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著者	Nozaki Yusuke, Harada Kenichi, Sanzen Yasun, Nakanuma Yasuni
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PPARγ ligand attenuates portal inflammation in the MRL-lpr mouse –A new strategy to restrain cholangiopathy in primary biliary cirrhosis-

Yusuke Nozaki[1,2], Kenichi Harada[1], Takahiro Sanzen[2], and Yasuni Nakanuma[1]

1. Department of Human Pathology, Kanazawa University Graduate School of Medicine, Kanazawa, Japan
2. Drug Safety Research Laboratory, Toyama Chemical Company Limited, Toyama, Japan

Running head; PPARγ ligand in MRL/lpr mouse

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Address correspondence to:
Kenichi Harada, M.D.
Department of Human Pathology
Kanazawa University Graduate School of Medicine
Kanazawa 920-8640, Japan
FAX : (0)76-234-4229 (Japan)
TEL : (0)76-265-2199 (Japan)
E-mail : kenichih@med.kanazawa-u.ac.jp

ABSTRACT

Primary biliary cirrhosis (PBC) is characterized by chronic destructive cholangitis, which is associated with the reduced expression of an anti-inflammatory molecule, peroxisome proliferator-activated receptor-γ (PPARγ), in intrahepatic bile ducts. We previously demonstrated the anti-inflammatory effects of PPARγ ligands using cultured human biliary epithelial cells. In this study, we evaluated the effectiveness of PPARγ ligand against peribiliary inflammation *in vivo*. As an animal model of PBC, we used MRL/lpr mice in which a PBC-like cholangitis occurs naturally. Anti-inflammatory effects of the intraperitoneal administration of a PPARγ ligand, the prostaglandin D metabolite 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 (15d-PGJ2), were evaluated. In untreated mice, portal inflammation including cholangitis was found to some degree in the majority of portal tracts. In mice given a high-dose group, the degree of portal inflammation was significantly reduced and mice mostly lacking portal inflammation and cholangitis were also found. T cell numbers in portal tracts were markedly decreased in the high-dose group, compared with controls, whereas there was no significant difference in terms of B cells and macrophages. This study is the first to assess the therapeutic potential of a PPARγ ligand against portal inflammation including cholangitis. Anti-inflammatory effects of PPARγ ligands may prevent the progression of cholangiopathy in PBC patients.

INTRODUCTION

Primary biliary cirrhosis (PBC) is characterized by the progressive loss of bile ducts, mainly interlobular bile ducts.[1,2] Infectious and xenobiotics components and abnormal immune responses are implicated in the etiopathogenesis of PBC.[3-8] Our previous study showed that biliary epithelial cells (BECs) of the intrahepatic bile ducts possess an innate immune machinery consisting of bacterial recognition molecules, Toll-like receptors (TLRs), and can respond to lipopolysaccharide (LPS) followed by nuclear factor-κB (NF-κB) and inflammatory cytokines in BECs.[9,10] Under physiological conditions, however, the intrahepatic biliary epithelium lacks any inflammatory reactions *in vivo*, though several pathogen-associated molecular patterns (PAMPs) including bacterial DNA and LPS exist in bile,[9,11] suggesting that BECs possess the capacity to attenuate cytokine gene expression related to the innate immune system. We have reported that peroxisome proliferator-activated receptor γ (PPARγ), a nuclear receptor superfamily of ligand-activated transcription factors involved in lipid metabolism and anti-inflammatory activities, was down-regulated in the damaged bile ducts of PBC patients and speculated that increased susceptibility to biliary innate immunity is associated with the pathogenesis of cholangiopathy in PBC.[12]

In addition to the biliary epithelial cells of PBC, a reduction of PPARγ expression in the colonic epithelial mucosa is reportedly associated with the pathogenesis of inflammatory bowel diseases (IBD).[13-16] Furthermore, the expression of PPARγ is affected by the commensal intestinal flora and ligands (agonists) for PPARγ can attenuate colitis in IBD-model mice, supporting the notion that PPARγ ligands may have salutary effects on IBD.[14,16-18] As for anti-inflammatory activities, the activation of PPARγ by its ligands is shown to inhibit the expression of pro-inflammatory cytokines, the induction of which is mediated via NF-κB and mitogen activated protein kinase (MAPK).[17,19-23] Several PPARγ ligands have been identified, including the prostaglandin D metabolite 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 (15d-PGJ2) and thiazolidinedione derivatives. Our previous study demonstrated that 15d-PGJ2 treatment attenuated LPS-induced NF-κB activation and also TNF-α production via an NF-κB-dependent pathway using cultured intrahepatic cholangiocarcinoma cells and biliary epithelial cells.[12]

In this study, we examined anti-inflammatory effects of PPARγ ligands *in vivo*

using MRL/lpr mice with a naturally occurring PBC-like cholangitis to clarify possible therapeutic targets in biliary inflammation.

MATERIALS AND METHODS

PPARγ ligand

An endogenous ligand, 15d-PGJ2 (diluted by DMSO, Calbiochem, Darmstadt, Germany), was used as a PPARγ ligand.

Animals

MRL/lpr mice bearing the lymphoproliferative gene lpr spontaneously develop severe autoimmune diseases including a systemic lupus erythematosus (SLE) and CNSDC-like cholangitis and produce an anti-mitochondrial antibody.[24-26] This strain was used here as an animal model for autoimmune-mediated cholangitis similar to PBC. A total of 20 18-week-old MRL/lpr mice (male/female=10/10) were purchased from Japan SLC, Inc. (Shizuoka, Japan).

Animal treatments

15d-PGJ2 was administered at low (400μg/kg/day) and high (1,000μg/kg/day) doses (5 mice each) for 3 weeks by intraperitoneal injection. The control group (n=10) received only vehicle (DMSO). After the animals were sacrificed, major organs were obtained and 4μm-thick sections of neutral formalin-fixed paraffin-embedded tissues were prepared for routine histologic observation and immunohistochemistry. The manipulation of these mice was done according to the Guidelines for the Care and Use of Laboratory Animals at the Takaramachi Campus of Kanazawa University (approved number, 050273).

Histological examination

To evaluate the anti-inflammatory effect of PPARγ, major organs were examined histologically. For the liver, ten representative portal tracts containing interlobular bile ducts were chosen and portal inflammation including cholangitis in each was semiquantitatively evaluated as no or mild (score 0), moderate (score 1), or severe

(score 2).

Immunohistochemistry

The deparaffinized and rehydrated sections used for studying CD3, and monocytes/macrophages and CD8 were treated with proteinase K and trypsin, respectively, while those used for CD79α were microwaved in 10mM citrate buffer for 20min in a microwave oven. Following the blocking of endogenous peroxidase, these sections were incubated at 4ºC overnight with antibodies against CD3 (rat IgG, 10μg/ml, Millipore Headquarters, Billerica, MA), CD8 (rat IgG1, 2.5μg/ml, Chemicon, Tokyo), CD79α (rabbit IgG, 1.0μg/ml, Abcam Japan, Tokyo), and macrophage/monocyte (rat IgG, 2.5μg/ml, BMA Biomedicals, Augst, Switzerland) and then at room temperature for 1h with a Simple Staining Kit (Nichirei, Tokyo). After a benzidine reaction, sections were lightly counterstained with hematoxylin. As a negative control, isotype-matched immunoglobulin was used as the primary antibody.

Statistical analysis

Data were analyzed using Welch's t-test; $p<0.05$ was considered statistically significant.

RESULTS

Histology of MRL/lpr mice

Variation in the incidence and degree of systemic lymphadenopathy, sialoadenitis, nephritis, pneumonia, arthritis, and hepatitis including cholangitis was found in MRL/lpr mice. In particular, sialoadenitis always occurred and the inflammation was extensive and destructive in most mice. In the liver, portal inflammation involving mononuclear cells was detected and portal tracts were cellularly enlarged. Moreover, chronic cholangitis resembling CNSDC was also found to varying degrees (Fig.1).

