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# Stimulated Kupffer cells attract cytotoxic T lymphocytes (CTL) and increase the adhesion of CTL to sinusoidal endothelial cells

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**Abstract**

Kupffer cells secrete many kinds of cytokines, and Kupffer cell-derived substances regulate the recruitment of neutrophils and monocytes in the liver. To investigate the roles of Kupffer cells in the extravasation of cytotoxic T lymphocytes (CTL), we examined whether supernatants of lipopolysaccharide (LPS)-stimulated rat Kupffer cells have chemotactic activity for CTL and enhance adhesion of CTL to sinusoidal endothelial cells. The study using a modified Boyden's chamber demonstrated that the supernatants of LPS-stimulated Kupffer cells were chemoattractant for CTL and the concentration-response curve was bell shaped which is typical in in vitro chemotaxis assays. In addition, incubating CTL with the supernatants of LPS-stimulated Kupffer cells enhanced the adhesion of CTL to monolayers of sinusoidal endothelial cells to 2.3 times higher than controls in a 60-min assay. Anti-lymphocyte function-associated antigen-1$\alpha$ (LFA-1$\alpha$) antibody blocked the increased adhesion of CTL by LPS-stimulated Kupffer cells. These results indicated that Kupffer cells play important roles in infiltration of CTL regulated by LFA-1$\alpha$-associated bindings in the hepatic inflammation.

*Keywords:* Kupffer cell; Cytotoxic T cell; Chemotaxis; Adhesion; Sinusoidal endothelial cell

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## 1. Introduction

Leukocyte infiltration is an important feature of pathophysiological reactions in inflammatory diseases.The adherence of leukocytes to vascular endothelium is the first step in their passage from the circulation to inflammatory foci. Cytokines induce the adhesion and transendothelial migration of leukocytes [1–3] and Kupffer cell-derived cytokines are chemotactic for neutrophils and monocytes [4–6]. In chronic viral hepatitis, the infiltration of cytotoxic T lymphocytes (CTL) is essential for the host immune response to virus-infected hepatocytes. To clarify the role of Kupffer cells in the extravasation of CTL into the liver, we investigated whether supernatants of LPS-stimulated rat Kupffer cells have chemotactic activity for CTL and enhance the adhesion of CTL to sinusoidal endothelial cells.

## 2. Materials and methods

### *2.1. Isolation of hepatic sinusoidal cells and lymphocytes*

Kupffer cells and sinusoidal endothelial cells were obtained from male Wistar rats (200–250 g body wt.) by in situ collagenase perfusion followed by differential centrifugation and centrifugal elutriation [7]. In brief, rat livers were perfused with $Ca^{2+}$, $Mg^{2+}$ free Hanks' balanced salt solution (HBSS) containing 0.01% EDTA and heparin (10 units/ml), followed by perfusion with HBSS supplemented with 70 units/ml of collagenase. Livers were removed, cut into pieces, and passed through a mesh to remove cell debris. After low speed centrifugation for separation of hepatocytes, Kupffer cells and endothelial cells were separated from a suspension of hepatic non-parenchymal cells by a centrifugal elutriation rotor (Hitachi Koki Co., Ltd., Ibaragi, Japan) as described previously [7]. Cells were identified morphologically by electron microscopy and histochemically using the following cell markers: DiI-acetylated low density lipoprotein (Ac-LDL) for endothelial cells and endogeneous peroxidase activity for Kupffer cells. Rat lymphocytes were prepared by density gradient centrifugation with Nycoprep™ (Nycomed Pharma, Oslo, Norway). In brief, heparinized whole blood was mixed with an equal volume of $Ca^{2+}$, $Mg^{2+}$ free HBSS with 0.01% EDTA, and the mixture was layered onto Nycoprep and centrifuged at 600 × *g* for 15 min. The cell layer at the interface was aspirated. Peripheral blood mononuclear cells were washed three times and cultured in plastic dishes for 60 min at 37°C to remove adherent cells (monocytes). Non-adherent cells were collected and suspended at $1 \times 10^6$ cells/ml in RPMI 1640 supplemented with 10% fetal bovine serum and antibiotics (culture medium).

### *2.2. Preparation of supernatants from Kupffer cells*

Kupffer cells were resuspended at $1 \times 10^6$ cells/ml in culture medium. Cell suspension (1 ml) was incubated in a 24-well culture plate at 37°C in a 5% $CO_2$ incubator. After a 1-h incubation Kupffer cells were washed and stimulated with 100 ng/ml of lipopolysaccharide (LPS). After 4 h, the supernatants were harvested and stored at −30°C until use. The supernatants of non-stimulated Kupffer cells were harvested after a 4-h incubation without LPS-stimulation.

