# INVOLVEMENT OF KUPFFER CELLS IN THE INTERACTION BETWEEN NEUTROPHILS AND SINUSOIDAL ENDOTHELIAL CELLS IN RATS

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ABSTRACT—During endotoxic liver injury, large numbers of neutrophils infiltrate the liver, and serum levels of tumor necrosis factor-$\alpha$ (TNF-$\alpha$) become elevated. The object of this study was to assess the roles of TNF-$\alpha$ secreted by Kupffer cells in the interaction between neutrophils and sinusoidal endothelial cells (SECs). Rat neutrophils were perfused onto SECs that were stimulated with either TNF-$\alpha$ or supernatant from lipopolysaccharide (LPS)-stimulated Kupffer cells using an *in vitro* flow system. Numbers of adhered or migrated neutrophils were counted, and the effect of an antibody against intercellular adhesion molecule-1 (ICAM-1) was studied. Compared with controls ($200 \pm 21$ cells/mm$^2$), neutrophil adhesion to SECs was significantly increased by both TNF-$\alpha$ ($342 \pm 26$ cells/mm$^2$; $P < 0.05$) and LPS-stimulated Kupffer cell supernatant ($331 \pm 29$ cells/mm$^2$; $P < 0.05$). Anti-ICAM-1 significantly inhibited neutrophil adhesion ($139 \pm 10$ cells/mm$^2$; $P < 0.05$) and decreased the migration rate of neutrophils on SECs treated with LPS-stimulated Kupffer cell supernatant ($P < 0.05$). LPS-stimulated Kupffer cells secreted TNF-$\alpha$ in an LPS dose-dependent manner, and they significantly enhanced ICAM-1 expression on SECs ($P < 0.05$ vs. control). In addition, dexamethasone suppressed TNF-$\alpha$ production by LPS-stimulated Kupffer cells and decreased ICAM-1 expression and neutrophil adhesion on SECs. These findings suggest that Kupffer cells are involved in neutrophil adhesion and migration in hepatic sinusoids via TNF-$\alpha$ production and induction of ICAM-1 expression on SECs during liver injury associated with endotoxemia.

KEYWORDS—Endotoxin, liver injury, tumor necrosis factor-$\alpha$, intercellular adhesion molecule-1, dexamethasone

## INTRODUCTION

Tumor necrosis factor-$\alpha$ (TNF-$\alpha$) is an inflammatory cytokine produced in response to endotoxin and is considered to be an essential mediator in the development of endotoxic shock (1). It has been shown that administration of endotoxin increases serum levels of TNF-$\alpha$ (2). However, the mechanism of tissue injury due to endotoxic shock is still under investigation. In endotoxic liver injury, large numbers of neutrophils accumulate in the liver tissue and are associated with the development of hepatocyte necrosis (2). Neutrophils play a major role in the pathogenesis of such disorders. This is supported by a previous report showing that the depletion of neutrophils using anti-neutrophil antibody prevents endotoxin-induced liver dysfunction in rats (3). On the other hand, Kupffer cells contribute to septic liver injury by producing various chemical mediators, including inflammatory cytokines (4). Administration of lipopolysaccharide (LPS) in rats stimulates the production of TNF-$\alpha$ by Kupffer cells and increases serum concentrations of TNF-$\alpha$ (5).

The involvement of leukocyte adhesion in the pathogenesis of the hepatic injury related to endotoxemia has clinical relevance. Leukocytes interact with the endothelial cells and extravasate prior to tissue infiltration. These phenomena are mediated by specific adhesion molecules on these cells (6).

Intercellular adhesion molecule-1 (ICAM-1), a member of the immunoglobulin superfamily, is expressed on endothelial cells and is upregulated by interleukin 1 (IL-1) and TNF-$\alpha$ (7). The ligands of ICAM-1 are leukocyte integrins such as Mac-1 (CD11b/CD18) on neutrophils (8) or lymphocyte function-associated antigen 1 (LFA-1, CD11a/CD18) on lymphocytes (9). Modulation of adhesion molecules by monoclonal antibodies is reported to be useful in the inhibition of inflammatory reactions and in preventing rejection of organ allografts (10).

