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Hepatic macrophage activation and the LPS pathway in patients with different degrees of severity and histopathological patterns of drug induced liver injury

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Summary. Background. Inflammatory activation of hepatic macrophages plays a primary role in drug-induced liver injury (DILI). However, the exact mechanism underlying DILI remains unclear.

Methods. A total of 328 DILI patients and 80 healthy individuals were prospectively enrolled in this study. The DILI patients were categorized into subgroups based on either disease severity or histopathological patterns. Plasma soluble CD163 (sCD163) and hepatic CD163 were examined to determine hepatic macrophage activation, and CD8, CD20, and MUM-1 were assessed to determine cellular immunity using immunohistochemistry. The lipopolysaccharide (LPS) pathway proteins [e.g. LPS, soluble CD14 (sCD14), and LPS-binding protein (LBP)] were measured using enzyme-linked immunosorbent assay.

Results. Plasma sCD163 levels were nine-fold higher in DILI patients than in healthy controls at the baseline, but significantly decreased at the 4-week follow-up visit after treatment. The numbers of hepatic macrophages, B cells, and plasma cells were significantly higher in the liver tissues from DILI patients than those from healthy controls. Furthermore, the baseline levels of LPS pathway proteins in the DILI patients were significantly higher than those in the controls. Notably, these proteins significantly decreased at the 4-week follow-up visit but remained significantly higher than the levels for the controls.

Conclusions. Hepatic inflammation in DILI involves the activation of hepatic macrophages and cellular immunity, in which the LPS pathway likely plays a role, at least in part. As such, this study has improved our understanding of the pathological mechanisms for DILI and may facilitate the development of better treatments for patients with DILI.

Key words: Drug-induced liver injury, Hepatic macrophages, Cellular immunity, LPS, LBP, CD163

Introduction

Drug-induced liver injury (DILI) is among the leading causes of acute liver failure (ALF), accounting for more than 50% of ALF cases (Weaver et al., 2020). Notably, the incidence of DILI is on the rise, mainly attributed to the ever-increasing utilization of prescribed drugs, over-the–counter (OTC) medications, herbs, and dietary supplements worldwide. Although substantial progress has been made in the diagnosis, treatment, and prognosis of DILI, the pathological mechanisms for DILI remain largely unknown. Therefore, it is important to better understand the molecular mechanisms underlying DILI.

The activation of both innate and adaptive immune responses in DILI is well documented. In the liver, drug-induced immune activation is a response from resident hepatic macrophages, also known as Kupffer cells, to initial hepatic damage and cell death (Krenkel and Tacke, 2017). Heymann and colleagues demonstrated that stress or danger signals released from injured hepatocytes [e.g. danger-associated molecular patterns

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Abbreviations. DILI, Drug-induced liver injury; ALT, alanine aminotransferase; AST, aspartate transaminase; ALP, alkaline phosphatase; GGT, γ-glutamyl transpeptidase; TBIL, total bilirubin; DBIL, direct bilirubin; ALB, albumin; Glob, globulin; ANA, antinuclear antibody; IgG, immunoglobulin G.

(DAMPs) or alarmins] can be recognized by local Kupffer cells, resulting in the release of cytokines and chemokines, and an increase in the recruitment of leukocytes, amongst the monocytes, from the bloodstream to the liver (Heymann and Tacke, 2016). In consideration of the hepatic portal and bile secretion systems in the gut-liver axis, Kupffer cells are the first macrophages exposed to gut-derived toxins, including lipopolysaccharide (LPS). A previous study has shown that LPS binding protein (LBP) facilitates the formation of the LPS-LBP complex and that the interaction of LBP with CD14 receptors on Kupffer cells eventually elicits strong immune responses (Su et al., 2000). Findings in animal models indicated that an LPS-related significant decrease in bile salts results in more severe liver disease (Schrumpf et al., 2017). CD163, a scavenger receptor on monocytes and macrophages, functions through binding and internalizing haemoglobin-haptoglobin complexes after erythrolysis. Several previous studies have shown that CD163 is overexpressed in the proliferation and activation of macrophages (Moller et al., 2004; Schaer et al., 2005). Soluble CD163 (sCD163) is shed from the cells into the circulation after Toll-like receptors (TLRs) are activated by inflammatory stimuli and is considered a highly specific marker for the activation of macrophages (Moestrup and Moller, 2004; Weaver et al., 2006). In addition, T/B lymphocytes represent key cellular components of the cellular immunity in DILI and are essential to maintain tissue homeostasis and ensure rapid responses to hepatic injury (Uetrecht, 2019).

As the liver is constantly exposed to gut-derived microbial products (e.g. LPS), there is accumulating evidence that gut microbial products serve as pathogen-associated molecular patterns to elicit strong immune responses (Dhillon et al., 2019). Given that LPS is an integrated part of the liver immune system, we hypothesized that portal-derived LPS and the LPS pathway components may play a role in immune responses to DILI through the activation of hepatic macrophages in DILI patients.

In this prospective study of patients with DILI, we aimed to investigate the activation of hepatic macrophages, cellular immunity, and the potential roles of the portal-derived LPS and LPS pathway proteins in DILI. The findings obtained through conducting this study may better elucidate the pathological mechanisms for DILI and may facilitate the development of more effective treatments for DILI.

Materials and methods

Patients and study design

A total of 328 patients with DILI were prospectively recruited from the Third Hospital of Hebei Medical University (Shijiazhuang, Hebei, China) during the period from January 2014 to August 2020. The diagnosis of DILI and the classification of histopathological patterns as well as disease severity were based on the Chinese Society of Hepatology (CSH) guidelines for the diagnosis and treatment of DILI and the European Association for the Study of the Liver (EASL) clinical practice guidelines for DILI. In brief, the histopathological patterns of DILI were categorized into three patterns in accordance with the R ratio for liver injury, calculated as the upper limit of normal (ULN) for alanine aminotransferase (ALT ULN)/ULN for alkaline phosphatase (ALP ULN). The hepatocellular pattern (HC) was indicated by $R \geq 5$, the cholestatic pattern (CL) was indicated by $R \leq 2$, and the mixed pattern (Mix) was indicated by $2 < R < 5$, as defined by the International Consensus Meeting for Drug-induced Liver Injury. The severity of DILI was classified into three degrees: mild, moderately severe, and extremely severe based on the DILI severity index defined in the CSH and EASL guidelines for the diagnosis and treatment of DILI (European Association for the Study of the Liver, 2019).

