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List of abbreviations: ALF: Acute Liver Failure, ALI: Acute Liver Injury, ALT: Alanine Aminotransferase, AST: Aspartate Aminotransferase, BRAK (CXCL14): Breast and Kidney cancer, HBV: Hepatitis B Virus, HCC: Hepatocellular Carcinoma, HCV: Hepatitis C Virus, IP-10 (CXCL10): Interferon ϒ-inducible protein 10, I-TAC (CXCL11): Human Interferon inducible T cell alpha chemokine, SDF-1 (CXCL12): Stromal cell derived factor 1.

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Abstract

Background & Aims: The chemokines CXCL10 (interferon ϒ-inducible protein 10 [IP-10]), CXCL11 (Human interferon inducible T cell alpha chemokine [I-TAC]), CXCL12 (stromal cell derived factor 1 [SDF-1]), and CXCL14 (breast and kidney-expressed chemokine [BRAK]) are involved in cell recruitment, migration, activation, and homing in liver diseases and have been shown to be upregulated during acute liver injury in animal models. However, their expression in patients with acute liver injury is unknown. Here, we aimed to provide evidence of the presence of circulating CXCL10, CXCL11, CXCL12, and CXCL14 during human acute liver injury to propose new inflammation biomarkers for acute liver injury. **Methods:** We analyzed the serum concentration of the studied chemokines in healthy donors (n=36) and patients (n=163) with acute liver injuries of various etiologies. **Results:** Serum CXCL10, CXCL11 and CXCL12 levels were elevated in all the studied groups except biliary diseases for CXCL11. CXCL14 was associated with only acute viral infection and vascular etiologies. The strongest correlation was found between the IFN-inducible studied chemokines (CXCL10 and CXCL11) in all patients and more specifically in the acute viral infection group. **Conclusion:** These data provide evidence for the presence of circulating CXCL10, CXCL11, CXCL12, and CXCL14 during acute liver injury and are consistent with data obtained in animal models. CXCL10, CXCL11 and CXCL12 were the most highly represented and CXCL14 the least represented chemokines. Differential expression patterns were obtained depending on acute liver injury etiology, suggesting the potential use of these chemokines as acute liver injury biomarkers.

Keywords: Acute liver injury, CXCL10, CXCL11, CXCL12, CXCL14

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Key points

- These results provide the first evidence that circulating CXCL10, CXCL11, CXCL12, and CXCL14 are found in ALI patients in a large cohort of patients reflecting many types of disorders.
- CXCL10, CXCL11 and CXCL12 were the most highly represented and CXCL14 the least represented chemokines.
- We observed a distinct expression patterns depending on the etiologies, suggesting a potential use of these chemokines as ALI biomarkers.
- This study provides new data on circulating inflammation mediators during ALI, given the important roles of CXCL10, CXCL11, and CXCL12.

Introduction

Acute liver injury (ALI) may have different origins, including viral infection, alcohol consumption, and drug intake. Patients may spontaneously recover, depending on the severity of the insult and the etiology. However, in some cases, the injury can lead to acute liver failure (ALF). ALF leads to multiorgan system failure and the loss of hepatic function. The incidence of ALF is fewer than 10 cases per million people per year in the developed world, but in the absence of liver transplantation, mortality is up to 50% [1], making ALF a significant public health problem.

Inflammation that results from tissue injury contributes to the pathogenesis of most acute liver diseases and is triggered by host-derived microbial and danger signals [2]. Inflammation is characterized by increased blood flow and vascular permeability, with the accumulation of fluid and leukocytes in the injured tissue, and may be detrimental for patients if not managed. Cytokines and chemokines mediate inflammation by attracting and activating immune cells and orchestrating their interaction [3]. Chemokines play an important role in inflammation. Numbering more than 50, they are categorized into four families: CC, CXC, CX3C, and C [3]. Among them, CXCL10 (IP-10), CXCL11 (I-TAC), and CXCL12 (SDF-1) are well described in the field of liver pathogenesis. Indeed, CXCL10 and CXCL11 are both interferon-γ inducible chemokines involved in chronic liver injury and are linked to liver fibrosis and inflammation through their common receptor, CXCR3, suggesting a strong role during evolution of the disease [4-12]. CXCL12 is a strong lymphocyte chemoattractant that interacts with CXCR4, a pathway involved in many chronic liver diseases [13]. Apart from its chemotactic effect, activation through the CXCL12/CXCR4 axis leads to fibrogenesis through the activation and proliferation of hepatic stellate cells, as well as regeneration by recruiting mesenchymal stem cells from bone marrow to the injured liver [14-20]. Furthermore, both CXCL12 and CXCR4 are upregulated in cirrhotic patients infected with hepatitis C virus

