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Short Communication

Transient effects of empty liposomes on hepatic macrophage populations in rats

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Abstract: Liposomes have been used as a vehicle for encapsulating chemicals or toxins in toxicological studies. We investigated the transient effects of empty liposomes on hepatic macrophages by applying a single intravenous injection at a dose of 10 ml/kg body weight in 6-week-old male F344 rats. One day after injection, the numbers of hepatic macrophages reacting to CD163, CD68, Iba-1, MHC class II, Gal-3 and CD204 were significantly increased in liposome-treated rats. $CD163^+$ Kupffer cells and $CD68^+$ macrophages with increased phagocytic activity in hepatic lobules were most sensitive. The histological architecture of the liver was not changed following liposome injection; however, hepatocytes showed increased proliferating activity, demonstrable with proliferation marker immunostaining and by an increase in gene profiles related to the cell cycle. In the liposome-treated rats, interestingly, AST and ALT values were significantly decreased, and MCP-1, IL-1β and TGF-β1 mRNAs were significantly increased. Collectively, the present study found that hepatic macrophages activated by liposomes can influence liver homeostasis. This information would be useful for background studies on liposomes. (DOI: 10.1293/tox.2015-0082; J Toxicol Pathol 2016; 29: 139–144)

Key words: liposome, hepatic macrophages, immunohistochemistry, homeostasis, rats

Liposomes, which are prepared from lipids and lipid mixtures with phospholipids, represent one of the most efficacious and promising drug-carrier vehicles for intracellular delivery[1]. Injected liposomes are rapidly phagocytized by macrophages in the liver and spleen[2]. For this reason, liposomes have been chosen as a suitable vehicle for manipulation of macrophage functions by encapsulating toxins and chemicals[3, 4]. Hepatic macrophages can express a variety of immunophenotypes in normal and pathological conditions[5, 6]. However, the detailed effects of empty liposomes, particularly on different macrophage populations, have not yet been decided. The present study was undertaken to investigate the immunophenotypes of hepatic macrophages in rats after injection of empty liposomes, in relation to hepatic homeostasis. The results revealed that injection of empty liposomes increased the number of hepatic macrophages with different immunophenotypes and influenced hepatic homeostasis, presumably through enhanced hepatic macrophage functions.

Eight 6-week-old male F344 rats were purchased from Charles River Laboratories Japan (Hino, Shiga, Japan). Rats were maintained in a room at 21 ± 3°C with a 12 hour light-dark cycle. Food and water were provided *ad libitum*. Four rats were given a single intravenous injection of liposomes (http://www.clodronateliposomes.org) at a dose of 10 ml/kg body weight via the tail. The used liposomes were multilamellar, large and of various dimensions between 150 nanometers and 3 microns. They were prepared from one single phospholipid, e.g., phosphatidylcholine (egg lecithin) and cholesterol. The remaining four rats were used as untreated controls. One day after injection, all animals were euthanized under deep isoflurane anesthesia. One hour before euthanasia, they received an intraperitoneal injection of bromo-2-deoxyuridine (BrdU; Sigma-Aldrich Corporation, St. Louis, MO, USA) at a dose of 50 mg/kg body weight. Serum samples were analyzed for aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP). The animal experiments were conducted under the institutional guidelines approved by the ethical committee of Osaka Prefecture University for animal care.

Liver tissues were fixed in 10% neutral buffered formalin (NBF), Zamboni's solutions (0.21% picric acid and 2% paraformaldehyde in 130 mM phosphate buffer, pH 7.4) and periodate-lysine-paraformaldehyde (PLP) solutions processed by the PLP-AMeX (acetone, methyl benzoate and xylene) method[7]. NBF-fixed tissue sections cut at a thickness of 3–4 μm were stained with hematoxylin and eosin (HE). Tissue sections fixed in NBF, Zamboni's solution or PLP were used for immunohistochemistry with CD163, CD68,

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Table 1. Details of Antibodies and Immunostaining

