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Increased Expression of CTLA4 by T Cells, Induced by B7 in Sera, Reduces Adaptive Immunity in Patients With Acute Liver Failure

Short Title: CTLA4 is a negative regulator in ALF

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Abbreviations: (APAP), acetaminophen; (ALF), acute liver failure; (AALF), acetaminophen induced acute liver failure; (CLD), chronic liver disease; (CTLA4), Cytotoxic T lymphocyte-associated molecule-4; (INR), international normalized ratio; (MELD), model of end-stage liver disease.

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Drafting of the manuscript: WK, CGA.

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Abstract

BACKGROUND & AIMS: Patients with acute liver failure (ALF) have defects in innate immune responses to microbes (immuneparesis) and are susceptible to sepsis. Cytotoxic T-lymphocyte associated protein 4 (CTLA4), which interacts with the membrane receptor B7 (also called CD80 and CD86), is a negative regulator of T-cell activation. We collected T cells from patients with ALF and investigated whether inhibitory signals downregulate adaptive immune responses in patients with ALF. **METHODS**: We collected peripheral blood mononuclear cells from patients with ALF and controls from September 2013 through September 2015 (45 patients with ALF, 20 patients with acute-on-chronic liver failure, 15 patients with cirrhosis with no evidence of acute decompensation, 20 patients with septic shock but no cirrhosis or liver disease, and 20 healthy individuals). Circulating $CD4^+$T cells were isolated and analyzed by flow cytometry. $CD4^+$ T cells were incubated with antigen, or agonist to CD3 and dendritic cells, with or without antibody against CTLA4; T-cell proliferation and protein expression were quantified. We measured levels of soluble B7 molecules in supernatants of isolated primary hepatocytes, hepatic sinusoidal endothelial cells, and biliary epithelial cells from healthy or diseased liver tissues. We also measured levels of soluble B7 serum samples from patients and controls, and mice with acetaminophen-induced liver injury using ELISAs. **RESULTS:** Peripheral blood samples from patients with ALF had a higher proportion of $CD4^+$ $CTLA4^+$ T cells than controls; patients with infections had the highest proportions. $CD4^+$T cells from patients with ALF had a reduced proliferative response to antigen or CD3 stimulation compared to cells from controls; incubation of $CD4^+$T cells from patients with ALF with an antibody against CTLA4 increased their proliferative response to antigen and to CD3 stimulation, to the same levels as cells from controls. $CD4^+$ T cells from controls upregulated expression of CTLA4 following 24–48 hrs culture with sera from patients with ALF; these sera were found to have increased

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concentrations of soluble B7 compared to sera from controls. Necrotic human primary hepatocytes exposed to acetaminophen, but not hepatic sinusoidal endothelial cells and biliary epithelial cells from patients with ALF, secreted high levels of soluble B7. Sera from mice with acetaminophen-induced liver injury contained high levels of soluble B7 compared to sera from mice without liver injury. Plasma exchange reduced circulating levels of soluble B7 in patients with ALF and expression of CTLA4 on T cells. **Conclusions**: Peripheral $CD4^+$ T cells from patients with ALF have increased expression of CTLA4 compared to individuals without ALF; these cells have a reduced response to antigen and CD3 stimulation. We found sera of patients with ALF and from mice with liver injury to have high concentrations of soluble B7, which upregulates CTLA4 expression by T cells and reduces their response to antigen. Plasma exchange reduces levels of B7 in sera from patients with ALF and might be used to restore antimicrobial responses to patients.

KEY WORDS: immune regulation; liver disease; treatment; infection susceptibility

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Acute liver failure (ALF) occurs following a severe hepatic insult resulting in a rapidly progressive clinical syndrome characterized by jaundice, encephalopathy, coagulopathy and multiple organ dysfunction[1, 2]. Whilst the initiating event in ALF is acute hepatocellular death, mortality is attributable to a profound activation of systemic inflammatory responses (SIRS) and multiple organ dysfunction[1-3]. Recent studies identify defects in innate immune responses to microbial cues, termed immuneparesis, which cause an increased susceptibility to secondary infections, a leading cause of mortality in ALF[4-7].

Following acute tissue injury, intracellular components, defined as damage-associated molecular pattern molecules (DAMPS) or alarmins, are released by necrotic and apoptotic cells and act as "danger" signal molecules that trigger organ specific and systemic inflammatory responses[8, 9]. Studies identify profound elevations in these danger signals in patients with acute hepatic inflammatory disorders which recently have been shown to modulate the function of myeloid and possibly lymphoid cells[10-12].

Substantial evidence exists in non-hepatic inflammatory disorders (e.g. severe trauma, pancreatitis, septic shock) of suppression in T cell-mediated anti-microbial responses that account for the immuneparesis and infectious complications encountered in these patients[9, 13]. The acquired immune dysfunction reported in sepsis includes increased regulatory T cells (Tregs) with elevated levels of inhibitory receptor including PD-1 and CTLA4[13, 14] which correlate with reduced IFN-γ production and low T cell proliferative capacity[15]. In murine models of sepsis, CTLA4 was also reported to be elevated on $CD4^+$ and $CD8^+$ T cells[16]. Accordingly, blocking PD-1 and CTLA4-mediated negative regulatory pathways demonstrated improved survival in animal models of bacterial and fungal sepsis, increased pathogen clearance and reversed T cell dysfunction in patients with sepsis[16, 17]. However, it remains to be determined whether defects in peripheral adaptive immune responses play a

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role in the immuneparesis reported in ALF.

CTLA4 (CD152) is a well characterised negative regulator expressed on T cells that binds to the same ligands (CD80 and CD86) as CD28, but with a higher affinity[18]. Upon T cell activation, CTLA4 cycles to the cell surface and exerts its inhibitory effect that results in attenuated T cell responses and inhibition of IL-2 secretion, a critical cytokine for T cell expansion[19, 20]. In humans, CTLA4 has been implicated in numerous autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus (SLE)[21, 22]. In severe sepsis, CTLA4 is upregulated resulting in impairment in T cell activation and antimicrobial responses[17, 23]. CTLA4 was the first immune checkpoint receptor to be clinically targeted and several studies have demonstrated that antibody blockade of CTLA4 could result in anti-tumour immunity[24, 25].

In this study, we sought to assess the immunological competence of $CD4^+$ T cells in patients with ALF by examining their phenotype and function. Here, we identify adaptive immune dysfunction in $CD4^+$ T cells due to a sustained expression of the negative regulator of T cell activation, CTLA4, via mechanisms that involve soluble B7 ligands. The impairment of peripheral $CD4^+$ T cells adaptive responses in ALF may further contribute to the susceptibility to infections seen in these patients.

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Methods

Patients Characteristics

All patients were consecutively recruited from September 2013 to September 2015. Subjects were recruited to the study within 24 hours of admission to the liver intensive therapy unit (LITU) or liver wards. Patients were categorised into the following groups: ALF (n=45: 35 acetaminophen-induced ALF [AALF] and 10 non-acetaminophen induced [NAALF], patients with acute-on-chronic liver failure (ACLF; n=20), chronic Liver Disease patients with cirrhosis with no evidence of acute decompensation (CLD; n=15), patients with septic shock with no underlying cirrhosis/liver disease (sepsis group; n=20), healthy controls (HC; n=20). A subset of ALF patients underwent sequential sampling on day 3 (n=7), day 7 (n=8) and day 14 (n=5) following admission. In addition, a subset ACLF patients underwent sequential sampling on day 3 (n=5), day 7 (n=4) and day 14 (n=5). When plasma exchange was instituted in ALF patients recruited, further sampling was performed immediately prior to PE (pre-PE, n=7) and within 8hours following completion of plasma exchange (post-PE; n=7). All patients who fulfilled criteria for ALF were transferred to LITU based on published referral guidelines for both acetaminophen and non-acetaminophen induced ALF[26]. Patients with ACLF fulfilled the established diagnostic criteria developed by the EASL-CLIF consortium[27]. This study was approved by the King's College Hospital ethics committee (12/LO/0167). Informed consent was obtained by the next of kin if patients were not able unable to provide consent.

Phenotyping Using Flow Cytometry

PBMCs isolated through density-gradient centrifugation were surface stained for CD3, CD4, CD8, CD45RA, CD45RO, CCR-7, CTLA4, PD-1, CD25, CD62L, CD28, CD40L and CD127. Twelve-color flow cytometric analyses were performed using an LSR Fortessa

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flow cytometer and data were acquired using BD FACSDiva software (Becton Dickinson Ltd, Oxford, UK).

Antigen Recall Responses and CD4$^+$ T cell proliferation

Specific CD4$^+$ T-cell antigen-recall responses to a pool of HLA class II-restricted T cell epitopes at 10µg/ml (CTL Europe GmbH, Germany) were examined *in vitro* for T cell proliferation using either carboxyfluorescein succinimidyl ester (CFSE) or cell proliferation dye eFluor®670 (eBioscience, Hatfield, UK) labelling, in the presence or absence of anti-CTLA4 blocking antibody (10µg/ml) (eBioscience, Hatfield, UK). All blocking antibodies used in this study were referenced neutralizing antibodies from the manufacturers. CD4$^+$ T cell proliferation was tested by labelling PBMCs with CFSE prior to being placed in culture for 6 days. PBMCs were cultured in RPMI 1640 (Thermo Fisher Scientific, Hemel Hempstead, UK) supplemented with 10% human AB serum (PAA laboratories Ltd, UK). After stimulation for 6 days at 37°C in 5% CO_2, cells were harvested and stained for surface markers (CD3, CD4 and CD8). Data were collected using LSR Fortessa (BD) and analyzed using FlowLogic (Inivai Technologies Pty Ltd, Victoria, Australia).

DC-CD4$^+$T cell Co-culture

Allogeneic DCs were generated as previously described[28]. They were then co-cultured with CD4$^+$ T cells from ALF patients in the presence of anti-CD3 (α-CD3) antibody (0.5µg/ml) (eBioscience, Hatfield, UK) at optimal ratio of 1:40 (DC to CD4$^+$ T cells) ratio, which was selected following a dose response testing (ranging from a 1:1 to a 1:80 DC to CD4$^+$ T cells ratio). Cells were co-cultured for 5 days in the presence or absence of anti-CTLA4 (α-CTLA4) (10µg/ml). Cell division of CD4$^+$ T cell was measured by the dilution of CFSE dye using flow cytometry.

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Effect of Circulating sB7 on CTLA4 Expression

$CD4^+$ T cells isolated from HC were cultured in fresh medium for 24 and 48 hours at 5×10^5 cells per well in the presence of 10% ALF (n= 6) or HC (n=4) sera and screened for CTLA4 surface expression using flow cytometry. Supernatant were collected for subsequent detection of cytokines. Sera samples from both HC and ALF patients were pre-incubated with anti-human CD80 or CD86 neutralizing antibodies (α-CD80 or α-CD86) (R&D Systems, Abingdon, UK) for 45min at room temperature before addition to $CD4^+$ T cells isolated from healthy donors. Cell culture supernatants were collected for assessing cytokine secretion. Cells were harvested for phenotyping. Unless otherwise stated, sB7 molecules refer to sCD80 and sCD86.

Primary Human Hepatocytes and Kupffer Cells (KC)

Cryopreserved hepatocytes (Invivogen Paisley, UK) were cultured for 24 hours in the presence or absence of 20mM APAP (Sigma, Dorset, UK). Cells were stained using apoptotic/necrotic cell kit (PromoCell GmbH, Heidelberg, Germany). Apoptosis and necrosis were detected using Annexin V, Ethidium homodimer III (EthD-III), respectively according to the manufacturer's instructions. KCs (Invivogen Paisley, UK) were stimulated overnight in the presence of LPS (100ng/ml). Supernatants from hepatocyte and KC cultures were collected for assessment of sB7 molecules.

Isolation and Culture of Hepatic Sinusoidal Endothelial Cells (HSECs) and Biliary Epithelial Cells (BECs)

HSECs and BECs were isolated according to previously described methods[29] approved by the University of Birmingham ethics committee (06/Q2702/61). HSECs were cultured in the presence of sera from ALF (n=6) and HC (n=6) at 25%. Supernatant were then

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collected and assessed for sB7 molecules. Sera pre-conditioned HSEC were cultured for an additional 24 hours in the presence of 10ng/ml recombinant human tumour necrosis factor-α (TNF-α) and 10ng/ml interferon-γ (IFNγ) (PeproTech, UK). Supernatants were collected for assessment of sB7 molecules.

