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De novo donor-specific anti-HLA antibodies mediated rejection in liver-transplant patients

Arnaud Del Bello,[1,2] Nicolas Congy-Jolivet,[2,3,4] Marie Danjoux,[5] Fabrice Muscari,[2,6] Laurence Lavayssière,[1] Laure Esposito,[1] Isabelle Cardeau-Desangles,[1] Joëlle Guitard,[1] Gaëlle Dörr,[1,2] David Milongo,[1] Bertrand Suc,[2,6] Jean Pierre Duffas,[6] Laurent Alric,[2,7] Christophe Bureau,[2,8] Céline Guilbeau-Frugier,[2,5] Lionel Rostaing[1,2,9] and Nassim Kamar[1,2,9]

1 Department of Nephrology and Organ Transplantation, CHU Rangueil, Toulouse, France
2 Université Paul Sabatier, Toulouse, France
3 Molecular Immunogenetics Laboratory, EA 3034, Faculté de Médecine Purpan, IFR150 (INSERM), Toulouse, France
4 Department of Immunology, Hôpital de Rangueil, CHU de Toulouse, Toulouse, France
5 Department of Pathology, CHU Purpan, Toulouse, France
6 Department of Surgery and Liver Transplantation, CHU Rangueil, Toulouse, France
7 Internal Medecine-Digestive Department, UMR 152, IRD, CHU Purpan, Toulouse, France
8 Department of Hepatology, Federation Digestive, CHU Purpan, Toulouse, France
9 INSERM U1043, IFR–BMT, CHU Purpan, Toulouse, France

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Correspondence
Arnaud Del Bello MD, Department of Nephrology and Organ Transplantation, CHU Rangueil, TSA 50032, 31059 Toulouse Cedex 9, France.
Tel.: +33 5 61 32 39 23;
fax: +33 5 61 32 39 89;
e-mail: delbello.a@chu-toulouse.fr

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Summary

The incidence and consequences of *de novo* donor-specific anti-HLA antibodies (DSAs) after liver transplantation (LT) are not well known. We investigated the incidence, risk factors, and complications associated with *de novo* DSAs in this setting. A total of 152 *de novo* liver-transplant patients, without preformed anti-HLA DSAs, were tested for anti-HLA antibodies, with single-antigen bead technology, before, at transplantation, at 1, 3, 6 and 12 months after transplantation, and thereafter annually and at each time they presented with increased liver-enzyme levels until the last follow-up, that is, 34 (1.5–77) months. Twenty-one patients (14%) developed *de novo* DSAs. Of these, five patients had C1q-binding DSAs (24%). Younger age, low exposure to calcineurin inhibitors, and noncompliance were predictive factors for de novo DSA formation. Nine of the 21 patients (43%) with *de novo* DSAs experienced an acute antibody-mediated rejection (AMR). Positive C4d staining was more frequently observed in liver biopsies of patients with AMR (9/9 vs. 1/12, $P < 0.0001$). Eight patients received a B-cell targeting therapy, and one patient received polyclonal antibodies. Only one patient required retransplantation. Patient- and graft-survival rates did not differ between patients with and without DSAs. In conclusion, liver-transplant patients with liver abnormalities should be screened for DSAs and AMR.

Introduction

The impact of donor-specific anti-HLA antibodies (DSAs) after liver transplantation remains controversial. Until recently, human liver allografts were considered to be highly resistant to antibody-mediated rejection (AMR) for several reasons: secretion of soluble HLA class I molecules that form immune complexes with alloantibodies, phagocytosis of platelet aggregates, immune complexes, and activated complement components by Kupffer cells, limited distribution of HLA class II expression in the microvasculature, large liver size, and dual hepatic vasculature, and finally, important hepatocyte regenerative capacity after injury [1–3].

However, over the last couple of years, there has been increased evidence that DSAs can have harmful effects after liver transplantation. A higher rate of graft loss has been observed in patients with a positive cross-match compared to those who have undergone transplantation with a negative cross-match [4,5]. Patients undergoing liver transplantation with preformed DSAs are at increased risk of hyperacute rejection [6] and increased risk of AMR within the first weeks after transplantation [7–9]. In addition, DSAs have been associated with chronic rejection [10,11], accelerated fibrosis [12,13], and anastomotic biliary strictures [14].

To date, very few studies have assessed the incidence and effect of *de novo* DSAs in liver-transplant patients. In patients that are DSA-free at transplantation, Kaneku *et al.* [15] reported an incidence of 8.1% of *de novo* DSAs within the first year after transplantation. Patients who developed *de novo* DSAs had significantly lower patient- and graft-survival rates compared to those without DSAs [15]. Here, the aims of our study were to assess the incidence of *de novo* DSAs in liver-transplant patients and to analyze their effect on liver histology, to assess their impact on patient- and graft-survival rates, and to report on the treatment of antibody-mediated rejection in this setting.

Patients and methods

Patients

Between February 2008 (i.e., the date when the solid-phase Luminex assay was set up in our institution) and September 2013, a total of 211 adult patients received a liver transplantation in our center. We excluded patients who died within the first month after transplantation ($n = 42$) from the study, and those who received a transplant with a preformed DSA directed against HLA A, B, CW, DR, DQ, or DP ($n = 17$). Acute rejection was not the cause of death in any patient who died within the first-month post-transplantation. Hence, 152 patients with a functioning liver allograft at month 1 after transplantation were included in this study after having given their informed consent and after we had obtained Toulouse University IRB approval.

