Lymphocyte Profile and Immune Checkpoint Expression in Drug-Induced Liver Injury: An Immunophenotyping Study

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The identification of specific HLA risk alleles in drug-induced liver injury (DILI) points toward an important role of the adaptive immune system in DILI development. In this study, we aimed to corroborate the role of an adaptive immune response in DILI through immunophenotyping of leukocyte populations and immune checkpoint expressions. Blood samples were collected from adjudicated DILI (n = 12), acute viral hepatitis (VH; n = 13), acute autoimmune hepatitis (AIH; n = 9), and acute liver injury of unknown etiology (n = 15) at day 1 (recognition), day 7, and day >30. Blood samples from patients with nonalcoholic fatty liver disease (NAFLD; n = 20) and healthy liver controls (HLCs; n = 54) were extracted at one time point. Leukocyte populations and immune checkpoint expressions were determined based on cell surface receptors, except for CTLA-4 that was determined intracellularly, using flow cytometry. At recognition, DILI demonstrated significantly higher levels of activated helper T-cell (P < 0.0001), activated cytotoxic T-cells (P = 0.0003), Th1 (P = 0.0358), intracellular CTLA-4 level in helper T-cells (P = 0.0192), and PD-L1 presenting monocytes (P = 0.0452) than HLC. These levels approached those of HLC over time. No significant differences were found between DILI and VH. However, DILI presented higher level of activated helper T-cells and CTLA-4 than NAFLD and lower PD-L1 level than AIH. Our findings suggest that an adaptive immune response is involved in DILI in which activated CD4+ and CD8+ play an important role. Increased expression of negative immune checkpoints is likely the effect of peripheral tolerance regulation.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

☑ The underlying mechanism of drug-induced liver injury (DILI) is not yet fully elucidated. However, the identification of HLA risk alleles in DILI suggests an important role for the adaptive immune system.

WHAT QUESTION DID THIS STUDY ADDRESS?

☑ We compared immune activation status in DILI and other acute and chronic liver conditions by determining lymphocyte populations and immune checkpoint levels by flow cytometry in serial blood samples from patients and controls.

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?

☑ Activation of helper and cytotoxic T-cells is increased at DILI onset similar to acute viral hepatitis. However, DILI presented higher level of activated helper T-cells and CTLA-4 than nonalcoholic fatty liver disease and lower PD-L1 level than autoimmune hepatitis. This further supports the involvement of an adaptive immune response in DILI development. However, no specific "immune fingerprint" was detected that could distinguish DILI from other forms of liver injury.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?

☑ Defining the cellular mechanism in DILI facilitates specific biomarker development for DILI diagnosis and prognosis. Such biomarkers would enable faster and more reliable DILI management in clinical practice.

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Received June 15, 2021; accepted September 3, 2021. doi:10.1002/cpt.2423

Idiosyncratic drug-induced liver injury (DILI) is a rare but important condition, which can potentially cause acute liver failure leading to death or liver transplantation. It is also one of the main reasons behind postmarketing drug withdrawals and is consequently of importance for the pharmaceutical industry. The development of DILI is considered to be multifactorial, and dependent on a combination of drug properties, host factors, as well as environmental factors.[1] The immune system is believed to play a fundamental role in DILI development and progression.[2] This is supported by findings of specific human leukocyte antigen (HLA) alleles associated with DILI susceptibility to specific drugs.[3] Intermediate metabolites formed during drug metabolism may act as haptens and bind to endogenous proteins to form adducts that when presented on specific HLA molecules as neoantigens may trigger T-cell activation and an immune response. However, the liver is constantly exposed to foreign antigens and bacterial matters from the endogenous microbiota due to being a highly vascularized organ in which ~ 25–30% of the body's blood supply passes through every minute.[4] A large proportion of this comes via the portal vein from the gastrointestinal tract. To protect itself from chronic inflammation, the liver has a propensity for immune tolerance. The development of DILI is therefore believed to depend on an interruption of the liver's tolerogenic state.[5] In fact, many liver conditions have been associated with alterations in immune tolerance, such as autoimmune hepatitis, primary biliary cirrhosis, and primary sclerosing cholangitis.[6–8]

In addition to antigen presentation on HLA molecules, the generation of T-cell activation is likewise dependent on further signals from stimulatory and inhibitory immune checkpoints binding to their corresponding ligands (**Figure 1**). CD28 is the most important costimulatory membrane receptor for T-cell activation. It is constitutively expressed on resting and activated T-cells and binds to the B7-1 (CD80) and B7-2 (CD86) ligands present on antigen presenting cells (APCs). Another costimulatory receptor is ICOS (CD278), which when bound to its ligand ICOS-L (CD275) provides stimulatory signals for T-cell activation. It differs from CD28 in that it is an inducible receptor and its effect appears less potent than that of CD28. Nevertheless, ICOS co-stimulation is believed to play a complex role in dictating the course of adaptive immunity and is a critical component in the production of isotype-switched antibodies.[9]

In addition to positive co-stimulation, CTLA-4 (CD152) and PD-1 (CD279) exert inhibitory effects on T-cell activation. CTLA-4 is a direct antagonist of CD28 stimulation as it competes for the same ligands (B7-1 and B7-2), which it binds to with higher affinity than CD28. In contrast to CD28, CTLA-4 is only expressed on activated T-cells. This receptor is characterized by rapid endocytosis from the plasma membrane resulting in most of CTLA-4 being intracellular. Although both CTLA-4 and PD-1 provide inhibitory signals, CTLA-4 seems to be important for limiting T-cell activity at an earlier phase of T-cell activation, whereas the inhibitory effect of PD-1 when bound to its ligand PD-L1 (CD274) occurs later and limits T-cell activity in peripheral tissues.[10]