(HCV) or hepatitis B virus (HBV), demonstrating the fundamental role of this axis in chronic injury [20]. CXCL11 and CXCL12 share a more recently discovered common receptor, CXCR7, which is involved in cell survival, cell adhesion, and tumor development [21]. CXCL14 (BRAK) was initially identified from breast and kidney and is constitutively expressed in normal tissue [22]. No direct receptor has been identified for CXCL14 and it has been reported to interact with CXCR4, but with no modulatory effect [23], without interfering with the CXCL12-CXCR4 signaling axis [24]. Conversely, a recent study showed that the high-affinity interaction of CXCL14 with CXCR4 acts synergistically with CXCL12 in chemotaxis [25]. Very little concerning CXCL14 in liver pathogenesis has been reported, although it has been shown to have antitumor activity in hepatocellular carcinoma (HCC) [26].

Little is known concerning the role of CXCL10, CXCL11, CXCL12, and CXCL14 in human ALI, although their expression has been reported in many *in vivo* animal models of ALI and they have been shown to play important roles in human chronic liver diseases [27-33]. Here we aimed to provide evidence for the presence of these circulating chemokines during human ALI and to identify distinct chemokine expression patterns in ALI patients with various etiologies to propose new biomarkers.

Materials and methods

ALI patients and healthy donors

The study protocol was approved by the ethics committee of the Pontchaillou University Hospital and written informed consent was obtained from participants. Inclusion criteria were any ALI with ALT activity > 129 UI/L (equivalent to three times the upper normal limit, considered in this study to be 43 UI/L). Patients with acute-on-chronic liver injury were excluded or if another source of ALT activity was suspected (rhabdomyolysis, myocardial

infarction, etc.). None of the patients had HCC. A control group consisting of 36 healthy volunteers from the French Blood Institute, who were negative for HBV, HCV, and Immunodeficiency Virus (HIV), and had normal ALT activity (< 43UI/L), were also studied. The principal patient characteristics and those of the healthy controls (age, sex, and transaminase activity) are given in Table 1. ALI patients were divided into eight groups, depending on the cause of ALI (Table 2). In this cohort, ALI was caused by direct [acute viral infection, autoimmune disease, toxic injury (including drugs, mushrooms, or alcohol intoxication), and ischemia-reperfusion injury groups] or indirect mechanisms. Indirect injuries are collateral effects caused by vascular problems leading to blood stasis and/or liver ischemia (congestive hepatopathy and vascular and obstetrical groups), as well as bile fluid accumulation, due to obstruction of the bile duct (biliary group). Blood stasis, liver ischemia, and bile fluid accumulation generate tissue damage leading to ALI.

Chemokine detection

Serum was obtained at the time of entry into the study in separator tubes, centrifuged at 2000g at 4°C for 10 min, aliquoted, and stored at -80C° until further analysis. Serum concentrations of chemokines were measured using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Wiesbaden, Germany for CXCL10 and CXCL14 and Peprotech, Rocky Hill, USA for CXCL11 and CXCL12), according to the manufacturers' instructions. The lower detection limits were 31.2 pg/ml for CXCL10, 32 pg/ml for CXCL11, 63 pg/ml for CXCL12 and 62.5 for CXCL14.

Statistical analysis

Data are shown graphically as box-and-whisker plots. The box-and-whisker plots display a statistical summary of the median, quartiles, inter-quartile range and extreme values. The degree of association between two variables was assessed by Spearman's parametric test rank

correlation. Comparisons of parameters between two groups were analyzed by the Mann-Whitney u-test with $\alpha = 0.05$. All statistical analyses were performed using GRAPHPAD PRISM (GraphPad software Inc., La Jolla, CA, USA).