The patients' characteristics are presented in Table 1. There were 122 men and 30 women ranging in age from 18 to 72 years. Ninety-eight percent of patients received a first liver transplant. Only, three patients were undergoing retransplantation.

All patients were screened routinely for anti-HLA antibodies before, at transplantation, at 1, 3, 6, and 12 months after transplantation, and thereafter annually and at each time they presented with increased liver-enzyme levels until the last follow-up, that is, 34 (1.5–77) months. The date of death of any patients who died within the study period was considered as the last follow-up.

Noncompliance was assessed at each visit. Patients who acknowledged having forgotten to take their immunosuppressive drugs two or more successive doses between two visits or who had stopped any immunosuppressant without medical indication were considered to be noncompliant.

Immunological analyses

Luminex® assays determined the specificity of class I HLAs in A/B and class II in DR/DQ IgG antibodies in the recipients' sera (centrifuged at 10 000 *g* for 10 min) using Labscreen single Ag HLA class-I and class-II detection tests (One Lambda, Canoga Park, CA, USA), according to the manufacturer's instructions. The presence and specificity of antibodies were then detected using a Labscan 100® (One Lambda, Canoga Park, CA, USA), and the mean fluorescence (baseline) value for each sample in each bead was evaluated. The baseline value was calculated as follows: (raw sample mean fluorescence intensity [MFI] − raw negative serum control MFI) − (negative-bead raw MFI sample − negative-bead raw MFI negative serum control). A baseline value of >1000 was considered positive. The immunodominant DSA was defined as the DSA with the highest MFI.

The presence of C1q-binding donor-specific anti-HLA antibodies was determined using a single-antigen flow bead assay according to the manufacturer's protocol (C1qScreenTM, One Lambda), as described previously [16].

Liver biopsy

Each time an acute rejection was suspected and/or when a *de novo* anti-HLA DSA was detected, a liver biopsy was performed. No serial protocol biopsies were performed to assess subclinical antibody-mediated rejection. Liver-pathology lesions were classified according to the Banff criteria [17,18]. In addition, antibody-mediated rejection was defined as evidence of graft dysfunction in the presence of circulating DSAs, with acute rejection refractory to steroids, and histological changes related to antibody-mediated liver-injury proliferation of the small bile ducts, centrilobular hepatocyte swelling, single-cell necrosis, sinusoidal accumulation of neutrophils, and hepatocanalicular cholestasis, as well as an increased number of plasma cells in the portal infiltrate and diffuse positive C4d staining (>50%) of the portal microvasculature [1,12,19].

Statistical analyses

Reported values represent the means (±SD) or medians (ranges). Proportions were compared using Fisher's exact test. Quantitative variables were compared using the Mann–Whitney nonparametric test or Student's *t*-test. The

Table 1. Comparison between liver-transplant patients with and without donor-specific antibodies.

Variable	*De novo* DSAs (*n* = 21)	No DSAs (*n* = 131)	*P*-value
Median donor age (years)	44 (17–77)	51 (9–81)	NS
Median recipient age (years)	52 (18–63)	58 (20–72)	0.02
Recipient gender, male (%)	16 (76)	106 (81)	NS
Rank of transplantation	1	1 ± 0.1	NS
Median MELD score	22 (6–40)	23 (6–40)	NS
MELD score <15 (%)	6 (29)	38 (29)	NS
Median Child–Pugh score	10 (5–15)	8 (5–15)	NS
Cause of liver disease, *n* (%)			
Viral hepatitis	9 (43)	39 (30)	NS
Alcoholic liver disease	8 (38)	61 (46)	
Autoimmune disease (AIH, PSC, PBC)	3 (14)	9 (7)	
Other	1 (5)	22 (17)	
CMV infection, *n* (%)	5 (24)	27 (21)	NS
Hepatic artery thrombosis, *n* (%)	0 (0)	9 (7)	NS
Positive HCV RNA, *n* (%)	7 (34)	29 (22)	NS
Interferon use after transplantation, *n* (%)	2 (9.5)	14 (11)	NS
Induction therapy (%)	16 (76)	95 (73)	NS
Polyclonal antibodies (%)	3 (14)	11 (8)	NS
Anti-interleukin receptor blocker (%)	13 (62)	84 (65)	NS
Initial immunosuppressive therapy			
Calcineurin inhibitors (%)	21 (100)	123 (94)	NS
Tacrolimus (%)	19 (90)	123 (94)	NS
Cyclosporine A (%)	2 (10)	0	0.02
Mycophenolic acid (%)	21 (100)	131 (100)	NS
Belatacept (%)	0	8 (6)	NS
Steroids (%)	20 (95)	111 (85)	NS
Immunosuppressive therapy at 3 months*			
Calcineurin inhibitors (%)	19 (91)	118* (91)	NS
Tacrolimus (%)	14 (67)	109* (84)	NS
Tacrolimus trough level (ng/mL)	8.75 ± 2.9	8.6 ± 2.6	NS
Cyclosporine A (%)	5 (24)	9 (7)	0.02
C2 Cyclosporine A (ng/mL)	813 ± 264	960 ± 389	NS
Mycophenolic Acid (%)	21 (100)	126 (98)	NS
Mycophenolic acid dose (mg/day)	1381 ± 650	1333 ± 529	NS
Belatacept (%)	0	7* (5)	NS
Everolimus (%)	2 (9.5)	4* (3)	NS
Everolimus (ng/mL)	5.5 ± 0.3	5 ± 2.8	NS
Steroids (%)	20 (95)	107* (83)	NS
Steroid dose (mg/day)	9 ± 5	8 ± 4	NS
Immunosuppressive therapy at 1 year†			
Calcineurin inhibitors (%)	19 (91)	107† (87)	NS
Tacrolimus (%)	18 (86)	100† (81)	NS
Tacrolimus trough level (ng/mL)	7.3 ± 3.3	8.4 ± 2.7	NS
Cyclosporine A (%)	1 (5)	7† (6)	NS
C2 Cyclosporine A	905	933 ± 289	NS
Mycophenolic acid (%)	19 (91)	113† (92)	NS
Mycophenolic acid dose (mg/day)	1000 ± 408	1045 ± 280	NS
Belatacept (%)	0	7† (6)	NS
Everolimus (%)	1 (5)	9† (7)	NS
Everolimus trough level (ng/mL)	4.5	7.4 ± 3	NS
Steroids (%)	17 (81)	92 (75)	NS
Steroid dose (mg/day)	6 ± 2	5 ± 2	NS
Conversion from CsA to tacrolimus or vice versa (%)	9 (43)	21 (16)	0.007
Conversion from a CNI to an mTOR inhibitor (%)	4 (19)	10 (8)	0.1
Low exposure to CNIs or mTORi‡ (%)	12 (57)	13 (10)	<0.001
Noncompliance to immunosuppressive therapy	3 (14)	2 (0.7)	0.02

