Clinical & Experimental Immunology
The Journal of Translational Immunology

Clinical and Experimental Immunology ORIGINAL ARTICLE doi:10.1111/cei.12558

Comparative analysis of portal hepatic infiltrating leucocytes in acute drug-induced liver injury, idiopathic autoimmune and viral hepatitis

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Accepted for publication 16 November 2014
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Summary

Drug-induced liver injury (DILI) is often caused by innate and adaptive host immune responses. Characterization of inflammatory infiltrates in the liver may improve understanding of the underlying pathogenesis of DILI. This study aimed to enumerate and characterize leucocytes infiltrating liver tissue from subjects with acute DILI ($n = 32$) *versus* non-DILI causes of acute liver injury ($n = 25$). Immunostains for CD11b/CD4 (Kupffer and T helper cells), CD3/CD20 (T and B cells) and CD8/CD56 [T cytotoxic and natural killer (NK) cells] were evaluated in biopsies from subjects with acute DILI, either immunoallergic (IAD) or autoimmune (AID) and idiopathic autoimmune (AIH) and viral hepatitis (VH) and correlated with clinical and pathological features. All biopsies showed numerous $CD8^+$ T cells and macrophages. DILI cases had significantly fewer B lymphocytes than AIH and VH and significantly fewer NK cells than VH. Prominent plasma cells were unusual in IAD (three of 10 cases), but were associated strongly with AIH (eight of nine) and also observed in most with AID (six of nine). They were also found in five of 10 cases with VH. Liver biopsies from subjects with DILI were characterized by low counts of mature B cells and NK cells in portal triads in contrast to VH. NK cells were found only in cases of VH, whereas AIH and VH both showed higher counts of B cells than DILI. Plasma cells were associated most strongly with AIH and less so with AID, but were uncommon in IAD.

Keywords: autoimmunity, inflammation, drug-induced liver injury, viral hepatitis

Introduction

Acute liver failure (ALF) or fulminant hepatitis (FH) has a mortality rate >70% in the absence of supportive management or liver transplantation [1]. In the United States, ALF/FH is due mainly to drug-induced liver injury (DILI), viral hepatitis (VH) and idiopathic autoimmune hepatitis AIH). Reliable biomarkers are available to aid in the diagnosis of VH and AIH (e.g. serological testing for hepatitis A–E viral antigens, antibodies and nucleic acids [2], or evidence of autoantibodies and hyperglobulinaemia [3,4], respectively). Conversely, drug-induced liver injury (DILI) is more difficult to establish and is often missed. Currently, it is a diagnosis of exclusion and relies upon a history of exposure to an agent that causes liver injury (drug, herbal product or dietary supplement) and upon exclusion of

other potential causes, such as viral, autoimmune or ischaemic hepatitis or extrahepatic obstruction of the bile ducts.

The normal liver contains resident immune cells that differ in proportion from those in peripheral blood. These include a large number of natural killer (NK) and Kupffer cells. These specialized immune cells play essential roles in both liver immune homeostasis and the immunopathology of liver diseases. For example, NK cells participate with $CD8^+$ cytotoxic T cells in suppression of liver metastases [5]. Dramatic increases of hepatic NK cells have been described in hepatitis C virus (HCV)-infected livers, and early release of type I–III interferons (IFNs) by NK cells has been shown to be associated with effective clearance of the virus in those individuals who clear the infection spontaneously [6,7]. Kupffer cells produce large amounts of tumour necrosis factor (TNF)-α in response to microbial infection [8]. Activation of Kupffer cells, and subsequent nitric oxide (NO) production, are major determinants of peroxynitrite formation and, *inter alia*, severity of acetaminophen liver toxicity [9]. While the liver is a site where lymphocytes undergo apoptosis during the clearance/resolution phase of peripheral immune response, infiltrating activated B and T lymphocytes in diseased livers have also been associated with hepatic histopathology. For instance, the ratio between pro- and anti-inflammatory $CD4^+$ T helper lymphocytes in the liver can alter the balance in favour of or against fibrosis in *Schistosoma mansoni*-infected mice [10], and CCl_4-induced fibrosis is reduced in B cell-deficient mice [11].

To date, there have been few, if any, studies that have carefully enumerated or characterized the leucocytes that infiltrate the livers of patients with clinically important DILI, compared to liver injury of other causes. In this work we enumerated and characterized portal mononuclear cell infiltrates of liver biopsies from subjects with acute hepatitis of DILI (with autoimmune or immunoallergic features) or non-DILI origins (idiopathic autoimmune, viral), and correlated clinical and histopathological features with particular profiles of liver-infiltrating leucocytes. Hepatic immune profiles were also established, in an exploratory fashion, for cases of acute intrinsic-type DILI (e.g. triggered by acetaminophen) or liver injury associated with the use of herbal remedies. We have developed and optimized procedures for dual-immunohistochemical (IHC) staining of mononuclear cells of myeloid (macrophages/Kupffer cells) and lymphoid (T, B and NK cells) origin infiltrating the liver. In hepatic portal triads, macrophages ($CD11b^+$) and T cells ($CD3^+$) were predominant in injured liver, regardless of the causative drug or clinicopathological phenotype, and were associated with the degree of liver damage. T cytotoxic ($CD8^+$) cells represented more than 90% of total T cell infiltrates in the liver. While liver tissue from DILI subjects had few B cells ($CD20^+$), they were numerous in idiopathic AIH and acute VH. Ninety per cent of idiopathic AIH subjects and 70% of autoimmune DILI cases (AID) had significant portal hepatic plasma cells infiltrate. NK cells ($CD56^+$) were found only in liver biopsies obtained from subjects with VH.

Material and methods

Subjects studied

Liver tissues from 57 subjects with acute hepatitis were investigated in this study. Selection criteria for biopsy or explanted liver to be included in the study were: (i) well-characterized trigger of liver inflammation, (ii) complete pathology report available, (iii) complete laboratory results available (including blood count, hepatic/renal damage markers), (iv) complete medical history available (including medications and herbals taken) and (v) immune features and causative drug(s) available for DILI patients.

Diagnosis of acute VH was based on compatible history, physical examination, abdominal imaging studies and on seropositivity for acute HBV, HCV or HEV infection. Diagnosis of idiopathic AIH was based on compatible clinical presentation, exclusion of DILI or VH as cause and presence of circulating autoantibodies, as well as high prevalence of hepatic plasma cells. Eleven AIH cases and 14 VH cases (seven due to HBV, six to HCV and one to HEV) were included in the study (Table 1). Liver samples were retrieved from tissue blocks stored at Carolinas Healthcare System (CHS)'s Pathology Department of Pathology or provided by the acute liver failure (ALF) study group. They included 18 needle biopsies, six explanted livers and one from autopsy (selected by H.L.B., W.A.A. and C.J. from CHS and W.M.L. from the ALF study group). The IRB of CHS approved all studies. All liver biopsies and other liver samples studied had been obtained as part of the standard of care and not as part of the DILIN study, or of this follow-on study.

