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SERUM CYTOKINE PROFILES ASSOCIATED WITH EARLY ALLOGRAFT DYSFUNCTION IN PATIENTS UNDERGOING LIVER TRANSPLANTATION

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Abstract

Early allograft dysfunction (EAD) occurring in the first week post-liver transplantation is associated with increased graft failure and mortality and is believed to be largely due to ischemia/reperfusion injury. We anticipated that the presence of EAD would be reflected by alterations in expression of serum proteins associated with an inflammatory response in the peri-operative period, and hypothesized that a specific pattern of expression might correlate with the development of EAD. The serum levels of 25 cytokines, chemokines, and immunoreceptors were measured by Luminex multiplex assays pre- and post-liver transplantation. Levels of each cytokine biomarker were compared in adult recipients with or without EAD at serial time points using samples collected pre-operatively and at 1, 7, 14, and 30 days post-transplant. EAD was defined according to standard criteria as maximum alanine transferase (ALT) or aspartate transferase (AST) levels on days 1–7 of >2000 U/ml, day 7 bilirubin level ≥ 10 mg/dl, or a day 7 international normalized ratio (INR) ≥ 1.7 . Multivariable analyses showed that patients experiencing EAD had lower pre-operative IL-6 and higher IL-2R levels. Patients with EAD also showed higher MCP-1 (CCL2), IL-8 (CXCL8), and RANTES (CCL5) chemokine levels in the early post-operative period, suggesting up-regulation of the NF- κ B pathway, in addition to higher levels of chemokines and cytokines associated with T cell immunity, including Mig (CXCL9), IP-10 (CXCL10) and IL-2R. These findings identify several possible biomarkers and pathways associated with EAD, that may guide future validation studies and investigation of specific cellular and molecular mechanisms of graft dysfunction. Furthermore, if validated, our findings may contribute to perioperative prediction of the occurrence of EAD and ultimately lead to identification of potential interventional therapies.

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Keywords

immune monitoring; multiplex analysis; chemokines; immunobiology

Following deceased donor (DD) liver transplantation, 20–25% of recipients develop early allograft dysfunction (EAD), a condition associated with significantly decreased graft and patient survival (1, 2). A current simple definition of EAD was recently validated in a multicenter analysis, and was characterized by early high transaminases, persistent cholestasis, and prolonged coagulopathy (2). Patients with one or more of these characteristics had a significant risk of graft loss or death in the first 6 months. While the clinical parameters used to define EAD are usually indices of hepatocellular damage and synthetic impairment, the underlying mechanisms of EAD are still not clear.

The development of EAD is often thought to be secondary to ischemia/reperfusion (I/R) injury, which is associated with acute cellular damage, cell death, oxidative damage from the creation of reactive oxygen species, and a severe inflammatory response occurring within the liver (3–8). In addition, there are numerous clinical characteristics that may play a role. The extent of I/R injury may be related not only to the duration of cold ischemic time, but also to donor and recipient characteristics including graft quality, age, underlying illness, and surgical events. While complex molecular events are occurring within the liver graft, these processes most likely result in the release of circulating proteins that can be measured in the peripheral blood. The overall state of the recipient also provides an important contribution to post-operative graft function, and the pre-operative status of the intended recipient may be reflected by changes in the levels of inflammatory and/or immunologically associated proteins. Knowledge regarding serum biomarkers that are associated with poor graft function may promote understanding of the innate molecular mechanisms underlying allograft dysfunction and post-transplant mortality due to graft failure. Such knowledge may also assist in the development of new treatments for the prevention and therapy of EAD in liver transplant patients, and new diagnostic approaches for identification of patients or liver grafts at increased risk for developing EAD.

Several previous clinical studies have linked elevated peri- or intra-operative cytokine production to increased rates of complications such as infection and rejection in liver transplant patients, but none have studied the relationship of these patterns to EAD (7–9). We previously used Luminex assays to analyze serum cytokines and chemokines in lung transplant recipients, and found that primary graft dysfunction (PGD) was associated with significantly increased levels of MCP-1 and IP-10 (10). In the current study, we measured the serum levels of 25 cytokines, chemokines and immunoreceptors in liver allograft recipients. Our panel included pro-inflammatory cytokines involved in the activation of lymphocytes and neutrophils, anti-inflammatory and pleiotropic cytokines, and chemokines involved in the recruitment of neutrophils, lymphocytes and monocytes.

