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**Roles of platelets and macrophages in the protective effects of lipopolysaccharide
against concanavalin A-induced murine hepatitis**

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ABSTRACT

Platelets are reportedly causal in hepatitis. We previously showed that in mice, lipopolysaccharide (LPS) induces a reversible and macrophage-dependent hepatic platelet accumulation (HPA), including translocation of platelets into Disse spaces and their entry into hepatocytes. Concanavalin A (ConA), which induces hepatitis in mice via both T cells and macrophages, also induces HPA. Here, we examined the relationship between HPA and ConA-hepatitis. ConA-hepatitis and HPA were evaluated by serum transaminases, hepatic 5-hydroxytryptamine, and/or electron microscopy. Unlike LPS-induced HPA, ConA-induced HPA was only moderately dependent on phagocytic macrophages. Against expectations, platelet-depletion significantly exacerbated ConA-hepatitis, and anti-P-selectin antibody and P-selectin receptor blockade reduced both ConA-induced HPA and hepatitis. Prior induction of HPA by pretreatment with low-dose LPS powerfully reduced ConA-hepatitis. Such protection by LPS-pretreatment was not effective in mice depleted of phagocytic macrophages. In platelet-depleted mice, LPS-pretreatment severely exacerbated ConA-hepatitis. In mice depleted of both macrophages and platelets, neither ConA nor LPS-pretreatment + ConA induced hepatitis. In mice deficient in IL-1 α and IL-1 β (but not in TNF α), ConA-induced hepatitis was mild, and a protective effect of LPS was not detected. These results suggest that (i) there are causal and protective types of HPA, (ii) the causal type involves hepatic aggregation of platelets, which may be induced by platelet stimulants leaked from injured hepatocytes, (iii) the protective type is inducible by administration of prior low-dose LPS in a manner dependent on phagocytic (or F4/80-positive) macrophages, and (iv) IL-1 is involved in both the causal and protective types. (within 250 words, now 244)

Highlights

There are causal and protective types of hepatic platelet accumulation (HPA).

Lipopolysaccharide (LPS) induces a protective HPA depending on macrophages.

HPA by pretreatment with LPS reduces hepatitis induced by concanavalin A (ConA).

In platelet-depleted mice, LPS-pretreatment severely exacerbates ConA-hepatitis.

IL-1 is involved in both the causal and protective types of HPA.

Keywords macrophage; platelet; concanavalin A; lipopolysaccharide; liver; Kupffer cells

ACCEPTED MANUSCRIPT

1. Introduction

Increasing evidence suggests that in addition to their roles in various inflammatory diseases, platelets are important in defending the host against invasion by foreign organisms [1-4]. We previously found that at low doses, lipopolysaccharide (LPS) induces hepatic platelet accumulation (HPA) in mice, an effect that can be assessed by measuring the 5-hydroxytryptamine (5HT) level [1, 5-7]. In such LPS-induced HPA, translocation of platelets into Disse spaces and their entry into hepatocyte are frequently observed [8]. The LPS-induced HPA can be largely prevented by prior depletion of phagocytic, F4/80-positive macrophages (or Kupffer cells) [8]. We have also reported that concanavalin A (ConA), too, induces a marked increase in hepatic 5HT [1, 5, 6], although it has not been established whether this 5HT accumulation is indeed a reflection of HPA.

There are several murine hepatitis models [induced by, for example, injection of LPS after *Propionibacterium acnes* treatment, co-injection of LPS plus D-galactosamine (GalN), or injection of ConA alone]. It is generally recognized that ConA-induced hepatitis is T cell-mediated, because nude mice (mostly deficient in mature T-cells) are resistant to ConA-hepatitis [9]. On the other hand, both the hepatitis induced by *P. acnes* + LPS and that induced by GalN + LPS are mediated by macrophages (via production of inflammatory cytokines) [10-13]. However, it has been repeatedly reported that macrophage-depletion markedly reduces ConA-hepatitis [14-16], suggesting that in addition to T-cells, macrophages too are causally involved in ConA-hepatitis.

Platelet-depletion reportedly affords significant protection against the mortality induced by GalN + LPS [17], and indeed activation of platelets occurs in the hepatitis models induced by either GalN + TNF or ConA [18, 19]. Actually, GalN + LPS induces a marked HPA that precedes the development of severe congestion [20]. These results are all indicative of platelets playing causal roles in those hepatitis models, irrespective of whether the hepatitis

is T cell-mediated or macrophage-mediated. However, we recently observed that platelets are protective in Fas-mediated hepatitis, when the hepatitis is local or not severe [21]. A very interesting point to note is that although LPS induces HPA, as described above, LPS-pretreatment has been shown to protect mice against the hepatitis induced by either ConA or GalN + LPS [21-25]. As yet, the precise mechanisms underlying this protection remain unclear.

The above background led us to speculate that platelets or HPA might be involved in the development of hepatitis, but that a pre-accumulation of platelets in the liver (i.e., HPA) resulting from low-dose LPS-pretreatment might modulate this process. Here, in mice, we examined the relation between platelets and ConA-induced hepatitis and between platelets and the protective effects of LPS-pretreatment.

2. Materials and methods

2.1. Animals

Male BALB/c mice (6-7 weeks old) were used in experiments. BALB/c mice deficient in IL-1 α , IL-1 β , and/or TNF α were established as reported previously [26, 27] and raised in our laboratory. All experiments conformed to national requirements (Japanese law no. 105, notification no. 6) and complied with the *Guidelines for Care and Use of Laboratory Animals in Tohoku University*.

2.2. Materials

ConA, clodronate, low-molecular-weight heparin (LMWH), and LPS from *Escherichia coli* O55:B5 were obtained from Sigma (St. Louis, MO, USA). ConA and LPS were dissolved in sterile saline. Hybridoma cells producing a rat monoclonal anti-mouse platelet

antibody, Pm-1, were provided by Dr. Todokoro (RIKEN, Tsukuba, Japan) [28]. Pm-1 antibody (IgG fraction) was prepared by precipitation with ammonium sulfate and dialysis of the precipitant. MWReg30 [a rat monoclonal anti-mouse integrin α IIb (CD41) antibody] and anti-P-selectin antibody were obtained from BD Pharmingen (San Jose, CA, USA). Normal rat IgG was used as a control for these antibodies. Recombinant human IL-1 α and human IL-1 β were provided by Dainippon Pharmaceutical Co. (Osaka, Japan) and Ohtsuka Pharmaceutical Co. (Tokushima, Japan), respectively. Unless otherwise mentioned, reagents were injected intravenously (i.v.) via a caudal vein.

2.3. Platelet count and estimation of HPA

Platelets were counted as described previously [21], and HPA was assessed by a method involving the use of 5HT as a marker for platelets [21, 29, 30].

2.4. Depletion and detection of platelets and phagocytic macrophages

Clodronate-encapsulated liposomes (Clo-lip) specifically deplete phagocytic macrophages [31, 32]. The “original suspension” of Clo-lip was prepared as described previously [33]. Five-fold-diluted (with saline) original Clo-lip suspension was intravenously injected (0.2 ml/mouse) to eliminate F4/80-positive cells (macrophages) from the liver [34]. Macrophages were detected by immunohistochemical staining using an F4/80 antibody (Serotec, Kidlington, UK) as described previously [33]. Platelets were depleted either by a subcutaneous (s.c.) injection of Pm-1 (10 mg/kg) or by an i.v. injection of MWReg30 (0.2 mg/kg) [35]. Platelets in the liver were detected by immunohistochemical staining with Pm-1 [36].

2.5. Determination of hepatitis markers

Blood (several drops) was collected directly from the neck into test tubes after decapitation, and aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities in the plasma were measured photometrically using commercial kits (Wako Pure Chemical Ind., Osaka, Japan).

2.6. Electron microscopy

Livers were removed rapidly from decapitated mice. In normal mice and all experimental groups of mice, 50-60 % of the total blood was removed by the decapitation. The liver was chopped into small pieces (about 10 mm³), and the specimens were fixed in a mixture of 2.5 % glutaraldehyde and 2 % paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). After a thorough rinse with PBS, specimens were post-fixed in 2% osmium tetroxide, dehydrated through a graded series ethanol, passed through propylene oxide, and then embedded in EPON 812. Ultra-thin sections were stained with uranyl acetate and lead citrate. In this study, we did not use perfusion fixation because the perfusion would have removed blood, and therefore platelets, from the sinusoidal capillaries in the liver.

2.7. Data analysis

Experimental values are given as mean \pm standard deviation (SD). Unless otherwise mentioned, each value is the mean \pm SD from 5 mice. The statistical significance of differences was analyzed using Dunnett's multiple-comparison test, *P* values less than 0.05 being considered to indicate significance.

