American Journal of Transplantation xxx (xxxx) xxx

Contents lists available at ScienceDirect

American Journal of Transplantation

journal homepage: www.amjtransplant.org

Original Article

Donor-specific immune senescence as a candidate biomarker of operational tolerance following liver transplantation in adults: Results of a prospective, multicenter cohort study

Naoki Tanimine [1,†], James F. Markmann [2,†], Michelle A. Wood-Trageser [3], Anthony J. Demetris [3], Kristen Mason [4], Juliete A.F. Silva [5,6], Josh Levitsky [7], Sandy Feng [8], Abhinav Humar [9], Jean C. Emond [10], Abraham Shaked [1], Goran Klintmalm [11], Alberto Sanchez-Fueyo [12], Drew Lesniak [3], Cynthia P. Breeden [5,6], Gerald T. Nepom [6], Nancy D. Bridges [13], Julia Goldstein [13], Christian P. Larsen [5,6], Michele DesMarais [6], Geo Gaile [6], Sindhu Chandran [6,*]

[1] *Department of Gastroenterological and Transplant Surgery, Hiroshima University, Higashihiroshima, Japan*
[2] *Penn Transplant Institute, Philadelphia, Pennsylvania, USA*
[3] *Department of Pathology, University of Pittsburgh, Pittsburgh, Pennsylvania, USA*
[4] *Rho, Durham, North Carolina, USA*
[5] *Emory Transplant Center, Emory University, Atlanta, Georgia, USA*
[6] *Immune Tolerance Network, Seattle, Washington, USA*
[7] *Department of Medicine, Northwestern University Feinberg School of Medicine, Chicago, Illinois, USA*
[8] *Department of Surgery, University of California-San Francisco, San Francisco, California, USA*
[9] *Starzl Transplantation Institute, University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania, USA*
[10] *Department of Surgery, Columbia University Irving Medical Center, New York, New York, USA*
[11] *Department of Surgery, Baylor University Medical Center, Dallas, Texas, USA*
[12] *Institute of Liver Studies, King's College Hospital, Medical Research Council (MRC) Centre for Transplantation, King's College London University, London, UK*
[13] *National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, USA*

ARTICLE INFO

Keywords:
liver transplantation

ABSTRACT

Immunosuppression can be withdrawn from selected liver transplant recipients, although robust clinical predictors of tolerance remain elusive. The Immune Tolerance Network

Abbreviations: ai-iSYN, automated image detection of immune synapses; ALT, alanine aminotransferase; CNI, calcineurin inhibitor; DSA, donor-specific antibody; eGFR, estimated glomerular filtration rate; HCV, hepatitis C virus; HLA, human leukocyte antigen; IQR, interquartile range; ISW, immunosuppression withdrawal; KLRG1, killer-cell lectin like receptor G1; LAFSc, liver allograft fibrosis score; MMF, mycophenolate mofetil.

* Corresponding author. 535 Mission St, Suite 2650, San Francisco, CA 94105, USA.
E-mail address: schandran@immunetolerance.org (S. Chandran).

† These authors contributed equally: Naoki Tanimine and James F. Markmann.

https://doi.org/10.1016/j.ajt.2024.10.022
Received 10 May 2024; Received in revised form 16 October 2024; Accepted 28 October 2024
Available online xxxx

Please cite this article as: Tanimine N 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, American Journal of Transplantation, https://doi.org/10.1016/j.ajt.2024.10.022

immune tolerance
immunosuppression withdrawal
immune senescence
clinical trial

ITN056ST study (OPTIMAL; NCT02533180) assessed clinical outcomes and mechanistic correlates of phased immunosuppression withdrawal (ISW) in nonautoimmune, nonviral adult liver transplant recipients. Enrolled subjects were ≥3 years posttransplant with minimal/absent inflammation or fibrosis on a screening liver biopsy. The primary end point was operational tolerance at 52 weeks following complete ISW. Of 61 subjects who initiated ISW, 34 failed during ISW and 10 restarted immunosuppression after completing ISW due to clinically manifest acute rejection. Only 10 of 17 clinically stable subjects remaining off immunosuppression at 1 year were ultimately deemed tolerant by biopsy. There were no cases of chronic rejection or graft loss; 28.3% developed de novo donor-specific antibody during ISW, which persisted in 11.3%. The majority of subjects (78.6%), including those who experienced rejection, ended the study on same or less calcineurin inhibitor than at baseline. A minority (16.4%) of histologically and clinically stable long-term adult liver transplant recipients can successfully discontinue and remain off immunosuppression. Increased frequency of donor-specific T cell senescence, C4d deposition, and higher density of immune synapses on the screening liver biopsy emerged as potential candidate biomarkers for operational tolerance.

1. Introduction

A subset of highly selected adult liver transplant recipients, estimated at ~20%,[1-3] can successfully discontinue immunosuppression and yet sustain normal graft function, consistent with a state of operational tolerance. Prospective identification of such individuals could provide clues to mechanisms of tolerance and facilitate tolerance induction in recipients not naturally predisposed to its spontaneous development.

Although robust predictors of tolerance remain elusive, age and time from transplant have been found to be strongly correlated with outcome,[4] with an 80% success rate in those who initiated weaning at >10.6 years posttransplant compared with only 38% of those 5.7-10.6 years posttransplant.[5,6] These correlations may be a clue to the mechanistic underpinnings of tolerance. Time-dependent development of immune dysregulation, especially in the CD8 T cell subset, which occurs in the setting of chronic antigen exposure such as with chronic viral infections,[7-15] has been termed immune exhaustion.[16] Functionally, these changes are manifested by a sequential loss of interleukin-2 production, proliferation, and cytotoxicity. In one study, tolerance was associated with a higher proportion of exhausted PD1/CTLA4/2B4-positive hepatitis C virus (HCV)-specific circulating $CD8^+$ T cells at baseline,[17] a population later found to be expanded in HCV-positive liver transplant recipients and correlated with higher CXCL10 plasma levels.[18] Notably, decreased antidonor responses in T cell lines generated from HCV-infected liver transplant recipients were significantly reversed by HCV eradication.

We theorized that chronic residence of a liver allograft in a transplant recipient recapitulates the chronic exposure to a viral pathogen, leading to tonic stimulation of donor-reactive T cells and their functional silencing or elimination over time, a process rendered more efficient by the age-dependent reduction in thymic output. This prospective trial investigated whether operational tolerance is associated with detectable signal of donor-specific T cell exhaustion and/or senescence before weaning.

2. Materials and methods

2.1. Study design and population

The Immune Tolerance Network trial ITN056ST, Evaluation of Donor-Specific Immune Senescence and Exhaustion as Biomarkers of Operational Tolerance Following Liver Transplantation in Adults (OPTIMAL), was a multicenter, prospective, single-arm interventional cohort study of adult liver transplant recipients. Candidates had to be ≥3 years posttransplant (≥6 years if ≤50 years old),[5] on a calcineurin inhibitor (CNI), with stable graft function, no episodes of rejection ≤1 year, and direct bilirubin and alanine aminotransferase (ALT) levels of <2× the upper limit of normal. A screening liver biopsy had to meet specific histologic criteria (Supplementary Table 1). Subjects with a history of HCV or HIV, active hepatitis B, autoimmune liver disease, and medical conditions requiring systemic steroids, or with estimated glomerular filtration rate (eGFR) of <40 mL/minute/1.73 m^2, or in need of chronic uninterrupted anticoagulation were excluded. All subjects provided informed consent and the protocol conformed to ethical principles stated in the 1975 Declaration of Helsinki. The study was registered with clinicaltrials.gov (NCT02533180).

2.2. Immunosuppression withdrawal and follow-up

Eligible subjects underwent immunosuppression withdrawal (ISW) from a CNI-based immunosuppressive regimen according to a prespecified algorithm over 24 to 45 weeks (Supplementary Table 2). Subjects were followed up for 3 years after successful or failed ISW. Protocol-directed liver biopsies were obtained at screening and at 1 and 3 years post-ISW, and for-cause biopsies as needed to evaluate unexplained allograft dysfunction.

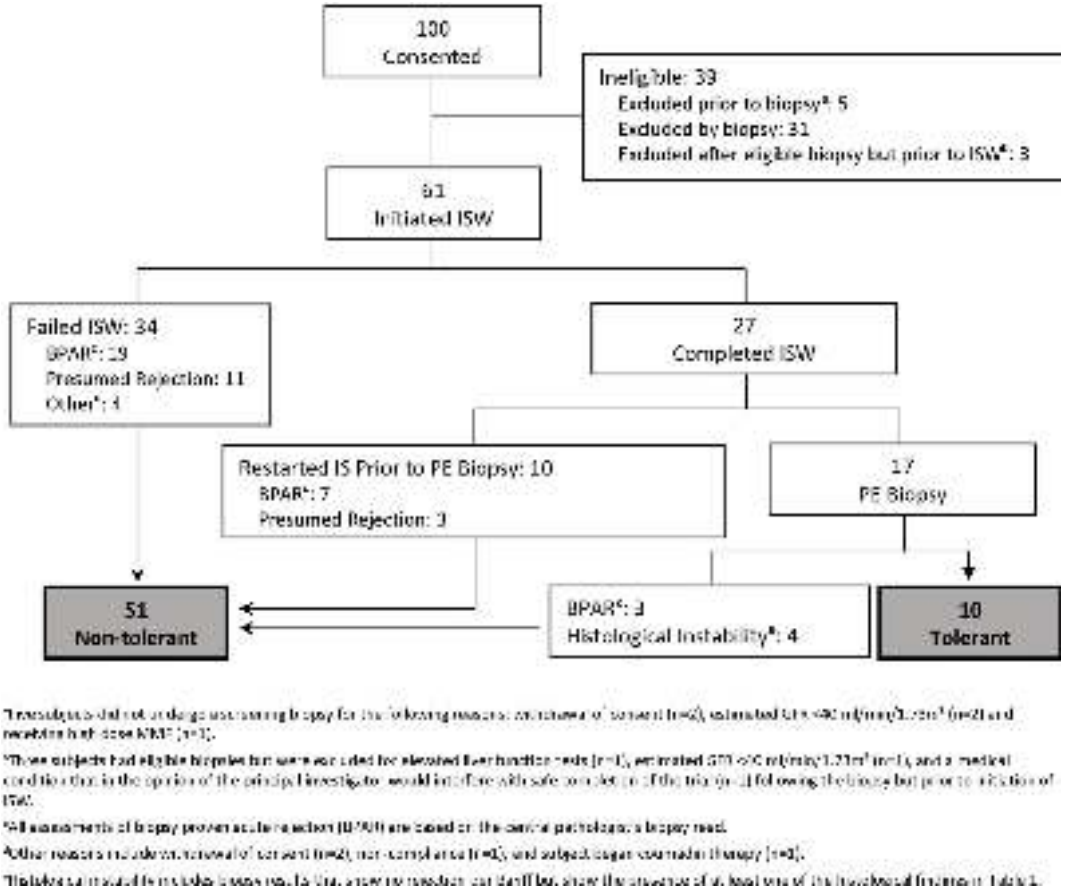

Figure 1. Flow of subjects from study entry through evaluation of the primary end point. IS, immunosuppression; ISW, immunosuppression withdrawal; MMF, mycophenolate mofetil; PE, primary endpoint.