The DILI patients were followed up for 4 weeks and blood samples were collected at the baseline (prior to the initiation of treatment) and three follow-up time points (2, 3, and 4 weeks after treatment). Blood samples from 60 age- and gender-matched healthy individuals were used as the control blood samples. In addition, we studied liver tissues from 209 DILI patients and normal liver tissue samples from 20 healthy liver donors for transplantation in our hospital. Plasma samples were stored at -80˚C for subsequent analysis in this study. Hematoxylin and eosin (H&E) and Masson-trichrome staining were used to assess the histopathological characteristics of DILI. Liver biochemical tests, such as ALT, aspartate transaminase (AST), ALP, γ-glutamyl transpeptidase (GGT), albumin (ALB), total bilirubin (TBIL), and direct bilirubin (DBIL) were performed in the DILI patients.

This study was reviewed and approved by the Ethics Committee of Hebei Medical University (Shijiazhuang, Hebei, China) and all participants provided written informed consent prior to participation in the study.

Plasma sCD163 measurements

The levels of plasma sCD163 in the study participants were measured using a human CD163 enzyme-linked immunosorbent assay (ELISA) kit (ZCI BIO, Shanghai, China) following the manufacturer's instructions. A standard curve was prepared from five human sCD163 standards with a lower threshold of detection of 1.0 pg/mL and a coefficient of variation (CV) of 0.99%. The absorbance (O.D. value) was determined at a wavelength of 450 nm and was used to calculate the concentration of plasma sCD163.

Measurements of plasma LPS and LPS pathway proteins

The concentrations of plasma LPS in the study participants were quantified using a human LPS ELISA

kit (ZCI BIO, Shanghai, China) according to the manufacturer's instructions. To avoid LPS contamination, the measurements were performed under pyrogen-free conditions, achieved using LPS-free reactants and equipment (ZCI BIO, Shanghai, China). The results were obtained with a standard curve prepared from five human LPS standards (lower threshold of detection, 0.1 pg/mL; CV, 1.0%). The concentrations of LPS were calculated after the absorbance (O.D. value) was read at a wavelength of 450 nm.

The plasma concentrations of the LPS pathway proteins, including LBP and sCD14, were measured using human LBP and sCD14 ELISA kits (ZCI BIO, Shanghai, China) following the manufacturer's manuals. Prior to the assays, each plasma sample was diluted five-fold with PBS.

Immunohistochemistry of activated hepatic macrophages, lymphocytes, and plasma cells in the liver sections

The protein levels of CD68, CD163 (alternatively for activated M2 macrophages), CD8, CD20, and MUM-1 were examined by immunohistochemistry (IHC). In brief, liver tissues in each group were dehydrated with xylene and ethanol, and then embedded in paraffin. The paraffin-embedded liver sections (5 μm thickness) were dewaxed and rehydrated. After endogenous peroxidase was quenched, the liver sections were incubated with the primary antibodies (ZSJB-BIO, Beijing, China), followed by incubation with the secondary antibodies. The integral optical density (IOD) of positive cells was analyzed using Image-Pro Plus v6.0 software. IOD = mean density * Area. The mean density represents the average reaction intensity of all selected objects (such as positive cells) in the field of vision. The IOD is the sum of the reaction intensity of all selected objects in the whole field of view. It can reflect the total amount of protein expression in the selected area. The mean IODs were calculated from ten images of each specimen.

Statistical analysis

Statistical analysis was conducted using SPSS version 17.0 software (SPSS Inc, Chicago, IL). Data were presented as the mean ± standard deviation (SD). Comparison between groups was performed using one-way analysis of variance (ANOVA) and the Student-Newman-Keuls test. The relationships between the expression levels of CD68, CD163, CD8, CD20, MUM-1, and liver biochemistry were analyzed using multivariate linear regression and correlation analysis. P values <0.05 were considered statistically significant.

Table 1. Demographic and clinical features of the DILI patients.

Characteristics	Numbers	Percentage %
Age (41~60 years)	176	53.7
Sex (Female)	190	57.9
Yellow urine	96	29.3
Fatigue	88	26.8
Lack of appetite	84	25.6
Icteric sclera	68	20.7
Liver pain	28	8.5
Nausea	22	6.7
Icteric skin	14	4.2
Edema in both legs	8	2.4
Nausea	2	0.6

Results

Demographic, laboratory, and clinical characteristics of the DILI patients

The demographical, laboratory, and clinical characteristics of the DILI patients are summarized in Table 1. The mean age was 45.99 (SD, 14.36) years, ranging from 11 to 77 years. Of these patients, 138 (42.07%) were men and 190 (57.93%) were women. The liver biochemical and function test results for patients with different patterns of liver injury and degrees of

Table 2. Liver biochemical test results for the DILI patients according to the pattern and severity of liver injury.

Features	Total (n=328)	Hepatocellular (n=153)	Cholestatic (n=94)	Mixed (n=81)
Median laboratory parameters (range)				
ALT, U/L	426.0 (569.0-794.0)	885.0 (610.0-1380.0)	193.0 (105.0-243.0)	326.0 (177.0-656.0)
AST, U/L	295.0 (177.0-506.0)	512.0 (321.0-874.0)	183.0 (116.0-275.0)	190.0 (78.0-370.0)
GGT, U/L	187.0 (103.0-364.0)	144.0 (84.0-225.0)	253.0 (144.0-563.0)	168.0 (70.0-284.0)
ALP, U/L	178.0 (137.0-267.0)	111.0 (88.0-147.0)	267.0 (217.0-396.0)	236.0 (203.0-298.0)
TB, μmol/L	117.3 (153.4-265.2)	124.3 (92.5-204.6)	188.5 (125.2-313.7)	164.5 (102.1-244.4)
Severity of liver injury (n)				
Mild	117	50	37	30
Moderately severe	94	43	26	25
Extremely severe	117	60	31	26

ALT, alanine aminotransferase; AST: aspartate transaminase; TB: total bilirubin; DB: direct bilirubin; ALP: alkaline phosphatase; GGT: γ-glutamyl transpeptidase.

severity are shown in Table 2. The histopathological patterns included 153 (46.65%) patterns for HC or drug-induced hepatocellular injury, 94 (28.66%) patterns for CL or cholestatic injury, and 81 (24.70%) mixed patterns (Fig. 1A). The causative drugs taken by the DILI patients were classified and are listed in Table 3. Several pharmacological agents, Chinese herbal medicines, and antibacterial agents were identified as the top three categories of causative drugs for DILI, accounting for 26.2%, 23.8%, and 9.7%, respectively.