Our primary goal was to assess the association of preoperative and postoperative cytokines and chemokines with the development of EAD, and secondarily, to assess temporal differences in these inflammatory agents between those patients that develop EAD and those that do not. We hypothesized that the presence of EAD would result in different levels of serum proteins in the peri-operative period, and that a specific pattern of expression would correlate with development of EAD. These investigations describe our preliminary findings of changes in serum proteins before and after liver transplantation comparing those patients who develop EAD to those who do not. While it is not possible to determine if the acute changes seen in these serum proteins are a result of the dysfunction, or a marker of a

pathogenic pathway leading to dysfunction, these explorations may provide clues to the mechanisms involved in EAD leading to future validation and studies.

PATIENTS AND METHODS

Study population

We performed a nested case-control study using a set of deceased donor liver recipient patients displaying EAD criteria transplanted from June 2006 to July 2008. All 29 EAD cases from this time period were included and population-matched with 44 controls to assure balances in age of the recipient, donor age, HCV status, and primary liver disease. All recipients were initially placed on tacrolimus-based immunosuppression and a steroid taper was begun postoperatively. Patients with preoperative renal dysfunction were also placed on mycophenolate mofetil.

As previously published (2), EAD in liver allograft recipients was defined using a validated clinical definition which included one or more of the following: maximum alanine transferase (ALT) or aspartate transferase (AST) levels from days 1–7 post transplant >2000 U/ml, day 7 bilirubin level ≥ 10 mg/dl, or a day 7 international normalized ratio (INR) ≥ 1.7 . Numerous EAD patients fitted more than one of these criteria (Table 1).

Data collection and management

Informed consent for this study was obtained prior to transplantation and approved by our Institutional Review Board. Blood samples were obtained pre-operatively on the day of transplant, and at day 1, day 7, day 14, day 30, and day 90 post-transplantation. As some patient serum samples were not available for each time point, the number of EAD and non-EAD patients at day 1, 7, 14, 30, and 90 were lower than the overall number (Table 2). There was an average of 3.9 samples per non-EAD patient and 4.8 samples from each EAD patient, with an overall average of 4.3 samples per patient. Serum was isolated from each blood sample and stored at -80°C until assessment by Luminex analysis. Clinical background variables describing the recipient and donor characteristics and operative data are presented in Table 3.

Luminex assays

A human 25-plex antibody bead kit (BioSource, Camarillo, CA) was used to measure the levels of 25 cytokines and chemokines in 50 μl of serum from each transplant patient at each available time point. Analytes included cytokines: IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-15, IL-17, TNF- α , IFN- α , IFN- γ , and granulocyte-macrophage colony-stimulating factor (GM-CSF); cytokine receptors: IL-1R α and IL-2R (CD25); and chemokines: eotaxin (CCL11), IFN- γ -inducible protein (IP-10, CXCL10), Mig (CXCL9), MCP-1 (CCL2), MIP-1 α (CCL3), MIP-1 β (CCL4), and regulated upon activation and normal T-cell expressed (RANTES, CCL5). Data were collected using a Luminex-100 array reader (Luminex Corp., Austin, TX).

Statistical Analysis

Analyses were carried out using R 2.9. (<http://www.R-project.org>). Demographic and clinical covariates at baseline were described using either proportions or medians and interquartile ranges (IQR). Hypothesis tests comparing the distribution of these variables between EAD and non-EAD patients were carried out using either a Wilcoxon-signed rank or a Chi-square test. The association between pre-operative cytokine levels and EAD status was first assessed using logistic regression, with each cytokine dichotomized into high and low levels at its median. We then used a random forests analysis to rank both the cytokine and clinical covariates as predictors of EAD status (11). Random forest analysis takes

repeated bootstrap samples to create training sets, and fits a classification tree to these samples; the “out of bag” samples are used for validation of the chosen model. Variables are ranked as potential predictors on the basis of importance scores that reflect the frequency of selection into the models, along with the success of the models in the validation. Random forest analysis avoids overfitting, which can yield results with poor generalization in subsequent studies (12). Since random forest analysis does not yield a single interpretable model, to present our findings we took seven variables based on either their importance in the random forests analysis, or in initial univariate analyses, and built a stepwise logistic regression model using AIC as the criteria for variable exclusion. For the two cytokines included in the final model, we assessed the association with recipient BMI and recipient age using Spearman’s rank correlation, and we tested for differences in the distribution of the cytokine among patients with and without ascites using a Wilcoxon Rank sum test, and among patients with different diagnoses using a Kruskal Wallis test. Differences between the distributions of cytokines were compared between EAD and non-EAD patients at each time point using Wilcoxon rank-sum tests. A type I error of 0.05 was used as the criterion for statistical significance; because our study was exploratory we did not formally adjust for multiple comparisons.