Diagnosis of acute DILI was one of exclusion (non-VH, non-AIH) and based on patient medication history; the process used by DILIN for assignment of causality was described previously [12]. Immunoallergic DILI (IAD) was defined as acute liver injury accompanied by symptoms of hypersensitivity such as rash, fever, lymphadenopathy, oedema and eosinophilia. Autoimmune DILI (AID) was defined as liver injury due to drugs known to cause such liver injury and accompanied by development of autoantibodies, a hepatocellular pattern of serum enzyme elevations [13,14]. Twelve IAD and nine AID cases enrolled into the prospective protocol of the US Drug Induced Liver Injury Network (DILIN) [15,16] and consented to participate in protocols approved by one of the eight clinical sites of the network were included in the study (Table 1). Unstained recuts from liver biopsies were made available for central pathological review [Laboratory of Pathology, NIH

Table 1. Selected demographic and laboratory results among subjects studied.

	Immunoallergic DILI ($n = 13$)		Autoimmune DILI ($n = 11$)		Idiopathic AIH ($n = 11$)		Viral hepatitis ($n = 14$)	
	Mean (± s.d.)	Min–max	Mean (± s.d.)	Min–max	Mean (± s.d.)	Min–max	Mean (± s.d.)	Min–max
Age (years)	44·8 (± 20·6)	12–81	48·9 (± 25·6)	19–84	17·0 (± 16·9)	1–65	38·0 (± 16·9)	14–63
Gender (M/F)	9F, 4M		9F, 2M		6F, 5M		8F, 6M	
Race	8W, 3B, 2O		8W, 1B, 2O		5W, 6B		9W, 3B, 2O	
BMI (kg/m^2)	26·3 (± 3·8)	20·5–31·6	26·1 (± 6·4)	20·5–41·0	19·7 (± 2·4)	15·1–22·9	26·4 (± 5·7)	16·9–36·7
Causality	2 lamotrigine, 7 amoxicillin/clavulanic acid, 4 other		6 nitrofurantoin, 4 minocyline, 1 other		Unknown		7 HBV, 6 HCV, 1 HEV	
Haemoglobin (g/dl)	12·6 (± 0·5)	8·2–15·9	13·9 (± 2·4)	7·8–16·7	12·6 (± 1·8)	9·6–14·8	13·0 (± 1·7)	9–15·5
WBC (k/ul)	7·3 (± 3·9)	1·1–17·2	9·5 (± 4·2)	4·9–16·4	8·6 (± 6·9)	3·3–26·4	7·6 (± 3·0)	3·2–15·0
Absolute eosinophil count (#/ul)	168 (± 246)	0–739	125 (± 127)	0–330	152 (± 235)	0–790	123 (± 187)	0–300
Platelets (k/ul)	254 (± 103)	78–448	268 (± 88)	146–444	257 (± 97)	112–428	193 (± 77)	64·9-327
T. Bili (mg/dl)	11·2 (± 9·6)	0·5–35·0	7·7 (± 8·5)	0·3–26·4	7·5 (± 6·1)	0·9–20·9	5·9 (± 5·8)	0·4–16·7
D. Bili (mg/dl)	6·1 (± 5·0)	0·1–14·5	3·2 (± 3·1)	0–7·7	3·4 (± 2·8)	0·2–8·1	3·4 (± 3·9)	0·1–9·9
Alk. Phos (IU/l)	241 (± 95)	79–389	219 (± 185)	55–678	238 (± 120)	50–475	134 (± 74)	36–262
GGTP (IU/l)	69·6 (± 68·7)	11–161	181 (± 1159)	16–356	90·5 (± 72·5)	24–267	48·0 (± 41·6)	11–113
ALT (IU/l)	378 (± 396)	23–1451	644 (± 799)	35–2333	1081 (± 777)	114–2652	1490 (± 2852)	20–10956
AST (IU/l)	350 (± 361)	18–1053	439 (± 449)	31–1240	1118 (± 971)	88–2784	657 (± 1084)	19–4126
Albumin (g/dl)	3·3 (± 0·9)	1·6-4·6	3·4 (± 0·7)	2·2–4·6	3·4 (± 0·6)	2·2–4·6	3·5 (± 0·5)	2·7-4·4
T. protein (g/dl)	6·4 (± 0·7)	4·8-7·3	7·4 (± 0·8)	5·9-8·7	7·4 (± 1·0)	6–9·1	6·3 (± 0·8)	5·1–7·5
INR	1·6 (± 0·7)	0·9-2·7	1·2 (± 0·3)	1-1·93	1·5 (± 0·8)	1–3·96	2·2 (± 2·5)	1–8·3
BUN (mg/dl)	10·0 (± 6·3)	2–19	14·2 (± 6·8)	6–26	8·4 (± 4·5)	2–17	9·5 (± 7·0)	2–28
Serum creatinine (mg/dl)	0·7 (± 0·1)	0·5-1·1	1·2 (± 1·0)	0·7-4·2	0·5 (± 0·1)	0·1-0·8	1·0 (± 0·8)	0·4-3·6
ANA	9 Neg, 3 Pos, 1 n.a.		1 Neg, 9 Pos, 1 n.a.		4 Neg, 7 Pos		7 Neg, 3 Pos, 4 n.a.	
ASMA	9 Neg, 2 Pos, 2 n.a.		8 Neg, 2 Pos, 1 n.a.		11 Pos		1 Neg, 8 Pos, 5 n.a.	
AMA	8 Neg, 5 n.a.		9 Neg, 2 n.a.		6 Pos, 5 n.a.		1 Neg, 3 Pos, 10 n.a.	
IgG (mg/dl)	1131 (± 147)	1026–1350	1627 (± 272)	1340–1960	2233 (± 1072)	1239–4054	1096 (± 358)	759–1600
IgA (mg/dl)	303 (± 169)	125–463	277 (± 191)	160–498	116 (± 41)	81–198	234 (± 84)	129–345
IgM (mg/dl)	135 (± 70)	38–200	278 (± 11)	170–392	103 (± 49)	57–193	127 (± 73)	56–252
Total globulin (g/dl)	3·3 (± 0·9)	2·1–5·2	4·0 (± 0·8)	3·3–5·9	4·0 (± 1·2)	2·5-5·8	2·7 (± 0·6)	1·7-3·7
MELD	18·7 (± 7·2)	7–27·7	16·4 (± 8·9)	6–37·6	11·2 (± 7·9)	3·7–28·4	15·8 (± 15·3)	0–52

Alk. Phos = alkaline phosphatase; ALT = alanine aminotransferase; AMA = anti-mitochondrial autoantibodies; ANA = anti-nuclear autoantibodies; ASMA = anti-smooth muscle autoantibodies; AST = aspartate aminotransferase; B = black; BMI = body mass index; BUN = blood urea nitrogen; D. Bili = direct bilirubin; DILI = drug-induced liver injury; F = female; GGTP = gamma glutamyl transpeptidase; INR = international normalized ratio; HBV = hepatitis B virus; HCV = hepatitis C virus; HEV = hepatitis E virus; Ig G/A/M = immunoglobulin G/A/M; M = male; MELD = model of end-stage liver disease; n.a. = not available; Neg = negative; O = other; Pos = positive; s.d. = standard deviation; T. Bili = total bilirubin; T. protein = total protein; W = white.

(DEK)]. The ALF study group biorepository provided tissue blocks from an additional 11 DILI subjects, including: one IAD, two AID, six intrinsic and two herbal DILI cases. From a total of 32 selected DILI cases (DILIN and ALF), seven were attributed to amoxicillin/clavulanic acid, two to lamotrigine, six to nitrofurantoin, four to minocycline, six to acetaminophen and seven to other drugs (Table 1 and Supporting information, Table S1).