Immunohistochemical studies have shown that several adhesion molecules are involved in the pathogenesis of various liver diseases. Volpes et al. (11) observed that the expression of ICAM-1 was increased on the sinusoidal endothelial cells (SECs) in inflamed lesions where LFA-1-positive lymphocytes had infiltrated. Thus far, there have been few studies on the interaction between neutrophils and SECs *in vitro*. We previously reported on the significant contribution of ICAM-1 and CD18 to neutrophil adhesion and migration in hepatic sinusoids using an *in vitro* flow system (12). This system allows for the dynamic analysis of the spatial and temporal details of cell-to-cell interactions under a programmed shear stress (13, 14). In the present study, we employed this system to address the hypothesis that TNF-$\alpha$, released by LPS-stimulated Kupffer cells, plays a major role in the adhesion of neutrophils to SECs in endotoxic liver injuries. We investigated the numbers of adhered or migrated neutrophils to SECs that were stimulated with either TNF-$\alpha$ or the supernatants of LPS-stimulated Kupffer cells. Additionally, it has been reported that corticosteroids improve neutrophil-mediated liver injury induced by endotoxin (15). To assess the mechanism of the

anti-inflammatory effects of dexamethasone under these patho-physiological conditions, we investigated its effects on TNF-$\alpha$ production by LPS-stimulated Kupffer cells and ICAM-1 expression on SECs.

## MATERIALS AND METHODS

### Animals

Male Wistar rats (Shimizu Animal Inc., Kyoto, Japan; 200 to 250 g) were used. They were fed a standard laboratory diet and water *ad libitum* under standard laboratory conditions. Procedures were performed according to the guidelines of the National Institutes of Health *Guide for the Care and Use of Laboratory Animals.*

### Isolation and culture of rat sinusoidal cells

Rat sinusoidal cells were obtained from male Wistar rats according to the method previously described (16) with a slight modification. Briefly, the liver was perfused via the portal vein with $Ca^{++}$ and $Mg^{++}$ free Hanks' solution containing 0.01% ethylenediaminetetraacetic acid and 10 U/mL of heparin. The flow rate was adjusted to 22 mL/min. After 7 min, the liver was perfused with Hanks' balanced salt solution (HBSS) supplemented with 0.05% collagenase. After 5 min, the liver was removed, cut into pieces, and passed through a mesh. Low-speed centrifugation (50$g$ for 1 min) was then performed twice to separate hepatocytes and cell debris. The suspension containing non-parenchymal cells was then introduced into a centrifugal elutriation rotor (Hitachi Koki Co., Ibaragi, Japan) to isolate the SECs and Kupffer cells. Cells were loaded at a flow rate of 14 mL/min onto the elutriation centrifuge head spinning at 2500 rpm at 4°C. To obtain SECs, the flow rate was kept up to 20 mL/min, and 100 mL of cell suspension was collected. The flow rate was then adjusted to 26 mL/min, and 100 mL of suspension was collected to obtain Kupffer cells. After centrifugation at 500$g$ for 5 minutes at 4°C, the SECs were resuspended at a density of $2 \times 10^6$ cells/mL in RPMI 1640 medium (Nissui Pharmaceutical Co., Tokyo, Japan) containing 10% fetal calf serum (Invitrogen, Carlsbad, CA), $10^{-7}$ M of dexamethasone (Sigma Chemical Co., St. Louis, MO), and 0.1% gentamycin sulfate (Sigma Chemical Co.). An aliquot (1.0 mL) of the endothelial cell suspension was incubated on type I C collagen-coated glass cover slips 24 × 50 mm (Matsunami Glass Industries, Osaka, Japan) for 2 h at 37°C in 5% $CO_2$. They were then rinsed and cultured in a medium free of dexamethasone for an additional 10 h before the assay. To measure the expression of ICAM-1 by ELISA, 100 $\mu$L of the endothelial cell suspension ($2 \times 10^5$ cells/well) was cultured on a 96-well plate coated with type I C collagen (Corning 25860 CPL 1; Iwaki Glass, Tokyo, Japan) as described above.

Kupffer cell suspension was centrifuged at 500$g$ for 5 min and was then resuspended at $1 \times 10^6$ cells/mL in the culture medium. This suspension (1.0 mL) was incubated in a 24-well culture plate at 37°C in 5% $CO_2$. After a 60-min incubation, nonadherent cells were removed with a pipette. Sinusoidal cells were identified morphologically by scanning electron microscopy, and histochemically using the following cell markers: fluorescently labeled low-density lipoprotein (LDL) for SECs (17) and anti-rat resident macrophage antibody (ED2; BMA Biochemicals, August, Switzerland) for Kupffer cells. About 92% of the cultured SEC fraction revealed the characteristic features of pores and sieve plates, and took up acetylated LDL. Flow cytometric analysis showed that ED2-positive cells exceeded 88% of the Kupffer cell fraction.