Co-culture of Monocytes with Apoptotic Neutrophils

Neutrophils were isolated by density gradient centrifugation as previously described[30]. Neutrophils were re-suspended (10^6 cells/ml) in fresh complete medium and incubated for 20 hours (37℃ in 5% CO_2) in 24-well plates (Corning, USA). Annexin-V kit (BD Biosciences, UK) was used to determine the percentage of apoptotic neutrophils, which was over 65%, as previously described[30]. Next, $CD14^+$ monocytes were re-suspended (0.5×10^6 cells/ml) and co-incubated in 1:2 ratio for 4 hours with apoptotic neutrophils. Supernatants were collected in order to measure sB7 levels by ELISA.

ELISA and MSD Multiplex Cytokine Detection System

Soluble B7 were assessed in cell culture supernatants, human sera and acetaminophen-induced (APAP) liver injury model murine models using ELISA. Cytokines were also assessed in cell culture supernatant using ELISA and MSD.

Statistical Analysis

Parametric statistical analysis was performed using the Student t test. Non-parametric analysis was carried out by using the Mann-Whitney U, Wilcoxon matched-pairs signed rank and Kruskal–Wallis tests and data are expressed as median [interquartile range]. For correlations of $CD4^+CTLA4^+$ T-cell frequency and clinical characteristics as well as correlations of sB7 ligands and disease severity indices, Spearman rank correlation

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coefficients were used. Statistical significance was assumed for P values of less than 0.05. All analyses were performed by using Graph Pad prism software.

Other details and additional experimental procedures are provided in the Supplementary Materials

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Results

Patient Characteristics

There was no significant difference in median ages of ALF patients when compared to HC, while pathological patients groups were significantly older (Supplementary Table 1). ALF patients have significantly higher biochemical and physiological indices of acute liver injury (MELD, INR, creatinine, and bilirubin) compared to CLD, ACLF and sepsis patients (Supplementary Table 1). The number of circulating lymphocytes was significantly reduced in ALF patients when compared to CLD and ALCF patients (Supplementary Table 1) while no differences were seen when compared with sepsis patients. Moreover, lymphocyte counts in AALF correlated negatively with indices of severity of liver injury (INR: r = -0.285, *P*=.04; AST: r = -0.465, *P*= .001; SIRS scores: r = -0.391, *P*= .009; and MELD scores: r = -0.557, *P*= .0001). The demographic and key clinical characteristics of patients are summarised in supplementary Table 1. Clinical data of ALF patients who underwent PE are summarised in supplementary Table 2.

Increased Frequency of Circulating $CD4^+$ T cells in ALF Patients with a Predominantly Naïve Phenotype

In ALF patients, there is an increase in the proportion of circulating $CD4^+$ T cells (median 64.01%, [56.51-71.61]) when compared to healthy (47.17%, [45.17-58.12]) (*P*=.003) and pathological controls (CLD 65.64%, [51.9-74.07], ACLF 57.30% [46.54-69.56] and sepsis patients (70.18%, [58.61-77.11]) (Figure 1*A*). No significant changes were seen in the proportion of circulating $CD8^+$ T cells in ALF when compared to HC and pathological control groups (Figure 1*A*). The distribution of naïve and memory subsets is markedly different, highlighted by a diminished proportion of memory population ($CD4^+CD45RO^+CD45RA^-$) and an increased proportion of naïve cells ($CD4^+CD45RO^-$

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$CD45RA^+$) in ALF compared with HC subjects (ratio memory to naïve: 0.4513%, [0.3328-0.7557] versus 0.7825%, [0.5961-1.338], *P*=.001) (Figure 1*B*). The reduction in the memory population was predominantly in the effector memory T cell (T_{EM}) subset ($CD4^+CCR7^-$ $CD45RO^+$) (*P*=.002) (Figure 1 *C*).

Circulating $CD4^+$ T Cells in ALF are Characterized by an Immunosuppressive CTLA4 Positive Phenotype

We assessed the surface expression of activation and inhibitory markers of T cell function. Compared to ACLF, CLD patients and healthy controls, CTLA4 surface expression was markedly elevated in $CD4^+$ T cells from ALF patients on admission to the liver intensive care unit (*P*<.0001) (Figure *2A&B*). The increase in CTLA4 expression is detected in both AALF and NAALF patients (1.390% [0.6800-6.418] in AALF and 1.985% [1.020-4.470] in NAALF compared to 0.1750% [0.0325-0.6225] in healthy controls). In line with previous reports, septic shock patients also showed high levels of CTLA4 expression when compared to healthy individuals (Figure 2*B*). However, levels in septic shock patients were significantly lower that detected in ALF patients (*P*=.001). Analyses of CTLA4 expression in sequentially collected ALF samples showed that the proportion of CTLA4 expressing $CD4^+$ T cells remained significantly higher than healthy controls throughout the course of admission (Figure 2*C*). At day 3 post-admission, CTLA4 levels of expression peaked, 2.5 fold above day 1 levels (*P*=.02) (Figure 2*C*). Detailed immunophenotypic analyses of $CD4^+$ T cells revealed no significant differences in the frequencies of cells expressing CD25, PD1, CD40L CD28 and CD62L among $CD4^+$ T cells in the studied groups (Supplementary Figure 2).

Distribution of CTLA4 Expression in Different $CD4^+$ T Cell Subsets

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We extended the observations reported above and investigated the distribution of CTLA4 expression on the $CD4^+$ naïve and memory T cell subsets distinguished based on expression of CD25, CD127 and CD45RA markers. The distribution of CTLA4 expression differed among ALF $CD4^+$ T cell subsets compared to HC. As illustrated in Figure 2D, CTLA4 expression was elevated in all $CD4^+$ T cells, most marked in the memory ($CD25^-CD127^-CD45RA^-$) when compared to the naïve ($CD25^{low}CD127^+CD45RA^+$) subset. In line with previously published studies in systemic inflammatory pathologies[31], levels were also high in the T regulatory subset ($CD25^+CD127^-$) (Supplementary Figure 4).

$CD4^+CTLA4^+$ T Cells Correlate With Disease Severity and Infectious Complications in ALF

CTLA4 expression by $CD4^+$ T cells was higher in patients who developed culture positive infections compared to non-infected patients (*P*=.04) (Figure 2*E*). When assessed for correlation with their corresponding clinical parameters, patients sampled on day 1 of admission who later developed culture-positive infection had elevated frequency of $CTLA4^+CD4^+$ T cell which correlated positively with MELD score (r=0.675, *P*=.01) (Figure 2*E*) and ammonia (r=0.771, *P*=.07) (Table 1). Analyses of the distribution of CTLA4 expression among naïve and memory subsets in patients following admission revealed that percentage of memory cells expressing CTLA4 peaked on day 3 following admission (Figure 2*E*), particularly within the infected cohort (1.330%, [0.1900-2.470] on day 1 compared to 9.320%, [0.800-17.84] on day 3) (Supplementary Figure 5). No differences were noted in the distribution of CTLA4 when infected ACLF groups were compared to non-infected ACLF (Supplementary Figure *3A&B*).

Defects in $CD4^+$-Mediated T Cell Responses are Restored through Blocking CTLA4

To investigate whether phenotypic changes reflect a change in the functional capacity in $CD4^+$ T cells in ALF, we assessed the proliferative capacity of $CD4^+$ T cells using both

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antigen-dependent and independent systems. Firstly, in response to MHC-II restricted recall antigens, we reveal that T cell proliferation and IL-2 secretion were significantly reduced in ALF (*P*=.008 and *P*=.02, respectively) when compared to HC (Figure 3 *A&B*). T cell proliferation was reversed following blockade of the CTLA4 pathway (*P*=.007) (Figure 3*C*). Significant increases in T cell proliferation following CTLA4 pathway blockade in ACLF or CLD patient groups was not detected (Figure 3C). IL-2 production was induced following CTLA4 blockade in ALF (12.50 pg/ml [9.810-40.25]) (*P*=.01). IFN-γ levels were also restored, but did not reach statistical significance (Supplementary Figure 7*A*). Furthermore, the improvement in proliferative capabilities was most pronounced in memory $CD4^+CD45RO^+$ subset (Figure 3*D*). Then, we determined whether $CD4^+$ T cells from ALF patients responded to TCR-mediated activation with α-CD3 antibody in the presence of allogeneic healthy donor-monocyte-derived DC. $CD4^+$ T cells isolated from HCs underwent more cell divisions in co-culture with allogeneic DC following α-CD3 antibody stimulation than the high expressing CTLA4 $CD4^+$ T cells derived from ALF patients (Figure 3*E*). To confirm the suppressive effect of CTLA4 in $CD4^+$ T cells from ALF patients, co-cultures were carried out in the presence of anti-CTLA4 antibody. Blocking CTLA4 activity restored $CD4^+$ T cell proliferation (*P*=.03) and IL-2 production (114.8 pg/ml [61.51-172.4] compared to 244.1pg/ml [165.9- 294.6] after blocking CTLA4) (*P*=.04). Analyses of $CD4^+$ T cell function following CTLA4 blockade revealed no significant differences in the ACLF and CLD patient groups (Figure *3E*).

Soluble Circulating Mediators in ALF Regulate CTLA4 Expression

In view of the importance of the inflammatory microenvironment in modulating immune cell function in ALF[4, 6, 7, 32], we investigated the effects of sera, derived from ALF patients, on CTLA4 expression in $CD4^+$ T cells from HC. Exposure to sera from ALF patients

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resulted in up-regulation of CTLA4 expression on $CD4^+$ T cells following 24 and 48 hours in culture (*P*=.01) (Figure 4*A*). Similar to the effect of sera from ALF patients, we report an elevation in CTLA4 expression following exposure to sera from sepsis patients. However, these levels were still significantly lower than what we detect following exposure to sera derived from ALF patients (Figure 4*B*). No effect on CTLA4 up-regulation was detected in cultures of $CD4^+$ T cells in sera derived from ACLF and CLD patients (Figure 4*B*).

In order to provide a mechanistic explanation for the activation of the CTLA4 pathway in ALF, we sought to investigate the role of circulating soluble B7 molecules (sCD80 and sCD86). Here, we detected a marked elevation in circulating concentrations of sCD80 and sCD86 in ALF compared with sera from ACLF, CLD and HCs (Figure *4C-E*). Similar to the CTLA4 levels, sB7 concentrations peaked at day 3 of admission (*P*=.03) and remained persistently elevated until day 14 (Figure 4*D*).

We hypothesised that high levels of circulating sB7 detected in ALF are responsible for the activation of the CTLA4 pathway in circulating $CD4^+$ T cells. To test this hypothesis, we assessed the effect of neutralising sCD80 and CD86 in ALF sera prior to exposure to $CD4^+$ T cells from HCs. As shown in Figure 4F, elevations in CTLA4 expressing $CD4^+$ T cells were significantly reduced whilst proliferation and IL-2 secretion were augmented (*P*=.01 and *P*=.002, respectively) following neutralisation of sCD80 and sCD86 in sera derived from ALF patients when compared to HC (Figure 4*F*). Similarly, we report an increase in levels of IFN-γ, but this did not reach statistical significance (Supplementary Figure 7*B*)

sB7 are Secreted by Injured Hepatocytes in ALF

In order to investigate the source of the increased circulating levels of sB7 molecules in the circulation, we assessed the ability of hepatic parenchymal (hepatocytes and biliary epithelial cells) and non-parenchymal cells (Kupffer cells, monocytes) to release sB7

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following *in vitro* and in an *in vivo* model of APAP-induced acute liver injury. Whilst immune cells, HSECs and biliary epithelial cells in ALF did not release increased concentrations of sB7, APAP-treated necrotic primary human hepatocytes (Figure 5*A*) released high levels of sB7 (Figure 5*B*). No detectable levels of sB7 ligands were seen in all tested activated and non-activated immune and non-immune cell types. Epithelial cell death was further validated by assessing levels of cell death markers (caspase-cleaved [CK-18 (M30)] and total cytokeratin [CK-18 (M65)] (Figure 5*B*). In addition to elevated levels of sB7, primary human hepatocytes secreted elevated levels of M30 and M65 following APAP-treatment (Figure 5*B*). We further assessed sera concentrations of M30 and M65 in ALF patients. Levels of M30 and M65 were significantly elevated in ALF patients (Figure 5 *C*) and strongly correlated with release of sCD86 (M30: r = 0.492, *P*=.01; M65: r = 0.420, *P*= .03).