Immunohistochemical (IHC) staining and histopathological scores

Unstained slides or paraffin-embedded liver blocks were sectioned and stained at the research histology laboratory at Carolinas Medical Center (CMC), where the specialized dual-staining technique for leucocytes was developed, and applied to the biopsies. After deparaffinization and rehydration, heat-induced epitope retrieval was performed using ethylenediamine tetraacetic acid (EDTA) citrate buffer pH 7·5–8·0 (Polyscientific, Bay Shore, NY, USA). To demonstrate positive staining of each single-stain antibody, a tonsil slide and an HBV-positive explanted liver slide were stained with each antibody individually. Antibodies were incubated for 1 h at room temperature with the exception of that for CD4, which was incubated overnight at 4°C. ImmPRESS peroxidase polymer detection systems for either rabbit or mouse were used to detect bound antibody following the manufacturer's instructions (Vector Laboratories, Burlingame, CA, USA). Visualization of antibody–polymer complex was performed using either the diaminobenzidine (DAB) or Vector VIP® peroxidase substrate. Slides were counterstained with methyl green, dehydrated and mounted with a coverslip and Permount. Dual staining was carried out according to the Multiple Antigen Labeling guide published by Vector Laboratories using the recommended ImmPRESS peroxidase detection systems described above.

Sections (4 μm) were immunostained for CD11b/CD4 (myeloid Kupffer and T helper cells), CD3/CD20 (T and B cells) or CD8/CD56 (T cytotoxic and NK cells) (Supporting information, Table S2). Whenever possible, five portal triads were scored for each liver biopsy or explanted tissue. Numbers of cells in portal tracts that stained positively for CD11b, CD4, CD8, CD3, CD20 or CD56, normalized to 10 000 μm^2 surface area, were recorded by two observers (D.F. and K.C.) and validated independently by two additional observers (D.E.K., T.W.). Among the 49 subjects with immunoallergic/autoimmune DILI, AIH and VH enrolled into the study, 14 needle biopsies had only three to four observable portal triads.

Hepatic histopathological features were recorded for all liver biopsies or explanted livers. Fibrosis stage, necroinflammatory grade, extent of hepatic iron and fat deposit were scored as described previously [17,18]. Portal plasma cells and neutrophils were evaluated on haematoxylin and eosin (H&E)-stained liver sections and scored by D.E.K. or C.J. as 0 (rare or nil) or 1 [prominent (focal aggregates, numerous)] as described previously [18].

Statistical methods

Descriptive statistics, including means and standard deviations (SDs) or counts and percentages, were calculated for the overall data and by diagnostic category (immunoallergic DILI, autoimmune DILI, idiopathic AIH and VH). As multiple measurements were taken on each subject, repeated-measures analyses using generalized estimating equations (GEE) were performed to compare cell counts of leucocytes among the five disease groups. For each liver chemistry and each systemic immune marker, data were divided into three categories; <lower limit of normal (LLN), normal and >upper limit of normal (ULN), and GEE were used to compare cell counts among the groups. SAS® Enterprise Guide® version 5·1 was used for all analyses. A two-tailed *P*-value of less than 0·05 was considered statistically significant.

Results

Clinical data and pathological assessments

Demographics were similar across disease groups. Sixty-five per cent of subjects were women and 60% were Caucasians. By chance, six of 11 subjects with idiopathic AIH were children and had lower body mass indices (BMIs). As expected, high levels of serum alkaline phosphatase, γ-glutamyl transpeptidase (GGTP) and alanine/aspartate aminotransferase (ALT and AST) were present (Table 1). Of note, the non-DILI cohorts had ALT/AST values on average 2·4/1·8 times greater than the immunoallergic (IAD) or autoimmune (AID) DILI subjects. In addition, the model of end-stage liver disease (MELD) mean score of intrinsic DILI cases (ascribed to acetaminophen toxicity) was twice as high as that of other DILI subjects (Supporting information, Table S1). High serum globulins and in particular immunoglobulin (Ig)G concentrations were observed in both autoimmune DILI (AID) and idiopathic AIH groups. While blood haemoglobin, white blood cell and platelet counts were within the normal range for all four cohorts, half the DILI subjects had elevated circulating eosinophil counts, compared with <10% for non-DILI subjects (Table 1). Histologically, necroinflammatory grades were elevated in all subjects, but the Ishak fibrosis stage was low to intermediate (≤3), and only limited fat (≤2) and iron (≤1) deposits were observed (Table 2).

H&E staining highlighted specific patterns of tissue damage and local immunological features among the different disease groups (Table 2, Supporting information, Figs S2 and S3). Livers from subjects with idiopathic AIH

Table 2. Histopathological scoring and cell counts of leukocytes in livers of subjects studied.

	Immunoallergic DILI ($n = 13$)		Autoimmune DILI ($n = 11$)		Idiopathic AIH ($n = 11$)		Viral hepatitis ($n = 14$)		
	Mean (± s.d.)	Min–max	Mean (± s.d.)	Min–max	Mean (± s.d.)	Min–max	Mean (± s.d.)	Min–max	*P*-value
Necro-inflammatory grade (0–18)	9·5 (± 4·6)	2–17	12·0 (± 4·0)	5–17	7·4 (± 3·1)	4–15	7·6 (± 4·7)	3–18	0·2721
Ishak fibrosis stage (0–6)	1·3 (± 1·1)	0–3	2·2 (± 1·3)	0–4	1·7 (± 0·9)	1–4	1·3 (± 0·7)	0–3	0·3858
Pericellular fibrosis (0–2)	0·5 (± 0·7)	0–2	0·4 (± 0·8)	0–2	1·3 (± 0·8)	0–2	0·9 (± 0·4)	0–2	0·5055
Fat (0–4)	0·7 (± 0·7)	0–2	0·7 (± 0·6)	0–2	0·1 (± 0·4)	0–1	0·1 (± 0·3)	0–1	0·1850
Iron (0–4)	0·9 (± 0·7)	0–2	0·1 (± 0·3)	0–1	0·1 (± 0·3)	0–1	0·2 (± 0·4)	0–1	0·0757
Plasma cells (0–1)*	7 (0), 3 (1), 3 n.a.		3 (0), 6 (1), 1 n.a.		1 (0), 8 (1), 2 n.a.		5 (0), 5 (1), 3 n.a.		
Neutrophils (0–1)*	7 (0), 3 (1), 3 n.a.		7 (0), 2 (1), 2 n.a.		2 (0), 7 (1), 2 n.a.		6 (0), 4 (1), 3 n.a.		
Liver tissue analysed	10 needle biopsies, 3 explanted livers†		9 needle biopsies, 2 explanted livers†		7 needle biopsies, 4 explanted livers		11 needle biopsies, 3 explanted livers		
Portal triads scored	63		54		58		61		
$CD11b^+$ cells per 10 000 μm^2	3·5 (± 3·5)	0·0–14·1	4·6 (± 6·8)	0·0–24·5	6·3 (± 4·6)	0·2–20·5	4·7 (± 3·9)	0·0–17·6	0·4753
$CD3^+$ cells per 10 000 μm^2	15·9 (± 9·2)	1·9–41·0	15·4 (± 8·4)	0·0–38·5	10·2 (± 8·0)	0·0–32·5	13·2 (± 6·6)	1·7–28·6	0·2689
$CD8^+$ cells per 10 000 μm^2	9·4 (± 5·5)	0·9–26·2	13·0 (± 6·2)	1·3–28·6	12·6 (± 5·0)	3·2–26·6	10·3 (± 3·0)	5·9–18·9	0·1652
$CD4^+$ cells per 10 000 μm^2	0·5 (± 0·7)	0·0–2·3	0·6 (± 0·7)	0·0–2·3	1·2 (± 1·2)	0·0–4·9	0·4 (± 0·6)	0·0–1·6	0·2033
$CD20^+$ cells per 10 000 μm^2	1·2 (± 1·3)	0·0–5·1	2·2 (± 1·9)	0·0–8·5	5·8 (± 6·9)	0·0–44·6	4·2 (± 3·5)	0·0–15·8	**<0·0001**
$CD56^+$ cells per 10 000 μm^2	0·5 (± 1·1)	0·0–7·4	0·4 (± 0·6)	0·0–3·0	0·3 (± 0·6)	0·0–2·7	3·1 (± 3·3)	0·0–14·2	**<0·0001**