Suspected rejection was confirmed by liver biopsy, unless medically contraindicated. Biopsies were prospectively analyzed and scored as per the Banff global assessment criteria.[19,20] The central pathologist's assessment was also prospective, which effectively blinded the assessment to outcome and was used for data analysis and reporting. Rejection diagnosis on for-because biopsies was made by the site pathologist and treatment determined by the site investigator. Subjects treated with increased immunosuppression for graft dysfunction without a confirmatory biopsy were considered to have clinical rejection.

2.3. Study end points

The primary end point was the proportion of subjects who achieved operational tolerance at 52 weeks after completion of ISW, defined as no acute rejection episodes and a liver biopsy demonstrating histologic stability consistent with operational tolerance per Banff 2012 criteria[21] and absence of rejection per the Banff global assessment criteria.[19,20] Secondary safety end points included the following: (1) proportion of subjects who developed de novo donor-specific antibodies (DSAs); (2) incidence, severity, and timing of acute, steroid-resistant, and chronic rejection; (3) incidence and progression of graft fibrosis; (4) incidence of graft loss; (5) incidence of all-cause mortality; and (6) incidence of study-related serious adverse events. Secondary efficacy end points included changes in renal function at 1 and 3 years post-ISW and predictive value of recipient age and the time since transplant for operational tolerance.

A key mechanistic end point was assessment of the predictive value of phenotypic or molecular markers of immune senescence and/or exhaustion in circulating T cells for operational tolerance. We also examined the predictive value of DSA and intra-allograft C4d at baseline, as well as de novo DSA during the study.

2.4. Human leukocyte antigen typing, mismatch, and antibody analysis

Recipient and donor human leukocyte antigen (HLA) typing data were obtained from United Network for Organ Sharing. DNA samples for HLA typing were obtained from all recipients and those living donors who agreed to participate. Batched class II HLA antibody analyses were performed centrally on serum samples collected at baseline and during, and after ISW. Mean fluorescent intensity of >1000 was considered a positive result. Additional details are in Supplementary Table 3.

2.5. Alloreactive T cell detection assay

Alloreactive T cells were detected following 1-way mixed lymphocyte reaction.[22] Briefly, 1-day rested recipient peripheral blood mononuclear cells were cocultured with carboxyfluorescein

Table 1
Baseline characteristics of study subjects by histologic eligibility and tolerance outcome.

Characteristic			Underwent screening biopsy (n = 95)			Initiated IS withdrawal (n = 61)		
			Total (n = 95)	Biopsy ineligible (n = 31)	Biopsy eligible[a] (n = 64)	Total (n = 61)	Nontolerant (n = 51)	Tolerant (n = 10)
Donor	Age (y), median (IQR)		39 (25-53)	38 (21-50)	42 (26-54)	42 (25-54)	42 (25-54)	46 (30-53)
	Male sex, n (%)		52 (54.7)	15 (48.4)	37 (57.8)	35 (57.4)	28 (54.9)	7 (70.0)
	Race, n (%)	White	69 (72.6)	21 (67.7)	48 (75.0)	46 (75.4)	39 (76.5)	7 (70.0)
		Black	4 (4.2)	0	4 (6.3)	3 (4.9)	3 (5.9)	0
		Other	14 (14.7)	4 (12.9)	10 (15.6)	10 (16.4)	8 (15.7)	2 (20.0)
	Ethnicity, n (%)	Hispanic or Latino	4 (4.2)	2 (6.5)	2 (3.1)	2 (3.3)	2 (3.9)	0
		Not Hispanic or Latino	41 (43.2)	15 (48.4)	26 (40.6)	26 (42.6)	21 (41.2)	5 (50.0)
		Unknown/not reported	40 (42.1)	9 (29.0)	31 (48.4)	28 (45.9)	24 (47.1)	4 (40.0)
	Type, n (%)	Deceased	70 (73.7)	24 (77.4)	46 (71.9)	44 (72.1)	36 (70.6)	8 (80.0)
		Living, related	20 (21.1)	6 (19.4)	14 (21.9)	14 (23.0)	12 (23.5)	2 (20.0)
		Living, unrelated	5 (5.3)	1 (3.2)	4 (6.3)	3 (4.9)	3 (5.9)	0
Recipient	Age at transplant (y), median (IQR)		53 (43-59)	47 (20-55)	55 (46-62)[b]	56 (46-62)	56 (46-62)	55 (46-59)
	Age at enrollment (y), median (IQR)		61 (53-66)	54 (32-62)	63 (55-68)[b]	63 (56-68)	63 (56-68)	65 (55-69)
	Male sex, n (%)		63 (66.3)	18 (58.1)	45 (70.3)	43 (70.5)	37 (72.5)	6 (60.0)
	Race, n (%)	White	80 (84.2)	27 (87.1)	53 (82.8)	50 (82.0)	43 (84.3)	7 (70.0)
		Black	1 (1.1)	1 (3.2)	0	0	0	0
		Asian	6 (6.3)	0	6 (9.4)	6 (9.8)	3 (5.9)	3 (30.0)
		Native Hawaiian or Pacific Islander	2 (2.1)	1 (3.2)	1 (1.6)	1 (1.6)	1 (2.0)	0
		Other	6 (6.3)	2 (6.5)	4 (6.3)	4 (6.6)	4 (7.8)	0
	Ethnicity (%)	Hispanic or Latino	6 (6.3)	1 (3.2)	5 (7.8)	4 (6.6)	4 (7.8)	0
		Not Hispanic or Latino	80 (84.2)	29 (93.5)	51 (79.7)	49 (80.3)	39 (76.5)	10 (100)
		Unknown/not reported	9 (9.5)	1 (3.2)	8 (12.5)	8 (13.1)	8 (15.7)	0
	Body mass index (kg/m^2), median (IQR)		28 (25-33)	27 (24-31)	28 (26-34)	28 (26-33)	28 (26-34)	27 (25-29)
	Blood type, n (%)	A	37 (38.9)	10 (32.3)	27 (42.2)	26 (42.6)	22 (43.1)	4 (40.0)
		AB	4 (4.2)	1 (3.2)	3 (4.7)	3 (4.9)	3 (5.9)	0

(*continued on next page*)

		B	10 (10.5)	5 (16.1)	5 (7.8)	5 (8.2)	5 (9.8)	0
		O	39 (41.1)	10 (32.3)	29 (45.3)	27 (44.3)	21 (41.2)	6 (60.0)
Transplant	Indication, n (%)	Alcoholic liver disease	30 (31.6)	7 (22.6)	23 (35.9)	21 (34.4)	19 (37.3)	2 (20.0)
		Hepatitis B	9 (9.5)	3 (9.7)	6 (9.4)	6 (9.8)	4 (7.8)	2 (20.0)
		Liver tumor	6 (6.3)	1 (3.2)	5 (7.8)	5 (8.2)	5 (9.8)	0
		NASH	8 (8.4)	0	8 (12.5)	8 (13.1)	6 (11.8)	2 (20.0)
		Inherited	2 (2.1)	2 (6.5)	0	0	0	0
		Biliary atresia	5 (5.3)	4 (12.9)	1 (1.6)	1 (1.6)	1 (2.0)	0
		Metabolic liver disease with cirrhosis	8 (8.4)	3 (9.7)	5 (7.8)	4 (6.6)	4 (7.8)	0
		Metabolic liver disease without cirrhosis	1 (1.1)	1 (3.2)	0	0	0	0
		Other	26 (27.4)	10 (32.3)	16 (25.0)	16 (26.2)	12 (23.5)	4 (40.0)
	Graft, n (%)	Whole	66 (69.5)	21 (67.7)	45 (70.3)	43 (70.5)	35 (68.6)	8 (80.0)
		Partial	28 (29.5)	9 (29.0)	19 (29.7)	18 (29.5)	16 (31.4)	2 (20.0)
At study entry	Time from transplant (y), median (IQR)		8 (5-12)	9 (6-16)	7 (5-11)	7 (5-11)	7 (5-11)	9 (4-13)
	Antibody depleting induction medication, n (%)		7 (7.3)	3 (9.7)	4 (6.3)	4 (6.6)	2 (3.9)	2 (20.0)
	Maintenance IS, n (%)	CNI + MMF compound	28 (29.5)	9 (29.0)	19 (29.7)	18 (29.5)	15 (29.4)	3 (30.0)
		CNI + prednisone	6 (6.3)	4 (12.9)	2 (3.1)	2 (3.3)	2 (3.9)	0
		CNI only	61 (64.2)	18 (58.1)	43 (67.2)	41 (67.2)	34 (66.7)	7 (70.0)
	Tacrolimus dose (mg/d), median (IQR)		3 (2-3)	3 (2-4)	3 (2-3)	2 (2-3)	3 (2-3)	2 (2-3)
	Cyclosporine dose (mg/d), median (IQR)		150 (75-150)	138 (88-150)	150 (75-200)	150 (75-200)	175 (113-225)	50 (50-50)
	Alanine aminotransferase (U/L), median (IQR)		22 (16-30)	21 (16-28)	22 (17-32)	21 (17-28)	22 (17-29)	20 (17-25)
	γ-Glutamyl transferase (U/L), median (IQR)		23 (17-55)	32 (17-71)	22 (16-50)	23 (17-48)	25 (17-52)	19 (17-24)
	Donor-specific antibody[c]		NA	NA	NA	7 (11.5)	6 (11.8)	1 (10.0)

CNI, calcineurin inhibitor; IS, immunosuppression; IQR, interquartile range; MMF, mycophenolate mofetil; NA, not applicable; NASH, non-alcoholic steatohepatitis.

[a] Three subjects underwent a screening biopsy, were eligible based on results of the screening biopsy, but were ineligible for IS withdrawal due to clinical reasons (subsequently elevated liver enzymes, estimated glomerular filtration rate of <40 mL/min/1.73 m^2, and a medical condition that in the opinion of the principal investigator would interfere with safe completion of the trial).

[b] $P < .01$ for comparison between biopsy eligible and biopsy ineligible.

[c] Donor-specific antibody at baseline was unavailable for 16 subjects who initiated withdrawal (13 nontolerant and 3 tolerant).

Table 2
Characteristics of tolerant subjects.

