Gastrointestinal, Hepatobiliary and Pancreatic Pathology

FasL Expression in Hepatic Antigen-Presenting Cells and Phagocytosis of Apoptotic T Cells by FasL⁺ Kupffer Cells Are Indicators of Rejection Activity in Human Liver Allografts

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Fas-Fas ligand (FasL) interaction and apoptosis are important in the mechanism of allograft rejection. However, the interaction between donor and recipient cells, specifically focusing on antigen-presenting cells (APCs), under various conditions is poorly understood in human liver allografts. FasL expression on APCs, its association with apoptosis, and the origin of apoptotic lymphocytes in human liver allografts were assessed by immunohistochemistry and *in situ* hybridization. We found increased expression of FasL on Kupffer cells (KCs) and endothelium in acute cellular rejection ($n = 20$) and to lesser extent in chronic rejection ($n = 6$) and septic cholangitis ($n = 5$) compared with stable grafts and normal controls. In addition, the graft specificity of infiltrating T cells was confirmed by polymerase chain reaction examination of T-cell receptor- γ loci. T-cell apoptosis occurred at a higher rate in acute cellular rejection than in chronic rejection or septic cholangitis. The number of apoptotic bodies derived from recipient lymphocytes correlated with the severity of rejection and was reversed by treatment. FasL⁺ KCs phagocytosed CD4⁺ interferon- γ ⁺ T cells, rather than CD4⁺ interleukin-4⁺ T cells, suggesting a role of KCs in regulating CD4⁺ T-cell subset differentiation. In conclusion, our data suggest that FasL expression on APCs and phagocytosis of apoptotic T cells by FasL⁺ KCs are indicators of rejection activity in human liver allografts.

tion activity in human liver allografts. (*Am J Pathol* 2007, 171:1499–1508; DOI: 10.2353/ajpath.2007.070027)

There are few studies regarding the relationship between antigen-presenting cells (APCs) and alloreactive recipient cells in human liver allografts.¹ Kupffer cells (KCs) are a type of resident macrophage in the liver that function as effective APCs and interact with lymphocytes, resulting in T-cell proliferation and cytokine synthesis.^{2,3} They are localized in the portal area and within the sinusoidal lumen, adhering to sinusoidal endothelial cells. It is known from animal models that apoptosis of activated T cells occurs in liver transplantation.^{4,5} In addition to activated T-cell apoptosis, a recent study demonstrated the ability of KCs to induce the apoptosis of alloreactive T cells as well as regulate T-cell differentiation in an allogeneic liver transplant animal model.⁶ It has been proposed that Fas-mediated apoptosis plays an important role in allograft rejection.^{7–10} It is well known that Fas ligand (FasL) expressed on the surface of the effector cells binds to Fas on the target cells and causes apoptosis by activating caspases.^{11,12}

Afford and colleagues⁸ documented the expression of FasL on sinusoids, hepatocytes, bile ducts, and inflammatory cells during acute cellular rejection (ACR) and during chronic rejection (CR) in human liver allografts. Apoptosis mediated via the Fas/FasL pathway has been described mainly in hepatocytes, which are in close contact to lymphocytes.^{8–10,13} The apoptosis of hepatocytes has been thought to be involved in the pathogenesis of liver allograft rejection or tolerance.^{8–10} Although apopto-

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Table 1. Clinical Characteristics of Patients

