

# Five-Year Histological and Serological Follow-up of Operationally Tolerant Pediatric Liver Transplant Recipients Enrolled in WISP-R

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Pediatric liver transplant recipients arguably have the most to gain and the most to lose from discontinuing immunosuppression (IS). Whereas IS undoubtedly exerts a cumulative toll, there is concern that insufficient or no IS may contribute to allograft deterioration. Twelve pediatric recipients of parental living donor liver grafts, identified as operationally tolerant through complete IS withdrawal (WISP-R; NCT00320606), were followed for a total of 5 years (1 year of IS withdrawal and 4 years off IS) with serial liver tests and autoantibody and alloantibody assessments. Liver biopsies were performed 2 and 4 years off IS, and, at these time points, immunoglobulin G (IgG) subclass and C1q binding activity for donor-specific antibodies (DSAs) were determined. There were no cases of chronic rejection, graft loss, or death. Allografts did not exhibit progressive increase in inflammation or fibrosis. Smooth-muscle actin expression by stellate cells and CD34 expression by liver sinusoidal endothelial cells remained stable, consistent with the absence of progressive graft injury. Three subjects never exhibited DSA. However, 3 subjects showed intermittent *de novo* class I DSA, 4 subjects showed persistent *de novo* class II DSA, and 5 subjects showed persistent preexisting class II DSA. Class II DSA was predominantly against donor DQ antigens, often of high mean fluorescence intensity, rarely of the IgG3 subclass, and often capable of binding C1q. *Conclusion:* Operationally tolerant pediatric liver transplant recipients maintain generally stable allograft histology in spite of apparently active humoral alloimmune responses. The absence of increased inflammation or progressive fibrosis suggests that a subset of liver allografts seem resistant to the chronic injury that is characteristic of antibody-mediated damage. (HEPATOLOGY 2017;65:647-660)

## SEE EDITORIAL ON PAGE 411

**O**peration tolerance—the maintenance of stable allograft function and histology in the complete absence of immunosuppression

(IS)—has now been demonstrated through clinical trials of IS withdrawal conducted for both adult and pediatric liver transplant recipients.<sup>(1)</sup> These trials have typically enrolled stable, long-term liver transplant recipients and gradually reduced IS dosing in a

*Abbreviations:* AE, adverse event; ALT, alanine aminotransferase; DSA, donor-specific antibody; GGT, gamma-glutamyl transpeptidase; HLA, human leukocyte antigen; HR, hazard ratio; IgG, immunoglobulin G; IL, interleukin; IS, immunosuppression; LSEC, liver sinusoidal endothelial cell; MFI, mean fluorescence intensity; MHC, major histocompatibility complex; NRH, nodular regenerative hyperplasia; SAE, serious adverse event; SMA, smooth-muscle actin.

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structured manner under close supervision. With the framework of a clinical trial, IS withdrawal can be attempted safely. The episodes of acute rejection that occurred, with prompt diagnosis and treatment, were readily reversed and thus did not appear to exert a negative impact beyond the transient exposure to increased IS. Treatment has typically consisted of increased doses of IS, occasionally bolus corticosteroids, and rarely administration of an antibody preparation.

Although there is now general acceptance that reducing IS can be safely attempted with close monitoring, the long-term impact of IS minimization or discontinuation on allograft health remains controversial. Within the IS withdrawal trials, assessment of tolerance typically occurs 1 year after the last dose of IS and is based on biochemical profile with or without histological assessment. For adult liver transplant recipients, there has been only a single publication delineating the histological status of eight tolerant allografts for a mean (range) of 78 (57-109) months after IS discontinuation.<sup>(2)</sup> This experience, however, has limited generalizability because all subjects were adults with hepatitis C infection.

