All-trans retinoic acid suppresses liver injury induced by Propionibacterium acnes and lipopolysaccharide in rats

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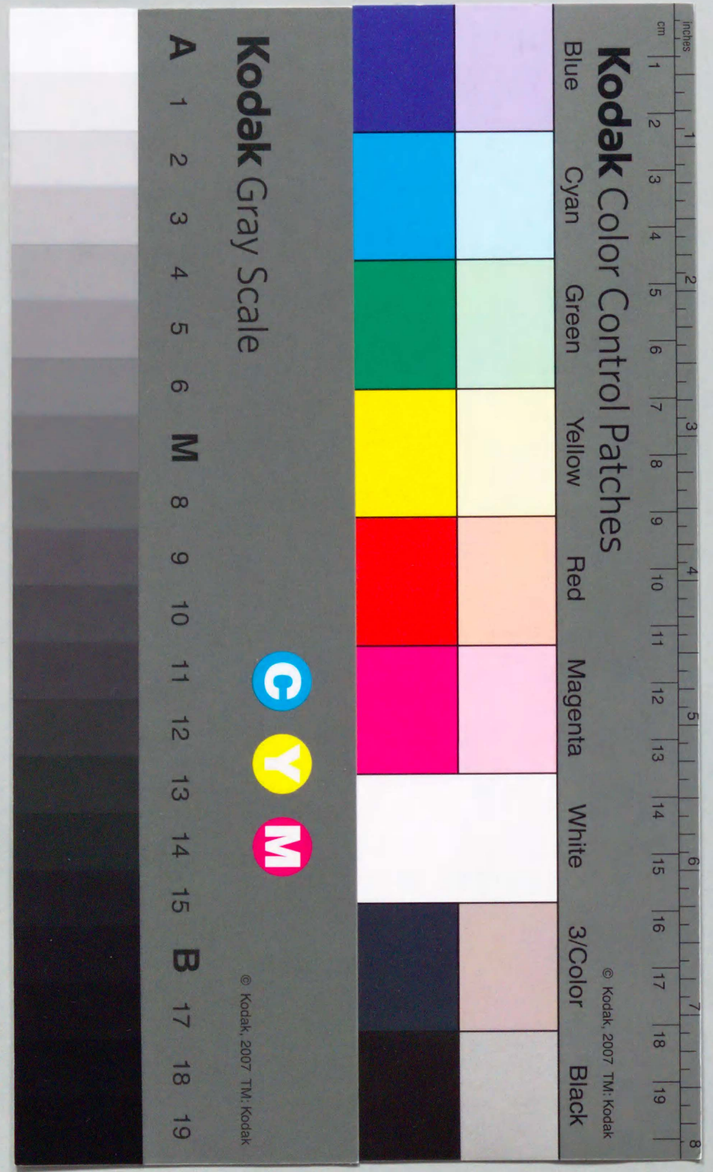

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All-*trans* retinoic acid suppresses liver injury induced by *Propionibacterium acnes* and lipopolysaccharide in rats

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Abstract All-*trans* retinoic acid (ATRA) has been reported to exert major effects on the immune system, including monocytes/macrophages. The present study was designed to determine whether ATRA would modulate macrophage-associated liver injury induced by *Propionibacterium acnes* and lipopolysaccharide (LPS) in rats. All-*trans* retinoic acid administration alleviated the liver injury and reduced the incidence of death following hepatic failure. Serum alanine aminotransferase (ALT) levels 5 h after, and survival rates within 12 h after the administration of LPS were significantly lower in the ATRA-treated group (134 ± 119 IU/L and 72.7%) compared with the control group (713 ± 411 IU/L and 18.2%; $P < 0.05$). Histological findings supported these results. These effects may be due to suppression of tumour necrosis factor-α (TNF-α) and superoxide anions produced by activated macrophages. Serum levels of TNF-α 1 h after LPS administration were significantly lower in the ATRA-treated group (60.5 ± 7.0 ng/mL) as compared with the control group (105.2 ± 39.3 ng/mL; $P < 0.05$). Formazan deposition that was generated by the perfusion of the liver with nitroblue tetrazolium, also suggested suppression of the release of superoxide anions from hepatic macrophages. These results suggest that ATRA acts as an immunomodulator in liver injury by suppressing the activation of liver macrophages.