3. Results

3.1. ConA-induced increase in liver 5HT reflects HPA

ConA induced an increase in liver 5HT that peaked at around 6 h, preceding the peak in the hepatitis markers AST and ALT (Fig. 1A). ConA induced this increase even at 5 mg/kg (Fig. 1B), a dose that did not increase the two hepatitis markers (data not shown). If the increase in liver 5HT were due to an accumulation of 5HT itself (i.e., not platelets), pargyline (an irreversible inhibitor of the 5HT-metabolizing enzyme, monoamine oxidase) might be expected to augment it [37]. However, pargyline did not have such an effect in mice given ConA (data not shown). Histochemical staining using Pm-1 confirmed that platelet accumulation was localized around sinusoidal vessels in the livers of mice given ConA (Fig. 1C). These results suggest that (i) the hepatic 5HT increase is indeed a reflection of HPA, (ii) ConA induces HPA at low doses without inducing hepatitis, and (iii) the ConA-induced HPA peaks before the peak increases in hepatitis markers.

3.2. Depletion of neither platelets nor phagocytic macrophages affects hepatitis markers

Subcutaneous injection of Pm-1 (10 mg/kg) largely depleted platelets from the blood within 12 h (Fig. 2A). The platelet count and 5HT level in the blood decreased in parallel, supporting the notion that 5HT is a marker for platelets (and thus for HPA). At 24 h after the Pm-1 injection, the levels of platelets and 5HT in the blood were less than 5 % of their initial levels. The transient 5HT increase in the liver (Fig. 2A) may suggest that damaged (or stimulated) platelets are transiently trapped in the liver during the course of their degradation. Despite this profound thrombocytopenia, there was no apparent illness in the mice, and the platelet levels recovered progressively (starting 3 days after the Pm-1 injection) (data not shown). Control IgG (10 mg/kg, s.c.) produced no significant changes in the basal levels of platelets and 5HT (data not shown). In control mice (saline-injected), F4/80-positive cells are seen within sinusoidal spaces (Fig. 2B). Injection of Clo-lip almost completely eliminated F4/80-positive cells from the liver within 24 h of the injection (i.e.,

no F4/80-positive cells were detected) (Fig. 2B). The phagocytic macrophage-depleted mice looked completely healthy, and there was no apparent change in their activities. We used saline-injected mice as a control because liposomes containing saline alone modulate the responses of macrophages [32]. Injection of Pm-1 mostly (95 % or more) depleted the liver of 5HT (Fig. 2C), indicating that the basal level of liver 5HT is also due to the presence of platelets. The absence of platelets in livers 24 after the injection of Pm-1 was also confirmed by electron-microscopic observations (see Section 3.13). Neither Pm-1 nor Clo-lip by themselves affected AST or ALT, and Clo-lip had no significant effect on the basal 5HT level in the liver (Fig. 2C).

3.3. ConA-induced HPA is partially phagocytic macrophage-dependent

In the following experiments, an increase in liver 5HT is termed “HPA” for convenience. In Clo-lip-treated mice, the ConA-induced HPA was significantly reduced, but not abolished (Fig. 3A). As described in Introduction, LPS also induced HPA (Fig. 3B), and as previously reported [8] the LPS-induced HPA was largely prevented by Clo-lip-pretreatment (Fig. 3B). These results suggest that although both LPS and ConA induce HPA, the LPS-induced HPA is *largely* phagocytic macrophage-dependent, while the ConA-induced HPA is *moderately* phagocytic macrophage-dependent (i.e., the latter is less dependent on phagocytic macrophages than the former).

3.4. LPS-pretreatment reduces ConA-induced hepatitis and HPA

It should be noted that LPS by itself (at 0.1 mg/kg or less) did not increase AST or ALT in the blood (data not shown). On the contrary, as described in Introduction, LPS-pretreatment reportedly reduces ConA-hepatitis. Thus, if HPA is causal for hepatitis, LPS-pretreatment might be expected to reduce ConA-induced HPA. Indeed, LPS-pretreatment

markedly reduced ConA-induced HPA (Fig. 3C), suggesting that platelets are causally involved in ConA-hepatitis, and that prior depletion of platelets may be protective against such hepatitis.

3.5. Platelets are not causal for ConA-hepatitis, but phagocytic macrophages are

However, contrary to the above expectation, Pm-1 (i.e., platelet-depletion) slightly, but significantly, augmented the ConA-induced increase in AST, although it had no significant effect on ALT (Fig. 4A). In contrast, Clo-lip (i.e., phagocytic macrophage-depletion) reduced the ConA-induced HPA (Figs. 3A and 4A) and dramatically reduced the effect of ConA on hepatitis markers (Fig. 4A). In mice depleted of both phagocytic macrophages and platelets, the ConA-induced increases in hepatitis markers were minimal (Fig. 4B). These results suggest that contrary to our initial expectation, platelets are not causal, and instead macrophages are critically important for the development of ConA-hepatitis.

3.6. LPS-pretreatment protects against ConA-hepatitis via phagocytic macrophage-dependent HPA

As shown in Figs. 3B and 3C, a low dose of LPS (10 µg/kg) induces HPA within a few hours of its injection in normal mice. Thus, in the following experiments, we examined the effect of pretreatment with 10 µg/kg of LPS (i.e., the effect of a prior induction of HPA) on the development of ConA-hepatitis.

The LPS-induced HPA was still detectable at 14 h after the LPS injection (see S + LPS + S group in Fig. 5A). LPS markedly reduced the ConA-induced HPA (Fig. 5A), and this reduction was repeatedly confirmed in other experiments (see Figs. 3C, 5B and 7B). In line with previous reports (see Introduction), LPS-pretreatment was found to be highly protective against ConA-induced hepatitis (see AST and ALT data for the S + LPS + ConA group in

Fig. 5A). In contrast, in phagocytic macrophage-depleted mice (viz. the group given Clo-lip), LPS + ConA induced a marked HPA, although LPS by itself did not induce HPA. In addition, the protective effect of LPS-pretreatment observed in normal mice was completely absent in these macrophage-depleted mice: i.e., the LPS + ConA-induced hepatitis in phagocytic macrophage-depleted mice (Clo-lip + LPS + ConA) was quantitatively similar to that induced by ConA alone in normal mice (S + S + ConA) (Fig. 5A). These results suggest that (i) the protective effect of LPS-pretreatment is phagocytic macrophage-dependent, and prior induction of HPA may be involved in this protection, and (ii) there might be a pathway by which LPS + ConA induces hepatitis without the involvement of phagocytic macrophages (see Discussion).

3.7. LPS-pretreatment exacerbates ConA-hepatitis in platelet-depleted mice

As shown in Fig. 4A, platelets themselves were not causal for ConA-hepatitis (actually, they were slightly protective). Surprisingly, in a pilot experiment in which platelet-depleted mice were injected with LPS and then with ConA, the mice soon weakened, and some mice (2/6) died within 6 h of the ConA injection. Thus, in the experiment shown in Fig. 5B we measured hepatitis markers at 2 h (instead of the 12 h measurement-point employed in most other experiments) after injection of ConA. Even at this time-point, hepatitis markers were markedly increased in the Pm-1 + LPS + ConA group. These results (i) suggest that LPS-pretreatment strongly exacerbates ConA-hepatitis in platelet-depleted mice (in complete contrast to its protective effect in normal mice), and (ii) support the above-described idea that the protective effect of LPS in platelet-non-depleted mice depends largely on platelets or HPA.

3.8. In mice depleted of both phagocytic macrophages and platelets, ConA with LPS-pretreatment induces no significant hepatitis

As described above, LPS + ConA induces both hepatitis and HPA in phagocytic macrophage-depleted mice (Fig. 5A), and this combination strongly exacerbates ConA-hepatitis in platelet-depleted mice (Fig. 5B). In contrast to these effects, in mice depleted of both phagocytic macrophages and platelets, LPS-pretreatment before ConA injection exhibited no significant hepatotoxic effect. This result, too, supports the ideas described above that (i) phagocytic macrophages are critically important for the induction of ConA-hepatitis, and (ii) the protective effect of LPS-pretreatment depends largely on platelets or HPA.

3.9. Anti- α IIb antibody has essentially the same effects as Pm-1

To try to confirm the effects of platelet-depletion on ConA-hepatitis described above, we tested the effects of MWReg30, another monoclonal anti-platelet antibody. This recognizes platelet-specific integrin α IIb (CD41) and depletes platelets from the circulation [35]. Indeed, at the dose used in the present study, MWReg30 reduced the platelet count to about 30 % of its normal level, but such treatment with MWReg30 had no significant effects on the ConA-induced increases in hepatitis markers (Fig. 7A). However, in a pilot experiment, MWReg30-treated mice became severely weakened within a few hours of ConA injection (as also observed in Pm-1-treated mice). Thus, in the experiment shown in Fig. 7B, we measured hepatitis markers at 4.5 h (not at 12 h as in most other experiments) after injection of ConA. As also observed in mice pretreated with Pm-1 (Fig. 5B), the ConA-induced increases in hepatitis markers were powerfully augmented in mice pretreated with MWReg30 (Fig. 7B). These results demonstrate that platelet-depletion, whether by Pm-1 or by MWReg30, does indeed strongly exacerbate ConA-hepatitis.

