American Journal of Transplantation xxx (xxxx) xxx

Contents lists available at ScienceDirect

American Journal of Transplantation

journal homepage: www.amjtransplant.org

Original Article

Randomized trial investigating the utility of a liver tissue transcriptional biomarker in identifying adult liver transplant recipients not requiring maintenance immunosuppression

Julien Vionnet [1,2], Jorge Torres-Yaguana [1], Rosa Miquel [1,3], Juan G. Abraldes [4,5], Jurate Wall [1], Elisavet Kodela [1], Juan-Jose Lozano [6], Pablo Ruiz [7], Miguel Navasa [7], Aileen Marshall [8], Frederik Nevens [9], Will Gelson [10], Joanna Leithead [10], Steven Masson [11], Elmar Jaeckel [12], Richard Taubert [12], Phaedra Tachtatzis [13], Dennis Eurich [14], Kenneth J. Simpson [15], Eliano Bonaccorsi-Riani [16], James Ferguson [17], Alberto Quaglia [8], Anthony J. Demetris [18], Andrew J. Lesniak [18], Maria Elstad [19], Marc Delord [19], Abdel Douiri [19], Irene Rebollo-Mesa [19], Marc Martinez-Llordella [1,20], Juliete A.F. Silva [21,22], James F. Markmann [23], Alberto Sánchez-Fueyo [1,*]

[1] *Institute of Liver Studies, School of Immunology and Microbial Sciences, King's College London University and King's College Hospital, London, UK*

[2] *Transplantation Center, Service of Immunology and Allergy, and Servide of Gastroenterology and Hepatology, University Hospital of Lausanne, Lausanne, Switzerland*

[3] *Liver Histopathology Laboratory, King's College Hospital, London, UK*

[4] *Liver Unit, Division of Gastroenterology, University of Alberta, Edmonton, Canada*

[5] *Centre of Excellence for Gastrointestinal Inflammation and Immunity Research, University of Alberta, Edmonton, Canada*

[6] *Bioinformatic Platform, Biomedical Research Center in Hepatic and Digestive Diseases (CIBEREHD), Instituto de Salud Carlos III, Barcelona, Spain*

[7] *Hospital Clinic Barcelona, Instituto de Investigaciones Biomédicas August Pi Sunyer (IDIBAPS), University of Barcelona, Barcelona, Spain*

[8] *Department of Cellular Pathology, Royal Free London NHS Foundation Trust, London, UK*

[9] *Department of Hepatology, University Hospital Leuven, Leuven, Belgium*

[10] *Cambridge Liver Unit, Cambridge University Hospitals NHS Foundation Trust, Cambridge, UK*

[11] *Newcastle National Institute for Health and Care Research (NIHR) Biomedical Research Centre, Newcastle upon Tyne Hospitals NHS Foundation Trust, Translational and Clinical Research Institute, Faculty of Medical Sciences, Newcastle University, Newcastle upon Tyne, UK*

[12] *Department of Gastroenterology, Hepatology, Infectious Diseases and Endocrinology, Hannover Medical School, Hannover, Germany*

[13] *Leeds Teaching Hospitals National Health Service (NHS) Trust, Leeds, UK*

[14] *Charité, Berlin, Germany*

[15] *Edinburgh Royal Infirmary, Edinburgh, UK*

[16] *Liver Transplant Unit, Cliniques Universitaires St-Luc, Brussels, Belgium*

Abbreviations: ALT, alanine aminotransferase; BSL, baseline; CI, confidence interval; CM, central memory; CNI, calcineurin inhibitor; DSA, donor-specific antibody; EM, effector memory; GGT, γ-glutamyl transpeptidase; IFNγ, interferon gamma; IS, immunosuppression; LIFT, Liver Immunosuppression Free Trial; LT, liver transplantation; Non-TOL, nontolerant; PBMC, peripheral blood mononuclear cells; PPV, positive predictive value; TEMRA, terminally-differentiated effector memory; Th2, T helper-2; TOL, tolerant; Tregs, regulatory T cells.

* Corresponding author. King's College London University, Denmark Hill campus, James Black Centre 1st floor, London SE5 9NU, UK.
E-mail address: sanchez_fueyo@kcl.ac.uk (A. Sánchez-Fueyo).

https://doi.org/10.1016/j.ajt.2024.12.002
Received 1 October 2024; Received in revised form 23 November 2024; Accepted 3 December 2024
Available online xxxx

Please cite this article as: Vionnet J et al., Randomized trial investigating the utility of a liver tissue transcriptional biomarker in identifying adult liver transplant recipients not requiring maintenance immunosuppression, American Journal of Transplantation, https://doi.org/10.1016/j.ajt.2024.12.002

[17] Liver Unit, University Hospitals Birmingham NHS Foundation Trust and National Institute for Health and Social Care Research (NIHR) Birmingham Biomedical Research Centre (BRC), Centre for Liver and Gastrointestinal Research, University of Birmingham, Birmingham B15 2TT, UK
[18] Department of Pathology, Division of Transplantation, University of Pittsburgh, Pittsburgh, Pennsylvania, USA
[19] School of Population Health and Environmental Sciences, King's College London, London, UK
[20] Quell Therapeutics Ltd, London, UK
[21] Immune Tolerance Network, Seattle, Washington, USA
[22] Emory University, School of Medicine, Department of Surgery, Division of Transplantation, Atlanta, USA
[23] Penn Medicine, University of Pennsylvania, Philadelphia, Pennsylvania, USA

ARTICLE INFO

Keywords:
LT
tolerance
biomarker
gene-expression profiling
immunosuppression minimization
DSA

ABSTRACT

The maintenance of stable allograft status in the absence of immunosuppression (IS), known as operational tolerance, can be achieved in a small proportion of liver transplant recipients, but we lack reliable tools to predict its spontaneous development. We conducted a prospective, multicenter, biomarker-strategy design, IS withdrawal clinical trial to determine the utility of a predictive biomarker of operational tolerance. The biomarker test, originally identified in a patient cohort with high operational tolerance prevalence, consisted of a 5-gene transcriptional signature measured in liver tissue collected before initiating IS weaning. One hundred sixteen adult stable liver transplant recipients were randomized 1:1 to either arm A (IS withdrawal regardless of biomarker status) or arm B (IS withdrawal in biomarker-positive recipients). Immunosuppression withdrawal was initiated in 82 participants, rejection occurred in 54 (67.5%), and successful discontinuation of IS was achieved in 22 (27.5%), but only 13 (16.3%) met operational tolerance histologic criteria (10 in arm A; 3 in arm B). The biomarker test did not yield useful information in selecting patients able to successfully discontinue IS. Operational tolerance was associated with time posttransplant, recipient age, presence of circulating exhausted $CD8^+$ T cells, and a reduced number of immune synapses within the graft.

1. Introduction

The unique immunoregulatory and regenerative properties of the human liver allow the minimization of maintenance immunosuppression (IS) in a large proportion of liver transplantation (LT) recipients, and even the complete discontinuation of IS in a small, highly selected, group of patients.[1] The latter phenomenon, referred to as operational tolerance, has been the focus of several IS withdrawal clinical trials over the past 2 decades.[1-4] These studies have outlined the natural history of operational tolerance following LT and provided clinical and histologic parameters conducive to the success of IS withdrawal. However, the identification of accurate biomarkers of operational tolerance has remained elusive. In 2012, we reported the results of a multicenter European trial in which LT recipients underwent exhaustive molecular and cellular analyses of peripheral blood mononuclear cells (PBMC) and liver tissue samples prior to initiating IS withdrawal.[3,5] Approximately 40% of study participants successfully discontinued IS and were considered operationally tolerant (TOL). Although the cellular and molecular markers derived from PBMCs lacked reproducibility and could not reliably predict the outcome of IS withdrawal, the analysis of the liver tissue transcriptome was very accurate in discriminating recipients who eventually became TOL from those who developed rejection.[5-7] The transcriptional changes associated with operational tolerance corresponded to biological processes involved in the regulation of iron metabolism and specific inflammatory pathways (eg, interferon gamma [IFNγ], macrophage inhibitory factor [MIF]). Accordingly, TOL recipients exhibited higher hepcidin levels and increased iron accumulation as compared to patients who failed to discontinue IS (nontolerant [Non-TOL]), both in blood and liver tissue. To facilitate a diagnostic application, the microarray-based transcriptional results were translated into a real-time polymerase chain reaction–based 5-gene logistic regression model (*TFRC, PEBP1, MIF, CDHR2, SOCS1*). This test, which was derived from a cohort of 48 patients and validated in an independent group of 21 patients, was highly accurate at predicting the success of IS discontinuation, with an area under the curve of 0.85 (Supplementary Fig. S1).[5]