### Isolation of rat neutrophils

Rat neutrophils were obtained from the peritoneal fluid 4 h after an intraperitoneal injection of 15 mL of 0.1% glycogen (Sigma Chemical Co.). After washing with HBSS, the cells were resuspended in HBSS at a density of $1 \times 10^6$ cells/mL and were maintained at 4°C for 1 h. Giemsa staining showed >95% of the isolated cells to be neutrophils. Flow cytometric analysis showed that 96% of the cell fraction isolated from the peritoneal fluid expressed CD18 (WT.3; Seikagaku Corporation, Tokyo, Japan) on their own surfaces (data not shown).

### Supernatants of LPS-stimulated Kupffer cells and assay of TNF-$\alpha$ production

After incubation for 1 h, cultured Kupffer cells were washed and stimulated with serial concentrations of LPS (*Escherichia coli*, 055:B5, Difco Laboratories, Detroit, MI) with or without $10^{-7}$ M of dexamethasone. Supernatants were harvested after 1, 2, 6, and 12 h of incubation. To avoid the effects of residual LPS in the supernatants on the following assays, polymyxin B (Difco Laboratories), which neutralizes biological function of LPS (18), was added to all of the supernatants after LPS stimulation. They were stored at −30°C until the adhesion assay or the assay of TNF-$\alpha$ production using an ELISA kit for rat TNF-$\alpha$ (Factor Test Rat TNF-$\alpha$ kit; Genzyme Co., Cambridge, MA).

### Adhesion assay

The adhesive interaction between neutrophils and SECs was assessed using an *in vitro* flow system where the fluid mechanics are well known, and surfaces can be easily coated with cultured endothelial cells (13, 14). The chamber consisted of a collagen-coated cover slip with a confluent monolayer of SECs that was attached to a polycarbonate base. The space between the cover slip and the base was held approximately 250 $\mu$m apart by a silastic rubber gasket. The flow rate was controlled with a syringe pump (Iwaki Glass Co.). Suspensions of neutrophils were passed through the chamber at a wall shear stress of 0.5 dyn/cm² and 37°C. Shear stress, $\tau$ in dyn/cm², was calculated from the equation: $\tau = 3 \mu Q/2ba^2$, where $\tau$ is the wall shear stress, $\mu$ is the viscosity of water at 37°C, which is used as an approximation of the viscosity of HBSS (0.007 poise), Q is volumetric flow rate, a is the half channel height, and b is the channel width. The wall shear stress in the hepatic sinusoid was lower than that in the venule because of its slow blood flow. A wall shear stress of 0.5 dyn/cm² is supposed to be within pathophysiological limits in the hepatic sinusoids, based on the shear stress measured with intravital fluorescence microscopy by Vollmar et al. (19). SECs on the coverslips were stimulated with 100 U/mL of rat TNF-$\alpha$ (Genzyme Co.) or the supernatants of LPS-stimulated Kupffer cells for 4 h, mounted in flow chambers, and perfused for 1 min with HBSS (rinse step), followed by an 8-min perfusion with a suspension of neutrophils (perfusion step). The interaction between neutrophils and SECs was observed using phase-contrast microscopy (Olympus Co., Tokyo, Japan) and was recorded on a videotape. In some experiments, an antibody against rat TNF-$\alpha$ (100 $\mu$g/mL; Genzyme Co.) was added. The number of neutrophils that remained in contact with the endothelial cell monolayer was determined in six digitized frames of different fields of view from the last minute (7–8 min) of the perfusion step and was expressed as the number of cells that adhered or migrated per square millimeter. The neutrophils that adhered to the monolayer possessed spherical geometry and appeared as phase bright objects, whereas those that migrated beneath the monolayer were flat and appeared as phase dark objects, as previously described (14). The migration rate was quantitated as follows:

the migration rate (%) = (migrated neutrophils × 100)/(adhered neutrophils + migrated neutrophils)

In some experiments, the SECs were pretreated with anti-rat ICAM-1 monoclonal antibody (1A29; 5 $\mu$g/mL, Seikagaku Corporation) for 20 min at 37°C.

