



The Effect of Endotoxin Administration on Cytokine Production in Obstructive Jaundiced Rats

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Abstract. The aim of this study was to evaluate the effect of lipopolysaccharide (LPS) administration, which mimics a surgical intervention, on the immune status of obstructive jaundiced (OJ) and sham-operated control rats. Rats were given 20 µg LPS intraperitoneally on day 13 following bile duct ligation or sham surgery. We determined serum levels of tumor necrosis factor α (TNF- α) and interleukin 6 (IL-6) on day 14 after surgery, and spontaneous as well as LPS-induced production of these cytokines in splenocyte and peritoneal exudate cell (PEC) cultures. We found that IL-6, but not TNF- α , serum concentrations were significantly elevated (4-fold) in OJ rats treated with LPS compared with LPS-untreated OJ rats. In sham-operated rats the differences between the respective groups were not significant. The production of TNF- α by splenocyte and PEC cultures was depressed in OJ rats treated with LPS; in particular, a very deep decline was observed in the case of spontaneous TNF- α production in PEC cultures. In contrast, TNF- α production in LPS-untreated and LPS-treated sham-operated rats did not differ. In the case of IL-6 production by splenocytes and PEC cultures, we observed a significant suppression of this cellular function in both OJ and sham-operated rats treated with LPS when compared with the respective controls. In conclusion, the results indicate that the already depressed cytokine production in OJ rats leads to even deeper hyporeactivity following LPS challenge. Lack of TNF- α suppression upon LPS treatment in sham-operated rats suggests that surgery-elicited hyporeactivity is mediated by a different mechanism than that leading to immune hyporesponsiveness in OJ. Our findings may explain the relatively high mortality rates observed of OJ patients subjected to surgery.

Key words: obstructive jaundice; LPS; splenocytes; peritoneal cells; TNF- α ; IL-6.

Introduction

Surgical procedures in obstructive jaundice patients result in significantly increased mortality^{2, 10, 16}. A number of reports have indicated the significance of lipopolysaccharide (LPS) and translocating bacteria in the pathogenesis of obstructive jaundiced (OJ)^{4, 5, 11, 15,}

^{18, 21, 22}. Disturbances in metabolism in OJ may include a reduced expression of adhesion molecules and an impaired response of neutrophils to bacterial products¹⁸, acute circulatory failure⁵, decreased wound healing⁶, fall in renal blood flow and blood pressure²², renal failure and overproduction of nitric oxide¹⁵ as well as enhanced production of hydroxyl radicals and liver

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damage²¹. A second clinical insult in OJ patients is frequently fatal¹⁰. To gain a better insight into the mechanism of the apparent lack of defense mechanisms in such patients, an experimental model in rats was designed which mimicks surgery by the administration of small doses of endotoxin to OJ rats^{17, 20}. These studies revealed a decrease in hepatic energy metabolism²⁰ and liver damage¹⁷. However, data evaluating the immune status of OJ rats treated with LPS are still incomplete, particularly with regard to cytokine production by immunocompetent cells. Therefore, the aim of this study was to determine the effect of LPS administration on 13-day OJ rats and sham-operated controls by measuring serum levels of and the ability to produce TNF- α and IL-6 spontaneously and upon contact with LPS in splenocyte and peritoneal exudate cell cultures.

Materials and Methods

Animals. Male rats of the Buffalo strain (170–270 g, mean 230 g) were used. The rats were maintained in stable conditions, fed a standard commercial diet with free access to food and water.

Surgical procedure. Procedures were performed under light oxygen-ether anesthesia in clean, but not sterile conditions. The abdominal cavity was opened with a midline incision after disinfecting the skin. The common bile duct (CBD) was located and OJ induced by a double ligation with 5/0 silk and transection of the CBD in the supraduodenal part between the lowermost tributary of the bile duct and the uppermost tributary of the pancreatic duct. The control group rats underwent opening of the abdominal cavity and dissection of the CBD without ligation.

At the end of the experiment, after opening the abdominal cavity, blood for bilirubin measurement was taken from all rats by direct aortic puncture.

In rats with OJ, the bilirubin level was high, the liver was markedly enlarged with characteristic discoloration, and the CBD was significantly dilated.