3-10. IL-1 is involved in ConA-induced hepatitis

We previously reported that among the cytokines tested, IL-1 (both α and β types) and TNF α (but not IL-2, IFN γ , IL-12, IL-8, G-CSF, or GM-CSF) each induced HPA (or a hepatic 5HT increase) [8]. Hence, we tested the effects of ConA in mice deficient in IL-1 and/or TNF α . The levels of the hepatitis markers induced by ConA in IL-1 KO mice were significantly lower than those induced by ConA in WT mice (Fig. 8A), although there was no significant difference between such mice in their HPA levels. These results suggest that IL-1 is causally involved in ConA-induced HPA and hepatitis.

3-11. IL-1 is also involved in LPS-induced protection against ConA-induced hepatitis

We previously reported that IL-1 (but not TNF α , IL-2, IL-6, IL-12, or IFNs) is protective against the hepatitis induced by LPS + GalN [23], and that a very low dose of IL-1 (1 μ g/kg) can induce a transient hepatic 5HT increase (or HPA) within a few hours of its intraperitoneal injection [38]. Thus, IL-1 might be expected to be involved in the protective effect of LPS-pretreatment. Indeed, LPS was not effective in IL-1-KO mice (Fig. 8B). Having obtained these results, we examined the effect of an intraperitoneal injection of human recombinant IL-1 α or IL-1 β (0.001-1 μ g/kg, 0.5 - 2 h before ConA-injection). However, we failed to obtain a protective effect of IL-1 like that induced by LPS-pretreatment (data not shown). These results suggest that although IL-1 is involved in the LPS-induced protection against ConA-induced hepatitis, exogenously administered IL-1 may not be effective.

3-12. P-selectin is involved in ConA-induced HPA

P-Selectin (PS) is known to be expressed on the surface of activated platelets and vascular endothelial cells [39]. Further, PS is reportedly [40] involved in the development of ConA-hepatitis since: (i) PS-deficient mice are resistant to ConA-hepatitis, and (ii) in normal mice, ConA-hepatitis is significantly reduced by an anti-PS antibody [40]. Low-molecular-weight heparins (LMWH) reportedly inhibit PS-dependent cell adhesion [41]. Thus, we tested whether PS is involved in ConA-induced HPA by using LMWH. As shown in Fig. 9A, LMWH markedly reduced both the HPA and the levels of hepatitis makers induced by ConA. Next, we examined the effect of anti-PS antibody on the maximal HPA that precedes maximal increases in AST and ALT (see Fig. 1A). At the dose used here, anti-PS antibody significantly reduced both the HPA and hepatitis markers induced by ConA without a detectable effect on the platelet count in the circulation (Fig. 9B). These results suggest that P-selectin is involved in both the HPA and hepatitis induced by ConA.

3-13. Electron-microscopic observations of livers

The above findings suggest that platelets may be protective against ConA-hepatitis. Hence, finally, we observed platelet movement or behavior within livers in mice given LPS and/or ConA. For this purpose, we made electron-microscopic observations of livers taken at two time-points, at 3 and 12 h after ConA injection: the former is an early time in the liver SHT increase (or HPA) (see Fig. 1A) and the latter is the peak time for hepatitis markers (see Fig. 1A).

At 12 h after ConA treatment: As shown in Figs. 10A-C, hepatic lesions are evident at 12 h after ConA injection, and LPS-pretreatment protects against such lesions. It was notable that non-aggregated platelets could be detected in the livers of mice pretreated with LPS, while aggregated platelets frequently detected in the livers of mice not given LPS-pretreatment (data not shown).

At 3 h after ConA treatment: As shown in Fig. 5B, LPS + ConA rapidly exacerbates ConA-hepatitis in Pm-1-treated (i.e., platelet-depleted) mice. Platelets were not detected in the livers of mice pretreated with Pm-1 (Fig. 10D). In these platelet-depleted mice, the rapid exacerbation of ConA-hepatitis by LPS + ConA was confirmed by electron-microscopic observations (Fig. 10D). Surprisingly; (i) expansion of Disse spaces was noted in platelet-depleted mice, (ii) the cell membrane of hepatocytes was ruptured, and (iii) the cell organelles (such as mitochondria) of hepatocytes were scattered within the expanded Disse spaces. Although congestion was evident in the liver at 3 h after ConA injection (Fig. 10E), the degree of hepatitis in such livers was mild compared with those shown in Figs. 10B and 10D. In LPS-treated mice, there were many platelets in contact with the endothelial cells of sinusoids (Figs. 10C and 10F).

Platelet behaviors in liver: We previously reported that LPS and IL-1 induce translocation of platelets into Disse spaces and their entry into hepatocytes [8]. In the present study, we confirmed such translocation of platelets into Disse spaces (Figs. 11A and 11B) and their entry into hepatocytes in the livers of mice treated with LPS followed by ConA (Fig. 11C).

4. Discussion

The present findings concerning the roles of platelets and/or phagocytic (F4/80-positive) macrophages in ConA-hepatitis and in the protective effect of LPS-pretreatment are summarized in Table 1. As described previously [14-16] and confirmed in the present study (Fig. 4A), ConA-hepatitis depends on the presence of phagocytic macrophages, this possibly being due to macrophages being required to stimulate T-cells. In addition, our data suggest that P-selectin (exogenous or injected) and endogenous IL-1 may be causally involved in ConA hepatitis (Figs. 8A and 9), while endogenous IL-1 may be involved in the protective

effect of LPS-pretreatment (Fig. 8B). Incidentally; (i) LPS and ConA each induce a marked increase in liver 5HT even in nude mice (mostly deficient in mature T-cells) [1], indicating that the HPA induced by these substances is independent of T-cells, and (ii) LPS by itself does not induce hepatocyte lesions in normal mice or in mice depleted of PLTs and/or phagocytic macrophages (data not shown), possibly because of its inability to stimulate T cells. Our findings may seem very complicated. However, cellular events may be multifactorial and dependent on timing in actual hepatitis (i.e., actual hepatitis may consist of various aspects or phases). So, it is quite possible that there is a phase in which platelets and/or macrophages are exacerbating and/or protective. Our supposition is that the present findings may reflect such a phase, and may help toward an understanding of that phase during the development of hepatitis.

In the present study, platelet-depletion did not reduce the severity of ConA-hepatitis (actually, there was if anything an exacerbation). In addition, pretreatment with LPS (i.e., prior induction of HPA) markedly reduced such hepatitis, suggesting that platelets are not causal, but protective, against ConA-hepatitis. It should be noted that in mice depleted of platelets (by either Pm-1 or MWReg30), LPS-pretreatment greatly exacerbated ConA-hepatitis. These results support the idea that the prior HPA induced by LPS is highly protective against ConA-hepatitis. In other words, LPS induces “protective HPA” (Fig. 12). However, in PLT-depleted mice, LPS + ConA induces lethal hepatitis because: (i) LPS stimulates phagocytic (F4/80-positive) macrophages so as to augment their interaction with T-cells, and (ii) such mice are unable to manifest the “protective HPA”. It was a striking finding that in platelet-depleted mice, LPS-pretreatment followed by ConA injection induced severe rupture of the cell membranes of hepatocytes and leakage of their constituents (Fig. 10D).

The precise mechanism underlying the above-mentioned protective HPA remains to be clarified. However, we speculate as follows on the basis of a unique behavior shown by platelets. Platelets translocate into Disse spaces (the spaces between vascular endothelial cells and hepatocytes) in response to LPS, and cell-to-cell contact between platelets and Kupffer cells may induce the migration of platelets from sinusoidal spaces into Disse spaces [7]. Surprisingly, some of these platelets enter hepatocytes [8]. Nevertheless, there is no visible damage to the hepatocytes. In fact, many polysomes can be seen around those platelets that are located within hepatocytes, suggesting an enhancement of protein synthesis [8]. Such translocation of platelets and their entry into hepatocytes have also been observed in the livers of mice given an agonistic anti-Fas antibody [21], and we confirmed these effects in the livers of mice given LPS followed by ConA (Fig. 11). The liver, in cooperation with various other cells, may play important roles in the initial defense against toxic materials or pathogens since, having entered the blood via the digestive tract, these pass first to the liver via the portal vein. Platelets may assist in this hepatic defense after gathering rapidly in the liver, although the mechanism underlying their protective effects remains to be elucidated.