Patient group	Median age at transplantation (range)	Median posttransplant day of biopsy (range)	Original disease
Acute rejection (<i>n</i> = 20)	1 (0 to 19)	12 (5 to 369)	Biliary atresia (<i>n</i> = 16) Primary sclerosing cholangitis (<i>n</i> = 2) Fulminant hepatic failure Caroli's disease
Chronic rejection (<i>n</i> = 6)	3 (0 to 19)	74 (33 to 406)	Biliary atresia (<i>n</i> = 4) Caroli's disease Byler's disease
Septic cholangitis (<i>n</i> = 5)	1 (0 to 18)	19 (15 to 67)	Biliary atresia (<i>n</i> = 3) Byler's disease Autoimmune hepatitis
Stable grafts (<i>n</i> = 5)	1 (0 to 1)	82 (38 to 382)	Biliary atresia (<i>n</i> = 5)

tic bodies are often observed in biopsy specimens in human liver transplant, hepatocyte damage is not a conspicuous feature of rejection.¹⁴ A very low number of apoptotic hepatocytes has been found in ACR despite high Fas antigen expression.^{8,10} Furthermore, direct contact between passenger lymphocytes and hepatocytes is believed to be prevented by sinusoidal endothelial cells and KCs.² It has recently been shown that graft-infiltrating CD3⁺ T-cell apoptosis occurs in human liver allografts.¹ The significance of Fas/FasL expression on APCs, its relationship to apoptotic lymphocytes, and the phenotype of apoptotic cells in human liver allografts with rejection is not fully understood.

In this study, we quantified T-cell apoptosis and determined its relationship to Fas/FasL expression on APCs. We also evaluated T-cell apoptosis and APCs that phagocytose apoptotic T cells as an indicator of rejection activity along with clinical course before and after steroid treatment. Finally, we examined the influence of apoptosis on T-cell differentiation in the transplanted liver. To determine whether lymphocyte apoptosis could be attributable to interaction with donor KCs retained in the graft, the phenotype of apoptotic lymphocytes was determined in sex-mismatched grafts.

Materials and Methods

Patients

We obtained liver allograft biopsies from 31 pediatric patients undergoing living donor liver transplantation, of which 20 had ACR, six CR, and five septic cholangitis (SC). Five stable grafts and six wedge liver biopsies from living donors were used as controls. The following exclusion criteria were applied to prevent participation in this study: i) ABO blood type-incompatible transplantation; ii) transplantation for liver diseases with known possibility of disease recurrence early after transplant, such as hepatitis C virus infection; and iii) ACR resistant to anti-rejection treatment.

Liver allograft rejection was diagnosed and graded in accordance with the Banff classification.¹⁴ The sum of all components, including portal inflammation, bile duct damage, and venous endothelial inflammation, which were ranked from 0 for none to 3 for severe, was as-

sessed to score rejection. The three categories were added together to produce a rejection activity index (RAI) of 0 to 9, which was converted to a rejection grade as follows: 3 to 5, mild; 6 to 7, moderate; and 8 to 9, severe acute rejection. Diagnosis of CR was made by the loss of bile ducts from at least 50% of portal tracts in the needle biopsy specimens, which may represent a late stage of CR.^{15,16} SC was diagnosed when a histological pattern comprising cholestasis and portal changes (including edema, predominantly neutrophilic infiltration, cholangiolar proliferation, and cholangiolitis with or without ductular cholestasis) was found in the liver biopsy in conjunction with clinical evidence of sepsis or local biliary infection.¹⁷

The median postoperative days when biopsies were taken were within 3 months (range, 12 to 74 days) for the 31 allografts. Indications for the liver transplantation are shown in Table 1. All were in the pediatric population with patients younger than the age of 20. In liver specimens from 20 patients with ACR, diagnosis was made on days 5 to 369, with a median of 12 days. The distribution of ACR grade was as follows: 7, mild; 10, moderate; and 3, severe. Rejection resolved after steroid treatment with additional dose increases of tacrolimus. In liver specimens from six patients with CR, the timing of the liver biopsies ranged from 33 to 406 days after living donor liver transplantation (median, 74 days). Of five patients with histological SC, all had either sepsis or biliary infection caused by Gram-negative bacilli as confirmed by systemic cultures. The timing of the diagnosis of SC was made on liver biopsies between 15 and 67 days after living donor liver transplantation (median, 19 days).