Having demonstrated *in vitro* that APAP-treated hepatocytes represented the main source of sB7, we postulated that sB7 are released as early mediators from damaged hepatocytes and play a key role in initiating and maintaining elevated CTLA4 expression levels on circulating $CD4^+$ T cells. We examined this *in vivo* using a time course APAP-induced acute liver injury murine model[33] (Figure 5*D*). Consistent with the *in vitro* findings, APAP-injury resulted in the release of increased concentrations of sB7, which were detected in the sera as early as 8 hours post APAP-treatment (initiation phase) (*P*=.007), remained elevated between 8 and 24 hours (peak liver injury) and significantly decreased at 48 hours onwards (resolution phase) (*P*=.0.03) to return to baseline levels after 5 days (Figure 5*E*).

Plasma Exchange Modulates CTLA4 Activation through Clearance of Circulating sB7 Molecules in ALF

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Plasma exchange (PE) has been previously reported to be an important therapeutic intervention capable of modulating innate immune responses in ALF but its effects on adaptive immunity are not known [12]. We assessed the levels of sCD80 and sCD86 and showed that these were significantly reduced following PE (Figure 6*A*). In contrast, no reductions in sB7 levels were detected in ALF patients who did not undergo PE (Figure 6*B*). To assess whether these reductions in circulating titres of sB7 molecules attenuate CTLA4 expression, we performed *in vitro* cultures quantifying the CTLA4 expression on $CD4^+$ T cells following incubation of HC derived $CD4^+$ T cells in sera obtained from ALF patients prior (pre-PE) and following (post-PE) plasma exchange. Exposure of $CD4^+$ T cell to pre-PE sera resulted in a significant increase in CTLA4 levels whereas exposure to post-PE sera did not (Figure 6*A*). These results indicate that PE sera drive a markedly different phenotype of the $CD4^+$ T cell population characterised by reduced levels of CTLA4.

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Discussion

This study identifies adaptive immune dysfunction, mediated through CTLA4 that is triggered by soluble co-stimulatory sB7 molecules released from the acutely inflamed liver in patients with ALF. Here, we identify not only a numerical reduction in circulating lymphocytes but also an increased proportion of $CD4^+$ T cells bearing the inhibitory, CTLA4 positive, phenotype specifically in AALF and NAALF patients and not in other acute and chronic hepatic inflammatory diseases. CTLA4 expression was particularly elevated in memory $CD4^+$ T cell, an immune cell subset responsible for protective immunity against microbial pathogens[34]. These findings are likely to be of pathogenic significance in ALF given that higher level of CTLA4 expressing cells on admission are detected in patients who proceed to develop culture positive secondary infections. Therefore, the elevated CTLA4 levels in the memory $CD4^+$ T cell compartment is likely to reflect an impaired capacity to protect against re-infection and a defect in producing robust effector function responses upon microbial challenge. The increased proportion of CTLA4 bearing cells in ALF indicates a central role for CTLA4 pathway in the predisposition to infection and that blockade of this immunosuppressive pathway may be beneficial in restoring anti-microbial responses. We reveal that blocking activation of the CTLA4 pathway significantly restores antigen-specific responses particularly in the memory $CD4^+$ T cell subset; with similar findings reported in CTLA4 expressing memory $CD8^+$ T cells[35]. It is interesting to note that infected ALF patients had persistent elevated CTLA4 expression on $CD4^+$ T cells. In this regard, several experimental and clinical studies showed the involvement of CTLA4-mediated negative regulation in infections[36, 37]. CTLA4 was reported to be expressed at higher levels in patients with sepsis than in non-sepsis critically ill patients and was associated with impairment in T cell responses[38, 39]. Furthermore, in severe sepsis T cell apoptosis and dysfunction were associated with an up-regulation of CTLA4 on $CD4^+$ T cells[40, 41]. Similarly, increased levels

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of CTLA4 were implicated in viral infection-associated complications[42, 43]. Accordingly, in murine models of bacterial and fungal sepsis, blocking CTLA4 improved pathogen clearance and survival[16, 17].

The immune regulatory functions of the B7/CD28/CTLA4 pathway are well recognized in acquired immunodeficiency and autoimmune disorders[44, 45]. It is known that membrane bound CD80 and 86 represent the shared ligands for CD28 and CTLA4, [46]. However, it has also been shown that soluble forms of B7 molecules represent an alternative powerful mechanism by which antigen presenting cells could modulate the signals normally generated via membrane bound forms of B7. Experimental studies indicate that recombinant soluble B7 molecules represent a putative mechanism for modulating T cell responses through either inhibition or enhancement of immune responses[47, 48]. In this study, we provide a novel mechanistic explanation as to the pathogenesis of adaptive immune dysfunction in patients with ALF. Here, we report a marked and persistent elevation in circulating titres of soluble B7 molecules, in particular sCD86, which strongly correlate with indices of severity of acute hepatic injury and more specific markers of hepatocellular death (M30/M65). In view of these findings, we hypothesized that the origin and release of these molecules was from the acutely injured liver. We screened for sB7 release in parenchymal and non-parenchymal cells and we identify that the likely origin of sB7 molecules is from hepatocytes which have undergone necrotic cell death following APAP administration. This finding is corroborated by *in vivo* data from the murine model of APAP- induced liver injury where highest circulating titres of sB7 are detected at peak hepatoxicity. Taken together, we highlight a novel role for soluble B7 molecules, released following hepatocyte cell death, that negatively regulate the adaptive immune responses through activation of the CTLA4 pathway. Although our data support that epithelial cell death is responsible for increased levels of sB7, in particular sCD86, it cannot be excluded that during ALF, renal replacement

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therapy (RRT) alone would be a sufficient modality in clearing the sudden increase in concentrations of these circulating proteins form the circulation. This is supported by our findings where we identify that plasma exchange that does result in significant reductions in the concentrations of sCD86 levels in patients with acute liver injury. Further studies are required to determine whether this mechanism of adaptive immune dysfunction is common to other hepatic and non-hepatic systemic inflammatory pathologies characterised by sudden and overwhelming acute tissue injury[9].

This work has identified a novel therapeutic target to reverse immune dysfunction in patients with ALF. CTLA4 inhibitory strategies are established immune checkpoint inhibitors in malignant and non-malignant inflammatory pathologies[16, 25, 49]. However, there would be concern regarding the use of this immunotherapeutic strategy in ALF (e.g. ipiluminab) given its significant side effect profile (e.g. colitis, dermatitis and autoimmune hepatitis[24]) and potential risk in impairing hepatic regenerative responses.

Plasma exchange has been recently shown to be of benefit in patients with ALF through clearance of damage associated molecular patterns (DAMPs) and other intracellular products released from the injured liver[12]. Given the fact that soluble B7 molecules, released from necrotic hepatocytes, are responsible for activation of the CTLA4 pathway, we postulated that plasma exchange would attenuate CTLA4 levels through removal of soluble B7 molecules. Here we demonstrate that plasma exchange significantly reduces circulating titres of soluble B7 molecules, in particular sCD86, and CTLA4 expression. This data indicate that plasma exchange has beneficial effects in modulating not only innate, but also adaptive immune responses and therefore may represent a plausible therapeutic strategy to restore adaptive immune responses, reverse immuneparesis whilst the injured liver undergoes regeneration in ALF. Future prospective studies are required to address this important question.

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In summary, we show that activation CTLA4 pathway is responsible for adaptive immune dysfunction, immuneparesis and infection susceptibility in patients with ALF. Furthermore, we highlight the role of soluble B7 molecules, released from acutely injured liver, in upregulating this suppressive pathway in circulating $CD4^+$ T cells. Plasma exchange may represent a credible immunotherapeutic strategy aimed at restoring adaptive immune responses against microbial pathogens by reducing concentrations soluble B7 molecules and CTLA4 bearing T cells.

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Table 1. Clinical and physiological characteristics of culture positive infected and non-infected AALF patients.

Parameter	**AALF Infected**	**AALF Non-infected**
Number of patients	11	24
Age, yr	31.00 [27.00-49.00]	36.00 [27.00-48.00]
WBC ($\times10^9$/L)	6.700 [5.910-8.130]	8.710 [5.925-11.61]
Monocytes ($\times10^9$/L)	0.2000 [0.1300-0.2600]	0.3200 [0.1250-0.6050]
Lymphocytes ($\times10^9$/L)	0.5100 [0.3000-0.7400]	0.7400 [0.3200-1.105]
SIRS SCORE	3.000 [2.000-4.000]	3.000 [2.000-4.000]
MELD score	39.00 [37.00-43.00]	39.00 [31.00-41.00]
Bilirubin (µmol/L)	70.00 [44.00-140.0]	90.00 [69.00-162.0]
INR	4.040 [2.870-6.250]	4.480 [2.480-6.340]
Creatinine (µmol/L)	259.0 [90.00-360.0]	93.00 [63.00-239.5]
Urea (mmol/L)	11.30 [5.700-14.00]	7.000 [4.900-8.700]
AST (IU/ml)	4703* [2521-8861]	1665 [951.5-3756]
Ammonia (µmol/L)	87.50 [54.75-138.5]	99.00 [67.50-150.3]
Encephalopathy score	3.000** [3.000-4.000]	2.000 [1.000-3.000]
CTLA4 (%)	3.005** [0.8575-6.418]	1.200 [0.6750-3.015]
Outcome[#] (number)	OLT(2) Survivors (6) Non-survivors (3)	OLT(8) Survivors (16) Non-Survivors (0)

Note. Values represent medians [interquartile range]. Abbreviations: SIRS score: Systemic Inflammatory Response Syndrome criteria score; MELD: Model for End stage Liver Disease; INR: international normalised ratio; AST: aspartate aminotransferase. ALD: acute liver disease; OLT: orthotopic liver transplantation; NA: not applicable; ND: Not determined. [#]outcome at 28 days post admission. *P=0.01 and ** P<0.002, compared to non-infected

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Figure Legends:

Figure 1. Phenotypic characterisation of T cell subsets in PBMCs of ALF patients. Gating strategy to define lymphocyte subsets is described in supplementary materials (Supplementary Figure 1). (*A*) Immunophenotyping of circulating lymphocytes using flow cytometry. Data show the percentages of circulating $CD3^+$, $CD4^+$ and $CD8^+$ T cells in ALF compared with HC and pathological control groups. (*B*) Distribution of naïve $CD45RO^-RA^+$ and memory $CD45RO^+RA^-$ subpopulations within the expanded $CD4^+$ T cells population in ALF compared with healthy and pathological control groups. Data show ratios of percentage of expression. (*C*) $CD4^+$ T cells were sub grouped into T_{EM} ($CD3^+CD4^+CD8^-CCR7^+CD45RO^-$) and T_{cm} ($CD3^+CD4^+CD8^-CCR7^+CD45RO^+$) populations. Percentages of expression are shown (Healthy controls (HC), n=20; ALF, n=45; CLD; n=15, ACLF, n=20; patients with septic shock with no underlying cirrhosis/liver disease (sepsis), n=20).

Figure 2. Percentages of CTLA4 expressing $CD4^+$ T cells are elevated in ALF patients. (*A*) Representative flow cytometry plots to determine CTLA4 expressing $CD4^+$ T cells in HC (left), ALF (middle) and pathological controls (right). (*B*) Data show that percentages of circulating $CD4^+$ T cells expressing CTLA4 are significantly elevated in ALF compared to HCs (*P*<.0001). (*C*) CTLA4 levels were determined in sequential samples at day 3 (n=7), 7 (n=8) and 14 (n=5) following admission and compared to HC levels, represented by the dashed line. (*D*) *Left*. Representative plot to define $CD3^+CD4^+$ T regs, naïve and memory subsets using CD25 and CD127 markers. *Right.* Distribution of CTLA4 expression in different $CD4^+$ T cell subsets, mainly naïve and memory subsets on day 1 of submission (n=15). (*E*) CTLA4 expression was assessed in ALF patients who developed infections (n=11) and the ones who did not develop infections (n=23). (*E*) Distribution of $CTLA4^+CD4^+$

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among memory and naïve T cell subsets assessed in longitudinal samples on days 1 (n=6), 3 (n=7), 7 (n=8) and 14 (n=5) following admission.