The concern for long-term allograft health is of particular concern for pediatric liver transplant recipients who require optimal graft longevity. It is now widely recognized that children maintained on standard-of-care IS experience clinically silent deterioration of liver histology over time. Multiple cross-sectional, single-center studies have consistently shown that liver allografts in children exhibit a higher prevalence of inflammation/hepatitis and fibrosis with increased time after transplantation.<sup>(3-8)</sup> Moreover, a cohort of operationally tolerant pediatric living donor liver transplant recipients, compared to a cohort maintained on IS, exhibited significantly higher fibrosis stages, although

the cohorts differed in several demographic parameters, such as age at and time after transplantation.<sup>(9)</sup> Risk factors for fibrosis identified by more than one study include deceased donor grafts, prolonged cold ischemia time, and presence of autoantibodies. The early reports of children maintained on standard-of-care IS have not correlated history of rejection and the nature of the IS regimen, including the use of corticosteroids, with the development of fibrosis. In more-recent reports, some of which include children who have undergone IS minimization, detection of donor-specific antibodies (DSAs) and positive staining for C4d has been associated with fibrosis, implicating a role for humoral allo-immune responses.<sup>(5,10-12)</sup> Finally, reinstatement of IS for those who have undergone withdrawal or intensification of IS for those maintained on standard IS each have been reported to stabilize and even reverse fibrosis, implicating insufficient IS as a potential mechanism driving chronic allograft damage.<sup>(6,9,13)</sup>

We have conducted and reported on a prospective pilot trial of IS withdrawal for pediatric recipients of living donor liver allografts (WISP-R; NCT00320606).<sup>(14)</sup> Among the 20 subjects enrolled at three centers, 12 were operationally tolerant, 7 experienced acute rejection, and 1 was withdrawn from the study secondary to a violation of inclusion/exclusion criteria. We now report on the 5-year follow-up of the 12 tolerant children. Serial allograft biopsies demonstrate architectural preservation without increased inflammation or progressive fibrosis. However, longitudinal testing shows frequent DSA in the majority of tolerant subjects. Juxtaposition of the histological and the alloantibody data raises the intriguing possibility that the liver, compared to other organs, may possess intrinsic mechanisms that can resist allograft deterioration by an ongoing or active allo-immune response.

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# Patients and Methods

## SUBJECTS AND ASSESSMENTS

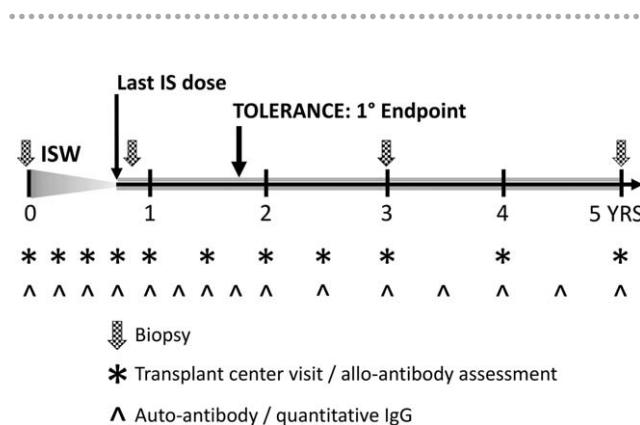
WISP-R (NCT00320606) was a prospective trial of IS withdrawal conducted at three pediatric liver transplant centers in the United States. Written informed assent (as appropriate) and/or consent were obtained from all subjects and/or their legal guardian, respectively. The clinical trial protocol was reviewed and approved by the institutional review board of participating centers. None of the participating transplant centers utilize organs procured from executed prisoners.

WISP-R identified 12 operationally tolerant pediatric liver transplant recipients who maintained stable liver test profiles (alanine aminotransferase (ALT), aspartate aminotransferase, total and direct bilirubin, gamma-glutamyl transpeptidase (GGT), and alkaline phosphatase) for 12 months after complete IS discontinuation. From the time of primary endpoint assessment and the determination of operational tolerance (year 2; Fig. 1), the primary trial extended for 3 years. A single participant withdrew consent 33.3 months after achieving the primary endpoint.

After determination of operational tolerance, liver tests were performed monthly with visits to the transplant center biannually for 1 year. Participants then transitioned to liver tests every 2 months with annual clinic visits. Two protocol biopsies were required, 2 and 4 years after the last IS dose (year 3 and year 5, respectively; Fig. 1). Alloantibodies were monitored biannually for 1 year and then annually. Autoantibodies and quantitative immunoglobulin G (IgG) were monitored quarterly for 1 year, then biannually for 2 years, and then annually thereafter.