### Expression of ICAM-1 on the SECs

The expression of ICAM-1 on the SECs was measured by cellular ELISA (3, 12). SECs cultured on a 96-well plate coated with type I C collagen were incubated with 100 U/mL of rat TNF-$\alpha$ or the supernatants of Kupffer cells for 4 h and fixed in 4% paraformaldehyde. In some experiments, an antibody against rat TNF-$\alpha$, or $10^{-7}$ M of dexamethasone was added. Three washes were done in PBS containing 0.1% bovine serum albumin (BSA). Nonspecific reactions were blocked by incubation for 30 min with 1% normal goat serum (Sigma Chemical Co.). After an additional wash, the cells were incubated for 1 h with 50 $\mu$L of anti-rat ICAM-1 monoclonal antibody at a dilution of 1:1000. After three washes with PBS containing BSA, the cells were incubated for 1 h with a peroxidase-labeled antibody against mouse IgG (Seikagaku Corporation). Peroxidase staining was then carried out with $o$-phenylenediamine (Sigma Chemical Co.) and $H_2O_2$. Absorbance at 490 nm was measured by an Immunoreader (Multiskan; Labosystems, Helsinki, Finland). The microplates were subsequently washed in tap water before 50 $\mu$l of 0.5% crystal violet was added for 5 min. After a thorough wash in tap water, 100 $\mu$L of 33% acetic acid was used to solubilize the nuclear stain, and absorbance at 570 nm was measured, representing the actual cell count per well. The expression of ICAM-1 was determined as the ratio between the specific protein measurement and cell density.

### Statistical analysis

Values are expressed as means ± SE. Data were analyzed by analysis of variance and Student's $t$ test, with Bonferroni's correction for multiple comparisons. An unpaired two-tailed Student's $t$ test was used to compare the migration rates. A level of $P < 0.05$ was considered to be significant.

## RESULTS

### TNF-$\alpha$ production by LPS-stimulated Kupffer cells

The production of TNF-$\alpha$ from Kupffer cells stimulated with serial concentrations of LPS for 6 h is shown in Figure 1A. Although non-stimulated Kupffer cells showed no significant TNF production, LPS stimulation induced a dose-dependent

increase in the production of TNF-$\alpha$. Figure 1B shows the time course of TNF-$\alpha$ production by Kupffer cells stimulated with 100 ng/mL of LPS, which peaked at 6 h. Administration of dexamethasone at a concentration of $10^{-7}$ M significantly inhibited TNF-$\alpha$ production from LPS-stimulated Kupffer cells.

### Adhesion assay

Compared with controls ($200 \pm 21$ cells/mm$^2$), neutrophil adhesion to SECs was significantly increased by both TNF-$\alpha$ ($342 \pm 26$ cells/mm$^2$; $P < 0.05$) and LPS-stimulated Kupffer cell supernatant ($331 \pm 29$ cells/mm$^2$; $P < 0.05$; Fig. 2). These were inhibited by the addition of neutralizing anti-TNF-$\alpha$ antibody ($212 \pm 28$ cells/mm$^2$; $P < 0.05$ and $249 \pm 22$ cells/mm$^2$; $P < 0.05$, respectively). Moreover, TNF-$\alpha$ and the supernatant of LPS-stimulated Kupffer cells significantly increased the number of neutrophils that migrated beneath the SEC. The supernatants of non-stimulated Kupffer cells had no effect on neutrophil adhesion or migration. The addition of 5 $\mu$g/mL of the anti-ICAM-1 significantly suppressed the increase in the number of neutrophils adhered to SECs stimulated with the LPS-stimulated Kupffer cell supernatant ($139 \pm 10$ cells/mm$^2$; $P < 0.01$; Fig. 2). In addition, the migration rate of neutrophils through SECs treated with the supernatant of LPS-stimulated Kupffer cells (21.2%) was significantly suppressed by the addition of anti-ICAM-1 antibody (migration rate of 7.4%).

To investigate the effect of dexamethasone on Kupffer cell-mediated neutrophil-SEC interaction, we examined neutrophil adhesion and migration on SECs treated with the supernatant from LPS-stimulated Kupffer cells in the presence of $10^{-7}$ M of dexamethasone. The numbers of both adhered and migrated neutrophils ($215 \pm 20$ and $28 \pm 7$ cells/mm$^2$, respectively) were significantly decreased compared with those in the absence of dexamethasone ($P < 0.05$).