Five patients underwent liver biopsy at the time of surgical repair for biliary stricture in the absence of biochemical graft dysfunction, and these biopsy specimens were used as stable graft controls. They were obtained at a median of 82 days after transplant (38 to 382 days). Histological diagnoses included mild portal inflammation in three case and mild steatosis and hepatocyte ballooning in one each (Table 1). As a control group, we studied living donor wedge biopsies with no steatosis. At the time of the study, none of the patients in the ACR, CR, or control groups showed signs of clinical or histological infection.

Double Immunohistochemical Labeling

Tissue specimens were fixed with 10% neutral formalin and embedded in paraffin. Biopsy samples were processed routinely, cut to 4- μ m thickness, and stained with hematoxylin and eosin. Serial sections were used for the immunohistochemical studies. Immunohistochemistry was performed using the catalyzed signal amplification system (DakoCytomation, Carpinteria, CA) according to the manufacturer's instructions, with minor modifications. After deparaffinization, antigen retrieval was performed using Target-Retrieval solution (DakoCytomation). The tissue sections were then incubated with avidin and biotin solutions for tissue block (DakoCytomation), followed by protein block (DakoCytomation). Sections were incubated overnight at 4°C with 1:50 to 1:100 diluted primary antibody. Monoclonal antibodies against Fas (SM1/1; Wako, Osaka, Japan) and FasL (N-20; Santa Cruz Biotechnology, Santa Cruz, CA) were used as primary antibody reagents. Dual staining was performed using Alexa Fluor 568 streptavidin (Invitrogen, Eugene, OR)-labeled anti-Fas, or -FasL, and fluorescein streptavidin (Vector Laboratories, Burlingame, CA)-labeled CD3 (F7.2.38; DakoCytomation), CD4 (RB204; DakoCytomation) and CD8 (C8/144B; DakoCytomation) for infiltrating lymphocytes; CD34 (My10⁺8G12; Becton-Dickinson, Mountain View, CA) and CD31 (JC/70A; DakoCytomation) for vascular and sinusoidal endothelium; and CD68 (PG-M1; DakoCytomation) for KCs, interferon (IFN)- γ (Becton-Dickinson), and interleukin (IL)-4 (Becton-Dickinson). High-magnification sections of at least three portal tracts and perivenular areas were analyzed by confocal laser-scanning microscopy (BX50 and AX80TR; Olympus, Tokyo, Japan) in each section.

Scoring of Fas and FasL Stainings

The liver tissues were graded with respect to Fas/FasL expression as follows: 0, no expression; +1, sporadic expression in a few cells; +2, up to 30% positive cells; +3, 31 to 60% positive cells; +4, more than 61% positive cells. The distribution of staining on liver cell types was recorded on dual-labeled tissue. Because it was difficult to recognize cell borders, particularly of the endothelium, a semiquantitative scale was used for Fas/FasL stainings.

Evaluation of Th1/Th2 Population in Rejection

Because CD4 helper T cell (Th) 1 effectors are characterized by the production of IL-2, IFN- γ , tumor necrosis factor- α , and tumor necrosis factor- β and Th2 effectors of IL-4, -5, -6, -10, and -13, Th1 cells were determined as CD4⁺ IFN- γ ⁺ cells and Th2 as CD4⁺ IL-4⁺ cells.¹⁸ The association with apoptotic fragments and the Th1/Th2 population were assessed by the combination of double immunostaining and morphology.

Terminal dUTP Nick-End Labeling (TUNEL) Staining

For the detection of apoptotic cells, an *in situ* apoptosis detection kit (Wako) was used according to manufacturer's protocol. Phycoerythrin-labeled streptavidin (Vector Laboratories) or diaminobenzidine was used to visualize the reaction.

In Situ Hybridization for Y Chromosome

The methods used for tissue preparation and *in situ* hybridization have been described in detail previously.¹⁹ The probe was derived from the sequence of the sex-determining region gene (SRY) located on the Y chromosome.