Results

Serum concentrations of CXCL10, CXCL11, and CXCL12, but not CXCL14, are higher in ALI patients than healthy donors.

We first compared the serum concentrations of the four chemokines in ALI patients to those of the healthy donors. The mean age and gender ratio between healthy donors and ALI patients were not statistically different (Table 1). Serum CXCL14 level was not significantly higher in ALI patients than healthy controls (Figure 1). On the contrary, the serum concentrations of CXCL10, CXCL11, and CXCL12 were significantly higher in the ALI cohort than healthy donors ($p < 0.0001$ for CXCL10, CXCL11 and CXCL12) (Figure 1).

CXCL10, CXCL11, CXCL12, and CXCL14 serum concentrations show distinct profiles among the various groups of ALI patients.

We next compared the serum concentration of the four chemokines of the various ALI patient groups with those of the healthy donor subjects (Table 3). The level of CXCL10 was highly significantly elevated ($p < 0.001$) in all groups of ALI patients compared to healthy donors except for auto-immune group which showed lower significance ($p = 0.0168$). The level of CXCL11 was highly significantly elevated for ischemia-reperfusion post liver graft, toxic, congestive hepatopathy, acute viral infection and obstetrical groups. Other etiologies showed either moderate ($p = 0.0013$ for the auto-immune group), slight ($p = 0.0101$ for the vascular group), or non-significant (biliary group) elevation of CXCL11 levels. Regarding CXCL12, the level was highly significantly elevated for all groups except for auto-immune, obstetrical

and biliary groups which showed a moderate significance ($p = 0.0018$, $p = 0.0018$ and $p = 0.0060$ respectively). The level of CXCL14 was highly significantly elevated for the acute viral infection and vascular groups only. Other groups didn't show any significantly levels compared to healthy donors. Data from auto-immune, vascular and obstetrical groups should be taken cautiously due to the low number of patients in each group. Furthermore, we found no correlation between the extent of necrosis (measured by ALT and AST activity) and circulating chemokine levels.

CXCL10, CXCL11 and CXCL12 chemokines correlate each other's in the different groups of patients.

We first evaluated all correlations among the upregulated chemokines in all patients (Table 4). The best correlation was found for CXCL10 and CXCL11, which was moderate ($r = 0.4852$), but highly significant ($p < 0.0001$). However, CXCL10 and CXCL12 ($r = 0.2213$; $p = 0.0045$), but also CXCL11 and CXCL12 ($r = 0.3369$; $p < 0.0001$) were also correlated. Then we looked for any correlation of these 3 chemokines among each group of ALI patients (Table 4). The results showed that the highest and most significant correlation was found for CXCL10 and CXCL11 in acute viral infection group ($r = 0.6905$; $p < 0.0006$). Other groups showed lower degree of correlation or even no significant correlation (Table 4).

Discussion

Our results show that CXCL10, CXCL11, CXCL12, and CXCL14 levels are elevated in the blood of ALI patients, consistent with results obtained in animal models.

Indeed, *in vivo* studies have shown that CXCR3 ligands are upregulated in mouse models of ALI using Concanavalin A [27, 28]. Furthermore, the serum CXCL10 concentration rises after a single administration of CCl4, reaching a peak at day 1, and neutralization of CXCL10 using a monoclonal anti-CXCL10 antibody promotes hepatocyte proliferation from an early

phase [29]. In our cohort, circulating CXCL11 was elevated for all etiologies except for biliary group. CXCL10 was highly or moderately elevated in all groups, and to a less extent in the auto-immune group. The serum levels of CXCL10 and CXCL11, which are interferon-ϒ inducible chemokines correlated with each other in the ALI cohort and with the highest degree in the acute viral infection group compared to the other upregulated chemokines. The level of CXCL10 was recently shown to be elevated in the blood of ALI patients infected by hepatitis A virus, consistent with our results [34].