Table 1. continued

Variable	*De novo* DSAs (n = 21)	No DSAs (n = 131)	*P*-value
HLA class I and II mismatches,	5.52 ± 1.53	5.55 ± 1.73	NS
HLA-A, HLA-B mismatches	2.95 ± 1.11	3.04 ± 1.03	NS
HLA-DR, HLA-DQ mismatches	2.57 ± 1.16	2.51 ± 1.18	NS
Pretransplant non-DSA anti-HLA antibodies (%)	2 (12)	21 (16)	NS
Pretransplant anti-HLA antibodies DSAs (%)	0	0	NS
Initial positive CDC T- or B-cell cross-match (%)	0	0	NS
Time between transplantation and the last follow-up (months)	39 (6–71)	33 (1.5–77)	NS
De novo non-DSA anti-HLA antibodies (%)	10 (48)	12 (9)	<0.001
De novo non-DSA anti-HLA class I antibodies (%)	7 (34)	10 (8)	<0.001
De novo non-DSA anti-HLA class II antibodies (%)	7 (43)	4 (3)	<0.001
Acute rejections (until last follow-up) (%)	11 (52)	28 (21)	0.005
Steroid-sensitive rejection episodes/patient	2 (12)	22 (16)	NS
Non-steroid-sensitive rejection episodes/patient	1 (5)	8 (6)	NS
Antibody-mediated rejection, *n* (%)	9 (53)	0	<0.0001

DSA, donor-specific antibodies; MELD, Model for End-Stage Liver Disease; AIH, autoimmune hepatitis; PSC, primary sclerosing cirrhosis; PBC, primary biliary cirrhosis; CMV, cytomegalovirus; HCV, hepatitis C virus; C2, 2 h after intake concentration; CsA, cyclosporin A; HLA, human leukocyte antigen; CDC, complement-dependent cytotoxicity, CNI, calcineurin inhibitors; mTORi, mTOR inhibitors; NS, not significant.

*Two patients in the non-DSA group died within the first 3 months after liver transplantation.

†Eight patients in the non-DSA group died within the first 12 months after liver transplantation.

‡Low exposure was defined as a tacrolimus trough level <5 ng/mL or a cyclosporin A C2 level <500 ng/mL or an everolimus trough level <5 ng/mL for at least 1 week.

predictive factors for developing a DSA were determined by univariate and multivariate regression analyses. Factors associated, by univariate analyses (at a significance of $P < 0.05$), with the detection of DSAs after transplantation were selected for inclusion in multivariate analyses. Survival rates were presented in Kaplan–Meier curves. A *P*-value of <0.05 was considered statistically significant.

Results

Incidence of *de novo* DSAs

At the last follow-up, that is, 34 (1.5–77) months after liver transplantation, 21 patients (14%) had developed *de novo* DSAs. Of these, only five patients had C1q-binding DSAs (24%). Three patients (14%) had developed only anti-class I DSAs: that is, one patient had developed an anti-A antibody, one patient had developed an anti-B antibody, and one patient had developed two anti-B antibodies. Sixteen patients (76%) developed only anti-class II DSAs [anti-DR (n = 3), anti-DQ (n = 11), and both anti-DR and anti-DQ (n = 2)]. The two remaining patients (10%) developed both anti-class I and II DSAs (one anti-A and anti-DQ, and the other an anti-A, anti-B, and anti-DR). Twelve patients developed one DSA, eight patients have developed two DSAs, and one patient presented with five DSAs.

Seven patients (34%) developed at least one DSA within the first 6 months post-transplantation, of which six cases developed within the first-month post-transplant. Six other patients (29%) developed DSAs between months 6 and 12 post-transplant. The eight remaining patients (37%) developed DSAs after 1-year post-transplant (Fig. 1a). None of the three patients who received a second liver transplant developed a *de novo* DSA.

In 10 patients, DSAs were detected at the routine annual screening for anti-HLA antibodies, for the 11 remaining patients DSAs were assessed and found to be positive because they presented with liver tests abnormalities. Ten of the last 11 patients have developed an acute humoral rejection.