Preparation of peritoneal and spleen cell cultures and induction of cytokines in cell cultures. In brief, the peritoneal cavities of the rats were lavaged with pre-cooled Hanks' solution using a syringe. The cells were washed 2 times with Hanks' solution and resuspended at a concentration of 10^6 /ml in a culture medium (RPMI 1640, 5 mM L-g glutamine, sodium pyruvate and antibiotics, supplemented with 10% fetal calf serum (FCS)). When harvesting the peritoneal cells, the spleens were removed and a single cell suspension was prepared by pressing the organs through a plastic

screen, then washed 2 times with Hanks' solution and resuspended in the culture medium at a concentration of 10^7 /ml. The peritoneal exudate cell (PEC) (10^6 /ml) and splenocytes (10^7 /ml) were incubated overnight in 24-well plates with or without 5 μ g LPS/ml from *E. coli* strain No. 011: B4 lot 5711148 (Sigma). The supernatants were harvested and kept frozen until cytokine determination.

Determination of IL-6 activity. The assay was performed according to VAN SNICK et al.²². Briefly, 7TD1 indicator cells were washed 3 times with Hanks' solution and resuspended in Iscove's medium supplemented with 10% FCS, HEPES buffer, glutamine and antibiotics with density of 2×10^4 cells/ml. Then, the cells were distributed in 100 μ l aliquots into 96-well flat-bottom plates containing 100 μ l of serially diluted plasma or supernatant in triplicate. After 72 h of culture the proliferation of 7TD1 cells was determined using the MTT colorimetric method¹². The results of IL-6 activity are presented in pg/ml, a concentration of IL-6 which corresponds to the activity of IL-6 expressed in units per 1 ml²². One unit of IL-6 activity was calculated as the inverse dilution of a plasma sample where a half-maximal proliferation of 7TD1 cells was registered. The sensitivity limit in this assay is 0.5 pg IL-6 when tested a recombinant IL-6. The 7TD1 line responds by proliferation only to IL-6²².

Determination of TNF- α activity. For the determination of TNF- α activity⁷, a highly specific indicator clone, WEHI 164.13, was used. The cells were washed 3 times with Hanks' solution and resuspended in RPMI 1640, supplemented with 10% FCS, glutamine and antibiotics at a concentration of 2×10^5 /ml. The cells were then distributed into 96-well, flat-bottom plates (2×10^4 /well). Serially diluted plasma samples were prepared on separate plates and transferred to microtiter plates containing WEHI 164.13 cells. The medium contained in addition 1 μ g/ml actinomycin D to increase the sensitivity of the assay. After overnight incubation, the survival of cells was determined using the MTT colorimetric assay¹². The results of TNF- α activity are presented in pg/ml, where 10 pg of TNF- α corresponds to 1 unit of activity when tested a recombinant human TNF- α . The sensitivity limit of this assay is 2.5 pg of TNF- α .

Statistics. The results are presented as mean values from several rats (as indicated in figure legends $\pm$ standard deviation). In the statistical evaluation of the results with regard to TNF- α and IL-6, one-way analysis of variance for completely randomized block design for 3 levels (influence of the studied subgroups of rats, presence of OJ and its persistence) was applied. In other studies, Student's *t*-test, Cox-Cochran's test and

paired data were applied. Each time the results were verified with the non-parametric Mann-Whitney test. The differences were regarded as significant when $p < 0.05$.

Results

TNF- α and IL-6 serum activities in 14-day OJ and sham-operated rats treated or untreated with LPS

The serum levels of TNF- α and IL-6, expressed in pg/ml, in 14-day OJ rats and sham-operated control animals, treated or untreated on day 13 of OJ with 20 μ g of LPS from *E. coli* strain No. 011: B4 lot 5711148 (Sigma), are shown in Fig. 1. Differences in cytokine serum levels between the experimental groups were found only in the case of IL-6. First, in the rats untreated with LPS, there was no difference in IL-6 serum levels between OJ and control rats. Second, OJ rats treated with LPS had a 5-fold higher serum IL-6 concentration compared with their control counterpart. Third, control LPS-treated rats demonstrated higher IL-6 levels compared with the appropriate control, and these levels were significantly lower (by 70%) than those in OJ rats treated with LPS.