## ROUTINE AND SPECIALIZED HISTOPATHOLOGY STUDIES

High-resolution 40 $\times$  whole-slide images of formalin-fixed, paraffin-embedded, and hematoxylin-eosin-stained 4-mm tissue sections were prospectively scored for 42 histopathological criteria (Supporting Table S1). Glass slides were also reviewed at the year 0 and year 5 points. Portal, lobular, and perivenular inflammation along with portal/periportal, Disse, and perivenular fibrosis were graded and staged, respectively (0 = none; 1 = mild [detectable, above baseline]; 2 = moderate; 3 = severe) with a total score range of 0 to 9 for both.



**FIG. 1.** WISP-R timeline for transplant center visits, antibody assessments, and liver biopsies. Schematic showing timeline of IS withdrawal, assessment of primary endpoint, and long-term follow-up, including timing and frequency of transplant center visit and liver biopsies

C4d deposition on snap-frozen tissue was evaluated blindly using both single immunofluorescence (mouse monoclonal, A213; Quidel, San Diego, CA) and multiplex quantum dot immunostaining (rabbit polyclonal BI-RC4d, 1:30; Alpco Diagnostics, Salem, NH), CD31 (mouse monoclonal JC/70A; ThermoFisher, Pittsburgh, PA), and major histocompatibility class complex (MHC) class II (mouse monoclonal CR3/43; MO775; Dako, Carpinteria, CA). Four vascular endothelial compartments (portal capillary and vein, sinusoidal, and central vein) were separately scored (0 = none; 1 = minimal; 2 = focal; 3 = diffuse) and summed for total C4d and MHC II scores (range, 0-12).

Changes in liver sinusoidal endothelial cell (LSEC)<sup>(15)</sup> and stellate cell phenotype<sup>(16)</sup> before and after IS withdrawal was studied by comparing similarly sized portal tracts, central veins, and sinusoids in the preweaning versus year 5, formalin-fixed, paraffin-embedded biopsy multiplex-stained for CD34 (mouse monoclonal, QBE-10; Dako) and smooth-muscle actin (SMA; mouse monoclonal, 1A4; Dako).

## HUMAN LEUKOCYTE ANTIGEN TYPING, ALLO-ANTIBODY DETECTION, IgG SUBCLASS DETERMINATION, AND C1q ASSAY

Human leukocyte antigen (HLA) typing was performed by automated DNA sequencing (University of California San Francisco, San Francisco, CA). HLA antibody screening and specificity determination were performed using FlowPRA Screening and LabScreen

**TABLE 1. Characteristics of 12 Operationally Tolerant Participants from WISP-R**

| Characteristic                        | N = 12                               |
|---------------------------------------|--------------------------------------|
| Age*                                  | 0.6 (0.3, 2.4)                       |
|                                       | At transplant                        |
|                                       | At study entry                       |
| Sex†                                  | 9.0 (5.2, 12.1)                      |
|                                       | Male                                 |
| Liver disease†                        | 9                                    |
|                                       | Biliary atresia                      |
|                                       | A-1 antitrypsin                      |
|                                       | Familial cholestasis (Byler disease) |
|                                       | Neonatal sclerosing cholangitis      |
| Calcineurin inhibitor at study entry† | 1                                    |
|                                       | Tacrolimus                           |
| ALT (U/L)*                            | 7                                    |
| GGT (U/L)*                            | 5                                    |
| Presence of DSA at study entry†       | 31 (18, 48)                          |
|                                       | Class I                              |
|                                       | Class II                             |
|                                       | DQ alone                             |
|                                       | DR alone                             |
|                                       | DQ+DR                                |
|                                       | 27 (12, 88)                          |
|                                       | 0                                    |
|                                       | 4                                    |
|                                       | 1                                    |
|                                       | 1                                    |
|                                       | 2                                    |

\*Median (range) at study entry.

