Fucoidan prevents concanavalin A-induced liver injury through induction of endogenous IL-10 in mice.

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Abbreviations: Con A, concanavalin A; MSR, macrophage scavenger receptor; ALT, alanine aminotransferase; IL, interleukin; TNF-α, tumor necrosis factor-alpha; IFN-γ, interferon gamma; MIP, macrophage inflammatory protein; ELISA, enzyme-linked immunosorbent assay.

Abstract

Fucoidan is a complex of sulfated polysaccharides derived from non-mammalian origin such as marine brown algae and induces cytokine expression. We investigated the effect of fucoidan on concanavalin A (Con A)-induced liver injury in mice. Liver injury was induced by an intravenous injection of Con A (18.5 mg/kg). Various doses of fucoidan (1-30 mg/kg) were intravenously administered 30 minutes before Con A injection. The plasma alanine aminotransferase (ALT) and several cytokines levels were determined, and hepatic histological changes were also assessed. The effect of fucoidan administration by itself on induction of interleukin (IL)-10 in plasma and liver tissue was investigated. Con A administration induced an elevation of plasma ALT level, and fucoidan administration dose-dependently prevented the Con A-induced elevation of plasma ALT. Con A administration increased plasma TNF-α and IFN-γ levels, and fucoidan pretreatment significantly inhibited these alterations and increased plasma IL-10 level. The inhibitory effect of fucoidan on Con A-induced liver injury and production of proinflammatory cytokines were reversed by anti-mouse IL-10 antibody pretreatment. Fucoidan induced the IL-10 production in plasma and liver tissue. These findings suggest that fucoidan prevents Con A-induced liver injury by mediating the endogenous IL-10 production and the inhibition of proinflammatory

cytokine in mice.

Keywords: Concanavalin A, Fucoidan, Liver injury, Cytokine, Kupffer cell, Scavenger receptor

Introduction

In many liver diseases, including viral hepatitis, autoimmune hepatitis, and allograft rejection, activated T-cells appear to play responsible roles. An intravenous injection of mice with concanavalin A (Con A), a plant lectin known to mitogenically activated T-cells, leads to polyclonal T-cell activation and to liver-selective necrotic injury (1). It has been described that various proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interferon gamma (IFN-γ), and interleukin (IL)-2 produced by Con A-activated T-cells, natural killer T cells, and macrophages, play critical roles in the development of massive hepatocellular apoptosis and necrosis (2-13). We have recently found that macrophage inflammatory protein (MIP)-2 and MIP-1α, one of the mouse CXC and CC chemokines, respectively, play a pivotal role in Con A-induced liver injury (11-14). On the other hand, IL-10 is a potent anti-inflammatory cytokine derived from macrophages and Th2 cells and proposed to inhibit the development of Con A-induced liver injury through suppressing proinflammatory cytokine production, including TNF-α and IFN-γ (6, 15). These proinflammatory cytokines and chemokines are also implicated in ischemia/reperfusion-, lipopolisaccharide-, and sepsis-induced liver injury (16-20). Therefore, the inhibition of these proinflammatory cytokines and chemokines or the induction of anti-inflammatory

cytokine has been considered to be a possible treatment strategy for human inflammatory liver disorders.

Fucoidan is a group of sulfated fucose-containing polysaccharides that derived from non mammalian origin such as a marine brown algae, the jelly coat from sea urchin eggs, and the sea cucumber body wall (21). Many studies have shown that fucoidan has a wide spectrum of activity in biological systems. Fucoidan has anticoagulant and antithrombotic activity, acts on the inflammatory and immune systems, has antiproliferative and antiadhesive effect on cells, and protects cells from viral infection (22-29). Several studies also report that fucoidan is one of ligand for macrophage scavenger receptors (MSR) and induced cytokine expression such as TNF-α, IL-1α, IL-8, IL-10, and IL-12 through MSR (30-33).

In the present study, we investigated the effect of fucoidan on Con A-induced liver injury, and whether the production of cytokines such as TNF-α, IFN-γ, IL-10, and MIP-2 were responsible for the effect of fucoidan on Con A- induced liver injury.

Materials and Methods

Reagents

Con A type IV (Jack Bean), fucoidan (Fucus vesiculosus), aprotinin, antipain, leupeptin, pepstatin A, and gadolinium chloride ($GdCl_3$) were obtained from Sigma-Aldrich Co. (St. Louis, MO). Monoclonal anti-mouse CD4 antibody (rat IgG 2b class) was purchased from R&D systems, Inc. (Minneapolis, MN), purified rat monoclonal IgG 2b from EYMED Laboratories, (San Francisco, CA), rat monoclonal anti-mouse IL-10 antibody from R&D systems, Inc., and rat monoclonal antibody (rat IgG 2b class) against MSR type I and II (2F8) from BMA Biomedicals AG (Rheinstrasse, Switzerland), respectively.

Animals

Female specific pathogen-free BALB/c mice (7-8 weeks old, 18-22 g body weight) were purchased from Japan SLC Inc. (Shizuoka, Japan). Mice were housed under condition of controlled temperature (22-24^{o}C) and illumination (12 hours light cycle starting at 06:00) for at least 7 days before experiments. Protocols describing the use of mice were approved by the Animal Care Committee of Aasahikawa Medical College and were in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Experimental protocols

Examination on the liver injury

A single dose of 18.5 mg/kg Con A was injected intravenously in a volume of 0.2 ml pyrogen-free saline via the tail vein. Fucoidan was dissolved in pyrogen-free saline (1, 5, 10, 20, and 30 mg/kg in a volume of 0.2 ml) and intravenously administered 30 minutes before Con A injection. At 2, 4, 8, and 24 hours after Con A injection, the murine abdomen was opened under ether anesthesia and the peripheral blood was obtained from the vena cava inferior with heparinized syringe. The blood was centrifuged at 3,000 rpm for 10 minutes, and plasma was kept at -70^{o}C until assay. Plasma alanine aminotransferase (ALT) level was determined enzymatically using commercially available kit (Wako Pure chemical industries Ltd., Osaka, Japan).

Histological examination

The liver samples were removed 8 and 24 hours after Con A injection, fixed in 20% neutral-buffered formalin and embedded in paraffin. The specimens were stained with hematoxylin and eosin for an assessment of hepatic histological change. Hepatic neutrophils were stained by the naphthol AS-D chloroasetate esterase technique using a commercially available kit (Sigma-Aldrich), and 10 randomly chosen high-power fields

(HPF; magnification of x400) of each sample were counted by blind fashion.

Evaluation of cytokine production

Fucoidan (10 mg/kg) was intravenously administered in a volume of 0.2 ml pyrogen-free saline 30 minutes before Con A injection. To evaluate the effect of fucoidan on the production of cytokines, plasma TNF-α, IFN-γ, IL-10, and MIP-2 levels were determined 2, 4, and 8 hours after Con A injection. Cytokines levels were determined by enzyme-linked immunosorbent assay (ELISA) using commercially available ELISA kits (TNF-α, IFN-γ, and IL-10 were purchased from R&D systems, and MIP-2 was purchased from IBL Co., LTD., Gunma, Japan) according to the manufacturer's instruction. In the ELISAs, the sensitivities of detection were 5.1 pg/ml for TNF-α, 2.0 pg/ml for IFN-γ, 4.0 pg/ml for IL-10, and 1.0 pg/ml for MIP-2, respectively.

Cytoprotective role of IL-10

The rat monoclonal anti-mouse IL-10 antibody (100 μg/mouse, in 0.2 ml pyrogen-free saline) was intravenously administered 90 minutes before fucoidan (10 mg/kg in 0.2 ml pyrogen-free saline) injection. Pyrogen-free saline (0.2 ml) or rat

monoclonal IgG 2b (100 μg/mouse, in 0.2 ml pyrogen-free saline) were also administered 90 minutes before fucoidan injection. After 30 minutes of fucoidan injection, Con A (18.5 mg/kg) was intravenously administered. After Con A injection, the plasma levels of ALT and IFN-γ at 8 hours, and TNF-α at 2 hours were determined.

Expression of IL-10

Fucoidan was dissolved in pyrogen-free saline (1, 5, 10, 20, and 30 mg/kg in a volume of 0.2 ml) and administered intravenously via the tail vein. The IL-10 level of plasma was determined 1 hour after fucoidan injection. Furthermore, the time course of IL-10 levels in plasma and liver were determined 0, 1, 2, 4, and 8 hours after fucoidan (10 mg/kg) injection. Liver was cut into small pieces and 0.2 g of wet tissue was homogenized in 1 ml 10 mM PBS (pH7.5) containing 1 μg/μl each of aprotinin, antipain, leupeptin and pepstatin A using a Ultra-Turax homogenizer (IWAKI, CO., Japan). The collected homogenate was centrifuged at 3,000 rpm for 10 minutes and supernatant were then centrifuged again at 12,000 rpm for 10 minutes. The IL-10 levels in the liver lysates were determined by ELISA and represented per liver wet weight.