Interestingly, despite the individual abilities of LPS and ConA to induce HPA in normal mice, LPS-pretreatment + ConA induced no detectable HPA (Fig. 5A) [this result was repeatedly confirmed in other experiments (Figs. 5B and 7B)]. However, in phagocytic macrophage-depleted mice, LPS-pretreatment + ConA induced hepatitis and a marked HPA (Fig. 5A). Hepatocytes are known to contain or produce some stimulants of platelets, such as ADP and prothrombin, which induce platelet aggregation. Thus, hepatocytes, when injured (as typically observed in Fig. 10D), may leak these substances [42]. Therefore, we speculate that in addition to the protective HPA (which is reversible and phagocytic macrophage-dependent), there exists a “causal HPA” (or platelet aggregation), which (i) is induced by the

platelet-stimulating substances leaked as a consequence of hepatocyte injury, and (ii) may be reduced by anti-P-selectin antibody or blockade of P-selectin receptors (Fig. 12). The causal type of HPA may be irreversible and thus result in aggregation of platelets, leading to an impaired hepatic circulation [18], and subsequently to an exacerbated hepatitis. Such a “vicious circle” (hepatocyte injury and release of platelet-stimulating substances → platelet aggregation → additional hepatocyte injury and further leakage of platelet-stimulating substances) may lead to severe hepatitis. Indeed, in a hepatitis model induced by LPS + GalN, the hepatic 5HT increase was markedly larger in livers displaying congestion than in livers with no congestion [20]. We, therefore, speculate that in normal mice, LPS-pretreatment can prevent ConA-induced hepatocyte injury via a reversible prior protective HPA, and thus can prevent a subsequent causal HPA (or platelet aggregation) being induced by injured hepatocytes (Fig. 12).

The experiments shown in Fig. 8 suggested that IL-1 may be involved both causally in ConA-hepatitis and protectively following LPS-pretreatment. Unfortunately, however, our exogenous injection of IL-1 was not effective at protecting against ConA-hepatitis. Thus, we speculate that in addition to IL-1, another factor(s) may be involved in the protective effects of LPS or in the effect of protective HPA. Thus, the protective mechanism remains to be fully elucidated.

As described in Introduction, ConA-hepatitis is dependent on both T-cells and macrophages. However, we noted that LPS + ConA (but not LPS alone or ConA alone) induced hepatitis even in phagocytic macrophage-depleted mice (Fig. 5A). This result suggests the possible presence of Clo-lip-resistant macrophage-like (F4/80-negative and not phagocytic) cells (Fig. 12). We speculate that in mice depleted of phagocytic macrophages, but not platelets, LPS cannot induce any protective HPA, and instead the LPS may stimulate the Clo-resistant, macrophage-like cells and promote their interaction with T-cells, thereby

inducing hepatocyte injury and triggering the vicious circle described above. However, in mice deficient in both phagocytic macrophages and platelets, this pathway by itself may be very weak and therefore not able to induce hepatitis.

In conclusion, the findings made in the present study, on mice, suggest that: (i) there are causal and protective types of HPA, (ii) the causal type may involve platelet aggregation, and this may be induced by platelet stimulants leaked from injured hepatocytes, (iii) the protective type is phagocytic (or F4/80-positive) macrophage-dependently inducible by prior low-dose administration of LPS, and (iv) IL-1 is involved in both the causal and protective types. Thus, a brief summary might be that LPS-pretreatment is able powerfully to protect against such hepatitis via a prior induction of HPA, but in the absence of platelets LPS-pretreatment strongly exacerbates ConA-hepatitis.

Acknowledgements

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ACCEPTED MANUSCRIPT

References

- [1] Y. Endo, M. Nakamura, Active translocation of platelets into sinusoidal and Disse spaces in the liver in response to lipopolysaccharides, interleukin-1 and tumor necrosis factor, *Gen Pharmacol*, 24 (1993) 1039-1053.
- [2] C.M. Herd, C.P. Page, Pulmonary immune cells in health and disease: platelets, *Eur Respir J*, 7 (1994) 1145-1160.
- [3] D.N. Mannel, G.E. Grau, Role of platelet adhesion in homeostasis and immunopathology, *Mol Pathol*, 50 (1997) 175-185.
- [4] A.S. Weyrich, G.A. Zimmerman, Platelets: signaling cells in the immune continuum, *Trends Immunol*, 25 (2004) 489-495.
- [5] Y. Endo, A lipopolysaccharide and concanavalin A induce variations of serotonin levels in mouse tissues, *Eur J Pharmacol*, 91 (1983) 493-499.
- [6] Y. Endo, Induction of hypoglycaemia and accumulation of 5-hydroxytryptamine in the liver after the injection of mitogenic substances into mice, *Br J Pharmacol*, 81 (1984) 645-650.
- [7] Y. Endo, M. Nakamura, The effect of lipopolysaccharide, interleukin-1 and tumour necrosis factor on the hepatic accumulation of 5-hydroxytryptamine and platelets in the mouse, *Br J Pharmacol*, 105 (1992) 613-619.
- [8] M. Nakamura, M. Shibasaki, Y. Nitta, Y. Endo, Translocation of platelets into Disse spaces and their entry into hepatocytes in response to lipopolysaccharides, interleukin-1 and tumour necrosis factor: the role of Kupffer cells, *J Hepatol*, 28 (1998) 991-999.
- [9] G. Tiegs, J. Hentschel, A. Wendel, A T cell-dependent experimental liver injury in mice inducible by concanavalin A, *J Clin Invest*, 90 (1992) 196-203.
- [10] M.A. Freudenberg, D. Keppler, C. Galanos, Requirement for lipopolysaccharide-responsive macrophages in galactosamine-induced sensitization to endotoxin, *Infect Immun*, 51 (1986) 891-895.
- [11] S. Seki, Y. Habu, T. Kawamura, K. Takeda, H. Dobashi, T. Ohkawa, H. Hiraide, The liver as a crucial organ in the first line of host defense: the roles of Kupffer cells, natural killer (NK) cells and NK1.1 Ag+ T cells in T helper 1 immune responses, *Immunol Rev*, 174 (2000) 35-46.
- [12] R. Silverstein, D-galactosamine lethality model: scope and limitations, *J Endotoxin Res*, 10 (2004) 147-162.
- [13] H. Tsutsui, K. Matsui, H. Okamura, K. Nakanishi, Pathophysiological roles of interleukin-18 in inflammatory liver diseases, *Immunol Rev*, 174 (2000) 192-209.
- [14] A. Morita, Y. Itoh, T. Toyama, H. Fujii, K. Nishioji, T. Kirishima, A. Makiyama, N. Yamauchi, T. Okanoue, Activated Kupffer cells play an important role in intra-hepatic Th1-associated necro-inflammation in Concanavalin A-induced hepatic injury in mice, *Hepatol Res*, 27 (2003) 143-150.
- [15] T. Okamoto, O. Maeda, N. Tsuzuike, K. Hara, Effect of gadolinium chloride treatment on concanavalin A-induced cytokine mRNA expression in mouse liver, *Jpn J Pharmacol*, 78 (1998) 101-103.
- [16] J. Schumann, D. Wolf, A. Pahl, K. Brune, T. Papadopoulos, N. van Rooijen, G. Tiegs, Importance of Kupffer cells for T-cell-dependent liver injury in mice, *Am J Pathol*, 157 (2000) 1671-1683.
- [17] P.F. Piguet, C. Vesin, J.E. Ryser, G. Senaldi, G.E. Grau, F. Tacchini-Cottier, An effector role for platelets in systemic and local lipopolysaccharide-induced toxicity in mice, mediated by a CD11a- and CD54-dependent interaction with endothelium, *Infect Immun*, 61 (1993) 4182-4187.