Variations in peripheral leukocyte populations can occur during disease development as different leukocyte populations are recruited to specific tissues and organs, including the liver, during injury and infections. The goal of these migrating populations is to eliminate the inflammatory trigger and facilitate tissue repair, and is associated with both innate and adaptive immune responses.[11] The rate of leukocyte recruitment and the nature of the recruited cells in response to inflammatory signals will shape the severity of the conditions.[12]

In this study, we aimed to determine the immunological fingerprint of DILI through flow cytometry analysis of peripheral blood and compare that to those of other hepatic conditions and population controls without liver injury. We also set out to determine the level of leukocytes presenting immune checkpoint receptors and their corresponding ligands to corroborate that an adaptive immune response is fundamental in DILI development.

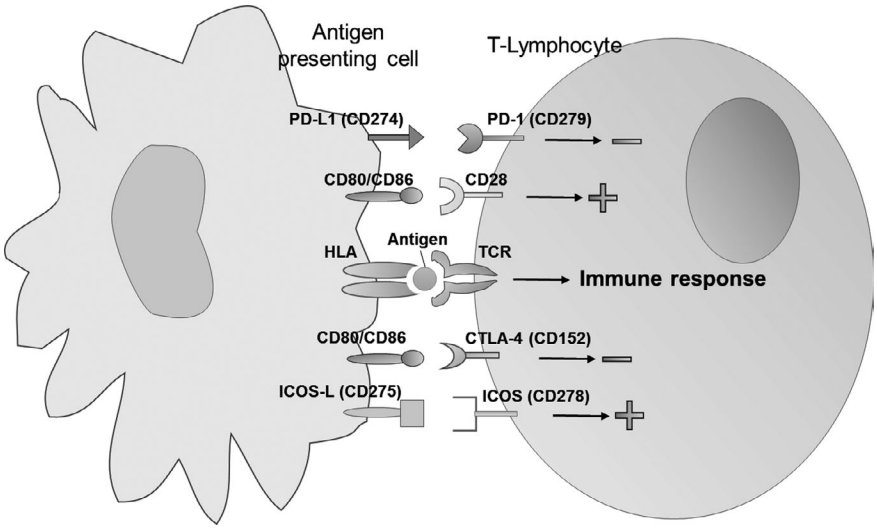

Figure 1 Schematic presentation of immune checkpoint receptors and their corresponding ligands. Antigens bound to the human leukocyte antigen (HLA) molecules on antigen presenting cells are recognized by T-cell receptors on T-cell lymphocytes. The checkpoint receptors CD28 and ICOS (CD278) when bound to their ligands CD80/CD86 and ICOS-L (CD275) provide a costimulatory signal for T-cell activation. In contrast, CTLA-4 (CD152) and PD-1 (CD279) bind to the CD80/86 and PD-L1 ligands to exert an inhibitory signal, which limits T-cell activation.

MATERIALS AND METHODS

Subjects and study protocol

Acute idiosyncratic DILI cases were obtained from those submitted to the Spanish DILI Registry; a collaborative network established in 1994 to prospectively identify cases of DILI in a standardized manner. All patients with DILI fulfilled at least one of the following 3 biochemical DILI criteria at the time of study recruitment: (i) alanine aminotransferase (ALT) ≥ 5 times the upper limit of normal (ULN), (ii) ALT ≥ 3 times the ULN + total bilirubin (TBL) ≥ 2 times the ULN, and (iii) alkaline phosphatase (ALP) ≥ 2 times the ULN.[13] Most cases fulfilled one of the ALT-based criteria. Only three cases met the ALP-based criterion alone without fulfilling either of the two ALT-based criteria. Serological and biochemical tests were performed to rule out liver damage from viral hepatitis (hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), hepatitis E virus (HEV), cytomegalovirus (CMV), and Epstein-Barr virus (EBV)) and imaging tests to rule out bile duct disorders. Alternative causes of liver injury, such as autoimmune hepatitis, were also ruled out. All submitted cases were evaluated for causality assessment, initially by clinical assessment and later by applying the Roussel Uclaf Causality Assessment Method (RUCAM), also known as the "Council for International Organizations of Medical Science (CIOMS) scale." The acute liver injury control groups viral hepatitis (VH), autoimmune hepatitis (AIH), and unknown etiology (UKE) used in this study met the same biochemical inclusion criteria as the DILI cases, whereas the patients with chronic non-alcoholic fatty liver disease (NAFLD), with lower liver profile elevations, were identified based on detection of steatosis in liver biopsies and/or abdominal ultrasound examinations. The VH group consisted of patients infected with HAV (46%), HBV (23%), HCV (7.7%), HEV (7.7%), CMV (7.7%), and EBV (7.7%). The UKE group consisted of acute liver injury cases that were initially suspected to be DILI, but could not be diagnosed as DILI with sufficient certainty based on the available information. Healthy liver controls (HLCs) presented a normal liver profile at the time of sample collection and had previously not suffered from any known DILI episode. This group was formed of university employee volunteers who underwent their yearly medical examination at the Área de medicina del trabajo de la Universidad de Málaga at Malaga University. The study protocol was approved by the local Ethics Committee at the Virgen de la Victoria University Hospital in Málaga, Spain, and all subjects gave informed consent.