Risk factors for *de novo* DSAs

We looked for the predictive factors for the development of *de novo* DSAs. Results from the univariate analysis are presented in Table 1. The following variables were included in the multivariate analysis: recipient's age at transplantation, the use of cyclosporin A at transplantation (versus no cyclosporine A), the use of cyclosporin A at month 3 post-transplantation (versus no cyclosporine A), conversion from tacrolimus to cyclosporine A or vice versa, low exposure to calcineurin inhibitor levels (defined as a tacrolimus trough level <5 ng/mL or cyclosporin A at a 2-h concentration of <500 ng/mL), or to everolimus (trough level <5 ng/mL) for at least 1 week after transplantation and a history of noncompliance. Low exposure to calcineurin inhibitors or to mammalian target of rapamycin inhibitors [OR 14.2; 95% CI (4.3–46.9), $P < 0.0001$], and noncompliance [OR 17; 95% CI (1.7–175.9), $P = 0.01$] have been identified as predictive factors for *de novo* DSA formation. Conversely, older age of the recipients at transplantation had a signifi-

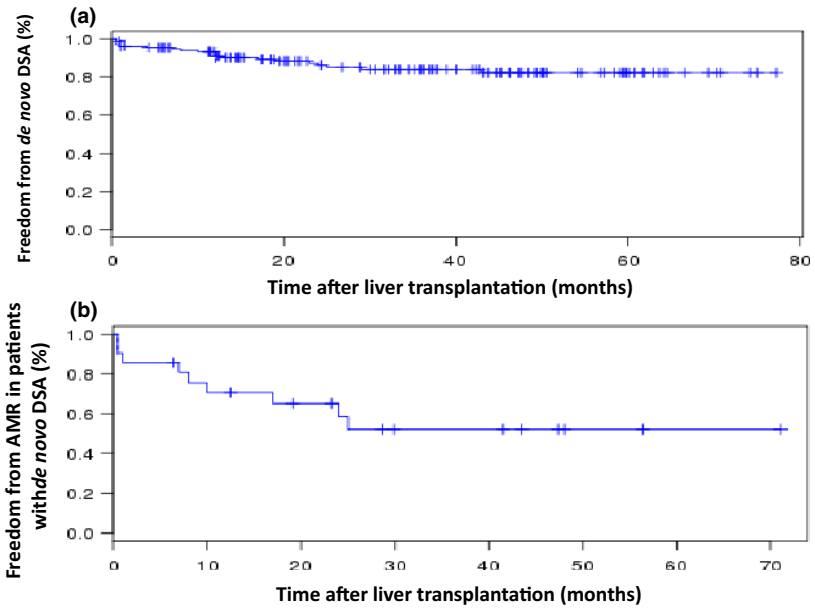

Figure 1 (a) Occurrence of donor-specific antibodies in *de novo* liver-transplant patients. (b) Occurrence of antibody-mediated rejection among *de novo* liver-transplant patients who developed donor-specific antibodies. DSA, donor-specific antibodies; AMR, antibody-mediated rejection.

cantly lower likelihood of *de novo* DSA occurrence [OR 0.9; 95% CI (0.88–0.97), $P = 0.002$].

Incidence of antibody-mediated rejection

Patients with de novo DSAs

Nine of the 21 patients who developed *de novo* anti-HLA DSAs (43%) presented with an episode of rejection that met the criteria for acute antibody-mediated rejection, that is, sudden alteration of liver-function tests, *de novo* anti-HLA DSAs, histological changes that were compatible (Table 2) with antibody-mediated rejection, with diffuse positive C4d staining and non-steroid-sensitive rejection. All other causes of acute liver disruption were ruled out. HEV RNA, HCV RNA, HBV DNA, CMV DNA, HHV6 RNA were negative in all patients. Liver ultrasonography was considered as normal. Toxin and drug causes on anamnesis. Six of the nine AMR episodes occurred at 3 months after transplantation (Fig. 1b). Seven of the nine patients had combined T-cell and antibody-mediated rejection, and the last two patients had isolated AMR.

Two other patients presented with steroid-sensitive rejection (10%). It occurred in one patient before DSAs were detected and in another patient after a DSA had developed. Both these acute rejection episodes were successfully treated by pulses of steroids.

One additional patient presented with non-steroid-sensitive cellular rejection. The histological findings did not meet the criteria for AMR, that is, absence of optical AMR-related microvasculitis and negative C4d staining. However, there was a marked T-cell infiltration in the portal tract and in the bile ducts, as well as a subendothelial infiltration involving most of the portal venules.

The nine remaining patients had no alteration of liver-function and normal liver biopsies. However, afterward, no serial liver biopsies were performed to detect subclinical AMR.

Patients without DSAs

During the follow-up, 22 of the 131 (16%) patients without DSAs presented with steroid-sensitive rejection (P = NS, compared to the group of patients with DSAs). Eight other patients presented an acute rejection episode that required the use of polyclonal antibodies (6%; P = NS compared to the group of patients with DSAs).

Comparison between patients with DSAs who did or did not experience an AMR episode

We compared the patients' characteristics, and clinical biological and histological findings between patients with *de novo* DSAs who did or did not experience AMR (Table 3). The proportion of patients that had positive C4d staining was significantly higher in patients experiencing AMR (9/9: 100%) compared to those who did not (1/12: 8.3%; $P < 0.0001$). This was the sole statistical difference between the two groups.