Antibody	Type	Fixative	Dilution	Pretreatment	Source
CD163	Mouse monoclonal	PLP	1/300	100 μg/ml Proteinase K, 10 minutes	AbD Serotec, Oxford, UK
CD68	Mouse monoclonal	PLP	1/500	Microwaving in citrate buffer, 20 minutes	AbD Serotec, Oxford, UK
Iba-1	Rabbit polyclonal	Zamboni's solution	1/1000	Microwaving in citrate buffer, 20 minutes	Wako Pure Chemical Industries, Japan
MHC class II	Mouse monoclonal	PLP	1/1000	Microwaving in citrate buffer, 20 minutes	AbD Serotec, Oxford, UK
Galectin-3 (Gal-3)	Rabbit polyclonal	Zamboni's solution	1/500	Microwaving in citrate buffer, 20 minutes	Santa Cruz Biotechnology Inc., Dallas, Taxas, USA
CD204	Mouse monoclonal	Zamboni's solution	1/1000	Microwaving in citrate buffer, 20 minutes	TransGenic Inc., Kumamoto, Japan
BrdU	Mouse monoclonal	NBF	1/500	4 N HCl and 100 μg/ml Proteinase K, 10 minutes	Dako Denmark A/S, Glostrup, Denmark

PLP, periodate-lysine-paraformaldehyde; BrdU, bromo-2-deoxyuridine; NBF, neutral buffered formalin.

Iba-1, Galectin-3 (Gal-3), MHC class II, CD204 and BrdU staining (Table 1). After pretreatment, tissue sections were stained with Histostainer (Nichirei Biosciences Inc., Tokyo, Japan). Briefly, sections were treated with 5% skimmed milk for 10 minutes and allowed to react with primary antibodies for 1 hour at room temperature (RT). After incubation in 3% H_2O_2 for 15 minutes, a horseradish peroxidase-conjugated secondary antibody (Histofine Simple Stain MAX PO®, Nichirei Biosciences Inc., Tokyo, Japan) was applied for 30 minutes at RT. The sections were then incubated with 3, 3′-diaminobenzidine (Nichirei Biosciences Inc., Tokyo, Japan) and counterstained with hematoxylin. The number of immunopositive cells with clear nuclei was counted per 40× field in five randomly selected areas in the perivenular (PV), periportal (PP) or Glisson's sheath (GS) areas including interlobular connective tissues and the portal triad. The number of BrdU-positive hepatocytes was also counted per 40x field and compared with the total number of hepatocytes.

Liver tissues were immersed in RNAlater reagent (Qiagen, Hilden, Germany), kept overnight at 4°C and stored at −80°C until use. Total RNA was extracted with an SV total RNA isolation system (Promega, Fitchburg, WI, USA). RNA was reverse transcribed to cDNA with SuperScript VILO reverse transcriptase (Life technologies, Carlsbad, CA, USA). Real-time PCR was performed using TaqMan gene expression assays (Life Technologies) in a PikoReal Real-Time PCR System (Thermo Fisher Scientific, Sunnyvale, CA, USA). The TaqMan probes specific for the cytokines used were as follows (Assay IDs): monocyte chemoattractant protein-1 (MCP-1), Rn00580555_m1; interleukin-1β (IL-1β), Rn00580432_m1; colony stimulating factor-1 (macrophage) (CSF-1), Rn00696122_m1; colony stimulating factor-2 (granulocyte-macrophage) (CSF-2), Rn01456850_m1; interferon-γ (IFN-γ), Rn00594078_m1; tumor necrosis factor-α (TNF-α), Rn01525859_g1; tumor growth factor-β1 (TGF-β1), Rn00572010_m1; interleukin-4 (IL-4), Rn01456866_m1; interleukin-6 (IL-6), Rn01410330_m1; interleukin-10 (IL-10), Rn00563409_m1; and β-actin,