PCR for Assessment for Graft Specificity of Graft-Infiltrating T Cells

To study the clonality of activated T cells infiltrating allografts undergoing ACR, paraffin-embedded liver biopsy specimens with ACR from three pediatric patients were subjected to PCR amplification. These biopsies were taken at a median of 12 days (range, 7 to 12 days) after transplant. Lymph nodes from T-cell lymphoma and reactive lymphadenopathy were used as controls. The paraffin-embedded tissues were deparaffinized and treated with proteinase K, phenol/chloroform extracted, and ethanol precipitated. Genomic DNA was extracted.

Because the *T-cell receptor (TCR)- γ* gene is thought to be rearranged in most T cells, even if the cells express TCR- α/β and not TCR- γ/δ , we used TCR- γ chain variable (V), and joining (J) segments to demonstrate the clonal expansion of T cells on the allograft. A 50- μ l reaction using a primer to the TCR- γ chain V region (TV- γ) (5'-AGGGTTGTGTTGGAATCAGG-3') as well as the J region (TJ- γ) (5'-CGTCGACAACAAGTGTGTTGCCAC-3') was performed for 30 cycles (94°C for 45 seconds, 55°C for 45 seconds, 72°C for 45 seconds) on a thermal cycler (Takara, Shiga, Japan) to amplify a 140- to 190-bp sequence.^{20,21} Primers for human β -actin (Invitrogen) served as the internal control.

Statistical Analysis

Results are expressed as mean $\pm$ SD of mean unless otherwise stated. All statistical analyses were performed using StatView software (Abacus Concepts, Berkeley, CA). Comparisons between these data and controls were analyzed using one-factor analysis of variance and Fisher's protected least significant difference test. Spearman rank correlation was used to test the association between RAI scores and the number of apoptotic bodies or the degree of FasL staining in KCs and endothelial cells in ACR. Significance was taken at $P < 0.05$.

Figure 1. FasL staining on KCs in the allograft and in normal liver. Dual staining for anti-FasL (red) and anti-CD68 (green) antibodies in representative cases of ACR (**A**), CR (**B**), stable graft (**C**), and normal liver (**D**). FasL staining on endothelial cells in the allograft and in normal liver. Sinusoidal endothelial cells were stained with anti-FasL (red)/anti-CD34 (green) antibodies in representative cases of the allograft undergoing ACR (**E**), CR (**F**), stable graft (**G**), and normal liver (**H**). Doubly stained cells were yellow. Original magnifications, $\times 400$.

Table 2. FasL Expression on Kupffer Cells in Liver Allograft and Normal Liver

	4+	3+	2+	1+	0
ACR	5/20 (25%)	9/20 (45%)	4/20 (20%)	2/20 (10%)	
CR		2/6 (33%)	4/6 (67%)		
SC		1/5 (20%)	2/5 (40%)	2/5 (40%)	
Stable grafts				5/5 (100%)	
Normal liver				1/6 (17%)	5/6 (83%)

0, no expression; 1+, sporadic expression in a few cells; 2+, up to 30% positive cells; 3+, 31 to 60% positive cells; 4+, more than 61% positive cells. ACR, acute cellular rejection; CR, chronic rejection; SC, septic cholangitis.