Of note, the proportion of patients with C1q-binding DSAs did not differ significantly between patients who did or did not experience AMR ($P = 0.1$).

Table 2. Description of presentations and outcomes of patients who experienced an antibody-mediated rejection.

Patients	Time between LT and AMR (months)	Type of DSA	Immunodominant MFI/MFI sum	IS at AMR	Banff score	Histological feature	Positive C4d staining	AMR treatment	AMR to last FU (mo)	DSAs at last FU	Outcome
1	17	DR4, DR15	10 000/18 000	CsA (C2: 100 ng/mL), S	7	Portal and central eosinophilic venulitis, ductopenia, cholestasis, hepatocyte ballooning	Portal	PP, R, S pulses, IvIg, RATG	3	Positive (MFI 9000)	Liver failure, retransplantation
2	25	DR4, DQ3	15 000/23 000	CsA (C2: 350 ng/mL), S	3	Hepatocyte ballooning, cholestasis, portal vein endothelial cell hypertrophy	Portal, sinusoidal	R, S pulses, IvIg	48	Positive (MFI 15 000)	Normal liver-enzyme tests
3	10	DQ7	12 000/12 000	Tac (C0: 5.8 ng/mL), S	4	Cholestasis, hepatocyte ballooning, portal eosinophilic venulitis	Portal	PP, R, S pulses, IvIg	3	Negative	Normal liver-enzyme tests
4	1	DR3	1500/1500	Tac (C0: 4 ng/mL), MPA, S	3	Cholestasis, hepatocyte ballooning	Portal, sinusoidal	PP, R, S pulses, IvIg	33	Negative	Normal liver-enzyme tests
5	1	A1, A24, B51, B44, DR53	5000/14 500	CsA (C2: 400 ng/mL), MPA, S	6	Portal vein endothelial cell hypertrophy, portal eosinophilic venulitis, cholestasis	Portal	PP, R, S pulses, IvIg	43	Negative	Normal liver-enzyme tests
6	8	A1, A30, DQ5	8000/22 000	Tac (C0 < LQ) MPA, S	6	Cholestasis, portal eosinophilic venulitis	Portal, sinusoidal, central vein	PP, R, S pulses, IvIg	18	Positive (MFI 10 000)	Persistent cholestasis
7	7	DQ5, DQ6	5200/8800	CsA (C2: 300 ng/mL), S	6	Cholestasis, portal and central eosinophilic venulitis	Portal	R, S pulses, IvIg	41	Negative	Stable liver enzymes tests
8	24	DQ3	5000/5000	Tac (C0 < LQ), MPA	4	Cholestasis, portal and central eosinophilic venulitis, portal vein endothelial cell hypertrophy, hepatocyte ballooning	Portal	R, S pulses	36	Positive (MFI 1500)	Normal liver-enzyme tests
9	1	B55, B57	1500/2700	Tac (C0: 10 ng/mL), MPA, S	6	Hepatocyte ballooning, portal vein endothelial cell hypertrophy, portal and central eosinophilic venulitis	Portal, sinusoidal	S pulses, RATG	65	Negative	Normal liver-enzyme tests

LT, liver transplantation, AMR, antibody-mediated rejection; MFI, mean immunofluorescence intensity; CsA, cyclosporine A; Tac, tacrolimus; MPA, mycophenolic acid; S, steroids; C0, trough level; C2, 2 h after intake concentration; PP, plasmapheresis; R, rituximab; IvIg, intravenous immunoglobulins; RATG, rabbit antithymocyte globulins; FU, follow-up; LQ, limit of quantification.

Table 3. Comparison between liver-transplant patients with donor-specific antibodies who did or did not experience antibody-mediated rejection.