Baseline immunosuppression (total daily dose)	Days to ISW completion	ALT/GGT (U/L)			Days off IS
		Baseline	52 wk after ISW	End of study	
Tacrolimus: 0.5 mg	126	23/17	25/15	20/20	1115
Tacrolimus: 1.5 mg	162	12/17	21/63	17/90	1211
Tacrolimus: 2 mg	168	19/18	32/17	24/14	1232
Tacrolimus: 2 mg	162	25/19	29/18	24/38	1266
Tacrolimus: 2.5 mg	168	20/14	30/19	16/16	1141
Tacrolimus: 4 mg	161	13/16	17/14	17/41	1111
Tacrolimus: 1 mg and MMF: 500 mg	228	17/23	18/25	22/25	1115
Tacrolimus: 3 mg and MMF: 250 mg	221	19/24	21/13	40/21	1097
Tacrolimus: 3 mg and MMF: 1500 mg	273	31/48	29/56	30/42	1045
Cyclosporine: 50 mg	144	40/29	25/17	48/25	1215

Allograft dysfunction was defined as an unexplained elevation in alanine aminotransferase (ALT) or γ-glutamyl transferase (GGT) tests relative to baseline: a value greater than 2× upper limit of normal (ULN; ALT: 41 U/L; GGT: 58 U/L) if the baseline value was less than the ULN or a value greater 2× baseline value if the baseline was greater than the ULN. IS, immunosuppression; ISW, immunosuppression withdrawal; MMF, mycophenolate mofetil.

succinimidyl ester–labeled irradiated stimulators activated for 48 hours with multimeric CD40L (100 ng/mL; Adipogen) and rIL-4 (10 ng/ml; Peprotech) in the presence of allophycocyanin-conjugated anti-CD154 monoclonal antibodies. After 20 hours of incubation, CD4 and CD8 alloreactive T cells were identified as $CD69^+CD154^+CD4^+$ and $CD69^+CD137^+CD8^+$ in responder $CD3^+$ cells, respectively. To identify third party-reactive T cells, we used the same batch stimulators that mixed allogeneic peripheral blood mononuclear cell after preconditioning (activation, carboxyfluorescein succinimidyl ester label, and irradiation). Senescence markers CD57 and killer-cell lectin like receptor G1 (KLRG1) were analyzed in samples from baseline, dose taper level 3 (33%-50% baseline), and 26 and 52 weeks post-ISW completion or failure. Antibody details are in Supplementary Table 4.

2.6. Histologic assessments

C4d staining was conducted on fresh-frozen tissues.[23] Multiplex immunohistochemistry for automated image detection of immune synapses (ai-iSYNs; CD34/CD45/HLA-DPB1/CD8/MAC387) was conducted as previously described[24] on formalin-fixed paraffin-embedded slides of the screening biopsy. Briefly, ai-iSYN counting was performed following nuclear segmentation and fully automated tissue-tethered cytometry using NearCYTE (http://nearcyte.org).[23-25] Detailed methods are in Supplementary Tables 3 and 5.

2.7. Statistical analysis and sample size calculation

Data were summarized using descriptive statistics for categorical variables (counts and percentages) and continuous variables (medians and interquartile ranges [IQR]). Statistical comparisons were made using a Fisher exact test (categorical data) and Student paired *t* test or Wilcoxon rank sum test (continuous data). An exact 2-sided 95% binomial confidence interval was computed for the primary end point. Analyses were performed using Statistical Analysis System (SAS), version 9.4, or R, version 4.0.3. A sample size of 60 subjects with an expected tolerance rate $\geq$40%[5] was estimated provide $\geq$80% power to detect a mean difference between tolerant and nontolerant subjects of −0.1155 in the proportion of $CD8^+IFN\gamma^+CTLA4^+PD1^+2B4^+$ donor-specific cells, a potential biomarker of tolerance.

3. Results

3.1. Study enrollment and subject disposition

In total, 100 subjects enrolled in the study from 201 to 2018 and proceeded as depicted in Figure 1; 95 subjects underwent screening liver biopsies: 31 were ineligible by histology and 3 became clinically ineligible or withdrew; 61 initiated ISW; and 27 (44.3%) completed ISW successfully. Of these, 17 (63.0%) remained off immunosuppression for 52 weeks, and 10 (37.0%) restarted immunosuppression within 52 weeks due to acute rejection. Further, 10 of the 17 subjects who remained off immunosuppression for 52 weeks met the primary end point.

3.2. Baseline demographics, clinical characteristics, and liver pathology

Subjects' baseline characteristics are provided in Table 1; 31 (32.6%) had unfavorable histology due to inflammation (n = 24), fibrosis (n = 12), bile duct damage (n = 3), and/or arteriopathy (n = 3). Ineligible subjects tended to have exclusionary findings in >1 category (Supplementary Fig. S1). Eligible subjects were older at transplant (median age, 55 vs 47 years; $P = .002$) and enrollment (median age, 63 vs 54 years; $P = .002$), and more likely to have hepatic steatosis with or without low-grade steatohepatitis (51.6% vs 9.7%; $P \leq .001$). No other significant differences were observed (Table 1).

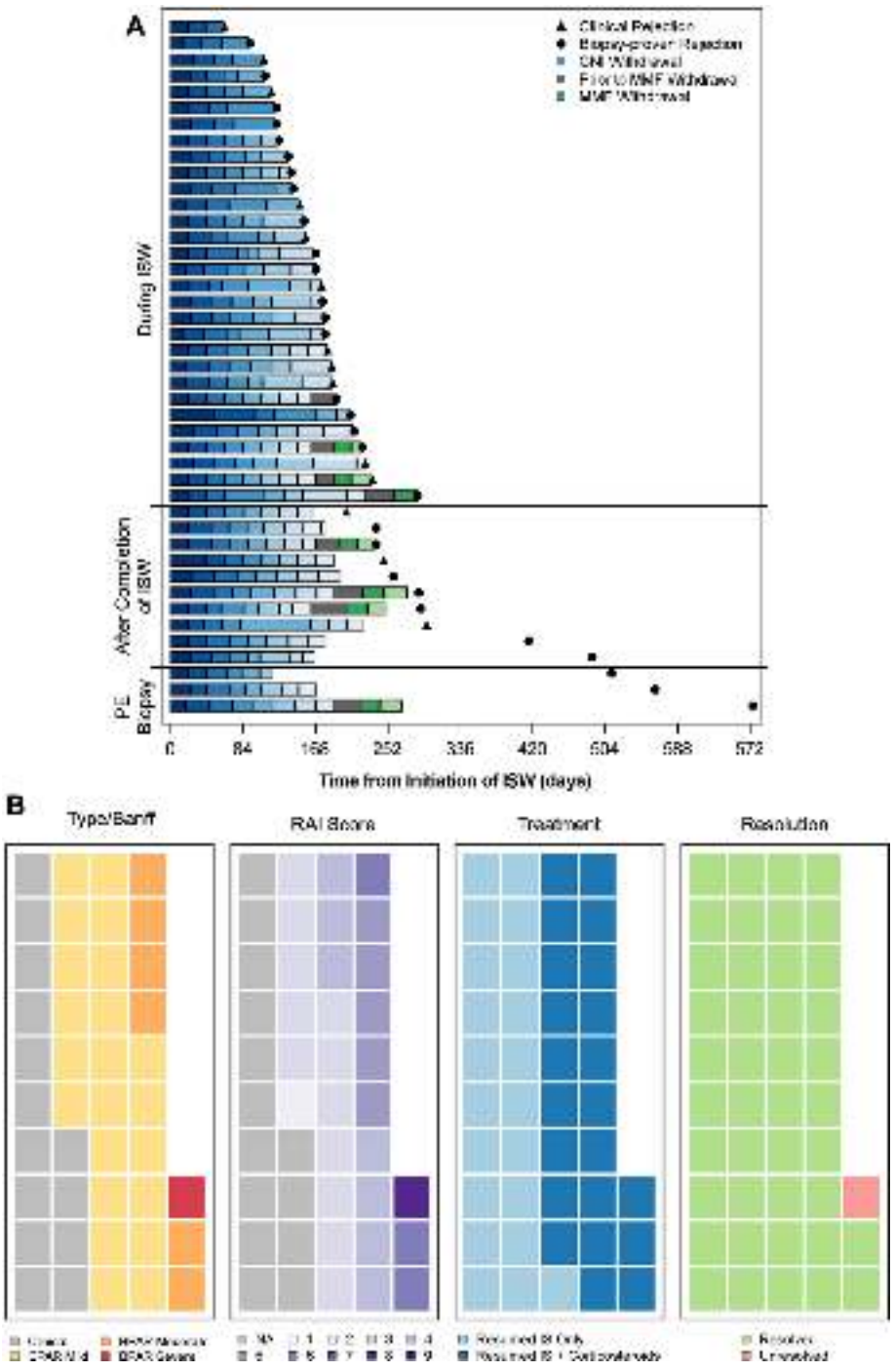

Figure 2. Acute rejection in the study: severity, treatment, and resolution. (A) Panel of subjects with acute rejection represented temporally by each taper level (up to 8 levels for those on CNI in blue and up to 2 additional levels for those also on a mycophenolic compound in green). The width of the box corresponds to the length of time spent at that taper level. Symbols indicate if the acute rejection event was clinical (triangle) or biopsy proven (circle). (B) Distribution plots summarize the 43 acute rejection episodes by severity according to Banff criteria and RAI score (as determined by the central pathologist) for biopsy proven acute rejection (BPAR) episodes, by treatment required, and by resolution of liver tests by the end of study participation. The unresolved acute rejection episode was in the participant who died due to suspected cardiac arrhythmia. At the time of death, liver tests were improving. CNI, calcineurin inhibitor; IS, immunosuppression; ISW, immunosuppression withdrawal; MMF, mycophenolate mofetil; NA, not available; PE, primary endpoint; RAI, rejection activity index.

3.3. Operational tolerance in the study

Characteristics of the 10 tolerant subjects are listed in Table 2. Another 7 completed ISW and maintained stable liver tests for 52 weeks but were deemed nontolerant on the primary end point biopsy. Six of the 7 resumed immunosuppression; 1 subject showed mild histologic changes, not meeting criteria for rejection, and remained off immunosuppression at the discretion of the treating physician. Altogether 51 subjects were considered nontolerant; their characteristics are listed in Supplementary Table 6.