Sample collection and preparation

Serial blood samples were prospectively collected from patients with DILI, VH, AIH, and UKE at days 1, 7, and ≥ 30 from liver injury detection. In the case of patients with NAFLD and HLC, blood samples were extracted at a single visit. After extraction, blood samples were immediately stained with combinations of monoclonal antibodies conjugated with specific fluorophores (**Table S1**) and incubated for 30 minutes in darkness at room temperature. Samples were subsequently treated with lysing buffer (Becton Dickinson, Franklin Lakes, NJ) to remove erythrocytes, and finally washed and suspended in phosphate-buffered saline buffer prior to flow cytometry measurements. Due to difficulties in detecting cells presenting extracellular CD152 membrane receptors, the presence of CD152 was determined intracellularly. Hence, cells were treated with permeabilizing solution (Becton Dickinson) according to the manufacturer's instructions prior to monoclonal antibody staining, similar to Matsubara *et al*.[14]

Multicolor flow cytometry

Multicolor flow cytometry was performed using a BD FACSVerse Universal Loader cytometer. Measurement data were collected and analyzed using the Kaluza Flow Cytometry Analysis Software (Beckman Coulter, Indianapolis, IN). Gatings were performed based on size and complexity to select and identify specific lymphocyte and monocyte populations. The different leukocyte populations were detected based on the expression of specific membrane receptors as outlined in **Table 1**.

Statistics

Sizes of the different leukocyte populations were compared between patient and control groups using nonparametric Kruskal-Wallis tests followed by post hoc analyses using Mann-Whitney tests. All statistical tests were 2-sided hypotheses performed at the 0.05 level of significance. Due to the exploratory nature of the study, the presented *P* values are not corrected and we considered a type I error (α) of 0.05 to reject the hypothesis testing.[15] The statistical analyses were performed using the GraphPad Prism 9.0.2 software (GraphPad Software, San Diego, CA).

RESULTS

Study cohort description

In this study, we analyzed serial blood samples from 12 patients with DILI, 13 with VH, 9 with AIH, and 15 with UKE and a single blood sample from 20 patients with NAFLD. In addition, 54 HLCs without any liver conditions were included as a control group for comparison. **Table 2** shows demographics

Table 1 Combination of markers used to analyze different lymphocyte populations

Lymphocyte populations	
Helper T-cells	CD4+/CD45+
Naive	CD4+/CD45RO-/CD45+
Memory	CD4+/CD45RO+/CD45+
Activated	CD4+/HLA-DR+/CD45+
Cytotoxic T-cells	CD8+/CD45+
Naive	CD8+/CD45RO-/CD45+
Memory	CD8+/CD45RO+/CD45+
Activated	CD8+/HLA-DR+/CD45+
B cells	CD4-/CD20 + /CD45+
Regulatory T-cells	CD4+/$CD25^{bright}$/$CD127^{dim}$/CD45+
NK cells	CD3−/CD16+/CD56+/CD45+
NKT-cells	CD3+/CD16+/CD56+/CD45+
Th1	CD4+/CD183+
Th2	CD4+/CD194+/CD196-
Th9	CD4+/CD194-/CD196+
Th17	CD4+/CD194+/CD196+
Th22	CD4+/CD194+/CD196+/CCR10+
CD28+	CD4+/CD28+
CTLA-4 (CD152)+[a]	CD4+/CD152+
ICOS (CD278)+	CD4+/CD278+
PD-1 (CD279)+	CD4+/CD279+
Monocyte populations	
CD80+	CD11b/Mac-1+/CD80+
CD86+	CD11b/Mac-1+/CD86+
ICOS-L (CD275)+	CD11b/Mac-1+/CD275+
PD-1L (CD274)+	CD11b/Mac-1+/CD274+

Markers used to analyze cells expressing immune checkpoint receptors and ligands in both lymphocytes and monocytes are also indicated.
[a]Intracellularly after permeabilization.

Table 2 Comparison of demographics and clinical characteristics between patients with DILI, acute VH, acute AIH, UKE, NAFLD, and HLCs

	DILI *N* = 12	VH *N* = 13	AIH *N* = 9	UKE *N* = 15	NAFLD *N* = 20	HLCs *N* = 54
Age (y), mean ± SD	55 ± 15	44 ± 20	57 ± 19	55 ± 24	53 ± 13	50 ± 11
Female, %	58	31	67	40	40	59
BMI (kg/m^2), mean ± SD	23.8 ± 5.6	24.5 ± 4.0	26.9 ± 5.2	24.9 ± 2.8	30.4 ± 3.8	24.6 ± 4.3
Diabetes mellitus, %	17	23	0	13	N/D	3.8
Hypertension, %	25	15	33	33	N/D	19
Liver episode characteristics, %						
Jaundice	58	62	100	67	0	N/A
Hospitalization	67	77	100	87	0	N/A
Type of liver injury, %						
Hep	55	62	100	53	N/A	N/A
Chol	36	15	0	20	N/A	N/A
Mix	9.1	23	0	27	N/A	N/A
Laboratory parameters at visit 1						
TBL, mg/dL (range)	6.3 (0.3–21)	7.4 (0.4–19)	17 (7.3–34)	9.5 (0.3–28)	0.7 (0.3–1.2)	0.6 (0.2–0.8)
AST, IU/L (range)	240 (41–656)	671 (79–2697)	846 (122–1875)	638 (15–2154)	48 (20–77)	24 (9–29)
ALT, IU/L (range)	417 (37–1213)	1279 (115–5162)	906 (217–2273)	783 (50–2257)	79 (24–205)	22 (15–27)
ALP, IU/L (range)	257 (93–768)	240 (92–509)	233 (103–309)	237 (96–555)	88 (40–194)	59 (30–89)
Severity, %						
Mild	33	7.7	0	13	N/A	N/A
Moderate	58	85	67	60	N/A	N/A
Severe	0	0	11	6.7	N/A	N/A
Death/liver transplantation	8.3	7.7	22	20	N/A	N/A