Figure 3. Attenuated ALF recall responses to HLA class II-restricted T cell epitopes. (*A*) PBMCs from HCs (n=5) and ALF (n=5) were labelled with CFSE and stimulated for 6 days with HLA class II-restricted T cell peptide pool at 10μg/ml and examined *in vitro* in the presence or absence of α-CTLA4 blocking antibody (10μg/ml). Recall responses induced in the $CD3^+CD8^-CD4^+$ T cell population were assessed and expressed as percentages of proliferating cells. Representative flow cytometry histograms of the proliferation responses (shown within the marker gate) in HCs (left) and ALF (right) and assessed in the absence (top panels) or the presence (bottom panels) of α-CTLA4 AB. (*B*) The resulting proliferation (B, left) and IL2 production (B, right) were determined by flow cytometry and ELISA, respectively. (*C*) Proliferation in $CD4^+$ T cells in ALF (n=8), CLD (n=4), ACLF (n=4) and sepsis (n=6) following neutralization of CTLA4. (*D*) CTLA4 blockade using α-CTLA4 antibody restored proliferation particularly in the memory subset (n=5). (*E*) Representative flow cytometry histograms of proliferation of isolated $CD4^+$T cells from ALF patients labelled with CPD efluor 670 and co-cultured with monocyte-derived healthy donor DCs (1:40 DC:$CD4^+$ T cell ratio) in the presence of α–CD3 antibody (left histogram). Data show the resulting proliferation at day 5 (right panel) in the presence or absence of α-CTLA4 in ALF (n=8), CLD (n=4), ACLF (n=4) and sepsis (n=6).

Figure 4. Effect of circulating soluble mediators on CTLA4 expression. (*A*) CTLA4 levels in purified $CD4^+$ T conditioned in media supplemented with HC (n=12) or ALF sera (n=14) were analysed using flow cytometry following 24 and 48 hr culture. (*B*) CTL-4 levels in purified $CD4^+$ T cells conditioned in media supplemented with sera from ACLF (n=15), CLD (6) or sepsis (n=10) sera in comparison to levels induced by culture in the presence of ALF and normal sera *(C)* Detection of levels of soluble costimulatory molecules sCD80 and CD86

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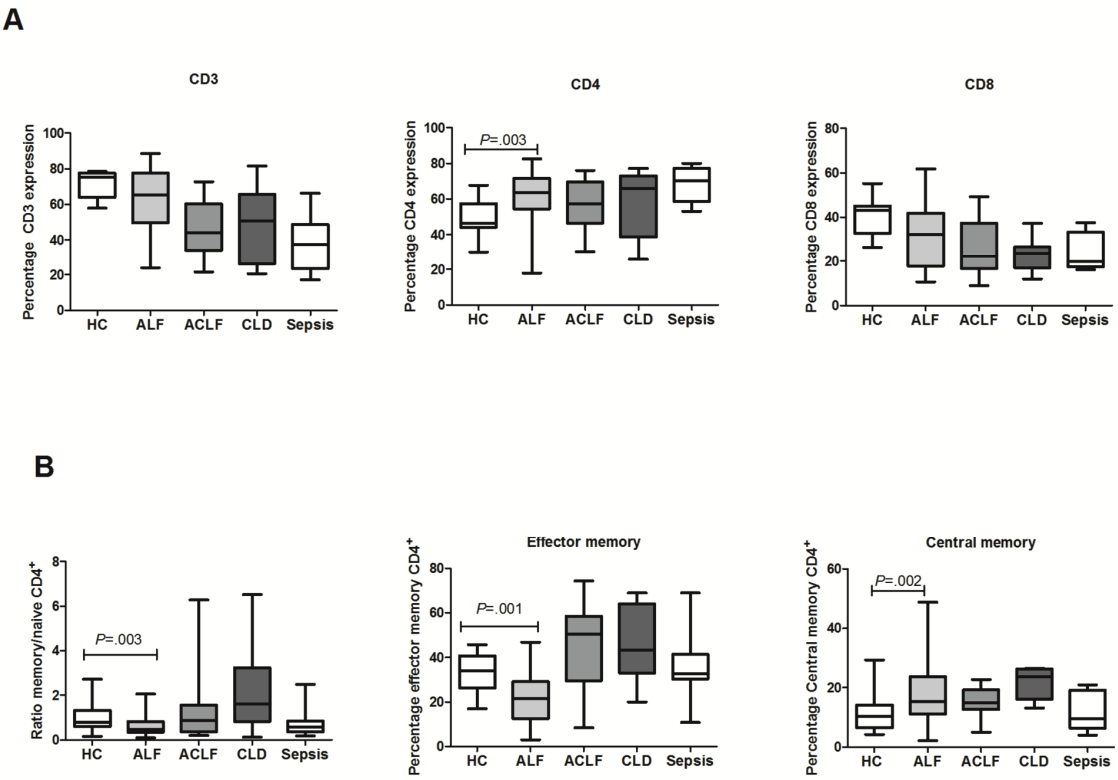
in sera samples from ALF (n=20) and HC (n=10) by ELISA. (*C*) sCD80 and sCD86 in sequential samples on day 1 (n=12), 3 (n=6), 7 (n=7) and 14 days (n=5) following admission. (*D*) Healthy and ALF sera were preincubated with anti-CD80 or CD86 to block soluble CD80 or CD86, respectively. Results are representative of 8 independent experiments. (*E*) circulating levels of sCD86 and sCD80 in sera from ALF (n=25), ACLF (n=20), CLD (n=15) and sepsis (n=20) patients determined by ELISA (*F*) Representative histograms gated on $CD3^+$ T cells demonstrating proliferation percentages of CFSE-labelled $CD4^+$ T cells preconditioned in ALF sera in the absence (top histogram) or presence (bottom histogram) of pre-treatment with αCD80 or CD86 blocking antibodies. Proliferation (middle panel) and IL-2 secretion (right panel) are from 5 independent experiments.

Figure 5. Human and murine sources of soluble sCD80 and sCD86. (*A*) Hepatocytes were first assessed for apoptosis and necrosis. Apoptotic cells are stained brightly green and necrotic brightly red. (*B*) Primary human hepatocytes were tested for their ability to secrete sCD86 hepatocytes following APAP treatment. Supernatants from 24 hour post-APAP-treated hepatocytes were assessed by ELISA for concentrations of sCD86 (top panel) and for soluble cell death markers (M30 and M65) (bottom panel). (*C*) Circulating levels of M30 (left panel) and M65 (right panel) in ALF (n=25), ACLF (n=20), CLD (n=15) and sepsis (n=20) patients determined in sera by ELISA (*D*) Representative H&E stained histology sections of liver tissue from murine model of APAP-induced liver injury highlighting different stages in the evolution and recovery from APAP-induced liver injury**.** Liver injury initiation (characterized by centrilobular hepatocyte necrosis) (8 hrs), peak (24 hrs) and resolution (within 5 days). (*E) Left.* Levels of sCD86 measured in APAP-injury murine sera at 0 hrs, 8 hrs, 24 hrs, 48hrs and 5 days post APAP-induced liver injury and *Right* corresponding ALT sera levels (n=5 per group).

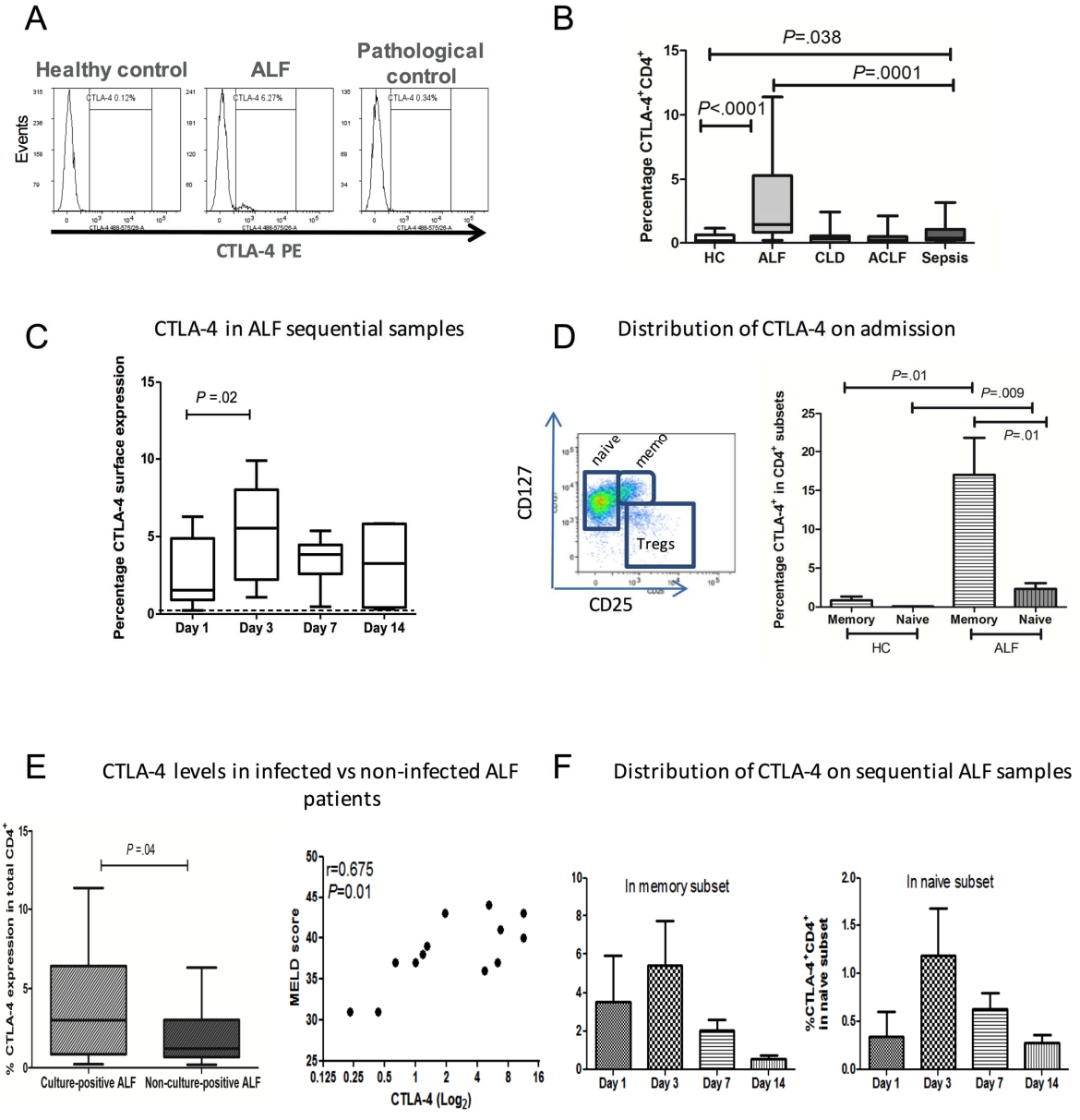
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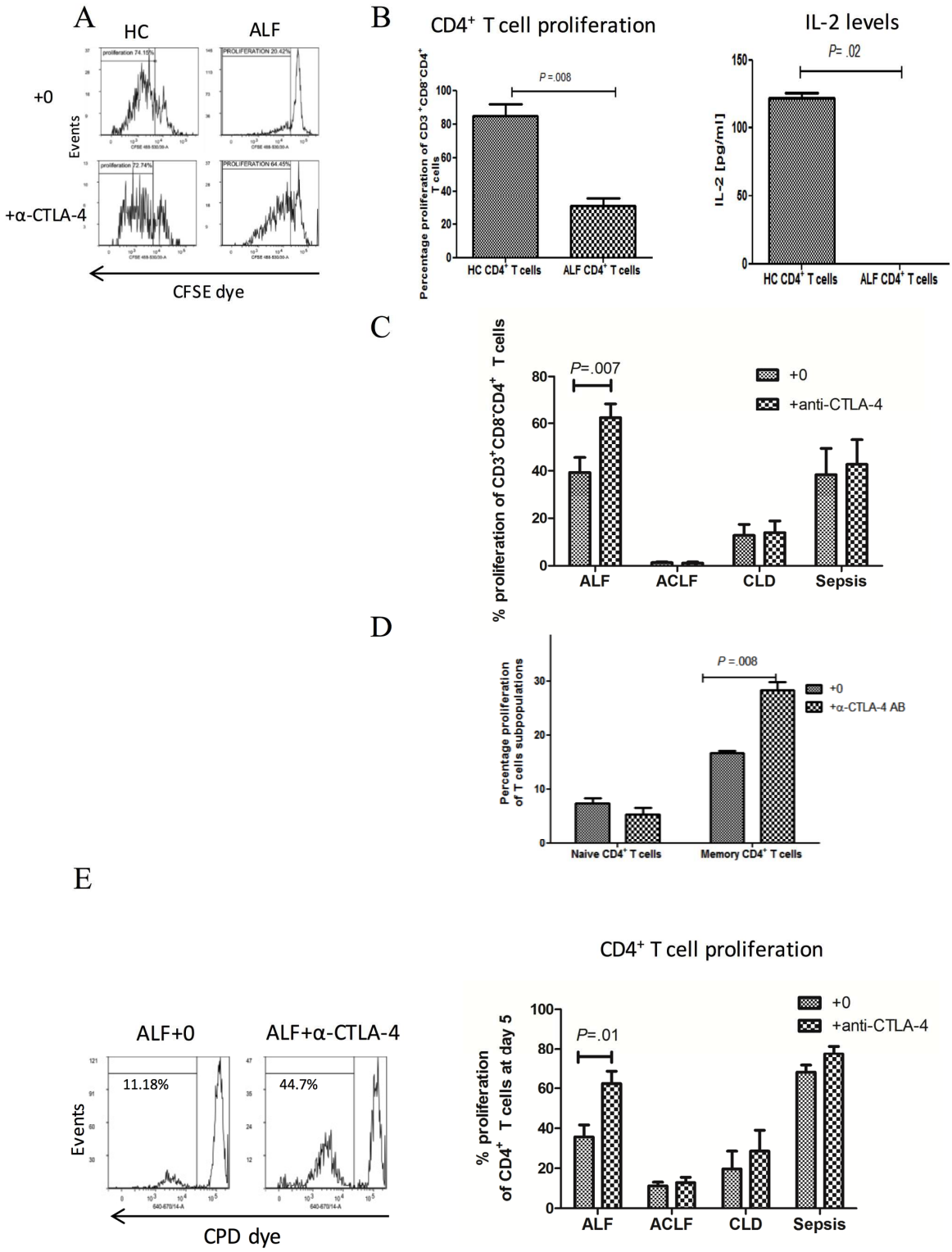
Figure 6. Plasma exchange results in the removal of circulating soluble CD80 and sCD86 and the reduction of CTLA4 levels on $CD4^+$ T cells. (*A*) Levels of soluble CD86 (Left panel) and sCD80 (middle panel) were assessed by ELISA in sera from pre and post PE patients and (*B*) in natural course samples. (*A) Right* CTLA4 levels were assessed by flow cytometry following cultures of purified $CD4^+$ T cells from HCs in media supplemented with sera from pre and post PE patients (n=7) and (*B) Right* sera from natural course patients group who did not undergo PE (n= 7).