Results

Expression of FasL on KCs and Endothelial Cells in Liver Allograft

An analysis of surface antigens in APCs was performed by immunohistochemistry using anti-HLA A, B, C, anti-HLA-DR, anti-CD68, anti-CD80, anti-CD86, and anti-FasL. As a result, it was found that the expression of FasL, an apoptosis-inducing molecule, on APCs was one of the available phenotypic markers for evaluation of variable allograft status. In ACR ($n = 20$), FasL stained strongly on KCs (Figure 1A), on vascular and sinusoidal endothelial cells, extending diffusely from the periportal to the centrilobular area (Figure 1E), and on portal lymphocytes. CD3⁺ FasL⁺-activated lymphocytes were observed in the portal tract. In CR ($n = 6$), mild to moderate FasL staining was seen on KCs (Figure 1B), vascular and sinusoidal endothelial cells (Figure 1F), and portal lymphocytes. The distribution of immunoreactivity on vascular and sinusoidal endothelial cells was similar to that seen in ACR, but the staining pattern was generally weaker. In contrast, generally moderate FasL staining was seen on KCs, and mild staining was seen on vascular and sinusoidal endothelial cells and portal lymphocytes in SC ($n = 5$) (Tables 2 and 3). Immunostaining for FasL on vascular and sinusoidal endothelial cells was confined to the periportal area in SC. As a control, there was only weak and focal expression of FasL on KCs (Figure 1C) and endothelial cells (Figure 1G) in stable allografts. Normal liver specimens displayed no detectable FasL expression (Figure 1, D and H).

RAI Score and FasL Expression on APCs

We examined whether the degree of FasL expression increased with rejection severity. Although not statistically significant, there was a tendency for the degree of FasL expression on KCs to increase with RAI score in

ACR ($P = 0.052$) (Figure 2A). FasL expression increased significantly with the severity of the rejection process in endothelial cells ($P = 0.0045$) (Figure 2B). In addition, a significantly higher ratio of KCs was positive for FasL in ACR [mean, 74.8 ± 12.8 (SD)%] ($n = 20$) than in CR [mean, 22.5 ± 7.6 (SD)%] ($n = 6$) ($P < 0.01$) (Figure 2C). These data indicated that FasL expression on APCs is a useful marker for rejection activity and rejection type.

FasL⁺ KCs Phagocytose Apoptotic Fragments in the Portal Area

Next, we investigated the phagocytosing activity of FasL⁺ KCs. We found that FasL⁺ KCs phagocytosed apoptotic nuclear fragments in the portal area undergoing ACR (Figure 3, A1 and A2). *In situ* hybridization, using a probe for the human Y chromosome on a female donor allograft transplanted to a male patient revealed that the apoptotic fragments were positive for the *SRY* signal, indicating that they were recipient-derived (Figure 3A3). The nuclei of KCs were negative for the *SRY* probe, indicating that they were likely of donor origin. The *SRY*-positive apoptotic fragments were detected in the cytoplasm of KCs (Figure 3A4).

The count of apoptotic fragments per portal area was significantly higher in ACR ($n = 8$) than in CR ($n = 6$), SC ($n = 5$), and stable status (ACR versus CR, $P < 0.001$; ACR versus SC, $P = 0.001$; ACR versus stable, $P < 0.001$) (Figure 3B). After the dose of steroid was increased to >30 mg/day in eight cases of ACR, the count of apoptotic bodies fell within 48 hours ($P < 0.001$; ACR, $n = 8$, onset versus 48 hours after administration) (Figure 3B). In addition, the count of apoptotic bodies was closely correlated with the RAI score (ACR, $n = 20$, $P = 0.0002$) (Figure 3C). In addition, the count of phagocytosing FasL⁺ KCs, which had apoptotic fragments in the cytoplasm, was significantly higher in ACR than other conditions (CR versus ACR, $P < 0.001$; SC versus ACR,

Table 3. FasL Expression on Endothelial Cells in Liver Allograft and Normal Liver

	4+	3+	2+	1+	0
ACR	4/20 (20%)	5/20 (25%)	8/20 (40%)	3/20 (15%)	
CR		4/6 (67%)	2/6 (33%)		
SC			2/5 (40%)	3/5 (60%)	
Stable grafts				5/5 (100%)	
Normal liver					6/6 (100%)

0, no expression; 1+, sporadic expression in a few cells; 2+, up to 30% positive cells; 3+, 31 to 60% positive cells; 4+, more than 61% positive cells. ACR, acute cellular rejection; CR, chronic rejection; SC, septic cholangitis.