*Portal hepatic plasma cell and neutrophil counts were performed on haematoxylin and eosin (H&E)-stained tissue sections and scored as follows (0): none, rare, occasional (1), focal aggregates, numerous (n.a. = data not available). †Tissue provided by the Acute Liver Failure (ALF) study group. Bolded characters highlight *P*-values <0.05. AIH = idiopathic autoimmune hepatitis; DILI = drug-induced liver injury; s.d. = standard deviation.

(not due to DILI) had scattered apoptotic hepatocytes with portal and lobular hepatitis with mixed inflammation, including lymphocytes, eosinophils, neutrophils and appreciable plasma cell infiltrates. Those from subjects with immunoallergic DILI (IAD) were characterized by scattered apoptotic hepatocytes with diffuse portal infiltrates with prominent eosinophils. Those from subjects with drug-induced liver injury with autoimmune features (AID) had extensive necrosis with portal inflammation with a mix of lymphocytes, macrophages and eosinophils. Of note, 70% of tissue sections from subjects with AID also displayed focal aggregates of plasma cells. Livers from subjects with acute VH had frequent apoptotic hepatocytes, panacinar hepatitis with mixed inflammatory infiltrates, including lymphocytes, eosinophils, plasma cells and neutrophils.

Characterization of liver infiltrating leucocytes

Despite heterogeneous immunological features among disease groups, all the livers studied had portal inflammation with mixed immune infiltrates of myeloid and lymphoid origin.

Because Kupffer cells represent the main resident myeloid cell subset in normal liver [19], we performed CD11b and CD68 single IHC stains on control tonsil and VH liver tissue (Supporting information, Fig. S2). Whether tissues were stained with CD11b or CD68, similar results were obtained when we performed absolute cell counts of portal infiltrating myeloid cells (data not shown). Because we observed less background/non-specific staining with CD11b, we used it in preference to CD68. Similarly, CD3 and CD20 single IHC stains were performed on control tonsil and VH liver tissues to identify T and B lymphocytes, respectively. T lymphocytes were subdivided further into $CD4^+$ and $CD8^+$ cell subsets. These four markers for IHC staining of liver-infiltrating lymphocytes showed low background staining and a high degree of specificity (Fig. 1). Finally, we performed CD56 and CD57 single IHC stains on control tonsil and VH liver tissues to label a fifth lymphoid cell type, i.e. NK cells (Supporting information, Fig. S2). While tonsil control tissue had only CD57-positive cells, viral hepatitis control liver tissue showed scattered CD56- and CD57-positive cells. Parenchymal $CD57^+$ cells consisted of a mix of small lymphoid (consistent with NK cells) and larger irregularly shaped bodies (consistent with dendritic cells). Parenchymal $CD56^+$ cells consisted mainly of bile ductules, which were distinguishable from infiltrating $CD56^+$ lymphoid NK cells. Therefore, CD56 was used for subsequent dual-IHC stains that enumerated portal NK cell infiltrates during acute hepatitis.

For each subject, three dual-IHC stains were performed separately: CD11b/CD4 stained for macrophages and $CD4^+$ T cells, CD20/CD3 stained for B/T lymphocytes and CD56/$CD8^+$ stained for NK/CD8 T cells (Fig. 1). Overall $CD11b^+$ macrophages and $CD3^+$ T cells were found throughout the

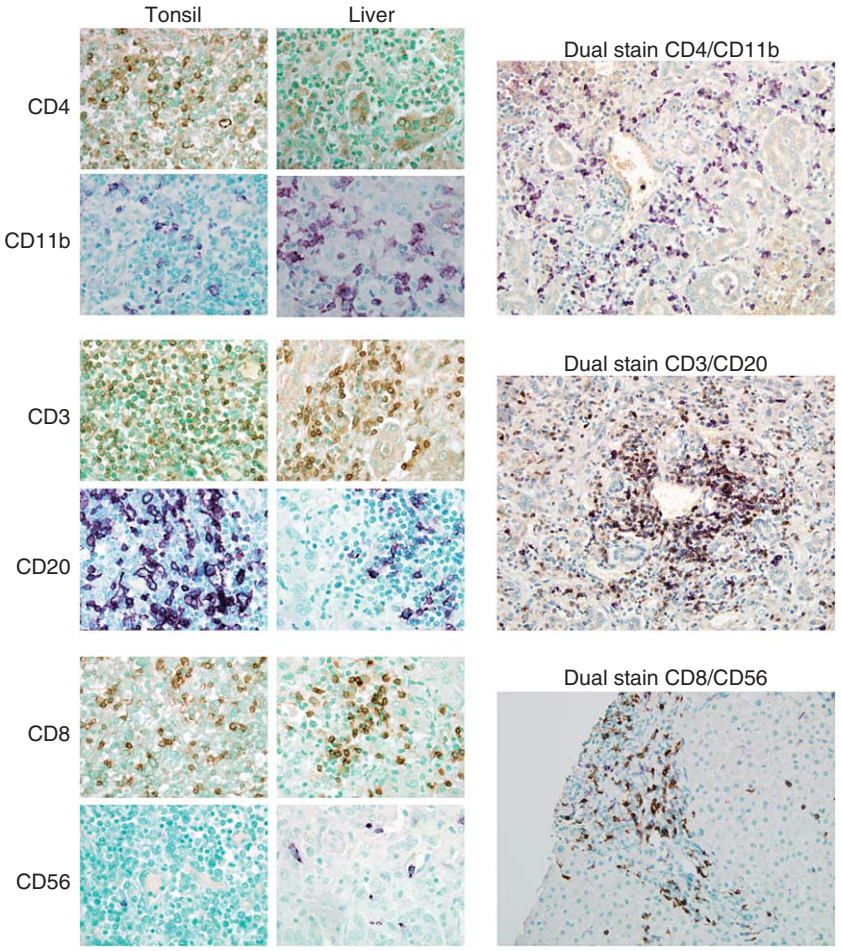

Fig. 1. Haematoxylin and eosin (H&E) histochemical, single and dual-immunohistochemistry stains of control lymphoid tissue (tonsil) and hepatits B virus $(HBV)^+$ liver tissue. Staining for Kupffer cells (CD11b), B cells (CD20) and natural killer (NK) cells (CD56) used visual immunoprecipitate assay (VIP) (purple) colorimetric substrate, whereas staining for T cells (CD3) and T cell subsets (CD4–CD8) used diaminobenzidene (DAB) (brown) colorimetric substrate.

liver and represented the main components of myeloid and lymphoid portal hepatic infiltrating leucocytes. Subcategorization of T cells showed that $CD4^+$ T lymphocytes were rare and that portal hepatic T cells were mainly $CD8^+$. Mature ($CD20^+$) B cells and $CD56^+$ NK cells were relatively rare and distributed unevenly across the livers. Parenchymal B cells were widely scattered single cells and small clusters, while portal B cells tended to form small aggregates when present at all.