Increases in the CD68+ and CD163+ hepatic macrophages in liver sections from DILI patients

The liver sections were stained with macrophage markers (CD68 and CD163) and the resulting images were analyzed. As shown in Fig. 1B,C, CD68+ and CD163+ hepatic macrophages in the liver sections of DILI patients (brown color) were frequently observed in the hepatic sinusoid and portal areas. We especially found that CD68+ and CD163+ hepatic macrophages in patients with a mixed pattern for DILI were significantly higher compared with the levels in the control group (P<0.01, Fig. 1D,E).

Elevated levels of plasma sCD163, a marker of hepatic macrophage activation, in DILI patients

As circulating sCD163 is well documented as a marker of hepatic macrophage activation, we examined the plasma levels of sCD163 in DILI patients and performed comparative analysis between the groups. Strikingly, the plasma sCD163 concentrations were nine-fold higher in the DILI patients compared with the levels in the healthy controls (P<0.01; Fig. 2A). Furthermore, the plasma sCD163 levels were positively correlated with the severity of DILI (r=0.436, P<0.01; Fig. 2B). It was noteworthy that the plasma levels of sCD163 were significantly reduced following treatment at the 4-week follow-up visit (P<0.01; Fig. 2C), reflecting the dynamic alterations in hepatic macrophage activation.

Relationship between hepatic macrophage activation and liver biochemistry in DILI patients

The relationship between hepatic macrophage activation and liver biochemistry was assessed, and the resulting data are presented in Table 4. The IODs of CD68+ hepatic macrophages in the liver tissues were negatively correlated with the serum levels of ALP and GGT. However, CD163+ hepatic macrophages in the

Table 3. Causative drugs for different histopathological patterns of liver injury in DILI patients.

Drug properties	Patterns of liver injury			Total	
	Hepatocellular	Cholestatic	Mixed	n	%
Multiple Drugs	60	16	10	86	26.2
Chinese herbal medicines	50	14	14	78	23.8
Antibiotics	26	2	4	32	9.7
Cardiovascular drugs	14	2	6	22	6.7
Dietary supplements	10	4	6	20	6.1
Environmental toxins	12	-	4	16	4.9
Analgesic-antipyretics	10	-	2	12	3.7
Endocrine system drugs	6	2	2	10	3
Hormone drugs	4	-	-	4	1.2
Anxiolytics	2	-	-	2	0.6
Unknown drugs	18	6	16	40	12.2
Others	4	2	-	6	1.8
Total	216	48	64	328	100

Table 4. Relationships between CD68/CD163 expression and hepatic biochemistry.

Variables	CD68 integral optical density		CD163 integral optical density	
	r	P	r	P
ALT	-0.040	0.729	0.068	0.547
AST	0.002	0.989	0.063	0.584
TB	-0.191	0.102	0.342	0.003
DB	-0.202	0.102	0.381	0.001
ALP	-0.412	0.001	0.410	0.001
GGT	-0.244	0.043	0.183	0.130

r, correlation coefficient; ALT, alanine aminotransferase; AST: aspartate transaminase; TB: total bilirubin; DB: direct bilirubin; ALP: alkaline phosphatase; GGT: γ-glutamyl transpeptidase.

Table 5. Multivariate regression analyses of the relationships between sCD163, LPS pathway protein expression, and severity of DILI.

Disease severity[a]	Predictor variables	Coefficient	S.E.	P value	OR	95% CI
Mild	Intercept	7.935	2.284	0.001		
	LPS	-0.205	0.113	0.069	0.815	0.653 to 1.016
	LBP	-0.013	0.006	0.040	0.987	0.974 to 0.999
	sCD163	-0.005	0.002	0.018	0.995	0.991 to 0.999
Moderately severe	Intercept	3.855	2.195	0.079		
	LPS	0.104	0.123	0.396	1.110	0.872 to 1.411
	LBP	-0.001	0.007	0.892	0.999	0.986 to 1.012
	sCD163	-0.007	0.002	0.003	0.993	0.989 to 0.998

[a], reference category: extremely severe; SE, standard error; OR, odds ratio; CI, confidence interval.