AIH, autoimmune hepatitis; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; Chol, cholestatic; DILI, drug-induced liver injury; Hep, hepatocellular; HLCs, healthy liver controls; Mix, mixed; N/A, not applicable; NAFLD, non-alcoholic fatty liver disease; TBL, total bilirubin; UKE, unknown etiology; VH, viral hepatitis.

and clinical characteristics of the study populations. The mean age varied from 44 years for the VH group to 58 years for the AIH group. Women predominated among patients with DILI, AIH, and HLC, while men were more frequent among patients with VH, NAFLD, and UKE. Of the patients with DILI, 83% developed DILI due to conventional medications and 17% occurred due to use of herbal and dietary supplements. A detailed description of the causative agents and corresponding HLA risk alleles identified to date can be found in **Table S2**. With regard to liver profile elevations, substantial ALT elevations were apparent in the VH, AIH, and UKE groups, somewhat lower in the DILI group and relatively low in the NAFLD group. Similarly, substantial TBL elevations were detected in the AIH and UKE groups, whereas those of the patients with DILI and VH were lower but substantially higher than those of the patients with NAFLD. Of the patients with NAFLD, 33% were considered to have a fibrosis stage F0, 13% were stage F1, 6.7% were stage F2, 20% were stage F3, and 27% were stage F4. The highest proportion of death/liver transplantation cases occurred among the AIH cases followed by UKE, DILI, and VH (**Table 2**).

Variations in leukocyte populations

Significant differences in leukocyte (P = 0.0047), neutrophil (P = 0.004), and monocyte (P < 0.0001) counts, but not lymphocyte counts, were detected among the groups (**Table 3**). However, the DILI group only differed from the HLCs (P = 0.037) and AIH (P = 0.023) in monocyte count and from AIH (P = 0.0404) in leukocyte count. The most noticeable cohort was that of AIH, which had significantly higher leukocyte (P = 0.0027), neutrophil (P = 0.0006), and monocyte (P < 0.0001) counts than those of HLCs.

We compared the proportion of specific lymphocyte populations between different liver injury groups and HLCs using flow cytometry, and evaluated the presence of specific immune checkpoint receptors and ligands on both lymphocytes and monocytes in the different groups (**Figure 2**). **Table 3** presents mean percentages for each of the cell populations in the different study groups at day 1. Similar proportions of helper T-cells were found at day 1 in the study groups. However, differences were detected in the proportion of activated helper T-cells (P = 0.0003), with DILI presenting a significantly higher proportion than HLCs (14% vs. 6.3%, P < 0.0001) and NAFLD (14% vs. 8.2%, P = 0.0150). In

Table 3 Absolute count and percentage of leukocyte populations in peripheral blood samples from patients with DILI, acute VH, acute AIH, and UKE at liver injury detection compared with patients with NAFLD and HLCs analyzed by flow cytometry