RESULTS

Patient characteristics

Table 3 shows donor age and BMI, cold ischemic time of the DD liver, recipient characteristics at baseline, and post-transplant indicators of liver injury. EAD patients had significantly higher BMI levels ($p=0.031$) and were more likely to have at least one liter of abdominal ascites ($p=0.045$). While not statistically significant, the age of both EAD recipients and their donor grafts appeared to be higher than both non-EAD recipients and their donor grafts. Cold ischemic times were 12% longer in EAD than in controls, but differences likewise did not achieve statistical significance. The distribution of gender, race, primary diagnosis and MELD score were similar among groups. Alanine transaminase (ALT, $p<0.001$), aspartate transaminase (AST, $p<0.001$), and bilirubin ($p=0.010$) were significantly higher in EAD patients; these were expected as these criteria are part of the case definition.

Cytokine levels

Table 4 demonstrates that a number of cytokines were at or below the lower detection limit throughout the much of the study. Cytokines with 85% or more of the samples at or below the lower detection limit included GM-CSF, IFN- α , IFN- γ , IL-13, IL-15, IL-17, IL-4 and IL-5.

Multivariable analysis indicates several cytokines pre-operatively associated with the occurrence of EAD

We first examined the association of pre-operative cytokine levels with subsequent development of EAD. Of the original 25 cytokines, we removed IFN- γ and IL-4 from the analysis because there was insufficient variation in levels at baseline, and we removed IL-1 β because 13 values were not available. Table 5 indicates that in the initial univariate models considering the dichotomized variables, IL-6 had an association with EAD, with higher values of IL-6 being protective for development of EAD. The odds ratio for developing EAD was 0.28 (95% CI=0.10, 0.77) for patients with IL-6 levels above versus below the median of 28 pg/ml.

The random forest analysis using all of the cytokine, demographic and clinical variables as potential predictors ranked recipient BMI and IL-6 most highly. In contrast, IL-10 and GM-

CSF, which approached significance in the univariate models in Table 5, had very low rankings. For the stepwise model we included a total of seven variables from the random forests analysis with the largest importance scores. The candidates in the stepwise model included five cytokines (IL-6, IL-2R, RANTES, MCP-1, IP-10) along with recipient BMI and recipient age. IL-10 and GM-CSF were additionally included as potential predictors because of the evidence of a possible association in the univariate model ($p < 0.20$). Variables were included in their dichotomized form, above versus below the median, or in the case of IL-10, above or at the level of detection. Table 6 shows that the final stepwise model included recipient BMI, IL-6 and IL-2R. Consistent with the univariate results, higher BMI values, higher pre-operative IL-2R, and lower values of IL-6 were all associated with the development of EAD.

Table 7 further explores the association between levels of pre-operative IL-6 and IL-2R and patient characteristics that may be associated with development of EAD. Lower levels of IL-6 were correlated with younger age in an association that approached significance ($p = 0.054$), and with the absence of ascites ($p = 0.012$). IL-6 also differed among patient diagnoses ($p = 0.027$); patients with HCV tended to have the lowest levels of IL-6 at baseline. Associations between IL-2R and patient characteristics did not achieve significance.

Cytokines involved in cellular immune responses and cell recruitment are increased in EAD

Since EAD, by definition, affects patients within the first week after liver transplantation, it was expected that pro-inflammatory cytokines and chemokines would be increased in the serum of the EAD patients during this period. Figure 1 shows examples of proteins implicated in T-cell responses and EAD. IL-2R, IP-10, Mig and IL-7 have effects on T cell activation, chemo-attraction, development and survival. IL-2R trended toward higher levels in EAD patients at all time points, and achieved statistical significance at Days 1, 7 and 14 (Fig. 1A). The chemokines IP-10 and Mig also displayed statistically significant differences at day 1 in EAD patients versus controls ($p < 0.01$, Fig. 1B & C). IP-10 levels tended to be higher in EAD patients across the study with values at day 14 that approached statistical significance ($p = 0.066$). As detailed in Table 3, over half of the data were at the lower limits of detection, and there were a number of outliers in the non-EAD patients; differences in IL-7 between EAD and non-EAD patients approached significance at 90 days ($p = 0.063$; data not shown).