Here, we report on a prospective multicenter randomized controlled trial (RCT) conducted at 12 transplant centers across

Europe (Liver Immunosuppression Free Trial [LIFT]) to determine the clinical utility of the liver tissue–derived tolerance biomarker test in stratifying adult long-term LT recipients prior to IS withdrawal. Additional, complementary, mechanistic and biomarker data from a parallel, harmonized, multicenter trial conducted in the US (OPTIMAL trial, NCT02533180) are provided as well.[8]

2. Materials and methods

2.1. Trial design

LIFT (NCT024989977) was a prospective, multicenter, phase IV, biomarker-strategy trial with a randomized control group in which stable adult long-term LT recipients underwent IS withdrawal. Enrolled participants were randomized 1:1 to the following: (1) nonbiomarker-based IS weaning (arm A); or (2) biomarker-based IS weaning (arm B). In participants allocated to arm A, IS was progressively withdrawn regardless of the result of the tolerance biomarker ("weaning all" strategy). Among participants allocated to arm B, only those found to be biomarker-positive (arm B+) were offered IS withdrawal, whereas biomarker-negative participants (arm B–) remained on their baseline (BSL) maintenance IS throughout the trial (Fig. 1). The study was approved by the research ethics committees of each participating country (United Kingdom, Germany, Belgium, and Spain) and informed consent was obtained from all subjects. The trial protocol is submitted as Supplementary Material (trial registration: ISRCTN 47808000 and EudraCT 2014-004557-14).

2.2. Selection of participants

Local investigators from 12 European liver transplant centers screened all adult (aged ≥18 years) LT recipients who visited their outpatient clinics for potential enrollment during the inclusion period, which spanned from November 2015 to May 2019. The key eligibility criteria were as follows: >3 years posttransplant if aged >50 years or ≥6 years posttransplant if ≤50 years; stable allograft function; no history of autoimmunity or active viral disease; no recent episodes of rejection; absence of significant comorbidities; screening liver biopsy without significant abnormalities as per Banff criteria (Supplementary Table 1).[9,10] The detailed list of inclusion/exclusion criteria is provided as Supplementary Material.

2.3. Trial interventions, endpoints, sample size, statistical analysis, and experimental methods

The details are provided as Supplementary Material.

3. Results

3.1. Trial conduct and patient characteristics

Between November 2015 and May 2019, 2239 LT recipients were screened for eligibility, 567 were offered participation in the trial, 204 consented, 190 underwent the liver biopsy required at study entry, and 116 were randomized (58 patients being allocated to each of the arms A and B) (Fig. 1). Participants in arm A initiated gradual IS withdrawal. In arm B, 24 biomarker-positive patients (arm B+) were progressively weaned of IS, whereas 34 biomarker-negative patients had their IS maintained throughout the trial (arm B–). Demographic and clinical characteristics of the arm A and B study participants were comparable (Table 1). Patient recruitment was prematurely halted in May 2019 following an interim analysis that included the first 48 study participants and showed that the 95% confidence interval (CI) of the positive predictive value (PPV) of the biomarker test did not meet the criteria prespecified in the study protocol.

3.2. Study participant clinical course and outcomes

In arm A, 57 participants initiated IS weaning, 10 (17.5%) reached the primary endpoint (TOL), 43 (75.4%) were considered Non-TOL, and 1 did not undergo the 1-year post-IS withdrawal protocol biopsy (Fig. 2). Of note, among the participants

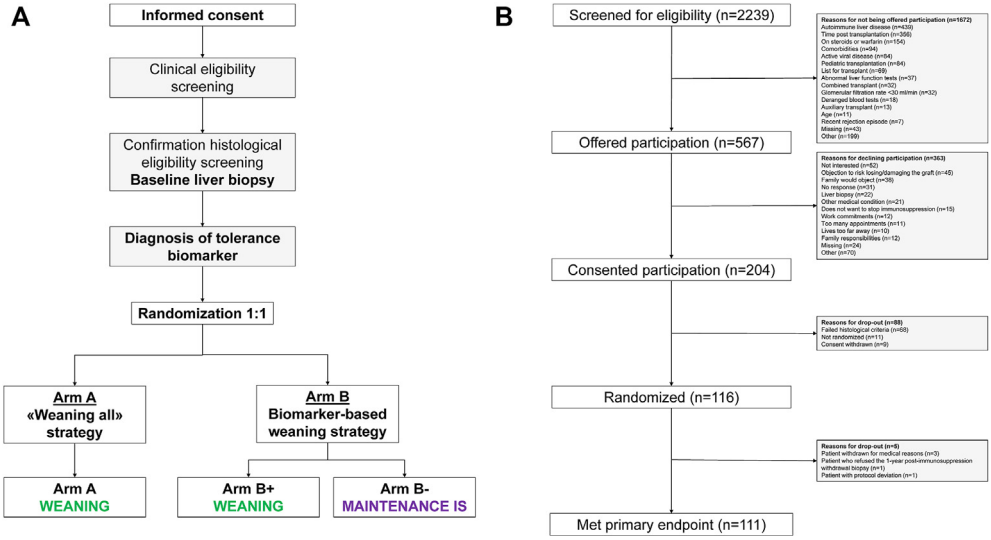


Figure 1. Liver Immunosuppression Free Trial study design (A) and Consolidated Standards of Reporting Trials (CONSORT) flow diagram (B). IS, immunosuppression.

Table 1
Characteristics of 116 randomized liver transplant recipients by trial allocation arm.

Characteristic			Nonbiomarker-based IS withdrawal (arm A, n = 58)	Biomarker-based IS withdrawal (arm B, n = 58)	Total (n = 116)	*P* value[a]
Recipient	Age (y)		60 (53-67)	62 (57-67)	61 (56-67)	.914
	Time since transplantation (y)		8 (6-13)	8 (5-11)	8 (6-11)	.331
	Male sex		39 (67%)	45 (78%)	84 (72%)	.299
	BMI (kg/m^2)		28 (24-30)	27 (24-31)	28 (24-31)	.757
	Race	Caucasian	56 (97%)	57 (98%)	113 (97%)	1.000
		Black	1 (2%)	0 (0%)	1 (1%)	
		Other	1 (2%)	1 (2%)	2 (2%)	
Liver allograft	Donor	Deceased	58 (100%)	57 (98.3%)	115 (99%)	1.000
	Type	Whole organ	52 (90%)	57 (98%)	109 (94%)	.114
	Primary transplant indication	Alcohol	17 (29%)	20 (35%)	37 (32%)	.890
		Hepatitis B	7 (12%)	2 (3%)	9 (8%)	
		Hepatitis C	11 (19%)	10 (17%)	21 (18%)	
		Hepatocellular carcinoma[b]	12 (21%)	12 (21%)	4 (3%)	
		MASLD	3 (5%)	2 (3%)	5 (4%)	
		Other[c]	18 (31%)	22 (38%)	40 (34%)	
At trial entry	Liver enzymes	ALT (U/L)	20 (15-26)	19 (15-25)	19 (15-25)	.697
		AST (U/L)	23 (20-27)	21 (19-24)	22 (19-25)	.154
		ALP (U/L)	76 (64-89)	78 (63-92)	77 (64-90)	.943
		GGT (U/L)	24 (16-38)	21 (16-31)	22 (16-34)	.260
		Total bilirubin (μmol/L)	6 (1-12)	7 (1-11)	7 (1-11)	.878
		Direct bilirubin (μmol/L)	2 (0.3-4)	2 (0.2-4)	2 (0.3-4)	.555
	IS	Combination containing CNI	26 (45%)	22 (38%)	48 (41%)	.572
		Tacrolimus monotherapy	16 (28%)	26 (45%)	42 (36%)	.082
		Tacrolimus trough level	3.9 (2.9-5.0)	4.5 (3.2-5.7)	4.1 (3.0-5.3)	.202
		Cyclosporine monotherapy	0 (0%)	2 (3%)	2 (2%)	.496

(*continued on next page*)

Table 1 (*continued*)

Characteristic			Nonbiomarker-based IS withdrawal (arm A, n = 58)	Biomarker-based IS withdrawal (arm B, n = 58)	Total (n = 116)	*P* value[a]
		Mycophenolate/ azathioprine monotherapy	2 (3%)	1 (2%)	3 (3%)	1.000
		mTOR inhibitors monotherapy	0 (0%)	1 (2%)	1 (1%)	1.000
	Iron parameters	Serum ferritin (μg/L)	123 (44-237)	110 (49-239)	120 (49-239)	.778
		Hepatocytic iron[11]	0 (0-6)	0 (0-6)	0 (0-6)	.561
		Mesenchymal iron[11]	0 (0-1)	0 (0-1)	0 (0-1)	.652
		Total iron score[11]	0 (0-7)	0 (0-6)	0 (0-7)	.912

Continuous variables are summarized using median and interquartile range. Categorical variables are summarized by counts and percentages.
ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CNI, calcineurin inhibitor (tacrolimus or cyclosporine); GGT, γ-glutamyl transpeptidase; IS, immunosuppression; MASLD, metabolic dysfunction–associated steatotic liver disease; mTOR, mammalian target of rapamycin.