*2.3. Chemotactic activity for rat CTL in the supernatants of Kupffer cells*

CTL chemotaxis was assessed with a modified Boyden's chamber method [8] using a Chemotaxicell (Kurabou, Osaka, Japan) with a pore size 5 μm. The upper compartment contained 0.5 ml of a lymphocyte suspension, and the lower compartment contained culture medium or the supernatants of Kupffer cells with or without LPS stimulation. After a 1-h incubation the membranes of the chambers were fixed with methanol for 1 min and CTL was stained using anti-rat CD8 monoclonal antibody (MAB1495, Chemicon International Inc., CA, USA) with avidin-biotin method. Chemotactic activity was assayed as the number of CTL that migrated into the pore in ten random fields. The chemotaxis index (C.I.) was expressed as the ratio of active migration to the supernatants relative to the random migration to culture medium alone.

*2.4. CTL-endothelial cell adhesion assay*

Monolayers of sinusoidal endothelial cells were prepared on glass coverslips coated with collagen in 24-well culture plates. The monolayers of sinusoidal endothelial cells were ascertained to be a full sheet by Ac-LDL staining and by silver staining [9] which stains the cell border along each endothelial cell. Rat lymphocytes were preincubated with or without the supernatants of LPS-stimulated Kupffer cells for 30 min, then $1 \times 10^6$ lymphocytes/wells were cultured on the monolayer of endothelial cells for 15, 30, 60, and 90 min at 37°C. Thereafter, the plates were washed three times to remove non-adherent cells, fixed with methanol, and stained using anti-rat CD8 monoclonal antibody as described above. The numbers of adherent CTL to endothelial cells per five fields were counted. To investigate whether lymphocyte function-associated antigen-1α (LFA-1α) participates in Kupffer cell mediated-CTL adhesion to sinusoidal endothelial cells, blocking assays were performed using anti-rat LFA-1α antibody (WT1; R&D systems Europe Ltd.,. Oxford, United Kingdom). Ten micrograms of anti-LFA-1α antibody was added to 1 ml of lymphocyte suspension and CTL adhesion assessed as described above.

*2.5. Statistical analysis*

Statistical analysis was performed by Student's $t$ test. $P < 0.05$ was considered to be statistically significant.

## 3. Results

*3.1. Chemotactic activity for CTLs in the supernatants of Kupffer cells*

The supernatants of Kupffer cells stimulated with LPS had chemotactic activity as shown in Fig. 1. The number of migrated CTL into the filter of the chamber was significantly greater in a $10^{-2}$ and $10^{-1}$ dilution of the supernatants of LPS-stimulated Kupffer cells than those of non-stimulated Kupffer cells or medium. Maximal activity was seen at a $10^{-2}$ dilution of the supernatants of stimulated Kupffer cells, and the chemotactic response decreased at both higher and lower concentrations.

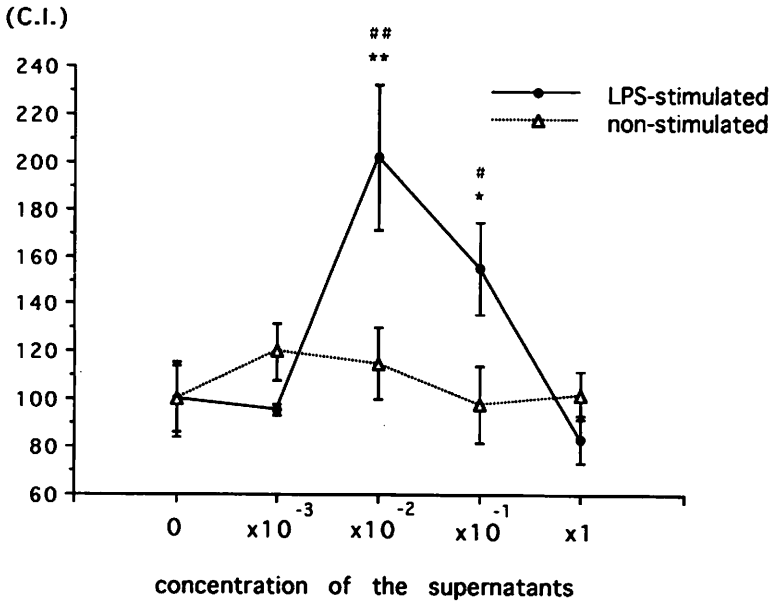


Fig. 1. Effect of the supernatant of Kupffer cells on the chemotactic activity for CTL. Chemotactic activity is expressed as the chemotaxis index (C.I.), namely the ratio to random migration by culture medium. Vertical lines show standard errors (S.D.). The supernatants of LPS-stimulated Kupffer cells were most chemotactic for CTLs at a $10^{-2}$ dilution. $^{*}P < 0.05$ vs. 0 (supernatant free); $^{**}P < 0.01$ vs. 0 (supernatant free); $^{\#}P < 0.05$ vs. non-stimulated; and $^{\#\#}P < 0.01$ vs. non-stimulated.