†N.

Single Antigen assays (One Lambda Inc., Canoga Park, CA) (Emory University, Atlanta, GA). A mean fluorescence intensity (MFI) threshold of 2,000 was used to identify a DSA. Class II DSA subtypes were determined using phycoerythrin-conjugated, IgG subclass-specific, anti-human IgG. C1q binding assays were performed and data were acquired and analyzed as described.<sup>(17)</sup> All IgG subtype and C1q binding activity assays were batched.

## Results

### CLINICAL STATUS, LABORATORY PROFILES, AND ADVERSE EVENTS OF OPERATIONALLY TOLERANT PEDIATRIC LIVER TRANSPLANT RECIPIENTS

Twelve pediatric recipients (8 male; 9 biliary atresia; Table 1) of parental living donor liver allografts were identified as operationally tolerant through gradual reduction and ultimate discontinuation of IS.<sup>(14)</sup> One participant (subject 7) withdrew from the trial 45.3 months after their last dose of IS; the remaining 11 completed the 5-year study. ALT and GGT profiles for all 12 operationally tolerant subjects are shown in Figure 2A-C.

No instances of death, graft loss, or chronic rejection occurred. During the 5-year follow-up, 1 subject experienced one study-related adverse event (AE): an

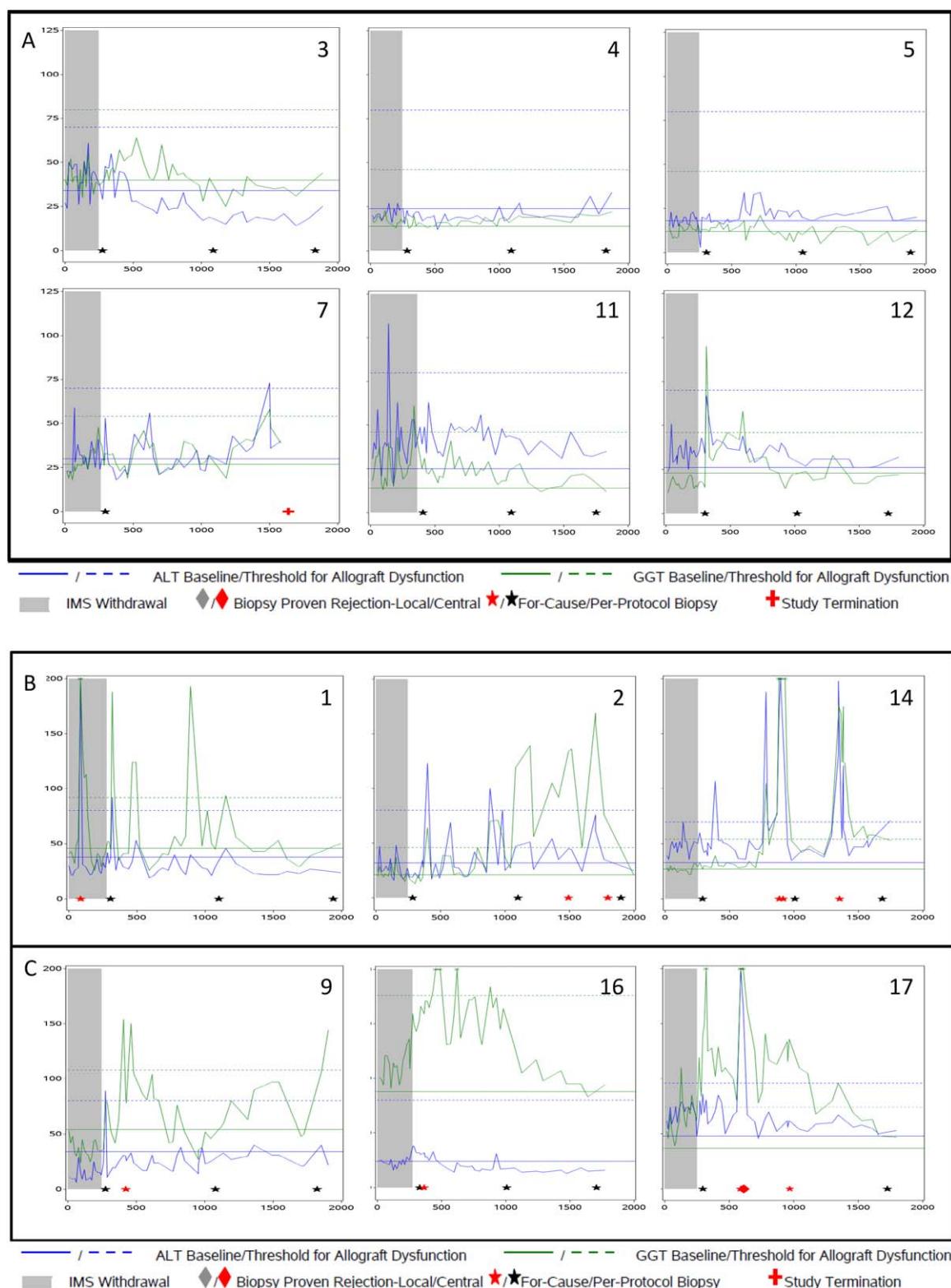
episode of cholangitis precipitated by a protocol biopsy (subject 1; *Supporting Table S2*). Six subjects experienced a total of 13 study-unrelated serious adverse events (SAEs): seven SAEs were related to biliary stricture/obstruction in 3 subjects (previously reported in Feng et al.<sup>(14)</sup>), including one caused by an incarcerated diaphragmatic hernia (subject 2); one SAE was secondary to portal vein stenosis (subject 17; also previously reported in Feng et al.<sup>(14)</sup>).