3.4. Acute rejection and graft fibrosis in the study

In total, 43 subjects had acute rejection in the study; 30 during ISW, 10 after completing ISW but before the primary end point assessment, and 3 diagnosed on the primary end point biopsy despite stable liver tests (Fig. 2A); 29 were biopsy confirmed. Most cases were mild (n = 22) or moderate (n = 6) per Banff criteria; there was 1 case of severe rejection. In 22 subjects, treatment consisted of resumption of immunosuppression plus corticosteroids, with a median total dose of 3335 mg (IQR, 2170-4975 mg)

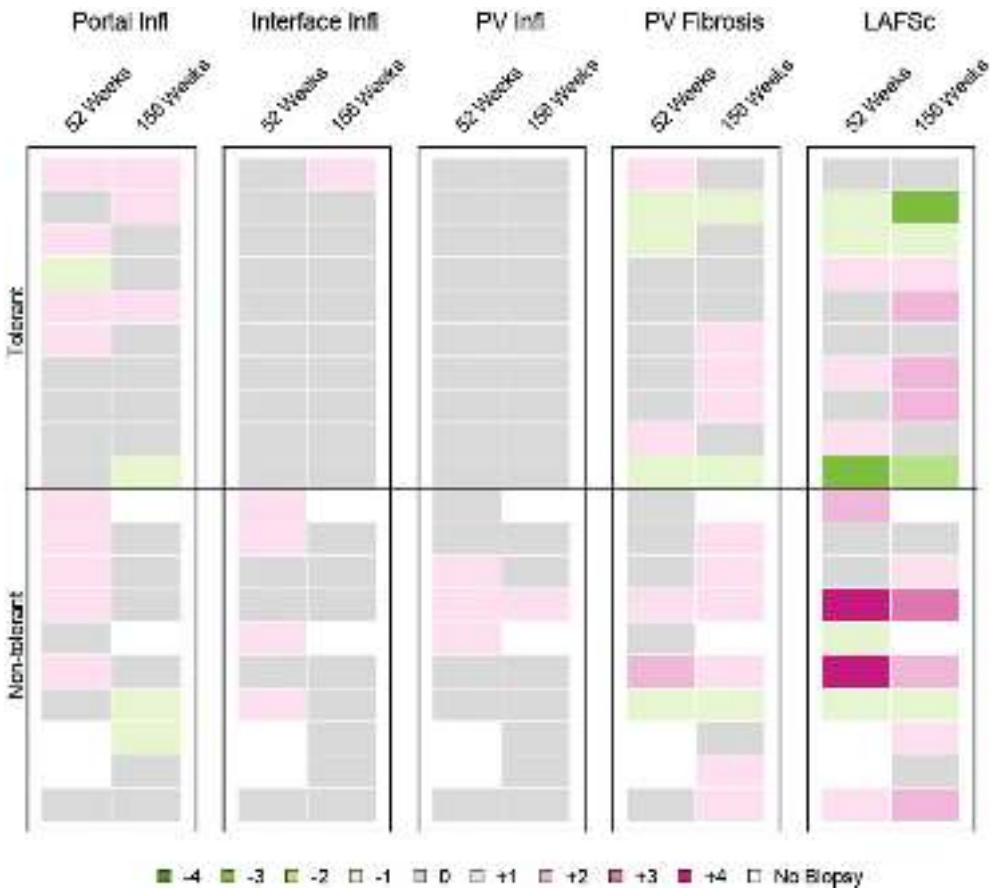

Figure 3. Changes in inflammation and graft fibrosis on follow-up compared to baseline biopsies. Biopsy data for tolerant (n = 10) and nontolerant (n = 10) subjects are shown; only 20% (10/51) of nontolerant subjects had biopsies collected at either one of these time points; 52 weeks represents the primary end point biopsy that was performed 52 weeks following successful completion of ISW and 156 weeks represents the biopsy collected 2 years after the primary end point (end of study). The 34 subjects who failed ISW did not have a biopsy at 52 or 156 weeks. Additionally, 7 subjects who failed following completion of ISW but before 52 weeks did not have a biopsy at 52 or 156 weeks. Each row represents a single subject and subjects are presented in the same order in all panels, sorted by tolerant and nontolerant status. To calculate change over time, absolute scores at baseline were subtracted from scores at follow-up for the following parameters: portal inflammation, interface inflammation, perivenular inflammation, perivenular fibrosis, and LAFSc. All score scales range from 0 to 3 except the LAFSc scale, which ranges from 0 to 9. Pink indicates an increase in score and green indicates a decrease in score; increasing intensity of either pink or green indicates larger magnitude of change. Gray indicates no change and blank (white) indicates no biopsy. ISW, immunosuppression withdrawal; LAFSc, liver allograft fibrosis score; PV, portal vein.

over a median of 169 days (IQR, 99-865 days); 21 subjects were treated with resumption of immunosuppression alone. Treatment resulted in resolution (by ALT) in all but 1 subject in a median of 78 days (IQR, 41-133 days). There were no cases of steroid-resistant or chronic rejection, or of graft loss. Figure 2B depicts the spectrum of rejection grades, treatment and resolution. Supplementary Figure S2 shows the trajectory of liver enzymes during and after rejection.

Figure 3 shows changes in graft inflammation and fibrosis including the liver allograft fibrosis score (LAFSc)[26] in tolerant vs nontolerant subjects with biopsies at 1 and 3 years post-ISW. Portal inflammation showed small increase (1 point) in 4 of the 10 tolerant subjects and 5 of the 10 nontolerant subjects at 52 weeks; scores improved to baseline values or lower in 2 tolerant and all nontolerant subjects at 156 weeks. Four of the 10 tolerant subjects showed slight increase (1-2 points) in the LASFSc at 156 weeks; scores were unchanged or improved in the remaining 6. Five of the 10 nontolerant subjects showed increases as well. The significance of these changes is unclear given that an increase or decrease $\geq$2 grades in in LAFSc in $\geq$1 analyzed compartment is usually required to be considered clinically significant.[27] By these measures, 6 of the 20 subjects (3 tolerant and 3 nontolerant) demonstrated worsening of fibrosis. Presence of DSA at baseline or emergence during the study were not correlated with worsening or fibrosis or inflammation on the follow-up biopsies.

3.5. Change in kidney function during the study

In tolerant subjects (off immunosuppression for 1045-1266 days at study completion), the median change in eGFR from baseline was +6.7 mL/min/1.73 m^2 (IQR, −0.1 to 9.4 mL/min/1.73 m^2) at 1 year and −3.8 mL/min/1.73 m^2 (IQR, −9.1 to 10.4 mL/min/1.73 m^2) at 3 years. Median change in nontolerant subjects' eGFR was −0.9 mL/min/1.73m^2 (IQR, −7.7 to 8.4 mL/min/1.73 m^2) and −6.0 mL/min/1.73m^2 (IQR, −11.8 to 9.3 mL/min/1.73 m^2) at comparable time points. The degree of change was not significantly different between the 2 groups.

3.6. Immunosuppressive therapy at baseline and end of study

All subjects were on a CNI at the time of enrollment; 11 subjects remained off immunosuppression at the end of the study. Of the 50 subjects who failed during or after ISW, 31 (62%) were on less CNI the end of the study compared with study entry. Figure 4 displays CNI doses in individual subjects over the timeline of the study. Overall, 48 of 61 subjects ended the study on same or lower dose of CNI than at study entry or on no CNI. Of the 50 subjects, 15 who failed were also on mycophenolate mofetil (MMF) at baseline. Of these, 7 (47%) were on less MMF at the end of the study. Two of the 50 subjects were on prednisone at baseline and ended the

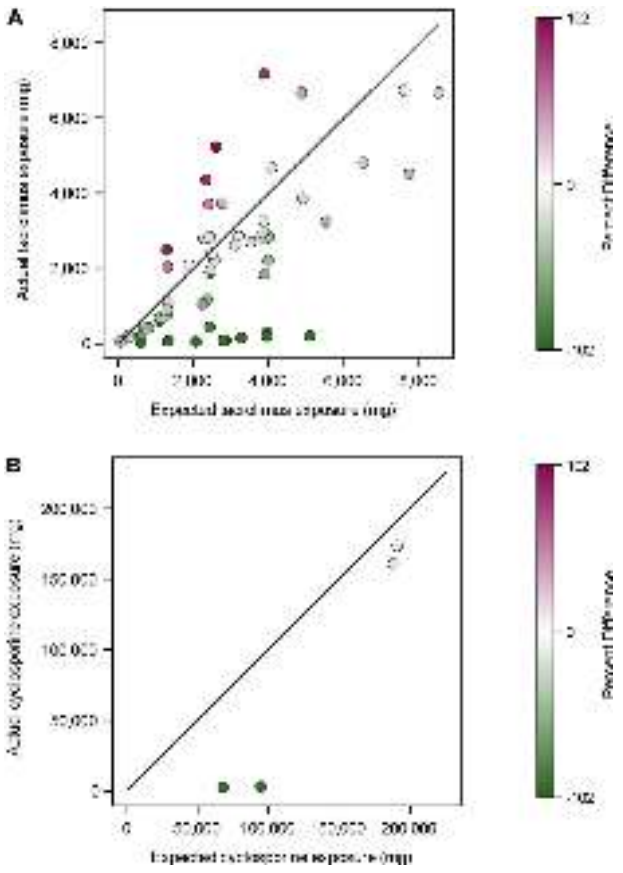

Figure 4. Immunosuppression exposure in the study. Immunosuppression exposure over the trial timeline is shown for subjects who initiated ISW. (A) Tacrolimus exposure for the 56 subjects who were on tacrolimus at study entry; (B) cyclosporine exposure for the 4 subjects who were on cyclosporine at study entry. One subject who was on cyclosporine at study entry and then switched to tacrolimus for the treatment of rejection is excluded. Expected exposure (x-axis) was calculated assuming that the subject was maintained on the dose at trial entry and plotted against actual exposure (y-axis). Pink circles (n = 12) identify subjects with higher actual than expected exposures, although green circles (n = 48) identify subjects with lower actual than expected exposures. Color intensity increases with larger differences between actual and expected exposures. ISW, immunosuppression withdrawal.

study on a lower dose. Finally, 7 (14%) subjects on CNI monotherapy at baseline ended the study with an additional immunosuppressant.

3.7. Safety

In total, 67 serious adverse events were reported in 27 subjects; 13 (in 10 subjects) were deemed related to study treatment (Supplementary Table 7). There were 2 deaths. One subject, a 70-year-old male, died from COVID-19, 605 days after ISW failure due to mild acute rejection, treated with steroids. He was on tacrolimus 0.5 mg daily, a dose lower than at study entry. Death was deemed not related to study participation. The second subject was a 76-year-old woman with diabetes mellitus and hypertension who died 60 days after treatment with corticosteroids and resumption of tacrolimus and MMF for mild acute rejection post-ISW completion. ALT and γ-glutamyl transferase were improving at her last visit. An autopsy showed moderate hypertensive and atherosclerotic cardiovascular disease, without other significant pathologic findings. Death was deemed possibly related to study participation, as the resumption of tacrolimus might have contributed to a cardiac arrhythmia.