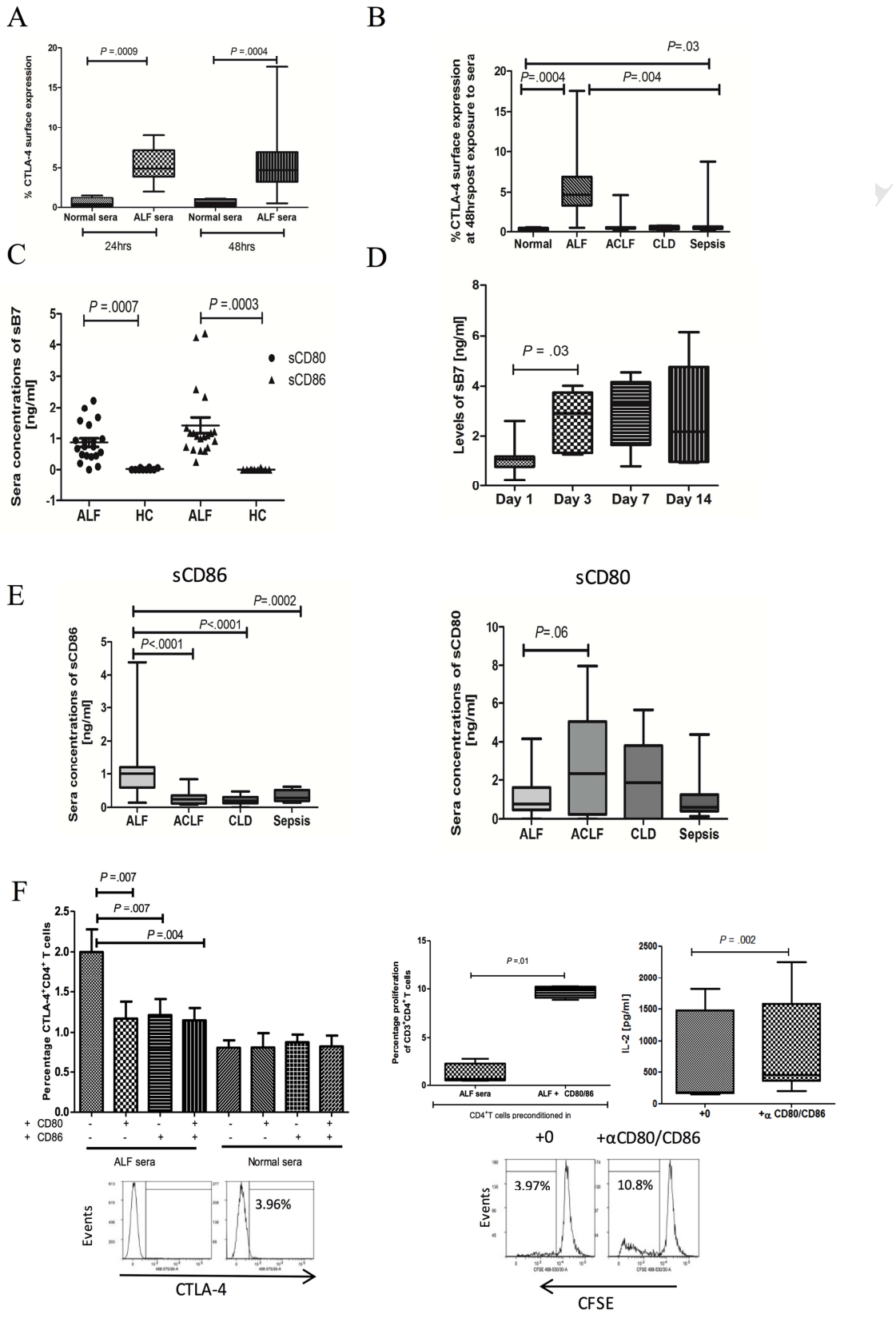
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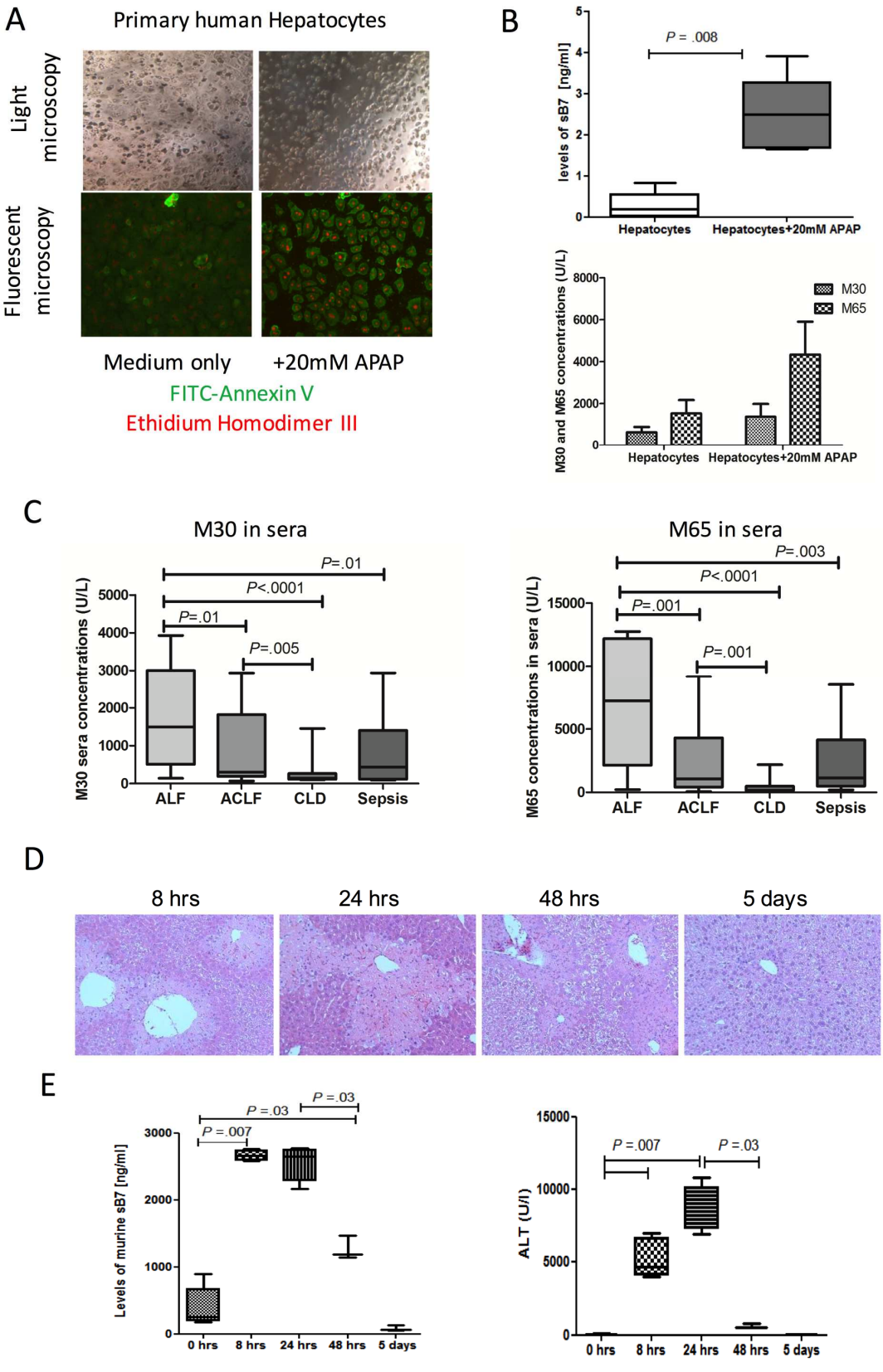


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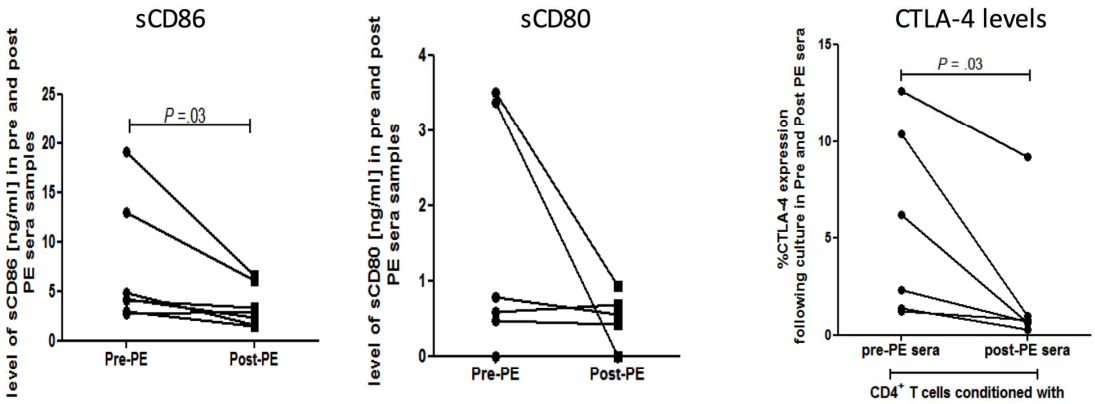