Associations among leucocyte infiltrates and causes of acute hepatitis

To correlate qualitative and quantitative assessments of portal hepatic infiltrating leucocytes with underlying causes of acute hepatitis, stained cells were counted within up to five portal areas per slide, normalized to 10 000 μm^2 areas, and averaged. Infiltrates of macrophages ($CD11b^+$) were highly heterogeneous among patients with acute hepatitis, but no pattern emerged that was associated with any particular underlying liver condition (Fig. 1, Table 2 and Supporting information, Table S1). Portal T lymphocytes ($CD3^+$) were highly represented among all disease groups and were mainly cytotoxic T cells ($CD8^+$). CD4 T lymphocytes were a comparatively rare cell subset (<10% of total T cells) (Fig. 2, Table 2 and Supporting information, Table S1). Of note, T cell portal infiltrates were correlated inversely with age and BMI: paediatric subjects (aged 1–18 years) and/or subjects with a BMI <20 kg/m^2 had fewer numbers of $CD3^+$ infiltrating cells (data not shown). While the idiopathic AIH cohort had a demographic bias towards the paediatric population with lower BMI, the degree of portal $CD3^+$ infiltrating T cells was similar across all disease groups.

B cells ($CD20^+$) were found rarely in biopsies of DILI subjects, with averages of only $1{\cdot}2 \pm 1{\cdot}3$ (IAD), $2{\cdot}2 \pm 1{\cdot}9$ (AID) and $0{\cdot}6 \pm 1{\cdot}4$ (intrinsic DILI) cells per 10 000 μm^2 portal areas, respectively (Fig. 3, Table 2 and Supporting information, Table S1). Conversely, the biopsies of non-DILI subjects were characterized by significantly greater B cell infiltrates regardless of the cause of acute hepatitis ($P < 0{\cdot}0001$ compared with DILI cohorts). The number of NK cells ($CD56^+$) allowed further discrimination among non-DILI subjects: subjects with idiopathic AIH had rare $CD56^+$ infiltrating cells ($0{\cdot}3 \pm 0{\cdot}6$), while patients with VH had significantly higher frequencies of such cells ($3{\cdot}1 \pm 3{\cdot}3$ per 10 000 μm^2, $P < 0{\cdot}0001$) (Fig. 3, Table 2).

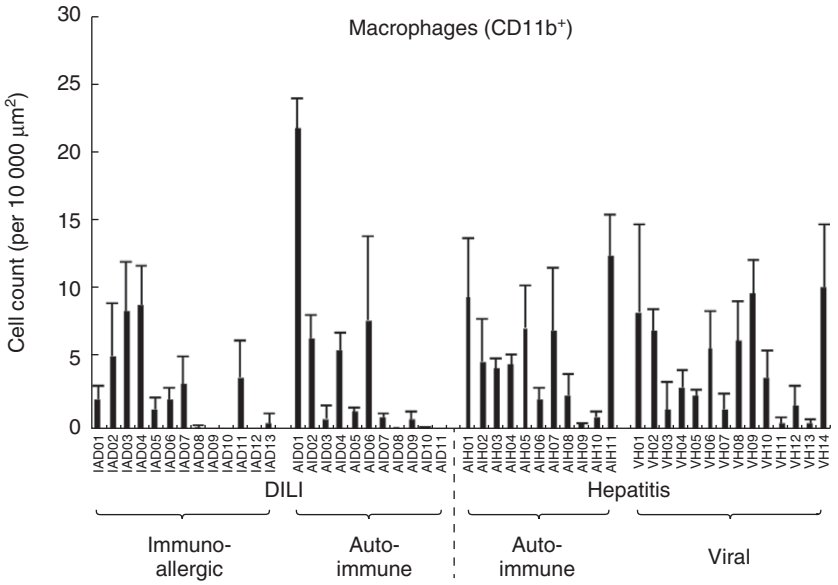

Fig. 2. Portal-infiltrating Kupffer cells (CD11b$^+$) were found in all livers studied. Cell counts were normalized to the size of portal triads and averaged for each subject. Results are means ± standard deviation.
AID = autoimmune drug-induced liver injury (DILI); AIH = idiopathic autoimmune hepatitis; IAD = immune-allergic DILI; VH = viral hepatitis.

Comparative analyses of liver infiltrates with liver damage/function

Macrophages and T lymphocytes represented the two main types of leucocytes in portal tracts during acute hepatitis. While the degree of infiltration did not correlate with specific underlying causes of acute hepatitis, both cell types tended to be more prevalent in liver specimens that showed enlarged portal triads and/or greater tissue damage (data not shown). To explore a potential link between overall liver damage and leucocyte infiltrates, CD11b$^+$ and CD3$^+$ scores were compared with results of selected liver test measurements (Table 3). Degrees of macrophage portal infiltrates were much higher in patients with altered hepatic function. For instance, subjects with elevated serum total bilirubin had, on average, four times more CD11b$^+$ cells than did their counterparts with normal serum bilirubin levels. A similar pattern was observed for subjects with ALT and AST values >ULN (respectively, 3·7- and 2·3-fold greater CD11b infiltration, compared with those with levels within normal range). T cell (CD3$^+$) analysis showed that patients with elevated ALT or AST levels had approximately 30% fewer portal hepatic CD3$^+$ cells than did those with levels of these analytes that were within the normal range.

Comparative analyses of liver infiltrates with peripheral markers of immunity

We did not find associations between any particular portal immune cell infiltrate and non-specific markers of systemic immunity, such as absolute eosinophil or white blood cell counts. To estimate the extent to which liver infiltrating leucocytes are representative of systemic immune events, we performed analyses that compared intrahepatic cell counts with serum markers of immune activation (presence of circulating autoantibodies and circulating immunoglobulin levels) (Table 4). Markers of autoimmunity such as presence and titres of circulating anti-nuclear (ANA), anti-smooth muscle actin (ASMA) or anti-mitochondrial (AMA) autoantibodies were associated directly with greater than normal levels of infiltrating CD20$^+$ B cells ($P < 0{\cdot}0001$) (Table 4). Eosinophilia, used commonly as a marker of allergic reactions, was associated with an overall lesser degree of liver inflammation. Patients with greater than normal circulating eosinophil counts had fewer liver infiltrating CD11b$^+$ macrophages ($P = 0{\cdot}006$), CD4$^+$ T cells ($P < 0{\cdot}0001$) and CD20$^+$ B cells ($P < 0{\cdot}0001$). In contrast, in subjects with hypergammaglobulinaemia (another peripheral marker for autoimmunity), the opposite trend was observed for macrophages (CD11b$^+$ cells) accumulated within portal areas ($P < 0{\cdot}0001$) (Table 4).