3.8. HLA antibody

In total, 61 recipients and 17 live donors provided samples for HLA typing. Historical HLA typing data (of variable quality) were available on 39 of the 44 deceased donors. At baseline, 13 of the 61 subjects had class II HLA antibody. During the study, 17 subjects developed de novo class II HLA antibody (including 1 subject who had class II HLA antibody at baseline). Donor specificity could not be determined in 8 subjects (6 with baseline class II HLA antibody and 2 with de novo class II antibody) due to inadequate historical deceased donor typing data. Among 53 subjects in whom HLA antibody could be reliably assigned as DSA or non-DSA, 7 (13.2%) had DSA at baseline and 15 (28.3%) developed de novo DSA during the trial, which persisted in 11.3% (6/53) (Fig. 5). Rates of operational tolerance were 9.7% in those without baseline or de novo DSA, 14.2% in those with baseline DSA, and 20% in those with de novo DSA; differences were not statistically significant.

3.9. Alloreactive T cell detection

Donor-reactive %CD4 and %CD8 T cells detected using a newly developed assay (Fig. 6A) were lower than those of third party-reactive cells at baseline (Fig. 6B). However, neither correlated with successful operational tolerance (Fig. 6B). Percentage of donor-reactive regulatory T cells ($CD137^{+}CD154^{+}CD4^{+}$) at baseline also did not correlate with tolerance status. On the contrary, the fraction of $CD57^{+}KLRG1^{hi}$ cells at baseline was higher in donor-reactive CD8 T cells compared with third party-reactive T cells in tolerant subjects (32.6% vs 8.58%; $P = .044$), although these fractions were similar in nontolerant subjects (12.1% vs 13.9%) (Fig. 6C). Looking at the ratio of donor vs third party-reactive T cells over time, we found the $\%CD57^{+}KLRG1^{hi}$ in donor-reactive vs third party-reactive CD8 T cells to be 58% higher in tolerant than those in nontolerant subjects at baseline ($P = .020$) and 66% higher at taper level 3 ($P = .021$) (Fig. 6D). Kinetic analyses revealed stable expression of CD57 and KLRG1 regardless of tolerance status over time.

3.10. Histologic findings

3.10.1. C4d staining

Overall, 54 subjects had acceptable C4d staining quality on baseline biopsy specimens. C4d was absent/minimal in the majority (48/54, 88.9%), including all 8 subjects ultimately deemed tolerant. Of these 48 subjects, 10 (20.8%) had baseline class II HLA antibody, 3 of which were identified as DSA. These DSA were directed against HLA-DRw53 and HLA-DQ7, HLA-DRw53 alone, and HLA-DQ5 alone, respectively. Conversely, all 7 subjects with focal or diffusely positive C4d staining (driven

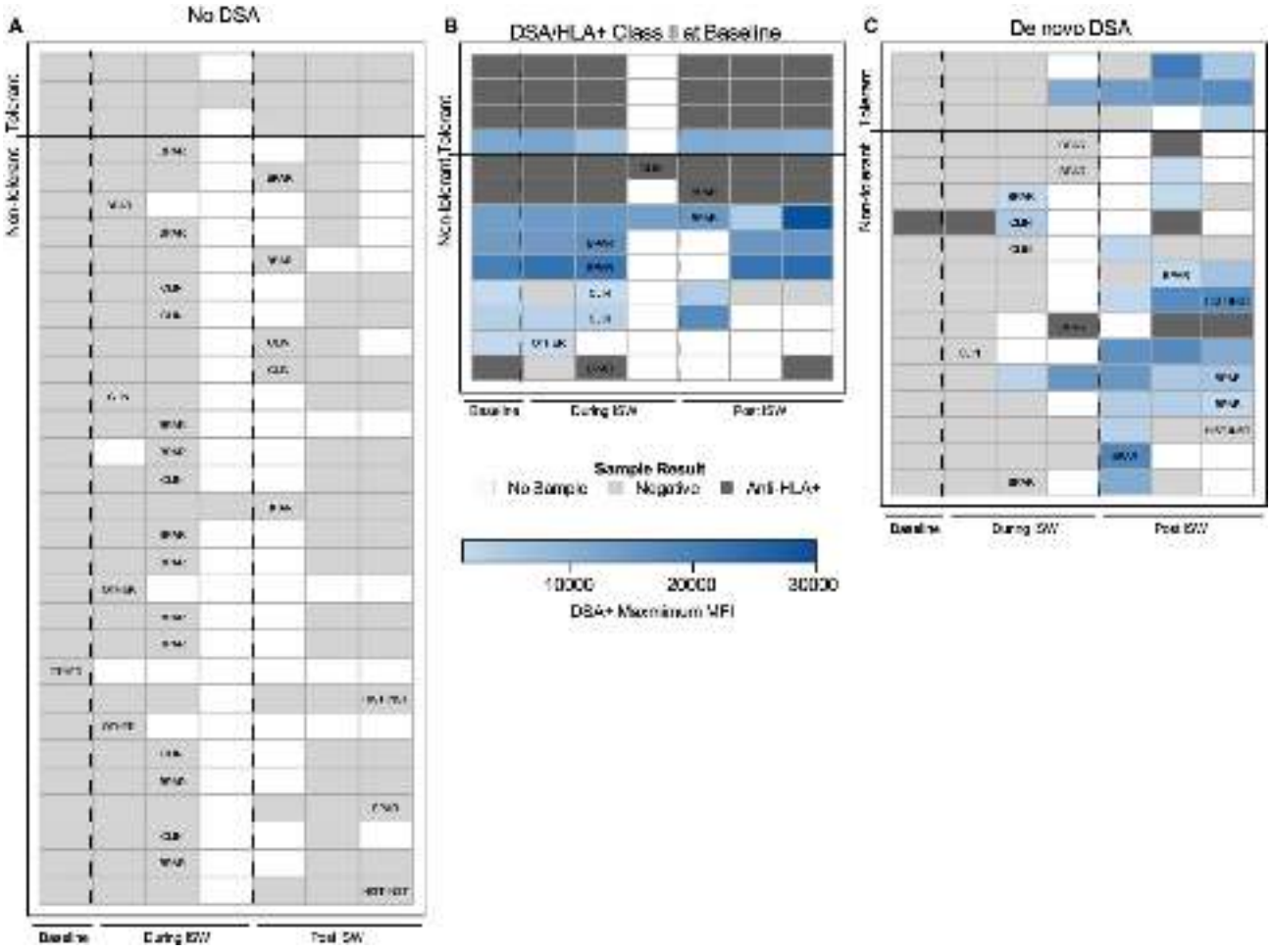

Figure 5. Evolution of donor-specific antibodies (DSAs) during the study. This figure displays heat maps of DSA in subjects who underwent ISW. (A) Subjects who did not have baseline DSA nor de novo DSA detected during the study. (B) Subjects who had DSA at baseline but did not develop de novo DSA during the study. (C) Subjects who developed de novo DSA during the study. Each row represents a single subject. Horizontal bold black lines separate nontolerant from tolerant subjects. Nontolerant reason is overlaid at the visit closest to the event: BPAR, clinical rejection, histologic instability (HIST INST), and other. Vertical dotted lines separate the phases: baseline, during ISW, and post-ISW. Blue cells indicate samples positive for DSA, light gray indicate no DSA, and dark gray cells indicate samples in which HLA antibody was detected but could not be categorized as DSA due to missing or inadequate donor typing data. White cells indicate that no sample was collected. Rates of tolerance were not significantly associated with presence or absence of DSA at baseline or with the emergence of de novo DSA during the study. BPAR, biopsy proven acute rejection; CLIN, clinical rejection (no biopsy); HLA, human leukocyte antigen; ISW, immunosuppression withdrawal; MFI, mean fluorescent intensity.

by portal capillary and sinusoidal positivity) were ultimately deemed nontolerant (Fig. 7). Three of these 7 (42.8%) had baseline class II HLA antibody identified as DSA to HLA-DR7 and HLA-DR53, HLA-DQA05 alone, and HLA-DQ2 alone, respectively.

3.10.2. Multiplex immunohistochemistry and analysis of ai-iSYN

ai-iSYN were characterized and defined in the baseline biopsies (Fig. 8A, B). Density (in cells per square millimeter) of lobular $CD8^+$ cells, previously shown to predict nontolerance,[28] was higher in biopsies of nontolerant subjects when compared with that in tolerant subjects (Fig. 8C),[24] but these differences were not statistically significant. No correlation was observed between increased metabolic dysfunction-associated liver disease fibrosis scores and ai-iSYN density. Stratification of the $CD8^+$ cell population into $CD8^+/CD45^{hi}$ and $CD8^+/CD45^{lo}$ demonstrated an increased proportion of $CD8^+/CD45^{hi}$ cells (but not $CD8^+/CD45^{lo}$ cells) in baseline biopsies of nontolerant subjects vs those of tolerant subjects ($P = .002$) (Fig. 8E, F).

The density of ai-iSYNs in the baseline biopsy, also previously shown by us to predict nontolerance,[25] was higher in nontolerant subjects than that in tolerant subjects ($P = .009$) in this study (Fig. 8D). As shown in Figure 8G, we were able to segregate tolerant subjects, contained inside the 3D scatter plot (green circles; n = 9) and nontolerant subjects (brown circles; n = 45) according to the number of lobular $CD8^+$ cells/mm^2 (T effector cells; x-axis), $MAC387^+$ cells/mm^2 (calprotectin/infiltrating macrophages; y-axis), and lobular $CD45^{hi}MHCII^+$pairs/mm^2 (leukocyte/antigen-presenting cell pairs; z-axis). The inner shaded cube identifies thresholds that simultaneously maximize the number of tolerant subjects (8 of 9; 88.9%) and minimize the number of nontolerant subjects (19 of 43; 42.2%). Plots only show subjects for whom values of all 3 parameters were available.