Effect of 15d-PGJ2

In the group injected intraperitoneally with a low-dose of 15d-PGJ2, portal inflammation including cholangitis in interlobular bile ducts remained, the incidence and degree being similar to those in the controls (vehicle) (Fig.2CD). However, in the

high-dose group, the portal inflammation and cholangitis were attenuated and their incidence and degree were significantly reduced (Fig.2A). Almost no portal inflammation or cholangitis was found in one of the five mice in the high-dose group (Fig.2B). Semiquantitative evaluation demonstrated that inflammation was improved in the high-dose group (Fig.3), but not low-dose group. For sialoadenitis and pancreatitis, although some inflammatory damage remained, it was significantly less severe in the low-dose as well as high-dose group. For glomerulonephritis, there was no significant anti-inflammatory effect in 15d-PGJ2-injected mice.

Analysis of inflammatory cells in portal tracts

To analyze the population of inflammatory cells in portal tracts of MRL/lpr mice and changes in the high-dose group, T cells, B cells, and macrophages were examined by immunohistochemistry. Few CD79α-positive B cells were found irrespective of 15d-PGJ2-treatment, but many scattered CD3-positive T cells and monocytes/macrophages were detected (Figs.4 and 5). In particular, the number of CD3-positive T cells was clearly decreased in 15d-PGJ2-administered mice and this reduction was significant in portal tracts without inflammation (Fig.4). However, a few CD8-positive T cells were found irrespective of 15d-PGJ2-treatment, indicating the decreased of CD3-positive T cells is caused by that of CD4-positive T cells. A summary of the population of inflammatory cells in portal tracts is given in Table 1.

DISCUSSION

We reported previously that PPARγ showing anti-inflammatory effects was constantly expressed in human biliary epithelial cells of intrahepatic small bile ducts including interlobular bile ducts and cultured human biliary epithelial cells.[27] Moreover, the expression of PPARγ has been reported to be down-regulated by a Th1-dominant cytokine milieu and reduced in the damaged bile ducts of PBC patients.[27] Based on these findings, we speculated that a Th1-dominant periductal cytokine milieu caused by CD4-positive T cells, following the reduction of PPARγ expression and increased susceptibility to several proinflammatory factors including innate immune responses is closely associated with the pathogenesis of cholangiopathy in PBC. Studies *in vitro*

have shown that functional PPARγ ligands suppress inflammatory responses by limiting the production of cytokines and chemokines secreted from macrophages and epithelial cells.[16,28-30] PPARγ ligands are divided into endogenous types and thiazolidinedione derivatives. As one of the former, the prostaglandin D metabolite 15d-PGJ2 is well known and reported to attenuate the activation of NF-κB, a master regulator of inflammation, by preventing the phosphorylation of its inhibitor protein (I-κB).[31] Our recent study demonstrated that 15d-PGJ2 treatment attenuated LPS-induced NF-κB activation and also TNF-α production via an NF-κB-dependent pathway in cultured intrahepatic cholangiocarcinoma cells and biliary epithelial cells.[9,27]

In this study, to verify whether PPARγ ligands show anti-inflammatory effects against portal inflammation including cholangitis *in vivo*, we used autoimmunity-prone mice, MRL/lpr. These mice have lymphoproliferative lesions caused by a deficiency of Fas (CD95) and spontaneously develop various forms of autoimmune disease in the same individuals, including glomerulonephritis, polyarteritis, arthritis and sialoadenitis associated with excessive production of autoantibodies.[25] Therefore, MRL/lpr mice have been used as a model for the study of SLE, but also reported as a potentially suitable animal model of PBC.[24,26] Although several clinical features including serum levels of total bilirubin and hepatobiliary enzymes including alanine aminotransferase (ALT), leucine aminopeptidase (LAP), and gamma-glutamyl transpeptidase (G-GTP) are incompatible with PBC, the serological and histopathological features including anti-mitochondrial antibody (AMA), cholangitis, and bile duct loss, indicate that MRL/lpr mice can be used as an experimental immune-mediated cholangitis model for PBC.[24,26] This study demonstrated that the administration of 15d-PGJ2 (high-dose) could attenuate the degree of portal inflammation in MRL/lpr mice. Because the incidence of cholangitis was originally low and individual differences were significant, the anti-inflammatory effect was considered relatively mild as shown in Fig.3. However, the cholangitis and portal inflammation were completely absent in the mice given a high-dose of 15d-PGJ2, suggesting an anti-inflammatory effect of PPARγ on portal inflammation. Moreover, an evaluation of the proportion of inflammatory cells in portal tracts revealed as significant reduction in T cells in PPARγ-administered mice. Moreover, although we could not directly confirmed the decreased of CD4-positive T cells, because of no antibodies against mouse CD4 which are commercially available

and usable for formalin-fixed, paraffin-embedded section, the decreased of CD3-positive T cells was speculated to be caused by that of CD4-positive T cells from results of immunohistochemistry of CD3 and CD4. T cells, particularly autoreactive CD4-positive T cells, play an important role in the pathogenesis of cholangiopathy in cases of PBC.[32] Therefore, because the PPARγ ligand had anti-inflammatory effects caused by the attenuation of CD4-positive T lymphocytes, it is likely to show as anti-inflammatory effect on cholangiopathy in PBC patients as well as MRL/lpr mice.

In addition to cholangitis, sialoadenitis and pancreatitis in MRL/lpr mice were also improved by the low-dose as well as high-dose of 15d-PGJ2, suggesting that the sialoadenitis and pancreatitis are closely associated with PPARγ-dependent mechanism and show more susceptibility to PPARγ ligand, compared with cholangitis. However, as for the severity of glomerulonephritis, there was no significant anti-inflammatory effect in 15d-PGJ2-injected mice. In SLE, the deposition in kidney tissue of immune complexes and their interaction with macrophages is thought to trigger the inflammatory response leading to glomerulonephritis. Moreover, macrophage-dependent destruction in MRL/lpr mice is demonstrated in the pathogenesis of glomerulonephritis.[33] As shown in Table 1 concerning the immunohistochemistry, the inflammation of monocyte/macrophage was less attenuated by the 15d-PGJ2 treatment, compared with that of T cells, speculating that 15d-PGJ2 offers little benefit to the macrophage-dependent destruction such as glomerulonephritis in MRL/lpr mice.

Other PPARγ ligands include rosiglitazone, pioglitazone, troglitazone, and ciglitazone. Pioglitazone has been recently administered for diabetic mellitus and non-alcoholic steatohepatitis. Troglitazone had been used to treat diabetic mellitus and in one notable case, improved liver dysfunction in a patient with AMA-negative PBC.[34] It is no longer used because of its severe hepatotoxicity, but several effects of Troglitazone on inflammatory bowel diseases, carcinogenesis in the colon, diabetic mellitus, chronic pancreatitis, and sepsis in animal models have been reported [18,35-37]. In this study, in addition to the anti-inflammatory activity of PPARγ in cultured human biliary epithelial cells, we demonstrated effected *in vivo* using MRL-lpr mice. This study is the first to assess the therapeutic role of a PPARγ ligand in inflammatory biliary diseases, particularly PBC. PPARγ ligands are potentially a new tool to restrain the

progression of cholangiopathy in PBC patients.