Spontaneous and LPS-induced TNF- α production by splenocyte and peritoneal exudate cell cultures of 14-day OJ and control rats treated with LPS on day 13 of OJ

Spontaneous and LPS-induced TNF- α production by splenocyte and peritoneal exudate cell cultures are

shown in Fig. 2 and 3. Analysis of Fig. 2 revealed that there was no change in TNF- α in splenocyte cultures of OJ versus control rats in LPS-untreated animals. However, following LPS administration there was a profound decline of both spontaneous (statistically significant) and LPS-induced (not significant) TNF- α production by splenocytes of OJ rats compared with LPS-treated control rats. Also, TNF- α production by LPS-treated OJ rats was significantly lower than in the respective OJ control rats untreated with LPS. A somewhat different picture was observed in PEC cultures, unstimulated or stimulated with LPS for TNF- α production. It was of interest that both spontaneous and LPS-induced TNF- α production did not differ between OJ and control rats in the LPS-untreated category of animals. The responses of cells from OJ rats were approximately one third higher compared with control rats. However, in the category of LPS-treated rats, the spontaneous TNF- α production in both OJ and sham-operated rats was very low compared with LPS-untreated rats (highly significant differences). In addition, LPS-induced TNF- α production in LPS-treated OJ rats was lower than in the relevant control group of the LPS-untreated category (a significant depression).

Spontaneous and LPS-induced IL-6 production by splenocyte and peritoneal exudate cell cultures of 14-day OJ and control rats treated with LPS on day 13 of OJ

The ability of spleen and peritoneal exudate cells from OJ and control rats to produce IL-6 spontaneously

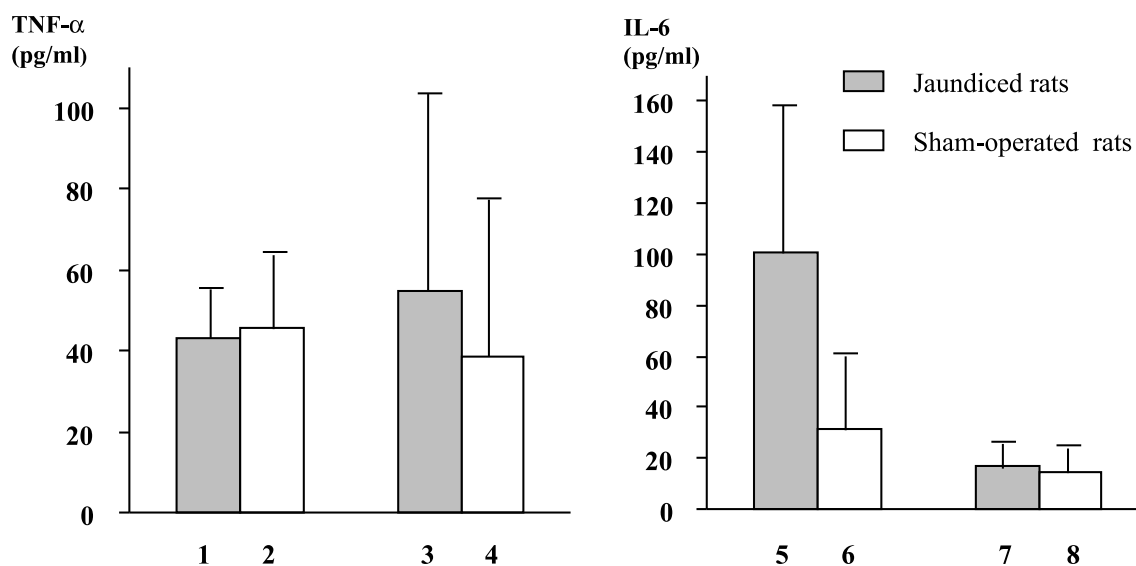


Fig. 1. TNF- α and IL-6 serum activities in 2-week obstructive jaundiced rats and in sham-operated controls treated on the 13th day or untreated with LPS. Rats treated with LPS (1, 2, 5, 6), untreated rats (3, 4, 7, 8). Statistically significant differences 5:6 ($p < 0.01$) and 5:7 ($p < 0.001$), other relations 1:2, 3:4, 7:8, 1:3, 2:4 and 6:8 not statistically significant