Key words: all-*trans* retinoic acid, immunomodulator, Kupffer cell, liver injury, LPS, *Propionibacterium acnes*, superoxide, TNF-α.

INTRODUCTION

Retinoids, natural and synthetic derivatives of vitamin A, exert marked effects on cellular proliferation and differentiation, and also on the immune system. The anti-inflammatory effects of retinoids have been demonstrated in dermatological diseases in humans[1] and in animal models of inflammatory diseases, such as adjuvant arthritis[2] and ultraviolet-induced erythema.[3]

Most of the effects of vitamin A (retinol) are linked to the oxidized form, all-*trans* retinoic acid (ATRA), via the ligand-dependent activation of two families of nuclear hormone receptors, retinoic acid receptors (RAR)[4–6] and retinoid X receptors (RXR).[7] We previously studied the effects of ATRA, retinal and retinol on the function of isolated rat Kupffer cells, which are resident macrophages in the liver. Of the three retinoids, ATRA is most potent in suppressing the production of tumour necrosis factor-α (TNF-α) and nitric oxide (NO) by lipopolysaccharide (LPS)-stimulated Kupffer cells.[8] This suggests that ATRA may inhibit inflammation in some types of liver injury by suppressing the production of inflammatory cytokines and free radicals from Kupffer cells or hepatic macrophages. The present study was designed to determine whether ATRA would exert any effect on experimental liver injury in the rat. *Propionibacterium acnes* (*P. acnes; Corynebacterium parvum*) and LPS were used to induce liver injury in this model. Severe liver injury and circulatory shock can be induced by the injection of a low dose of LPS to mice or rats pretreated with heat-killed *P. acnes*.[9–11] Hepatic macrophages are considered to be mainly involved in the development of the liver injury in this model.[9–11]

METHODS

Animals

Male Wistar rats (170–200 g, Laboratory of Animal Experiments, Kyushu University, Japan) were maintained on a basal pelleted diet and water *ad libitum* in a room under normal laboratory lighting conditions. Protocols

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for these studies were reviewed by the Committee of the Ethics on Animal Experiment in Faculty of Medicine, Kyushu University and carried out under the control of the Guidelines for Animal Experiments in the Faculty of Medicine, Kyushu University and the law (No.105) and notification (No.6) of the Japanese Government.

Experiment 1

Eleven rats received ATRA (Sigma Chemical Co., St Louis, MO, USA) at a daily dose of 20 mg/kg bodyweight. Preliminary experiments employing control rats confirmed that ATRA had no significant side effect at this dose. The drug was given orally as a 4 mg/1 mL mixture with olive oil (Wako Pure Chemical Industries Ltd, Osaka, Japan), daily for 8 days (ATRA-treated group). Another 11 control rats similarly received 5 mL/kg bodyweight of olive oil per day. All rats received an injection of 40 mg/kg bodyweight of heat-killed *P. acnes* (*C. parvum* ATCC11827; provided by Otsuka Pharmaceuticals, Tokushima, Japan) as a 20 mg/mL suspension in saline via the tail vein on day 1. Seven days later, they received an intravenous injection of 400 μg/kg bodyweight of LPS (from *Escherichia coli* 055: B5; Difco Laboratories, Detroit, MI, USA) as a 200 mg/mL solution in saline after the final administration of ATRA or olive oil. Rats were observed for 12 h after the injection of LPS to determine the survival rates.

Experiment 2

Twelve rats were divided into two groups and treated as described in Experiment 1. To determine the severity of liver injury, blood was collected from the inferior caval vein under anaesthesia with diethyl ether (Wako) 5 h after the LPS injection. The specimens of liver for light microscopic study were rapidly excised and fixed in 10% formalin. The paraffin-embedded sections were stained with haematoxylin and eosin (HE) for histological evaluation.

Experiment 3

Twenty-four rats were divided into two groups (14 rats for control, and 10 for ATRA administration) and were treated as in Experiment 1. Before the administration of LPS, four rats in each group were killed for the evaluation of granuloma formation in the liver by light microscopy. The specimens of liver were excised, fixed, embedded in paraffin and stained with HE. One hour after the injection of LPS, another six rats from each group were subjected to liver perfusion with nitroblue tetrazolium (NBT). Blood was collected for the determination of serum concentrations of TNF-α. Another further four control rats were examined by liver perfusion with NBT and superoxide dismutase (SOD) and liver specimens from them were compared with those perfused without SOD to confirm whether formazan deposition would be produced by superoxide anion.