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Table 1

Summary of the population of inflammatory cells in portal tracts

MRL/lpr mice	T cells		B cells	Monocyte/
	CD3	CD8	CD79a	Macrophage
Controls (DMSO)	++	±	±	++
15d-PGJ2-treated (high-dose)	± ～ +	±	±	+ ～ ++

± : none or a few, ＋ : some, ＋＋ : many

FIGURES and FIGURE LEGENDS

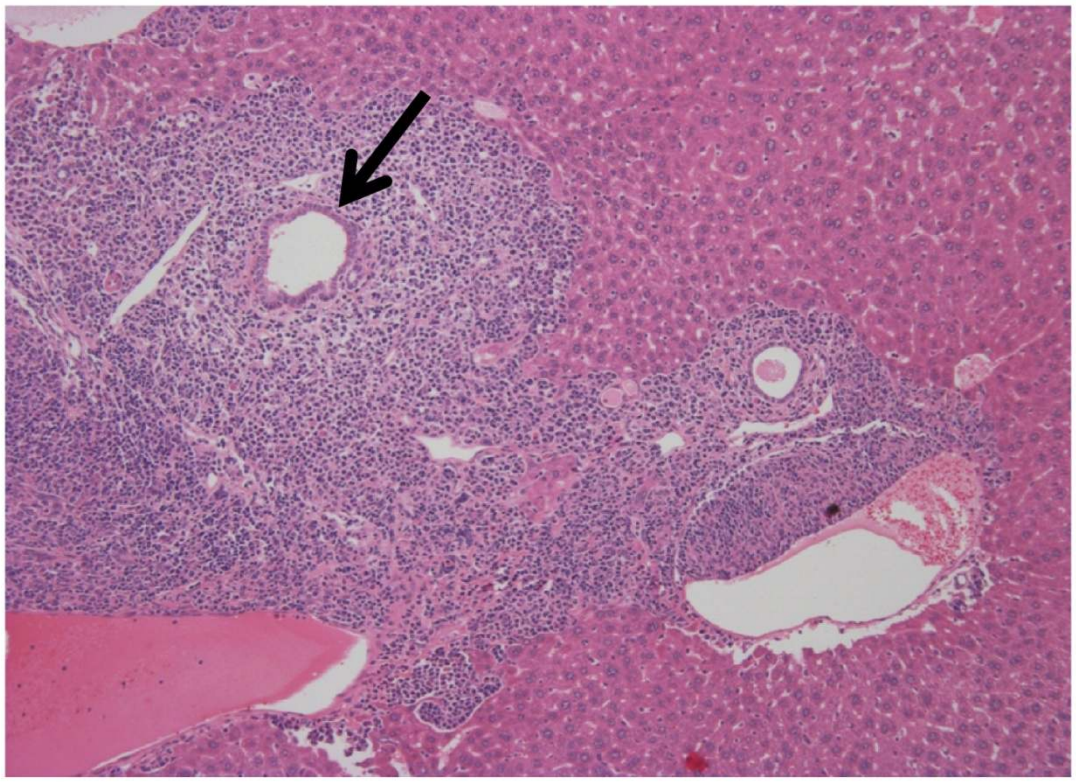
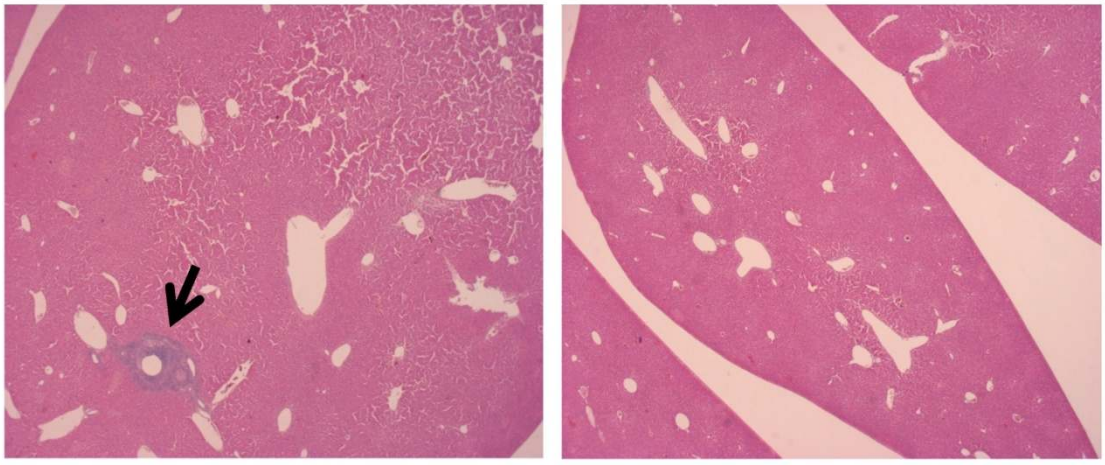


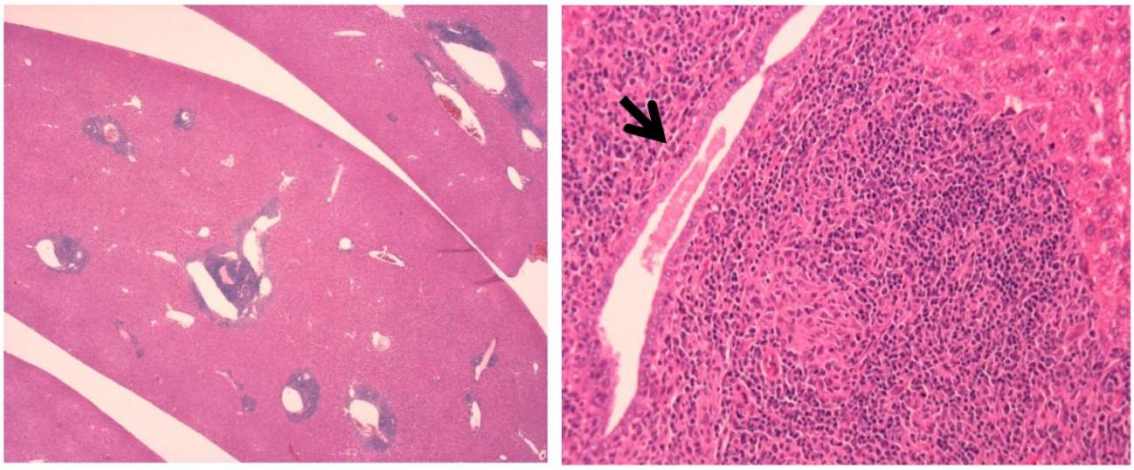
Fig.1

Fig.1 The MRL/lpr mouse at 21 weeks. Marked portal inflammation involving lymphoid cells and non-suppurative destructive cholangitis-like lesions with irregular biliary epithelial polarity (arrow) are found.



A B

Fig.2AB



C D

Fig.2CD

Fig.2 MRL/lpr mice 21 weeks after the administration of high-dose 15d-PGJ2 (1,000μg/kg/day) for 3 weeks (A and B) and controls (DMSO, C and D). A and B show the high-dose group in which portal inflammation remains (arrow) and has completely disappeared, respectively. C and D are controls. Most portal tracts exhibit significant inflammation (C) and mild bile duct damage (arrow, D) with infiltration by lymphocytes and macrophages.

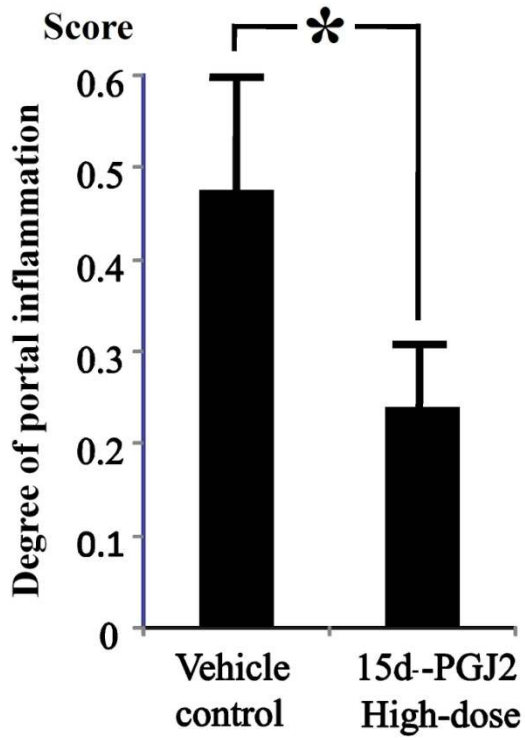


Fig.3

Fig.3 Semiquantitative evaluation of portal inflammation. Ten representative portal tracts were chosen in each mouse and the inflammation in each was evaluated (score 0, 1, or 2). The average score of MRL-lpr mice treated with high-dose 15d-PGJ2 (0.48±0.11 [mean±S.E.M]) is significantly reduced, compared with the control (DMSO, 0.24±0.07). *, $p<0.05$.