- [18] Y. Miyazawa, H. Tsutsui, H. Mizuhara, H. Fujiwara, K. Kaneda, Involvement of intrasinusoidal hemostasis in the development of concanavalin A-induced hepatic injury in mice, *Hepatology*, 27 (1998) 497-506.
- [19] W. Van Molle, J. Vanden Berghe, P. Brouckaert, C. Libert, Tumor necrosis factor-induced lethal hepatitis: pharmacological intervention with verapamil, tannic acid, picotamide and K76COOH, *FEBS Lett*, 467 (2000) 201-205.
- [20] M. Shibazaki, M. Nakamura, Y. Endo, Biphasic, organ-specific, and strain-specific accumulation of platelets induced in mice by a lipopolysaccharide from *Escherichia coli* and its possible involvement in shock, *Infect Immun*, 64 (1996) 5290-5294.
- [21] Y. Ohtaki, K. Yamaguchi, Z. Yu, H. Kumamoto, H. Shimauchi, Y. Iwakura, S. Sugawara, Y. Endo, Hepatic platelet accumulation in Fas-mediated hepatitis in mice, *Int Immunopharmacol*, 9 (2009) 1071-1078.
- [22] Y. Endo, T. Kikuchi, M. Nakamura, Ornithine and histidine decarboxylase activities in mice sensitized to endotoxin, interleukin-1 or tumour necrosis factor by D-galactosamine, *Br J Pharmacol*, 107 (1992) 888-894.
- [23] Y. Endo, M. Shibazaki, K. Yamaguchi, K. Kai, S. Sugawara, H. Takada, H. Kikuchi, K. Kumagai, Enhancement by galactosamine of lipopolysaccharide(LPS)-induced tumour necrosis factor production and lethality: its suppression by LPS pretreatment, *Br J Pharmacol*, 128 (1999) 5-12.
- [24] M.A. Freudenberg, C. Galanos, Induction of tolerance to lipopolysaccharide (LPS)-D-galactosamine lethality by pretreatment with LPS is mediated by macrophages, *Infect Immun*, 56 (1988) 1352-1357.
- [25] T. Nishikage, S. Seki, S. Toyabe, T. Abo, Y. Kagata, T. Iwai, H. Hiraide, Inhibition of concanavalin A-induced hepatic injury of mice by bacterial lipopolysaccharide via the induction of IL-6 and the subsequent reduction of IL-4: the cytokine milieu of concanavalin A hepatitis, *J Hepatol*, 31 (1999) 18-26.
- [26] Y. Tagawa, K. Sekikawa, Y. Iwakura, Suppression of concanavalin A-induced hepatitis in IFN-gamma(-/-) mice, but not in TNF-alpha(-/-) mice: role for IFN-gamma in activating apoptosis of hepatocytes, *J Immunol*, 159 (1997) 1418-1428.
- [27] R. Horai, M. Asano, K. Sudo, H. Kanuka, M. Suzuki, M. Nishihara, M. Takahashi, Y. Iwakura, Production of mice deficient in genes for interleukin (IL)-1alpha, IL-1beta, IL-1alpha/beta, and IL-1 receptor antagonist shows that IL-1beta is crucial in turpentine-induced fever development and glucocorticoid secretion, *J Exp Med*, 187 (1998) 1463-1475.
- [28] Y. Nagata, Y. Shozaki, H. Nagahisa, T. Nagasawa, T. Abe, K. Todokoro, Serum thrombopoietin level is not regulated by transcription but by the total counts of both megakaryocytes and platelets during thrombocytopenia and thrombocytosis, *Thromb Haemost*, 77 (1997) 808-814.
- [29] Y. Ohtaki, H. Shimauchi, T. Yokochi, H. Takada, Y. Endo, In vivo platelet response to lipopolysaccharide in mice: proposed method for evaluating new antiplatelet drugs, *Thromb Res*, 108 (2002) 303-309.
- [30] L. Zhao, Y. Ohtaki, K. Yamaguchi, M. Matsushita, T. Fujita, T. Yokochi, H. Takada, Y. Endo, LPS-induced platelet response and rapid shock in mice: contribution of O-antigen region of LPS and involvement of the lectin pathway of the complement system, *Blood*, 100 (2002) 3233-3239.
- [31] N. Van Rooijen, A. Sanders, Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications, *J Immunol Methods*, 174 (1994) 83-93.
- [32] N. van Rooijen, A. Sanders, Elimination, blocking, and activation of macrophages: three of a kind?, *J Leukoc Biol*, 62 (1997) 702-709.

- [33] Y. Endo, M. Nakamura, Y. Nitta, K. Kumagai, Effects of macrophage depletion on the induction of histidine decarboxylase by lipopolysaccharide, interleukin 1 and tumour necrosis factor, *Br J Pharmacol*, 114 (1995) 187-193.
- [34] M. Shibazaki, M. Nakamura, Y. Nitta, Y. Endo, Displacement of platelets from blood to spleen following intravenous injection of liposomes encapsulating dichloromethylene bisphosphonate, *Immunopharmacology*, 39 (1998) 1-7.
- [35] B. Nieswandt, B. Echtenacher, F.P. Wachs, J. Schroder, J.E. Gessner, R.E. Schmidt, G.E. Grau, D.N. Mannel, Acute systemic reaction and lung alterations induced by an antiplatelet integrin gpIIb/IIIa antibody in mice, *Blood*, 94 (1999) 684-693.
- [36] K. Yamaguchi, Z. Yu, H. Kumamoto, Y. Sugawara, H. Kawamura, H. Takada, T. Yokochi, S. Sugawara, Y. Endo, Involvement of Kupffer cells in lipopolysaccharide-induced rapid accumulation of platelets in the liver and the ensuing anaphylaxis-like shock in mice, *Biochim Biophys Acta*, 1762 (2006) 269-275.
- [37] Y. Endo, Evidence that the accumulation of 5-hydroxytryptamine in the liver but not in the brain may cause the hypoglycaemia induced by 5-hydroxytryptophan, *Br J Pharmacol*, 85 (1985) 591-598.
- [38] Y. Endo, Parallel relationship between the increase in serotonin in the liver and the hypoglycaemia induced in mice by interleukin-1 and tumour necrosis factor, *Immunol Lett*, 27 (1991) 75-79.
- [39] P. Andre, P-selectin in haemostasis, *Br J Haematol*, 126 (2004) 298-306.
- [40] A. Massaguer, S. Perez-Del-Pulgar, P. Engel, J. Serratosa, J. Bosch, P. Pizcueta, Concanavalin-A-induced liver injury is severely impaired in mice deficient in P-selectin, *J Leukoc Biol*, 72 (2002) 262-270.
- [41] N. Maugeri, G. Di Fabio, M. Barbanti, G. de Gaetano, M.B. Donati, C. Cerletti, Parnaparin, a low-molecular-weight heparin, prevents P-selectin-dependent formation of platelet-leukocyte aggregates in human whole blood, *Thromb Haemost*, 97 (2007) 965-973.
- [42] P.G. Langley, A. Forbes, R.D. Hughes, R. Williams, Thrombin-antithrombin III complex in fulminant hepatic failure: evidence for disseminated intravascular coagulation and relationship to outcome, *Eur J Clin Invest*, 20 (1990) 627-631.

Table 1. Effects of depletion of platelets (PLTs) and/or phagocytic macrophages (M ϕ *), with or without prior LPS-treatment (pLPS), on the hepatitis and HPA induced by

ConA. The effects of ConA in normal mice were graded as +++. Figures that include relevant data are indicated in the lowermost row for convenience.

Depletion	None	PLT	M ϕ *	PLT+M ϕ *	None	PLT	M ϕ *	PLT+M ϕ *
pLPS ^a	No	No	No	No	Yes	Yes	Yes	Yes
Hepatitis	+++	+++ ^b	±	-	±	++++ ^c	+++ ^d	- ^e
HPA	+++	-	±	-	-	+++ ^d	- ^e	
Figs		4A	4A	4B	5A	5B	5A	6

-: no significant effect ±: minor effect + ~ ++++: grades of effect

- a) LPS by itself did not induce hepatitis in normal mice or in PLT- and/or M ϕ *-depleted mice (data not shown).
- b) In PLT-depleted mice, ConA-induced hepatitis was slightly augmented (plasma AST was significantly increased) (Fig. 4A).
- c) In PLT-depleted mice, LPS + ConA induced lethal hepatitis (Fig. 5B).
- d) In M ϕ *-depleted mice, LPS + ConA induced both hepatitis and HPA, which were similar to those induced in normal mice (Fig. 5A).
- e) In mice depleted of both PLTs and M ϕ s*, LPS + ConA induced almost no hepatitis (Fig. 6).

Figure legends

Fig. 1. Effects of ConA. (A) Time-dependent changes in hepatic 5HT and in serum AST and ALT activities. ConA (10 or 30 mg/kg) was injected at time 0, and the livers and blood were taken at the times indicated. (B) Dose-dependent increases in hepatic 5HT. ConA was injected at the doses indicated, and livers were taken 6 h later. (C) Histochemical detection of HPA in mice given ConA. Livers were taken 6 h after injection of ConA (5 mg/kg), subjected to immunostaining using an anti-mouse platelet monoclonal antibody (Pm-1), and visualized using fluorescein isothiocyanate (green fluorescence) or diaminobenzidine (brown spots). Results shown in C are representative of 5 mice.

Fig. 2. Depletions of platelets and macrophages. (A) Effects of Pm-1 on platelets and on 5HT in blood and liver. Pm-1 was injected (s.c.) at time 0, and blood and livers were taken at the indicated times. (B) Effects of Clo-lip on macrophages. Saline or Clo-lip was injected, and 24 h later livers were taken. The livers were subjected to immunostaining with F4/80 antibody, and visualized using diaminobenzidine (brown spots). Results shown are representative of three mice. (C) Effects of Pm-1 and Clo-lip on liver 5HT and hepatitis markers. Saline, Pm-1, or Clo-lip was injected as described above, and 24 h later blood and livers were taken.

Fig. 3. Effects of macrophage-depletion and LPS-pretreatment on the HPA induced by ConA or LPS. (A) ConA (30 mg/kg) was injected 24 h after injection of saline (S) or Clo-lip, and livers were taken at the indicated times. (B) S or Clo-lip was injected, followed 24 h later by injection of S or LPS (10 or 100 µg/kg). Livers were taken 4 h after the second

injection. (C) S or LPS (10 µg/kg) was injected, followed 2 h later by injection of S, ConA (30 mg/kg), or LPS (10 µg/kg). Blood and livers were taken 4.5 h after the second injection.

Fig. 4. Effects of depletion of platelets and/or macrophages. (A) Effects of depletion of either platelets or macrophages. Saline or ConA (30 mg/kg) was injected 24 h after injection of S, Pm-1 (s.c.), or Clo-lip. Livers and blood were taken 12 h after the second injection. (B) Effects of depletion of both platelets and macrophages. S or Pm-1 was injected (s.c.), followed 12 h later by injection of S or Clo-lip. Finally, S or ConA (30 mg/kg) was injected 6 h after the second injection, and 12 h later livers and blood were taken.