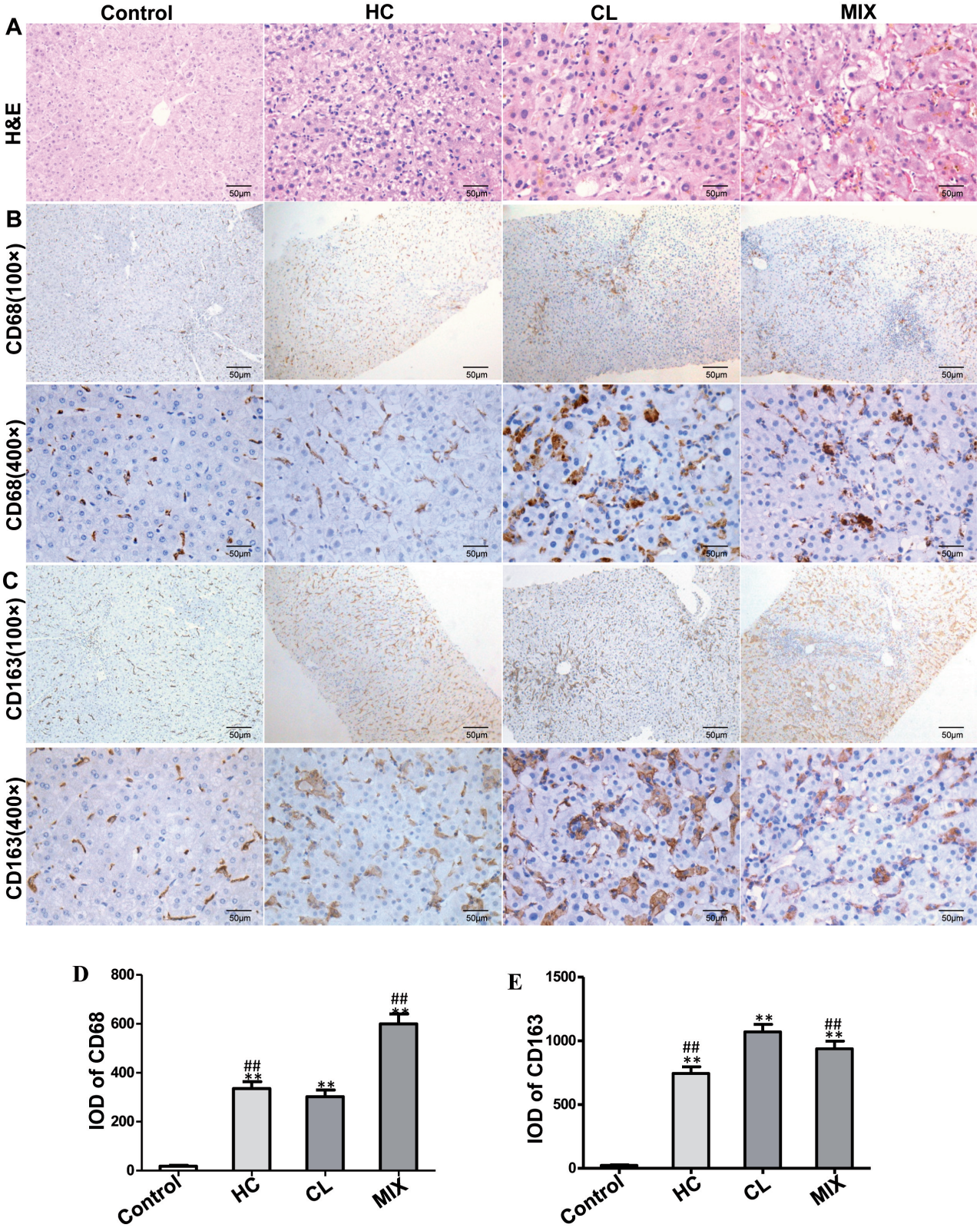

Fig. 1. Histology and immunohistochemistry of the liver sections in patients with DILI. HE staining of liver tissue sections **(A)**. The expression levels of hepatic CD68 **(B)** and CD163 **(C)** were detected with immunohistochemistry (immunohistochemical staining). The use of the antiCD68 and antiCD163 antibodies allowed us to visualize the moderate increase in the macrophage cells, especially in the areas where the hepatocyte lesions were more intense. Macrophages were found in large numbers and there was an increase in the size of macrophage cells in the mixed and cholestatic groups, suggesting an increase in the intensity of the phagocytosis process. The data are represented as the mean ± SD **(D, E)**. **, P<0.001 vs control group; ##, P<0.001 vs CL group. x 400.

liver tissues were positively correlated with the serum levels of ALP, TBIL, and DBIL. Either CD68 or CD163 expression had a correlation with the serum levels of liver biochemistry (Table 4). Multivariate regression analysis revealed that abnormally high plasma concentrations of sCD163 were associated with severe liver injury in DILI patients (Table 5).

Increases in CD8+ T lymphocytes, CD20+ B lymphocytes, and MUM-1+ plasma cells in liver sections from DILI patients

Consistently, CD8+ T cells were markedly increased in the liver sections from the DILI patients compared to those from the controls, particularly in the necrotic and portal areas (Fig. 3A,D).

CD20+ B cells and MUM-1+ plasma cells were significantly higher in the liver tissues from DILI patients compared with those from the controls. The IODs of these cells were significantly higher than those of the controls. As shown in Fig. 3B,C,E,F, the IODs of CD20+ B cells and MUM-1 plasma cells in the portal area were substantially elevated in patients with hepatocellular injury compared with the levels in patients with mixed or cholestatic liver injury ($P<0.01$).

LPS and the LPS pathway proteins were elevated in DILI patients

LPS analyses were performed using the peripheral venous blood samples of the DILI patients. As shown in Fig. 4A, the levels of LPS were eight-fold higher in the samples from DILI patients compared with the levels in samples from healthy controls ($P<0.01$). Notably, plasma-LBP was nearly 16-fold higher in the DILI patients than in control individuals ($P<0.01$, Fig. 4B), and sCD14 was nearly quadruple ($P<0.01$, Fig. 4C). In addition, the levels of LPS, LBP, and sCD14 declined during the 4 weeks of follow-up ($P<0.01$, Fig. 4A2-C2), whereas all the three indicators were higher in DILI patients in comparison with the levels in healthy individuals.

Discussion

The main novel findings of this study are summarized as follows: 1) Hepatic macrophage activation was associated with DILI. This was demonstrated by multiple lines of evidence, including strikingly higher plasma sCD163 and hepatic CD163 levels in DILI patients versus healthy controls, a positive correlation between plasma sCD163 and the severity of DILI, and a positive correlation between hepatic CD163 and serum TBIL, DBIL, and ALP; 2) CD20+ lymphocytes and MUM-1+ plasma cells were significantly higher in liver biopsy samples from DILI patients compared with the levels in the samples from healthy controls. The number of these cells in the portal area was significantly elevated in patients with an HC pattern versus those with mixed or cholestatic patterns of DILI, suggesting a potential role of the cellular immunity responses in the pathogenesis of DILI; 3) The LPS and LPS pathway proteins were significantly higher at the baseline. Their levels significantly decreased during the 4 weeks of follow-up but remained higher than those of the controls. These findings suggest that the hepatic inflammation observed in DILI patients involves the activation of hepatic macrophages and cellular immunity and that the LPS pathway may play a role, at least in part, in the responses.

The histopathological patterns and severity of DILI largely depend on the characteristics of the patients and