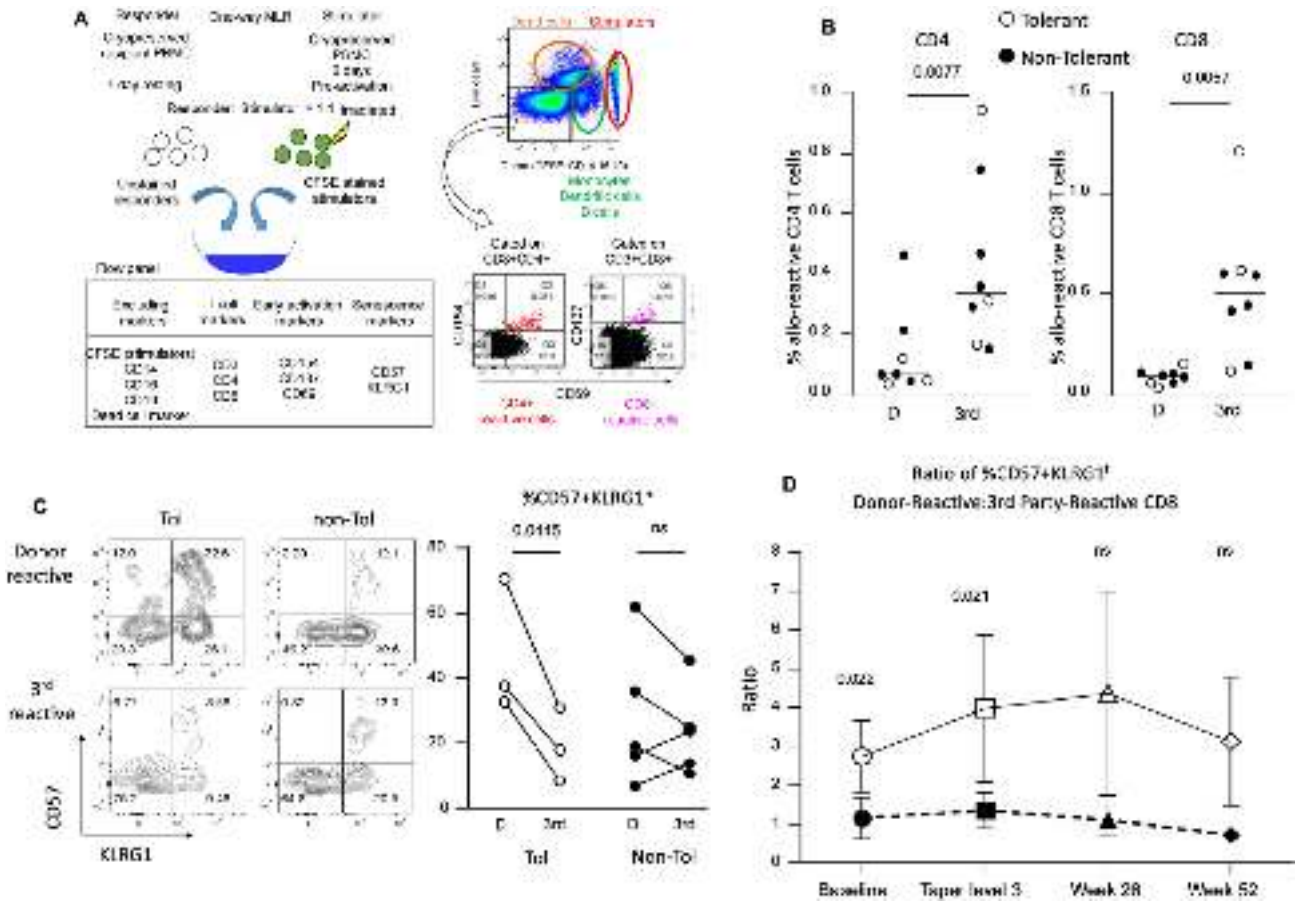

Figure 6. Accumulation of donor-specific senescence in alloreactive CD8$^+$ T cells characterize recipients who achieved operational tolerance prior to initiation of ISW. (A) Alloreactive T cell detection assay identified donor-reactive CD4/CD8 T cells in 1-way mixed lymphocyte reaction. (B) The dots plots show the percentage of alloreactive CD4 and CD8 T cells in PBMC from tolerant (open) and nontolerant (closed) subjects at baseline. (C) Representative flow plots demonstrate a CD57$^+$KLRG1^{hi} population accumulating in donor-reactive CD8$^+$ T cells (upper plots) compared with third party-reactive CD8 (lower plots) in tolerant recipients (Tol). No greater accumulation is found in donor-reactive CD8$^+$ T cells in nontolerant (non-Tol) subjects. The paired plots demonstrate the status of CD8$^+$ T cells at baseline. In tolerant subjects, the proportion of CD57$^+$KLRG1^{hi} in donor-reactive CD8$^+$ T cells (D) is significantly higher than third party-reactive cells (3rd) at baseline. Statistical analyses were performed using Student paired *t* tests. (D) Stable accumulation of the senescence phenotype is found in donor-reactive CD8$^+$ T cells in tolerant recipients. The line graph shows the mean ratio of %CD57$^+$KLRG1^{hi} in donor-reactive vs third party-reactive CD8$^+$ T cells from tolerant and nontolerant recipients. The bars and symbols indicate standard deviation and time points, respectively: baseline: circle (Tol, n = 3/non-Tol, n = 5); taper level 3: square (Tol, n = 3/non-Tol, n = 5); and 26 weeks: triangle (Tol, n = 3/non-Tol, n = 4) and 52 weeks: diamond (Tol, n = 2/non-Tol, n= 3) after ISW completion/rejection). Tolerant, open/empty symbol; nontolerant, closed/filled symbol. Statistical analyses were performed using unpaired *t* tests at each time point. CFSE, carboxyfluorescein succinimidyl ester; ISW, immunosuppression withdrawal; KLRG1, killer-cell lectin like receptor G1; MLR, mixed lymphocyte reaction; PBMC, peripheral blood mononuclear cell.

4. Discussion

We conducted an ISW trial in stable long-term liver transplant recipients and found a rate of operational tolerance of ~16%, lower than that reported by Benitez et al,[5] despite stringent candidate selection in OPTIMAL that included both normal graft function based on liver tests and graft biopsy to exclude occult pathology. The differences in outcome of ISW between the 2 studies may be related to differences in baseline cohort characteristics including a higher prevalence of viral etiology of liver disease and active HCV viremia as well as lower level of starting immunosuppression in the cohort described by Benitez et al.[5] On the other hand, the rate of tolerance in OPTIMAL is comparable to that observed (11%) in another ITN study, A-WISH, although in that study, the earlier timing of ISW was thought to have been a contributing factor.[29]

The low rate of tolerance in OPTIMAL prompts a reexamination of the risk: benefit calculus of ISW. Subjects in OPTIMAL underwent stringent screening and close monitoring, including serial biopsies. The large majority of acute rejection cases was mild and nearly half responded to resumption of immunosuppression alone. At the end of the study 62% were on same or lower doses of CNI than at study entry and 18% were free of immunosuppression. This may reflect an untapped potential for immunosuppression minimization in stable liver transplant recipients, as also indicated in the A-WISH study. Conversely, 7 of 17 subjects (41%) with

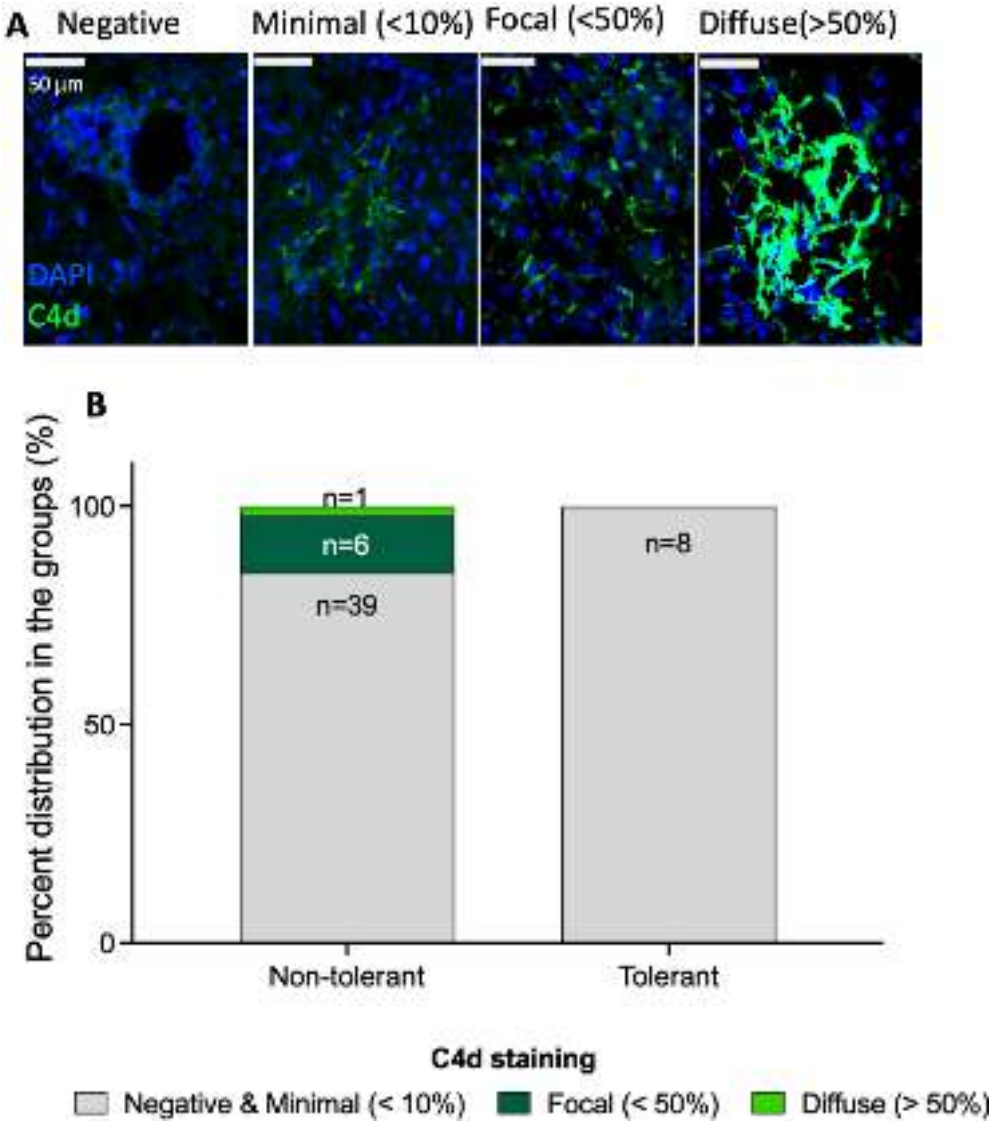

Figure 7. C4d staining on baseline biopsy in tolerant and nontolerant subjects. (A) Representative images of immunofluorescence staining for C4d (green) separated according to score. Nuclei are shown in blue. Bars = 50 µm. (B) Graphic representation of the percentage distribution of C4d deposition according to the score (negative 0%, minimal <10%, focal <50%, and diffuse >50% C4d deposition stained in the respective areas) in nontolerant (n = 46) and tolerant (n = 8) subjects. C4d staining quality was inadequate in biopsy samples from 2/10 tolerant and 5/51 nontolerant subjects.

stable liver enzymes for 1 year post-ISW were found to have either acute rejection or histologic instability on biopsy, highlighting the need for surveillance biopsies. Additionally, a small but significant proportion (28.3%) of subjects developed de novo DSA during ISW, which persisted in in 11.3%, including 3 subjects deemed tolerant. Although no correlation was identified between DSA and the change in LAFSc, longer follow-up may be needed to truly identify the impact of ISW on the evolution of DSA and liver fibrosis and to monitor those who show worsened inflammation or fibrosis after weaning; transient elastography could be considered for monitoring fibrosis progression.