Role of Kupffer cells on IL-10 production

Either 10 mg/kg of $GdCl_3$ or 100 μg of anti-mouse CD4 antibody, or both of them were intravenously administered 24 and 48 hours before fucoidan injection. Fucoidan (10 mg/kg) was intravenously administered in a volume of 0.2 ml pyrogen-free saline, and plasma level of IL-10 was determined 1 hour after fucoidan injection.

Role of MSR on IL-10 production

The rat monoclonal anti-mouse macrophage scavenger receptor (50 μg/mouse, in 0.2 ml pyrogen-free saline) or 0.2 ml of pyrogen-free saline was intravenously administered 1 hour before fucoidan. Fucoidan (10 mg/kg) was intravenously administered in a volume of 0.2 ml pyrogen-free saline, and plasma level of IL-10 was determined 1 hour after fucoidan injection.

RT-PCR

Total RNA was prepared from liver tissue after the homogenization using RNeasy kit (QIAGEN, Inc. Valencia, CA) according to the manufacturer’s instruction. The obtained samples were quantified by absorbance at 260 nm. cDNA was synthesized using a Monoclonal Murine Leukemia Virus reverse transcriptase (M-MLV RT) in a

20-μl reaction 2.5 μM of oligo $(dT)_{20}$ primer, 40 U RNase inhibitor, 0.5 mM of deoxynucleotide triphosphate, 2 μL of 10x reaction buffer, 5 mM $MgCl_2$, 200 U M-MLV RT (all from Invitrogen Co., Carlsbad, CA), and 1 μg of total RNA. The reactions were incubated at 65°C for 5 minutes, 50°C for 50 minutes, 85°C for 5 minutes, and finally cooled at 4°C. cDNA was amplified using commercially available system (Takara Bio Inc., Shiga, Japan) in 25 μl of the final volume, which was 10% of the reverse transcription products and 0.5 U of Taq DNA polymerase with neutralizing monoclonal antibody to Taq DNA polymerase. IL-10 and GAPDH were amplified using specific primers for mouse IL-10 and housekeeping gene for GAPDH, purchased from R&D systems by using 35 cycles at 94°C for 1 minutes, at 54°C for 1 minutes, and 72°C for 1 minute. PCR amplification reactions were evaluated through electrophoresis of 5 μl of PCR product on 2% agarose gels containing 1 μg/μl ethidium bromide and visualized by UV transilumination.

Statistical analysis

All results were expressed as means ± SEM. Multiple group comparisons were performed by ANOVA followed by Fisher's protected least significant difference test. $P < 0.05$ was considered statistically significant.

Results

Effect of fucoidan on Con A-induced liver injury

Con A administration induced an elevation of plasma ALT level from 15 ± 2 to 2055 ± 259 KU/l 8 hours after the administration. The elevation of plasma ALT level 8 hours after Con A injection was dose-dependently inhibited by fucoidan pretreatment (Figure 1). Although plasma ALT level after Con A injection was markedly decreased at 8 hours by fucoidan pretreatment, this inhibitory effect was weaken at 24 hours (mean ± SE, KU/l; 2204 ± 272 in vehicle and 1044 ± 289 in fucoidan pretreatment, respectively, p=0.019). Histological evaluation showed that sinusoidal congestion was reduced at 8 hours in the liver of fucoidan-pretreated mice compared with those of vehicle pretreated mice. The midzonal hepatocellular necrosis was also markedly reduced at 24 hours in the liver of fucoidan-pretreated mice (Figure 2). On the other hand, infiltration of hepatic neutrophils was not attenuated by fucoidan pretreatment (mean ± SE, /HPF; 15 ± 1.1 in vehicle and 13 ± 0.84 in fucoidan pretreatment, respectively, p=0.37).

Effect of fucoidan on plasma cytokine and chemokine productions after Con A injection

Plasma TNF-α, IFN-γ, IL-10, and MIP-2 were not detected before Con A

treatment. Plasma TNF-α level increased and reached a peak level 2 hours after Con A injection (18.5 mg/kg), and plasma IFN-γ level increased and reached a peak level 4-8 hours after Con A injection. The elevated TNF-α level after Con A injection was significantly reduced by fucoidan (10 mg/kg) injection at 2 and 4 hours, and the elevated plasma IFN-γ levels 2 and 8 hours after Con A injection was also significantly attenuated by fucoidan pretreatment (Figure 3a, 3b). Although the plasma IL-10 level was gradually elevated following Con A injection, plasma IL-10 level 2 hours after Con A injection was significantly increased by fucoidan pretreatment compared with vehicle pretreatment (Figure 3c). Plasma MIP-2 level reached peak level 2 hours after Con A injection, whereas no significant changes were seen between fucoidan- and vehicle-pretreatment at any time points (Figure 3d).

Effect of anti-mouse IL-10 antibody on the cytoprotective effect of fucoidan

The elevation of plasma ALT level 8 hours after Con A injection was significantly reduced by fucoidan (10 mg/kg) pretreatment. However, this cytoprotective effect of fucoidan on Con A-induced liver injury was completely reversed by anti-mouse IL-10 antibody (100 μg/mouse) pretreatment (Figure 4a).

Furthermore, the inhibitory effect by fucoidan on plasma TNF-α level at 2 hours was partially and plasma IFN-γ level at 8 hours was completely reversed by IL-10 neutralization antibody, respectively (Figure 4b and 4c).

Effect of fucoidan on plasma and hepatic IL-10 production

Although plasma IL-10 was not detected before fucoidan administration, plasma IL-10 level increased and reached a maximal level 1-2 hours after intravenous fucoidan (10 mg/kg) administration (Figure 5). The stimulatory effect of fucoidan on plasma IL-10 level was dose-related ranging from 5 to 30 mg/kg, assessed 1 hour after fucoidan administration (Figure 6). On the other hand, hepatic IL-10 content was increased by intravenous administration of fucoidan (10 mg/kg), and this stimulatory effect was reached maximum 1 hour after fucoidan injection (Figure 7). Moreover, although the IL-10 mRNA was not detected in the liver from control mice, IL-10 mRNA was detected 1 hour after fucoidan and Con A injection (Figure 7).

The roles of Kupffer cells and MSR on IL-10 production by fucoidan

Plasma IL-10 level 1 hour after fucoidan (10 mg/kg) injection was slightly decreased by anti-mouse CD4 antibody pretreatment. The plasma IL-10 level was

markedly decreased by $GdCl_3$ pretreatment, while no further inhibitory effect on the plasma IL-10 was shown by simultaneous treatment by $GdCl_3$ and anti-mouse CD4 antibody (Figure 8). Furthermore, the elevation of plasma IL-10 level after fucoidan injection was significantly decreased by anti-mouse MSR antibody pretreatment (Figure 9).

Discussion

An intravenous injection of mice with Con A, a plant lectin known to mitogenically activated T-cells, leads to polyclonal T-cell activation and to liver-selective necrotic injury (1). Several studies showed that $CD4^+$ T-cells were stimulated, and various cytokines and chemokines such as TNF-α, IFN-γ, IL-2, IL-6, IL-10, MIP-2, and MIP-1α were produced by Con A injection (2-14). Among these cytokines, TNF-α and IFN-γ were described to play a critical role in the development of massive hepatocellular apoptosis and necrosis by several studies using neutralization antibodies against these cytokines and the target gene knockout mice (3-5, 7). On the other hand, IL-10 is a potent anti-inflammatory cytokine derived from macrophages and T-cells and proposed to inhibit the development of Con A-induced liver injury through suppressing proinflammatory cytokine production, including TNF-α and IFN-γ (6, 15).

In the present study, we demonstrated that fucoidan, a complex of sulfated polysaccharides derived from marine blown algae, had a cytoprotective effect on Con A-induced liver injury. Pretreatment of fucoidan dose dependently inhibited the elevation of plasma ALT levels after Con A injection (Figure 1). Fucoidan (10 mg/kg) pretreatment reduced sinusoidal congestion and midzonal hepatocellular necrosis in the liver after Con A injection (Figure 2). Furthermore, plasma TNF-α and IFN-γ

production by Con A administration was significantly reduced by fucoidan pretreatment compared with those of vehicle pretreatment (Figure 3a and 3b). In contrast, the plasma IL-10 level was significantly increased by fucoidan pretreatment at 2 hours compared with that of vehicle pretreatment (Figure 3c). The inhibitory effect of fucoidan on Con A-induced liver injury and production of proinflammatory cytokines (TNF-α and IFN-γ) were reversed by anti-mouse IL-10 neutralization antibody (Figure 4), suggesting that fucoidan prevents Con A-induced liver injury by mediating through IL-10 production and inhibition of proinflammatory cytokines in mice. Adverse effects of fucoidan were not shown in this study, and the administration of fucoidan 1 hour after Con A injection also attenuated the elevation of plasma ALT level (mean ± SE, KU/l; 2067 ± 677 in vehicle and 504 ± 156 in fucoidan treatment, respectively, $p=0.054$). These results propose that fucoidan is one of the useful therapeutic agents in T cell-mediated liver injury such as viral hepatitis and autoimmune hepatitis.