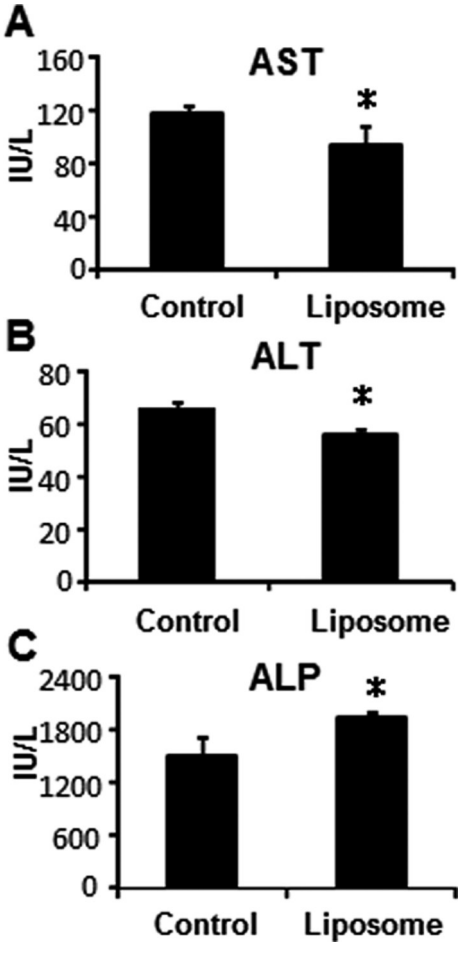

Fig. 1. A–C: Blood biochemical analyses in control and liposome-treated rats. Aspartate transaminase (AST) (A), alanine transaminase (ALT) (B) and alkaline phosphatase (ALP) (C). Student's *t*-test. **P*<0.05, significantly different from control rats.

(Rn00667869_m1). The mRNA expression was normalized against the expression of β-actin mRNA as the internal controller gene. The data were analyzed using the comparative Ct method (ΔΔCt method). Expression profiles of mRNAs were analyzed with a SurePrint G3 Rat GE8x60K Microarray (Agilent Technologies, Santa Clara, CA, USA).

Obtained data were expressed as the mean ± standard deviation (SD). Statistical analysis was performed using Student's *t*-test. $P<0.05$ was considered significant.

In liposome-treated rats, the AST and ALT levels were significantly decreased (Fig. 1A and B), and the ALP level was significantly increased (Fig. 1C) in comparison with the controls. There were no significant differences in histopathology of livers between control and liposome-treated rats (Fig. 2A and B). However, BrdU-positive hepatocytes were diffusely distributed in the liver parenchyma, which significantly increased in the liposome-treated rats (Fig. 2C–E), indicating increased proliferation activity of hepatocytes.

Macrophages expressing different immunophenotypes in the PV, PP and GS areas of livers were evaluated by using different antibodies such as CD163, CD68, Iba-1, MHC class II, Gal-3 and CD204 (Fig. 3A–F). In the PV and PP areas, the numbers of macrophages reacting to CD163, CD68, Iba-1, MHC class II, Gal-3 and CD204 were significantly increased in liposome-treated rats (Fig. 3A-F). In addition, $CD163^+$ macrophages were also increased significantly in number in the GS area (Fig. 3A). There were no macrophages positive for Gal-3 (Fig. 3E) and CD204 (Fig. 3F) in the GS area. Among macrophages expressing different antigens, $CD163^+$ and $CD68^+$ macrophages showed the greatest