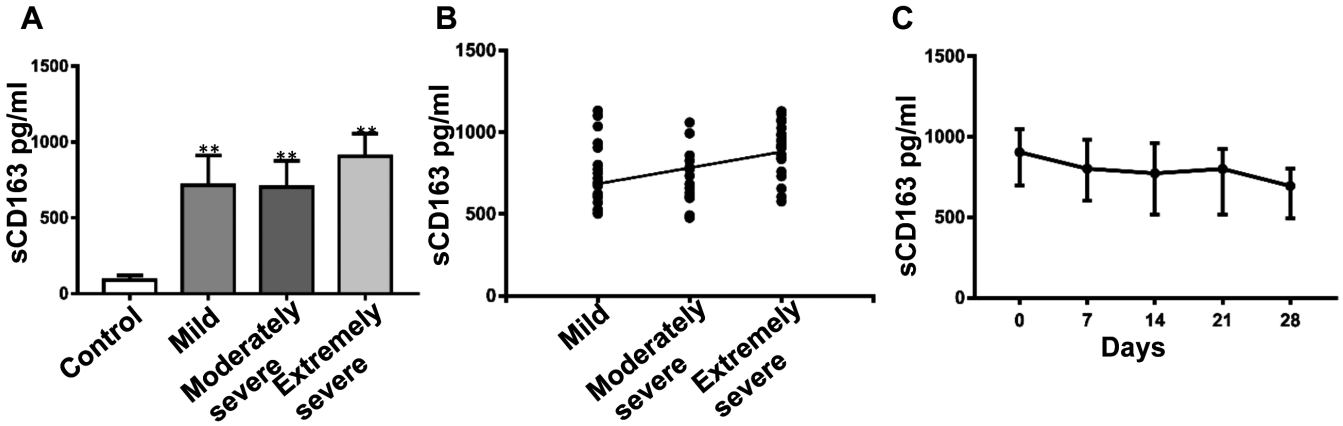

Fig. 2. Increase in hepatic macrophage activation marker levels in DILI patients and correlation with disease severity. The levels of plasma sCD163 detected in DILI patients were grouped by disease severity and compared to the levels in healthy controls. The data are shown as the mean ± SD, *$P<0.01$ compared to the controls **(A)**. The plasma levels of sCD163 were plotted according to the severity of DILI. Spearman's correlation coefficient ($r=0.436$) was determined to measure the direction (positive or negative) and the significance ($P<0.01$) of the association between sCD163 levels and disease severity **(B)**. sCD163 levels in DILI patients during 4 weeks of follow-up (median, interquartile range (IQR)). ANOVA, analysis of variance **(C)**.

the causative drugs (Andrade et al., 2019). In the present study, the demographic characteristics (e.g. middle age, female gender) and the use of multiple causative drugs that were found may be predisposing factors for DILI. Our previous study showed that the use of three or more agents was strongly related to the onset and persistence of DILI (Zhao et al., 2016). In this study, 26.2% of DILI patients developed liver injury due to the use of multiple medications. It was also noted that 23.8% of DILI cases were associated with herbs and herbal medications. Herbal medicine products have long been used as effective treatments for a broad range of chronic diseases in East Asian countries and are increasingly applied in Western countries (Wai et al., 2007; Aiso et al., 2019). Herbs and herbal medicines are erroneously considered to be side-effect-free, mainly due to the common belief that herbal medicines are derived from natural plants (Shen et al., 2019). Given the growing evidence of potential hepatotoxicity, herbs and herbal medicines should be used cautiously, especially by patients at a higher risk for DILI. In our study, antibiotics as causative drugs accounted for 9.7% of DILI cases. Therefore, reasonable use and proper management of antibiotics remain important strategies against antibiotics-induced liver damage. Furthermore, 6.1% of DILI cases resulted from the usage of dietary

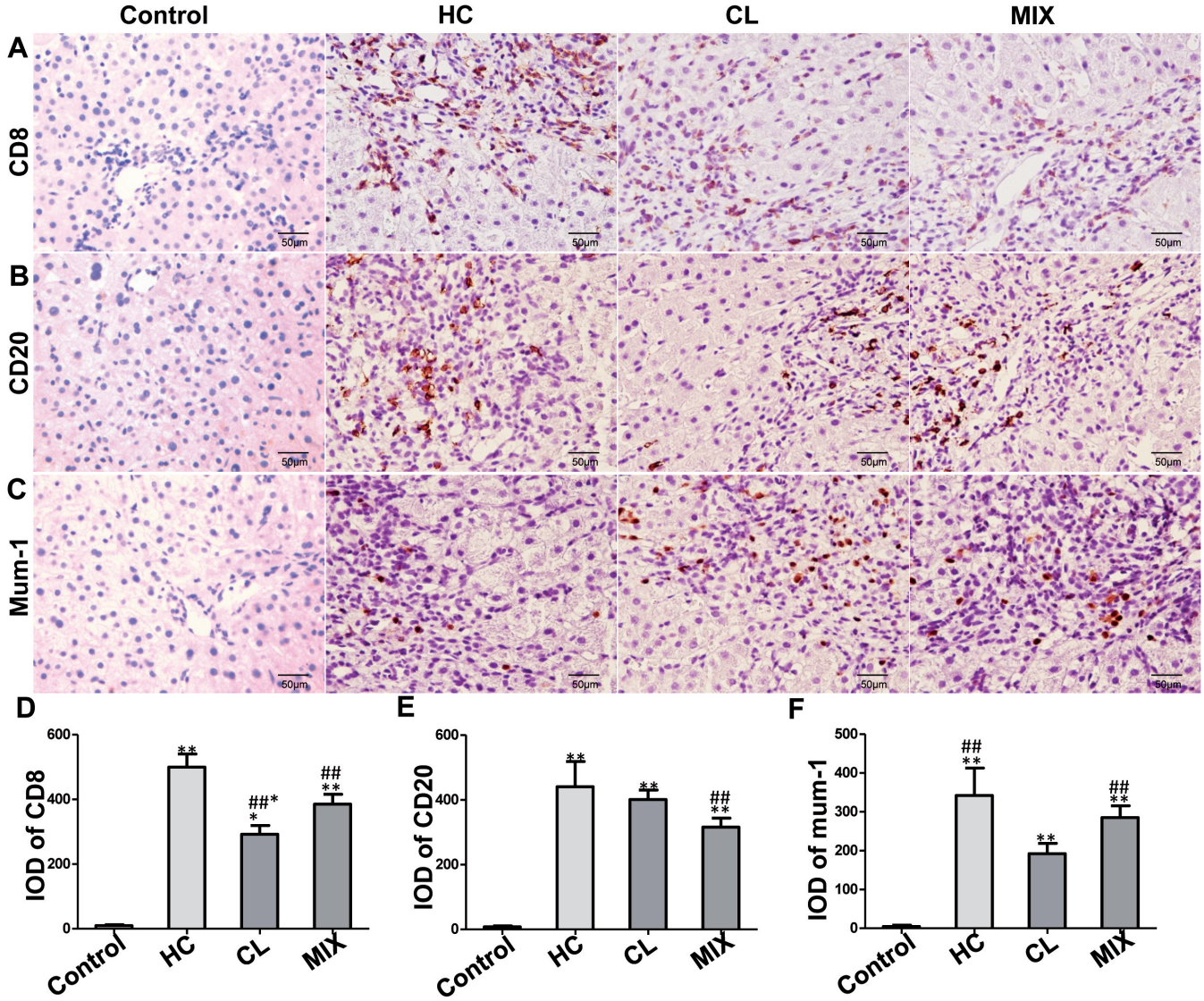

Fig. 3. T/B lymphocyte activation in the liver tissues of DILI patients. The expression of CD8 was detected using immunohistochemistry (immunohistochemical staining. The data are represented as the mean ± SD **(A)**. **, P<0.001 vs control group; ##, P<0.001 vs HC group. The expression levels of CD20 **(B)** and MUM-1 **(C)** were detected with immunohistochemistry (immunohistochemical staining). The data are expressed as the mean ± SD **(D-F)**. **, P<0.001 vs control group; ##, P<0.001 vs CL group. x 400.

supplements, similar to those from cardiovascular drugs (6.7%), but higher than those from environmental toxins (4.9%). These findings suggest that both users and providers should be aware of the potential hepatotoxicity of dietary supplements.