### *3.2. CTL-endothelial cell adhesion*

Incubation of lymphocytes with the supernatants of stimulated Kupffer cells increased the adhesion to endothelial cells (Fig. 2). A time course study showed that the number of adherent lymphocytes reached a maximum at 60 min. At 60 min, the supernatants of stimulated Kupffer cells increased lymphocyte adhesion 2.3-fold over that of control medium. Anti-LFA-1α antibody blocked the increased adhesion induced by LPS-stimulated Kupffer cells to the level of CTL adhesion induced by medium alone.

## 4. Discussion

Macrophages play important roles in the host defense mechanism [10]. Kupffer cells, which are liver macrophages, are responsible for secreting cytokines, such as interleukin-1 (IL-1), IL-6, leukotrien $B_4$ ($LTB_4$) and tumor necrosis factor-α (TNF-α) [4,11,12]. Kupffer cell-derived substances regulate the recruitment of neutrophils and monocytes into the damaged hepatic lesion [4–6]. In this study we investigated whether the supernatants of stimulated Kupffer cells had chemotactic activity for CTL and whether they enhanced the adhesion of CTL to sinusoidal endothelial cells.

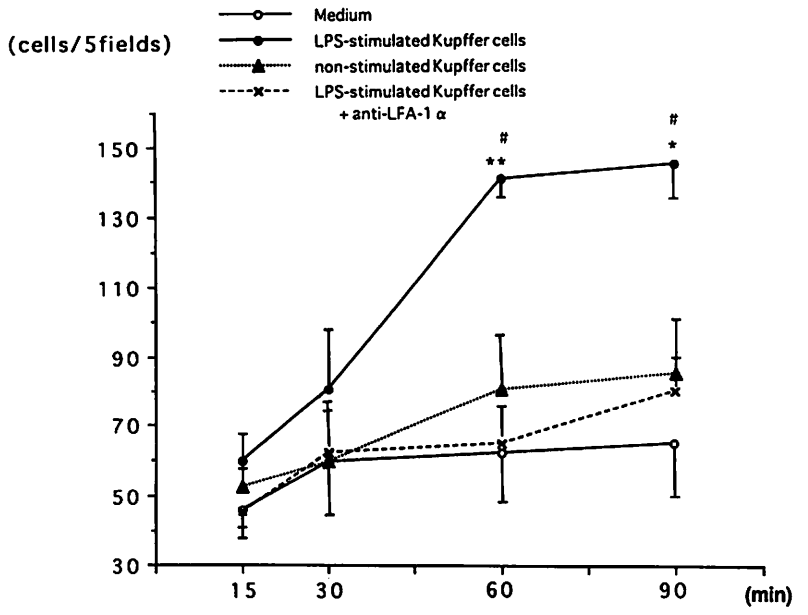


Fig. 2. Time course of CTL adhesion to sinusoidal endothelial cells. Vertical lines show standard errors (S.D.). The supernatants of LPS-stimulated Kupffer cells significantly increased CTL adhesion to endothelial cells after 60 min. $^{*}P < 0.05$ vs. Medium; $^{**}P < 0.01$ vs. Medium; $^{\#}P < 0.05$ vs. non-stimulated.

The supernatants of LPS-stimulated Kupffer cells were chemoattractant for CTL, and they increased the binding of CTL to the endothelial cell monolayer. The chemotaxis was maximal at a $10^{-2}$ dilution of the supernatants from LPS-stimulated Kupffer cells and the concentration-response curve was bell-shaped, which is typical in in vitro chemotaxis assays [8]. Some of the macrophage-secreted substances, such as macrophage inflammatory protein-1$\alpha$ and $\beta$, and IL-6, are chemoattractant for lymphocytes [8,13]. These chemical mediators might be secreted by LPS-stimulated Kupffer cells and might show chemotactic activity in this assay. In addition, the supernatants of stimulated Kupffer cells enhanced CTL-adhesion to sinusoidal endothelial cells. In chronic liver diseases lymphocytes leave the blood stream through the sinusoidal endothelial cells to accumulate in areas of piecemeal necrosis and spotty necrosis in intralobular areas. Intercellular adhesion molecule-1 (ICAM-1) is strongly up-regulated on sinusoidal endothelial cells in areas of hepatic inflammation, where LFA-1 positive lymphocytes are accumulated [14]. These findings indicate that the adhesion molecules on sinusoidal endothelial cells and lymphocytes play important roles in the modulation of liver injury. Anti-LFA-1$\alpha$ antibody significantly reduced the increased adhesion of CTL by LPS-stimulated Kupffer cells, indicating that Kupffer cells might modulate the adhesion of CTL by the regulation of ICAM-1-LFA-1 bindings. The present study demonstrated that Kupffer cells might play important roles in the recruitment of CTL in the damaged liver.

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