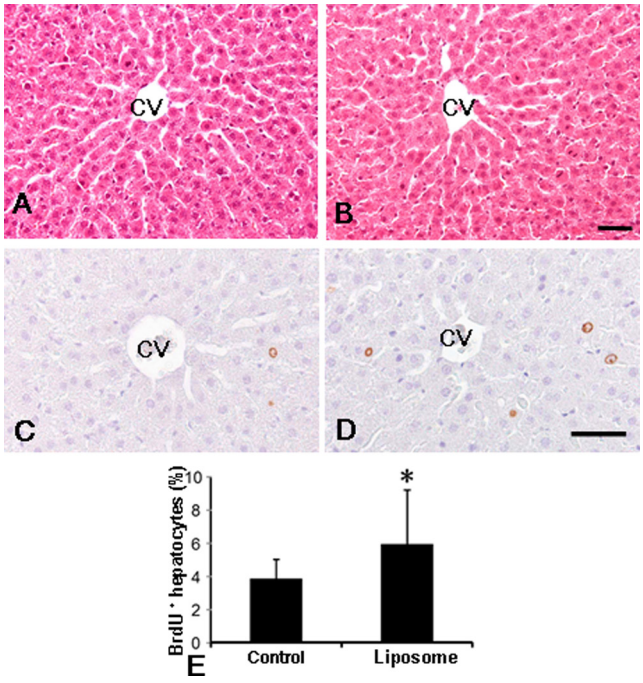

Fig. 2. A and B: Histopathology of the liver in control and liposome-treated rats. The hepatic architecture is normal in rats of both groups. C and D: BrdU-positive hepatocytes are seen in livers of control (C) and liposome-treated rats (D). E. The kinetics of BrdU-positive hepatocytes in the liver. The number of positive hepatocytes was significantly increased in liposome-treated rats. CV, central vein; BrdU, bromo-2-deoxyuridine. Student's *t*-test. *$P<0.05$, significantly different from control rats. Bar = 50 μm.

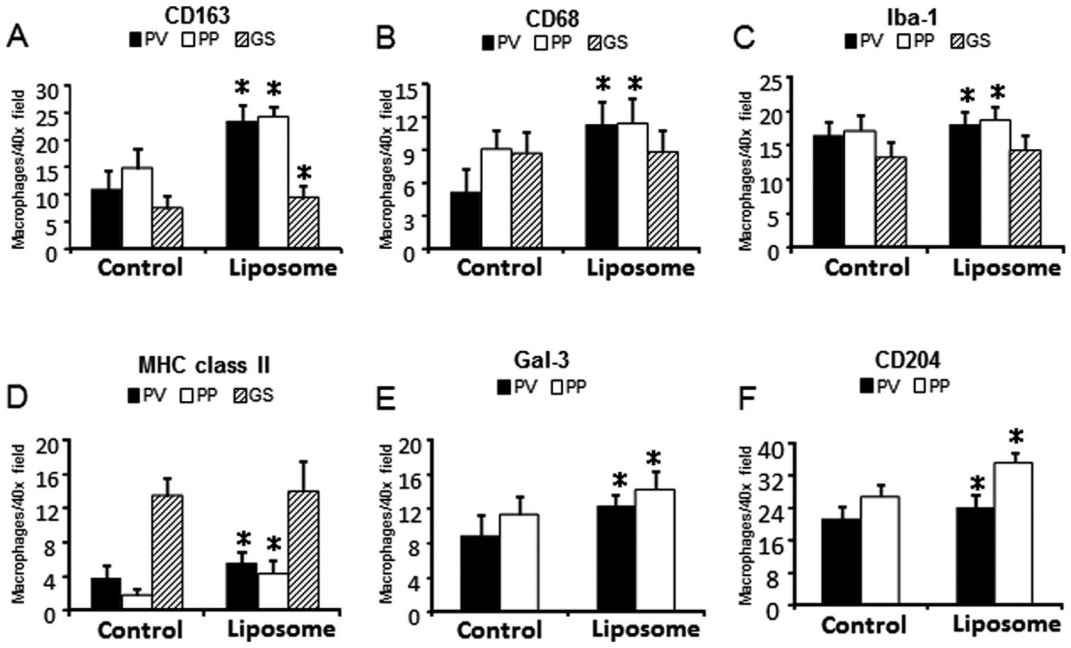

Fig. 3. The kinetics of macrophages reacting to CD163 (A), CD68 (B), Iba-1 (C), MHC class II (D), Gal-3 (E) and CD204 (F) in the perivenular (PV), periportal (PP) and Glisson's sheath (GS) areas of the liver in control and liposome-treated rats. Student's *t*-test. *$P<0.05$, significantly different from control rats.