It may merit attention in this study that the hepatic inflammation observed in DILI patients involved the activation of hepatic macrophages, cellular immunity responses, and the LPS pathway. Although the exact pathological mechanisms for DILI are largely unknown, it has been proposed that one of the key mechanisms for drug-related immune activation is the response of hepatic macrophages, lymphocytes, and plasma cells to damage-associated molecular patterns (DAMPs), resulting in the release of cytokines and chemokines as well as the recruitment of leukocytes, amongst the monocytes, from the bloodstream into the liver (Kaplowitz, 2005; Roth et al., 2017). In support of these hypothetical mechanisms, our findings suggest that hepatic macrophages were likely activated by LPS. Hepatic macrophage activation was indicated by the high plasma concentration of sCD163 and was confirmed based on the hepatic macrophage activation in the liver tissue. The expression of CD8, CD20, and

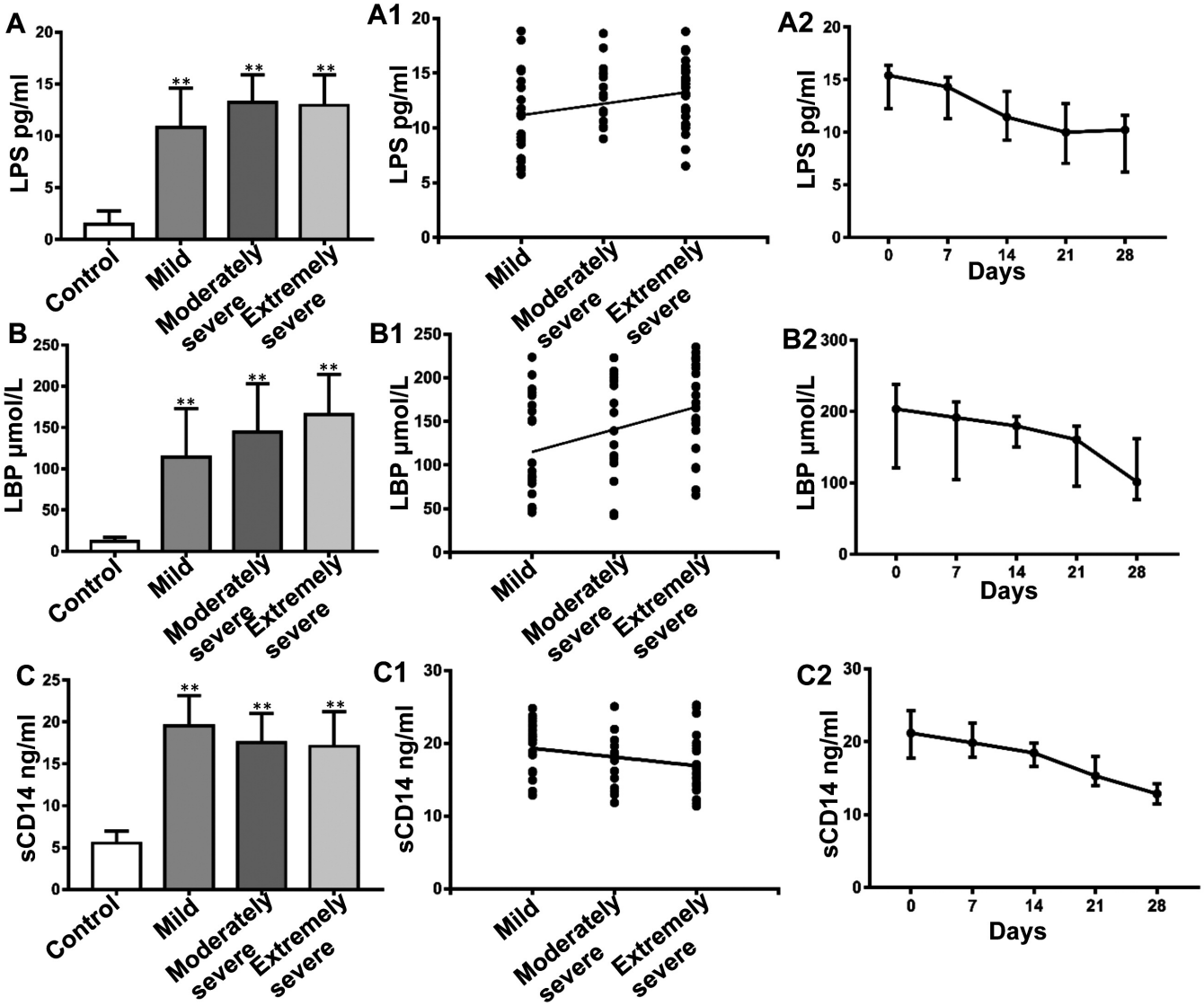

Fig. 4. Increases in levels of LPS and LPS pathway proteins in DILI and their correlation with disease severity. The levels of LPS, LBP, and sCD14 were determined in the plasma of DILI patients and compared to those of healthy controls. The data are shown as the mean ± SD, *P<0.01 compared to the controls **(A-C)**. The plasma concentrations of LPS and LPS pathway proteins were plotted according to the severity of DILI. Spearman's correlation coefficient (r) was determined to measure the direction (positive or negative) and significance (P) of the association between these variables and disease severity **(A1, B1, C1)**. LPS, LBP, and sCD14 levels in DILI patients during 4 weeks of follow-up (median, interquartile range (IQR)). ANOVA, analysis of variance **(A2, B2, C2)**.