Cytokines induced by NF- κ B signaling are significantly increased in EAD

Figure 2 displays serum levels of four NF- κ B-dependent cytokines. Levels of MCP-1, RANTES, IL-8 were each significantly higher in EAD patients at day 1 compared to non-EAD patients (Fig. 2A–C). Unlike preoperative levels of IL-6, post-operative levels of IL-6 were higher in EAD patients compared to non-EAD patients at Day 1 but the difference did not achieve significance (Figure 2D, $p = 0.072$). The median of IL-6 was also higher in EAD compared to non-EAD patients at two weeks post-transplant, with levels that approached statistical significance ($p = 0.086$). With the exception of RANTES, median serum levels of all four cytokines tended to be higher at Day 1 in EAD patients compared to non-EAD patients. Between day 1 and 7, the non-EAD serum levels of MCP-1, RANTES, IL-8, and IL-6 were observed to those of patients with EAD.

Association of cytokines with anti-inflammatory effects with EAD

Figure 3 shows four cytokines that were more abundant in control patients. Figure 3A displays serum levels of the IL-1 α , an anti-inflammatory mediator that can block IL-1 mediated signaling (13). Serum IL-1 α levels were significantly higher in EAD than non-EAD patients at day 1 and day 14. IL-10 is another anti-inflammatory cytokine that was

marginally higher in controls at the pre-operative time point (Fig. 3B). GM-CSF and TNF- α were also higher in the sera of control patients (Fig. 3C & D). Unexpectedly, a pro-inflammatory cytokine, TNF- α , was also significantly higher in non-EAD patients at day 14 ($p < 0.01$).

DISCUSSION

This study determined serum levels of peripheral blood cytokines, chemokines and immunoreceptors pre- and post-operatively in patients with or without development of EAD following liver transplantation. Correlations were found between preoperative and post-operative expression of several cytokines and chemokines and the development of EAD, identifying several unique expression patterns of inflammatory and immunomodulatory proteins.

While it is often thought that EAD is mostly due to factors related to IR injury in the graft, we also found an association between preoperative levels of cytokines in recipients who developed EAD after transplantation. Multivariable modeling identified a significant correlation between the development of EAD and preoperative IL-6 expression. Lower levels of IL-6 were linked to a stronger likelihood of developing EAD, though it is unclear why patients had higher or lower levels of IL-6 pre-transplant. Interestingly, studies in experimental I/R models have shown IL-6 to be hepatoprotective (14–16). In contrast, higher pre-transplant IL-2R levels were a risk factor for subsequent development of EAD, and also correlated with post-operative serum elevation of IL-2R during EAD. These data suggest that a recipient's underlying illness and pre-operative condition may contribute to the risk of developing EAD independent of graft characteristics. The identification of high risk recipients before transplantation would be of clinical relevance, given that EAD is associated with increased morbidity and mortality. While the correlation of preoperative IL-6 and IL-2 levels is interesting, further work with an independent validation population is needed to test the utility of these potentially predictive measures.

At several postoperative timepoints, the development of EAD correlated with higher levels of IL-2R, IL-7, which are cytokines that are centrally involved in the activation or recruitment of host T cells, as well as IP-10 and Mig, chemokines whose signaling recruits T cells, NK cells or B-cells, depending on biological context (17, 18). Hepatic I/R injury induces CD4⁺ T cell infiltration, possibly via antigen-independent events at least in some systems (19–21). Chemoattraction of CXCR3⁺ CD4⁺ T-cells via IP-10 and MIG, both ligands for CXCR3, was observed following cold I/R in rat liver allografts (20). Likewise, studies in our laboratory using partial murine grafts and prolonged cold preservation times identified IP-10 as a potent signal leading to neutrophil and T-cell recruitment and subsequent graft dysfunction (22). Therefore, these data support the concept established in previous studies that expression of T-cell related chemokines in injured liver tissue is an early event that follows I/R injury. Determining whether altered expression of these chemokines correlates with a true cellular infiltrate in EAD patients, and determining the cellular makeup of such an infiltrate, would require histopathology or cytometric studies of tissue biopsies, which were not possible in this study.