[a] *P* values refer to the nonparametric Mann-Whitney *U* test for continuous variables and the Fisher exact test for categorical variables.

[b] Hepatocellular carcinoma cases are counted whenever present, also as a secondary or tertiary indication.

[c] Others: α1-antitrypsin deficiency (n = 3), hemochromatosis (n = 4), Wilson disease (n = 1), drug-induced liver injury (n = 4), polycystic liver disease (n = 3), Budd-Chiari syndrome and other vascular liver diseases (n = 1), cryptogenic (n = 5), other metabolic liver disorders (n = 3), undefined (n = 16).

who initiated IS weaning, the primary endpoint could not be assessed in 4 (1 did not undergo the 1-year post-IS withdrawal biopsy, 3 were withdrawn from the study before IS weaning was completed due to comorbidities [cholangiopathy, lung cancer, lymphoma, respectively]; Fig. 2). Among Non-TOL participants, 37 developed clinically apparent acute rejection during IS weaning and 6 were successfully weaned but did not meet the histologic criteria for tolerance at the 1-year post-IS withdrawal biopsy (2/6 reinstituted IS, 4/6 were kept off IS) (Supplementary Table 2).

In arm B, 23 participants initiated IS weaning (arm B+) and 34 were maintained on IS (arm B–) (Fig. 2). Three of 23 (13.0%) were considered TOL and 19 (82.6%) Non-TOL. Seventeen Non-TOL participants underwent an acute rejection episode whereas 2 discontinued IS but did not meet the tolerance histologic criteria (no IS reinstitution was necessary). One patient refused the 1-year protocol biopsy after successfully discontinuing IS. None of the 34 patients kept on maintenance IS developed rejection.

Altogether, out of the 80 participants included in the intention-to-treat analysis who initiated IS withdrawal, 23

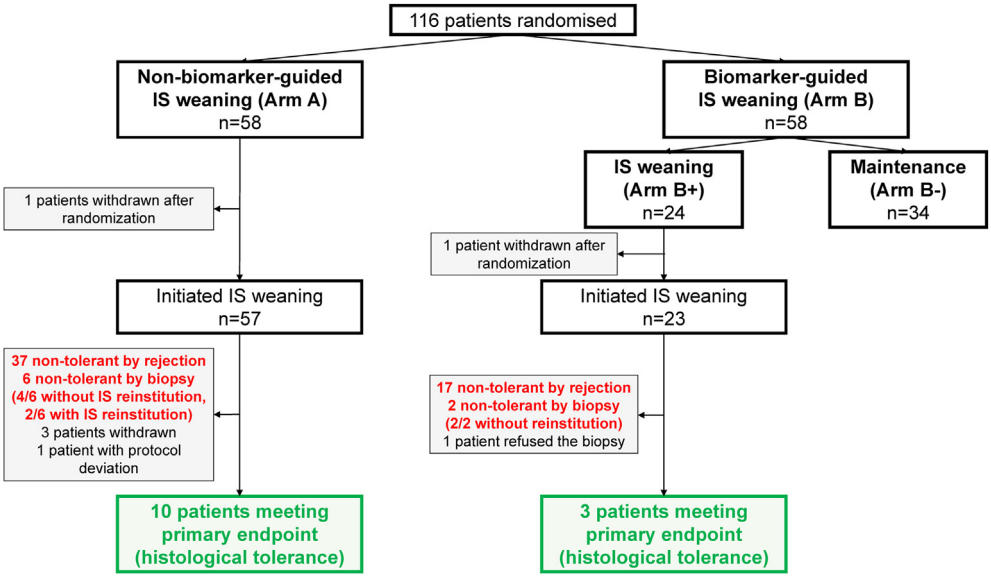


Figure 2. Clinical outcomes of Liver Immunosuppression Free Trial study participants according to the study allocation arm. IS, immunosuppression.

(28.8%) discontinued IS for >1 year, 21 (26.3%) underwent the 1-year post-IS withdrawal protocol biopsy, but only 13 (16.3%) met the histologic criteria for operational tolerance at 1-year postwithdrawal and were classified as TOL (Fig. 2). Among the 8 (10%) participants who successfully discontinued IS but were considered Non-TOL based on the 1-year histologic findings, 2 reinitiated IS and 6 developed mild fibro-inflammatory lesions that did not require IS reinstitution. On reassessment 2 years after IS discontinuation, 2 of the 6 participants were considered TOL as per histologic criteria, 2 did not meet the histologic criteria of operational tolerance but remained off IS, and 2 did not meet histologic criteria and had to restart IS (Supplementary Table 2).

Table 2 shows the BSL characteristics of the 75 patients who completed the study protocol. TOL patients were significantly older than Non-TOL (67.0 [59.0-70.0] vs 59.5 [52.0-63.8] years, respectively; $P = .021$) and had a longer time period between LT and the start of IS withdrawal (13.3 [9.8-14.0] vs 7.4 [5.7-11.2] years, respectively; $P = .007$). TOL patients were also more likely than Non-TOL participants to be on tacrolimus monotherapy at trial entry (7 [53.8%] and 15 [24.2%], respectively; $P = .046$). The cumulative probability of IS weaning failure over time in the per protocol population is displayed in Figure 3.

No differences in liver test levels between TOL and maintenance IS participants were detected over the course of the study (Supplementary Material and Supplementary Table 3).

3.3. Severity and outcome of rejection episodes occurring during the study period

Fifty-four (67.5%) patients developed acute cellular rejection during (n = 49, 74.1%) or after (n = 14, 25.9%) IS withdrawal, 37 of which were biopsy-proven, and 5 were classified as histologically severe. Rejection episodes occurred at a median time of 168 (116-258) days after the start of IS withdrawal (68.1% when IS doses were at ≤25% of the entry dose). Median liver function tests at the time of rejection were as follows: aspartate aminotransferase 68 (52-118) IU/L, alanine aminotransferase (ALT) 116 (85-242) IU/L, alkaline phosphatase 113 (89-185) U/L, γ-glutamyl transpeptidase (GGT) 82 (55-235) U/L, and total bilirubin 9 (5-13) μmol/L.

Corticosteroids (prednisolone or equivalent) were used to treat rejection in 34 cases, with a median dose of 20 (20-50) mg and a median treatment duration of 55 (30-94) days. Two patients were still taking low-dose corticosteroids at the end of the trial. No corticosteroid-resistant rejection episodes were reported.

Biochemical resolution, defined as ALT and GGT < 1.5 times the BSL occurred in 69.0% of patients at a median time of 135 (85-434) days after diagnosis. ALT and GGT <50 IU/L were achieved in 78.6% of patients at a median time of 126 (52-336) days. At the end of the trial, patients who underwent rejection were receiving similar IS regimens to those at BSL (55.6% on monotherapy IS, 43.4% on 2-agent IS).

3.4. The liver tissue–derived transcriptional biomarker of operational tolerance was not useful in predicting the outcome of IS withdrawal

No association was found between arm A and arm B allocation and the likelihood of operational tolerance (odds ratio, 0.68; 95% CI, 0.11-3.07; $P = .74$). The diagnostic performance metrics of the biomarker included: sensitivity 54%, specificity 42%, PPV 16%, and negative predictive value 81%, with an accuracy of 44%. The standard error of the PPV was 9.1% and the 95% CI for PPV was 15.6% to 51.0%.

We performed an exploratory post hoc analysis of the biomarker performance in arm A only, by plotting the probability of operational tolerance as determined by the biomarker test (continuous variable from 0 to 1) against the observed rate of operational tolerance. This confirmed that the test failed to adequately predict operational tolerance (Supplementary Fig. S2A).