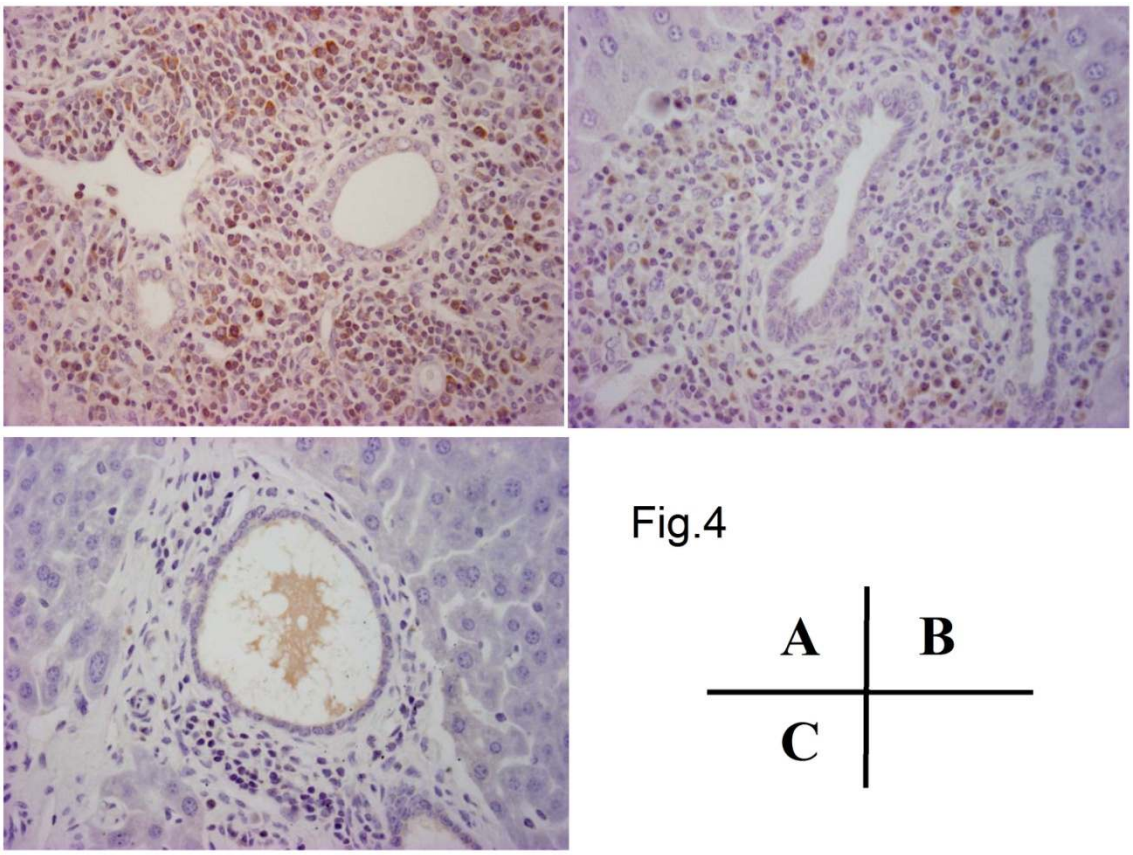


Fig.4 Immunohistochemistry of CD3 in MRL-lpr mice treated with vehicle (DMSO, A) and high-dose 15d-PGJ2 (B and C). In the control groups, CD3-positive T cells are found in enlarged portal tracts (A). In the high-dose 15d-PGJ2 group, marked inflammation remains in portal tracts, but CD3-positive T cell numbers are significantly decreased (B), compared with controls (A). The portal tracts of the high-dose 15d-PGJ2 group contain few CD3-positive T cells (C).

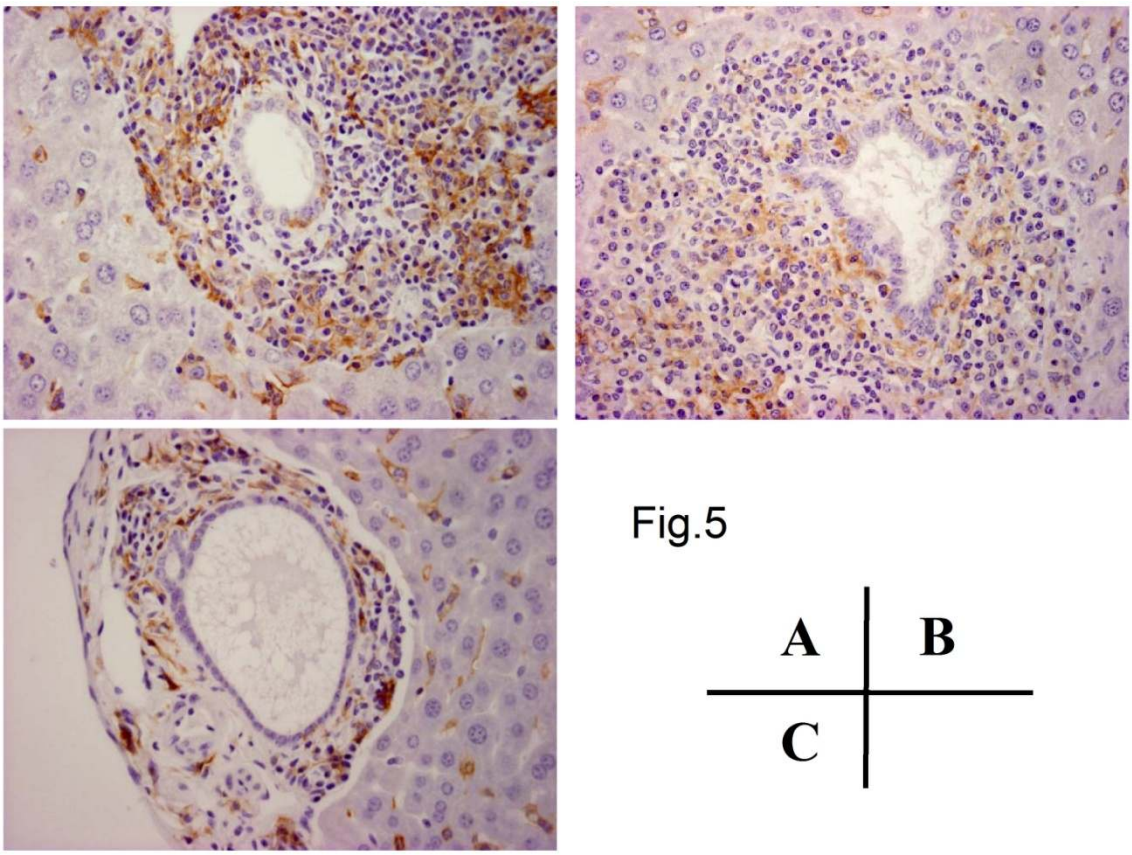


Fig.5 Immunohistochemistry of monocytes/macrophages in MRL-lpr mice treated with vehicle control (DMSO, A) and high-dose 15d-PGJ2 (B and C). Many monocytes/macrophages are found in inflammed portal tracts in both the control and high-dose 15d-PGJ2 groups (A and B, respectively). The portal tracts of the high-dose 15d-PGJ2 group still contain several monocytes/macrophages (C).