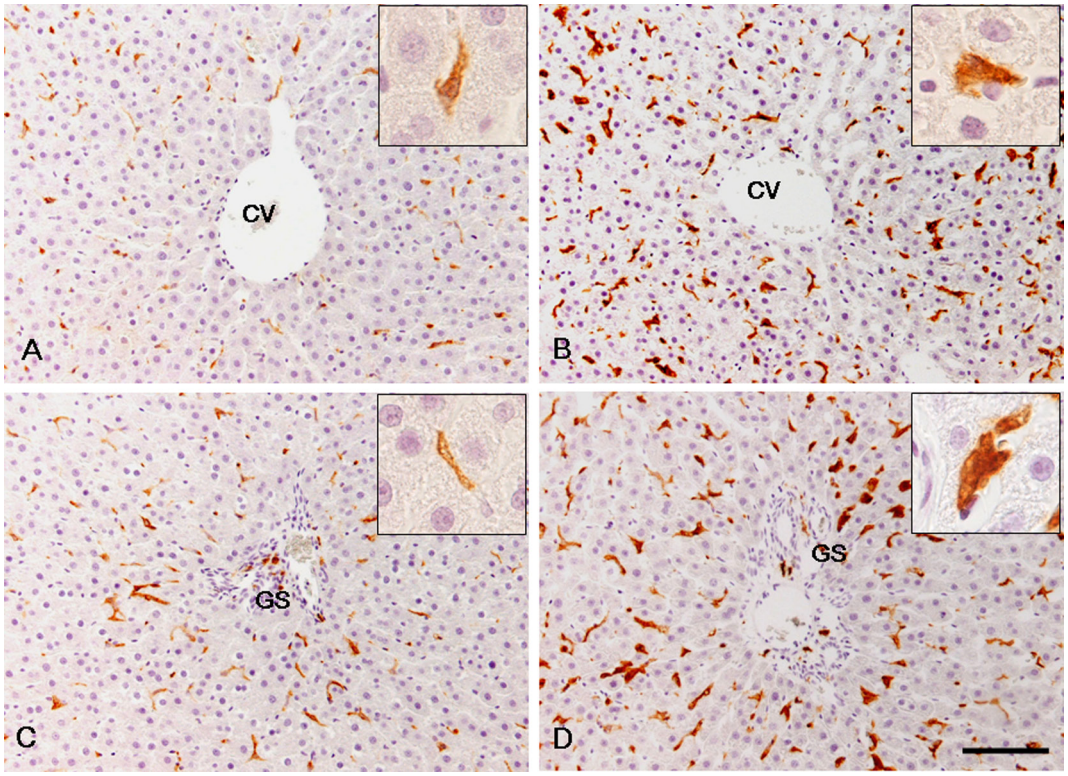

Fig. 4. Immunohistochemistry with CD163 in control and liposome-treated rats in the perivenular (PV) and periportal (PP) areas of the liver. Macrophages reacting to CD163 are seen in the control rat liver in the PV (A) and PP (C) areas; the positive cells have a round shape with processes (inset). Increased numbers of macrophages are seen in the PV (B) and PP (D) areas in liposome-injected rats; $CD163^{+}$ cells appear more swollen and enlarged in size, with fewer processes (inset). CV, central vein; GS, Glisson's sheath. Bar = 50 µm.

Table 2. Upregulated (more than 2-fold) and Downregulated (less than 0.5-fold) Genes in the Liver of Liposome-treated rats Versus Control rats