Given that time after transplant and patient's age at the time of study entry were both significantly associated with tolerance (Table 2), we used logistic regression to investigate if the biomarker provided additional predictive information at different levels of these 2 parameters (Supplementary Fig. S2B, C). Again, no additional value of the biomarker was found (Supplementary Table 4). Of note, time after transplant, previously employed as a tool to stratify patients into 3 groups with a low, medium, and high probability of tolerance, respectively,[3] was also found to be useful in LIFT, particularly when considering patients who successfully discontinued IS regardless of the histologic outcome (Supplementary Fig. S2D, E).

We also investigated blood and intrahepatic iron markers, previously reported to be higher in TOL than in Non-TOL recipients,[5] but found no association with the outcome of IS withdrawal (Tables 1 and 2, Supplementary Material, and Supplementary Fig. S3A-D).

3.5. Development of de novo donor-specific antibodies was associated with failure to successfully discontinue IS

Sequential serum anti-Human Leukocyte Antigen (HLA) antibodies were available from 65 participants initiating IS withdrawal (Fig. 4). Sixteen of 65 patients had positive anti–class II donor-specific antibodies (DSA) at study entry (15/16 anti-HLA-DQ); 1/16 also exhibited anti–class I DSA. Their cumulative mean fluorescence intensity (MFI) either remained stable (3/16) or decreased (13/16) throughout the duration of the trial. Their outcomes were rejection in 8 and successful IS withdrawal in 8 (out of whom 4 reached operational tolerance at 1 year). Fourteen of the 49 patients with negative DSA at study entry subsequently developed de novo DSA (all of them anti–class II). None of the patients developing de novo DSA during the study period reached operational tolerance (11 experienced clinical rejection and 4 displayed worsened inflammatory changes in the 1-year protocol biopsy

Table 2
Characteristics of 75 subjects undergoing IS withdrawal and completing primary endpoint by tolerance status (per protocol).

Characteristic			Tolerant (n = 13)	Nontolerant (n = 62)	Total (n = 75)	*P* value[a]
Recipient	Age (y)		67 (59-70)	60 (52-64)	60 (53-66)	.021
	Time since transplantation (y)		13 (10-14)	7 (6-11)	8 (6-13)	.007
	Male sex		11 (85%)	39 (63%)	50 (67%)	.198
	BMI (kg/m^2)		28 (24-32)	27 (24-30)	27 (24-31)	.378
	Race	Caucasian	12 (92%)	61 (98%)	73 (97%)	.319
		Black	0 (0%)	1 (2%)	1 (1%)	
		Other	1 (8%)	0 (0%)	1 (1%)	
Liver allograft	Donor	Deceased	13 (100%)	62 (100%)	75 (100%)	1.000
	Type	Whole organ	11 (85%)	59 (95%)	70 (93%)	.205
	Primary transplant indication	Alcohol	3 (23%)	22 (36%)	25 (33%)	.028
		Hepatitis B	5 (39%)	2 (3%)	7 (9%)	
		Hepatitis C	2 (15%)	10 (16%)	12 (16%)	
		Hepatocellular carcinoma[b]	3 (23%)	11 (18%)	14 (19%)	
		MASLD	1 (8%)	3 (5%)	4 (5%)	
		Other[c]	1 (8%)	25 (40%)	26 (35%)	
At trial entry	Liver enzymes	ALT (U/L)	22 (16-33)	20 (15-25)	20 (15-26)	.378
		AST (U/L)	24 (24-28)	22 (20-26)	23 (20-26)	.040
		ALP (U/L)	75 (67-77)	76 (64-88)	75 (64-88)	.604
		GGT (U/L)	34 (19-49)	23 (17-32)	23 (17-34)	.153
		Total bilirubin (μmol/L)	4 (1-9)	6 (1-12)	6 (1-12)	.811
		Direct bilirubin (μmol/L)	0.4 (0.3-2.0)	2.0 (0.2-4.0)	2.0 (0.3-4.0)	.676
	IS	Combination containing CNI	5 (39%)	28 (45%)	33 (44%)	.764
		Tacrolimus monotherapy	7 (54%)	15 (24%)	22 (29%)	.046
		Tacrolimus trough level	2.3 (1.8-4.8)	3.9 (3.4-5.3)	3.9 (3.0-5.2)	.026
		Cyclosporine monotherapy	0 (0%)	0 (0%)	0 (0%)	1.000
		Mycophenolate/azathioprine monotherapy	1 (8%)	1 (2%)	2 (3%)	.319
		mTOR inhibitors monotherapy	0 (0%)	0 (0%)	0 (0%)	1.000
	Iron parameters	Serum ferritin (μg/L)	179 (56-298)	121 (49-243)	124 (51-251)	.459
		Hepatocytic iron[11]	0 (0-6)	0 (0-6)	0 (0-6)	.985
		Mesenchymal iron[11]	0 (0-1)	0 (0-1)	0 (0-1)	.443
		Total iron score[11]	2 (0-9)	1 (0-7)	1 (0-7)	.857

Continuous variables are summarized using median and interquartile range. Categorical variables are summarized by counts and percentages.
ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CNI, calcineurin inhibitor (tacrolimus or cyclosporine); GGT, γ-glutamyl transpeptidase; IS, immunosuppression; MASLD, metabolic dysfunction–associated steatotic liver disease; mTOR, mammalian target of rapamycin.

[a] *P* values refer to the nonparametric Mann-Whitney test for continuous variables and the Fisher exact test for categorical variables.

[b] Hepatocellular carcinoma cases are counted whenever present, also as a secondary or tertiary indication.

[c] Others: α1-antitrypsin deficiency (n = 1), hemochromatosis (n = 3), Wilson disease (n = 1), drug-induced liver injury (n = 3), polycystic liver disease (n = 2), Budd-Chiari syndrome and other vascular liver diseases (n = 1), cryptogenic (n = 4), other metabolic liver disorders (n = 1), undefined (n = 11).

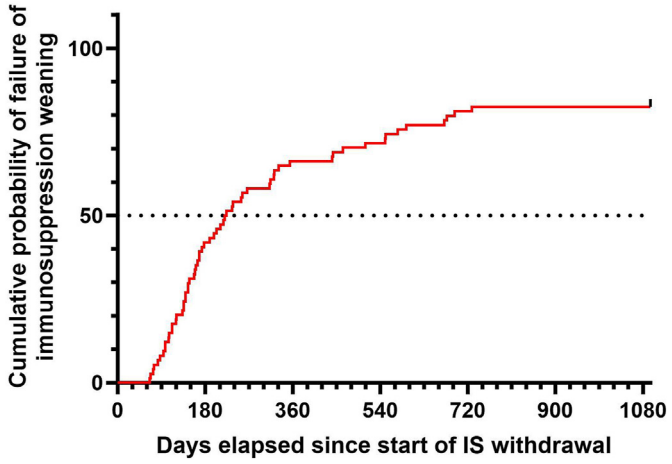


Figure 3. Cumulative probability of immunosuppression discontinuation failure over time. IS, immunosuppression.

that required IS reinstitution in 2). Finally, 35/65 remained DSA-negative throughout the duration of the study (7 reached operational tolerance and 28 developed clinical rejection or developed worsened liver inflammation). Altogether, although the presence or absence of DSA at study entry had no predictive value, the development of de novo DSA was strongly associated with the failure of IS withdrawal (Fisher exact test, $P = .037$).

3.6. Changes in circulating lymphocyte subpopulations over time

We conducted spectral flow cytometry experiments on cryopreserved PBMC specimens from 22 study participants who underwent IS weaning (10 TOL and 12 Non-TOL). PBMCs were sequentially collected at BSL, 6 months after initiating weaning, and 12 months after discontinuing IS or after the episode of rejection (Supplementary Fig. S4).