A
Healthy control
ALF
Pathological control
Events
CTLA-4 PE
B
Percentage CTLA-4+CD4+
P=.038
P=.0001
P<.0001
HC
ALF
CLD
ACLF
Sepsis
C
CTLA-4 in ALF sequential samples
Percentage CTLA-4 surface expression
P =.02
Day 1
Day 3
Day 7
Day 14
D
Distribution of CTLA-4 on admission
CD127
CD25
naive
memo
Tregs
Percentage CTLA-4+ in CD4+ subsets
P=.01
P=.009
P=.01
Memory
Naive
Memory
Naive
HC
ALF
E
CTLA-4 levels in infected vs non-infected ALF patients
% CTLA-4 expression in total CD4+
P =.04
Culture-positive ALF
Non-culture-positive ALF
MELD score
r=0.675
P=0.01
CTLA-4 (Log2)
F
Distribution of CTLA-4 on sequential ALF samples
In memory subset
In naive subset
%CTLA-4+CD4+ in naive subset
Day 1
Day 3
Day 7
Day 14


A
HC
ALF
+0
+α-CTLA-4
Events
CFSE dye
B
CD4+ T cell proliferation
P=.008
Percentage proliferation of CD3+CD8-CD4+ T cells
HC CD4+ T cells
ALF CD4+ T cells
IL-2 levels
P=.02
IL-2 [pg/ml]
C
P=.007
+0
+anti-CTLA-4
% proliferation of CD3+CD8-CD4+ T cells
ALF
ACLF
CLD
Sepsis
D
P=.008
+0
+α-CTLA-4 AB
Percentage proliferation of T cells subpopulations
Naive CD4+ T cells
Memory CD4+ T cells
E
ALF+0
ALF+α-CTLA-4
11.18%
44.7%
Events
CPD dye
CD4+ T cell proliferation
P=.01
+0
+anti-CTLA-4
% proliferation of CD4+ T cells at day 5
ALF
ACLF
CLD
Sepsis


A
% CTLA-4 surface expression
P =.0009
P =.0004
Normal sera
ALF sera
Normal sera
ALF sera
24hrs
48hrs
B
% CTLA-4 surface expression at 48hrspost exposure to sera
P=.03
P=.0004
P=.004
Normal
ALF
ACLF
CLD
Sepsis
C
Sera concentrations of sB7 [ng/ml]
P =.0007
P =.0003
sCD80
sCD86
ALF
HC
ALF
HC
D
Levels of sB7 [ng/ml]
P = .03
Day 1
Day 3
Day 7
Day 14
E
sCD86
Sera concentrations of sCD86 [ng/ml]
P=.0002
P<.0001
P<.0001
ALF
ACLF
CLD
Sepsis
sCD80
Sera concentrations of sCD80 [ng/ml]
P=.06
ALF
ACLF
CLD
Sepsis
F
Percentage CTLA-4+CD4+ T cells
P =.007
P =.007
P =.004
+ CD80
+ CD86
ALF sera
Normal sera
Events
3.96%
CTLA-4
Percentage proliferation of CD3+CD4+ T cells
P =.01
ALF sera
ALF + CD80/86
CD4+T cells preconditioned in
IL-2 [pg/ml]
P = .002
+0
+α CD80/CD86
+0
+αCD80/CD86
Events
3.97%
10.8%
CFSE

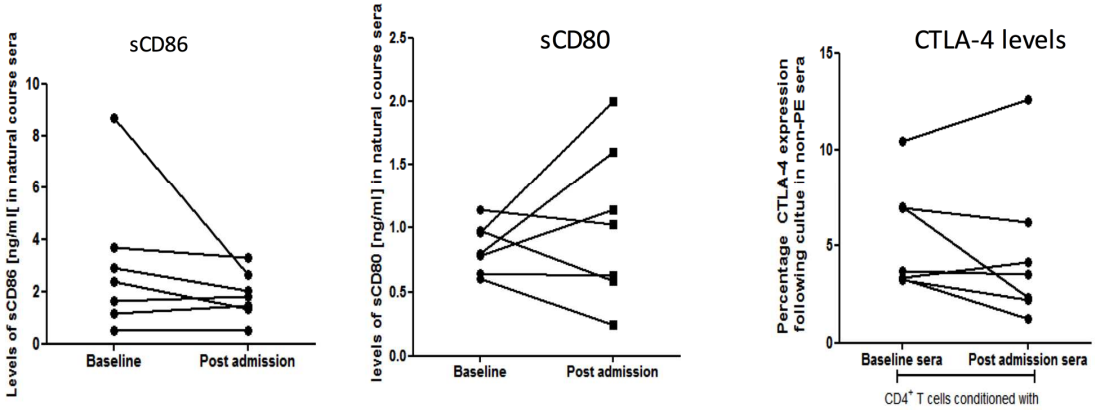

A
Primary human Hepatocytes
Light microscopy
Fluorescent microscopy
Medium only
+20mM APAP
FITC-Annexin V
Ethidium Homodimer III
B
P = .008
levels of sB7 [ng/ml]
Hepatocytes
Hepatocytes+20mM APAP
M30
M65
M30 and M65 concentrations (U/L)
C
M30 in sera
M30 sera concentrations (U/L)
P=.01
P<.0001
P=.01
P=.005
ALF
ACLF
CLD
Sepsis
M65 in sera
M65 concentrations in sera (U/L)
P=.003
P<.0001
P=.001
P=.001
D
8 hrs
24 hrs
48 hrs
5 days
E
Levels of murine sB7 [ng/ml]
P =.03
P =.03
P =.007
0 hrs
8 hrs
24 hrs
48 hrs
5 days
ALT (U/l)
P =.007
P =.03

A Pre and Post PE samples



B

Natural course samples



Supplementary Materials and Methods

Collection of Peripheral Blood Mononuclear Cells (PBMCs), Isolation of $CD4^+$ T Cells and Generation of Monocyte-Derived Dendritic Cells (DCs)

PBMCs were isolated from whole blood through Ficoll-paque ™ Plus (GE Healthcare Bio-Sciences AB, Sweden) gradient centrifugation according to the manufacturer's instructions. $CD4^+$T cells and $CD14^+$ monocytes were isolated by negative and positive magnetic bead selection, respectively (Miltenyi Biotec, Surrey, UK) according to the manufacturer's instructions. Purified cells were routinely tested for purity using flow cytometry and were greater than 98%. Monocyte-derived DCs were generated from isolated $CD14^+$ cells as previously described[26]. Briefly, DCs were generated after 7 days of culture in the presence of recombinant human (rHu) IL-4 and granulocyte-macrophage colony-stimulating factor (rHu GM-CSF) (eBioscience, Hatfield, UK). DCs were characterized by the expression of CD1a, CD11c, HLA-DR, CD80, CD83, CD86, CD14. On day 6, DCs were matured by addition of Lipopolysaccharide (LPS) from *Escherichia coli* (*E. coli*) (1µg/ml) (Source BioScience, Nottingham, UK). DCs were cultured in RPMI medium supplemented with 10% fetal calf serum (FCS) and 2 mmol L-glutamine (Invitrogen, Paisley, UK). Viable-cell counts were determined by trypan blue exclusion.

Phenotyping Using Flow Cytometry

PMBCs were surface stained for markers using the following pre-titrated fluorochrome-labelled mouse anti-human MAbs: CD3-eFluor®450, CD4-Brillaint violet 510, CD45RA-Brilliant violet 605, PD-1-Brilliant violet 786, CD45RO-FITC, CTLA4/CCR7-PE, CTLA4-PE, CD25 BD HorizonTM PE-CF594, CD62L PerCP-eFluor®710 (All from eBioscience, Hatfield, UK) and CD28/CD127 PE-Cy7, CD8/CD40L APC, (Becton Dickinson Ltd, Oxford, UK). Fluorescence minus one (FMO) controls for each marker were

used to set the gates to define the different subpopulations of lymphocytes. Cell Viability Dye eFluor®780 was used for the exclusion of dead cells. PBMCs were incubated in the presence of the antibodies or the corresponding FMO controls in the dark for 20 min (except for CCR7 which was incubated for 45mnin) at 4°C. Single-color controls for compensation were performed using UltraComp eBeads (eBioscience, Hatfield, UK). The single stain compensation control for the cell viability dye eFluor®780 was performed using ArC beads (Thermo Fisher Scientific, Hemel Hempstead, UK) following the manufacture's protocol. Twelve-color flow cytometric analyses were performed using Fortessa flow cytometer. Data were acquired using BD FACSDiva software (Becton Dickinson Ltd, Oxford, UK). A total of 10,000-20,000 gated events were analyzed for each cell population. Data was analyzed using FlowLogic software (Inivai Technologies Pty Ltd, Victoria, Australia). When assessed for T cell proliferation using CPD dye eFluor®670 labelling, cells were labelled with CD3-eFluor®450, CD4-Brillaint violet 510, CD8 APC, CD45RA-Brilliant violet 605 and CD45RO-FITC antibodies to assess proliferation of $CD4^+$ T cells memory vs naïve populations.

Primary Human Hepatocytes and Kupffer Cells (KC)

Cryopreserved hepatocytes were obtained from Life technologies Ltd (Paisley, UK) and cultured following the manufacturer's instructions. Hepatocytes were first thawed using Cryopreserved Hepatocyte Recovery Medium (CHRM), cell count and viability were determined using trypan blue staining. Viability was greater than 97%. Cells were then plated using Cryopreserved Hepatocyte Plating Medium (CHPM) in 6 chamber slides collagen coated plates (Life technologies Ltd, Paisley, UK) at a lot-specific predetermined seeding density (0.9×10^6 cells /ml) for 4 hours to allow adherence of the cells before washing and replacing the CHPM with Williams' E supplemented medium (Life technologies Ltd, Paisley, UK). Hepatocytes were allowed to further adhere overnight. Cultures were then

checked for their cell morphology and monolayer integrity under the microscope before being washed. Freshly supplemented media was replaced with medium only or medium containing 20mM APAP (Sigma, Dorset, UK) for 24 hour exposure. Non-APAP controls were included. Cells were stained using apoptotic/necrotic cell kit (PromoCell GmbH, Heidelberg, Germany) Annexin V, Ethidium homodimer III (EthD-III), respectively and according to the manufacturer's instructions. Cells were then fixed in 4% paraformaldehyde and mounted using PromoFluor antifade Reagent (PromoCell GmbH, Heidelberg, Germany). Wide-field epi-fluorescence microscopy was performed on a Leica DMI6000B equipped with a Hamamatsu ORCA-ER CCD camera operated by the MetaMorph software (Molecular Devices). Cryopreserved KCs were obtained from Invitrogen and were recovered in DMEM thawing and plating media (Life technologies, Paisley, UK). KCs were seeded at a miminum density of $0.2\text{-}0.4\times10^6$ cells /ml on 24 well Cell bind plates (VWR, Lutterworth, UK). Following 3 hours of culture, the KCs were washed and media refreshed. Following overnight stimulation, media was replaced with DMEM maintenance media (Life technologies Ltd, Paisley, UK). KCs were then stimulated in the presence of LPS (1µg/ml) for 2 hours, washed then co-cultured with APAP treated hepatocytes at a 1:4 cell ratio overnight. Supernatants from hepatocyte and KC cultures were collected for assessment of soluble B7 using ELISA.

Isolation and Culture of Human Biliary Epithelial Cells

Human biliary epithelial cells (BECs) were isolated from 150g of human diseased liver tissue derived from acute liver failure (ALF, n=3) and chronic liver disease (CLD, n=6) patients. The liver tissue was enzymatically digested with collagenase type 1A (Sigma, UK), filtered and purified further via density gradient centrifugation over Percoll (Amersham Biosciences, UK). BECs were then extracted from the mixed non-parenchymal population via magnetic selection, as previously described[27] and were cultured for one week (37˚C with

5% CO2) in rat-tail collagen coated flasks. After the first passage, BECs were cultured for 24h and BEC culture supernatants were collected to soluble B7 using ELISA.