We also investigated the influence of fucoidan by itself on IL-10 production in the liver and plasma. The plasma IL-10 level was significantly elevated and reached peak level 1-2 hours after fucoidan injection (Figure 5a), and the IL-10 level in the liver tissue was also significantly elevated and reached a peak level 1 hour after fucoidan treatment (Figure 5b). The IL-10 mRNA was detected in the liver tissue as early as 1

hour after fucoidan injection (Figure 7). These data suggest that plasma IL-10 induction by fucoidan is associated with the IL-10 production in the liver tissue.

In the present study, we also investigated the cellular source of IL-10 after fucoidan administration. Several studies have been shown that the liver is a major source of IL-10, in experimental peritonitis, systemic endotoxemia, and during reperfusion of human liver grafts (34-36). The potential cellular sources of IL-10 in the liver were reported to be macrophages, Kupffer cells, T and B lymphocytes (37, 38). We determined the IL-10 producing cells by depleting Kupffer cells using $GdCl_3$ and depleting $CD4^+$ T-cells using anti-mouse CD4 antibody, respectively. In consequence, plasma IL-10 level after fucoidan injection was markedly decreased following the depletion of Kupffer cells by $GdCl_3$ treatment, however, the effect of fucoidan on plasma IL-10 production was only slightly inhibited by the depletion of $CD4^+$ T-cells by anti-mouse CD4 antibody treatment and no further inhibitory effect was demonstrated by simultaneous treatment of $GdCl_3$ and anti-mouse CD4 antibody (Figure 8). These findings suggest that the main cellular source of IL-10 in the liver after fucoidan injection is Kupffer cells. It was reported that osteopontin, a phosphorylated glycoprotein, aggravated Con A-induced liver injury through induction of TNF-α and IFN-γ, and inhibition of IL-10 (39). Although we did not examine the osteopontin

expression in plasma and liver, present study could not show that the effect of fucoidan on the cytokine regulation was mediated by osteopontin expression. Further study is needed to demonstrate the relationship between the effects of fucoidan on the immune modulation and osteopontin expression in the liver. On the other hand, it was reported that Gum arabic was a polysaccharide which suppressed kupffer cell function and attenuated massive hepatic necrosis by endotoxin treatment after partial hepatectomy in rats (40). In this study, since the suppression of kupffer cell function by $GdCl_3$ decreased IL-10 production after Fucoidan administration, the inhibitory effect of fucoidan on production of proinflammatory cytokines and liver injury may be different from gum arabic.

Fucoidan is a complex of sulfated polysaccharides, derived from non-mammalian origin such as a marine brown algae, the jelly coat from sea urchin eggs, and the sea cucumber body wall (21). In this study, we used fucoidan from the common brown algae *Fucus vesiculosus* which has a polysaccharide based on L-fucose with mainly $\alpha(1\rightarrow3)$ glycosidic bonds and sulfate groups at position 4 (29). Although little is known about the role of fucoidan in marine organisms, fucoidan has described to have a wide spectrum of activity in biological systems. Fucoidan has anticoagulant and antithrombotic activity, acts on the inflammatory and immune systems, possesses

antiproliferative and antiadhesive activity, and protects cells against viral infection (22-29).

Fucoidan can act as a ligand for either L- or P-selectins, both of which interact with sulfated oligosaccharides (24). Several studies reported that fucoidan prevented the leukocyte accumulation by inhibiting the leukocyte-endothelial cell interaction and the inflammatory cytokines production, such as TNF-α, IL-1β, and IL-8 (41-43). Recent studies demonstrated that the liver infiltration of polymorphonuclear leukocytes (neutrophils) was involved in the development of Con A-induced liver injury (11, 44). We reported that MIP-2, one of the murine CXC chemokines, play a pivotal role in Con A-induced liver injury through mediating neutrophils accumulation and activation in liver (11). In the present study, we determined the effect of fucoidan on the plasma MIP-2 level after Con A injection and the accumulation of neutrophils in the liver at 8 hours. No significant changes were seen on plasma MIP-2 level between fucoidan and vehicle pretreatment at any time points after Con A injection, and the infiltration of hepatic neutrophils caused by Con A was not attenuated by fucoidan pretreatment. Fucoidan is indicated to prevent liver injury not through preventing the leukocyte accumulation in this study.

Furthermore, recent studies have shown that fucoidan is a ligand for MSR and

induced cytokine expression through MSR (30-33). MSRs are a group of proteins that are involved in the uptake of items for disposal, such as oxidized lipoproteins, damaged cells, or invading microorganisms by macrophages (21). It has been demonstrated that macrophages produce IL-10 after engulfing apoptotic cell through MSRs and producing IL-10 plays an important role in terminating cell death, including apoptosis, thereby suppressing excessive inflammatory reaction (45). Fucoidan is a ligand for at least some of MSRs, triggering a protein kinase-dependent signaling pathway (30, 46). In the present study, we demonstrated that anti-mouse MSR antibody reduced the plasma IL-10 production by fucoidan (Figure 9). This result suggests that fucoidan induces endogenous IL-10 production by MSR at least partially in mice.

In conclusion, the present study proposed that fucoidan have an inhibitory effect on Con A-induced liver injury through production of endogenous IL-10 by Kupffer cells, and these effects were mediated by MSR.

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Figure Legends

Figure 1 Effect of fucoidan on Con A-induced liver injury. Mice were intravenously injected with various doses of fucoidan 30 minutes before Con A (18.5 mg/kg) injection. Plasma ALT levels were determined 8 hours after Con A injection. *P < 0.05, and ***P < 0.001 compared with Con A treatment.

Figure 2 Effect of fucoidan on hepatic histological changes induced by Con A. Fucoidan (10 mg/kg) was dissolved in pyrogen-free saline in a volume of 0.2 ml and intravenously administered 30 minutes before Con A (18.5 mg/kg) injection. Vehicle mice were pretreated with pyrogen-free saline 30 minutes before Con A injection. The liver samples were obtained 8 hours (a: vehicle pretreated mouse, b: fucoidan pretreated mouse), or 24 hours (c: vehicle pretreated mouse, d: fucoidan pretreated mouse) after Con A injection. The livers were removed and stained with hematoxylin and eosin (x40).

Figure 3 Effect of fucoidan on plasma cytokines production after Con A administration. Fucoidan (10 mg/kg) was dissolved in pyrogen-free saline in a volume of 0.2 ml and intravenously administered 30 minutes before Con A

(18.5 mg/kg) injection. Plasma TNF-α (a), IFN-γ (b), IL-10 (c), and MIP-2 (d) levels were determined before and 2, 4, and 8 hours after Con A injection. *P < 0.05, **P < 0.01, and ***P < 0.001 compared with vehicle (pyrogen-free saline) pretreatment.

Figure 4 Effect of anti-mouse IL-10 antibody on the cytoprotective effect of fucoidan against Con A. Rat monoclonal anti-mouse IL-10 antibody (100 μg/mouse) or rat monoclonal IgG (100 μg/mouse) was intravenously administered 90 minutes before fucoidan injection. Fucoidan (10 mg/kg) was intravenously administered 30 minutes before Con A (18.5 mg/kg) injection. After Con A injection, plasma levels of ALT and IFN-γ at 8 hours, and TNF-α at 2 hours were determined. *P < 0.05 compared with saline plus rat IgG treatment or fucoidan plus IL-10 antibody.

Figure 5 Effect of fucoidan on plasma and liver IL-10 production. Fucoidan (10 mg/kg) was dissolved in pyrogen-free saline in a volume of 0.2 ml and intravenously administered. IL-10 levels in plasma (a) and liver (b) were determined before and 1, 2, 4, and 8 hours after fucoidan injection. *P < 0.05,

**P < 0.01, and ***P < 0.001 compared with pyrogen-free saline vehicle treatment.

Figure 6 Dose-related stimulating effect of fucoidan administration on plasma IL-10 level. Fucoidan was dissolved in pyrogen-free saline (1, 5, 10, 20, and 30 mg/kg in a volume of 0.2 ml), and intravenously administered. Plasma IL-10 level was determined 1 hour after fucoidan treatment. **P < 0.01, ***P < 0.001 compared with vehicle (pyrogen-free saline) treatment.

Figure 7 IL-10 expression in the liver after fucoidan injection. Fucoidan (10 mg/kg) was dissolved in pyrogen-free saline in a volume of 0.2 ml and intravenously administered. IL-10 mRNA expression was determined by using RT-PCR 1 hour after fucoidan treatment. In each panel, lane 1 is control, lane 2 is Con A (1 hour), and lane 3 is fucoidan (1 hour).