The CXCL12/CXCR4 axis was shown to be indispensable for liver regeneration in a partial hepatectomy mouse model [30]. Injection of AMD3100, a specific inhibitor of CXCR4, worsened ALI after CCl4 injection, providing additional clues to the hepatoprotective role of this axis through the recruitment of MSC from bone marrow to the injured liver [31]. In contrast, injection of AMD3100 resulted in increased hepatocyte proliferation and reduced necrosis after reperfusion in an ischemia-reperfusion injury mouse model, suggesting a specific mechanism following oxygen deprivation [32]. Serum CXCL12 levels were higher for every etiology in our cohort, consolidating the idea that CXCL12 is a major liver homeostatic chemokine. In addition, ALI patients of the obstetrical group, of whom six of seven suffered from preeclampsia, had elevated levels of circulating CXCL12, consistent with recent findings [35].

Circulating CXCL14 levels have been shown to be higher in a CCl4 ALI mouse model. Neutralization of CXCL14 using monoclonal antibodies reduced the severity of liver injury and steatosis with reduced mouse mortality [33]. Surprisingly, CXCL14 was the least represented chemokine in our ALI cohort. However, we detected elevated levels in the acute viral infection and vascular groups, suggesting a specific mechanism for its release following these injuries.

ALI patients belonging to the congestive hepatopathy and obstetrical groups suffer from indirect ALI, caused by heart failure in one and thrombosis in the other. Other organs may also be affected, and we cannot exclude that this may have been a source of the studied chemokines. Patients of the ischemia-reperfusion injury group were taking anti-rejection medication when samples were collected. It is likely that these drugs reduced the concentration of these circulating chemokines, as they are used to reduce the immune response and inflammation to avoid graft rejection. Besides, results obtained from obstetrical, vascular and auto-immune groups should be interpreted cautiously due to the low number of patients in each group.

In addition, blood was collected at different moments of the ALI for each patient. Indeed, some samples may have been collected at the most acute stage of the liver injury, whereas others may have been collected at a more distant time. This element must be considered in the interpretation of the results, because the release dynamics of these chemokines is unknown for the various etiologies studied, and the levels of the studied chemokines did not correlate with transaminase activity.

This study provides new data on circulating inflammation mediators during ALI, given the important roles of CXCL10, CXCL11, and CXCL12 mediated by their receptors (CXCR3 for CXCL10 and CXCL11, CXCR4 for CXCL12, and CXCR7 for CXCL12) in chronic liver injury shown by *in vitro* and animal model studies. It also adds new knowledge on CXCL14, as its role following interaction with CXCR4 is not yet well established. Additional data on lymphocyte or monocyte phenotypes during ALI could provide new complementary evidence concerning the inflammation processes that occur during these disorders.

In conclusion, these results provide the first evidence that circulating CXCL10, CXCL11, CXCL12, and CXCL14 are found in ALI patients in a large cohort of patients reflecting many

types of disorders. We observed distinct expression patterns depending on the etiologies, suggesting the potential use of these chemokines as ALI biomarkers.

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Figure legends

Figure 1. Box-and-whisker plots illustrating serum chemokine levels in patients (n=163) and healthy donors (n=36). The box extends from the 25^{th} to 75^{th} percentile. The line in the middle denote the median value, and the lines extending from either end of each box denote the extent of the data beyond the 25^{th} and 75^{th} percentiles and outliers, if any. (A-C) ELISA for CXCL10, CXCL11, CXCL12 and CXCL14 respectively. Concentrations are given in pg/ml. (*** p<0,001; statistical significance was determined by Mann-Whitney u-test).