Hepatic Endothelial Sinusoidal Cells (HSEC) Exposure to Plasma from ALF Patients

48-well cell culture plate (Costar, Corning) were pre-coated with Type 1 rat-tail collagen (Sigma-Aldrich, UK). Human sinusoidal endothelial cells (HSEC) from an explanted liver with liver cirrhosis were cultured until confluent. The cells were subsequently detached using cell-dissociating reagent TrypLE Express Enzyme (Gibco, Life Technologies, UK) for 5 minutes at 37°C, deactivated by addition PBS. A cell pellet was obtained by centrifugation at 2000rpm for 5 minutes. To each well was added 2×10^4 HSEC in a volume of 500µl of media (human endothelial serum free media (SFM), 10% human AB serum, recombinant vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) (PeproTech EC Ltd, London, UK) both at concentrations of 10ng/ml). Following 24-hour incubation, cells were washed and cultured in SFM media supplemented with 2% fetal bovine serum (FBS) and recombinant VEGF and HGF at 10ng/ml (eBioscience, Hatfield, UK). After 24-hour culture, cells were washed and further incubated for 24 hours in the presence of sera from patients with ALF (n=6) or healthy controls (n=6) at 25%. Cells were further activated using 10ng/ml recombinant human tumour necrosis factor-α (TNF-α, PeproTech, UK) and 10ng/ml interferon-γ (IFNγ, PeproTech, UK). Supernatants were then collected and analysed for sB7 molecules.

ELISA and MSD Multiplex Cytokine Detection System

Soluble CD80 and CD86 were assessed in cell culture supernatants and human sera of (HC; n=4); and ALF (n=6); Sequential samples (D1, n=12; D3, n=6; D7, n=7; D14, n=5) using ELISA (eBioscience, Hatfield, UK and Cusabio, MD, USA respectively). In addition, Levels of sCD86 were quantified in sera from acetaminophen-induced (APAP) liver injury

model murine models using ELISA (Cusabio, MD, USA). Quantikine ELISA was used for IL-2 detection (R&D Systems, UK). The MSD Human TH1/TH2 10 plex panel was used to assess the following cytokines: IFN-γ, IL-1β, IL-2, IL-4, IL-8, IL-10, IL-12p70, IL-13 and TNF-α. ELISA microplates were read using the SoftMax® Pro software (Molecular Devices LLC, USA). MSD plates were acquired on the Sector Imager 2400 apparatus (Gaithersburg, MD). All ELISA and MSD experiments were performed in duplicate and according to the manufacturer's instruction. Total and caspase-cleaved cytokeratin 18 (M65 and M30, respectively) were measured in sera on admission in ALF (n=25), ACLF (n=20), CLD (n=15) and non-hepatic sepsis (n=20) using ELISA (Peviva AB, Bromma Sweden) and according to manufacturer's instructions. Levels were assessed in supernatants from APAP untreated and treated primary human hepatocytes.

Murine Sera Samples

All research using live animals was approved by local ethics committees (Imperial College Central Animal Welfare Ethical Review Board (AWERB)) and was carried out under Home Office supervision in accordance with the Animal (Scientific Procedures) Act 1986 (UK). Procedures were performed under the authority of UK project licence 70/7578. All efforts were taken to minimise animal suffering. Mice were housed in specific pathogen free conditions in individually ventilated cages (Techniplast UK Ltd). The environment was controlled with a 12 hour light/dark cycle, ambient temperature of 21°C (+/-2°C) and humidity of 55% (+/-10%). Mice had access to irradiated diet (Special Diet Services UK) and water (25ppm chlorine) ad libitum except when food was withdrawn for defined experimental purposes. Mice were fasted overnight for 12 hours, prior to intraperitoneal dosing with 325mg/kg of APAP dissolved in saline by warming to 50°C for 45 minutes. One experimental group were culled at baseline, following the overnight fast alone. Other groups of were culled at 8 hours, 24 hours, 48 hours and 5 days after dosing with APAP. At necropsy

the liver was excised; half was place in formalin for fixation and half was snap frozen in liquid nitrogen. Formalin fixed liver tissue was paraffin embedded, sectioned at 10μm thickness and mounted onto glass slides. Sections were stained with haematoxylin and eosin (H&E).

Supplementary Figure Legends

Supplementary Figure 1. Gating strategy to define lymphocyte subsets. (*A*) Representative dot plots of the gating strategy. Lymphocytes were first gated according to the forward and side scatter profile. Doublets were excluded from the analyses using side scatter area versus width discrimination. Dead cells, which were determined by positive staining for the cell viability dye, were then excluded. (*B*) CD3 then CD4 and CD8 markers were used to determine the lymphocyte primary populations which were then gated into subsets according to the corresponding fluorescence-minus-one (FMO) control (middle and bottom panels). SSC: side scatter, FCS-A: forward scatter area.

Supplementary Figure 2. PBMCs were analysed for surface expression of co-stimulatory (CD28, C62L) and inhibitory molecules (PD1, CD25 and CD40L) in $CD3^{+}CD8^{-}CD4^{+}$ T cells by flow cytometry (Healthy controls (HC), n=20; ALF, n=45; CLD; n=15, ACLF, n=20; non-hepatic septic shock (sepsis), n=20).

Supplementary Figure 3. Distribution of $CTLA4^{+}CD4^{+}$ in ACLF. (*A*) Longitudinal distribution of CTLA4 expression in sequential samples on days 1 (n=5), 3 (n=5), day 7 (n=4) and day 14 (n=5) following admission. (A) On day 1 of admission (n=15) and (*B*) CTLA4 expression in culture-negative non-infected and culture-positive infected ACLF patients determined by flow cytometry.

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Supplementary Figure 4. Distribution of CTLA4 surface expression in the $CD127^{low}CD25^{high}$ T regulatory cells. (*A*) on day 1 of admission (n=15) and (*B*) in longitudinal samples on days 1 (n=6), 3 (n=7), 7 (n=8) and 14 (n=5) following admission.

Supplementary Figure 5. Distribution of CTLA4 surface expression in memory and naïve $CD4^+$ T cell subsets in sequential samples of ALF patients who (*A*) developed infections and (*B*) the ones who did not develop infections. *Left panels* Distribution of CTLA4 expression in memory $CD4^+$ T cell subset. *Right panels* Distribution of CTLA4 expression in the naïve $CD4^+$ T cell subset.

Supplementary Figure 6. IFN-γ levels assessed by MSD. (*A*) Supernatants from cultures in the presence or absence of α-CTLA4 blocking antibody. (*B*) IFN-γ levels in cultures preconditioned in sera treated with α-CD80 and CD86 (right panel). (n=5).

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Supplementary Table 1: Demographical and clinical parameters of acute liver failure (ALF), chronic liver disease (CLD), acute-on-chronic liver failure (ACLF), non-hepatic septic (sepsis) patients and healthy controls (HC)

Parameter	HC	ALF	CLD	ACLF	Septic
Number of patients	20	45	15	20	20
Age, yr	33.00 [27.25-36.75]	35.00*[abc] [26.50-48.5]	59.00 [44.5-61.60]	51.70 [39.00-61.50]	48.00 [32.25-61.25]
Aetiology (number)	NA	Acetaminophen (35) Autoimmune (2) Seronegative (3) Drug induced liver injury (5)	Alcoholic liver disease (13) HCV (1) Wilson's disease (1)	Alcohol related disease (16)[#] NAFLD (3)[#] Cryptogenic cirrhosis (1)[#]	Pneumococcal (7) Urological (2) Streptococcal Cellulitis (3) Streptococcal meningitis (1) Liver abscess (1) Fournier's (1) Chest sepsis (5)
WBC ($x10^9$/L)	NA	6.040*[a] [9.880-8.130]	6.110 [4.000-5.200]	8.100 [4.300-13.55]	9.335 [5.528-13.52]
Monocytes ($x10^9$/L)	NA	0.2800*[a,b] [0.1300-0.5100]	0.6000 [0.3000-0.4200]	0.5750 [0.4500-1.050]	0.3350 [0.1300-0.6850]
Lymphocytes ($x10^9$/L)	NA	0.6800*[a,b] [0.3300-1.190]	1.400 [0.700-1.770]	0.6700 [0.4525-1.350]	0.6400 [0.275-0.9300]
SIRS SCORE	NA	3.000*[c] [2.000-4.000]	ND	1.500 [1.000-2.000]	4.000 [3.00-5.000]
MELD score	NA	38.00*[a,b] [31.00-40.00]	11.00 [9.000-16.00]	22.50 [17.80-26.25]	NA
Child Pugh	NA	NA	8.000 [6.000-9.000]	10.00 [9.000-13.00]	NA
SOFA score (CLIF SOFA score in ACLF)	NA	13.50*[c] [7.750-17.00]	NA	10.00 [8.000-17.00]	6.000 [4.000-9.000]
Bilirubin (µmol/L)	NA	94.00*[a,b,c] [57.00-161.0]	28.00 [20.00-69.00]	218.0 [110.0-425.8]	14.50 [11.25-25.00]
INR	NA	3.840*[a,b,c] [2.450-6.295]	1.300 [1.200-1.440]	1.655 [1.475-1.950]	1.430 [1.190-1.918]
Creatinine (µmol/L)	NA	154.0*[a,b] [67.00-288.0]	64.00 [61.00-75.00]	79.00 [59.00-116.0]	101.0 [75.00-116.0]
CVVH % (n)	NA	60% (27)	NA	45% (9)	40% (8)
Urea (mmol/L)	NA	7.200*[a] [5.300-13.10]	4.300 [3.100-5.350]	5.500 [4.050-7.325]	5.850 [3.600-8.100]
AST (IU/ml)	NA	3584*[a,b,c] [1010-6073]	47.00 [39.00-245.0]	92.00 [58.00-128.0]	49.50 [34.00-241.0]
Ammonia (µmol/L)	NA	94.00 [65.00-127.0]	89.00 [88.00-90.00]	ND	ND
Outcome[#] % (number)	NA	Spontaneous Survivors 69% (31) OLT 24.4% (11) Non-survivors 6.6% (3)	Survivors 100% (15)	Spontaneous survivors 60% (12) OLT 15% (3) Non-survivors 25% (5)	Survivors 95% (19) Non-survivors 5% (1)

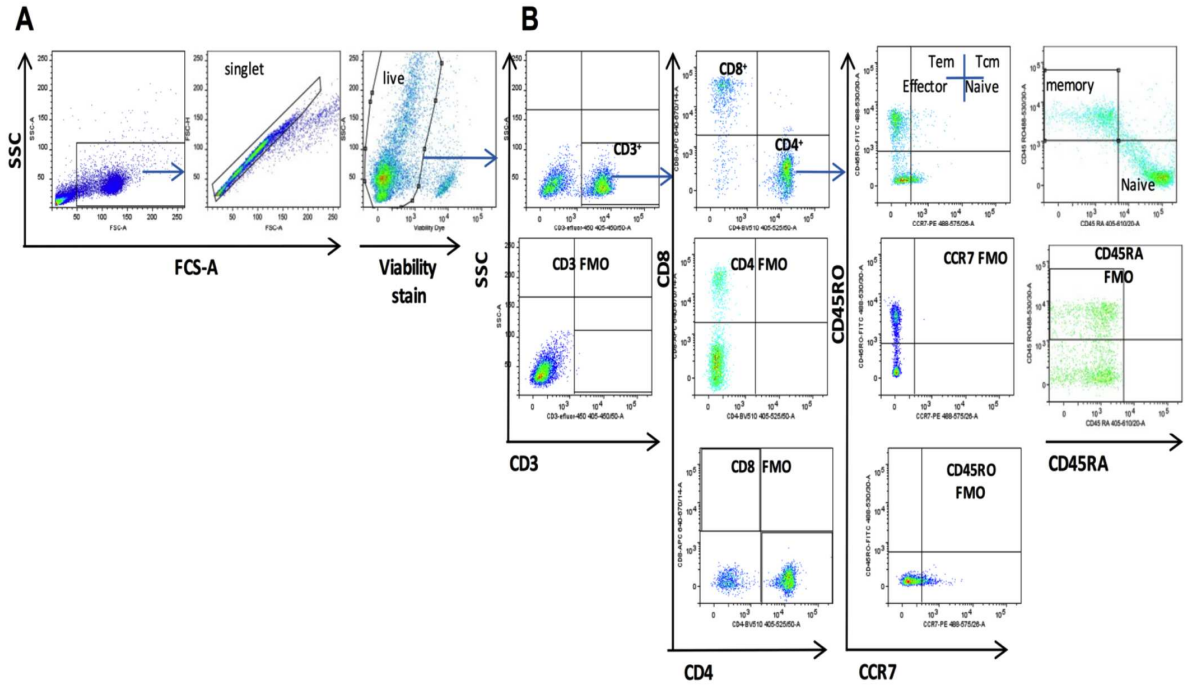
Note. Values represent medians [interquartile range]. Abbreviations: SIRS score: Systemic Inflammatory Response Syndrome criteria score; MELD: Model for End stage Liver Disease; INR: international normalised ratio; AST: aspartate aminotransferase. NAFLD: non-alcoholic fatty liver disease; CVVH: Continuous Veno-Venous Hemofiltration; OLT: orthotopic liver transplantation; NA: not applicable; ND: Not determined. #outcome at 28 days post admission. *P<0.05, aSignificant differences comparing ALF to CLD patients, bcomparing ALF to ACLF and c comparing ALF to sepsis patients. #cause for decompensation in ACLF: alcoholic hepatitis (n=11), variceal bleed (n=2), sepsis (n=7).