### Cellular ELISA for ICAM-1 expression on SECs

Stimulation with 100 U/mL of rat TNF-$\alpha$ significantly enhanced the expression of ICAM-1 on SECs compared with unstimulated controls (Fig. 3). When the SECs were incubated with the supernatants of LPS-stimulated Kupffer cells, the expression of ICAM-1 significantly increased. Anti-rat

TNF-$\alpha$ antibody attenuated this effect. The supernatants of non-stimulated Kupffer cells had no effect on ICAM-1 expression. Dexamethasone attenuated the constitutive ICAM-1 expression on inactivated SECs and significantly suppressed ICAM-1 expression on TNF-$\alpha$-stimulated SECs as well as on SECs treated with the supernatant of LPS-stimulated Kupffer cells.

## DISCUSSION

The pathogenesis of endotoxin-induced liver injury is under intensive investigation using various animal models. In galactosamine/LPS-induced liver injury, numerous neutrophils infiltrate the liver, and necrosis of hepatocytes is demonstrated (20). Pretreatment with anti-neutrophil antiserum attenuates the severity of the liver injury, indicating that neutrophils play a major role in this model (3). On the other hand, several reports show that Kupffer cells contribute to endotoxic liver injury by releasing several chemical mediators such as cytokines, proteases, and oxygen-derived free radicals (21, 22). Gadolinium chloride, which destroys Kupffer cells by damaging their plasma membranes, decreases endotoxin-related liver injury (23). Inflammatory cytokines including TNF-$\alpha$ have been implicated in this pathological condition. The administration of LPS induces the overproduction of TNF-$\alpha$ in a few hours in rats *in vivo* (24). In the present study, we assessed the role of both purified TNF-$\alpha$ and TNF-$\alpha$ present in LPS-stimulated Kupffer cell supernatants in neutrophil-SEC interactions.

We demonstrated that LPS induced a dose-dependent increase in the production of TNF-$\alpha$ from Kupffer cells that peaked 6 h after stimulation. In support of this finding, Chensue et al. (25) had reported that positive staining for TNF-$\alpha$ was observed in Kupffer cells after LPS exposure in mice *in vivo*. It has been suggested that TNF-$\alpha$ secreted from Kupffer cells stimulates other sinusoidal lining cells, including SECs. Our adhesion assay showed that the supernatant of LPS-stimulated Kupffer cells, as well as TNF-$\alpha$, increased the adhesion of the neutrophils to the SEC monolayers under the shear stress of hepatic sinusoids, and that anti-rat TNF-$\alpha$ antibody suppressed neutrophil adhesion induced by LPS-stimulated
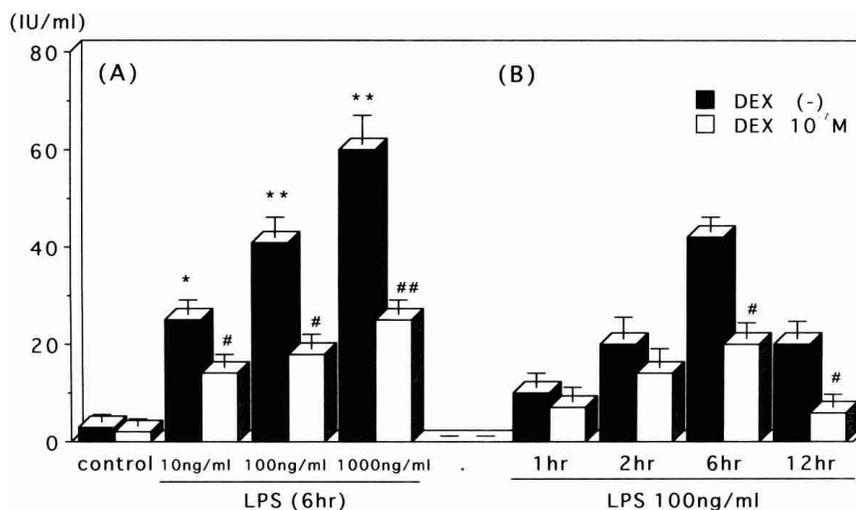