We did not observe significant differences over time in the kinetics of regulatory T cells (Tregs) ($CD25^+FOXP3^+$ Tregs), central memory (CM), effector memory (EM), and terminally-differentiated effector memory (TEMRA) $CD4^+$ or $CD8^+$ T cell subsets identified by manual Boolean gating (data not shown). The use of unsupervised hierarchical clustering of $CD4^+$ T cells revealed that Non-TOL recipients displayed significantly higher BSL percentages of CM, CD25-expressing CM T follicular helper cells, CM T helper-1 cells, activated Tregs, and senescent TEMRA ($TEMRA_{senes}$) as compared to TOL. In contrast, naive, exhausted EM $PD1^+CD57^+$ T helper-2 (EM $Th2_{exh}$) cells, and exhausted TEMRA $PD1^+CD57^+$ ($TEMRA_{exh}$) subpopulations were higher in the BSL samples of TOL patients (Fig. 5A-C). The unsupervised analyses of $CD8^+$ T cells (Fig. 5D-F) showed that, at BSL, Non-TOL recipients exhibited higher percentages of senescent EM (EMsenes), $TEMRA_{senes}$, and $Helios^+$ $TEMRA_{senes}$, whereas TOL patients exhibited higher frequencies of $PD\text{-}1^+CD57^+HELIOS^+$ EM_{exh}, TEMRA, and $PD\text{-}1^+CD57^+$ $TEMRA_{exh}$. Taken together, these results would suggest that TOL recipients exhibit an aged immune system.[12] Of note, recipient age was positively correlated with the proportion of $TEMRA_{exh}$ subpopulations and negatively correlated with the proportion of $TEMRA_{senes}$ populations (Fig. 5G). In contrast, no significant correlations with time after transplantation were observed (data not shown). Within the CD19+ B cell compartment, the unsupervised analysis revealed that Non-TOL recipients exhibited a significant increase of T1 cells post-IS withdrawal, whereas TOL where characterized by a reduction in antibody-secreting cells (Supplementary Fig. S5).

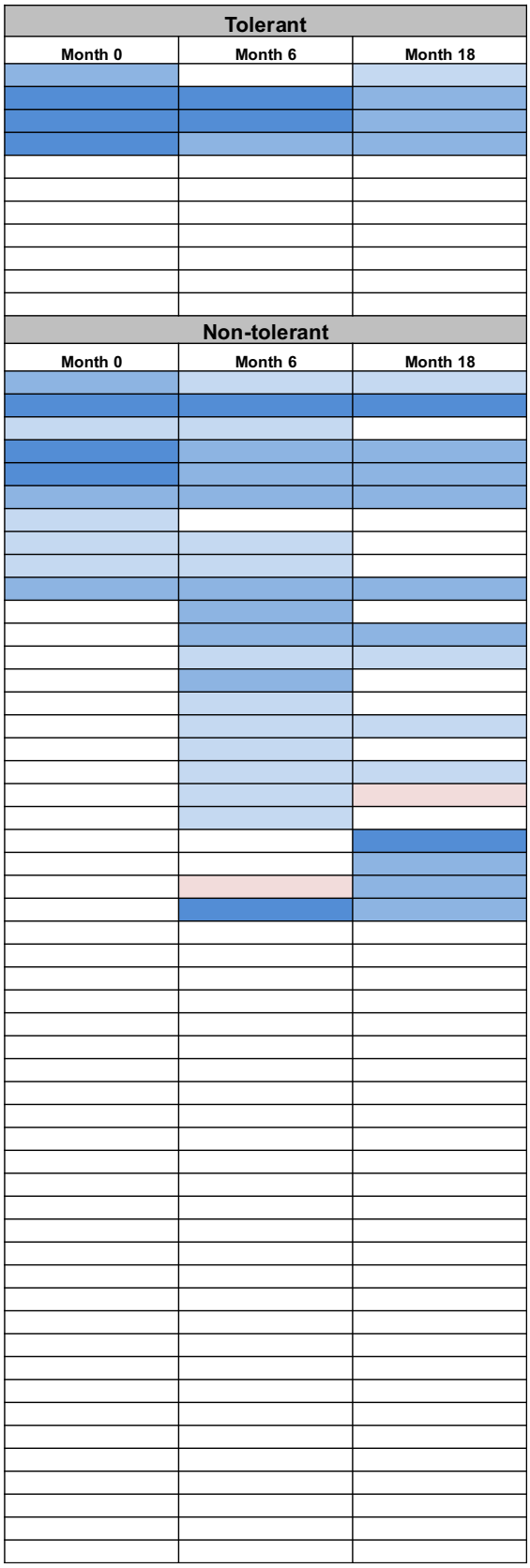
3.7. Transcriptional profiling of liver tissue specimens identifies an association between interferon-regulated pathways and operational tolerance

The integrated analysis of the LIFT and OPTIMAL RNASeq data sets revealed that 16 genes were differentially expressed with a false discovery rate $<10\%$ when comparing the BSL liver biopsies from TOL and Non-TOL recipients. Among them, there was a clear overrepresentation of interferon-stimulated genes (eg, *ISG15, ISG20, OASL, OAS1, IFI6, IFITM1, IFI35*), all of them overexpresssed in the TOL samples (Fig. 6A and Supplementary Table 5). Accordingly, the top 4 hallmark pathways significantly enriched in the TOL group corresponded to interferon-regulated pathways (Supplementary Table 5). In contrast, the transcriptome of Non-TOL samples was mostly enriched in metabolic pathways (Supplementary Table 5). Of note, a transcriptional pathway previously identified as being highly associated with T cell–mediated rejection in LT[13,14] was not significantly enriched in the liver tissue transcriptome of TOL patients (Supplementary Table 5). On the other hand, among the genes preferentially expressed in the TOL samples, we did not identify overrepresentation of iron-related pathways, nor the differential expression of genes previously associated with successful IS withdrawal.[5]

We next investigated the transcriptional changes associated with the discontinuation of IS by analyzing the sequentially collected protocol biopsies from LIFT and OPTIMAL (BSL and 12 months after IS discontinuation). In the follow-up samples obtained from TOL patients, we observed further enrichment in the interferon alpha pathway, with underrepresentation of pathways linked to Fc receptors, innate immunity, and complement system (Supplementary Table 6). In contrast, the comparison between BSL and follow-up samples collected from Non-TOL participants showed a marked enrichment in rejection-associated transcriptional pathways (Supplementary Table 7). In short, our results failed to confirm the association between iron metabolism and operational tolerance, in keeping with the poor predictive value showed by the polymerase chain reaction–based biomarker test. In contrast, we identified a link between interferon responses and operational tolerance.

3.8. Quantification of intragraft immunologic synapses

We conducted multiplex immunohistochemistry experiments on paraffin-embedded slides from TOL and Non-TOL specimens collected at BSL for automated image detection of immune



(caption on next column)

synapses, as previously described.[15] We plotted in 3D the number of lobular $CD8^+$ cells/mm^2 (T effector cells; x-axis), $MAC387^+$ cells/mm^2 (calprotectin/infiltrating macrophages; y-axis), and lobular $CD45^{high}$/Major Histocompatibility Complex (MHC)-II^+ pairs/mm^2 (leukocyte/antigen-presenting cell pairs; z-axis). We identified a cube (inner shaded cube; Fig. 6B) that contained 6/7 (86%) of TOL samples and 13/32 (41%) of Non--TOL samples.

4. Discussion

Removing life-long IS would transform long-term outcomes after LT but cannot be applied to clinical practice due to the lack of accurate predictive biomarkers and uncertainties regarding the true prevalence of operational tolerance when using stringent histologic criteria. We conducted a multinational prospective RCT using a biomarker-based strategy design to validate a predictive transcriptional signature of operational tolerance in stable adult LT recipients. The biomarker test failed to adequately discriminate between patients who developed rejection and those who tolerated IS discontinuation and/or achieved operational tolerance, and did not demonstrate clinical utility.

It is well recognized that only a very tiny fraction of published biomarkers eventually enter clinical practice. The laboratory test, target population, and eligibility criteria employed in LIFT were derived from a previous multicenter IS withdrawal trial in which the biomarker test proved to be highly accurate.[3,5] However, the 2 studies exhibited severaldifferences, which might have compromised the biomarker performance and could explain the negative results of LIFT. First, as a result of the Banff consensus guidelines introduced in 2012,[10] the 2 studies employed different histologic thresholds to determine participant eligibility and to adjudicate operational tolerance (both of which were more stringent in LIFT). Consequently, the liver biopsies employed in LIFT to assess the biomarker status had a lower inflammatory burden than those used in the original discovery study. Second, despite following almost identical clinical inclusion/exclusion criteria, the 2 cohorts differed in the proportion of participants with a history of resolved hepatitis C virus (HCV) infection, and in the type of immunosuppression at study entry (21% of the overall participants and 36% of those to be found to be TOL in the original study received no calcineurin inhibitors, compared to 3% and 8% in LIFT, respectively).[5]

Figure 4. Sequential changes in circulating anti-HLA class II donor-specific antibody profile in 63 patients who completed immunosuppression (IS) withdrawal. Heatmap including 65 participants with sequential serum anti-Human Leukocyte Antigen (HLA) antibodies measurements at month 0 (ie, before the start of IS withdrawal), month 6, and month 18 after the start of IS withdrawal. Each row corresponds to a study. The color scale displays donor-specific antibodies cumulative mean fluorescence intensity (MFI) as follows: 1000 $\leq$MFI $<$10 000 (light blue); 10 000 $\leq$MFI $<$20 000 (blue); MFI $\geq$20 000 (dark blue).