EAD was also associated with early post-operative increases in four proteins (MCP-1, RANTES, IL-8, and IL-6) whose expression is driven by NF- κ B activation. A link between NF- κ B regulation and EAD was anticipated because induction of the NF- κ B-associated genes in Kupffer cells is a known early event following I/R injury, and serves as a point of convergence for molecular signals inherent in many forms of chronic liver disease (23, 24). Activation of the NF- κ B pathway in Kupffer cells leads to the expression of many cytokines and chemokines, including those identified in this analysis, that in turn induce expression of

many other inflammatory mediators in injured liver tissue (24, 25). During toxic liver injury and oxidative stress, localized expression of MCP-1 attracts monocytes and activated lymphocytes to sites of injury (26). Likewise, IL-8 is secreted from Kupffer cells in response to I/R and causes sinusoidal neutrophil sequestration and resultant hepatocellular damage (27–30). Recently, a study of cytokine and chemokine responses in 26 patients following hepatectomy found that large increases in MCP-1, IL-6 and IL-8 correlated with higher rates of post-operative complications (bile duct leak, surgical site infection, liver failure) (31). Excessive activation of the NF- κ B pathway may be detrimental to early liver allograft function post-transplantation, or alternately NF- κ B-regulated cytokines and chemokines may simply be markers of increased injury and diminished graft function.

Our study has several inherent limitations. Clearly, this work is exploratory in nature and a descriptive case-control study, therefore cannot assign causality or mechanism to our findings, nor can we recommend specific clinical interventions or action based on our conclusions. The sample size is the major limiting factor, and a prospective validation set is still needed for more definitive conclusions and to confirm any clinical associations.

For the serum proteins we have identified as showing significant change associated with EAD, we are not able to state whether they represent the actual cause or effect of organ dysfunction, or part of a new pathogenic process altogether. Further studies will be required to validate that the factors we have identified are indeed part of a pathophysiologic mechanism that relates to EAD. We used a previously validated simple clinical definition of EAD based on clinical parameters during the first week post-transplant that correlated with worse outcome (2). An assumption of this model is that EAD, due to I/R injury and donor/host characteristics, is a pathological process distinct from early acute rejection and HCV recurrence. We acknowledge that we are unable to truly distinguish between EAD, early acute rejection and HCV recurrence without liver biopsy data, however these events are highly unlikely to affect the parameters to the defined levels within the first week post-op, except for hepatic artery thrombosis, which was excluded. In our center, it is relatively rare for patients to be biopsied within the first week, so biopsy data is not available for most patients.

We also recognize some limitations in sample size and missed time points. We did not have serum samples for all patients at all time points. We caution that because the sample size changed across time, the power to detect a given effect size also changed. Negative results should be examined with caution particularly for day 30, which had the smallest sample size. Also, many individual cytokine measurements were at or below the detectable minimum, leading to skewed distributions for some of the cytokines, such as IL-10. Therefore our inability to demonstrate differences in those mediators with 85% or more of the samples at or below the LDL (GM-CSF, IFN- α , IFN- γ , IL-13, IL-15, IL-17, IL-4 and IL-5) should not be interpreted as evidence of lack of pathophysiological involvement. There were differences in clinical variables between EAD and non-EAD groups, and we accounted for these in the multivariable analyses that included pre-operative clinical variables and cytokine levels. Because this is one of the first studies of cytokine levels associated with EAD in liver transplantation, we wanted to identify possible differences between patients with and without EAD, and for this reason we did not formally adjust for multiple comparisons, as to do so would reduce our statistical power. However, because we assessed multiple biomarkers at numerous times, many hypotheses tests were carried out, and there was a higher chance that some results were false positives. In a number of cases, patterns across time showed consistency, even when differences were statistically significant at only a subset of times, lending weight to our main conclusions. All patients were also on immunosuppressive therapy following transplantation, which likely altered the expression of cytokines and chemokines; this is an effect that cannot be directly measured in humans in a

controlled manner. Hence, this study was of an exploratory nature, and larger studies that include more patients with EAD and graft failure are important to validate our findings.