Figure 2. **A:** Scatter plots of the degree of FasL expression in CD68⁺ KCs by RAI score in ACR. There was a tendency for the degree of FasL expression to increase with RAI score ($P = 0.052$). **B:** Scatter plots of the degree of FasL expression in CD34⁺ endothelial cells by RAI score in ACR. The degree of FasL expression significantly correlated with RAI score ($P = 0.0045$). **C:** Scatter plots of the proportion of CD68⁺ KCs positive for FasL⁺ to all KCs in ACR and CR ($P < 0.01$, ACR versus CR).

$P < 0.001$; stable grafts versus ACR, $P < 0.001$) (Figure 3D). The phagocytosing FasL⁺ KCs decreased significantly within 48 hours after steroid administration except in one case (*) ($n = 8$) (Figure 3E). These data constitute evidence that the phagocytosing activity of KCs is an indicator of rejection activity and the effectiveness of immunosuppressant administration to control rejection.

Identification of the Phenotype of Apoptotic Nuclear Fragments

Next, we attempted to identify the origin of apoptotic fragments for the immunological significance of phagocytosis. We used serial sections of liver allograft with ACR (Figure 4, A–F). First, it was found that a mean value of 96.8 ± 2.3 (SD) % of apoptotic fragments were positive for CD3, indicating that they included T-cell-derived elements (Figure 4, A and B). FasL⁺ KCs phagocytosed the CD3⁺ Fas⁺ apoptotic fragments, probably derived from activated T cells (Figure 4, B–D). Furthermore, the apoptotic CD4⁺ T cells were positive for IFN- γ (Figure 4E) or IL-4 (Figure 4F). The recombination of the *TCR- γ* locus in T cells in the allografts with ACR exhibited several accumulating bands with a dominant recombination, suggesting that there was a dominant subpopulation of the infiltrating T cells. In contrast, only a single significant band appeared in T-cell lymphoma (Figure 4G). The phagocytosed apoptotic fragments were observed predominantly in IFN- γ -producing CD4⁺ T cells rather than IL-4-producing CD4⁺ T cells ($n = 20$) (Figure 4H). In ACR and CR, there were more IL-4⁺ CD4⁺ T cells than IFN- γ ⁺ CD4⁺ T cells (Figure 4I). IL-4⁺ CD4⁺ T cells were dominantly observed in the portal area in ACR (inset in Figure 4I). Notably, the percentage of IL-4⁺ CD4⁺ T cells to the total

CD4⁺ T cells was significantly higher in CR (* $P = 0.04$, ACR versus CR; Figure 4I).

Discussion

Although experimental studies have asserted that manipulation of the Fas/FasL system might provide apoptosis signals to induce allospecific immunosuppression,^{22,23} the interaction between donor and recipient cells on APCs and Fas/FasL expression is poorly understood in human liver allografts. Our study has demonstrated that FasL is abundantly expressed on KCs, endothelial cells in human liver allografts undergoing ACR and, to a lesser extent, allografts undergoing CR. We also found that apoptotic T cells were phagocytosed by FasL⁺ KCs, and the count was associated with rejection activity in human liver allografts. FasL expression on KCs and apoptotic T cells decreased after the administration of immunosuppressants. This provided evidence that FasL expression on APCs, T-cell apoptosis, and phagocytosed apoptotic T cells were useful parameters for assessing the severity of rejection and monitoring the effectiveness of therapeutic strategies. Our data suggested that FasL-expressing donor KCs and endothelial cells may recognize recipient cells and induce apoptosis in recipient Fas-expressing activated T cells, presumably by Fas-FasL interaction (Figure 4D).^{24,25}

Phagocytosis itself triggers macrophage release of FasL and induces apoptosis of bystander leukocytes.²³ The apoptotic recipient T cells in the study could be graft-specific cells. The findings of clonal T-cell proliferation were presumptive evidence of cellular responses to alloantigens. These results indicated that T cells infiltrat-