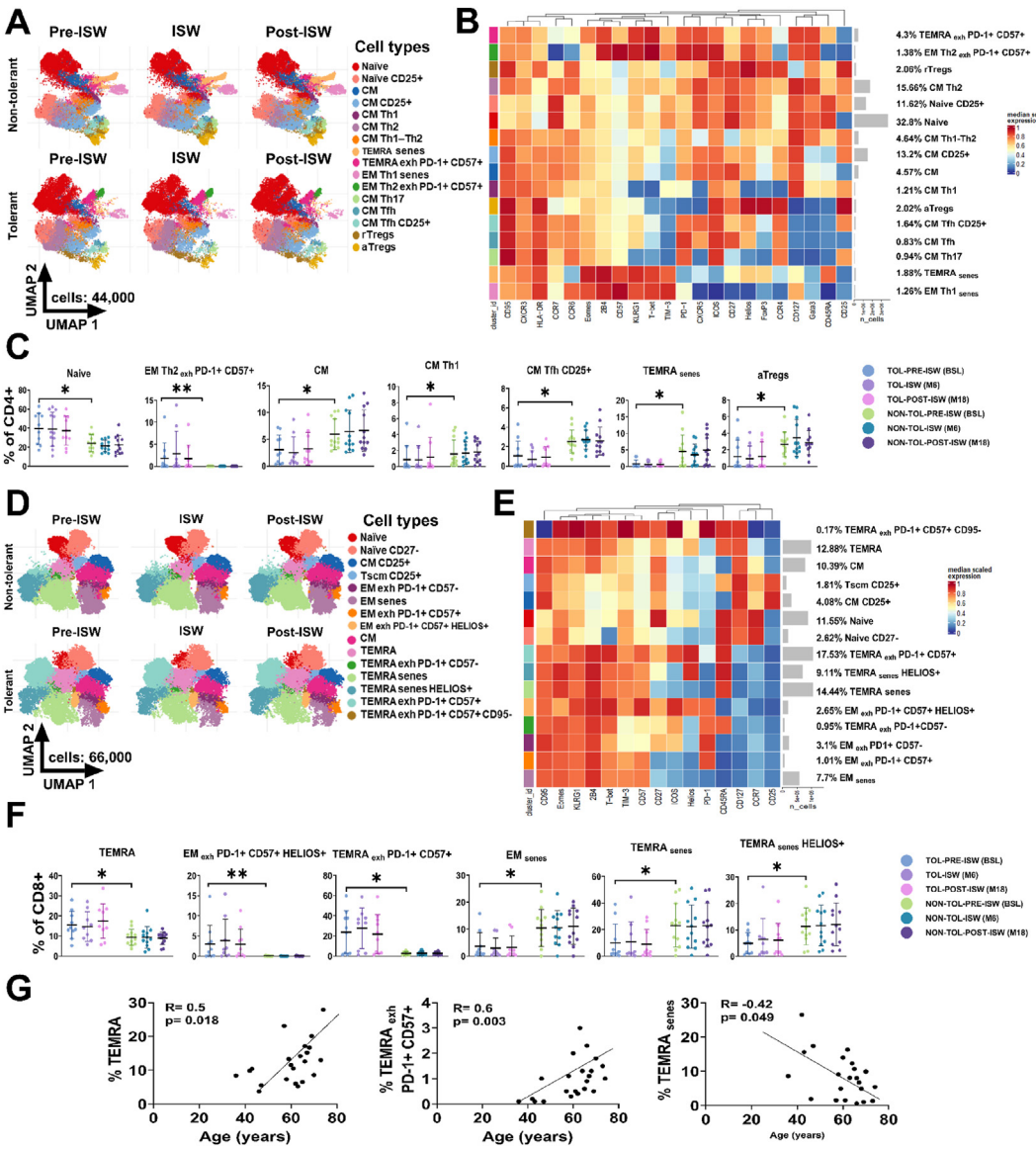


Figure 5. Unsupervised analysis of the spectral flow cytometry experiments corresponding to circulating CD4+ and CD8+ T cells from tolerant (TOL) and nontolerant (Non-TOL) study participants. (A) Uniform manifold approximation and projection (UMAP) plot depicting the 2D spatial distribution of 44 000 cells from TOL and Non-TOL recipients stratified by time point and embedded with FlowSOM clusters. (B) Heatmap showing the median intensity of 22 fluorescent markers across the 16 cell populations identified by FlowSOM after manual metacluster merging. The colors identifying each of the rows in the heatmap correspond to the same colors used to identify the clusters in the corresponding UMAP plots. The color scale used in the heatmap displays the median arcsinh of the marker expression (0-1 scaled) for all samples. The gray bar indicates the relative frequency (%) of each cluster. (C) Scatter plots of the frequency of specific CD4+ subsets found to be statistically different between the baseline (BSL) samples of TOL and Non-TOL patients. (D) UMAP of 66 000 CD8+ T cells from TOL and Non-TOL recipients stratified by time point and embedded with FlowSOM clusters. (E) Heatmap showing the median intensity of 15 fluorescent markers across the 15 cell populations identified by FlowSOM after manual metacluster merging. The colors identifying each of the rows in the heatmap correspond to the same colors used to identify the clusters in the corresponding UMAP plots. The color scale used in the heatmap cells displays the median arcsinh of the marker expression (0-1 scaled) for all samples. The gray bar indicates the relative frequency (%) of each cluster. (F) Scatter plots of the frequency of specific CD8+ subsets found to be statistically different between the BSL samples of TOL and Non-TOL patients. (G) Spearman correlation between the frequency of specific CD8+ T cell subpopulations and recipient age. n = 22 (10 TOL and 12 Non-TOL); $*P < .05$; $**P < .01$; C and F comparisons conducted using unpaired Mann-Whitney test. No statistically significant changes between BSL and 6 months after initiating immunosuppression weaning (M6) and M8 samples were observed. aTregs, activated regulatory T cells; CM, central memory; EM, effector memory; ISW, immunosuppression withdrawal; M6/M18, month 6/18 after initiating immunosuppression withdrawal; TEMRA, terminally-differentiated effector memory; Tfh, T follicular helper; Th1, T helper-1; Th2, T helper-2; Tregs, regulatory T cells.

These differences in IS regimens might have introduced a selection bias (ie, patients in whom CNI discontinuation resulted in rejection would not have been selected to participate in the trial). The type of IS might have also directly influenced the expression levels of the genes included in the test (although this was specifically investigated in the original study, the small sample size precludes reaching firm conclusions). Likewise, the use of CNIs could have negatively impacted some of the immunoregulatory networks promoting tolerance (eg, Treg homeostasis).[16] Of note, the exact same biomarker test used in LIFT exhibited a high discriminative accuracy when applied to patients who were receiving sirolimus monotherapy at the time of initiating IS withdrawal.[17] Third, the prevalence of operational tolerance in the 2 study cohorts was drastically different (44% vs 16%), which could have resulted in a miscalibration of the predictive model. On the other hand, given the strong association originally reported between the 5-gene signature and specific molecular pathways not detected in LIFT (eg, iron metabolism),[5] the poor accuracy of the test is unlikely to be attributable to technical/analytical inadequacies.