Despite these limitations, this study provides evidence for several associations between specific inflammatory mediators and EAD that can guide future investigation. It is of interest that there are significant differences in the cytokine and chemokine profiles, and associations with clinical characteristics of EAD and non-EAD recipients, both before and after transplantation, as this highlights the dual importance of both donor and recipient factors. We also found that the majority of the cytokine and chemokine differences between patients who or do not develop EAD occur very early in the post-operative period and, if validated, may provide a brief window of opportunity for clinical decision making or intervention. However, we caution that our current studies are both exploratory and descriptive by design, and are not intended to predict clinical course or explain pathophysiologic mechanisms. The molecules we have identified may be promising targets for further investigation as mediators of liver injury post-transplantation, and/or serve as potential clinical biomarkers for prediction or early detection of EAD and the possibility of graft loss. The ultimate goal would be to identify potential pathways for therapeutic intervention in order to prevent or minimize the development of EAD and thereby improve post-transplant graft function.

Abbreviations

ALT	alanine transferase
AST	aspartate transferase
DD	deceased donor
EAD	early allograft dysfunction
INR	international normalized ratio
I/R	ischemia/reperfusion
PGD	primary graft dysfunction

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Figure 1. Box plots of serum levels of cytokines important to cellular immune responses, shown by day of study for EAD (blue) and non-EAD (yellow) subjects

(A) IL-2R, (B) IP-10 and (C) Mig. Black squares represent the median and the box is the interquartile range; circles represent outliers. P-values are displayed for $p < 0.1$; note the natural log scale.

Figure 2. Box plots of serum levels of NF- κ B-stimulated chemokines that are higher in EAD patients in the early post-operative period, shown by day of study for EAD (blue) and non-EAD (yellow) subjects
 (A) MCP-1, (B) RANTES, (C) IL-8 and (D) IL-6. Black squares represent the median and the box is the interquartile range; circles represent outliers. P-values are displayed for $p < 0.1$; note the natural log scale.

Figure 3. Box plots of serum levels of cytokines that have anti-inflammatory effects or are higher in control patients, shown by day of study for EAD (blue) and non-EAD (yellow) subjects (A) IL-1 α , (B) IL-10, (C) GM-CSF, and (D) TNF α . Black squares represent the median and the box is the interquartile range; circles represent outliers. P-values are displayed for $p < 0.1$; note the natural log scale.

Table 1
Patients with different early allograft dysfunction defining characteristics

EAD Criteria	Patients
ALT/AST ^a	86%
Bilirubin ^b	10%
INR ^c	14%
ALT/AST and bilirubin	7%
ALT/AST and INR	3%
Bilirubin and INR	3%
Met all three criteria	3%

^a maximum post-operative day 1–7 ALT or AST >2000 U/ml

^b bilirubin ≥ 10 mg/dl

^c INR ≥1.7

Table 2
Number of sera available at each time-point for comparison of non-EAD, EAD, and EAD patients with graft loss

Day	non-EAD	EAD
0	44	29
1	25	22
7	29	23
14	33	24
30	21	22

Table 3
Donor characteristics and recipient characteristics pre-operatively or in the first seven days post-surgery (results are stratified by EAD status)

Values for continuous variables are medians and interquartile ranges; values for categorical variables are number of subjects with percent in parentheses; P-values >0.20 are shown only as non-significant (NS).

Variable	Level	Non-EAD (n=44)	EAD (n=29)	P-value
Donor				
Age	NA	42 (24,55)	47 (36,64)	.144
BMI	NA	25.6 (22.7,29.1)	28.1 (24.1,32.7)	.096
Recipient				
Gender	Female	9 (20%)	8 (28%)	NS
	Male	35 (80%)	21 (72%)	
Race	White	29 (66%)	19 (65%)	NS
	Black	11 (25%)	8 (28%)	
	Other	4 (9%)	2 (7%)	
Age	NA	53 (47,60)	57 (53,62)	.076
MELD score	NA	24.0 (19.0,27.5)	23.0 (17.0,28.0)	NS
BMI	NA	25.7 (22.2,28.6)	27.8 (25.6,29.3)	.031
Ascites ^a	Present	30 (70%)	16 (57%)	.045
	Absent	13 (30%)	12 (43%)	
Primary diagnosis	HCV	22 (50%)	16 (55%)	NS
	Alcoholic cirrhosis	10 (23%)	3 (10%)	
	Cryptogenic cirrhosis	5 (11%)	3 (10%)	
	PSC	3 (7%)	2 (7%)	
	NASH	1 (2%)	2 (7%)	
	Other	3 (7%)	3 (10%)	
Cold ischemic time ^b	NA	326 (279,408)	366 (304,436)	.185
ALT ^c		452 (248,760)	2331(1838,3043)	<0.001
AST ^c		622 (374,1156)	3547 (2321,3848)	<0.001
Bilirubin ^d		1.60(0.90,2.70)	3.0(1.50,6.70)	.010