Discussion

In this work we evaluated systematically the profiles of infiltrating leucocytes in DILI cases compared to other causes of acute liver injury. The major novel findings of this work are as follows: (i) we have developed and optimized methods for performing dual-IHC staining and for enumerating mononuclear inflammatory cells in liver biopsies of subjects with acute liver injury of diverse causes, including immune-mediated or 'intrinsic' (acetaminophen-induced) DILI, idiopathic AIH and viral hepatitis (Figs 1–4). (ii) In liver biopsies of subjects with clinically important acute DILI caused by any of five drugs commonly incriminated as causes of immunoallergic DILI (amoxicillin/clavulanic acid or lamotrigine), autoimmune DILI (minocycline or nitrofurantoin) or intrinsic DILI (acetaminophen), the predominant leucocytes present are T cells (CD3$^+$), not B cells (CD20$^+$) or NK cells (CD56$^+$) (Figs 3 and 4 and Supporting

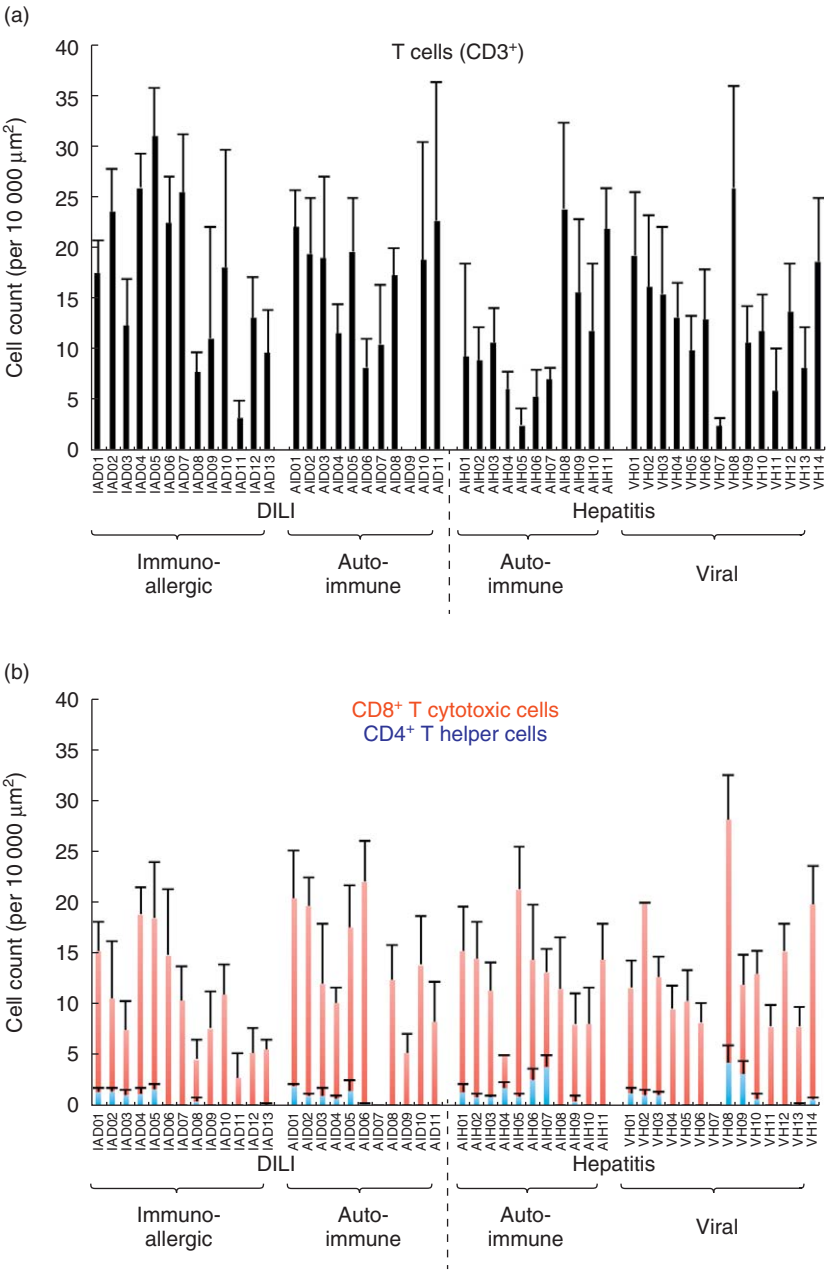

Fig. 3. CD8+ T cells were the main mononuclear cell type infiltrating the portal triads of injured livers. Total T cells (a) or CD4/CD8 subsets (b) counts were performed using a pan-T cell marker (CD3) or T helper (CD4)/T cytotoxic markers (CD8), normalized to the portal triads size, and averaged for each subjects. Results are means ± standard deviation.

information, Table S1). In all biopsies studied, regardless of underlying cause, the great majority (> 90%) of T cells are cytotoxic (CD8+), rather than T helper (CD4+) cells (Table 2 and Supporting information, Table S1, Fig. 3). (iii) B cells (CD20+) are few in number in acute DILI, regardless of cause or presence of positive markers of autoimmunity in serum (positive AMA, ANA or ASMA), whereas such B cells are commonly present in idiopathic AIH or VH (Table 2, Fig. 4). (iv) Natural killer (CD56+) cells were undetectable or rare in all forms of injury, except for VH (Table 2, Fig. 4b).

Our results extend knowledge of the numbers and types of mononuclear immune cells that occur in liver biopsies of subjects with acute DILI. Heretofore, such staining and characterization had been limited to individual case reports [20]. Our finding of a consistent predominance of cytotoxic (CD8+) T cells in the biopsies of DILI subjects is supportive of the hypothesis that, for several drugs well known as causes of DILI, the pathogenesis of liver injury involves drug-induced critical alterations of hepatocytes and/or cholangiocytes which, in turn, activate the susceptible hosts' immune systems to infiltrate their livers and to attack and damage or destroy hepatocytes (usually the primary foci of injury) and/or cholangiocytes (less frequently the primary foci of injury). The frequent occurrence of apoptotic bodies, of hepatocyte swelling and other typical changes of

Table 3. Associations between leucocyte cell counts in livers and liver chemistries.

		Normal		>ULN		
		n	Mean (± s.d.)	*n*	Mean (± s.d.)	*P*-value
D. Bili	CD11b	7	1·9 (± 1·0)	26	6·48 (± 5·4)	**<0·0001**
	CD3		14·4 (± 8·0)		12·7 (± 8·4)	**0·0003**
T. Bili	CD11b	12	1·5 (± 1·1)	37	6·0 (± 5·2)	**0·0002**
	CD3		14·6 (± 8·7)		13·1 (± 8·5)	0·1885
Alk. Phos	CD11b	13	3·1 (± 3·5)	35	5·8 (± 5·4)	0·1549
	CD3		13·6 (± 8·6)		13·7 (± 8·6)	0·1183
GGTP	CD11b	12	5·1 (± 4·5)	17	5·9 (± 5·8)	0·4193
	CD3		13·1 (± 9·6)		12·3 (± 7·4)	0·5054
ALT	CD11b	5	1·4 (± 1·0)	44	5·3 (± 5·1)	**0·0015**
	CD3		19·1 (± 7·8)		13·0 (± 8·5)	**0·0002**
AST	CD11b	7	2·3 (± 2·2)	42	5·3 (± 5·2)	0·0823
	CD3		18·5 (± 7·7)		12·8 (± 8·5)	**0·0012**