Variables	*De novo* DSAs with AMR (*n* = 9)	*De novo* DSAs without AMR (*n* = 12)	*P*-value
Median recipient age at transplantation (years)	52 (18–63)	52 (19–60)	NS
Median donor age (years)	45 (17–77)	44 (30–71)	NS
Median MELD score	28 (14–40)	22 (6–40)	NS
Median Child–Pugh score	11.5 (8–15)	9 (5–15)	NS
Gender, male (%)	6 (67)	10 (83)	NS
HLA mismatch class I and II	5 ± 1.7	5.9 ± 1.3	NS
Class I	2.9 ± 1.5	3 ± 0.9	NS
A	1.4 ± 0.9	1.4 ± 0.5	NS
B	1.4 ± 0.7	1.6 ± 0.5	NS
Class II	2.1 ± 1.4	2.9 ± 0.9	NS
DR	1.4 ± 0.5	1.5 ± 0.5	NS
DQ	1 ± 0.7	1.4 ± 0.6	NS
Induction therapy (%)	7 (78)	9 (75)	NS
Polyclonal antibodies (%)	3 (33)	0	NS
Anti-interleukin receptor blockers (%)	4 (45)	9 (75)	NS
Cause of liver disease (%)			
Viral hepatitis	2 (22)	7 (58)	NS
Alcoholic liver disease	5 (56)	3 (25)	NS
Autoimmune disease (AIH, PSC, PBC)	2 (22)	1 (13)	NS
Other	0	1 (13)	NS
CMV infection, *n* (%)	3 (33)	2 (17)	NS
Positive HCV RNA, *n* (%)	2 (22)	5 (42)	NS
Interferon use at post-transplantation, *n* (%)	0	2	NS
Initial immunosuppressive therapy			
Calcineurin inhibitors (%)	9 (100)	12 (100)	NS
Tacrolimus (%)	8 (89)	11 (92)	NS
Cyclosporine A (%)	1 (11)	1 (8)	NS
Mycophenolic acid (%)	9 (100)	12 (100)	NS
Steroids (%)	8 (89)	12 (100)	NS
Immunosuppression therapy at 3 months			
Calcineurin inhibitors (%)	9 (100)	10 (83)	NS
Tacrolimus (%)	6 (67)	8 (67)	NS
Tacrolimus trough level (ng/mL)	8.2 ± 4	9.1 ± 2	NS
Cyclosporine A (%)	3 (33)	2 (17)	NS
C2 cyclosporine A (ng/mL)	917 ± 288	658 ± 181	NS
Mycophenolic acid (%)	9 (100)	12 (100)	NS
Mycophenolic acid dose (mg/day)	1250 ± 584	1556 ± 726	NS
Everolimus (%)	0	2	NS
Everolimus trough level (ng/mL)	–	5.25 ± 0.3	–
Steroids (%)	8 (89)	12 (100)	NS
Steroid dose (mg/day)	11.8 ± 7	7.5 ± 2.6	NS
Immunosuppression therapy at 1 year			
Calcineurin inhibitors (%)	9 (100)	10 (83)	NS
Tacrolimus (%)	9 (100)	9 (75)	NS
Tacrolimus trough level (ng/mL)	8.8 ± 3.7	5.9 ± 2.3	NS
Cyclosporine A (%)	0	1	NS
C2 cyclosporine A (ng/mL)	–	905	–
Mycophenolic acid (%)	8 (89)	11 (92)	NS
Mycophenolic acid dose (mg/day)	1062 ± 620	954 ± 151	NS
Everolimus (%)	0	1 (8)	NS
Everolimus trough level (ng/mL)	–	4.5	–
Steroids (%)	7 (78)	10 (83)	NS
Steroid dose (mg/day)	5.7 ± 2	6 ± 2	NS
Immunosuppressive therapy at DSA detection			
Calcineurin inhibitors (%)	9 (100)	8 (67)	NS
Tacrolimus (%)	4 (44)	6 (50)	NS

Table 3. continued

Variables	*De novo* DSAs with AMR (n = 9)	*De novo* DSAs without AMR (n = 12)	*P*-value
Tacrolimus trough level (ng/mL)	8.4 ± 1.6	5.7 ± 2.4	NS
Cyclosporine A (%)	5 (56)	2 (17)	NS
C2 cyclosporine A (ng/mL)	474 ± 274	646 ± 387	NS
Mycophenolic acid (%)	6 (67)	11 (92)	NS
Mycophenolic acid dose (mg/day)	1584 ± 665	1000	NS
Everolimus (%)	0	3	NS
Everolimus trough level (ng/mL)	–	8.7 ± 4.3	–
Steroids (%)	8 (89)	10 (83)	NS
Steroid dose (mg/day)	16.2 ± 18	6.4 ± 4.5	NS
Triple immunosuppressive regimen	5 (56)	6 (50)	NS
Double immunosuppressive regimen	4 (44)	6 (50)	NS
Low exposure to CNIs or mTORi * (%)	4 (44)	8 (67)	NS
Noncompliance to immunosuppressive therapy	3 (33)	0	0.06
Time between transplantation and last follow-up (months)	39.5 (18–71)	35.7 (6.5–71)	NS
DSA type			
Class I	3 (33)	2 (17)	NS
A	2 (22)	1 (8.5)	NS
B	2 (22)	1 (8.5)	NS
Class II	8 (89)	10 (83)	NS
DR	4 (44)	1 (8.5)	NS
DQ	5 (56)	9 (75)	NS
Median number of DSAs (ranges)	2 (1–5)	1 (1–2)	0.1
MFI of the immunodominant DSAs	7000 ± 4500	7000 ± 5200	NS
MFI sum	12 000 ± 8000	9000 ± 7600	NS
C1q binding to DSA (%)	4 (45)	1 (8)	0.1
Positive C4d staining	9 (100)	1 (8)	<0.0001

DSA, donor-specific antibodies; MELD, Model for End-Stage Liver Disease; AIH, autoimmune hepatitis; PSC, primary sclerosing cirrhosis; PBC, primary biliary cirrhosis; CMV, cytomegalovirus; HCV, hepatitis C virus; C2, 2 h after intake concentration; CsA, cyclosporin A; HLA, human leukocyte antigen; CNI, calcineurin inhibitors; mTORi, mTOR inhibitors; MFI, mean fluorescence intensity; NS, not significant.
*Low exposure was defined as a tacrolimus trough level <5 ng/mL, or cyclosporin A C2 level <500 ng/mL, or everolimus trough level <5 ng/mL at for least 1 week.

Management of patients experiencing AMR

All nine patients who experienced an acute AMR were treated by pulses of steroids (10 mg/kg/day for 3 days; n = 9), and one or several of the following treatments: plasmapheresis (five sessions; n = 6), rituximab (375 m/m²/week for 2 weeks; n = 8), polyclonal antibodies (Thymoglobulins®, 1.5 mg/kg/day for 5 days; n = 2), and intravenous immunoglobulins (1 g/kg/day for 2 days; n = 6) (Table 2). In addition, all patients but one were given an immunosuppressive regimen based on tacrolimus (target trough level: 5–8 ng/mL), mycophenolic acid (1 g/day), and low-dose steroids (5 mg/day). After a median follow-up of 36 (3–65) months after DSAs were first detected, they became undetectable in five of the nine treated patients.