^a unknown for two patients;

^b minutes;

^c max postoperative day 1–7, U/ml;

^d day 7 mg/dl

Table 4
Lower detection limit for cytokines and fraction of all assays at this limit

Cytokine	Lower Detection Limit (pg/ml)	Fraction of Assays at Limit (%)
Eotaxin	5	6
GM-CSF	12	85
IFN- α	12	89
IFN- γ	14	99
IL-10	22	72
IL-12	10	2
IL-13	19	99
IL-15	26	88
IL-17	17	92
IL-2	6	58
IL-4	24	100
IL-5	10	98
IL-6	10	26
IL-7	13	60
IL-8	39	47
IL1- β	14	39
IL1-RA	30	0
IL-2R	30	0
IP-10	3	7
MCP-1	10	0
MIG	4	2
MIP-1 α	12	61
MIP-1 β	15	2
RANTES	15	0
TNF- α	2	77

Table 5
Univariate association between EAD status and cytokine level

Cytokines were dichotomized at the median into a high versus low category; cytokines included in the stepwise modeling procedure are shown in bold; P-values above 0.20 are designated as NS (not significant)

Cytokine	Median (pg/ml)	Odds Ratio ^a	95% CI	P-value
Eotaxin	77	1.02	(0.40,2.61)	NS
GM-CSF	12	0.33	(0.10,1.14)	0.079
IFNa	12	1.01	(0.26,3.96)	NS
IL-10	22	0.40	(0.13,1.27)	0.120
IL-12	213	0.93	(0.37,2.38)	NS
IL-13	19	1.52	(0.20,11.4)	NS
IL-15	26	0.62	(0.21,1.88)	NS
IL-17	17	0.54	(0.15,1.94)	NS
IL-2	6	0.64	(0.25,1.66)	NS
IL-5	10	1.01	(0.16,6.46)	NS
IL-6	28	0.28	(0.10,0.77)	0.013
IL-7	13	1.95	(0.76,5.06)	0.167
IL-8	39	0.74	(0.74,1.17)	NS
IL1-RA	3622	1.48	(0.29,1.90)	NS
IL2-R	1413	2.36	(0.90,6.18)	0.079
IP-10	28	0.93	(0.37,2.38)	NS
MCP-1	506	0.49	(0.19,1.29)	0.149
Mig	23	1.44	(0.55,3.77)	NS
MIP-1a	12	0.98	(0.38,2.55)	NS
MIP-1b	72	0.74	(0.29,1.90)	NS
RANTES	5247	1.86	(0.72,4.82)	0.199
TNFa	2	0.56	(0.19,1.59)	NS

^aOdds of developing EAD versus no EAD for high versus low values of each cytokine.

Table 6
Odds of developing EAD based on preoperative IL-6 and IL-2R in the multivariable model

Variable	Odds Ratio ^a (95% CI)	P-value
BMI	1.15 (1.01,1.31)	0.036
IL-6	0.20 (0.06,0.64)	0.006
IL-2R	3.77 (1.22,11.64)	0.021

^aOdds of developing EAD for a one-unit increase in BMI or for increased values of IL-6 or IL-2R above versus below the median.

Table 7
Association of pre-operative cytokines with clinical variables (NS indicates P>0.20)

Variable	Level	IL6		IL2R	
		Level ^a	P-value ^b	Level ^a	P-value ^b
BMI	NA	.065	NS	-.01	NS
Age	NA	-.23	.054	.10	NS
Ascites	Present	45.4 (19.1,253)		1360 (627,9418)	
	Absent	21.2 (10.48,8)	.012	1413 (345,2381)	.124
Primary diagnosis	HCV	19.2 (10.7,46.5)		1053 (403,3041)	
	Alcoholic cirrhosis	38.1 (22.7,64.8)		1413 (555,3480)	
	Cryptogenic cirrhosis	31.3 (19.5,87.8)		1639 (625,12046)	
	PSC	49.4 (27.4, 65.9)		1910 (1165,9524)	
	NASH	56.4 (36.4,174)	.027	2864 (1813,10233)	NS

^a Shown as Spearman's correlation coefficient for BMI and age, and medians (pg/ml) with IQR for ascites and primary diagnosis;

^b Test of Spearman's correlation coefficient of zero for BMI and age, and test of equality of medians among levels of the variable for ascites and primary diagnosis.