	DILI *N* = 12	VH *N* = 13	AIH *N* = 9	UKE *N* = 15	NAFLD *N* = 20	HLC *N* = 54	*P* value
Cell count, mean (×10^9/L) ± SD							
Leukocytes	6.5 ± 3.0	7.0 ± 2.9	9.2[a,b] ± 3.2	8.9[a] ± 6.6	ND	5.9 ± 1.8	0.0054
Lymphocytes	1.6 ± 0.7	2.3 ± 1.7	1.5 ± 0.5	1.8 ± 1.1	ND	1.8 ± 0.7	0.4295
Neutrophils	4.2 ± 2.2	3.7 ± 2.2	6.5[a] ± 2.9	6.1[a] ± 6.5	ND	3.4 ± 1.4	0.004
Monocytes	0.6[a] ± 0.3	1.4[a] ± 2.1	1.0[a,b] ± 0.3	0.8[a] ± 0.4	ND	0.4 ± 0.1	< 0.0001
Lymphocyte populations							
Helper T-cell (% CD45)	43 ± 11	42 ± 13	44 ± 6.0	43 ± 11	43 ± 8.6	46 ± 7.2	0.8247
Naive (% CD4)	25 ± 13	40 ± 13	31 ± 12	33 ± 16	27 ± 12	35 ± 13	0.0719
Memory (% CD4)	71 ± 12	58 ± 14	66 ± 11	65 ± 16	70 ± 12	63 ± 13	0.2851
Activated (% CD4)	14[a] ± 8.8	14[a] ± 8.2	9.8 ± 4.6	9.5 ± 7.5	8.2[a,b] ± 3.7	6.3 ± 3.1	0.0003
Cytotoxic T-cells (% CD45)	24 ± 9.9	29[a] ± 9.6	22 ± 7.4	25 ± 10	19 ± 7.1	21 ± 5.5	0.0277
Naive (% CD8)	17 ± 9.7	25 ± 19	21 ± 12	18 ± 16	23 ± 12	29 ± 16	0.1015
Memory (% CD8)	49 ± 12	57 ± 19	53 ± 15	49 ± 9	48 ± 14	46 ± 16	0.6656
Activated (% CD8)	31[a] ± 14	39[a] ± 24	32[a] ± 17	27 ± 21	22 ± 15	15 ± 10	0.0002
Regulatory T-cells (% CD4)	8.0 ± 1.9	8.1 ± 2.4	9.0 ± 3.0	8.9 ± 2.9	7.1 ± 2.2	7.2 ± 1.9	0.1275
Th1 (% CD4)	52[a] ± 13	44 ± 11	44 ± 7.9	41 ± 13	53[a] ± 14	42 ± 11	0.0311
Th2 (% CD4)	9.5 ± 4.0	14 ± 5.4	12 ± 6.5	13 ± 5.3	11 ± 4.4	12 ± 4.9	0.3997
Th9 (% CD4)	20 ± 7.5	13[a] ± 6.1	19 ± 7.1	13[a] ± 5.1	25[a] ± 11	18 ± 5.5	0.0026
Th17 (% CD4)	13 ± 5.6	14 ± 5.3	19 ± 9.5	14 ± 4.9	16 ± 6.6	13 ± 4.4	0.5051
Th22 (% CD4)	3.2 ± 1.6	3.3 ± 2.2	4.4 ± 2.8	4.3 ± 2.4	4.0 ± 1.4	4.4 ± 1.8	0.4178
B cells (% CD45)	11 ± 6.8	7.7 ± 3.6	11 ± 7.0	6.5[a] ± 4.8	11 ± 4.6	9.8 ± 4.3	0.0197
NK cells (% CD45)	14 ± 11	8.8 ± 6.6	9.6 ± 3.9	12 ± 6.2	12 ± 5.4	12 ± 5.9	0.2834
NKT-cells (% CD45)	0.5 ± 0.5	1.4 ± 1.5	0.6 ± 0.3	0.6 ± 1.0	0.5 ± 0.8	0.6 ± 0.8	0.1585
CD28+ (% CD4)	91 ± 9.3	91 ± 8.0	92 ± 5.4	93 ± 11	96 ± 5.9	96 ± 4.8	0.2230
ICOS+ (% CD4)	5.1 ± 4.2	8.7[a] ± 5.7	3.9[a] ± 1.1	2.7 ± 1.8	1.8 ± 0.7	2.3 ± 0.9	< 0.0001
CTLA-4+ (% CD4)	28[a] ± 8.0	30[a] ± 15	38[a] ± 8.7	24[a] ± 11	18[b] ± 4.6	18 ± 6.2	< 0.0001
PD-1+ (% CD4)	13 ± 8.5	23[a] ± 11	15 ± 7.2	12 ± 7.7	10 ± 5.9	9.3 ± 5.2	0.0131
Monocyte populations							
CD80+ (% CD11b)	7.9 ± 3.3	10 ± 6.3	12 ± 8.6	13 ± 8.5	10 ± 5.8	9.2 ± 4.4	0.6693
CD86+ (% CD11b)	9.5 ± 5.1	9.5 ± 2.3	9.1 ± 5.3	11 ± 3.9	12 ± 3.5	11 ± 3.5	0.2561
ICOS-L+ (% CD11b)	15 ± 6.2	15 ± 6.8	12 ± 8.6	15 ± 7.4	16 ± 3.9	13 ± 4.2	0.0797
PD-L1+ (% CD11b)	5.3[a] ± 1.8	9.1[a] ± 4.4	10[a,b] ± 5.2	8.1[a] ± 5.6	5.0[a] ± 1.3	3.9 ± 1.8	< 0.0001

AIH, autoimmune hepatitis; DILI, drug-induced liver injury; HLCs, healthy liver controls; NAFLD, non-alcoholic fatty liver disease; UKE, unknown etiology; VH, viral hepatitis.
Statistical tests: Kruskal-Wallis test followed by post hoc analyses using Mann-Whitney *U* test.
[a]Significantly different from HLCs ($P < 0.05$).
[b]significantly different from DILI ($P < 0.05$).

addition, VH (14% vs. 6.3%, $P = 0.0031$) and NAFLD (8.2% vs. 6.3%, $P = 0.031$) also presented significantly higher activated helper T-cells than HLCs. Only VH differed significantly from HLCs (29% vs. 21%, $P = 0.001$) in cytotoxic T-cell proportion. In contrast, several groups presented an increased level of activated cytotoxic T-cells compared with HLCs, including DILI (31% vs. 15%, $P = 0.0003$), VH (39% vs. 15%, $P < 0.0001$), and AIH (32% vs. 15%, $P = 0.0105$). With regard to helper T-cell subpopulations, the distributions of Th1 ($P = 0.0311$) and Th9 ($P = 0.0026$) cells differed significantly. Increased level of Th1 cells was detected in DILI (52% vs. 42%, $P = 0.0358$) and NAFLD (53% vs. 42%, $P = 0.0062$) compared with HLCs. The NAFLD group also demonstrated increased level of Th9 (25% vs. 18%, $P = 0.0267$), whereas the UKE and VH groups presented lower levels than HLCs (13% vs. 18%, $P = 0.0159$ and 13% vs. 18%, $P = 0.0112$, respectively).