Fig. 5. Effects of low-dose LPS-pretreatment. (A) Effects of LPS-pretreatment in macrophage-depleted mice. Saline (S) or Clo-lip was injected, followed 24 h later by injection of S or LPS (10 µg/kg). Then, S or ConA (30 mg/kg) was injected 2 h after the second injection, and 12 h later livers and blood were taken. (B) Effects of LPS-treatment in platelet-depleted mice. S or Pm-1 was injected (s.c.), followed 24 h later by injection of S or LPS (10 µg/kg). Then, S or ConA (30 mg/kg) was injected 2 h after the second injection, and *two hours* later livers and blood were taken.

Fig. 6. Effects of LPS-treatment in mice depleted of both platelets and macrophages. Saline (S) or Pm-1 was injected (s.c.), followed 12 h later by injection of S or Clo-lip. Then, S or LPS (10 µg/kg) was injected 24 h after the second injection. Finally, S or ConA (30 mg/kg) was injected at 2 h after the third injection. Livers and blood were taken 12 h after the last injection.

Fig. 7. Effects of MW-Reg, another monoclonal anti-mouse platelet antibody. (A)

Effects on platelets and hepatitis markers. Saline (S) or MWReg30 (0.2 mg/kg) was injected, followed 24 h later by injection of S or ConA (30 mg/kg). Blood and livers were taken 12 h after the second injection. **(B)** Effects on the protection afforded by LPS-pretreatment. S or MWReg30 was injected, followed 24 h later by injection of S or LPS (10 µg/kg). Then, S or ConA (30 mg/kg) was injected 2 h after the second injection, and *four and a half hours* later blood and livers were taken.

Fig. 8. Involvement of IL-1 in ConA-induced HPA and hepatitis, and in the protective effect of LPS. (A)

Effects of ConA in mice deficient in IL-1 and/or TNFα. Saline (S) or ConA (30 mg/kg) was injected into various mice [viz. control BALB/c wild-type (WT) mice, IL-1-KO mice (deficient in both IL-1α and IL-1β), TNFα-KO mice, or triple-KO (TKO) mice (deficient in IL-1α, IL-1β, and TNFα)], and 12 h later livers and blood were taken. **(B)** Effects of LPS-pretreatment in IL-1-KO mice. S or LPS (10 µg/kg) was injected into WT or IL-1-KO mice, and 2 h later ConA (30 mg/kg) was injected. Livers and blood were taken 12 h after the ConA injection.

Fig. 9. Involvement of P-selectin (PS) in the HPA and hepatitis induced by ConA. (A)

Effects of low-molecular-weight heparin (LMWH). Saline (S), ConA (30 mg/kg), or a mixture of ConA and LMWH (each 30 mg/kg) was injected, and 12 h later livers and blood were taken. **(B)** Effects of monoclonal anti-mouse PS antibody (anti-PS). S or ConA (30 mg/kg) was injected, and 15 min later S or anti-PS (1.6 mg/kg) was injected. Livers and blood were taken 6 h after the second injection.

Fig. 10. Electron-microscopic observations of livers from mice treated with ConA, LPS, and/or Pm-1. Saline (S) or Pm-1 was injected (s.c.), followed 24 h later by injection of S or LPS (10 µg/kg, i.v.). Then, S or ConA (30 mg/kg, i.v.) was injected 2 h after the second injection. Livers were taken 3 or 12 h after the last injection. **(A)** Normal liver (S→^{24h} S→^{2h} S→^{12h} sampling). Hepatocytes have abundant glycogen granules in their cytoplasm, indicating the normal status of the liver. It was hard to detect platelets in the liver. **(B)** ConA-injured liver. Liver was taken 12 h after ConA injection (S→^{24h} S→^{2h} ConA→^{12h} sampling). Severe congestion within sinusoids and decrease in electron density in the cytoplasm (asterisks) of hepatocytes were obvious. Aggregated platelets were also frequently detected (not seen in this field). **(C)** LPS-protected liver (S→^{24h} LPS→^{2h} ConA→^{12h} sampling). Both the congestion and decrease in electron density were mild in degree compared with those observed in B. In the sinusoidal lumen, several platelets (arrow) were detected, but aggregated platelets were not detected. **(D)** Exacerbation of ConA-hepatitis by platelet depletion (Pm-1→^{24h} LPS→^{2h} ConA→^{3h} sampling). Platelets were detected within neither sinusoidal lumens nor Disse spaces. Expansion of Disse space was evident. Cell membrane of hepatocytes was ruptured and the cell organelles of hepatocytes were scattered in the expanded Disse space, indicating severe hepatitis had occurred within only 3 h after ConA-injection. Arrows indicate mitochondria leaked from hepatocytes into the expanded Disse space. **(E)** Liver 3 h after ConA injection (S→^{24h} S→^{2h} ConA→^{3h} sampling). Congestion of liver was already evident, but the degree of hepatitis was mild compared with those observed in B and D. Non-aggregated platelets were sometimes detected (not seen in this field). **(F)** Effects of LPS (S→^{24h} LPS→^{2h} ConA→^{3h} sampling). Many platelets (arrows) are adhering to sinusoidal endothelial cells.

Fig. 11. Platelet behavior within the liver in mice treated with LPS + ConA. Livers were taken from the mice that provided the samples shown in Fig. 10F (i.e., S→^{24h} LPS →^{2h} ConA→^{3h} sampling). **(A and B)** Several platelets (arrows) were seen in the Disse space (asterisks), indicating their translocation into Disse spaces from blood. **(C)** Platelets (arrow) were sometimes detected in the cytoplasm of hepatocytes, indicating entry of platelets into hepatocytes from Disse spaces.

Fig. 12. Putative relationships between ConA-hepatitis and HPA, and effects of LPS.

Inferences and speculations made from the data are summarized in this figure. (i) ConA stimulates interaction between T-cells and phagocytic macrophages (or Kupffer cells), inducing hepatocyte injury and then resulting in causal HPA (platelet aggregation) via leakage of platelet stimulants from injured hepatocytes. This causal HPA may form part of a vicious circle and thereby exacerbate the hepatitis. (ii) LPS stimulates interaction between phagocytic macrophages and platelets, inducing protective HPA. (iii) Clo-lip-resistant, macrophage-like cells may also be present. In mice depleted of phagocytic macrophages, but not of platelets, LPS may stimulate the Clo-lip-resistant, macrophage-like cells and promote their interaction with T-cells, inducing hepatocyte injury and triggering the vicious circle mentioned in (i). However, in mice depleted of both phagocytic macrophages and platelets, this pathway by itself may be too weak to induce hepatitis. See text together with Table 1 for further details.