Although the low rate of success reduced our ability to identify robust clinical predictors of tolerance, we explored several candidate biomarkers. An interesting observation was that subjects with favorable histology were older and more likely to have hepatic steatosis or low-grade steatohepatitis than those with ineligible screening biopsies (50.8% vs 9.7%). The risk of hepatic steatosis/steatohepatitis in native livers increases with age[30]; whether this might be a surrogate marker for immune senescence is uncertain, but worth further study. Of additional interest were 7 subjects with positive C4d staining on their baseline biopsies who were all ultimately deemed nontolerant, similar to the WISP-R study,[6] although this difference was not statistically significant in our study. We were unable to find a statistical correlattion of DSA at baseline or its development during ISW with operational tolerance, unlike previous studies,[25,31] possibly due to the small number of tolerance events and the potential for antibody adsorption by the liver.

Two assays of immune reactivity revealed potentially informative associations with tolerance/nontolerance. First, a next-generation pathologic analysis was conducted to detect antigen-presenting cell-lymphocyte pairs (immune synapses: ai-iSYN) as indicators of ongoing alloimmune activity. A lower number of ai-iSYN was associated with the achievement of tolerant status, as previously reported in a pediatric cohort. The clinical usefulness of this assay may lie in its ability to exclude subjects with the lowest chances of success at screening, thereby creating a more favorable risk: benefit profile in the patients who undergo weaning. Although not directly tested in this study, the lobular ai-iSYN engaged antigen-presenting cells are likely to be recipient-derived Kupffer cells[32–34] and implicate

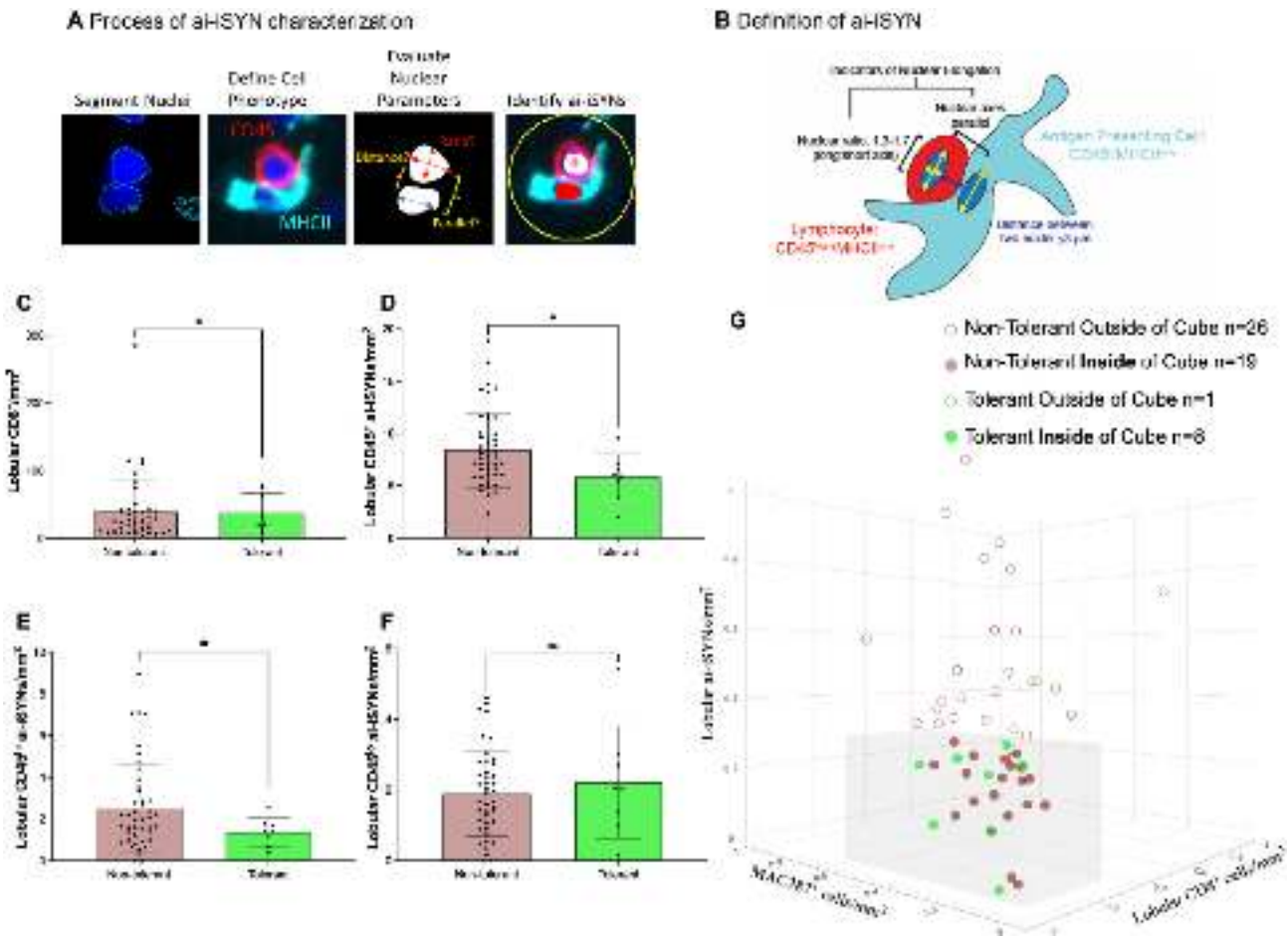

Figure 8. Identification of ai-iSYNs in baseline biopsies correlates with elevated risk of rejection. Images (A) and schematic diagram (B) explaining the process of ai-iSYNs characterization and definition as previously described.[24] Slides queried with multiplex IHC for CD45/MHCII/CD34/DAPI were subjected to unbiased digital analysis. Analysis first identified nuclei based on DAPI (blue) staining to create objects. Objects were then defined as different cell types based on the staining characteristics of CD45 (red) and MHCII (cyan). If CD45 and MHCII cells were found to be in proximity of each other, their nuclear characteristics were evaluated for distance (<3 μm), and elongation of the nucleus of the $CD45^+$ cells as a marker of flattening (ratio of 1.3-1.7 long-short axis). The axes of both nuclei were also compared to determine whether the long axes were parallel. Together, distance, nuclear flattening of $CD45^+$ cells, and parallel nuclei are used as surrogate markers of activated immunologic synapses. (C-G) Graphical representation of baseline biopsies subjected to multiplex IHC with CD34/CD45/CD8/MHCII/MAC387. (C) Lobular $CD8^+$ cells/mm^2 alone did not distinguish between nontolerant and tolerant subjects. (D) Detection of ai-iSYNs/mm^2 (defined in methods as $CD45^+$/MHCII any paired with MHCII only plus nuclear rules) was significantly increased in nontolerant subjects over tolerant subjects($^*P = .009$). When CD45 cells in ai-iSYNs were further stratified based on expression level into (E) $CD45^{high}$ vs (F) $CD45^{low}$, only $CD45^{high}$ containing ai-iSYNs were able to predict tolerant vs nontolerant subjects on baseline biopsies ($^*P = .002$). (G) Combining lobular $CD8^+$ cells/mm^2 (x-axis) with $MAC387^+$ cells/mm^2 (y-axis) and $CD45^{high}$ containing ai-iSYNs/mm^2 (z-axis) allows formulation of a prediction cube for this subset of subjects. The gray-shaded box maximizes the number of subjects who will ultimately become operationally tolerant (8/9 tolerant subjects within the box), although minimizing the number of subjects who fail ISW (19/43 subjects within the box). Ranges for the inner cube correspond to (1) lobular $CD8^+$: 0 to 79.4 cells/mm^2; (2) $MAC387^+$: 0 to 18.1 cells/mm^2; (3) lobular $CD45^{high}$ ai-iSYNs: 0 to 7.1 pairs/mm^2. Subjects within the inner cube are closed symbols; subjects outside the inner cube are open symbols. Brown are nontolerant although green are tolerant. ai-iSYN, automated image detection of immune synapses; IHC, immunohistochemistry; ISW, immunosuppression withdrawal; MHC, major histocompatibility complex.

indirect major histocompatibility complex allorecognition, as observed in previous liver allograft studies.[24,25] Conversely, the majority of ai-iSYN engaged lymphocytes are $CD8^+$/$CD45^{hi}$ in nontolerant recipients.[35] Similar approaches are being used to monitor immune activation in tumor microenvironments.[36]

Second, we explored whether the long-term residence of a liver allograft might predispose induction of senescence or exhaustion in graft-reactive T cells. A novel assay was developed to detect donor versus self-reactive and third party-reactive lymphocytes and to assess the reactive T cells for markers of senescence and exhaustion.[22] This was accomplished using early activation markers to reveal activation of donor-specific and third party–specific T cells in a short-term in vitro mixed lymphocyte reaction-like assay, a period during which the expression of several exhaustion and senescence markers remained unperturbed. This analysis identified an increase in the percentage of $CD57^+KLRG1^{hi}$ donor-reactive $CD8^+$ T cells, compared with third party-reactive $CD8^+$ T cells in tolerant subjects, with $CD57^+KLRG1^{hi}$ levels that remained relatively stable during the study. Expression levels of CD57 and KLRG1 did not

differ between donor and third party-reactive $CD4^+$ T cells. Although only a small number of samples was available for this analysis and we did not investigate whether these findings were replicated in intrahepatic T cells,[37] these observations are consistent with previous studies showing that tolerance in HCV-positive liver transplant recipients is associated with an expansion of exhausted PD1/CTLA4/2B4-positive HCV-specific circulating $CD8^+$ T cells[17] and intrahepatic overexpression of type I interferon and immunoregulatory genes.

Relevant here is an interesting set of parallel observations in studies of patients with type 1 diabetes mellitus, where $CD8^+$ T cell exhaustion profiles (both $CD57^+$ and $KLRG1^+$) appear to be induced by alefacept, a CD2 agonist.[38,39] Alefacept depletes T cells with high CD2 expression but exhibits an agonist function in naïve T cells and T regulatory cells with low CD2 expression, pushing both CD4 and CD8 cells toward terminal hyporesponsive states. The CD8 effect involves a $KLRG1^+TIGIT^+$ profile resembling exhaustion and evolving into either a $CD57^+$ or a KIR^+ terminal lineage, both of which correlate with tolerance.[38] Conceptually based, in part, on these results, we have initiated (1) studies to determine the prevalence and developmental stage of T cells with senescent/exhausted phenotypes within liver biopsy tissues to allow direct assay of graft tolerance within the organ rather than in circulating cell populations and (2) the development of a follow-on liver trial to ascertain whether, during ISW withdrawal, the administration of siplizumab, a monoclonal antibody to CD2 currently in clinical development, can enhance the proportion of successful weaning by augmenting donor-specific T cell exhaustion.