Variations were also found in the proportion of B cells, but only UKE differed significantly from HLCs (6.5% vs. 9.8%, $P = 0.0014$). No significant differences were found between the different study groups for any of the other lymphocyte populations. The detected variations in the aforementioned leukocyte populations approached HLC levels in the subsequent visits at days 7 and ≥ 30, as illustrated in **Figure 3**, with the exception of

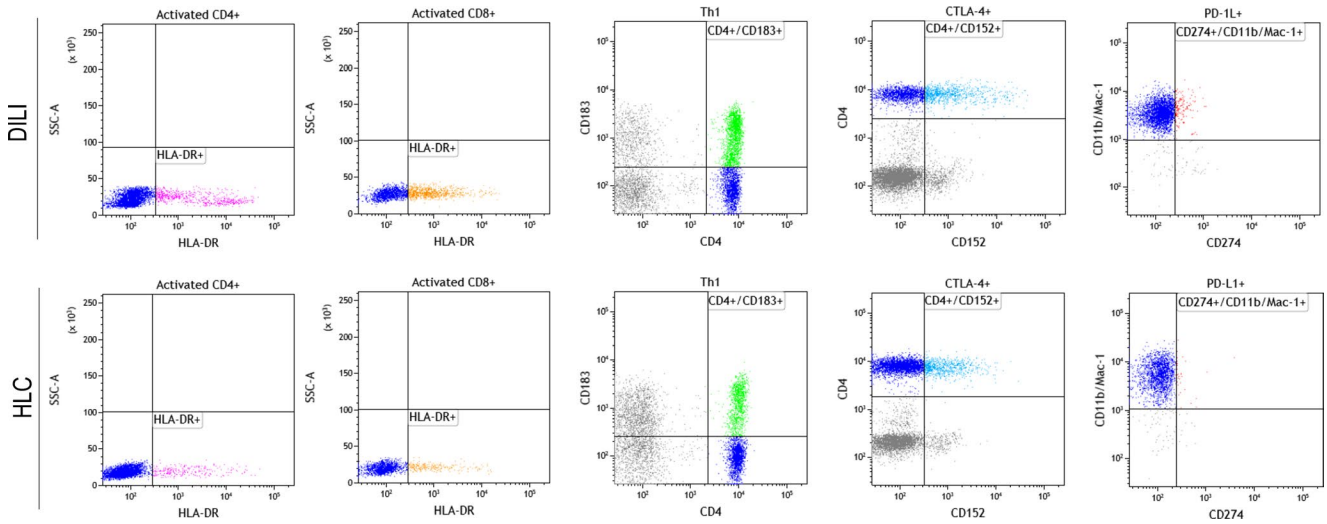

Figure 2 Flow cytometry results in a representative drug-induced liver injury (DILI) and healthy liver control (HLC) case of leukocyte populations (activated helper T-cells, activated cytotoxic T-cells, Th1, CTLA-4 presenting helper T-cells, and PD-1L presenting monocytes) found to be significantly higher in the DILI cohort. [Colour figure can be viewed at wileyonlinelibrary.com]

Th1 cells in DILI for which the mean decrease was less apparent in the last visit. Nevertheless, the difference between Th1 cells in DILI in the last visit and that of HLCs was no longer statistically significant.

Variations in immune checkpoints

We also determined the level of immune checkpoint receptors and ligands on helper T-cells and activated monocytes, respectively, to demonstrate the presence of a possible adaptive immune response (**Table 3**). Regarding the costimulatory immune checkpoints, no differences were detected between any of the patient groups and HLCs for CD28+ cells, which is consistent with this receptor being constitutively expressed. However, significantly higher levels of ICOS+ T-cells were detected in VH (8.7% vs. 2.3%, $P < 0.0001$) and AIH (3.9% vs. 2.3%, $P = 0.0001$). The level of ICOS+ T-cells in DILI was higher than HLCs (5.1% vs. 2.3%), but did not reach statistical significance. With regard to inhibitory immune checkpoints, we found that the intracellular level of CTLA-4 differed among the study groups ($P < 0.0001$). The level of CTLA-4 was significantly higher in DILI than HLCs (28% vs. 18%, $P = 0.0192$) and NAFLD (28% vs. 18%, $P = 0.0227$). Similarly, VH (30% vs. 18%, $P = 0.0068$), AIH (38% vs. 18%, $P < 0.0001$), and UKE (24% vs. 18%, $P = 0.0120$) demonstrated an increased level of CTLA-4 compared with HLCs. No significant difference was detected in the level of the additional inhibitory checkpoint receptor PD-1 between DILI and HLCs. Only VH presented significantly higher PD-1 level than HLCs (23% vs. 9.3%, $P = 0.0005$).

In terms of immune checkpoint ligands situated on activated monocytes, only the level of PD-L1 differed between the groups ($P < 0.0001$). All subgroups presented a higher level of PD-L1 expression than HLCs (DILI: 5.3% vs. 3.9%, $P = 0.0452$; VH: 9.1% vs. 3.9%, $P < 0.0001$; AIH: 10% vs. 3.9%, $P = 0.0004$; UKE: 8.1% vs. 3.9%, $P < 0.0001$; and NAFLD: 5.0% vs. 3.9%, $P = 0.0104$). In addition, the increased expression in AIH was borderline significant compared with DILI (10% vs. 5.3%, $P = 0.0418$).

To investigate any potential effect resulting from phenotype variations, the leukocyte population and immune checkpoint data were re-analyzed after removing three cases (one DILI and two UKE) which at the time of detection presented ALP > 2 times the ULN and whose ALT level did not exceed 5 times the ULN (or alternatively 3 times the ULN in the presence of TBL > 2 times the ULN) at any timepoint during the episode. No substantial differences were detected between the results obtained when using the original and the reduced cohort. The main findings associated with DILI remained when cases only fulfilling the ALP-based criterion were removed (**Table S3**).