Procedure for liver perfusion with nitroblue tetrazolium

The procedure for liver perfusion was carried out as previously described by Mochida *et al.*[12] with some modifications. Under anaesthesia with diethyl ether, the liver was perfused successively with Ca^{2+}/Mg^{2+}-free Hanks' balanced salt solution (HBSS) for 5 min, Dulbecco's minimal essential medium (Nipro, Tokyo, Japan) with 0.05% NBT (Wako) for 10 min or with NBT and 60 U/mL of SOD (from bovine erythrocytes; Sigma) as previously described.[12] Perfusion was performed at a flow rate of 20 mL/min at 37°C with a continuous supply of O_2. The perfusate contained 20 mmol/L HEPES, pH 7.4. The excised liver was then fixed in formalin and embedded in paraffin, and stained with nuclear fast red.

Measurement of tumour necrosis factor-α

The serum concentration of TNF-α was assayed by ELISA as previously described.[8] Briefly, 96-well microtitre plates were coated with hamster anti-murine TNF-α monoclonal antibody (Genzyme, Boston, MA, USA) and a blocker. Samples and standard recombinant murine TNF-α (no. TNF-M; Genzyme) were applied and incubated for 2 h, followed by the addition of rabbit anti-murine TNF-α polyclonal antibody (no. IP-400; Genzyme). Peroxidase-conjugated goat anti-rabbit IgG was used as a signalling antibody, and *o*-phenylenediammonium dichloride was used as a substrate. Spectrophotometric measurements were obtained with an ImmunoReader MJ2000 (InterMed, Tokyo, Japan) at an optical density of 490 nm. All steps in this assay were performed at room temperature. The ELISA system measured murine TNF-α in the range of 153–2500 pg/mL. Cross-reactivity between the murine and rat TNF-α was demonstrated by the manufacturer.

Aminotransferase assay

L-Alanine aminotransferase (ALT) activity in the serum was assayed by standard spectrophotometric methods using commercial test reagents (GPT-OA test; Wako).

Histological evaluation

The histological extent of liver injury and granuloma formation were evaluated by using formalin-fixed and paraffin-embedded liver specimens. All slides were evaluated at random by one of the authors on light microscopy or micrographs.

The extent of liver necrosis was classified using a four-degree score as previously described.[13] Granuloma formation was also evaluated as number and size in the randomly selected fields of liver specimens. These were performed as shown in the footnote of Table 1 with the HE-stained sections.

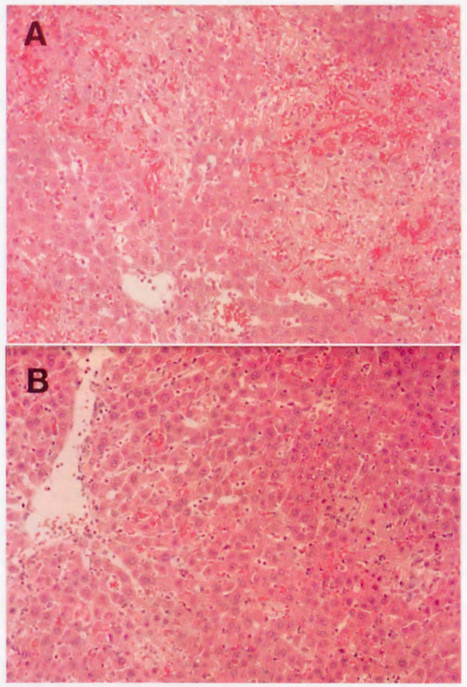


Figure 1 Histological appearance of the liver of *Propionibacterium acnes*-lipopolysaccharide-treated rats. (a), Control group; (b), all-*trans* retinoic acid (ATRA)-treated group. Paraffin-embedded sections stained with HE (×117). In the liver of ATRA-treated rats, coagulative necrosis was rarely observed and intrasinusoidal haemorrhage and hepatocellular degeneration were diminished as compared with control group.