Bolded characters highlight *P*-values <0.05. Alk. Phos = alkaline phosphatase; ALT = alanine aminotransferase; AST = aspartate aminotransferase; GGTP = γ-glutamyl transpeptidase; D. Bili = direct bilirubin; T. Bili = total bilirubin; s.d. = standard deviation; ULN = upper limit of normal.

hepatocyte injury (Supporting information, Fig. S1) [21] provides additional evidence for this pathogenic sequence. Still unknown for most cases of acute DILI are the precise molecular mechanisms whereby commonly used drugs, such as amoxicillin/clavulanic acid or nitrofurantoin, elicit injury in only a small percentage of people who take these drugs. However, generation of neoantigens produced by the drugs or their metabolites interacting with host proteins (e.g. albumin or other plasma/intracellular proteins) [22] may be the initiating event and, because antigens are displayed on the plasma membranes of hepatocytes and/or cholangiocytes, call forth activation and expansion of T cells, especially of the $CD8^+$ cytotoxic T cells, which predominate in the liver biopsies (Table 2, Figs 3 and 4). More likely than not, the innate immune status of the host (HLA type) is also important.

Similar mechanisms have been posited for autoimmune DILI, idiopathic AIH and VH [23], and the similarities in T

Table 4. Associations among cell counts and systemic immune markers.

		<LLN		Normal		>ULN		
		n	Mean (± s.d.)	*n*	Mean (± s.d.)	*n*	Mean (± s.d.)	*P*-value
Absolute eosinophil	CD11b			32	5·9 (± 5·3)	16	1·9 (± 2·3)	**0·0006**
	CD4				1·0 (± 1·0)		0·1(± 0·2)	**<0·0001**
	CD20				4·3 (± 5·3)		1·2 (± 1·3)	**<0·0001**
WBC	CD11b	8	3·3 (± 3·7)	31	5·4 (± 5·3)	10	5·2 (± 7·9)	0·6727
	CD4		0·9 (± 1·0)		0·8 (± 0·7)		0·7 (± 1·4)	0·6585
	CD20		4·3 (± 4·6)		2·8 (± 3·0)		4·9 (± 8·2)	0·7871
ANA*	CD11b			21	5·5 (± 4·6)	22	4·9 (± 5·6)	0·5639
	CD4				0·7 (± 0·7)		0·9 (± 1·1)	0·1787
	CD20				2·7 (± 3·3)		4·4 (± 5·9)	**0·0293**
ASMA*	CD11b			20	3·2 (± 3·6)	22	7·0 (± 5·8)	**0·0208**
	CD4				0·5 (± 0·6)		1·1 (± 1·1)	0·2451
	CD20				1·7 (± 1·8)		5·0 (± 6·0)	**<0·0001**
AMA*	CD11b			20	4·3 (± 5·9)	12	6·7 (± 4·9)	0·5195
	CD4				0·6 (± 0·7)		0·9 (± 0·8)	0·7812
	CD20				1·8 (± 1·8)		7·7 (± 3·9)	**<0·0001**
Total globulin	CD11b			10	1·9 (± 1·8)	31	5·4 (± 5·3)	0·1609
	CD4				0·9 (± 1·2)		0·8 (± 0·9)	0·3145
	CD20				4·8 (± 5·2)		3·5 (± 5·0)	0·4439
IgG	CD11b			5	0·5 (± 0·5)	14	5·3 (± 4·7)	**<0·0001**
	CD4				0·7 (± 0·7)		1·2 (± 1·2)	0·1640
	CD20				0·0 (± 0·0)		5·7 (± 7·1)	0·0639

*Negative or positive results instead of normal or > upper limit of normal (ULN), respectively. Bolded characters highlight *P*-values <0.05. AMA = anti-mitochondrial autoantibodies; ANA = anti-nuclear autoantibodies; ASMA = anti-smooth muscle autoantibodies; Ig = immunoglobulin; LLN = lower limit of normal; WBC = white blood cells.

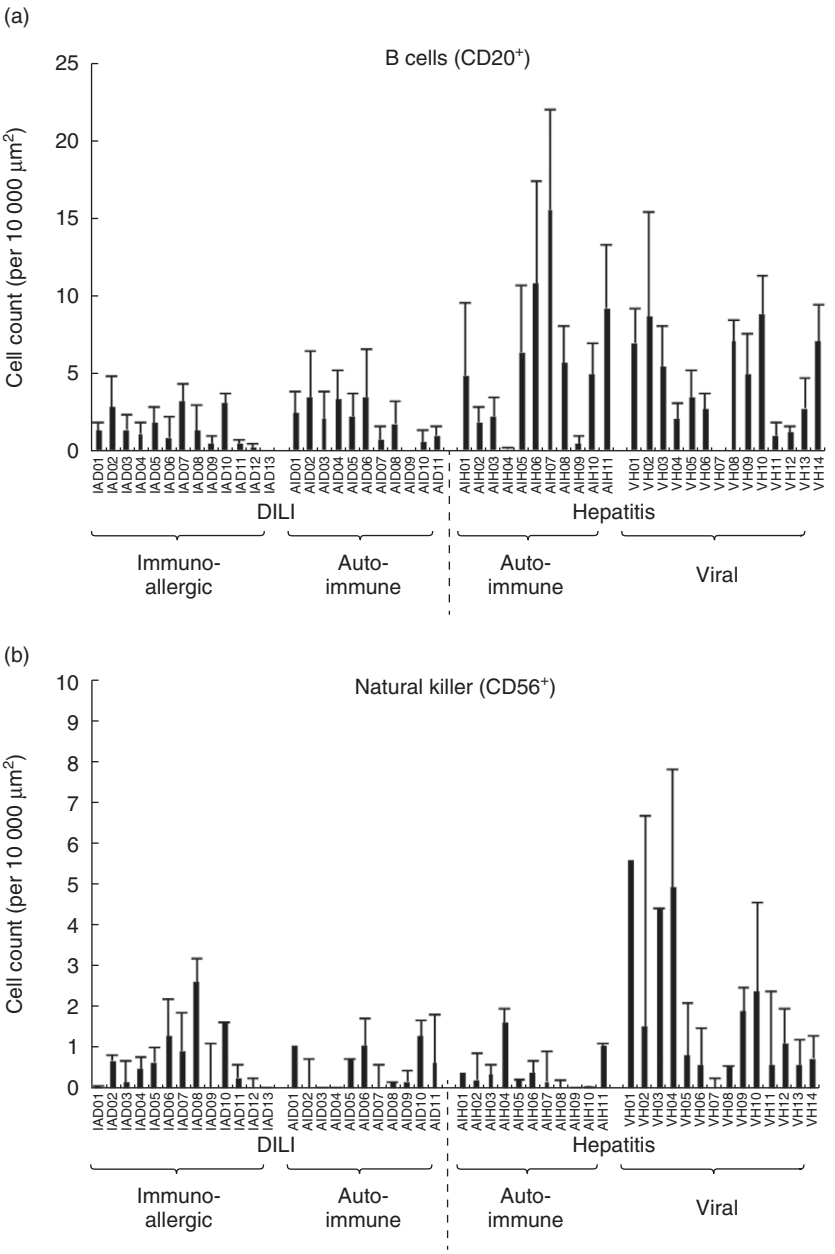

Fig. 4. Portal infiltrating B cells (CD20$^+$) were found primarily in non-drug-induced liver injury (DILI)-related injury; presence of natural killer (NK) cells (CD56$^+$) allowed further discrimination between viral hepatitis (VH) *versus* idiopathic autoimmune hepatitis (AIH) patients. (a) B cells (CD20$^+$) and (b) NK cells (lymphoid CD56$^+$) normalized to the size of portal triads and averaged for each subject. Results are means ± standard deviation.