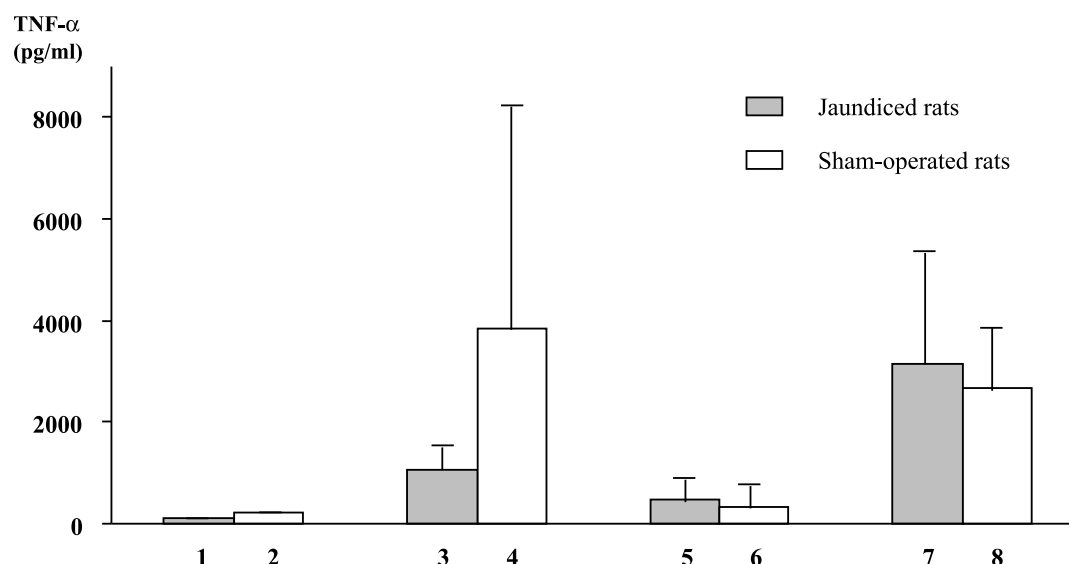


Fig. 2. TNF- α activities in culture of splenocytes isolated from 2-week obstructive jaundiced rats and sham-operated controls treated on the 13th day or untreated with LPS. Rats treated with LPS (1–4), untreated rats (5–8); cultures treated with LPS (3, 4, 7, 8), untreated cultures (1, 2, 5, 6). Statistically significant differences 1:5 ($p<0.001$), 2:6 ($p=0.013$), 1:2 ($p=0.03$) and 3:7 ($p=0.04$), other relations 3:4, 3:6, 7:8 and 4:8 not statistically significant

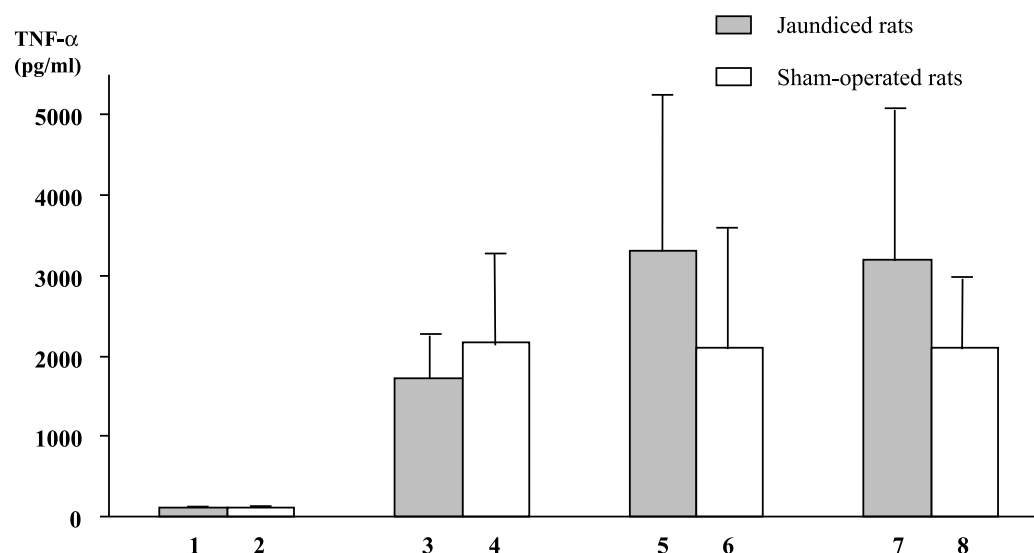


Fig. 3. TNF- α activities in culture of peritoneal exudates cells derived from 2-week obstructive jaundiced rats and sham-operated controls treated on 13th day or untreated with LPS. The rats treated with LPS (1–4), untreated rats (5–8); cultures treated with LPS (3, 4, 7, 8), untreated cultures (1, 2, 5, 6). Statistically significant differences 1:5 ($p<0.001$), 2:6 ($p<0.001$), and 3:7 ($p=0.02$), other relations 1:2, 3:4, 5:6, 7:8 and 4:8 not statistically significant

and upon LPS-stimulation in culture is presented in Fig. 4 and 5. In the category of LPS-untreated animals, no differences in both types of response between OJ and control rats were registered. Cells from LPS-treated rats, on the other hand, showed a significantly lower ability to produce IL-6 in comparison with the respective LPS-untreated control groups.