MUM-1 in liver biopsy samples was also elevated. The origin of the plasma sCD163, which was directly confirmed by an increase in sCD163 from the portal to the hepatic vein, was determined to be the liver in a cirrhotic portal hypertension study (Holland-Fischer et al., 2011). Hepatic macrophage activation could be a result of LPS and the activation of the LPS protein pathway because of the systematic and consistent interactions between LPS-related variables and sCD163 during the follow-up period.

Some previous studies have indicated that CD163+ and CD68+ Kupffer cells have pro-inflammation and anti-inflammation functions, respectively, in nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH) (Ramachandran et al., 2012; Tacke and Zimmermann, 2014; Mehal and Schuppan, 2015; Kazankov et al., 2019). It is well documented that Kupffer cells are activated under various pathological conditions, and can differentiate into classically activated macrophages (M1-like) and alternatively activated macrophages (M2-like) (Murray et al., 2014). Consequently, alterations in hepatic macrophage function are associated with liver injuries. Classical M1 macrophages are anti-inflammatory, whereas alternative M2 macrophages are pro-inflammatory and pro-fibrogenic (Tacke and Zimmermann, 2014; Eming et al., 2017). CD68 (alteration of hepatic macrophages) and CD163 (M2 alteration of hepatic macrophages) antigens are usually used as surface markers for different subtypes of M2 macrophages and activated hepatic macrophages (Tacke and Zimmermann, 2014).

In addition, we found that the expression of CD8 was significantly increased in liver biopsy samples from DILI patients and was particularly high in samples with the hepatocellular pattern of liver injury. The pathogenesis of liver injury involves drug-induced critical alterations of hepatocytes and/or cholangiocytes. The overexpression of CD8 can activate host immune factors that infiltrate the liver and kill hepatocytes (usually the primary foci of injury) and/or cholangiocytes (less frequently the primary foci of injury). Furthermore, in addition to T cell activation, B cell (CD20 positive) and plasma cell (MUM-1 positive) infiltration was also observed in the hepatic inflammatory and portal areas. This indicates that the humoral immune responses also participate in the pathogenesis of DILI and an abnormal autoimmune response might be one of the key mechanisms of severe and chronic DILI. The results of a hepatitis B virus study suggested that the impairment of CD8+ T cell responses is mediated by Kupffer cells and that the depletion of Kupffer cells leads to CD8+ T cell activation (Tian et al., 2016). This is consistent with our finding that CD8, CD163, and CD68 were highly expressed in liver biopsy samples from DILI patients. Flucloxacillin induces liver injury through CD8+ T cells, which mediate the cellular immune response and B cell-mediated immune responses have been observed in patients with liver injury induced by isoniazid (Monshi et al., 2013; Metushi et al., 2014). Our study suggested that the activation of cellular immunity, including hepatic macrophages and T/B cells, is likely to be involved in the onset and progression of DILI, but further study will be needed to investigate whether there are interactions between hepatic macrophages and T/B cells in DILI.

The results of our study may have clinical implications. For instance, the hepatic macrophage activation in DILI can be blocked or reversed. Immunosuppression with glucocorticoids is a common treatment option for patients with severe DILI. Research has shown that targeted delivery of glucocorticoids to hepatic macrophages for the treatment of autoimmune liver diseases as well as after liver transplantation is a promising approach for minimizing side effects. Our study provides another approach to reduce hepatic macrophage activation by altering gut microbes or blocking the LPS protein pathway, and thereby reducing liver damage in DILI patients.

In conclusion, hepatic macrophage activation, along with cellular immune responses, is involved in the onset and progression of DILI, and the LPS pathway is likely involved in the hepatic responses. Furthermore, this study has demonstrated, for the first time, to the best of our knowledge, that hepatic macrophage activation in DILI is positively correlated with disease severity, and plasma sCD163 holds potential as a non-invasive marker for assessing the severity of the disease. Therefore, the present study has improved our understanding of the pathological mechanisms underlying DILI and may facilitate the development of better treatments for DILI.

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Authors' contributions. YN and JZ designed the study; HD, SZ, WZ, NF, XN, and ZY performed the experiments; HD and NF analyzed the data; YN, DH and SZ wrote the paper.

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References

Aiso M., Takikawa H., Tsuji K., Kagawa T., Watanabe M., Tanaka A., Sato K., Sakisaka S., Hiasa Y., Takei Y., Ohira H., Ayada M., Hashimoto E., Kaneko S., Ueno Y., Ohmoto K., Takaki A., Torimura T., Matsuzaki Y., Tajiri K., Yoneda M., Ito T., Kato N., Ikejima K., Mochida S., Yasuda H. and Sakamoto N. (2019). Analysis of 307 cases with drug-induced liver injury between 2010 and 2018 in Japan. Hepatol. Res. 49, 105-110.

Andrade R.J., Chalasani N., Bjornsson E.S., Suzuki A., Kullak-Ublick G.A., Watkins P.B., Devarbhavi H., Merz M., Lucena M.I., Kaplowitz

N. and Aithal G.P. (2019). Drug-induced liver injury. Nat. Rev. Dis. Primers. 5, 58.

Dhillon A.K., Kummen M., Troseid M., Akra S., Liaskou E., Moum B., Vesterhus M., Karlsen T.H., Seljeflot I. and Hov J.R. (2019). Circulating markers of gut barrier function associated with disease severity in primary sclerosing cholangitis. Liver Int. 39, 371-381.

Eming S.A., Wynn T.A. and Martin P. (2017). Inflammation and metabolism in tissue repair and regeneration. Science 356, 1026-1030.

European Association for the Study of the Liver. Electronic address e. e. e., Clinical Practice Guideline Panel C., Panel m. and representative E.G.B. (2019). EASL Clinical Practice Guidelines: Drug-induced liver injury. J. Hepatol. 70, 1222-1261.

Heymann F. and Tacke F. (2016). Immunology in the liver--from homeostasis to disease. Nat. Rev. Gastroenterol. Hepatol. 13, 88-110.

Holland-Fischer P., Gronbaek H., Sandahl T.D., Moestrup S.K., Riggio O., Ridola L., Aagaard N.K., Moller H.J. and Vilstrup H. (2011). Kupffer cells are activated in cirrhotic portal hypertension and not normalised by TIPS. Gut 60, 1389-1393.

Kaplowitz N. (2005). Idiosyncratic drug hepatotoxicity. Nat. Rev. Drug Discov. 4, 489-499.

Kazankov K., Jorgensen S. M.D., Thomsen K.L., Moller H.J., Vilstrup H., George J., Schuppan D. and Gronbaek H. (2019). The role of macrophages in nonalcoholic fatty liver disease and nonalcoholic steatohepatitis. Nat. Rev. Gastroenterol Hepatol. 16, 145-159.