Figure 8 Effect of $GdCl_3$ and anti-mouse CD4 antibody on fucoidan-induced IL-10 production. Either 10 mg/kg of $GdCl_3$ or 100 μg/body of anti-mouse CD4 antibody, or both of them were intravenously administered 24 and 48 hours

before fucoidan (10 mg/kg) injection. Plasma IL-10 level was determined 1 hour after fucoidan injection. *P < 0.05, and ***P < 0.001 compared with fucoidan treatment.

Figure 9 Effect of anti-mouse MSR antibody on IL-10 production after fucoidan administration. Rat monoclonal anti-mouse MSR antibody (50 μg/mouse) was intravenously administered at 1 hour before fucoidan (10 mg/kg in 0.2 ml pyrogen-free saline) injection. Plasma IL-10 level was determined at 1 hour after fucoidan injection. *P<0.05 compared with vehicle (pyrogen-free saline) treatment.

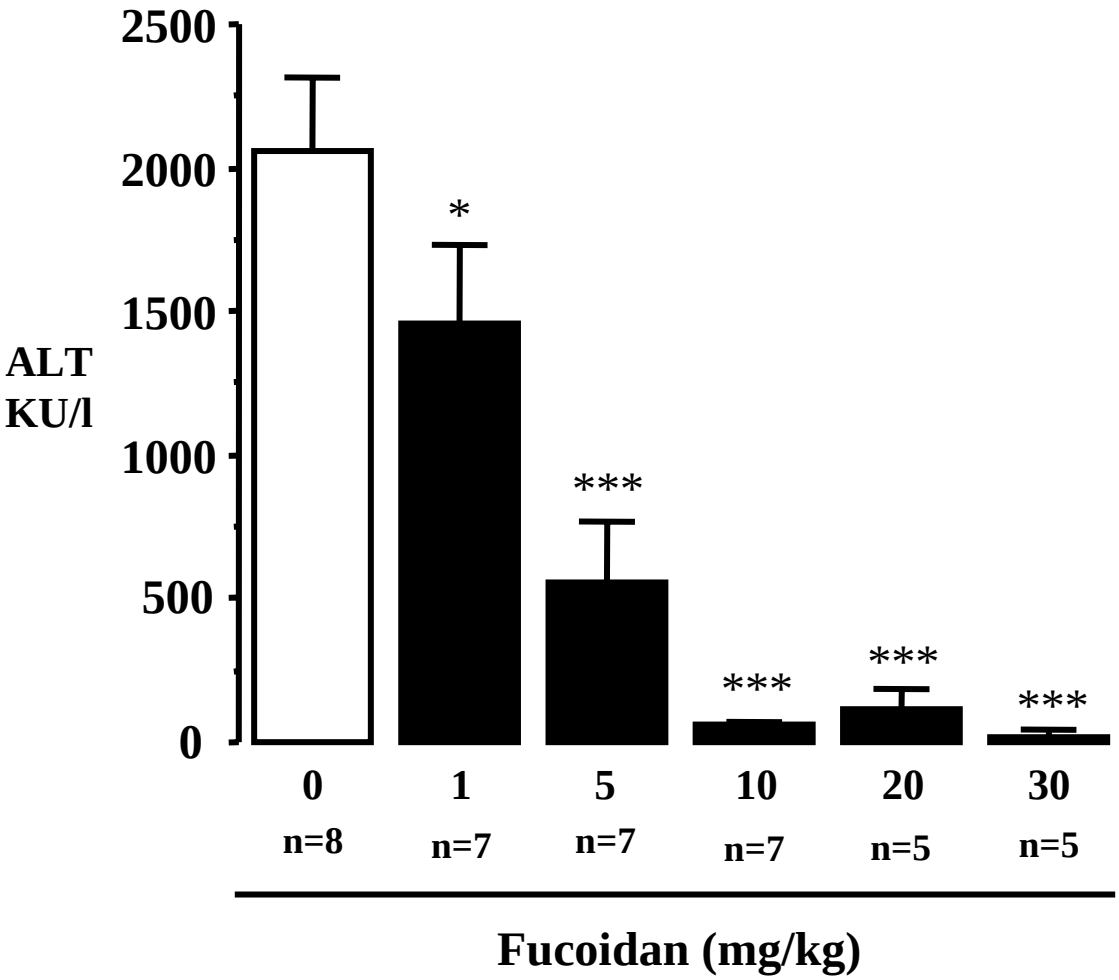
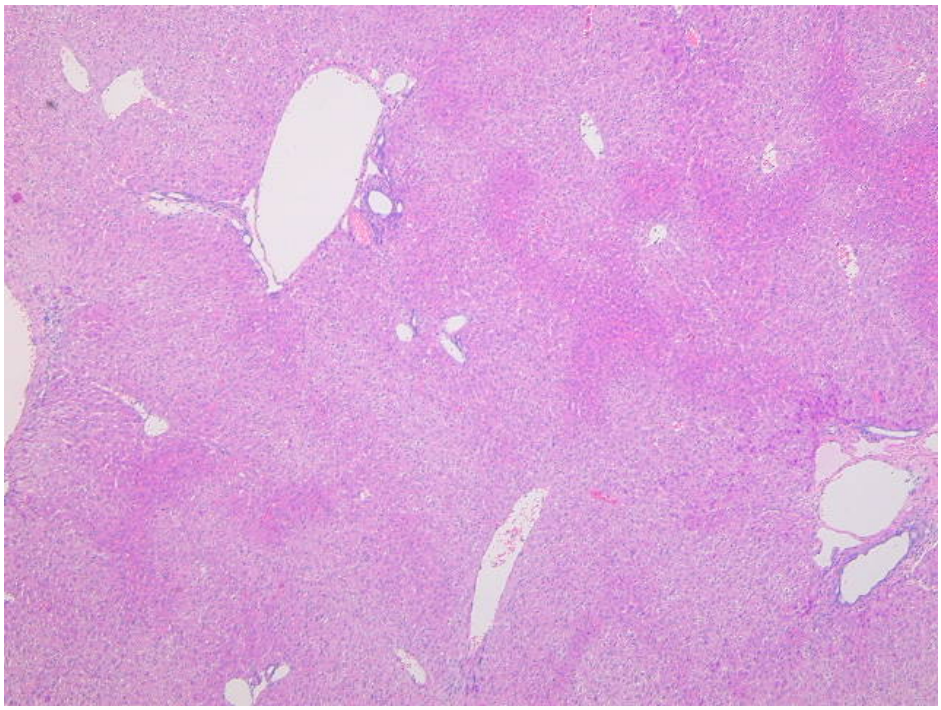

2500
2000
1500
1000
500
0
ALT
KU/l
*
***
***
***
***
0
n=8
1
n=7
5
n=7
10
n=7
20
n=5
30
n=5
Fucoidan (mg/kg)

Figure 1

a.



b.

