LMU München Dept. of Surgery, GHSF, University of California, San Francisco, California, USA. Dept. of Surgery, Klinikum Grosshadern, LMU München II. Medizinsche Klinik, Klinikum Grosshadern, LMU München
21. April 2000	**States Examination Part 3 ("*3. Staatsexamen*") and Graduation from LMU medical school.**
2000-2001	Research Fellowship, Johns-Hopkins-University School of Medicine, Dept. of Surgery, Div. of Pediatric Surgery, Baltimore MD, USA; Mentor: Antonio De Maio, Ph.D,.
December 2001 – June 2003	Internship ("*Arzt im Praktikum*"), Dept of Surgery, Klinikum Grosshadern, LMU München.
Since July 2003	Surgical Resident, Dept of Surgery, Klinikum Grosshadern, LMU München.

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