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Fig. 1

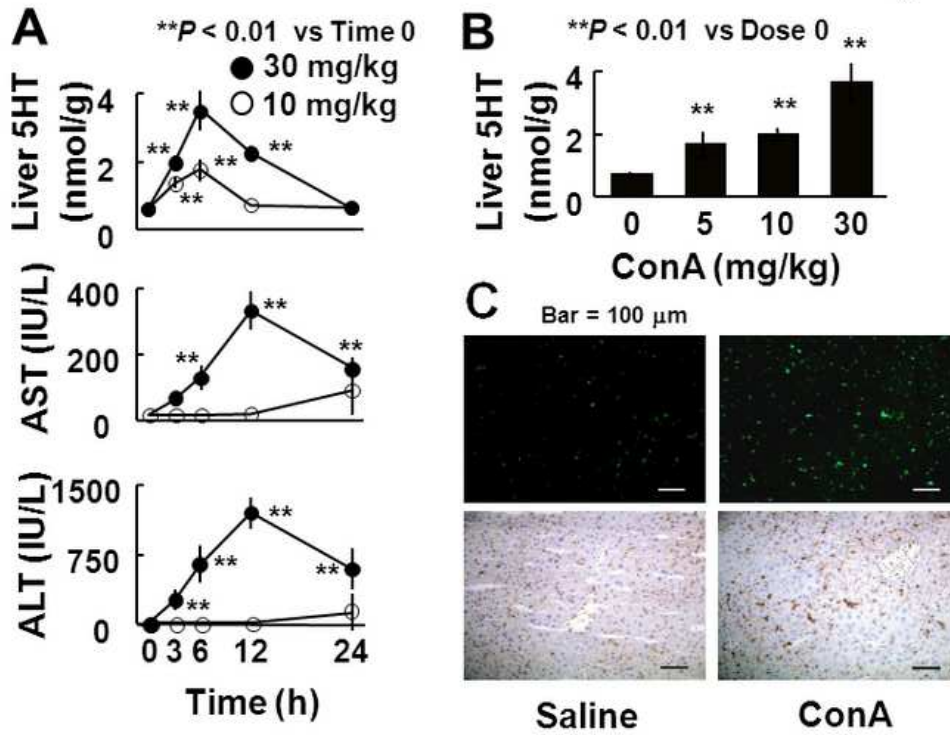


Fig. 2

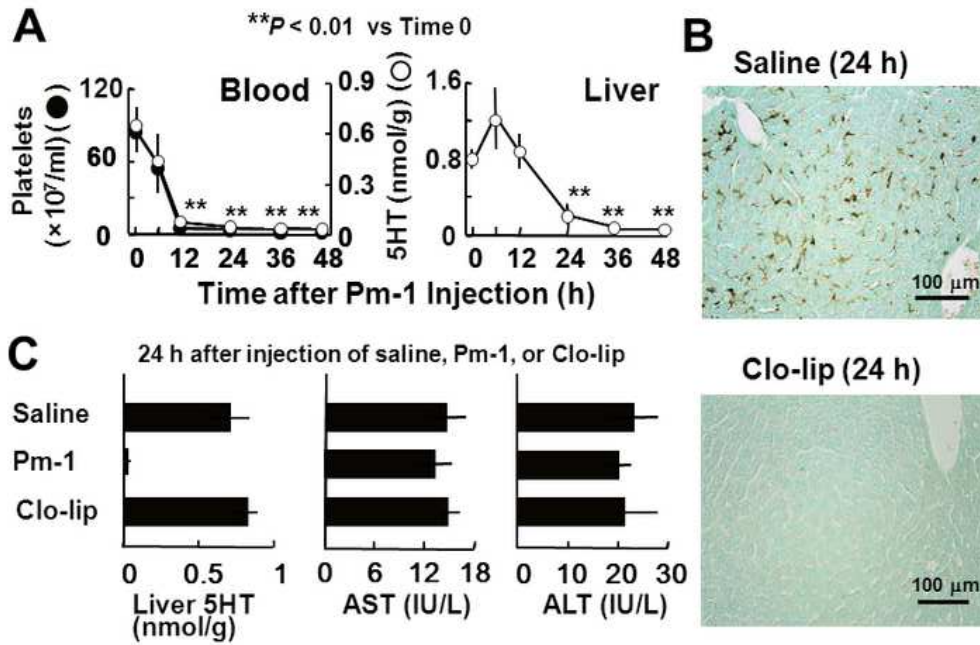


Fig. 3

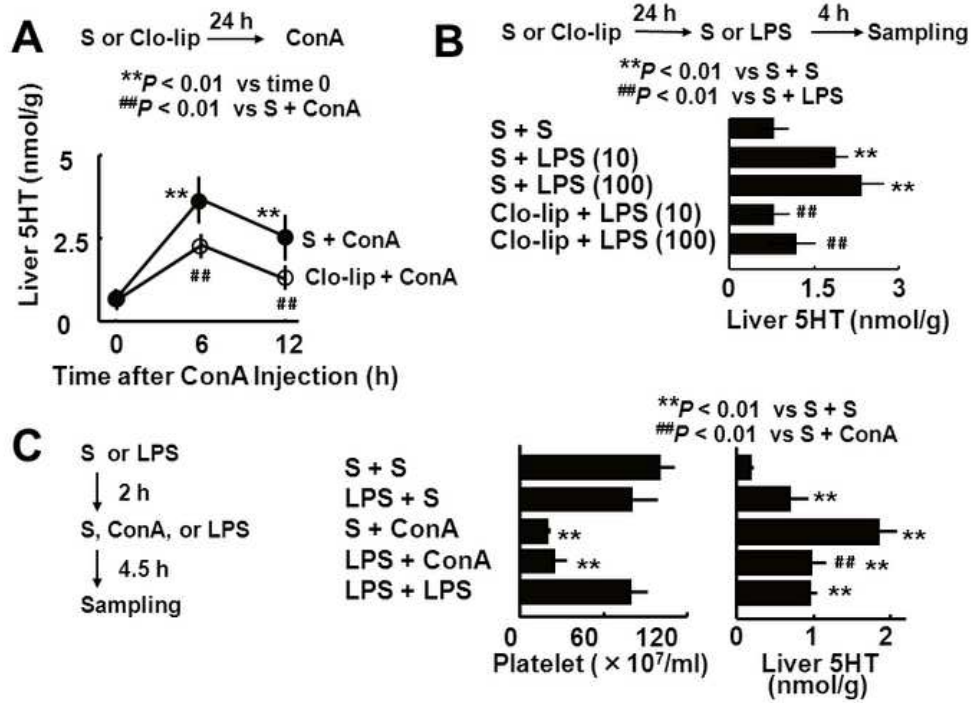
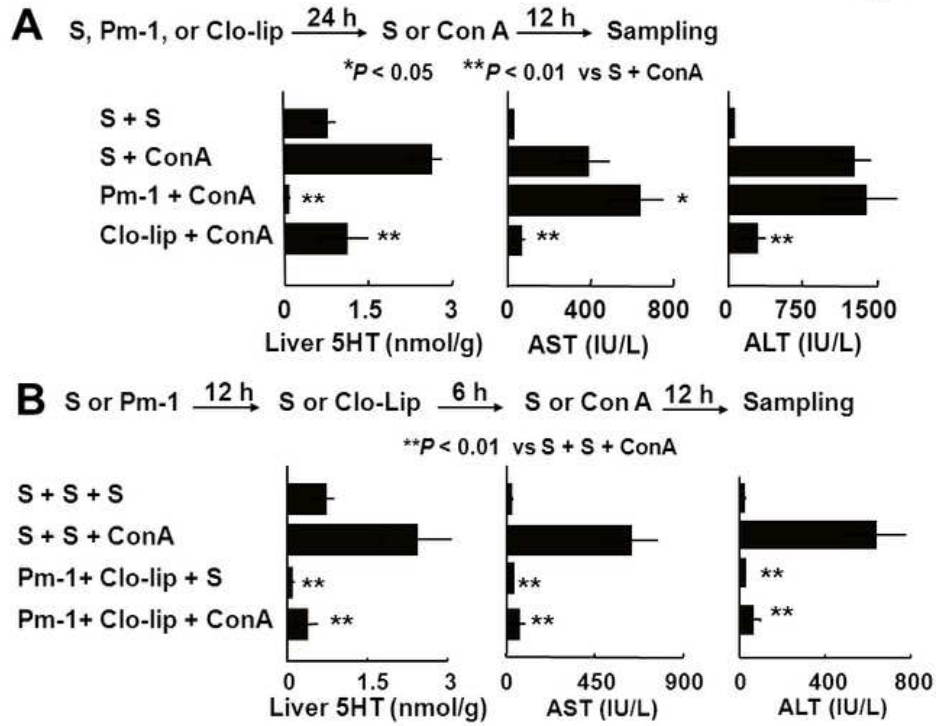


Fig. 4



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Fig. 5

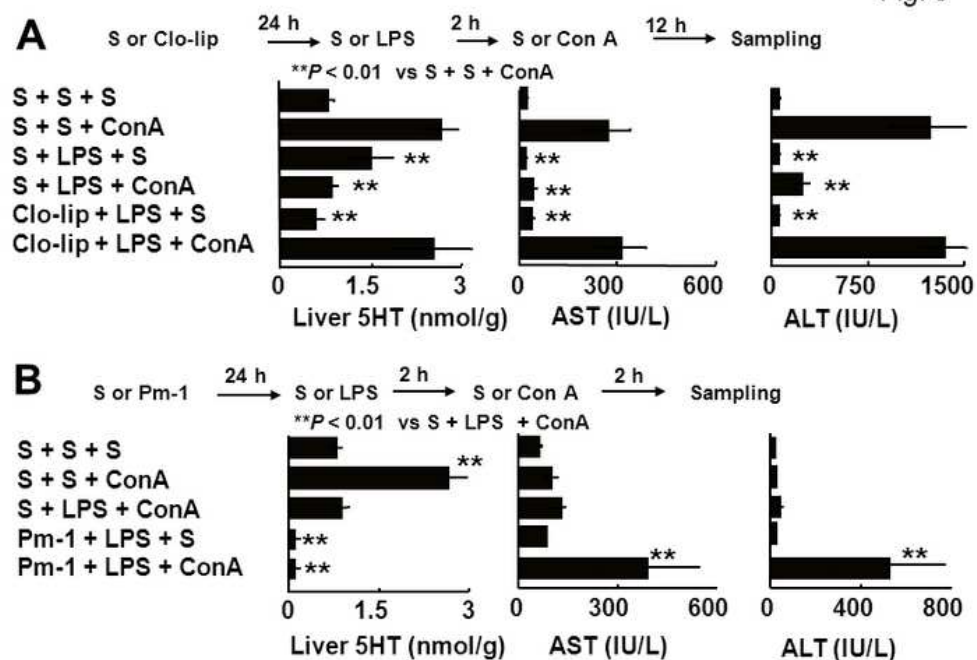
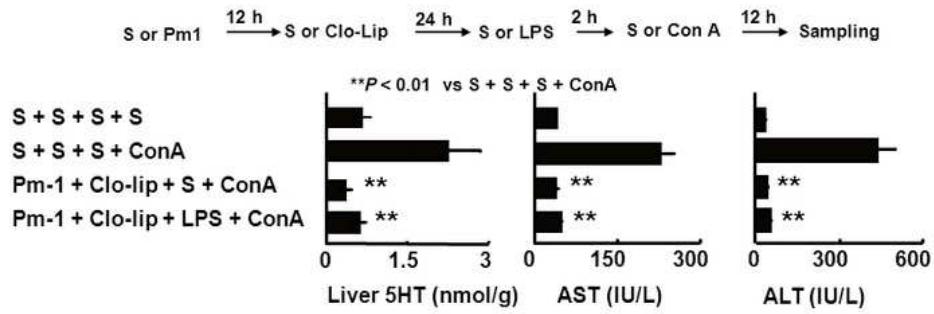