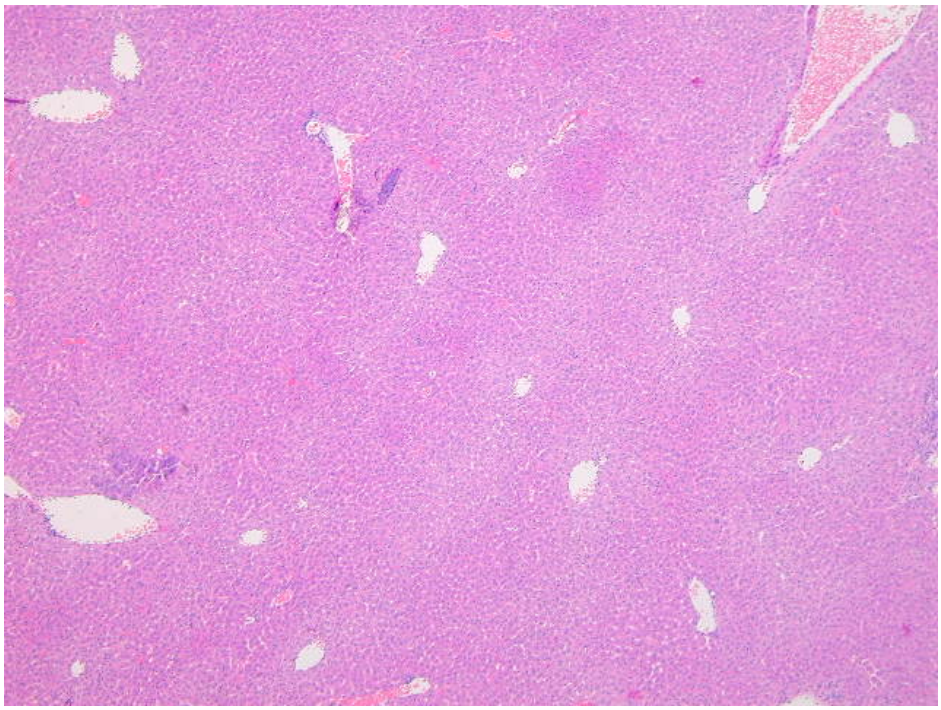
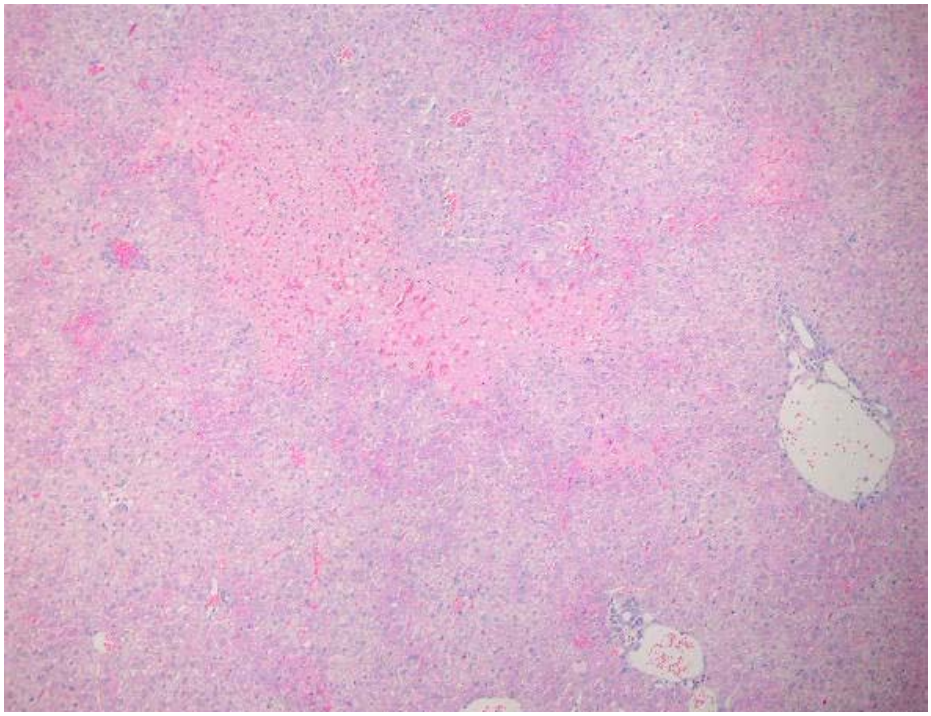


Figure 2

c.



d.

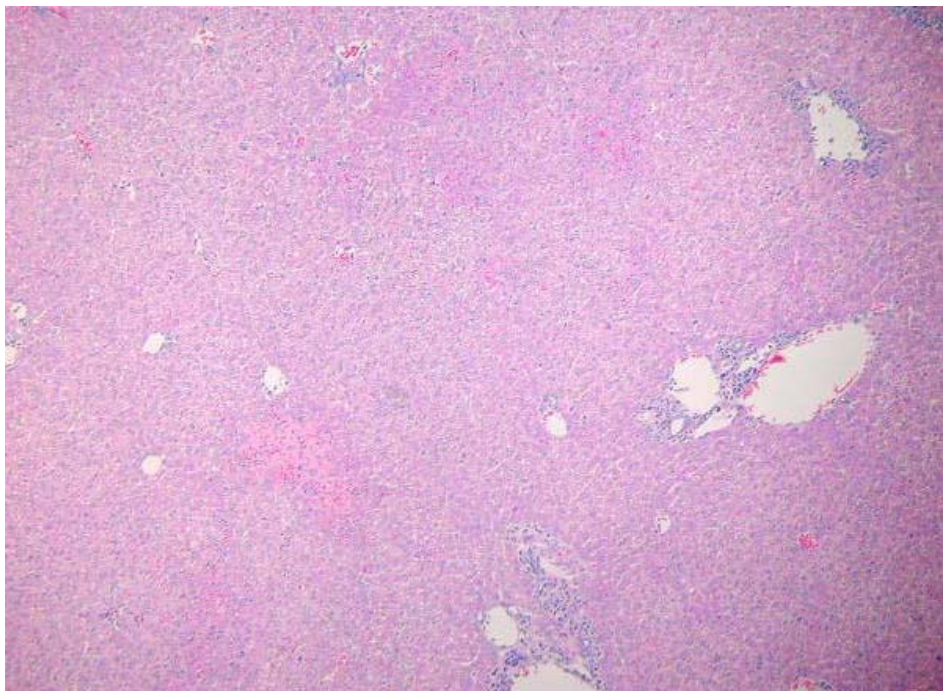
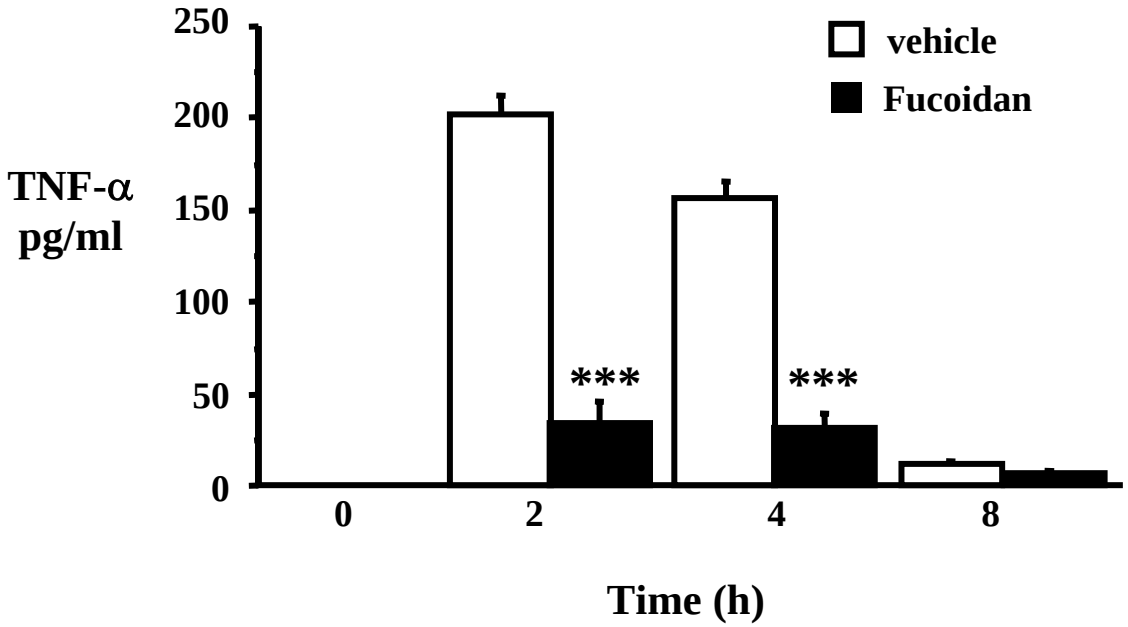


Figure 2

a.


250
200
150
100
50
0
TNF-α
pg/ml
vehicle
Fucoidan
***
***
0
2
4
8
Time (h)

b.