Functional Category	Gene symbol	Gene description	Fold change
Upregulated genes			
Cell cycle	Anapc1	Rattus norvegicus anaphase promoting complex subunit 1 (Anapc1), mRNA [NM_001107771]	3.55
	Tgfb1	Rattus norvegicus transforming growth factor, beta 1 (Tgfb1), mRNA [NM_021578]	2.67
	Tgfb3	Rattus norvegicus transforming growth factor, beta 3 (Tgfb3), mRNA [NM_013174]	2.63
	Pkmyt1	Rattus norvegicus protein kinase, membrane associated tyrosine/threonine 1 (Pkmyt1), mRNA [NM_001105766]	2.54
	Gadd45a	Rattus norvegicus growth arrest and DNA-damage-inducible, alpha (Gadd45a), mRNA [NM_024127]	2.07
Cytokine-cytokine interaction	Cxcl9	Rattus norvegicus chemokine (C-X-C motif) ligand 9 (Cxcl9), mRNA [NM_145672]	11.83
	Ccl2	Rattus norvegicus chemokine (C-C motif) ligand 2 (Ccl2), mRNA [NM_031530]	7.88
	Il18r1	Rattus norvegicus interleukin 18 receptor 1 (Il18r1), mRNA [NM_001106905]	3.84
	Il1b	Rattus norvegicus interleukin 1 beta (Il1b), mRNA [NM_031512]	3.42
	Epor	Rattus norvegicus erythropoietin receptor (Epor), mRNA [NM_017002]	2.86
	Tnfrsf21	Rattus norvegicus tumor necrosis factor receptor superfamily, member 21 (Tnfrsf21), mRNA [NM_001108207]	2.81
	Flt4	Rattus norvegicus fms-related tyrosine kinase 4 (Flt4), mRNA [NM_053652]	2.68
Downregulated genes			
Cytokine-cytokine interaction	Acvr2b	Rattus norvegicus activin A receptor, type IIB (Acvr2b), mRNA [NM_031554]	0.49
	Acvr2a	Rattus norvegicus activin A receptor, type IIA (Acvr2a), mRNA [NM_031571]	0.41
	Bmpr2	Rattus norvegicus bone morphogenetic protein receptor, type II (serine/threonine kinase) (Bmpr2), mRNA [NM_080407]	0.35
	Il1rap	Rattus norvegicus interleukin 1 receptor accessory protein (Il1rap), transcript variant 1, mRNA [NM_012968]	0.32
	Amh	Rattus norvegicus anti-Mullerian hormone (Amh), mRNA [NM_012902]	0.18
Apoptosis	Ntrk1	Rattus norvegicus neurotrophic tyrosine kinase, receptor, type 1 (Ntrk1), mRNA [NM_021589]	0.32

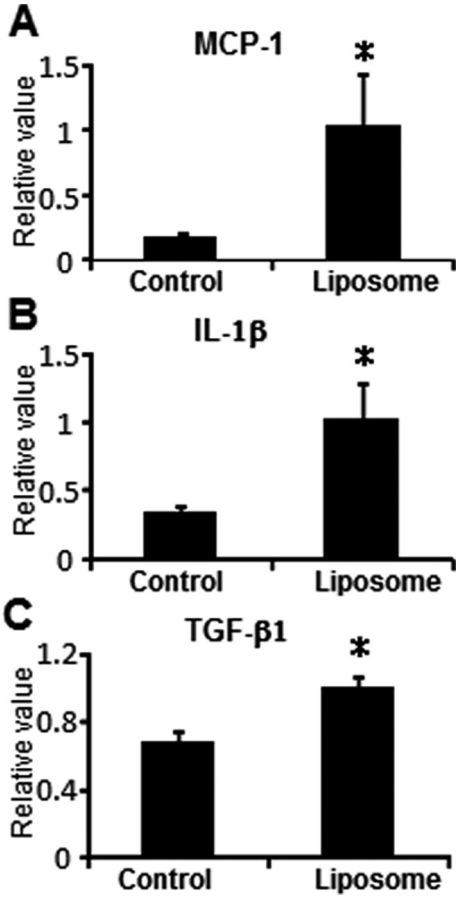

Fig. 5. Levels of mRNA expression of inflammatory cytokines MCP-1 (A), IL-1β (B) and TGF-β1 (C) in control and liposome-treated rats. Expression levels were normalized to the β-actin mRNA level. Student's *t*-test. **P*<0.05, significantly different from control rats.

increases in number after liposome injection. Interestingly, macrophages reacting to all antibodies in liposome-treated livers showed a larger cytoplasm than in controls; in particular, the morphological change was most prominent in CD163$^+$ hepatic macrophages in liposome-treated rats (Fig. 4A–D).

MCP-1, IL-1β and TGF-β1 mRNAs were significantly increased in liposome-treated rats (Fig. 5A-C). Other cytokines (CSF-1, CSF-2, TNF-α, INF-γ, IL-4, IL-6 and IL-10) examined did not show any significant change following liposome treatment (data not shown). A total of 721 genes were upregulated (more than 2-fold), and 703 genes were downregulated (less than 0.5-fold) in response to liposome treatment. These gene profiles were grouped into distinct differentially expressed functional groups (mainly, cell cycle, cytokine-cytokine interaction, and apoptosis groups) (Table 2) based on the gene ontogeny classification system[8]. In the cell cycle-related functional gene group, five genes showed upregulation, and there were no downregulated genes. Such genes related to the cell cycle might be corresponding to increase number of BrdU-positive hepatocytes. In the cytokine-cytokine interaction gene group, a total of seven genes showed upregulation and five genes were downregulated. Apoptosis-related genes were small in number, and in fact, few apoptotic cells, demonstrable with the TUNEL method, were detected (data not shown).