### ASSESSMENT OF TOLERANT ALLOGRAFTS FOR INFLAMMATION, FIBROSIS, C4d DEPOSITION, AND EVIDENCE OF SUBCLINICAL INJURY

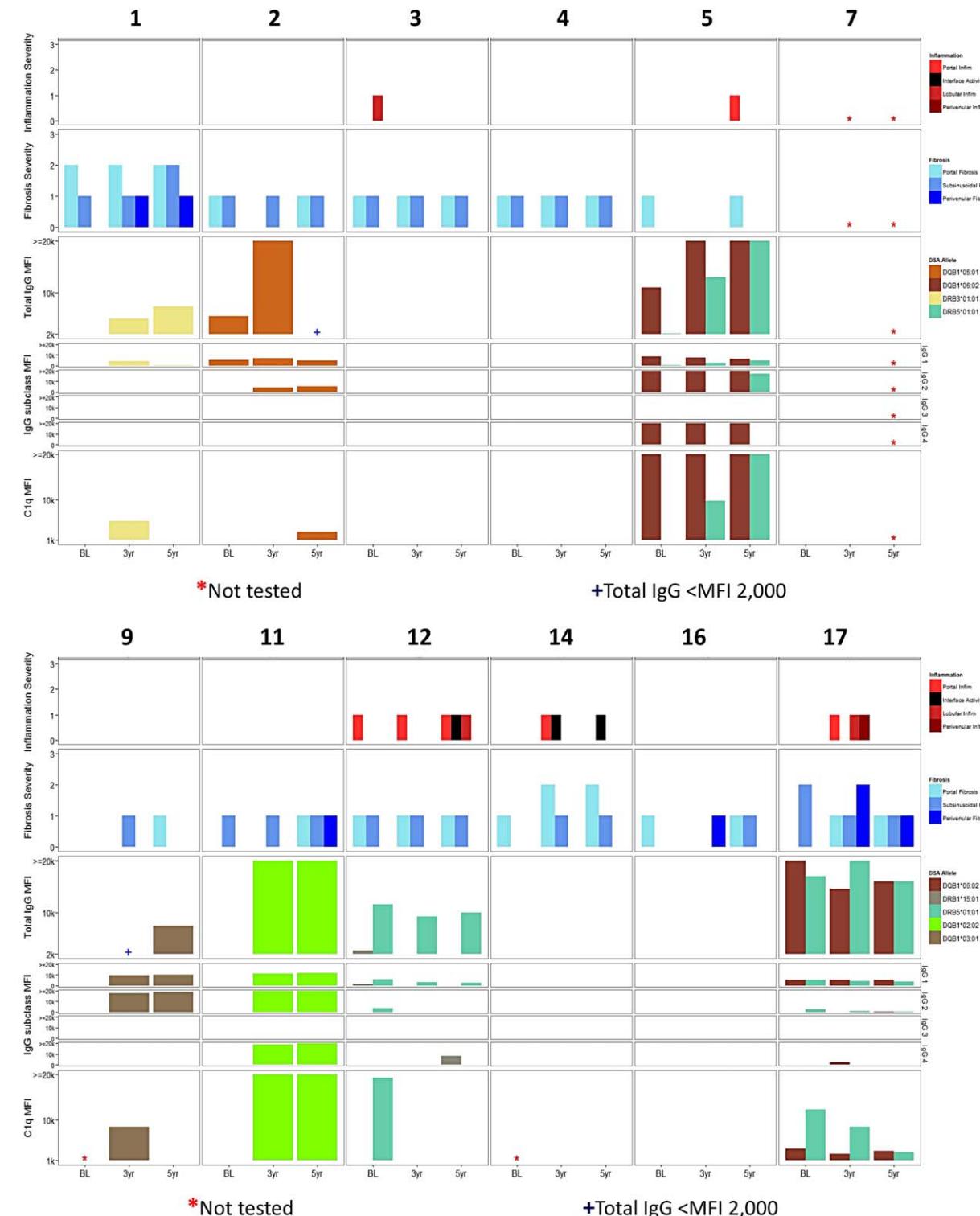
Protocol liver biopsies 2 (year 3) and 4 years (year 5) after IS discontinuation were compared to the baseline biopsy performed for trial eligibility (year 0; Fig. 1). Figure 3 displays sequential scores for inflammation (portal, lobular, and perivenular) and fibrosis (portal, Disse, and perivenular).