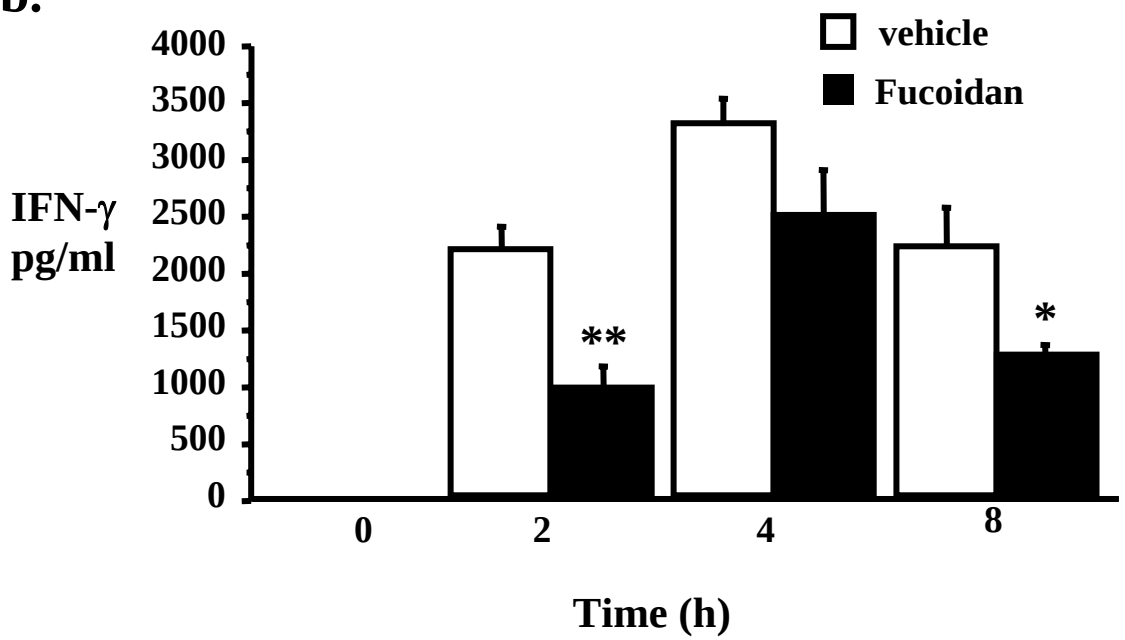
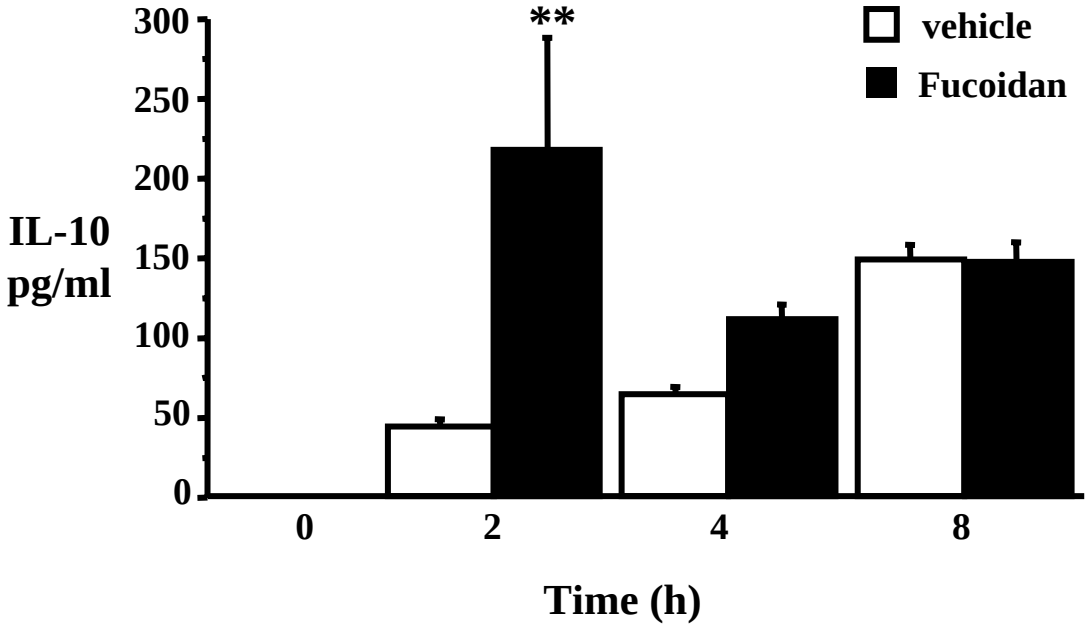

4000
3500
3000
2500
2000
1500
1000
500
0
IFN-γ
pg/ml
vehicle
Fucoidan
**
*
0
2
4
8
Time (h)

Figure 3

c.


300
250
200
150
100
50
0
IL-10
pg/ml
**
vehicle
Fucoidan
0
2
4
8
Time (h)

d.

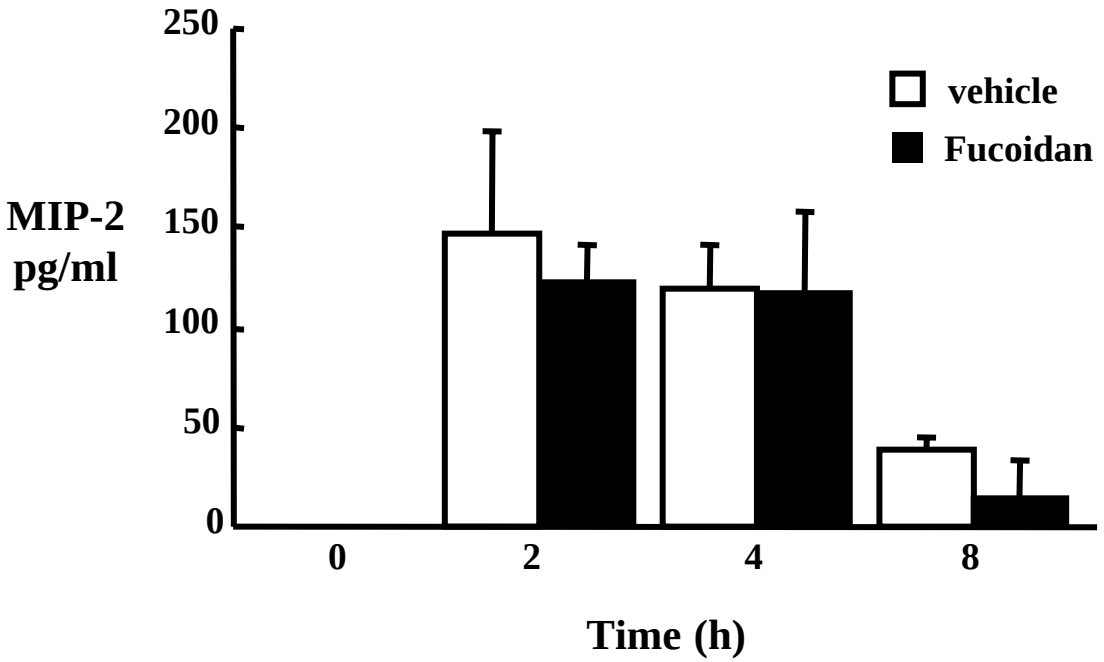

250
200
150
100
50
0
MIP-2
pg/ml
vehicle
Fucoidan
0
2
4
8
Time (h)

Figure 3

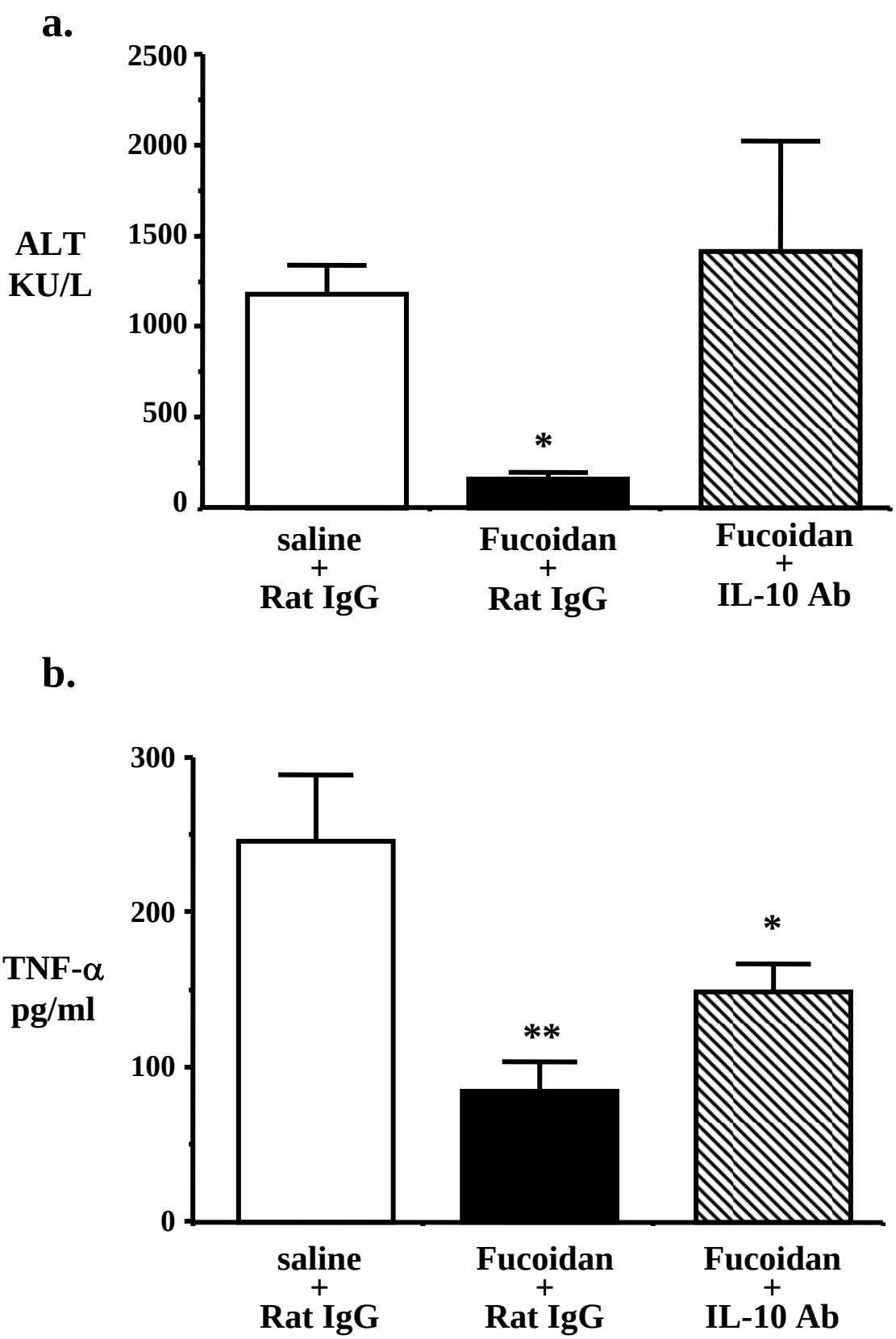

a.
2500
2000
1500
1000
500
0
ALT
KU/L
*
saline
+
Rat IgG
Fucoidan
+
Rat IgG
Fucoidan
+
IL-10 Ab
b.
300
200
100
0
TNF-α
pg/ml
**
*
saline
+
Rat IgG
Fucoidan
+
Rat IgG
Fucoidan
+
IL-10 Ab

Figure 4

c.