In summary, we were unable to identify robust predictors of tolerance in OPTIMAL due to a low rate of tolerant subjects (16%). However, we identified signals and markers meriting further investigation as follows: (1) absence of correlation of circulating DSA and tolerance outcome; (2) C4d deposition in the liver microvasculature at baseline, which was associated with DSA in 50%; (3) higher ai-iSYN density in the allograft, an indicator of ongoing occult alloreactivity that may be useful as a predictor of ISW failure; and (4) preliminary evidence that donor-specific $CD8^+$ T cell senescence may be a candidate marker for and a mechanism of operational tolerance. Collectively, these results suggest predictors of operational tolerance/nontolerance in liver transplant recipients that may aid in identifying candidates for ISW and guide the use of novel interventions to better foster spontaneous liver graft-induced tolerance in future studies.

Acknowledgments

The authors thank Brian Armstrong at Rho for lending additional statistical support to make revisions to the manuscript after initial review.

Funding

The study was funded by the Immune Tolerance Network, an international clinical research consortium headquartered at the Benaroya Research Institute and supported by the National Institute of Allergy and Infectious Diseases of the National Institutes of Health under Award Number UM1AI109565. Rho provided statistical support under Award Number UM2AI117870. 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 no conflicts of interest to disclose as described by the American Journal of Transplantation.

Data availability

The data that support the findings of this study are openly available in trial share at https://www.itntrialshare.org.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ajt.2024.10.022.

ORCiD

Naoki Tanimine https://orcid.org/0000-0003-2744-9097
James F. Markmann https://orcid.org/0000-0002-2762-6535
Michelle A. Wood-Trageser https://orcid.org/0000-0002-1880-4926
Anthony J. Demetris https://orcid.org/0000-0002-9582-3733
Kristen Mason https://orcid.org/0000-0002-4721-2639
Juliete A.F. Silva https://orcid.org/0000-0002-4543-5169
Josh Levitsky https://orcid.org/0000-0001-7527-6093
Sandy Feng https://orcid.org/0000-0002-2601-4350
Abhinav Humar https://orcid.org/0000-0002-6430-1000
Jean C. Emond https://orcid.org/0000-0003-1642-9242
Abraham Shaked https://orcid.org/0000-0002-2162-095X
Goran Klintmalm https://orcid.org/0000-0003-0916-6042
Alberto Sanchez-Fueyo https://orcid.org/0000-0002-8316-3504
Cynthia P. Breeden https://orcid.org/0000-0001-8716-4435
Gerald T. Nepom https://orcid.org/0000-0002-8063-1464
Nancy D. Bridges https://orcid.org/0000-0002-1140-5201
Christian P. Larsen https://orcid.org/0000-0001-6573-2649
Michele DesMarais https://orcid.org/0000-0002-0063-907X
Geo Gaile https://orcid.org/0009-0000-5929-5710
Sindhu Chandran https://orcid.org/0000-0003-4547-3381

References

1. Bohne F, Martínez-Llordella M, Lozano JJ, 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/JCI5941.
2. Lerut J, Sanchez-Fueyo A. An appraisal of tolerance in liver transplantation. *Am J Transplant*. 2006;6(8):1774–1780. https://doi.org/10.1111/j.1600-6143.2006.01396.x.
3. Mazariegos GV, Reyes J, Marino IR, et al. Weaning of immunosuppression in liver transplant recipients. *Transplantation*. 1997;63(2):243–249. https://doi.org/10.1097/00007890-199701270-00012.
4. Appenzeller-Herzog C, Rosat A, Mathes T, et al. Time since liver transplant and immunosuppression withdrawal outcomes: systematic

review and individual patient data meta-analysis. *Liver Int*. 2024;44:250–262. https://doi.org/10.1111/liv.15764.

5. 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.
6. 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.
7. Appay V, Almeida JR, Sauce D, Autran B, Papagno L. Accelerated immune senescence and HIV-1 infection. *Exp Gerontol*. 2007;42(5):432–437. https://doi.org/10.1016/j.exger.2006.12.003.
8. Campisi J, d'Adda di Fagagna F. Cellular senescence: when bad things happen to good cells. *Nat Rev Mol Cell Biol*. 2007;8(9):729–740. https://doi.org/10.1038/nrm2233.
9. D'Souza M, Fontenot AP, Mack DG, et al. Programmed death 1 expression on HIV-specific CD4+ T cells is driven by viral replication and associated with T cell dysfunction. *J Immunol*. 2007;179(3):1979–1987. https://doi.org/10.4049/jimmunol.179.3.1979.
10. Effros RB, Dagarag M, Spaulding C, Man J. The role of CD8+ T-cell replicative senescence in human aging. *Immunol Rev*. 2005;205:147–157. https://doi.org/10.1111/j.0105-2896.2005.00259.x.
11. Fletcher JM, Vukmanovic-Stejic M, Dunne PJ, et al. Cytomegalovirus-specific CD4+ T cells in healthy carriers are continuously driven to replicative exhaustion. *J Immunol*. 2005;175(12):8218–8225. https://doi.org/10.4049/jimmunol.175.12.8218.
12. Kono H, Rock KL. How dying cells alert the immune system to danger. *Nat Rev Immunol*. 2008;8(4):279–289. https://doi.org/10.1038/nri2215.
13. Pawelec G, Wagner W, Adibzadeh M, Engel A. T cell immunosenescence in vitro and in vivo. *Exp Gerontol*. 1999;34(3):419–429. https://doi.org/10.1016/s0531-5565(99)00002-9.
14. Spaulding C, Guo W, Effros RB. Resistance to apoptosis in human CD8+ T cells that reach replicative senescence after multiple rounds of antigen-specific proliferation. *Exp Gerontol*. 1999;34(5):633–644. https://doi.org/10.1016/s0531-5565(99)00033-9.
15. Voehringer D, Blaser C, Brawand P, Raulet DH, Hanke T, Pircher H. Viral infections induce abundant numbers of senescent CD8 T cells. *J Immunol*. 2001;167(9):4838–4843. https://doi.org/10.4049/jimmunol.167.9.4838.
16. Akbar AN, Henson SM. Are senescence and exhaustion intertwined or unrelated processes that compromise immunity? *Nat Rev Immunol*. 2011;11(4):289–295. https://doi.org/10.1038/nri2959.
17. Bohne F, Londoño MC, 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.
18. Merritt E, Londoño MC, Childs K, et al. On the impact of hepatitis C virus and heterologous immunity on alloimmune responses following liver transplantation. *Am J Transplant*. 2021;21(1):247–257. https://doi.org/10.1111/ajt.16134.
19. Banff schema for grading liver allograft rejection: an international consensus document. *Hepatology*. 1997;25(3):658–663. https://doi.org/10.1002/hep.510250328.
20. Demetris A, Adams D, Bellamy C, et al. Update of the International Banff Schema for Liver Allograft Rejection: working recommendations for the histopathologic staging and reporting of chronic rejection. An international panel. *Hepatology*. 2000;31(3):792–799.
21. Banff Working Group on Liver Allograft Pathology. 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.
22. Tanimine N, Burrell BE, Deng K, et al. Detection of alloreactive T cells from cryopreserved human peripheral blood mononuclear cells. *J Immunol Methods*. 2021;491:112987. https://doi.org/10.1016/j.jim.2021.112987.
23. Feng S, Bucuvalas JC, Demetris AJ, et al. Evidence of chronic allograft injury in liver biopsies from long-term pediatric recipients of liver transplants. *Gastroenterology*. 2018;155(6):1838–1851.e7. https://doi.org/10.1053/j.gastro.2018.08.023.
24. 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.
25. 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.
26. Venturi C, Sempoux C, Bueno J, et al. Novel histologic scoring system for long-term allograft fibrosis after liver transplantation in children. *Am J Transplant*. 2012;12(11):2986–2996. https://doi.org/10.1111/j.1600-6143.2012.04210.x.
27. Hübscher SG, Feng S, Gouw ASH, et al. Standardizing the histological assessment of late posttransplantation biopsies from pediatric liver allograft recipients. *Liver Transpl*. 2022;28(9):1475–1489. https://doi.org/10.1002/lt.26482.
28. Wong T, Nouri-Aria KT, Devlin J, Portmann B, Williams R. Tolerance and latent cellular rejection in long-term liver transplant recipients. *Hepatology*. 1998;28(2):443–449. https://doi.org/10.1002/hep.510280223.
29. Shaked A, DesMarais MR, Kopetskie H, et al. Outcomes of immunosuppression minimization and withdrawal early after liver transplantation. *Am J Transplant*. 2019;19(5):1397–1409. https://doi.org/10.1111/ajt.15205.
30. Bertolotti M, Lonardo A, Mussi C, et al. Nonalcoholic fatty liver disease and aging: epidemiology to management. *World J Gastroenterol*. 2014;20(39):14185–14204. https://doi.org/10.3748/wjg.v20.i39.14185.
31. Jucaud V, Shaked A, DesMarais M, et al. Prevalence and impact of de novo donor-specific antibodies during a multicenter immunosuppression withdrawal trial in adult liver transplant recipients. *Hepatology*. 2019;69(3):1273–1286. https://doi.org/10.1002/hep.30281.
32. Fan X, Lu P, Cui XH, et al. Repopulating Kupffer cells originate directly from hematopoietic stem cells. *Stem Cell Res Ther*. 2023;14(1):351. https://doi.org/10.1186/s13287-023-03569-0.
33. Gouw AS, Houthoff HJ, Huitema S, Beelen JM, Gips CH, Poppema S. Expression of major histocompatibility complex antigens and replacement of donor cells by recipient ones in human liver grafts. *Transplantation*. 1987;43(2):291–296. https://doi.org/10.1097/00007890-198702000-00025.
34. Steinhoff G. Major histocompatibility complex antigens in human liver transplants. *J Hepatol*. 1990;11(1):9–15. https://doi.org/10.1016/0168-8278(90)90264-r.
35. Courtney AH, Shvets AA, Lu W, et al. CD45 functions as a signaling gatekeeper in T cells. *Sci Signal*. 2019;12(604). https://doi.org/10.1126/scisignal.aaw8151.
36. Wang VG, Liu Z, Martinek J, et al. Computational immune synapse analysis reveals T-cell interactions in distinct tumor microenvironments. *Preprint. Posted online June 1*. 2023. https://doi.org/10.21203/rs.3.rs-2968528/v1. Res Sq rs.3.rs-2968528.
37. Mederacke YS, Nienen M, Jarek M, et al. T cell receptor repertoires within liver allografts are different to those in the peripheral blood. *J Hepatol*. 2021;74(5):1167–1175. https://doi.org/10.1016/j.jhep.2020.12.014.
38. Diggins KE, Serti E, Muir V, et al. Exhausted-like CD8+ T cell phenotypes linked to C-peptide preservation in alefacept-treated T1D subjects. *JCI Insight*. 2021;6(3). https://doi.org/10.1172/jci.insight.142680.
39. Wiedeman AE, Muir VS, Rosasco MG, et al. Autoreactive CD8+ T cell exhaustion distinguishes subjects with slow type 1 diabetes progression. *J Clin Invest*. 2020;130(1):480–490. https://doi.org/10.1172/JCI126595.