Fig. 6



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Fig. 7

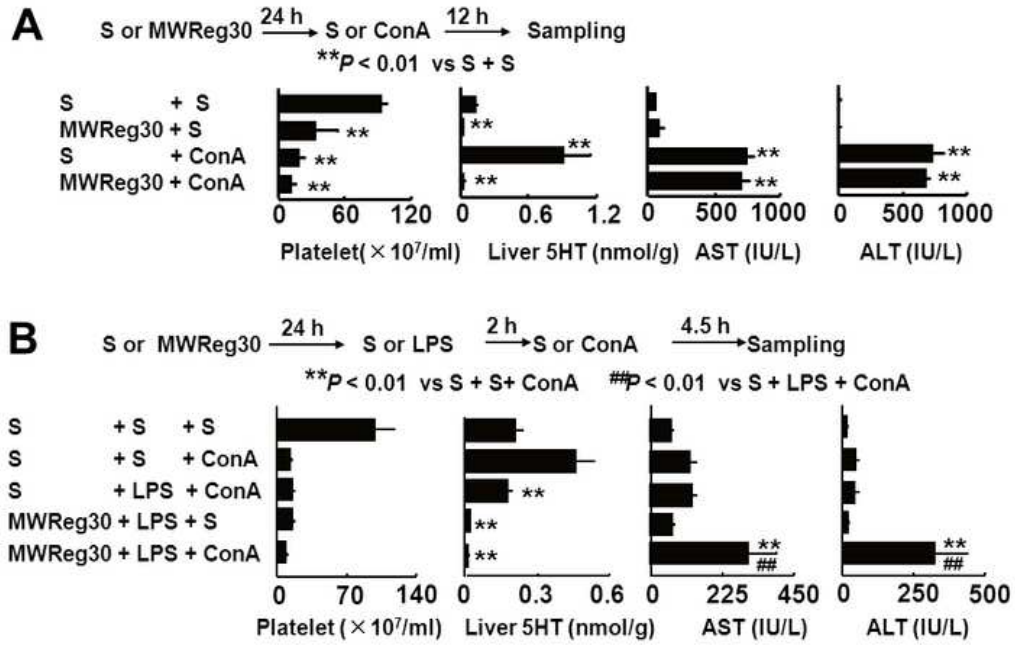
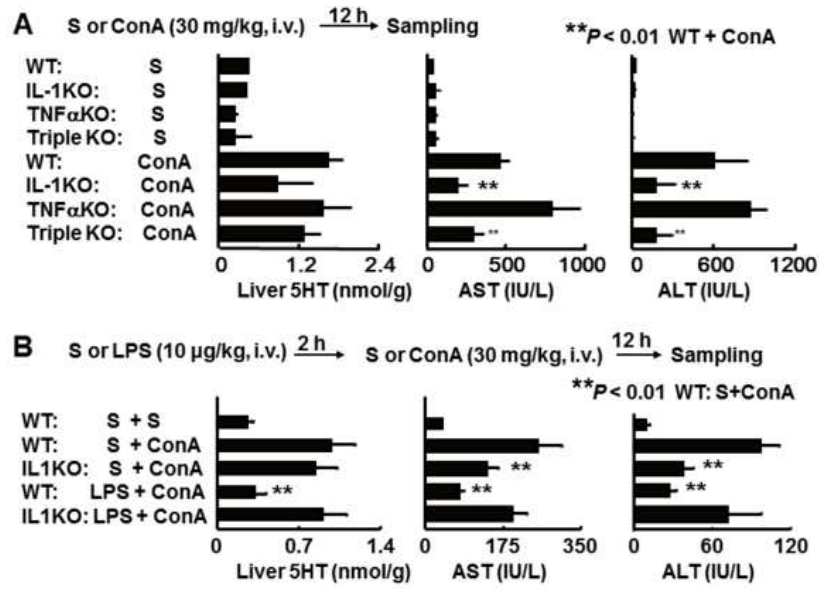




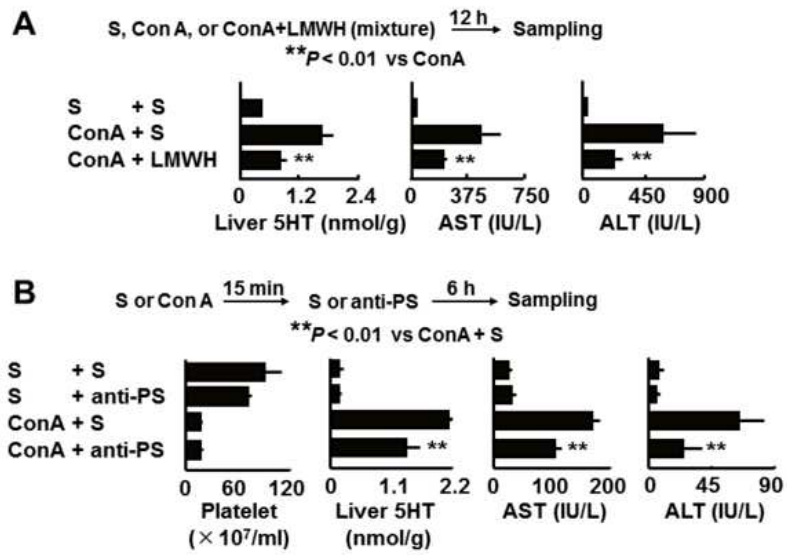
Fig. 8



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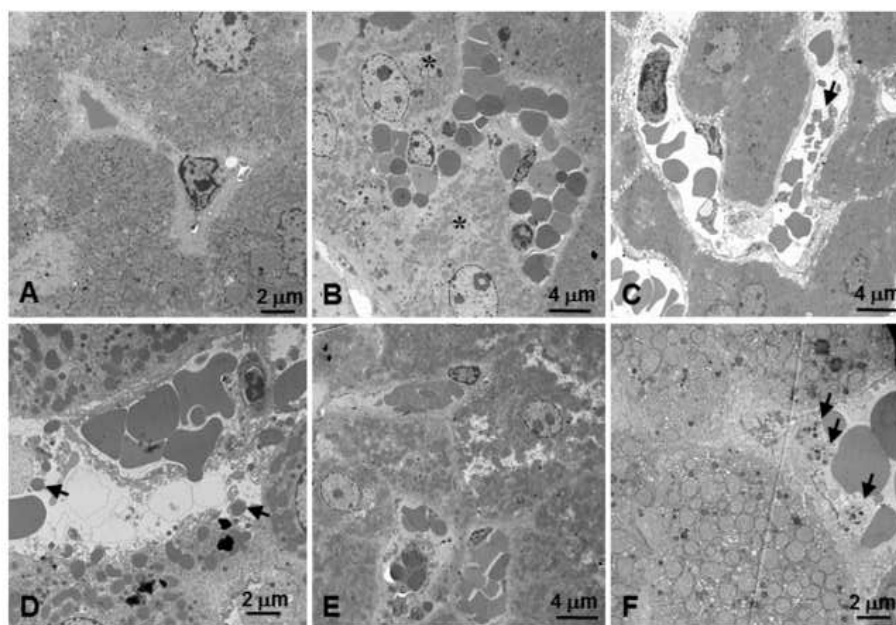
Fig. 9



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Fig. 10



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Fig. 11

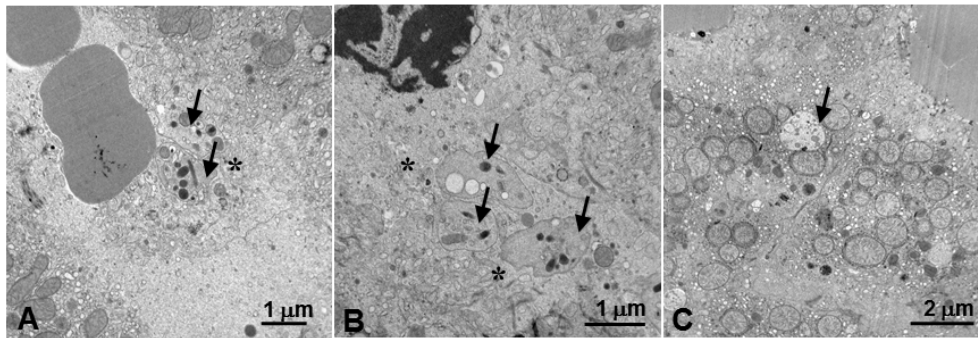


Fig. 12