Fig. 1. **TNF-$\alpha$ production from Kupffer cells.** Values are expressed as mean $\pm$ SE in three experiments in triplicate. TNF-$\alpha$ levels increased LPS dose dependently and peaked at 6 h (hr). Dexamethasone (DEX, $10^{-7}$ M) inhibited TNF-$\alpha$ production from LPS-stimulated Kupffer cells. *$P < 0.05$ and **$P < 0.01$ compared with control. #$P < 0.05$ and ##$P < 0.01$ compared with DEX(-).
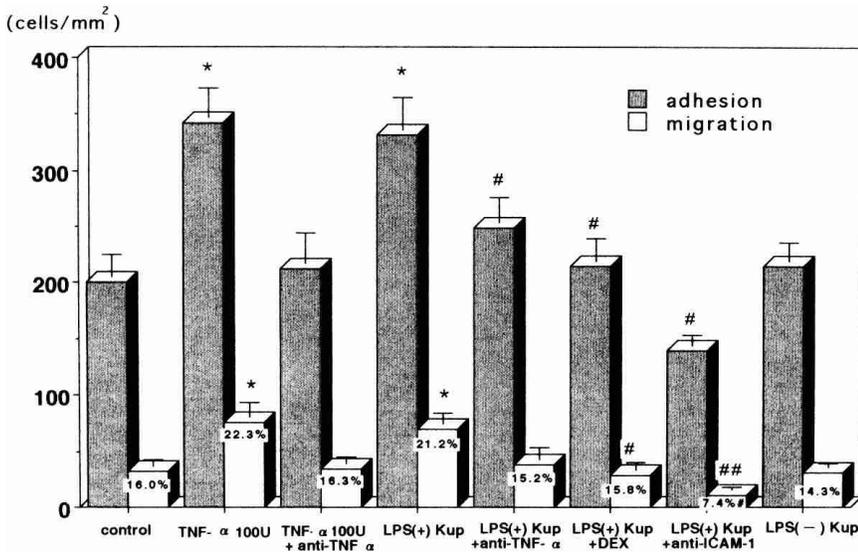
Fig. 2. **Neutrophil adhesion to and migration beneath SECs.** Values are expressed as mean ± SE in three experiments in duplicate. "%" shows migrated neutrophil ratio to adhered neutrophil (migration rate). TNF-$\alpha$ and the supernatant of LPS-stimulated Kupffer cells [LPS (+) Kupffer cell] increased neutrophil adhesion and migration, but the supernatant of non-stimulated Kupffer cells [LPS (−) Kupffer cell] did not. Dexamethasone (DEX) and anti–ICAM-1 antibody decreased the number of both adhered and migrated neutrophils. In addition, anti-ICAM-1 significantly inhibited the migration rate of neutrophils. *$P < 0.05$ compared with control. #$P < 0.05$ compared with TNF-$\alpha$ treated SECs. ##$P < 0.01$ compared with TNF-$\alpha$- treated SECs.

Kupffer cells. Kupffer cells secrete various kinds of inflammatory cytokines other than TNF-$\alpha$ (25, 27). However, our finding using anti-TNF-$\alpha$ suggests that TNF-$\alpha$ secreted from Kupffer cells, at least in part, promotes adhesion of neutrophils in hepatic sinusoids, which is the first step in a sequential cascade of inflammatory reactions. LPS-stimulated TNF-$\alpha$ production from our isolated Kupffer cells peaked at 6 h, but it peaks at about 2 h *in vivo* (24). Knolle et al. (26) also reported that isolated human Kupffer cells showed maximal secretion 6 h after LPS stimulation and that anti-inflammatory cytokine, IL-10, suppressed TNF-$\alpha$ secretion from LPS-stimulated Kupffer cells. The discrepancy of peak time between *in vivo* and *in vitro* may be due to anti-inflammatory cytokines secreted by other immune cells.

We previously reported that anti-ICAM-1 decreased migration rate of neutrophils on TNF-$\alpha$-stimulated SECs (12). In this study, we showed that LPS-stimulated Kupffer cells increased the number of neutrophils that migrated beneath the SEC monolayers, and that anti-ICAM-1 decreased the migration rate of neutrophils on SECs treated with the supernatants of LPS-stimulated Kupffer cells. These results indicate that ICAM-1 induced by LPS-stimulated Kupffer cells may be

crucial for neutrophil migration in hepatic sinusoids. Migrated neutrophils produce oxygen-derived free radicals, which injure hepatocytes (28). Inflammatory cytokines are also known to induce the expression of ICAM-1 on hepatocytes as well as SECs (29). Migrated neutrophils can recognize and cause injury to hepatocytes overexpressing ICAM-1 (24). Our findings suggest that ICAM-1 on SECs plays an important role in the migration step of neutrophils during the liver injury after LPS exposure.