Krenkel O. and Tacke F. (2017). Liver macrophages in tissue homeostasis and disease. Nat. Rev. Immunol. 17, 306-321.

Mehal W. Z. and Schuppan D. (2015). Antifibrotic therapies in the liver. Semin. Liver Dis. 35, 184-198.

Metushi I.G., Sanders C., Acute Liver Study G., Lee W.M. and Uetrecht J. (2014). Detection of anti-isoniazid and anti-cytochrome P450 antibodies in patients with isoniazid-induced liver failure. Hepatology 59, 1084-1093.

Moestrup S.K. and Moller H.J. (2004). CD163: a regulated hemoglobin scavenger receptor with a role in the anti-inflammatory response. Ann. Med. 36, 347-354.

Moller H.J., de Fost M., Aerts H., Hollak C. and Moestrup S.K. (2004). Plasma level of the macrophage-derived soluble CD163 is increased and positively correlates with severity in Gaucher's disease. Eur. J. Haematol. 72, 135-139.

Monshi M.M., Faulkner L., Gibson A., Jenkins R.E., Farrell J., Earnshaw C.J., Alfirevic A., Cederbrant K., Daly A.K., French N., Pirmohamed M., Park B.K. and Naisbitt D.J. (2013). Human leukocyte antigen (HLA)-B*57:01-restricted activation of drug-specific T cells provides the immunological basis for flucloxacillin-induced liver injury. Hepatology 57, 727-739.

Murray P.J., Allen J.E., Biswas S.K., Fisher E.A., Gilroy D. W., Goerdt S., Gordon S., Hamilton J. A., Ivashkiv L.B., Lawrence T., Locati M., Mantovani A., Martinez F.O., Mege J.L., Mosser D. M., Natoli G., Saeij J.P., Schultze J. L., Shirey K.A., Sica A., Suttles J., Udalova I., van Ginderachter J.A., Vogel S.N. and Wynn T.A. (2014). Macrophage activation and polarization: nomenclature and experimental guidelines. Immunity 41, 14-20.

Ramachandran P., Pellicoro A., Vernon M.A., Boulter L., Aucott R.L., Ali A., Hartland S.N., Snowdon V.K., Cappon A., Gordon-Walker T.T., Williams M.J., Dunbar D.R., Manning J.R., van Rooijen N., Fallowfield J.A., Forbes S.J. and Iredale J.P. (2012). Differential Ly-6C expression identifies the recruited macrophage phenotype, which orchestrates the regression of murine liver fibrosis. Proc. Natl. Acad. Sci. USA 109, E3186-3195.

Roth R.A., Maiuri A.R. and Ganey P.E. (2017). Idiosyncratic drug-induced liver injury: Is drug-cytokine interaction the linchpin?. J. Pharmacol. Exp. Ther. 360, 461-470.

Schaer D.J., Schleiffenbaum B., Kurrer M., Imhof A., Bachli E., Fehr J., Moller H.J., Moestrup S.K. and Schaffner A. (2005). Soluble hemoglobin-haptoglobin scavenger receptor CD163 as a lineage-specific marker in the reactive hemophagocytic syndrome. Eur. J. Haematol. 74, 6-10.

Schrumpf E., Kummen M., Valestrand L., Greiner T.U., Holm K., Arulampalam V., Reims H.M., Baines J., Backhed F., Karlsen T.H., Blumberg R. S., Hov J.R. and Melum E. (2017). The gut microbiota contributes to a mouse model of spontaneous bile duct inflammation. J. Hepatol. 66, 382-389.

Shen T., Liu Y., Shang J., Xie Q., Li J., Yan M., Xu J., Niu J., Liu J., Watkins P.B., Aithal G.P., Andrade R.J., Dou X., Yao L., Lv F., Wang Q., Li Y., Zhou X., Zhang Y., Zong P., Wan B., Zou Z., Yang D., Nie Y., Li D., Wang Y., Han X., Zhuang H., Mao Y. and Chen C. (2019). Incidence and etiology of drug-induced liver injury in mainland China. Gastroenterology 156, 2230-2241 e2211.

Su G.L., Klein R.D., Aminlari A., Zhang H.Y., Steinstraesser L., Alarcon W.H., Remick D.G. and Wang S.C. (2000). Kupffer cell activation by lipopolysaccharide in rats: role for lipopolysaccharide binding protein and toll-like receptor 4. Hepatology 31, 932-936.

Tacke F. and Zimmermann H.W. (2014). Macrophage heterogeneity in liver injury and fibrosis. J. Hepatol. 60, 1090-1096.

Tian Y., Kuo C.F., Akbari O. and Ou J.H. (2016). Maternal-derived hepatitis B virus e antigen alters macrophage function in offspring to drive viral persistence after vertical transmission. Immunity 44, 1204-1214.

Uetrecht J. (2019). Mechanisms of idiosyncratic drug-induced liver injury. Adv. Pharmacol. 85, 133-163.

Wai C.T., Tan B.H., Chan C.L., Sutedja D.S., Lee Y.M., Khor C. and Lim S.G. (2007). Drug-induced liver injury at an Asian center: a prospective study. Liver Int. 27, 465-474.

Weaver L.K., Hintz-Goldstein K.A., Pioli P.A., Wardwell K., Qureshi N., Vogel S.N. and Guyre P.M. (2006). Pivotal advance: activation of cell surface Toll-like receptors causes shedding of the hemoglobin scavenger receptor CD163. J. Leukoc. Biol. 80, 26-35.

Weaver R.J., Blomme E.A., Chadwick A.E., Copple I.M., Gerets H.H.J., Goldring C.E., Guillouzo A., Hewitt P.G., Ingelman-Sundberg M., Jensen K.G., Juhila S., Klingmuller U., Labbe G., Liguori M.J., Lovatt C.A., Morgan P., Naisbitt D.J., Pieters R.H.H., Snoeys J., van de Water B., Williams D.P. and Park B.K. (2020). Managing the challenge of drug-induced liver injury: a roadmap for the development and deployment of preclinical predictive models. Nat. Rev. Drug Discov. 19, 131-148.

Zhao S., Zhang Y., Tan P., Wang R. and Nan Y. (2016). Clinical fetures of drug-induced autoimmune hepatitis and drug-induced liver injury: a comparative analysis. Chin. J. Hepatol. 4, 302-306.

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