Discussion

The present study on the immune status of OJ and sham-operated rats provided an explanation for the exceptional vulnerability of OJ patients, subjected to clinical insult, to postoperative complications. In addition, our experiments revealed differences in responses to

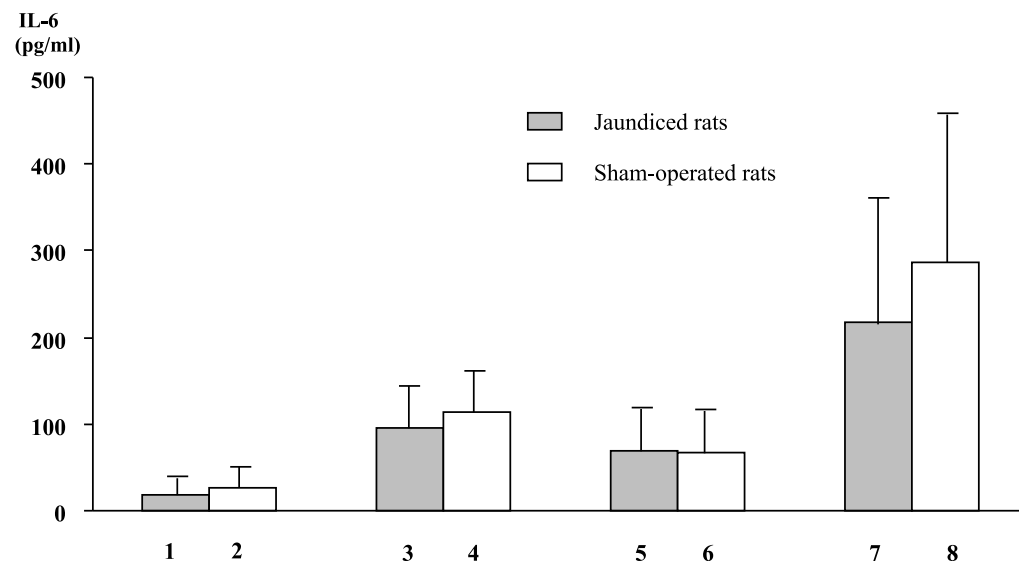


Fig. 4. IL-6 activity in culture of splenocytes isolated from 2-week obstructive jaundiced rats and sham-operated controls treated on the 13th day or untreated with LPS. Rats treated with LPS (1–4), untreated rats (5–8); cultures treated with LPS (3, 4, 7, 8), untreated cultures (1, 2, 5, 6). Statistically significant differences 1:5 ($p<0.001$), 2:6 ($p=0.001$), 3:7 ($p<0.001$) and 4:8 ($p<0.001$), other relations 1:2, 3:4, 5:6, and 7:8 not statistically significant