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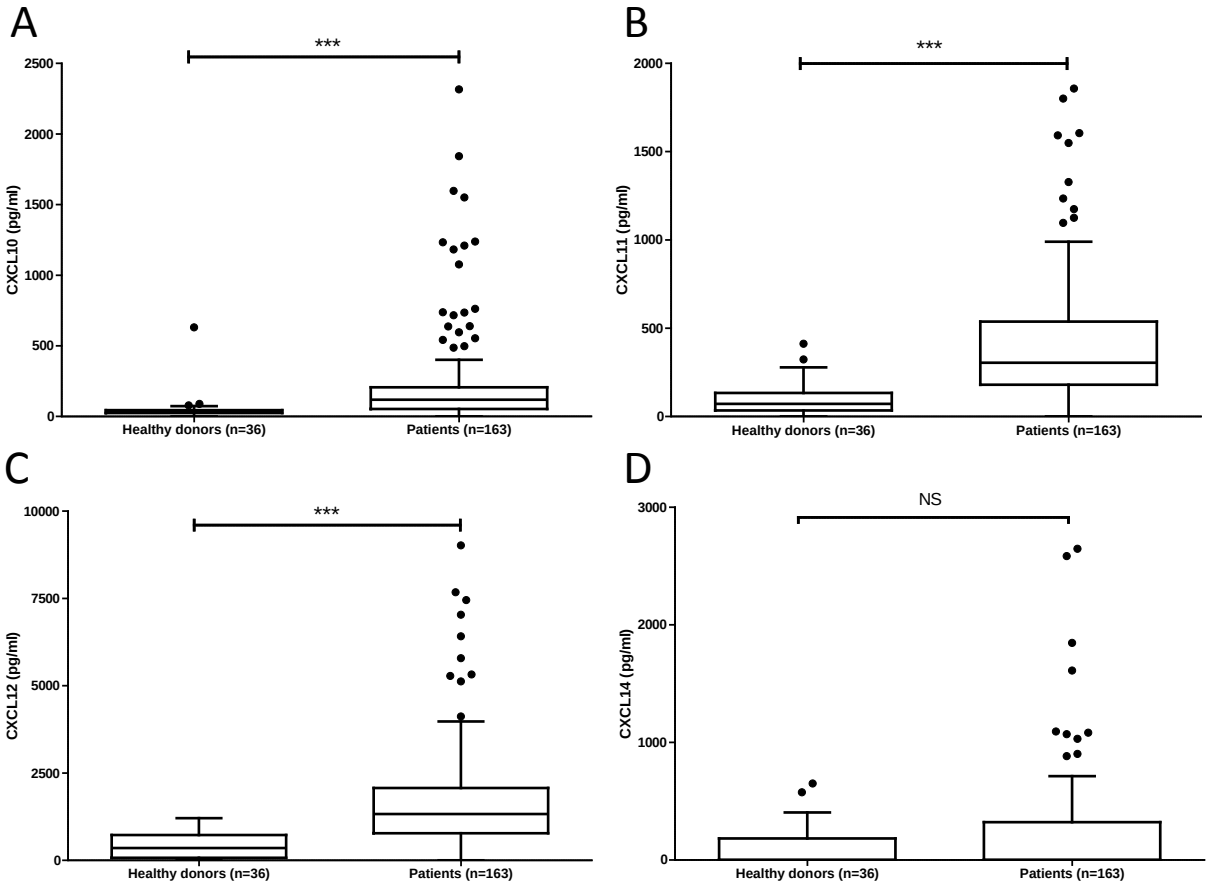

Table 1. ALI patients/healthy donors main characteristics and ALT/AST activity.

	Healthy donors	ALI patients
Case (n)	36	163
Sex (male/female) (n)	21/15	113/50
Age (year)	48 (33-65)	53 (13-99)
ALT (IU/L)	22,2 (12-42)	1205,2 (134,0-8587,0)
AST (IU/L)	25,3 (16,0-40,0)	1215,4 (32,0-11369,0)

Table 2. Description of the etiologies. ALI patients were divided into 8 groups of different injuries : auto-immune (n=6), vascular (n=7), obstetrical (n=7), Biliary (n=19), acute viral infection (n=22), Congestive hepatopathy (n=23), Toxic (n=27) and ischemia reperfusion post liver graft (n=52).