To assess the role of ICAM-1 in TNF-$\alpha$-induced neutrophil-SEC interactions, we investigated ICAM-1 expression on SECs after incubation with TNF-$\alpha$ or LPS-stimulated Kupffer cells. Our cellular ELISA showed that the supernatant of LPS-stimulated Kupffer cells, as well as 100 U/mL of TNF-$\alpha$, significantly increased ICAM-1 expression on SECs, and that anti-rat TNF-$\alpha$ antibody led to the inhibition of ICAM-1 expression. Supernatants from non-stimulated Kupffer cells had no effect on ICAM-1 expression. Additionally, the neutrophil adhesion assay revealed that anti-ICAM-1 antibody inhibited both adhesion and migration of neutrophils induced by LPS-stimulated Kupffer cell supernatant. These findings suggest that TNF-$\alpha$ released from LPS-stimulated Kupffer
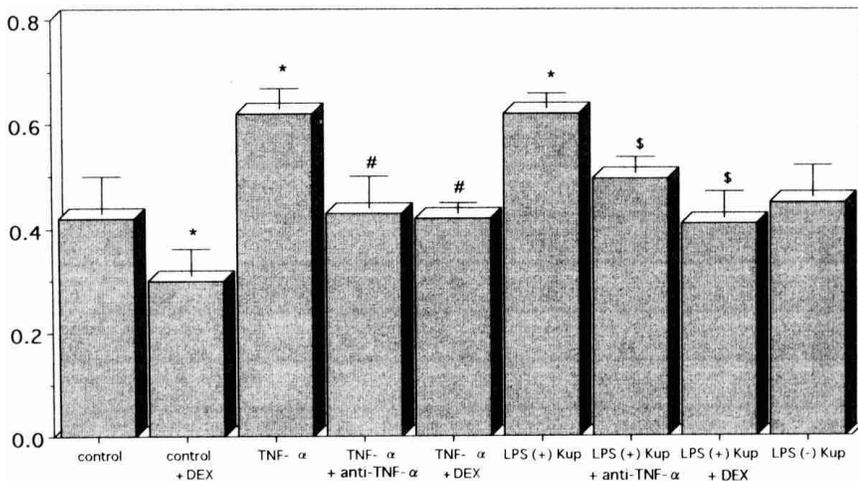


Fig. 3. **ICAM-1 expression on SECs.** Values are the mean ± SE of three experiments. TNF-$\alpha$ and LPS-stimulated Kupffer cells increased ICAM-1 expression. Dexamethasone significantly suppressed these effects. *$P < 0.05$ compared with control. #$P < 0.05$ compared with TNF-$\alpha$-treated SECs. $P < 0.05$ compared with SECs treated with LPS-stimulated Kupffer cell supernatant.

cells is crucial in the sinusoidal expression of adhesion molecules and that ICAM-1 may play a major role in hepatic infiltration of neutrophils in endotoxin-induced liver injury.