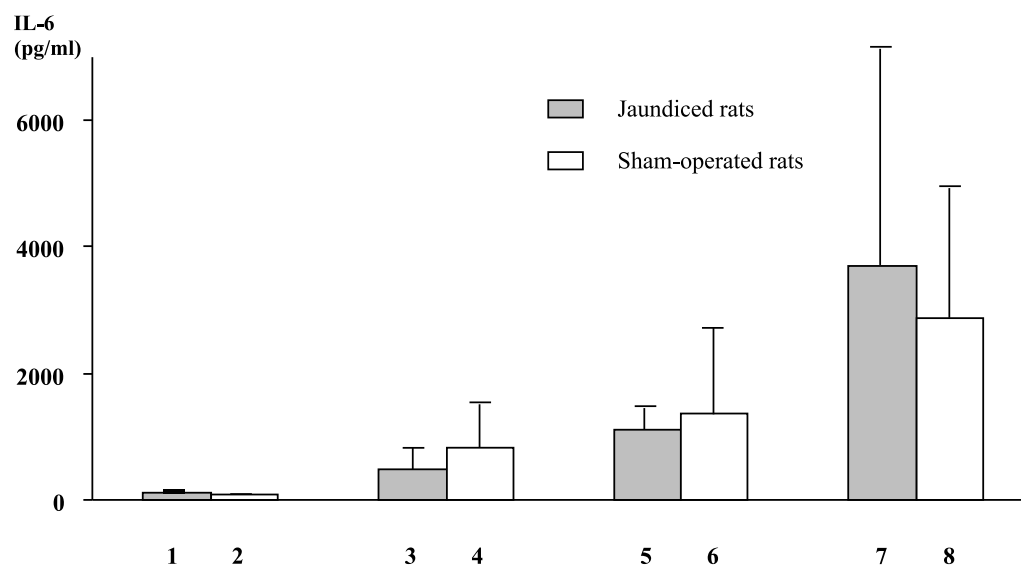


Fig. 5. IL-6 activity in cultures from peritoneal exudate cells derived from 2-week obstructive jaundiced rats and sham-operated controls treated on the 13th day or untreated with LPS. Rats treated with LPS (1–4), untreated rats (5–8); cultures treated with LPS (3, 4, 7, 8), untreated cultures (1, 2, 5, 6). Statistically significant differences 1:5 ($p=0.001$), 2:6 ($p<0.001$), 3:7 ($p<0.003$) and 4:8 ($p<0.001$), other relations 1:2, 3:4, 5:6 and 7:8 not statistically significant

LPS challenge between rats subjected to sham surgery and rats with fully developed, long-term OJ. In general, the immune status of rats which had been given LPS in the postoperative period was characterized by a deep hyporeactivity in terms of cytokine production (Fig. 2–5). This phenomenon could be the cause of the high mortality of OJ rats given 100 μ g LPS in our pilot experiments (not shown). To ensure 100% survival, we lowered the dose of LPS to 20 μ g. It was of interest

that the LPS challenge was not reflected by changes in TNF- α serum levels between OJ and control rats. On the other hand, IL-6 serum levels were 5-fold higher in OJ LPS-treated and by 2-fold in sham-operated LPS-treated rats. Elevated IL-6 serum levels could be a cause of depressed TNF- α production in LPS-treated rats, since one of the functions of IL-6 is to inhibit TNF- α production¹⁹. Not significantly elevated IL-6 concentrations in LPS-treated sham-operated rats could

also explain the lack of TNF- α suppression in the cells of sham-operated rats. Another reason of the low serum TNF- α level in LPS-treated OJ rats could be increased concentrations of soluble TNF- α receptor³. The concomitant presence of TNF- α and its soluble receptor may lower the activity of TNF- α measured by bioassays in this work. The phenomenon of reticuloendothelial system hyporeactivity following surgery or in septic shock^{1, 23} is well known and caused by monocyte/macrophage desensitization or exhaustion^{8, 23}. Although down-regulation of other LPS receptors may be associated with this phenomenon⁹, it seems likely that inhibition of MHC class II molecules could account for the described deep hyporeactivity. Particularly, the profound inhibition of spontaneous cytokine production by peritoneal cells may be a good indicator for such a mechanism, since PEC consist mostly of macrophages. One of us recently demonstrated that the spontaneous cytokine production in a mixed culture of syngeneic macrophages and T cells may be blocked by antibodies against MHC class II antigens on macrophages²⁴.

The hyporeactivity of cells in both categories of rats (OJ and sham-operated) was very deep. On the other hand, unmanipulated control animals expressed significant levels of cytokine production⁶, exceeding 5–10 times the values observed in surgical animals. The production of TNF- α by PEC cultures (mostly macrophages) could not be further increased upon LPS induction (Fig. 3). This endotoxin tolerance for TNF- α production in OJ was observed by others¹³, and the increased concentrations of soluble TNF- α receptor could play a role in this phenomenon³. Splenocytes, however, produced more TNF- α upon LPS induction, which indicates that other cell types (e.g. T cells) could be responsible for TNF- α synthesis. Also, in the case of IL-6, both splenocytes and PEC could be activated with LPS for enhanced production of this cytokine, suggesting the participation of other cell types beside macrophages.

Of particular interest was the finding revealing no further depression of TNF- α production after LPS treatment in sham-operated rats in contrast to OJ rats. This may indicate that, despite hyporeactivity, the cells may still react to bacterial products. This different type of cell hyporeactivity, after surgery, unassociated with septic conditions, may have important implications. This suggests that patients in the postoperative period may still develop adequate TNF- α response to potential infection or second surgery. In contrast, OJ patients would be unable to mount such a response, which could explain their exceptional susceptibility to developing postsurgical complications.

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