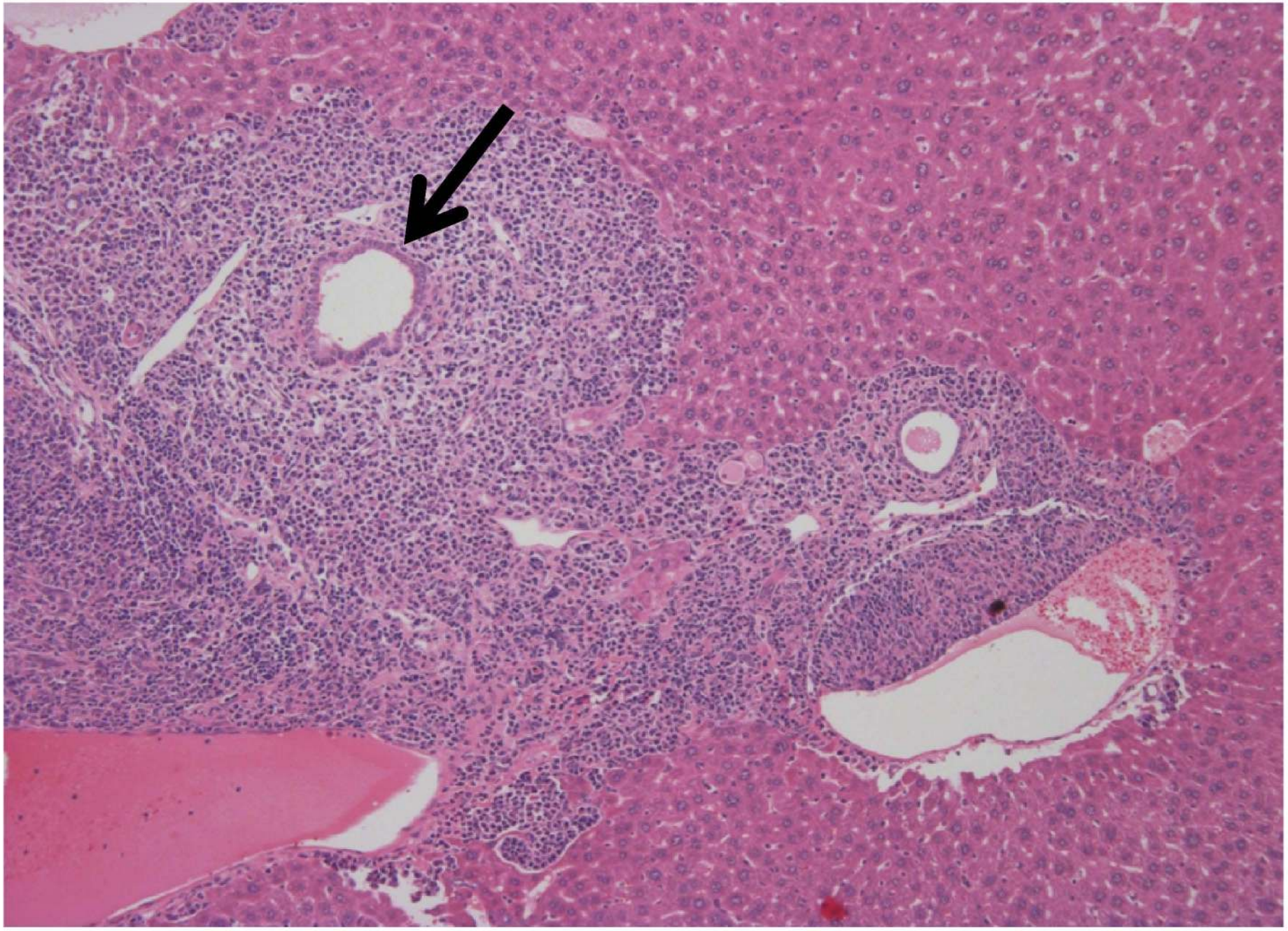
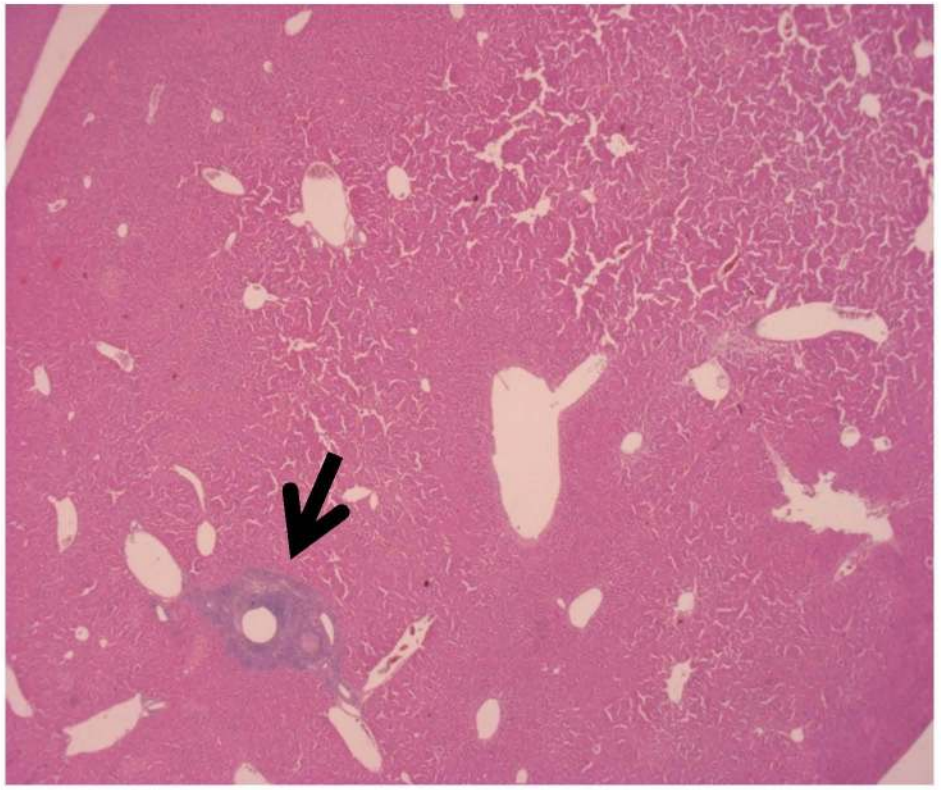
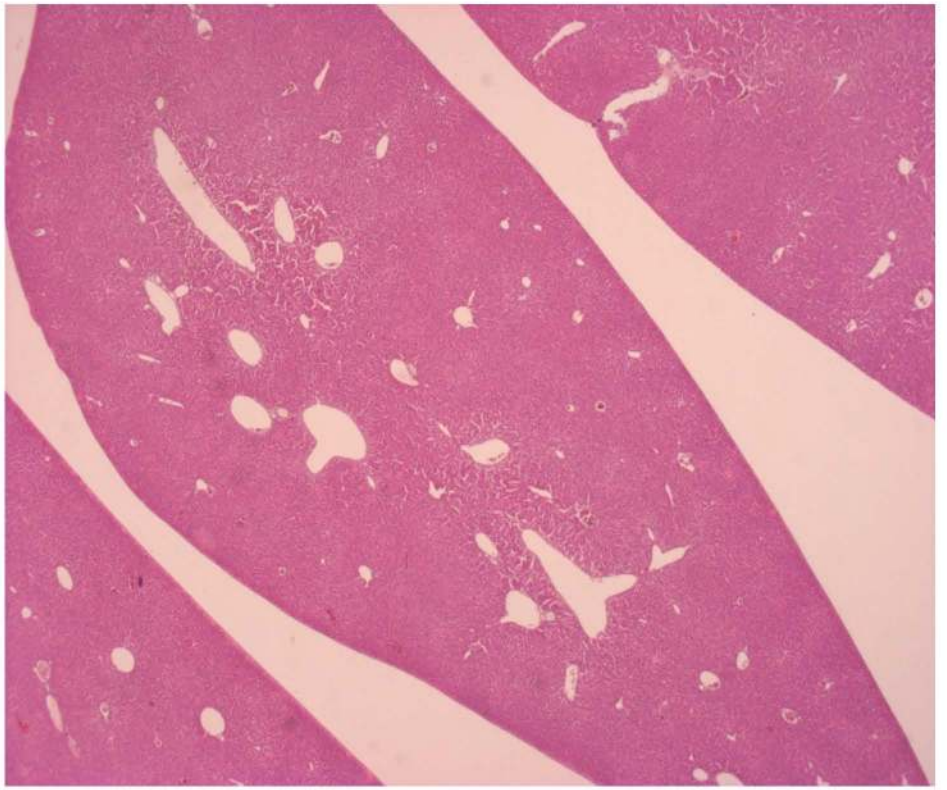