As a group, the baseline (year 0) biopsies were generally small (mean [range] 1.1 [0.4-3.4]cm) sampling a mean (range) of 8 (3-24) portal tracts. All prewithdrawal biopsies showed nodular regenerative hyperplasia (NRH) and portal venopathy of variable severity, which resulted in gross fragmentation of three year 0 biopsies (subjects 1, 2, and 4). For 2 participants (subjects 2 and 4; Fig. 4), fragmentation was likely exacerbated by use of a small (18-G) biopsy needle because biopsies obtained later in the course of the trial using a larger (16-G) biopsy needle later showed less fragmentation. However, for the remaining participant (subject 1) who experienced recurrent biliary obstruction requiring multiple interventions during the trial (Feng et al.<sup>(14)</sup>; *Supporting Table S2*), the year 5 biopsy for continued to show fragmentation (Fig. 5). NRH changes in the other subjects did not appreciably progress over time. More detailed accounting of the minor changes in inflammation, fibrosis, and overall architecture are detailed in *Supporting Table S3*. There was no evidence for progressive increase in inflammation or fibrosis among the tolerant subjects without biliary issues during the follow-up period.

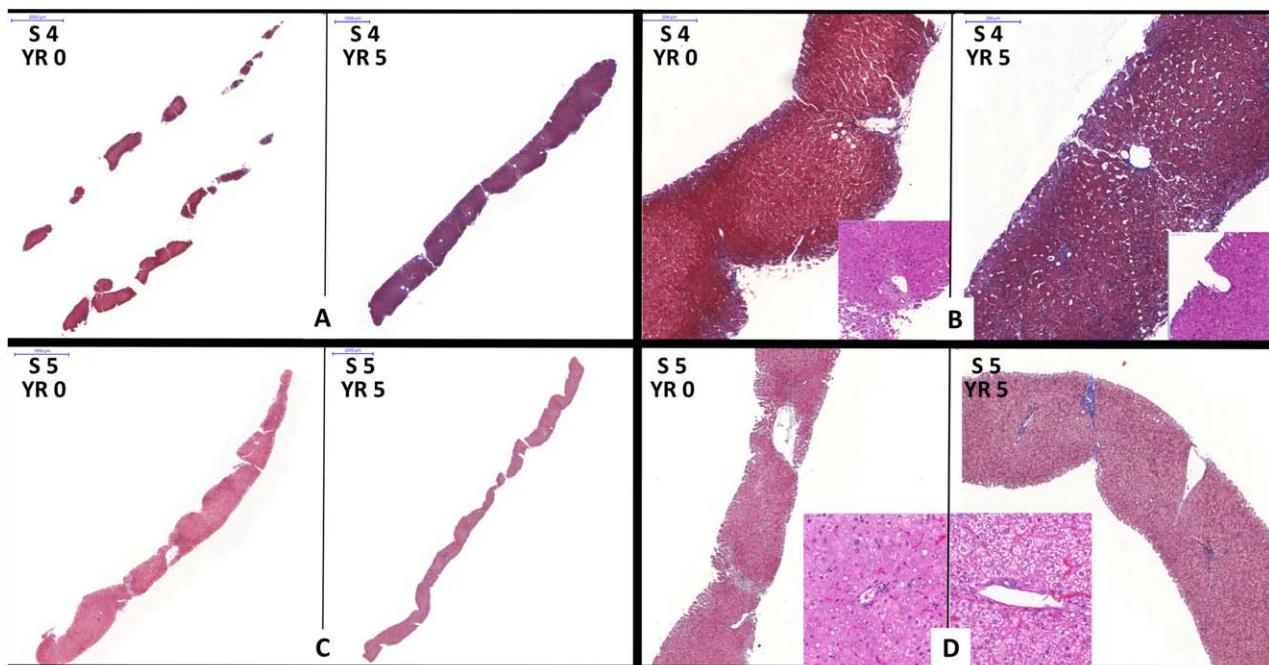
A separate needle biopsy fragment (0.5-1.0 cm) was snap-frozen for optimal assessment of C4d deposition. Tissue handling and preservation artifacts resulted in the availability of only eight paired baseline and year 5 specimens for comparison (Fig. 6). C4d scores