In contrast to previous studies, the LIFT study protocol stipulated for patients who discontinued IS but developed mild

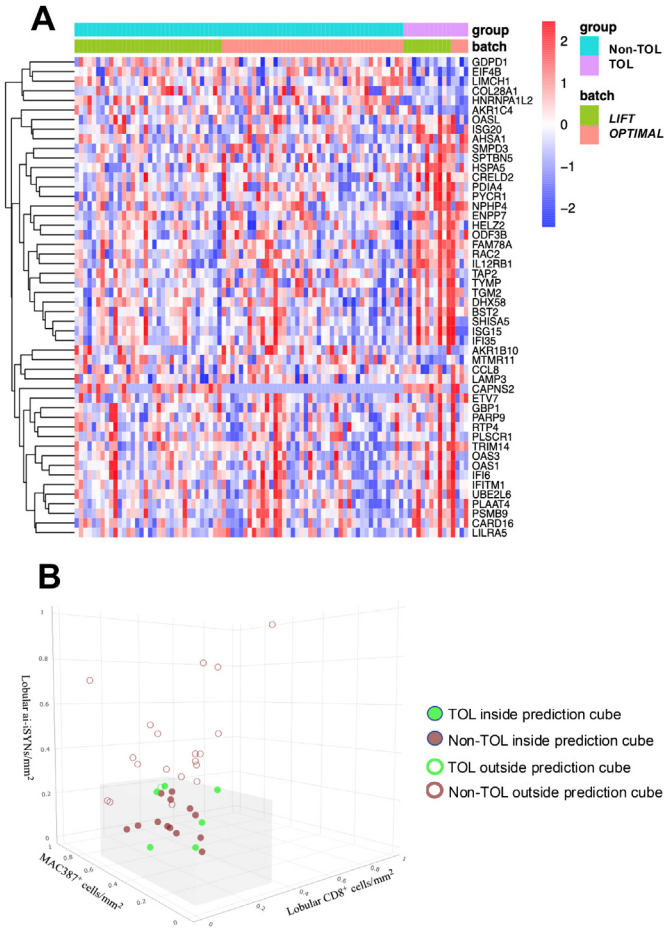


Figure 6. Transcriptional and immunohistochemistry differences of baseline liver biopsies from tolerant (TOL) and nontolerant (Non-TOL) study participants. (A) Integrated analysis of RNASeq data from liver tissue samples collected at baseline in study participants from the Liver Immunosuppression Free Trial (LIFT) and OPTIMAL trials stratified as TOL and Non-TOL. The heatmap displays the top 50 differentially expressed genes. Rows represent genes and columns represent samples. The intensity of each color denotes the standardized ratio between each value and the average expression of each gene across all samples. Red pixels correspond to an increased abundance, whereas blue pixels indicate decreased transcript levels. (B) Multiplex immunohistochemistry experiments on paraffin-embedded slides from TOL and Non-TOL specimens from LIFT study participants collected at baseline for automated image detection of immune synapses (ai-iSYNs). The slides were queried with multiplex immunohistochemistry for CD45/Major Histocompatibility Complex (MHC)II/CD34/CD8/4′,6-diamidino-2-phenylindole (DAPI) and were subjected to unbiased digital analysis. Lobular CD8+ cells/mm^2 alone did not distinguish between TOL and Non-TOL patients. Detection of ai-iSYNs/mm^2 (defined in Supplementary Methods as CD45+/MHC-II any paired with MHC-II only plus nuclear morphology and distance relationships) was significantly increased in Non-TOL patients over TOL patients (*$P = .016$; Welch 2 sample t-test). Combining lobular CD8+ cells/mm^2 (x-axis) with MAC387+ cells/mm^2 (y-axis) and lobular CD45+^{high} containing ai-iSYNs/mm^2 (z-axis) allowed the formulation of a prediction cube for this subset of patients. The gray shaded box maximizes the number of patients who will ultimately become operationally TOL (6 of 7 TOL patients within the box) although minimizing the number of patients who will fail immunosuppression withdrawal (13 of 29 Non-TOL patients within the box). Ranges for the inner cube correspond to: (1) lobular CD8+: 0 to 11.6 cells/mm^2; (2) MAC387+: 0 to 31.7 cells/mm^2; (3) lobular CD45+^{high} ai-iSYNs: 0 to 2.85 pairs/mm^2. Subjects within the inner cube are indicated by closed symbols; subjects outside the inner cube are indicated by open symbols.

histologic abnormalities in the 1-year post-IS withdrawal protocol to be kept off IS. This allowed us to confirm that operational tolerance is occasionally associated with transient, spontaneously-resolving, histologic changes. This is consistent with 2 previous reports describing the long-term histologic and molecular changes of LT off IS.[3,18] This observation, which has important clinical implications, indicates that the development of nonclinically apparent worsened histologic changes following IS discontinuation does not always require the reinstitution of IS, provided patients are carefully monitored.

In addition to the methodological shortcomings outlined above, a further limitation of the LIFT trial was the very low prevalence of tolerance observed, which reduced the study's overall power. This was partially overcome by the fact that the LIFT trial protocol was harmonized with OPTIMAL, a completely independent IS withdrawal trial conducted in the US.[8] Although OPTIMAL did not include randomization and all study participants were offered IS withdrawal, both studies employed the exact same eligibility criteria, IS discontinuation steps, and histologic definition of operational tolerance. Notably, the clinical outcomes of OPTIMAL and LIFT were almost identical, with 28% of participants in both studies discontinuing IS without developing allograft dysfunction, and 16% meeting the primary endpoint of operational tolerance at 1-year. The harmonization of the trial protocols allowed for the integration and/or direct comparison of the mechanistic experiments conducted as part of the 2 studies, partially overcoming the limitations of the small number of TOL participants available for analysis. The integrated RNASeq analyses revealed an association between operational tolerance and interferon responses. Type-1 interferons and IFNγ exert clearly distinct effects on the immune system and yet display similar, partially overlapping, downstream signaling programs that cannot be easily differentiated at the transcriptional level. The fact that the histologic features of TOL and Non-TOL biopsies were similar and that we failed to detect overexpression of a highly sensitive transcriptional signature of T cell–mediated rejection in liver allografts (known to be orchestrated by IFNγ), indicates that the transcriptional changes associated with operational tolerance are likely to be dominated by type-I interferon. This is in keeping with what we previously described in hepatitis C–infected operationally TOL recipients,[19] and consistent with a large body of literature in clinical and preclinical models linking type-1 interferon responses with immune exhaustion and inhibition of adaptive immune responses.[20,21] Of note, both *IFNG* and *SOCS1,* but not rejection-related genes such as CXCL9, were found to be overexpressed in the TOL specimens from the original discovery study.[5] These transcriptional changes detected in the grafts of TOL recipients provide a potential explanation for the increased number of potentially exhausted $CD8^+$ T cells observed in both studies (albeit it should be noted that that the markers employed in the 2 studies to discriminate between T cell exhaustion and senescence are not entirely comparable). Future studies will be needed to better define the contributions of immune exhaustion vs senescence in LT recipients.

An additional striking similarity between LIFT and OPTIMAL is the confirmation of the value of using automated image detection to quantify immune synapses in the screening liver biopsies as a means to predict the outcome of IS withdrawal. These findings are also in agreement with those of a previously reported study in pediatric LT (iWITH trial).[4,15] Of note, although in these 3 study cohorts TOL patients exhibited a similar range of intra-hepatic immunologic synapses, it was not possible to select a unique diagnostic threshold as a result of patient and histologic heterogeneity (eg, presence/absence of metabolic dysfunction–associated steatotic liver disease). Future studies will be required to develop rapid and simple morphologic immune synapse markers that the practicing pathologist can utilize via available imaging hardware and digital analysis software. If successful in ensuring accuracy when translated into such a widely applicable clinical test, the quantification of immune synapses would become a useful biomarker of operational tolerance in LT. Alternative strategies worthwhile exploring to assist in the management of patients undergoing IS withdrawal include the use of markers to detect early allograft damage before it becomes clinically apparent.[14,22]

Taken together, LIFT and OPTIMAL provide the most accurate description available to date of the prevalence of operational tolerance and the expected clinical outcomes when conducting IS withdrawal trials across a large number of clinical sites and using strict histologic criteria. These are important observations that should inform clinical practice as well as future clinical trials of IS withdrawal in LT. Furthermore, the 2 studies provide complementary evidence highlighting a mechanistic role for type-1 interferon signaling and immune exhaustion/senescence in modulating alloimmune responses and promoting operational tolerance.

Acknowledgments

The authors are grateful to all the patients who participated in the study. The authors also thank local research nurses for their invaluable help as well as current and past members of our laboratory at King's College London.

Author contributions

J.V.: Data collection, study supervision, clinical trial management, data analysis and laboratory data analysis, manuscript writing, critical review for intellectual content and approval of the manuscript; J.T.-Y.: Laboratory data analysis, manuscript writing, critical review for intellectual content and approval of the manuscript; R.M.: Concept and design of the study, histologic analysis, data collection, critical review for intellectual content and approval of the manuscript; J.G.A.: Concept and design of the study, data analysis, critical review for intellectual content and approval of the manuscript; J.W.: data collection, clinical trial management, approval of the manuscript; E.K.: Laboratory data analysis, approval of the manuscript; J.J.-L.: Statistical analysis, critical review for intellectual content and approval of the manuscript; P.R., M.N., A.M., F.N., W.G., J.L., S.M., E.J., R.T., P.T., D.E., K.J.S., E.B.-R., J.F.: Data collection, critical review for intellectual content and approval of the manuscript; A.Q.: Data collection, histologic analysis, critical review for intellectual content and

approval of the manuscript; A.J.D., A.J.L.: Histologic analysis, approval of the manuscript; M.E., M.D., A.D.: Statistical analysis, approval of the manuscript; I.R.-M.: concept and design of the study; M.M.-L.: concept and design of the study, laboratory data analysis, critical review for intellectual content and approval of the manuscript; J.A.F.S.: Critical review for intellectual content and approval of the manuscript; J.F.M.: Concept and design of OPTIMAL study, critical review for intellectual content and approval of the manuscript; A.S.-F.: Concept and design of the study, obtained funding, study supervision, data collection, data analysis, manuscript writing, critical review for intellectual content and approval of the manuscript.