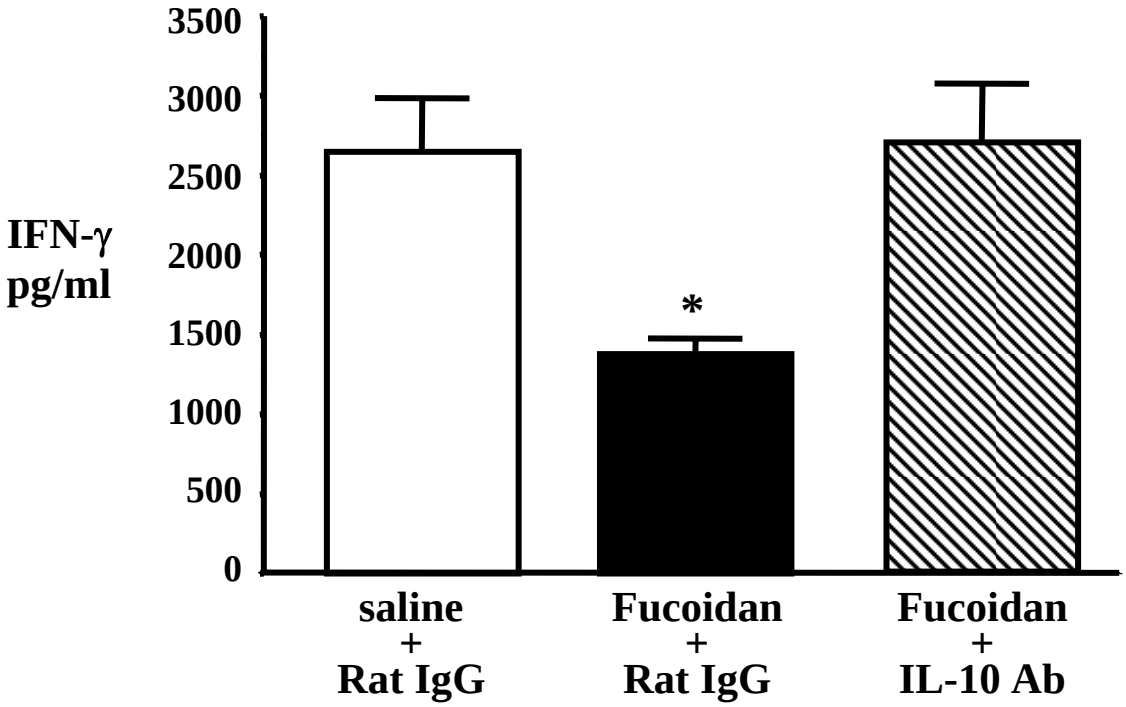

3500
3000
2500
2000
1500
1000
500
0
IFN-γ
pg/ml
*
saline
+
Rat IgG
Fucoidan
+
Rat IgG
Fucoidan
+
IL-10 Ab

Figure 4

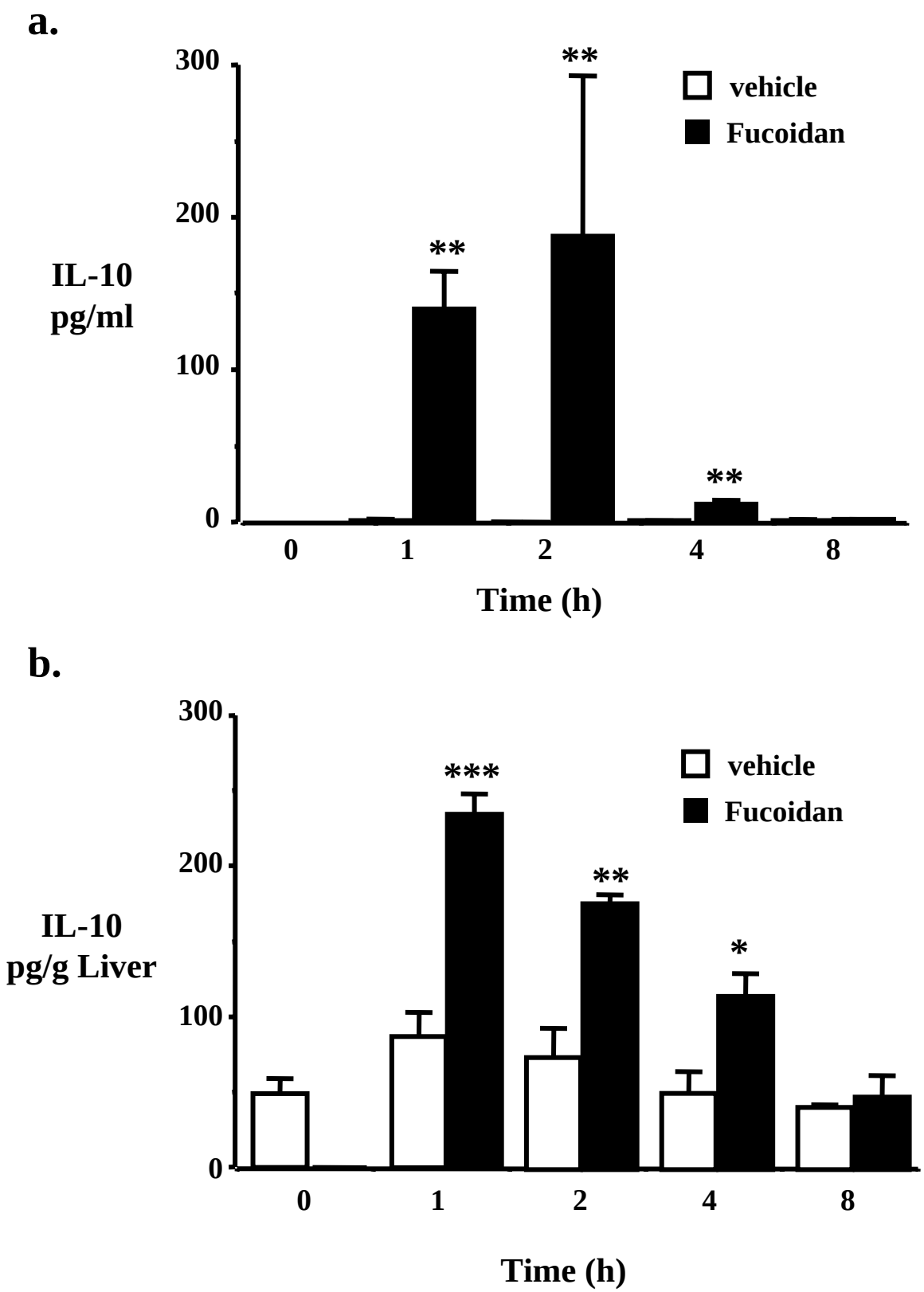

a.
300
200
100
0
IL-10
pg/ml
**
**
**
vehicle
Fucoidan
0
1
2
4
8
Time (h)
b.
300
200
100
0
IL-10
pg/g Liver
***
**
*
vehicle
Fucoidan
0
1
2
4
8
Time (h)