Fig.1

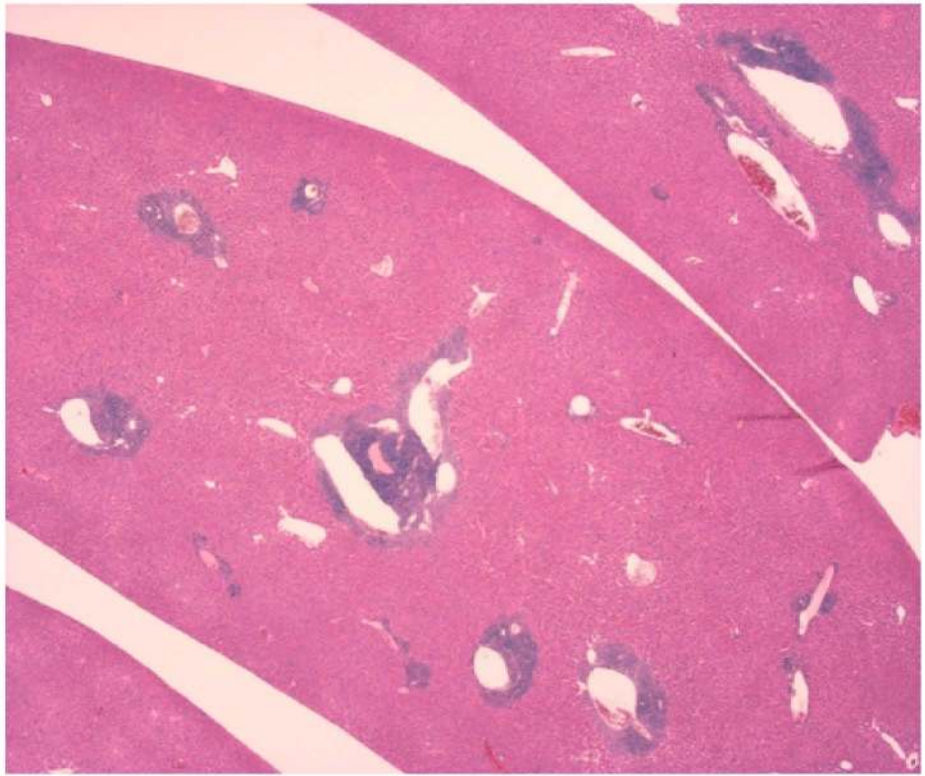


A

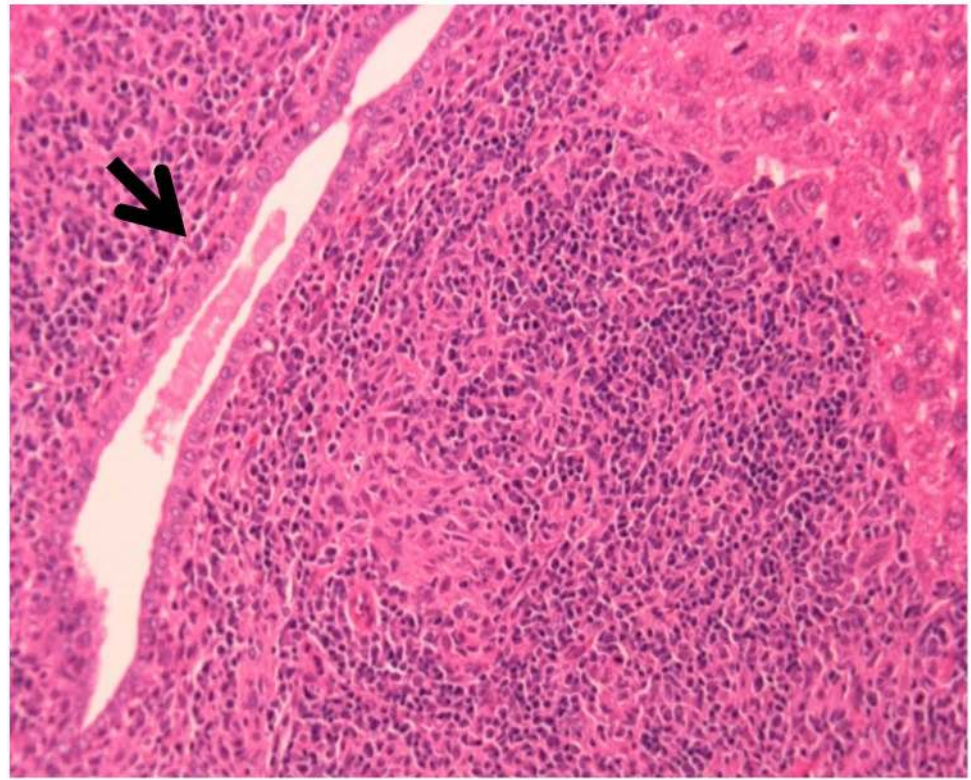


B

Fig.2AB



C



D

Fig.2CD

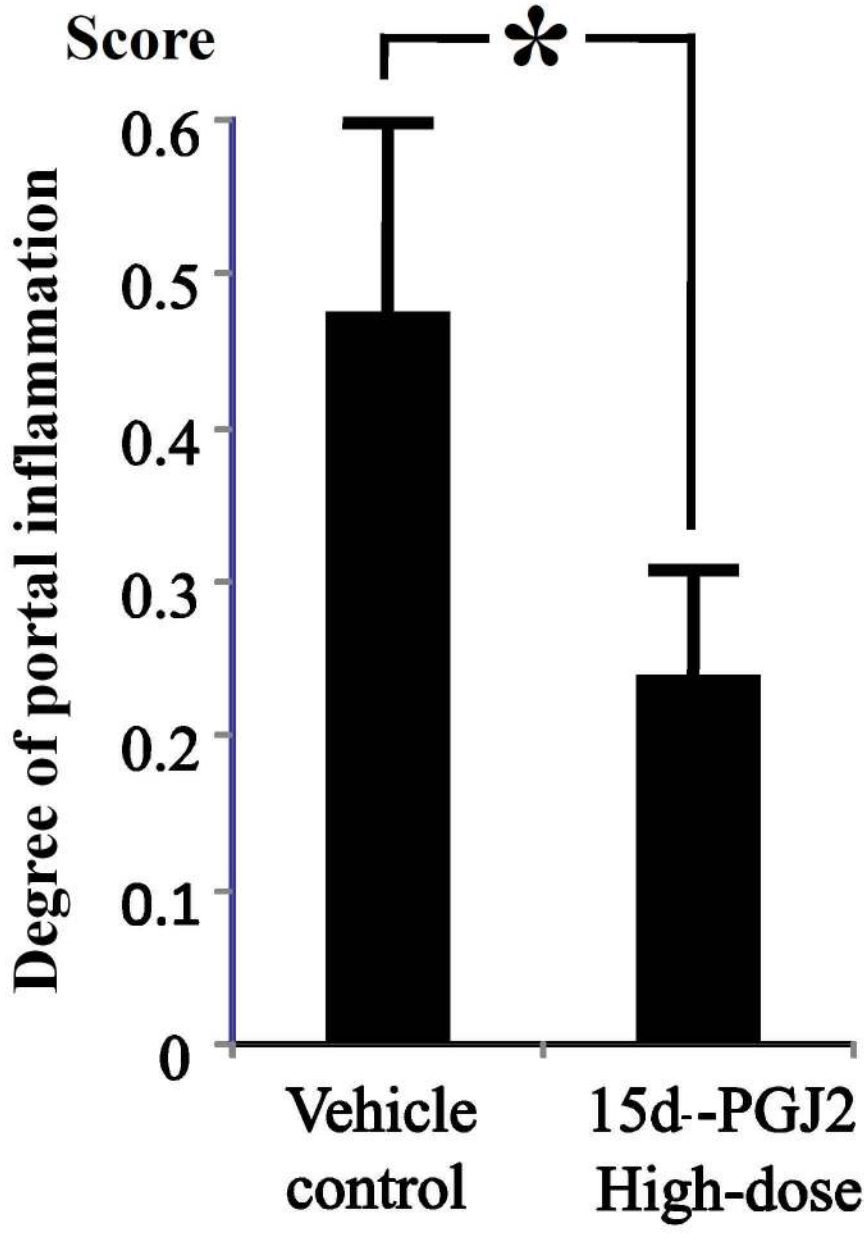