Funding

The work was supported by an award from the National Institute for Health and Care Research (NIHR) Efficacy and Mechanism Evaluation Programme (reference 13/94/55; to A.S.-F.), the Medical Research Council (MRC) Centre for Transplantation at King's College London, and the National Institute for Health Research Biomedical Research Centre (NIHR-BRC) at Guy's and St Thomas' National Health Service Foundation Trust and King's College London. J.V. was supported by the Swiss National Science Foundation (Early Postdoc.Mobility P2LAP3_181318 and Postdoc.Mobility P400PM_194501 grants), by the Fondation genevoise de bienfaisance Valeria Rossi di Montelera (Eugenio Litta grant), by the Société Académique Vaudoise and the Lausanne University Hospital. Research reported in this publication was supported by the National Institute of Allergy and Infectious Diseases (NIAID) of the National Institutes of Health (NIH) under Award Number UM1AI109565. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Declaration of competing interest

The authors of this manuscript have conflicts of interest to disclose as described by the American Journal of Transplantation. M. Martinez-Llordella and A. Sánchez-Fueyo are founders of Quell Therapeutics Ltd and Marc Martinez-Llordella is currently employed by Quell Therapeutics. The other authors of this manuscript have no conflicts of interest to disclose as described by the American Journal of Transplantation.

Data availability

To ensure the privacy of the participants, further research data remain confidential. The anonymized data set is available upon request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ajt.2024.12.002.

ORCiD

Julien Vionnet https://orcid.org/0000-0002-1654-0488
Jorge Torres-Yaguana https://orcid.org/0000-0001-5535-4154
Pablo Ruiz https://orcid.org/0000-0002-5810-8802
Steven Masson https://orcid.org/0000-0003-1041-9844
Richard Taubert https://orcid.org/0000-0001-9270-2496
Alberto Sánchez-Fueyo https://orcid.org/0000-0002-8316-3504

References

1. Thomson AW, Vionnet J, Sanchez-Fueyo A. Understanding, predicting and achieving liver transplant tolerance: from bench to bedside. *Nat Rev Gastroenterol Hepatol*. 2020;17(12):719–739. https://doi.org/10.1038/s41575-020-0334-4.
2. Feng S, Ekong UD, Lobritto SJ, et al. Complete immunosuppression withdrawal and subsequent allograft function among pediatric recipients of parental living donor liver transplants. *JAMA*. 2012;307(3):283–293. https://doi.org/10.1001/jama.2011.2014.
3. Benítez C, Londoño MC, Miquel R, et al. Prospective multicenter clinical trial of immunosuppressive drug withdrawal in stable adult liver transplant recipients. *Hepatology*. 2013;58(5):1824–1835. https://doi.org/10.1002/hep.26426.
4. Feng S, Bucuvalas JC, Mazariegos GV, et al. Efficacy and safety of immunosuppression withdrawal in pediatric liver transplant recipients: moving toward personalized management. *Hepatology*. 2021;73(5):1985–2004. https://doi.org/10.1002/hep.31520.
5. Bohne F, Martínez-Llordella M, Lozano J-J, et al. Intra-graft expression of genes involved in iron homeostasis predicts the development of operational tolerance in human liver transplantation. *J Clin Invest*. 2012;122(1):368–382. https://doi.org/10.1172/JCI59411.
6. Martínez-Llordella M, Puig-Pey I, Orlando G, et al. Multiparameter immune profiling of operational tolerance in liver transplantation. *Am J Transplant*. 2007;7(2):309–319. https://doi.org/10.1111/j.1600-6143.2006.01621.x.
7. Martínez-Llordella M, Lozano JJ, Puig-Pey I, et al. Using transcriptional profiling to develop a diagnostic test of operational tolerance in liver transplant recipients. *J Clin Invest*. 2008;118(8):2845–2857. https://doi.org/10.1172/JCI35342.
8. Tanimine N, Markmann JF, Wood-Trageser MA, et al. Donor-specific immune senescence as a candidate biomarker of operational tolerance following liver transplantation in adults: results of a prospective, multicenter cohort study. *Am J Transplant*. 2004. https://doi.org/10.1016/j.ajt.2024.10.022.
9. Demetris AJ, Bellamy C, Hübscher SG, et al. 2016 Comprehensive update of the Banff Working Group on Liver Allograft Pathology: introduction of antibody-mediated rejection. *Am J Transplant*. 2016;16(10):2816–2835. https://doi.org/10.1111/ajt.13909.
10. Banff Working Group on Liver Allograft Pathology, Demetris A. Importance of liver biopsy findings in immunosuppression management: biopsy monitoring and working criteria for patients with operational tolerance. *Liver Transpl*. 2012;18(10):1154–1170. https://doi.org/10.1002/lt.23481.
11. Deugnier YM, Loréal O, Turlin B, et al. Liver pathology in genetic hemochromatosis: a review of 135 homozygous cases and their bioclinical correlations. *Gastroenterology*. 1992;102(6):2050–2059. https://doi.org/10.1016/0016-5085(92)90331-r.
12. De Biasi S, Lo Tartaro D, Neroni A, et al. Immunosenescence and vaccine efficacy revealed by immunometabolic analysis of SARS-CoV-2-specific cells in multiple sclerosis patients. *Nat Commun*. 2024;15(1):2752. https://doi.org/10.1038/s41467-024-47013-0.
13. Vionnet J, Miquel R, Abraldes JG, et al. Non-invasive alloimmune risk stratification of long-term liver transplant recipients. *J Hepatol*. 2021;75(6):1409–1419. https://doi.org/10.1016/j.jhep.2021.08.007.

14. Bonaccorsi-Riani E, Pennycuick A, Londoño MC, et al. Molecular characterization of acute cellular rejection occurring during intentional immunosuppression withdrawal in liver transplantation. *Am J Transplant*. 2016;16(2):484–496. https://doi.org/10.1111/ajt.13488.
15. Wood-Trageser MA, Lesniak D, Gambella A, et al. Next-generation pathology detection of T cell-antigen-presenting cell immune synapses in human liver allografts. *Hepatology*. 2023;77(2):355–366. https://doi.org/10.1002/hep.32666.
16. Whitehouse G, Gray E, Mastoridis S, et al. IL-2 therapy restores regulatory T-cell dysfunction induced by calcineurin inhibitors. *Proc Natl Acad Sci U S A*. 2017;114(27):7083–7088. https://doi.org/10.1073/pnas.1620835114.
17. Levitsky J, Burrell BE, Kanaparthi S, et al. Immunosuppression withdrawal in liver transplant recipients on sirolimus. *Hepatology*. 2020;72(2):569–583. https://doi.org/10.1002/hep.31036.
18. Taubert R, Danger R, Londoño MC, et al. Hepatic infiltrates in operational tolerant patients after liver transplantation show enrichment of regulatory T cells before proinflammatory genes are downregulated. *Am J Transplant*. 2016;16(4):1285–1293. https://doi.org/10.1111/ajt.13617.
19. Bohne F, Londoño M-C, Benítez C, et al. HCV-induced immune responses influence the development of operational tolerance after liver transplantation in humans. *Sci Transl Med*. 2014;6(242):242ra81. https://doi.org/10.1126/scitranslmed.3008793.
20. Moreira-Teixeira L, Tabone O, Graham CM, et al. Mouse transcriptome reveals potential signatures of protection and pathogenesis in human tuberculosis. *Nat Immunol*. 2020;21(4):464–476. https://doi.org/10.1038/s41590-020-0610-z.
21. Sumida TS, Dulberg S, Schupp JC, et al. Type I interferon transcriptional network regulates expression of coinhibitory receptors in human T cells. *Nat Immunol*. 2022;23(4):632–642. https://doi.org/10.1038/s41590-022-01152-y.
22. Levitsky J, Kandpal M, Guo K, Kleiboeker S, Sinha R, Abecassis M. Donor-derived cell-free DNA levels predict graft injury in liver transplant recipients. *Am J Transplant*. 2022;22(2):532–540. https://doi.org/10.1111/ajt.16835.