Statistical analysis

Survival rates are expressed as alive/total ratio and were statistically analysed by the Chi-squared method. Scored histological extent of liver injury was statistically analysed by the Mann–Whitney test. Other data are expressed as mean values ± SD and were statistically analysed by the Student's *t*-test. A level of $P < 0.05$ was considered to be statistically significant.

RESULTS

Effects of all-*trans* retinoic acid on lethality and liver injury caused by lipopolysaccharide in rats pretreated with *P. acnes*

The 12 hour survival rate was significantly higher in the group treated with ATRA than in the control group (72.7% [8/11] *vs* 18.2% [2/11]; $P < 0.05$; Table 1). This indicates that *P. acnes* plus LPS-induced lethality was significantly reduced by the administration with ATRA. As shown in Table 1, ATRA also reduced significantly *P. acnes* plus LPS-induced increase in serum ALT activities.

On histological examination, rats in the control group showed diffuse intrasinusoidal haemorrhage, massive hepatocellular degeneration and scattered areas of coagulative necrosis mainly in the midzonal areas (Fig. 1a); five of six rats showed the necrosis of grade 3 (Table 1). In the group treated with ATRA, these findings were mild or rare (Fig. 1b); only two of six rats showed grade 2 necrosis and others showed grade 1 (Table 1).

Effect of all-*trans* retinoic acid on release of superoxide anion from activated liver macrophages

Seven days after the administration of *P. acnes*, the number and size of hepatic granulomas, which

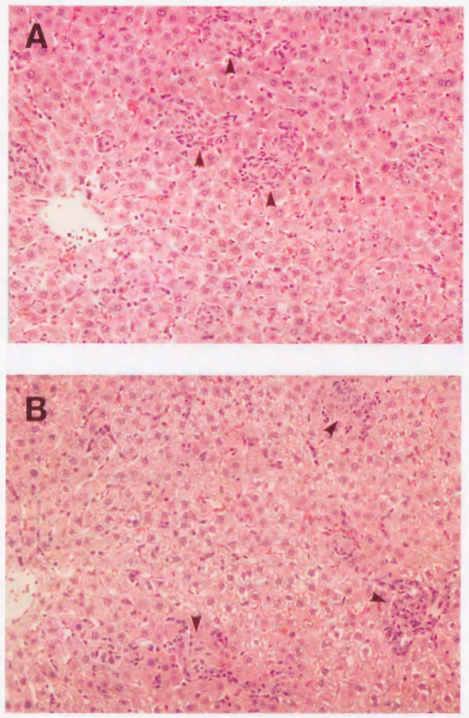
Table 1 Effects of treatment with all-*trans* retinoic acid (ATRA) on liver injury caused by administration of lipopolysaccharide in rats pretreated with *Propionibacterium acnes*

Group	Exp. 1 Survival rate (No. alive/total)	Exp. 2 Serum ALT (KU)	Exp. 2 Extent of liver injury Grade				Exp. 3 Serum TNF-α concentration (ng/mL)	Exp. 3 Granuloma	
			0	1	2	3		No.	Size (μm)
Control	2/11 (18.2%)	713.2 ± 411.4	0	0	1	5	105.2 ± 39.3	39.9 ± 9.8	68.9 ± 9.8
ATRA treated	8/11 (72.7%)*	134.4 ± 119.1†	0	4	2	0‡	60.5 ± 7.0§	36.13 ± 11.9	65.9 ± 8.7

Histological extent of liver injury is classified according to the area of coagulative necrosis in hepatic lobules as follows: grade 0, no coagulative necrosis; grade 1, coagulative necrosis covering < 5%; grade 2, 5% to 10%; grade 3, > 10%.

The number of granulomas is the total number counted from five fields randomly selected under magnification × 100. The granuloma size is the mean of largest diameter of 30 granulomas randomly selected on the printed picture magnified × 130. ALT, alanine aminotransferase; TNF, tumour necrosis factor. The values of serum ALT and TNF-α are expressed as mean ± SD ($n = 6$). The values of No. and size of granuloma are expressed as mean ± SD ($n = 4$).

*, Significantly different from control ($P < 0.05$, χ^2-test); †, significantly different from control ($P < 0.05$, Student's *t*-test); §, significantly different from control ($P < 0.05$, Student's *t*-test); ‡, significantly different from control ($P < 0.01$, Mann–Whitney *U*-test).