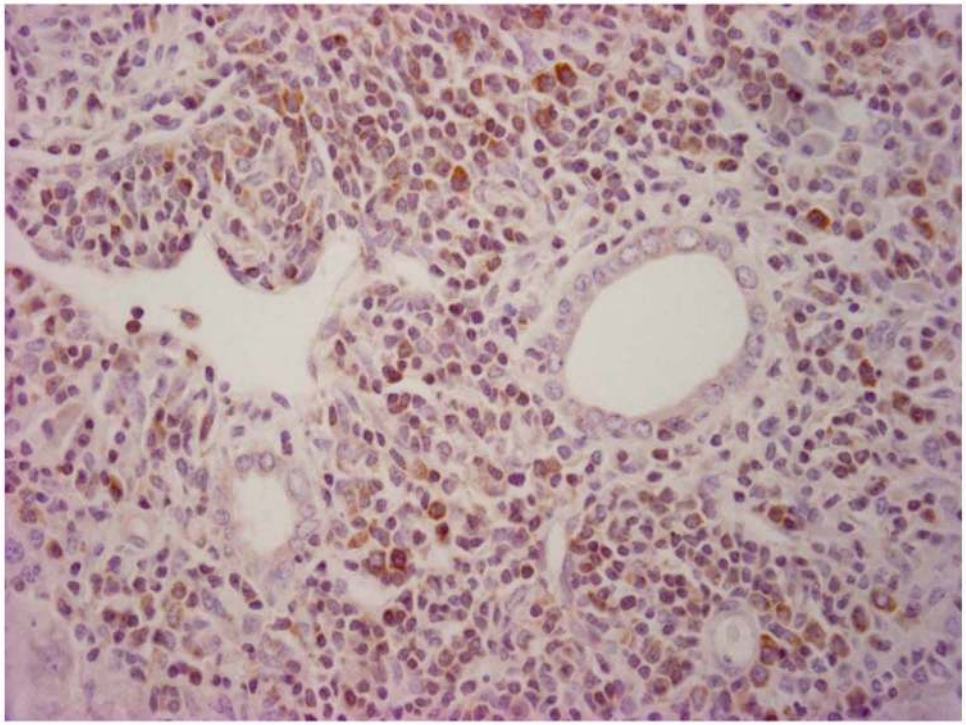
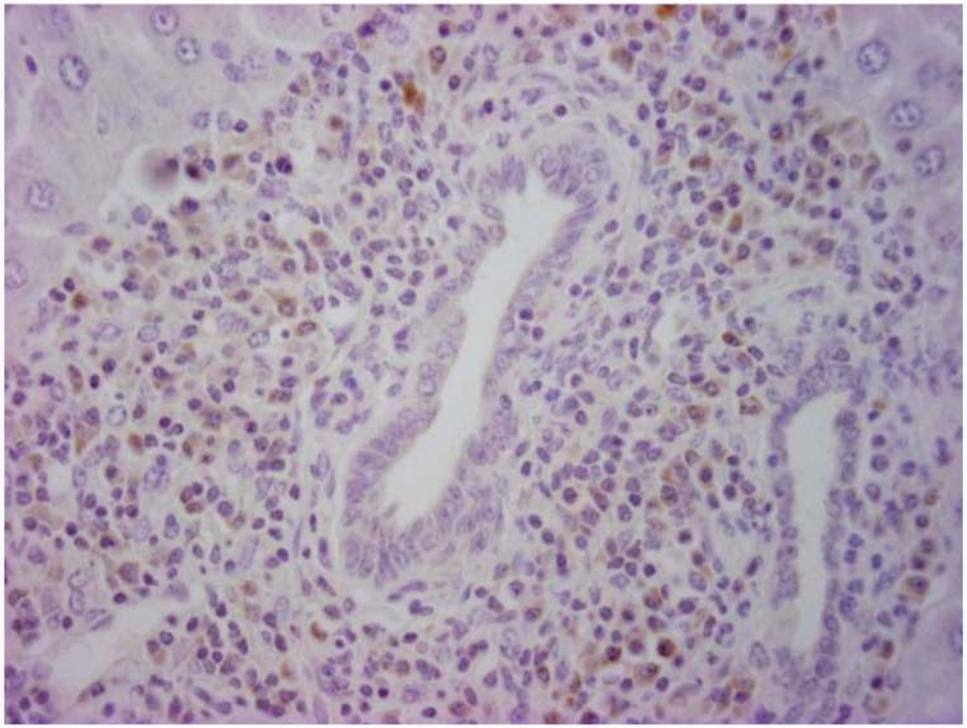

Score
*
0.6
0.5
0.4
0.3
0.2
0.1
0
Degree of portal inflammation
Vehicle control
15d--PGJ2 High-dose