Liver-enzyme levels returned to within normal ranges in six patients, remained stable in one patient, but severe cholestasis persisted in one patient without liver failure. One patient presented at 2 months after diagnosis of hepatorenal syndrome with liver failure: despite salvage therapy with polyclonal antibodies they had to undergo a second transplantation (Table 2, Fig. S1).

Of note, of the five of the 12 patients with a DSA that did not develop acute AMR (42%), DSAs became undetectable after a median follow-up of 7 (5–12) months after first detection. Four patients had anti-class II antibodies, and one patient had anti-class I antibodies. The mean MFI of the immunodominant DSA was 4100 ± 3500. None of the DSAs were C1q positive. The time since transplantation and the occurrence of *de novo* DSAs, the MFI of the immunodominant DSA, the sum of MFIs, and binding or not the C1q did not differ significantly between patients in whom DSAs became undetectable spontaneously or after specific therapy (data not shown).

Impact of *de novo* DSAs on patient- and graft-survival rates

Overall, during the follow-up period, 27 patients (17%) died. At last follow-up, the death rate did not differ significantly between patients who developed *de novo* DSAs and

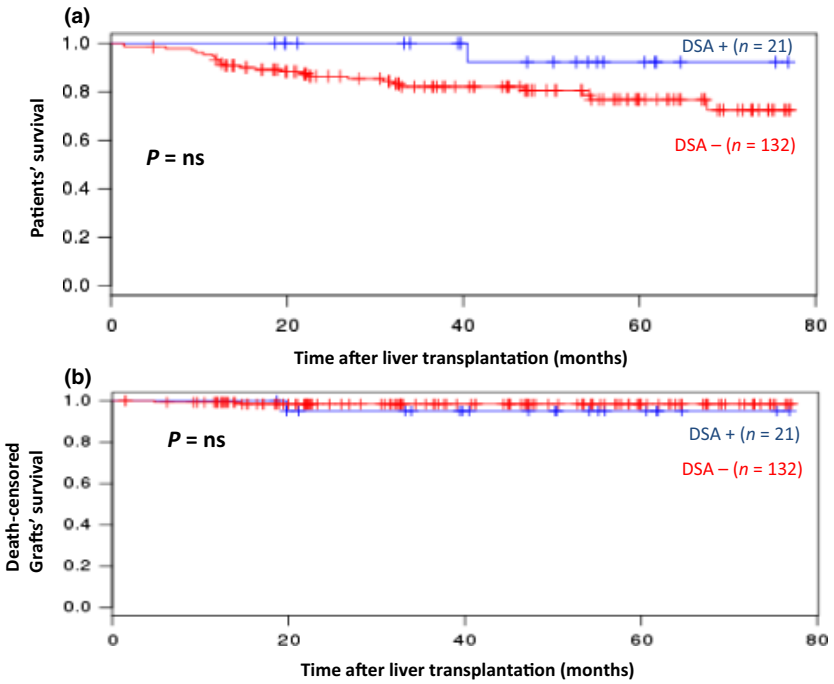

Figure 2 (a) Survival of patients according to the presence or not of donor-specific antibodies. (b) Death censored grafts' survival according to the presence or not of donor-specific antibodies. DSA, donor-specific antibodies; NS, not significant.

those who did not, that is, respectively, 1 of 21 (5%) and 26 of 131 (19.8%; $P = 0.12$) (Fig. 2a). In patients without DSAs, the deaths were related to infectious complications ($n = 8$), the recurrence of hepatocellular carcinoma or *de novo* cancer ($n = 11$), cardiovascular disease ($n = 4$), chronic rejection ($n = 1$), or death during retransplantation after hepatic artery thrombosis ($n = 2$). One patient with a DSA, but without a history of antibody-mediated rejection, died after recurrence of hepatocellular carcinoma.

At last follow-up, graft survival was similar between patients who developed DSAs (95.2%) and those who did not (98.5%) (Fig. 2b). Two patients without a DSA experienced a hepatic artery thrombosis that required retransplantation, and one patient with a *de novo* DSA, which caused an acute antibody-mediated rejection that did not reverse after specific therapy, developed hepatorenal syndrome and liver failure that required a retransplantation.

Discussion

After liver transplantation, several studies have shown that preformed DSAs and DSAs detected in maintenance liver-transplant patients are associated with an increased risk of acute rejection and reduced allograft survival [9,12,15,20–24]. To the best of our knowledge, only one study has assessed the incidence of DSAs in *de novo* liver-transplant patients. In the present study, we have studied the incidence and impact of *de novo* DSAs in a cohort of 152 *de novo* liver-transplant patients. Our findings are fivefold: (i) The incidence of DSAs was 14%; (ii) independent predictive factors for DSA formation were that they occurred in younger patients who had low exposure to immunosuppressants coupled with noncompliance; (iii) forty-three percent of patients with *de novo* DSAs experienced an AMR episode; (iv) no predictive factors for AMR were found; and (v) in all patients but two, AMR was successfully treated by plasmapheresis, and/or rituximab, and/or Intravenous immunoglobulins, and/or polyclonal antibodies.