Figure 3. **A:** Morphological characteristics of apoptotic bodies. **A1:** KCs were found to phagocytose apoptotic fragments in the liver allograft undergoing ACR (arrow). **A2:** Immunostaining of the inset in **A1** showed FasL⁺ (stained green with FITC). KCs phagocytosed these fragments (stained red with PE by TUNEL staining, arrow). **A3:** *In situ* hybridization of apoptotic bodies with the *SRY* probe (brown dot, arrow). The apoptotic fragments with *SRY* signal were recipient in origin. **A4:** Apoptotic bodies in the cytoplasm of KCs were stained with a FITC-labeled *SRY* probe (stained green). The KCs stained with PE-labeled CD68 (red). The nuclei of the KCs stained blue with DAPI and not by the *SRY* probe, indicating that the KCs were of female-donor origin. Arrows indicate the phagocytosing KCs. **B:** The count of apoptotic fragments per portal area: SC, ACR, and CR. In ACR, apoptotic fragments were significantly increased compared with CR, resolved status, and stable grafts. **C:** Correlation between the number of apoptotic bodies (y) and RAI score (x) in cases of ACR. Lines confirmed the positive correlation. **D:** The count of phagocytosing FasL⁺ KCs in a portal area. **E:** The count of phagocytosing FasL⁺ KCs in a portal area at the onset of rejection and 48 hours after steroid administration ($P < 0.001$). Original magnifications: $\times 400$ (**A1**); $\times 600$ (**A2–A4**).

ing liver allografts with ACR underwent proliferation and clonal expansion in response to a specific antigen that may be an alloantigen. Analogous analyses of TCR repertoires in peripheral blood lymphocytes or allografts in lung,^{26,27} kidney,^{28–30} and cardiac^{31,32} transplant patients have shown that clonal and oligoclonal T-cell expansions. ACR and CR are directed against a single dominant alloepitope of one of the donor's mismatched HLA-DR antigens in organ allografts, indicating that allopeptide-specific T-cell clones may play a role in rejection.^{33,34}

The development of tolerance is associated with an increased rate of apoptosis of T cells in the portal inflammatory infiltrate and the presence of an intra-graft Th2-like T-cell population.^{6,35,36} Sun and colleagues⁶ found that KCs express functional FasL, induce apoptosis of Fas⁺ cells, and can significantly suppress T-cell proliferation.

Our present data also suggest that KCs down-regulate the activity of T cells and reduce the survival of the IFN- γ -producing Th1-like T-cell population, leading to immunosuppression in the portal tract. The selective death of Th1 effectors, leading to selective Th2 survival, provides a unique mechanism for differential regulation of the two subsets of helper T cells in the allograft. This cytokine profile may drive the immune response in liver allograft toward a Th2 (suppressive) phenotype and may be associated with the pathogenesis of CR or tolerance (Figure 4I).^{6,35,36} Although the majority of studies report the predominant expression of type 1 cytokines in ACR and type 2 cytokines in CR and tolerance,³⁷ it is not known what factors are important in determining the outcome of the graft.

In addition to KCs, infiltrating cells or antigens passing through the hepatic sinusoids can contact the si-

Figure 4. Identification of phagocytosed apoptotic bodies. **A–F:** Immunostaining of apoptotic bodies and phagocytosing KCs using serial sections in ACR. **A:** Apoptotic bodies (arrows) stained by the TUNEL method. **B:** Combined staining of a KC with anti-CD68 (purple, stained with fuchsin; arrowhead) and phagocytosed CD3⁺ apoptotic bodies (stained brown, arrow) in the cytoplasm of a CD68⁺ KC. **C and D:** The KC was stained with anti-FasL (red) (**C**) and anti-Fas antibodies (green, an arrow indicates phagocytosed apoptotic fragments; **D**). **E and F:** The apoptotic bodies shown in **A** were doubly stained with anti-CD4 (red) and anti-IFN-γ (green, an arrow indicates the fragment seen in **A**; **E**) or IL-4 (green, an arrow indicates the fragment seen in **A**; **F**). **G:** Detection of TCR-γ clonal bands of graft infiltrating T cells in liver allografts with ACR (lanes 2 to 4, ACR1 to 3). Positive control (lane 1, TCL: T cell lymphoma) and negative control (lane 5, LN: reactive lymphadenopathy) were shown. β-Actin signals from the same samples are also shown. **H:** The phenotype of phagocytosed T cells in the allograft with ACR (*n* = 20). **I:** Cytokine productivity of the T cells in the allograft with ACR (*n* = 20) and CR (*n* = 6). The inset shows IL-4⁺ (stained red with PE) and CD4⁺ (stained green with FITC) T cells in the portal area in ACR. Original magnifications, ×600.