Shear stress is implicated in the interaction of leukocytes and endothelial cells mediated by adhesion molecules (13). Under conditions of physiological flow, firm adhesion of neutrophils to endothelial cells cannot be elicited without rolling of neutrophils, which involves selectins (30). In fact, anti-ICAM-1 failed to inhibit the adhesion of neutrophils to IL-1-stimulated endothelial cells at a shear stress of 4.0 dyn/cm$^2$ (31). However, at a shear stress below a physiological flow (1.0 dyn/cm$^2$), interactions between neutrophils and IL-1-stimulated endothelial cells are induced by ICAM-1/CD18, but do not involve the selectin-carbohydrate antigen (30). Shear stress in the hepatic sinusoids is very low because of its slow blood flow (19). The contribution of ICAM-1 to hepatic infiltration of neutrophils *in vivo* is under controversy. Xu et al. (32) reported that ICAM-1-deficient mice were resistant to lethal effects of high doses of LPS, and that this correlated with a significant decrease in neutrophil infiltration in the liver 24 h after stimulation. This is consistent with our finding showing that anti-ICAM-1 attenuated neutrophil adhesion to SECs under low shear stress of hepatic sinusoids. On the other hand, Jaeschke et al. (33) reported that anti-ICAM-1 inhibited liver injury, but had no significant effect on neutrophil accumulation in hepatic sinusoids 90 min after endotoxin exposure. Vollmar et al. (34) also stated that pretreatment with anti-ICAM-1 antibody was not effective in preventing intravascular sequestration of leukocytes in the liver 1 h after LPS administration. However, the overexpression of ICAM-1 on SECs was not observed until 6 to 8 h after LPS exposure (24, 35). This time frame *in vivo* required to observe overexpression of ICAM-1 on SECs is consistent with our *in vitro* data showing that it takes a few hours for Kupffer cells to generate TNF-α after LPS-stimulation, and that ICAM-1 expression on SECs was significantly increased at 4 h after treatment with TNF-α. Considering this temporal relationship, 1 h after LPS stimulation is supposed to be too early to estimate the role of ICAM-1 in the liver injury. In fact, normal and ICAM-1 deficient livers had approximately similar numbers of neutrophils at 2 h, whereas there were distinctly fewer neutrophils infiltrating the liver in ICAM-1-deficient mice 24 h after LPS exposure (32). This finding shows that the roll of ICAM-1 in the late stage of endotoxic liver injury differs from that in the early stage. In this study, we clearly demonstrated that anti-ICAM-1 could inhibit neutrophil adhesion to SECs with the overexpression of ICAM-1, such as SECs in the late stage. Taken together, blocking ICAM-1-mediated cell interaction could be a therapeutic strategy for preventing liver injury in endotoxic shock.

Corticosteroids have been used for the treatment in endotoxic liver injury, and may protect mice from LPS mortality in animal models of endotoxic shock (36). In this study, we investigated the effect of dexamethasone on the neutrophil-SEC interaction under a low-flow condition. Our adhesion assay showed that dexamethasone significantly inhibited the increase in the numbers of the adhered and migrated neutrophils on SECs treated with the supernatant of LPS-stimulated Kupffer cells. This finding supports the anti-inflammatory effect of

dexamethasone. To clarify the mechanism that dexamethasone decreases the neutrophil-SEC interaction, we examined the effect of dexamethasone on TNF-α production from LPS-stimulated Kupffer cells. Dexamethasone demonstrated a significant suppressive effect on the production of TNF-α from LPS-stimulated Kupffer cells. These findings were consistent with an immunohistochemical analysis by Chensue et al. (25) showing that dexamethasone decreased Kupffer cell expression of TNF-α. We previously reported that TNF-α increases ICAM-1 expression on SECs in a dose-dependent manner (12). Thus, our findings suggest that the suppression of TNF-α release is one of the mechanisms that dexamethasone uses to attenuate ICAM-1-mediated inflammatory reactions in the liver.

On the other hand, our investigation demonstrated that dexamethasone inhibited neutrophil adhesion and ICAM-1 expression on SECs to an extent similar to that in control, although dexamethasone decreased TNF-α production from LPS-stimulated Kupffer cells by only 50%. We also investigated the effect of dexamethasone on ICAM-1 expression on SECs. Cellular ELISA showed that dexamethasone ($10^{-7}$ M) significantly decreased ICAM-1 expression not only on SECs treated with the supernatant of LPS-stimulated Kupffer cells, but also on SECs stimulated with 100 IU/mL of TNF-α. This finding means that dexamethasone can directly inhibit ICAM-1 expression on SECs in the presence of TNF-α secreted from Kupffer cells. SECs have a constitutive expression of ICAM-1 *in vivo* and *in vitro* (3, 12, 24). Our cellular ELISA demonstrated that dexamethasone significantly attenuated the constitutive expression on unstimulated SECs *in vitro*. Thus, we speculated that $10^{-7}$ M dexamethasone could inhibit ICAM-1 expression on SECs stimulated with Kupffer cell supernatants to the extent similar to the control, whereas dexamethasone did not completely suppress TNF-α release. Dexamethasone might prevent neutrophils from adhering to SECs mainly by inhibiting the overexpression of ICAM-1 induced by TNF-α, in addition to the suppression of LPS-stimulated TNF-α release.

In summary, our studies suggest that TNF-α released from LPS-stimulated Kupffer cells might induce the overexpression of ICAM-1 on SECs *in vivo*, and ICAM-1, in turn, might play a major role in the hepatic infiltration of neutrophils in endotoxic liver injury.

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