Auto-immune (n=6)	
	Auto-immune hepatitis (n=5)
	Auto-immune cholangitis (n=1)
Vascular (n=7)	
	Liver ischemia (n=4)
	Portal veins thrombosis (n=2)
	Liver veno-oclusive disease (n=1)
Obstetrical (n=7)	
	Preeclampsia (n=6)
	Cholestasis of pregnancy (n=1)
Biliary (n=19)	
	Malignant biliary obstruction (n=6)
	Angiocholitis (n=4)
	Acute biliary pancreatitis (n=4)
	Biliary lithiasis migration (n=3)
	Cholecystitis (n=2)
Acute viral infection (n=22)	
	Hepatitis A (n=8)
	Hepatitis E (n=7)
	Hepatitis B (n=5)
	Herpes simplex virus 2 (n=2)
Congestive hepatopathy (n=23)	
	Heart failure (n=11)
	Cardiogenic shock (n=4)
	Pericardial tamponade (n=4)
	Dilated cardiomyopathy (n=3)
	Valvular cardiomyopathy (n=1)
Toxic (n=27)	
	Drug intoxication (other than acetaminophen) (n=9)
	AAH (Acute Alcoholic Hepatitis) (n=7)
	Acetaminophen intoxication (n=5)
	Mushrooms intoxication (n=3)
	AAH + acetaminophen intoxication (n=2)
	Drugs intoxication + AAH (n=1)
Ischemia reperfusion post liver graft (n=52)	

Table 3. Serum chemokine levels among the different groups of patients and healthy donors. Median and range (in parenthesis) for CXCL10, CXCL11, CXCL12 and CXCL14 are given. Statistical analysis of each group and for each chemokine compared to healthy donors (p-value of u-test are given).

		Concentrations (pg/ml)							
	n	CXCL10	P-value	CXCL11	P-value	CXCL12	P-value	CXCL14	P-value
Healthy donors	36	32,6 (0,0-630,9)	-	71,6 (0,0-411,6)	-	351,5 (0,0-1207,0)	-	0,0 (0,0-651,0)	-
Auto-immune	6	200,0 (20,9-1598,0)	0,0168	559,3 (75,2-1327,0)	0,0013	1523,0 (550,6-3426,0)	0,0018	0,0 (0,0-186,3)	NS
Vascular	7	220,2 (50,8-2316)	0,0006	403,1 (0,0-627,3)	0,0101	1352,0 (606,2-6416,0)	<0,0001	650,5 (0,0-902,5)	0,0009
Obstetrical	7	194,4 (98,8-297,6)	<0,0001	617,9 (365,0-1096,0)	<0,0001	1000,0 (603,8-4122,0)	0,0018	0,0 (0,0-259,9)	NS
Biliary	19	76,5 (21,7-342,1)	0,0002	147,5 (0,0-696,5)	NS	760,4 (0,0-2022,0)	0,0060	0,0 (0,0-460,3)	NS
Acute viral infection	22	162,9 (42,0-1211,0)	<0,0001	675,4 (0,0-1856,0)	<0,0001	1257,0 (164,0-3234,0)	<0,0001	386,8 (0,0-1030,0)	0,0005
Congestive hepatopathy	23	66,1 (35,5-638,1)	<0,0001	210,8 (32,5-829,4)	0,0001	1524,0 (463,9-7034,0)	<0,0001	0,0 (0,0-2584,0)	NS
Toxic	27	103,6 (0,0-1552,0)	0,0002	226,9 (0,0-1549,0)	0,0004	1832,0 (384,0-7679,0)	<0,0001	0,0 (0,0-2647,0)	NS
Ischemia reperfusion post liver graft	52	121,3 (0,0-1844,0)	<0,0001	324,3 (0,0-1604,0)	<0,0001	1260,0 (0,0-9018,0)	<0,0001	0,0 (0,0-183,2)	NS

Table 4. Correlation of CXCL10, CXCL11 and CXCL12 chemokines among the different groups of patients. Spearman's rank correlation test. Only significant results are shown. (r, correlation coefficient, p, p-value)

	CXCL10 vs CXCL11		CXCL10 vs CXCL12		CXCL11 vs CXCL12	
Etiology	r	p	r	p	r	p
Ischemia reperfusion post liver graft	0,4223	**		NS	0,4424	**
Toxic		NS		NS		NS
Congestive hepatopathy		NS	0,2144	*		NS
Acute viral infection	0,6905	***		NS	0,5991	**
Biliary		NS		NS	0,5542	*
All patients	0,4852	***	0,2213	**	0,3369	***