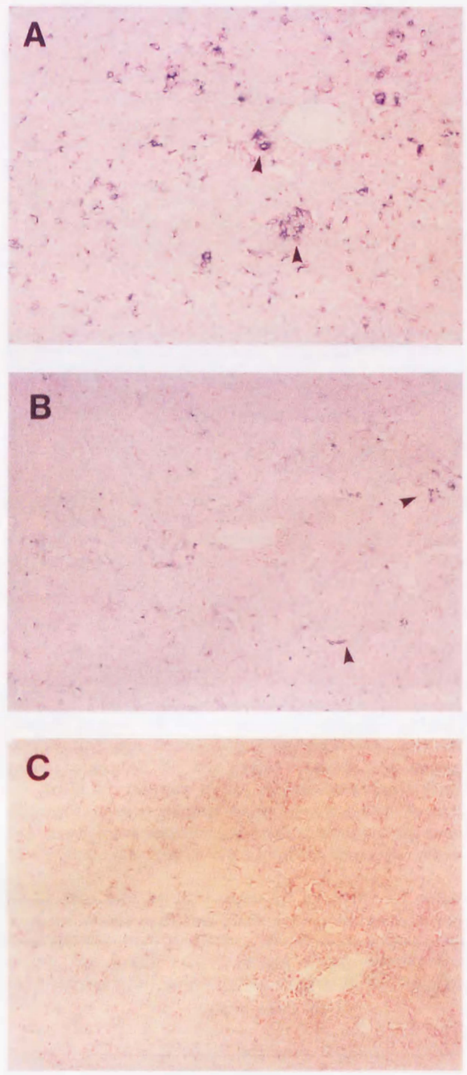


Figure 2 Histological appearance of the liver of *Propionibacterium acnes*-pretreated rats. (a), Control group; (b), all-*trans* retinoic acid (ATRA)-treated group. Paraffin-embedded sections stained with HE (×117). Size and distribution of the granulomas (arrowheads) in the livers were similar in each group as shown in Table 1.

Figure 3 Light micrographs showing formazan deposition of the liver perfused with nitroblue tetrazolium (NBT) in *Propionibacterium acnes*-lipopolysaccharide-administered rats. (a), Control group; (b), all-*trans* retinoic acid-treated group; (c), group perfused with NBT and superoxide dismutase. Paraffin-embedded sections stained with nuclear fast red (×117). (a), Formazan deposits are prominent in macrophages (arrowheads) throughout the liver of control group; (b), few deposits are found in macrophages (arrowheads); (c), the deposits are mostly diminished throughout the liver.

consisted of infiltrating macrophages,[9] were similar between the ATRA and control groups (Table 1, Fig. 2a,b). However, the deposition of formazan in the liver obviously differed between the two groups. In the control group, the marked deposition of formazan was observed not only surrounding granulomas but also in perisinusoidal areas throughout the liver (Fig. 3a). Fewer deposits of formazan were observed in the ATRA-treated group (Fig. 3b). These deposits of formazan nearly disappeared in the rats that were simultaneously perfused with NBT and SOD (Fig. 3c).

Effect of all-*trans* retinoic acid on serum level of tumour necrosis factor-α

The administration of LPS increased the serum concentration of TNF-α in the control group. This increase was significantly attenuated by treatment with ATRA ($P < 0.05$; Table 1).

DISCUSSION

Endotoxins are potent immunomodulators that are toxic to a number of tissues.[14] Because the liver is the major organ responsible for the clearance of soluble and particulate materials (including bacterial products such as LPS) from the circulation, this organ is highly susceptible to the deleterious effects of endotoxins.[15] As a consequence of the liver's functions, a wide array of bioactive substances are released. Among them are reactive oxygen intermediates which are involved in the pathogenesis of liver injury caused by endotoxaemia,[16] ischaemia-reperfusion,[17] and alcohol toxicity.[18] Protection against these radicals has been accomplished by the use of free radical scavengers.[10,16]

Cytokines such as TNF-α and interleukin-1 (IL-1) are also produced in large quantities during systemic inflammation, and are implicated in many of the pathophysiological responses that accompany endotoxaemia.[19] Tumour necrosis factor-α is believed to be the principal mediator of the deleterious effect of LPS.[20,21] Neutralization of TNF-α activity with TNF-α antibodies ameliorates many of the adverse effects of LPS and prevents death from severe bacteraemia.[20,21] Hepatic macrophages play important roles in the development of liver injury by producing proinflammatory cytokines[22] and free radicals.[10] Inhibition of these inflammatory mediators is an important target in the treatment of liver diseases,[23,24] including the primary non-functioning graft in liver transplantation.[25]

In the present study, the administration of ATRA reduced the severity of liver injury and the incidence of mortality caused by the administration of *P. acnes* and LPS.