cell counts and distributions across several aetiologies of acute hepatitis in our studies support this view (Table 2, Fig. 3). However, there are also some important differences in leucocyte patterns comparing, for instance, DILI and non-DILI causes; namely, the higher numbers of mature B (CD20$^+$) cells in non-DILI acute hepatitis (Fig. 4a). Hepatic infiltrating plasma cells (e.g. fully differentiated B cells), used commonly to establish the diagnosis of idiopathic AIH [24,25], lack CD20 expression. While plasma cell infiltrates indeed characterized idiopathic AIH subjects in our study, the majority of autoimmune cases caused by drugs also shared the same feature (Supporting information, Fig. S3). Combined analysis of portal CD20$^+$ mature B cells and plasma cells therefore provided a better discrimination between idiopathic AIH and AID patient cohorts. Similarly, among subjects with notable hepatic B cell infiltrates, frequent CD56$^+$ NK cells in biopsies allowed further discrimination among the idiopathic AIH and VH patient populations (Fig. 4b).

Our results will, of course, benefit from confirmation in larger numbers of subjects with acute DILI and with other aetiologies of hepatitis. Such studies are planned in the future, and we hope that other groups will study additional unique cohorts of subjects to ascertain whether our findings can be confirmed. We are not suggesting that similar findings will be observed in all instances of DILI. For example, DILI due to acetaminophen leads typically to variably severe zone 3-centred hepatocyte necrosis. Early in the

course of such injury, there is little hepatic infiltration with mononuclear inflammatory cells, owing probably to the fact that the injury and necrosis of hepatocytes are due to the intrinsic toxic effects of N-acetyl-p-benzoquinone imine (NAPQI). Inflammatory infiltrates, mainly neutrophils and macrophages, develop later. Recent studies suggest that they mediate injury as well as serve a 'clean up' function in acetaminophen toxicity [26,27]. This is in stark contrast to DILI caused by 17-alkylated anabolic steroids, which is characterized typically by prolonged and variably severe cholestasis. Liver biopsies of subjects with such DILI typically show 'bland' cholestasis with bile in hepatocytes, bile plugs in canaliculi and ductules, but with minimal inflammation. In liver injury caused by anabolic steroids, a primary alteration of bile salt and/or other transporters appears to be the key factor in pathogenesis [28,29]. Perhaps those susceptible to DILI due to such 17-alkylated anabolic steroids have genetic or acquired defects in these transporters, which predispose them to injury.

Among the strengths of the current work are that we have screened several primary and secondary antibodies, optimized staining conditions and protocols and established dual-IHC staining, which requires only three 4-μm-thick sections of each biopsy. We also studied 32 well-characterized subjects from the US DILIN or ALF study group adjudged definitely (>95% likelihood) or very likely (75–94% likelihood) to have had acute DILI due to the incriminated drugs. The US DILIN has developed and validated structured methods for case summarization and assessment of causality [30]. All other potential causes were searched for and excluded, including acute viral hepatitis A–E.

Our study also has limitations: thus far, we have studied only subjects with acute hepatitis. It would be of benefit to study larger numbers of subjects of both DILI and non-DILI causes and to study biopsies from subjects with DILI due to several other drugs in order to assess the extent to which our initial results extend to other drugs. In addition, extending our hepatic immune phenotyping methodology, using dual-IHC to cases of chronic hepatitis such as alcoholic or non-alcoholic steatohepatitis (ASH, NASH) could provide additional information regarding the ubiquity of interplay among the most prominent adaptive immune variables observed in our study ($CD8^+$ T cells and $CD20^+$ B cells).

Conclusions

In summary, we have developed and optimized procedures for dual-staining IHC of mononuclear immune cells in liver biopsies and have characterized the mononuclear cells that infiltrate the portal tracts of biopsies from subjects with immunoallergic, autoimmune and intrinsic DILI. Regardless of the causative drug or the clinicopathological phenotype, the predominant cell types are T cells ($CD3^+$), not B cells ($CD20^+$) or NK cells ($CD\ 11^+$ or $CD\ 56^+$), and nearly all the T cells are cytotoxic ($CD8^+$), rather than T helper cells ($CD4^+$). In contrast, B cells are numerous in idiopathic AIH, VH and NK cells are found in VH. Our findings provide insight into potential pathogenic mechanisms of hepatitis and, we speculate, will prove useful in establishing the correct aetiological agent responsible for acute hepatitis, especially in the still-problematic assessment of whether due to drug- or non-drug causes. Although not powered to establish thresholds of clinically relevant B, NK and plasma cell infiltrates, this study allowed us to develop a concept algorithm to help establish a correct diagnosis among IAD, AID, idiopathic AIH and VH (Supporting information, Fig. S4). While prominent mature B cells ($CD20^+$) differentiate DILI from non-DILI cases, NK cells ($CD56^+$) further differentiate AIH and VH subjects, and plasma cell counts help to distinguish autoimmune from immunoallergic DILI.

Acknowledgements

This study was supported by institutional funds from CHS, the Intramural Research Program of the NIH, National Cancer Institute, by the Acute Liver Failure Study Group, supported by a co-operative agreement (U01 DK-58369 with NIDDK); and by the Drug Induced Liver Injury Network (DILIN), which is supported by the following co-operative agreements with the National Institute of Diabetes and Digestive and Kidney Diseases: U01 DK-065193, U01 DK-065211, U01 DK-065238, U01 DK-065184, U01 DK-065201, U01 DK-83023, U01 DK-083020, U01 DK-08992 andU01 DK-083027.

Disclosure

The authors have no commercial or consultancy conflicts of interest.

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Supporting information

Additional Supporting information may be found in the online version of this article at the publisher's web-site:

Figure S1. Haematoxylin and eosin (H&E) histochemical stains highlighting immunological features of drug-induced liver injury (DILI), autoimmune hepatitis, hepatitis B virus (HBV) and cryptogenic hepatitis.
Figure S2. Representative single immunohistochemical staining output for macrophages (CD11b *versus* CD68) and natural killer (NK) cells (CD56 *versus* CD57) on control lymphoid tissue (tonsil) and hepatitis B virus $(HBV)^+$ liver tissue.
Figure S3. Percentile of acute hepatic cases with prominent portal hepatic infiltrating plasma cells and neutrophils.
Figure S4. Concept algorithm to help establishing differential diagnosis among immune-allergic drug-induced liver injury (DILI) (IAD), autoimmune DILI (AID), idiopathic autoimmune hepatitis (AIH) and viral hepatitis (VH) patients using portal hepatic-infiltrating monocular cell analysis.
Table S1. Demographic, laboratory results and histopathological findings in subjects with drug-induced liver injury (DILI) due to acetaminophen ('intrinsic') or herbal products.
Table S2. Primary antibodies used.