A spearman analysis was performed between quantitative variables (lymphocyte/checkpoint populations and biochemical variables) in search for immunological factors associated with the degree of liver injury. However, the limited number of patients in the study cohorts prevented any firm conclusions to be drawn (data not shown).

DISCUSSION

Flow cytometry enables the characterization of lymphocyte populations (immunophenotyping) in complex cell mixtures, such as peripheral blood. Given that lymphocyte infiltration into the liver occurs during many diseases, immunophenotyping of peripheral blood could reflect immune-related changes associated with a specific condition. In our study, we analyzed general lymphocyte populations (major functional lymphocyte cell subsets) and the expression of cellular markers involved in immune response regulation (immune checkpoint expressions) in DILI compared with non-drug-induced liver conditions and healthy controls, to support the involvement of an adaptive immune response in DILI development. The proportion of activated helper T-cells (CD4+/HLA-DR+) and activated cytotoxic T-cells (CD8+/HLA-DR+) were both significantly increased at an early stage in DILI, and

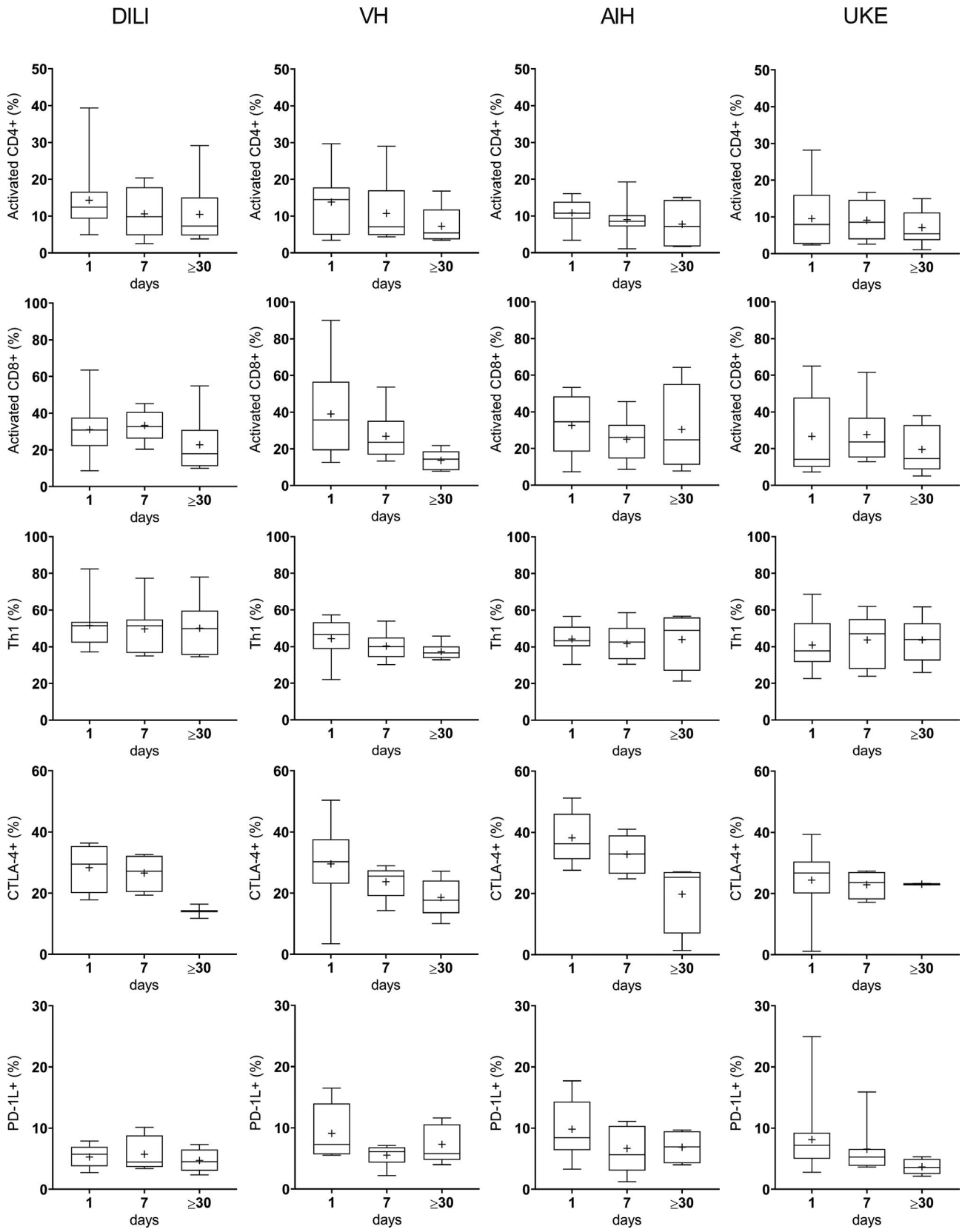

Figure 3 Longitudinal study of leukocyte populations with significant differences in peripheral blood of patients with drug-induced liver injury (DILI), acute viral hepatitis (VH), acute autoimmune hepatitis (AIH), and acute liver injury of unknown etiology (UKE). The median is represented as the horizontal line within the box and the mean as the + sign. The top and the bottom of the box represent the 75th and 25th percentiles, respectively.