This effect may be explained by the fact that serum TNF-α levels and the formazan deposition in the liver (which are the indicators of the activation of liver macrophages),[13] were apparently lessened in the ATRA-treated group. The formazan depositions were confirmed to be generated by extracellular superoxide anion release from the activated macrophages, because they nearly disappeared in the rats perfused with SOD; a result similar to one previously described.[12]

We previously confirmed that ATRA suppressed the production of TNF-α by LPS-stimulated rat isolated Kupffer cells *in vitro*.[8] Some researchers also reported that ATRA inhibited the synthesis of collagenase by monocytes,[25] the production of TNF and of NO by murine peritoneal macrophages,[26] and inhibited the IL-1-induced cytokine synthesis in human monocytes.[27] These results seem to be consistent with the *in vivo* effects of ATRA in the present study. However, it remains unclear how much TNF-α in serum was derived from hepatic macrophages since macrophages/monocytes throughout the body can also secrete TNF-α. It is well known that Kupffer cells occupy the largest population of resident macrophages in the whole body,[28] and that injected LPS is mainly absorbed by Kupffer cells.[15] Therefore, it seems reasonable to expect that activated hepatic macrophages are the major source of TNF-α in our model.

Macrophage activation in *P. acnes* and LPS-induced liver injury is known to include 'priming' and 'excitation' steps.[11,12] Interferon (IFN)-γ from T lymphocytes induced by *P. acnes* is required to prime macrophages ('priming' step) in order to activate those with LPS ('excitation' step).[29] Recently, it was reported that IFN-γ-inducing factor (IGIF) was secreted from Kupffer cells and activated macrophages in order to stimulate IFN-γ secretion from T cells.[30,31] Anti-IGIF antibody could prevent liver damage in this model.[31] It was also reported that activated hepatic macrophages produced endothelial cell destruction leading to massive liver necrosis through microcirculatory disturbance caused by fibrin deposition in the hepatic sinusoids.[32] Adhesion between endothelial cells and activated macrophages in the hepatic sinusoids is reported to be essential for the initiation of massive hepatic necrosis because a blockade of this adhesion can prevent liver injury without reducing TNF-α secretion from activated macrophages.[14] Further investigation is needed on the action of ATRA on the IGIF and IFN-γ system, and the adhesion between endothelial cells and activated macrophages. In the carbon tetrachloride (CCL_4)-induced liver injury model, different effects of retinoids were reported.[33] The main causes of CCL_4-induced liver injury is the oxidative stress in hepatocytes,[34] and the contribution of hepatic macrophages is different from that in *P. acnes* and LPS-induced liver injury.[12] Vitamin A can enhance the direct hepatotoxicity of CCL_4 under some conditions.[35] This enhancement is thought to be caused by cytochrome P450 activity stimulated by CCL_4, which is thought to metabolize vitamin A.[33] However, vitamin A deficiency is known to potentiate fibrosis induced by CCL_4.[35] It was also reported that retinyl palmitate inhibited the fibrosis without attenuating serum transaminase activity.[36] This effect is thought to be mediated by an action on stellate cells rather than hepatocytes.[36] Acyclic retinoid and β-carotene were also reported to prevent both liver injury and fibrosis by CCL_4.[37,38] These results were considered to be mediated by the cytoprotective effect of each retinoid on hepatocytes.[37,38] Because this study focused on the relationship between ATRA and the activation of macrophages through released TNF-α and superoxide anions, our results do not touch upon the action of ATRA on other cell types of liver. An evaluation of the effects of retinoids on each liver cell type and their functions in various experimental models is needed to clarify further their detailed mechanisms.

In conclusion, our present results indicate that ATRA is capable of acting as an anti-inflammatory agent in liver injuries that are caused primarily by the activation of the liver macrophages. Further investigation is required to clarify the effects of retinoids and the relationship between liver disease and vitamin A.

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