Liposomes are prepared as a nontoxic, nondegradable and nonimmunogenic vehicle. The administration of empty liposomes may activate macrophages by a phagocytic stimulus[9]; however, detailed characterization of the activated macrophages has not been reported. The present study showed that empty liposome treatment increased the number of hepatic macrophages with different immunophenotypes for all antibodies (CD163, CD68, Iba-1, MHC class II, Gal-3 and CD204) examined immediately (one day later) after injection; furthermore, it was found that CD163$^+$ and CD68$^+$ macrophages located in the PV and PP areas were most sensitive, showing an elongated/swollen, larger cytoplasm. The CD163 antibody is used to detect resident macrophages (Kupffer cells in the liver)[10], and CD68 expression implies phagocytic activity of macrophages[5]. CD204 is a scavenger receptor for lipid metabolism, and its expression may be also related to phagocytosis[11]. The findings for these antibodies indicated that the liposome treatment might activate resident macrophages with enhanced phagocytosis. Iba-1 expression may indicate cell migration of macrophages[12], and Gal-3 expression may be related to fibrosis after tissue injury[6]. MHC class II-expressing macrophages are regarded as antigen-presenting cells[13]. In addition to activated phagocytosis, liposome treatment could influence macrophage functions as shown in the immunohistochemistry for Iba-1, Gal-3 and MHC class II.

In contrast to the increased number of macrophages with different immunophenotypes, there was no significant change in histopathology of HE-stained sections between control and liposome-treated rats, indicating that liposomes are not toxic to hepatocytes. However, interestingly, that number of BrdU-positive hepatocytes was significantly increased following liposome treatment; this increased proliferating activity was supported by the upregulated the cell cycle-related functional gene group in the microarray analysis. Activation of hepatic macrophages due to phagocytosis of liposomes might give rise to an imbalance in the relationship between Kupffer cells and hepatocytes, presumably resulting in proliferation of hepatocytes. Presumably, the increased ALP in liposome-treated rats may reflect the slight effect of the biliary system due to the imbalance, as the bile canaliculi are located between hepatocytes.

On the other hand, it is interesting to note that the liposome treatment in this study decrease significantly AST and ALT values. It is reported that hepatic macrophages may play a role in the clearance of such hepatic deviation enzymes; in fact, AST and ALT levels in serum were increased under hepatic macrophage depletion[7, 14]. The opposite phenomenon (decreased levels) observed for AST and ALT in the present study might have been related to the increased number of activated hepatic macrophages.

During inflammation, activated macrophages produce

various inflammatory factors, which can induce cell injury in the early stages or reparative fibrosis in the late stages. The increased mRNA expression of IL-1β, TGF-β1 and MCP-1 following liposome treatment also indicated the activation of hepatic macrophages. The influence of cytokine-cytokine interaction on gene profiles might be related to changes in such inflammatory factors.

In conclusion, we showed that empty liposomes can activate hepatic macrophages with different immunophenotypes, presumably resulting in decreased AST and ALT levels and increased expression of inflammatory factors (IL-1β, TGF-β1 and MCP-1), as well as increased hepatocyte proliferation. Recently, liposomes have been widely used as a vehicle by encapsulating drugs or toxins. In particular, in order to determine the functions of hepatic macrophages, a lioposome-encapsulated clodronate capable of eliminating hepatic macrophages was utilized in chemically induced hepatic lesions[3]. The present study was conducted by using liver samples obtained one day after liposome injection, because we wanted to determine the transient influence of liposomes on rat hepatic macrophages. Further studies are needed to determine the dynamic influences of liposomes on hepatic macrophages over a longer term. Toxicologic pathologists should take these liposome effects on hepatic macrophages into consideration when using liposomes as a vehicle.

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Disclosure of Potential Conflicts of Interest: The authors declare that they have no conflicts of interest.

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