even more so in viral hepatitis compared to HLCs. Unlike other activation markers, HLA-DR is considered a late activation marker.[16] This suggests initiation of T-cell activation in DILI prior to disease detection and is in line with an adaptive immune response. HLA-DR molecules are constitutively expressed on B cells and antigen-presenting cells, but are also known to occur on activated T-cells.[17] In addition, HLA-DR expression has also been observed on different regulatory T-cell (Treg) subpopulations with suppressive activity, which is important in maintaining self-tolerance and preventing autoimmunity.[18] Although presence of HLA-DR on Treg cells was not determined in the current study, it is unlikely that the significant increase in CD4+/HLA-DR+ cells found corresponds to immune suppressive Treg cells as the level of Treg cells did not differ from that of HLCs. In addition, the levels of CD4+/HLA-DR+ cells were notably higher than the proportion of Tregs. This points toward an active role for both CD4+ and CD8+ T-cells in DILI development, and is in accordance with earlier findings from cell-based assays.[19,20] Surprisingly, we also detected an increase in Th1 cells, a lineage of CD4+ effector T-cells, in DILI, which was higher than that of VH. The reason for this is unclear, but could include differences in cytokine release between the two conditions and subsequent T-cell polarization.

One might hypothesize that the tendency toward lower lymphocyte count and level of T-cell activation in DILI compared with VH (although not reaching statistical significance) could indicate that the T-cell response in DILI is oligoclonal, whereas viral infections with various immunogenic antigens usually result in a polyclonal T-cell response. If the causative drug forms a neoantigen through haptenization with a unique endogenous peptide, a monoclonal/oligoclonal T-cell response is likely to occur. However, no information on T-cell receptor Vbeta repertoires implicated in DILI is currently available.

An increased proportion of helper T-cells expressing the costimulatory checkpoint receptor ICOS was also seen in DILI, although the increase was not statistically significant. ICOS is weakly expressed on naïve T-cells and quickly upregulated on activated helper and cytotoxic T-cells.[9] Hence, increased level of ICOS in DILI is in line with T-cell activation and an adaptive immune response, similar to the occurrence in the VH group.

Increased PD-L1 expression on monocytes were detected in all patient groups including DILI, whereas increased PD-1 expression on T-cells were most evident in patients with VH. PD-1 is upregulated during the symptomatic phase in acute viral hepatitis and downregulated after recovery.[21] Transient upregulation of PD-1 and PD-L1 also occurs during other forms of acute infections and is thought to be a way of regulating peripheral tolerance.[22] This could similarly be the case in DILI. Increased PD-1 expression is also associated with T-cell exhaustion (impaired proliferation and effector function), but this occurs generally during persistent infection/inflammation. The decreasing levels of PD-1 over time in the current study points toward peripheral tolerance regulation rather than T-cell exhaustion.

The checkpoint receptor CTLA-4 was quantified intracellularly as it undergoes rapid internalization. In fact, Qureshi *et al*. demonstrated that 80% of membrane bound CTLA-4 was internalized within 5 minutes and that this process does not change during T-cell activation, as initially believed.[23] Nevertheless, increased transient cell surface expression of CTLA-4 is known to occur on CD4+ T-cells during the early phase of T-cell responses. The increase in intracellular CTLA-4 detected in DILI (as well as VH, AIH, and UKE) could therefore reflect the accumulation of CTLA-4 expression since the initiation of the immune response, assuming that receptor expression is superior to intracellular degradation.[24] Hence, the observed reduction in intracellular CTLA-4 over time as the patient recuperates from the liver episode may reflect the reduction in antigen presentation and subsequent T-cell activation, based on findings from *in vitro* studies of T-cell receptor stimulation.[25]

This study has revealed new information on the role of the immune system in DILI, but is not without limitations. The use of peripheral blood samples may not fully reflect the immune reactions taking place within the liver. Using liver tissue would undoubtedly be more informative, but would in practice be more difficult, as biopsies are rarely done in patients with DILI who recuperate without any major problems. Furthermore, the populations used in this study are relatively small and the results require additional confirmation in independent cohorts.

In conclusion, this is, to our knowledge, the first presentation of data on different leukocyte populations in a longitudinal study on acute idiosyncratic DILI and patients with additional liver conditions. Our data indicate an increase in activated CD4+ and CD8+ T-cells during the acute phase of DILI that approaches normal values during normalization of the liver profile. These data support the involvement of an adaptive immune response in DILI in line with previous hypotheses. A better understanding of the underlying mechanism of DILI will enable the development of specific diagnostic and prognostic biomarkers, which will facilitate clinical assessment and management of this condition.

SUPPORTING INFORMATION

Supplementary information accompanies this paper on the *Clinical Pharmacology & Therapeutics* website (www.cpt-journal.com).

FUNDING

The present study has been supported by grants of Instituto de Salud Carlos III cofounded by Fondo Europeo de Desarrollo Regional – FEDER (contract numbers: PI19/00883, PI16/01748, P18-RT-3364-2020, and PT20/000127). CIBERehd and Plataforma ISCiii Ensayos Clínicos are funded by Instituto de Salud Carlos III. Funding for open access charge: Universidad de Málaga/CBUA. The funding sources had no involvement in the study design; in the collection, analysis, and interpretation of data; in the writing of the report, or in the decision to submit the manuscript for publication.

CONFLICTS OF INTEREST

The authors declared no competing interests for this work.

AUTHOR CONTRIBUTIONS

A.C.S., F.R.C., R.J.A., M.I.L., and C.S. wrote the manuscript. R.J.A., M.I.L., and C.S. designed the research. A.C.S., E.D.C.H., M.R.D., J.S.C., A.O.A., M.G.C., R.G.G., and M.J.P. performed the research. A.C.S., H.N., F.R.C., R.J.A., M.I.L., and C.S. analyzed the data.

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