Supplementary Table 2: Demographical and clinical parameters of pre and post plasma exchanged acute liver patients

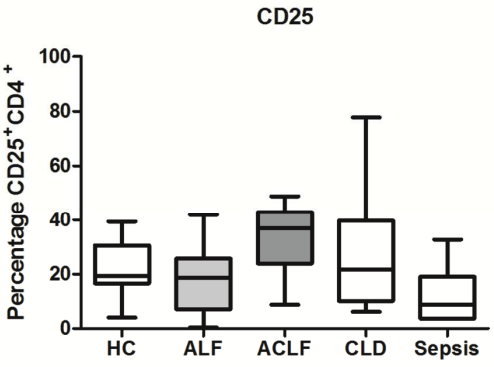
Parameter	Pre PE	Post PE
Number of patients	7	
Age, yr	46 [36.5-52]	
Aetiology (number)	Acetaminophen (5) Acute Wilson's disease (1) Liver trauma (1)	
WBC ($\times 10^9$/L)	11.4 [8.3-12.8]	7.2 [5.9-9.4]
Monocytes ($\times 10^9$/L)	0.1800 [0.1100-0.3300]	0.2400 [0.1500-0.3000]
Lymphocytes ($\times 10^9$/L)	0.8 [0.3-0.8]	0.4 [0.3-0.6]
SIRS SCORE	2 [2-3]	2 [2-3]
MELD score	40 [39-40]	37 [34-38]
Bilirubin (µmol/L)	87 [73.5-97.5]	95 [88-112]
INR	3.14 [3.01-9.12]	2.03 [1.64-2.32]
Creatinine (µmol/L)	148 [120-301]	125 [94.5-171]
Urea (mmol/L)	7.600 [4.800-9.400]	4.300 [2.500-6.400]
AST (IU/ml)	5420 [3424-8834]	2196 [1214-3587]
Ammonia (µmol/L)	58.00 [39.50-71.50]	45.00 [14.00-201.0]
Outcome	OLT (2) Survivors (3) Non-survivors (2)	

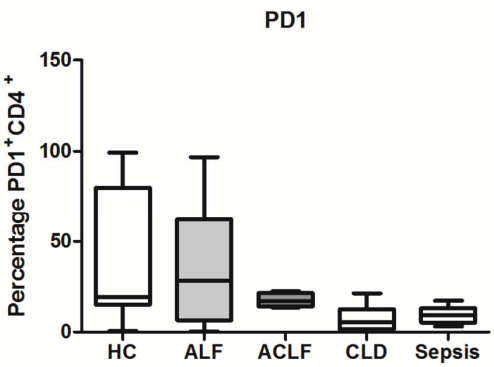
Note. Values represent medians [interquartile range]. Abbreviations: SIRS score: Systemic Inflammatory Response Syndrome criteria score; MELD: Model for End stage Liver Disease; INR: international normalised ratio; AST: aspartate aminotransferase. ALD: acute liver disease; OLT: orthotopic liver transplantation.

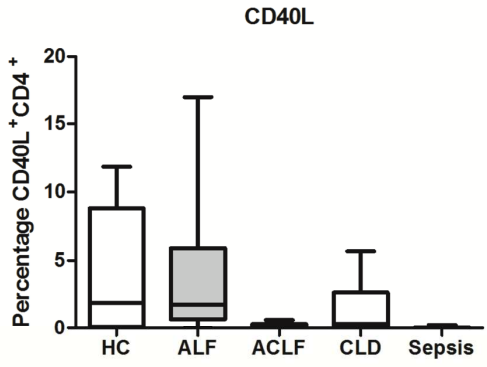
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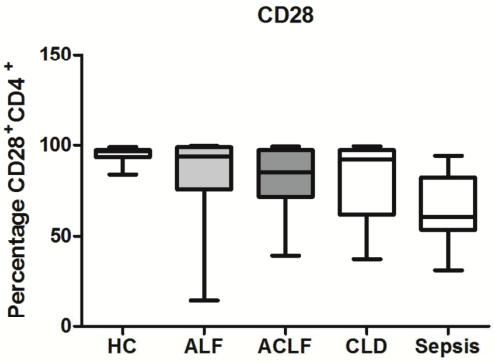


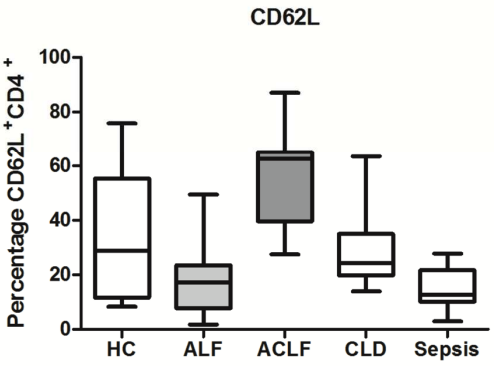
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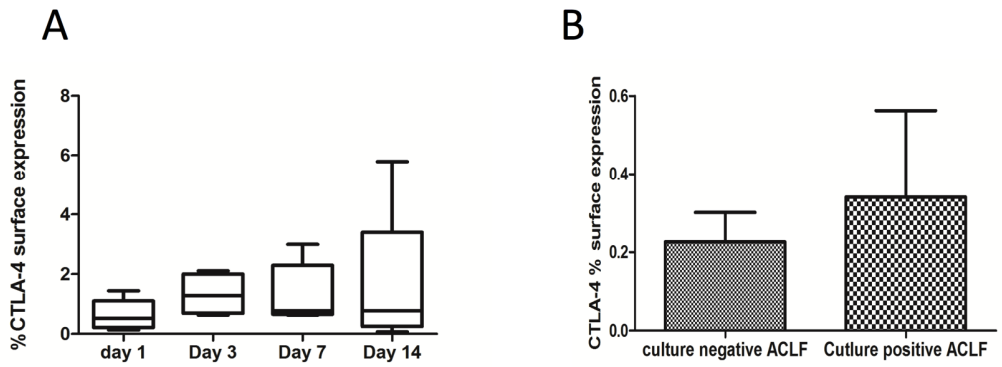

CD25
Percentage CD25+CD4+
100
80
60
40
20
0
HC
ALF
ACLF
CLD
Sepsis


PD1
Percentage PD1+CD4+
150
100
50
0
HC
ALF
ACLF
CLD
Sepsis

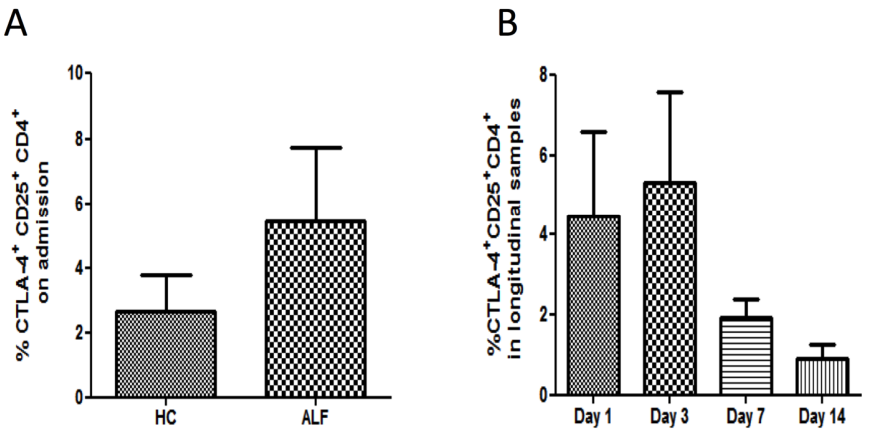

CD40L
Percentage CD40L+CD4+
20
15
10
5
0
HC
ALF
ACLF
CLD
Sepsis


CD28
Percentage CD28+CD4+
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100
50
0
HC
ALF
ACLF
CLD
Sepsis

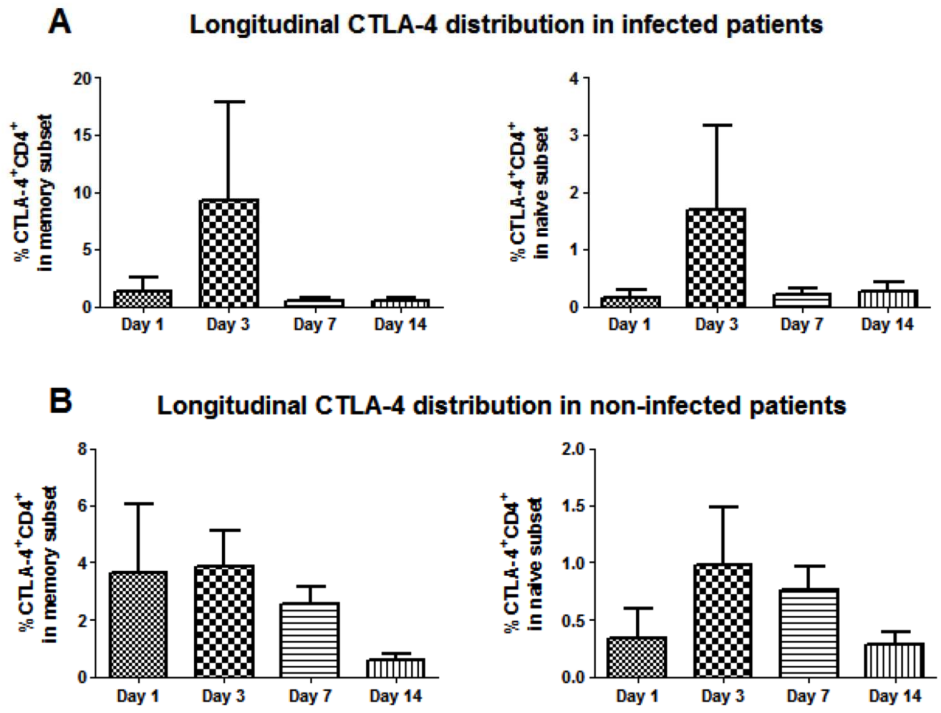

CD62L
Percentage CD62L+CD4+
100
80
60
40
20
0
HC
ALF
ACLF
CLD
Sepsis



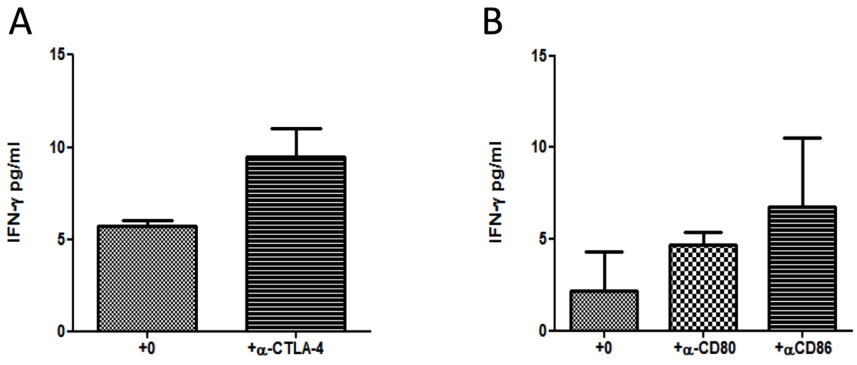
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