Fig.3





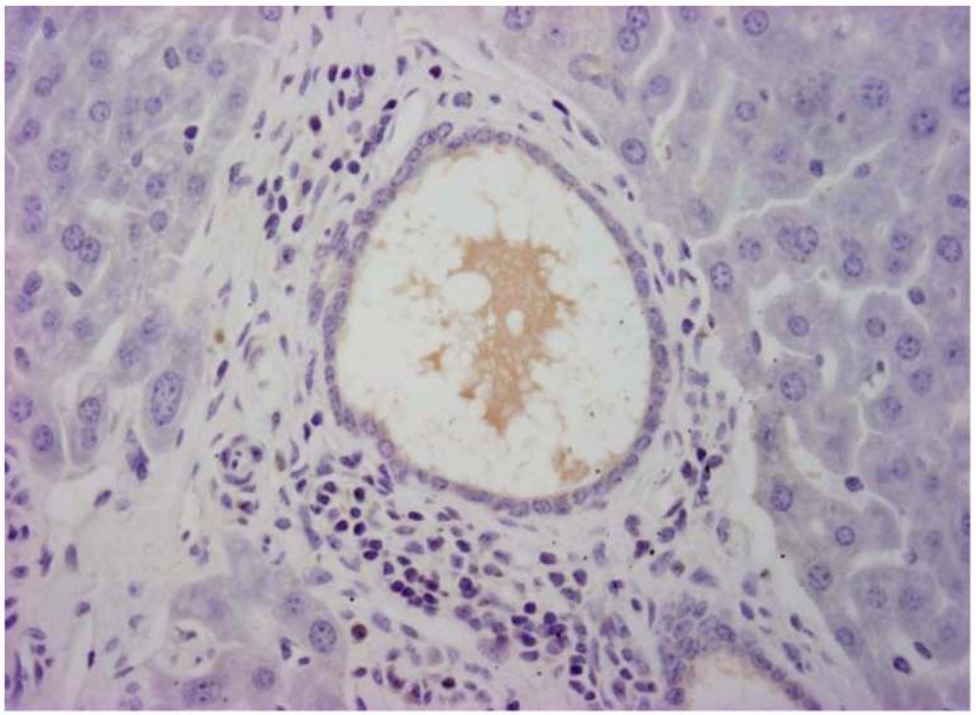
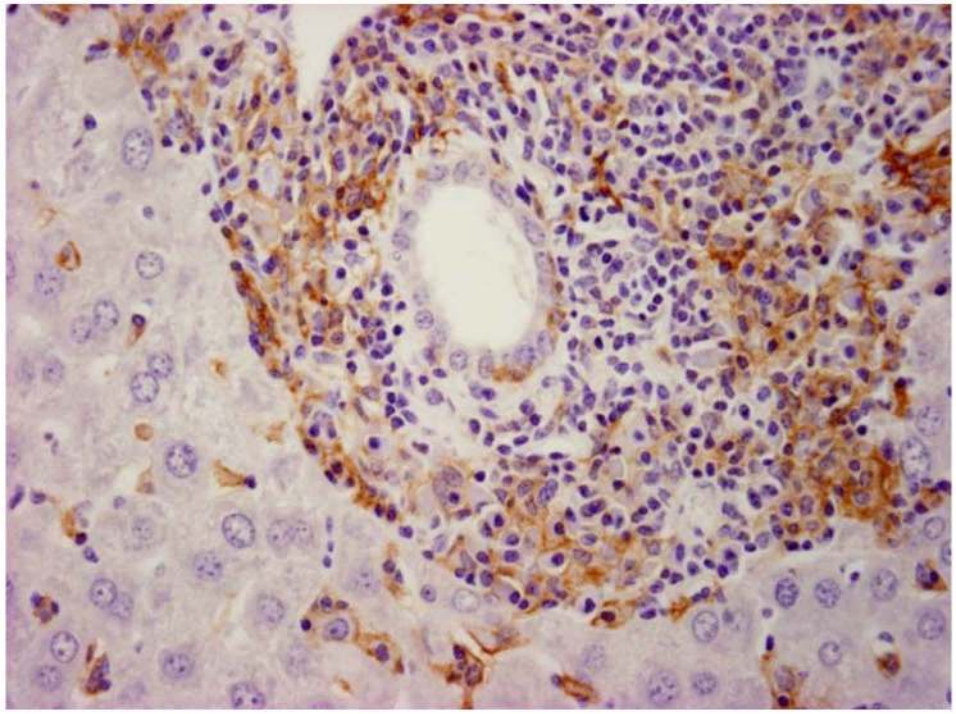
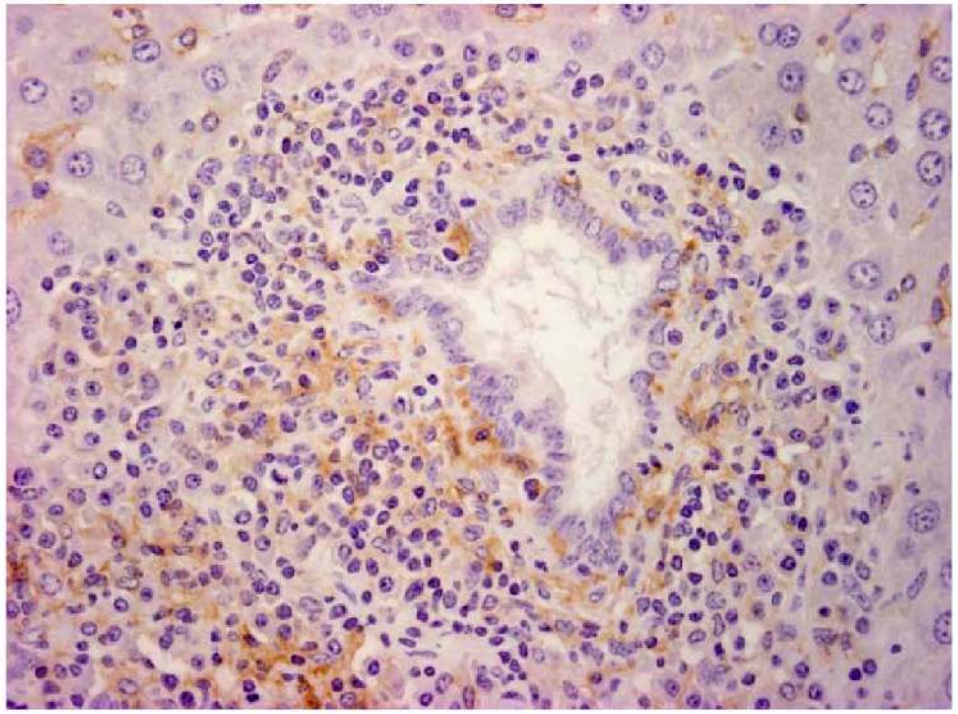


Fig.4

A B

C





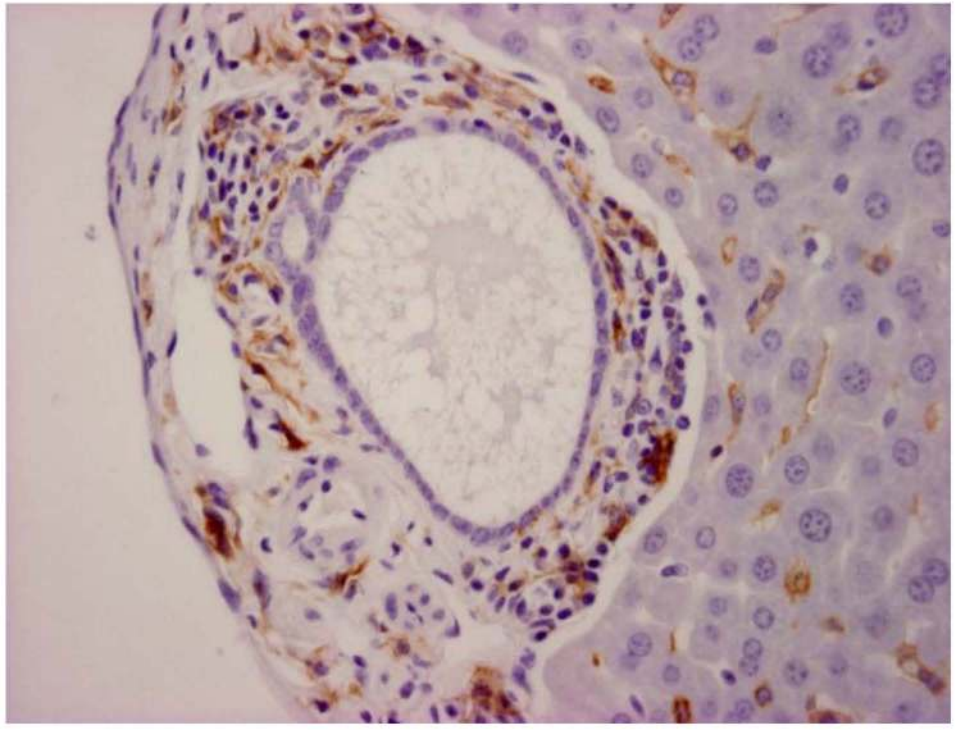


Fig.5

A B

C