In the present study, after a median follow-up of 34 (1.5–77) months, we observed an overall 14% incidence of DSAs. At 1-year post-transplantation, the incidence of DSAs was 9.3%. Although the cut-off MFI used for defining a DSA differed between the present study (MFI ≥1000) and the study by Kaneku el al. (MFI >5000) [15], the results from both studies are very similar. Kaneku *et al.* reported a 1-year incidence of *de novo* DSAs of 8.1% [15]. The majority of DSAs were directed against anti-class II antigens: 95% in their study [15] and 86% in our study. Nearly half of the patients (53%) developed one DSA in our study compared to 75% in their study [15]. As previously described [12,15], in the present study, the majority of *de novo* DSAs were directed against anti-DQ locus (52%). The mechanism of increased risk of anti-DQ DSAs is not well known.

However, our study is the first to look for C1q-binding *de novo* DSAs liver-transplant patients. Twenty-four percent of DSAs were bound with C1q. To note, five of the nine patients who developed antibody-mediated rejection were C1q negative. This observation is in line with recent reports documenting the existence of complement-fixing DSAs that are undetectable by standard C1q-binding assays [25,26]. Overall, the incidence of DSAs seems to be lower in liver-transplant patients compared to kidney-transplant patients [27,28]. This may be related to the ability of the liver to absorb anti-HLA antibodies, mainly anti-class I antibodies [27].

Quite similar risk factors for DSAs formation were observed in our study and the study by Kaneku *et al.* In this latter study, the use of cyclosporine A compared to tacrolimus was an independent predictive factor for *de novo* DSAs. In our study, patients treated by cyclosporine A were more likely to develop DSAs. However, this was not identified as an independent predictive factor for DSA formation. Conversely, in both studies, a younger age and under immunosuppression, that is, low exposure to calcineurin inhibitors or to mTOR inhibitors were independent predictive factors for DSAs. In addition, confirmed noncompliance was associated with the occurrence of DSAs. In maintenance liver-transplant patients, younger age has also been identified as a predictor for DSA formation [12]. These data suggest that adequate exposure to immunosuppressive drugs is required to avoid the development of DSAs, especially in younger recipients who are known to have a higher risk of noncompliance after both liver and kidney transplantation [12,28,29].

For the first time, we report the incidence of AMR in liver transplantation. It occurred in 6% of liver-transplant patients after a median follow-up of 22 (1–66) months post-transplantation, and in 43% of patients who developed a *de novo* DSA. All patients had increased liver-enzyme levels, histological features of AMR (as previously described by O'Leary *et al.*), and positive C4d staining. C1q-binding DSAs that were associated with increased kidney-allograft loss [30] were more likely to be associated with AMR ($P = 0.1$). However, the lack of statistical difference is probably related to the small number of patients with DSAs in our study. A larger sample is required to assess the impact of complement-binding DSAs on the development of AMR. Interestingly, some patients developed AMR in the presence of a DSA and a low MFI.

As previously reported for kidney- [31] and liver-transplant patients [32] presenting with AMR, the use of rituximab associated with the removal of anti-HLA antibodies by plasmapheresis and/or intravenous immunoglobulins was successfully used in seven of the nine patients with AMR. One patient had persistent cholestasis, and treatment failed in one patient who required retransplantation. At the last follow-up, five of the nine patients who presented with AMR had undetectable DSAs. Interestingly, among the 12 patients who developed DSAs without experiencing AMR, five had undetectable DSAs at the last follow-up without needing a specific therapy. This agrees with previous reports, which have shown that some liver-transplant patients with preformed DSAs or maintenance patients can become spontaneously cleared of DSAs. These data suggest that all liver-transplant patients who develop DSAs will not necessarily present with acute AMR. However, the long-term impact on liver histology is still unknown.

In contrast to Kaneku *et al.* [15], who found significantly decreased patient- and graft-survival rates in liver-transplant patients with DSAs, we did not observe a significant difference in the survival of patients either with or without DSAs. This could be related to the early diagnosis and treatment of AMR in our series. O'Leary *et al.* [23] have previously reported that early graft failure can be related to antibody-mediated rejection. Our data suggest that liver-transplant patients who present with liver dysfunction should be screened for DSAs, should undergo a liver biopsy that searches for AMR, and should receive early treatment if necessary.

Our study has several limitations. Firstly, we did not realize complete donor and recipient HLA-C and HLA DP typing. However, none of our patients who developed DSAs had anti-HLA antibodies directed against these two loci. Secondly, we did not analyze the DSA IgG subclasses. Kaneku *et al.* [33] have previously reported that DSAs IgG3 were more frequently observed in patients with chronic rejection and graft loss. Herein, we analyzed the capacity of DSAs to bind with C1q.

In conclusion, DSAs occurred in 14% of *de novo* liver-transplant patients, mainly in those with a low exposure to immunosuppressants. AMR occurred in 6% of cases. Plasmapheresis and immunosuppressive therapy that targeted B-cells was successfully used in this setting. Larger studies are required to confirm these data.

Authorship

ADB and NK: designed the study, analyzed the data, and wrote the paper. ADB: collected the data. NCJ: performed the immunological analysis. MD and CGF: performed the pathological work-up. FM, JPD and BS: did the liver transplantation. LL, LE, ICD, JG, GD, DM, LA, CB, CGF and LR: did the follow-up to patients. All authors revised the paper.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Outcome of the nine patients who developed antibody-mediated rejection. Abbreviations: ALT, alanine aminotransferase; GGT, gamma glutamyl transpeptidase; S, steroids; PE, plasma exchange; IvIg, intravenous immunoglobulin.

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