Figure 5

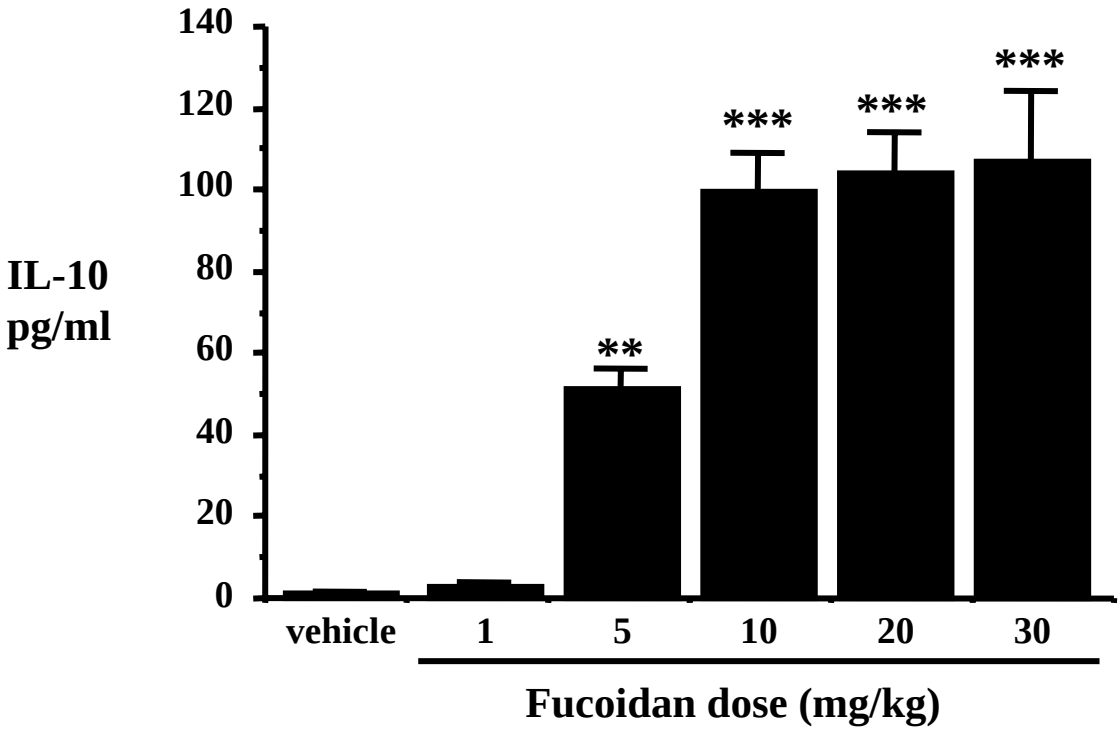

IL-10
pg/ml
140
120
100
80
60
40
20
0
**
***
***
***
vehicle
1
5
10
20
30
Fucoidan dose (mg/kg)

Figure 6

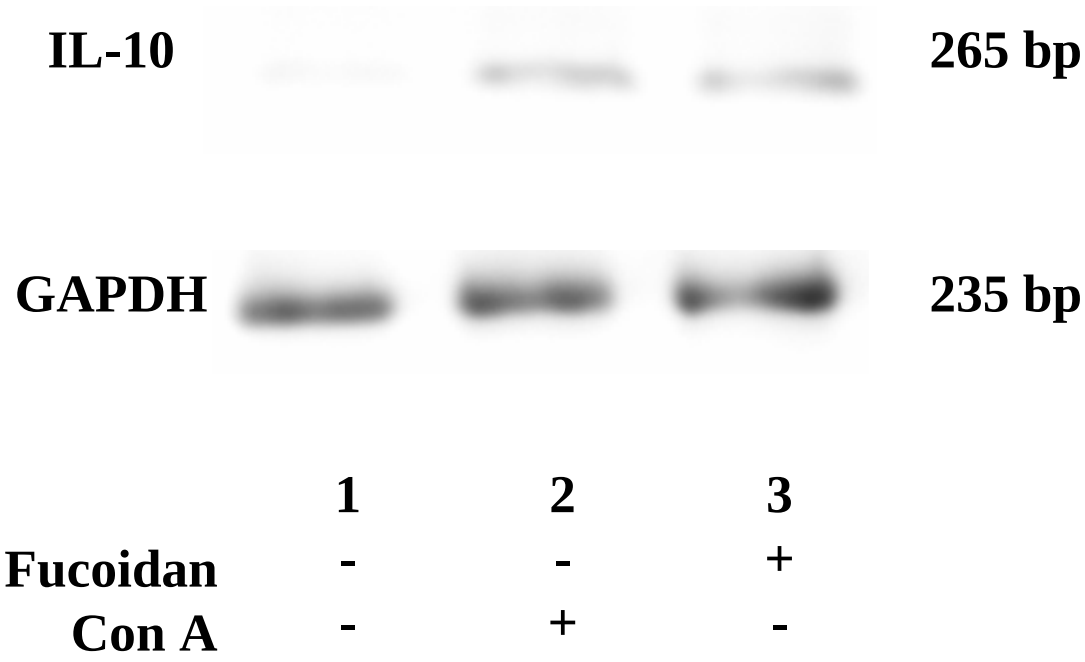


Figure 7

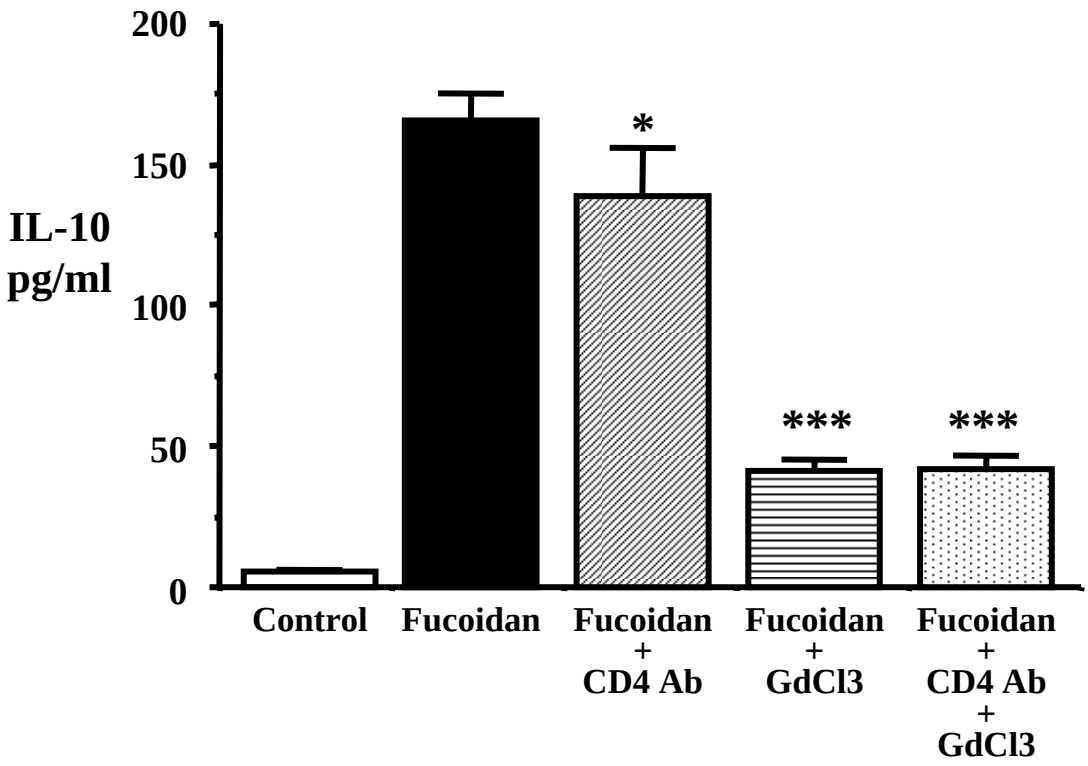

200
150
100
50
0
IL-10
pg/ml
*
***
***
Control
Fucoidan
Fucoidan + CD4 Ab
Fucoidan + GdCl3
Fucoidan + CD4 Ab + GdCl3

Figure 8

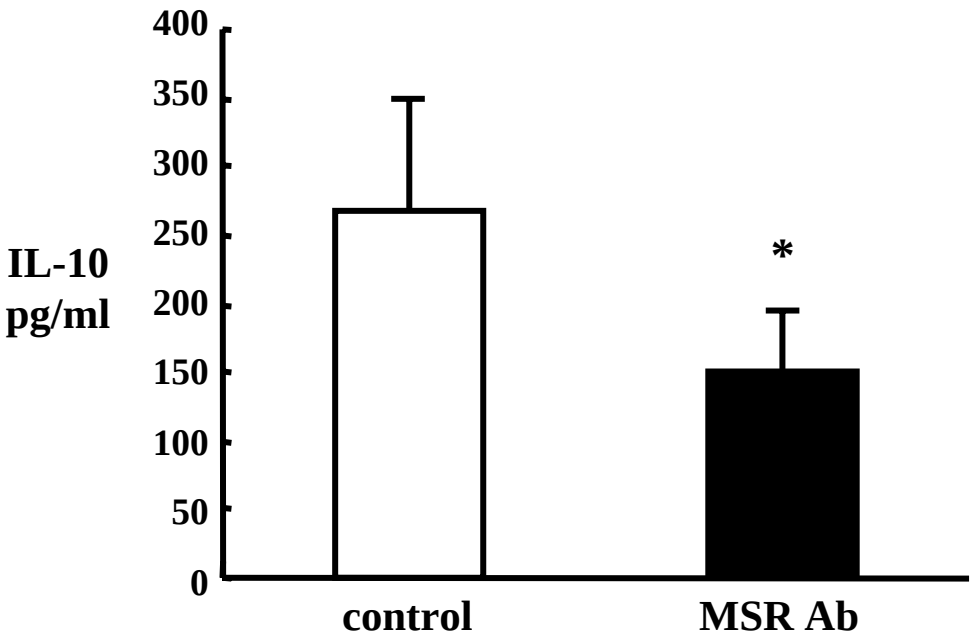


Figure 9