nusoidal endothelium, which forms a highly distinctive endothelium.^{2,3} The human sinusoidal endothelium does not constitutively express HLA class II or co-stimulatory molecules³⁸; however, other properties of the endothelium, such as the high capacity for antigen uptake and direct access to circulating lymphocytes, may enable it to contribute to the unique immunological function of the liver.^{38,39} Although we did not specifically determine whether apoptotic recipient T cells are cleared by sinusoidal endothelial cells,^{2,40} the high expression of FasL on vascular and sinusoidal endothelial cells in ACR and CR raises the possibility of its contributing to recognition of the antigen in the liver and full T-cell activation, with immune deviation leading to the differentiation of T cells to a suppressive or regulatory phenotype or of abortive activation leading to T-cell apoptosis. Although CR usually follows an earlier episode of ACR, it remains unclear whether the pathogenic mechanisms are identical. A possible mechanism is that cytokines, such as IL-1 and

transforming growth factor, would be continuously secreted by KCs and augment tissue injury. The activity of ACR might subside, switching the Th1 response to a Th2 response and leading to proliferation of endothelial and smooth muscle cells in CR.^{41,42}

In our study, CR was accompanied by persistent up-regulation of the expression of FasL in endothelial cells, which may promote expansion of donor T cells and recipient T-cell apoptosis.⁴³ Although donor-derived hematopoietic APCs are considered responsible for direct allorecognition and the intensity of the initial acute rejection process, they decrease substantially in number throughout time.⁴⁴ Endothelial replacement to recipient type has been demonstrated in sinusoidal endothelial cells and in central veins,⁴⁵ but most donor endothelial cells persist for the life of the graft and may provide a constant source of stimulation for directly alloreactive T cells.^{42,46}

In SC, FasL staining was less intense than in rejection. It is suggested that the polyclonal T-cell response

mounted against an infecting pathogen limits the efficiency of the induction of apoptosis, whereas these cell populations mediate apoptosis in an antigen-specific way in ACR and CR.⁸ In actual diagnosis, it is often difficult to detect apoptotic T cells in the portal tract without using FasL staining. Without this staining, apoptotic clusters often resembled leukocytoclastic cells, including the neutrophilic infiltrates seen in SC. Thus, FasL staining can provide evidence of active rejection and a way of evaluating the therapeutic effect of steroid administration.

Although the data do not preclude the possibility that hepatocytes could induce lymphocyte apoptosis by Fas/FasL interaction, we demonstrated Fas/FasL-dependent apoptosis of recipient T cells, likely induced by donor APCs. We also showed that apoptotic T cells were phagocytosed by FasL⁺ KCs. The functional role for APCs in the interaction with recipient T cells has been an area of speculation in our study, because it was retrospective and descriptive in design. Thus, we could not objectively evaluate the functional role of APCs.

The present study provided evidence that T-cell apoptosis and phagocytosed apoptotic T cells were useful parameters for assessing the severity of rejection and monitoring the effectiveness of therapeutic strategies. The results also provided supplementary evidence that KCs regulate T-cell subset differentiation and participate in the development of immune tolerance after liver transplantation.

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