**FIG. 2.** ALT and GGT profiles for operationally tolerant WISP-R subjects. The 12 operationally tolerant subjects are divided into three groups as described.<sup>(14)</sup> (A) Six subjects exhibiting generally stable profile throughout study follow-up. (B) Three subjects with discrete spikes in ALT and GGT, reflecting the diagnosis of biliary obstruction made during the study. (C) Three subjects exhibiting persistent and/or recurrent elevation of predominantly GGT during the study.



**FIG. 3.** Inflammation and fibrosis scores and class II DSA MFIs (single antigen bead, IgG 1-4 subclasses, and C1q) for operationally tolerant WISP-R subjects. For each operationally tolerant subject, data are shown at three time points: baseline (BL), before study entry and IS withdrawal; year 3, 3 years after study entry, corresponding to 2+ years after last dose of IS; and year 5, 5 years after study entry, corresponding to 4+ years after last dose of IS. The top two rows show inflammation and fibrosis scores (range, 0-3) by compartment. The remaining rows show single antigen bead MFIs, IgG 1, 2, 3, and 4 MFIs, and C1q binding activity MFIs.



**FIG. 4.** Biopsies from 2 representative WISP-R subjects with consistently normal liver test profiles as shown in Fig. 2A. Biopsies from 2 representative WISP-R participants (subjects 4 and 5; liver test profiles shown in Fig. 2A) are shown. A scale bar is in the upper left corner of the panels. Trichrome-stained sections of entire year 0 and year 5 biopsies captured at low magnification from subjects 4 (A) and 5 (C) show intact architecture. Higher magnification of representative areas of year 0 and year 5 biopsies are shown (B and D). Each inset shows higher magnification of representative portal tracts: all are devoid of inflammation. Finally, note that the thinner, 18-gauge year 0 biopsy is fragmented whereas the thicker, 16-gauge, year 5 biopsy is not (A).

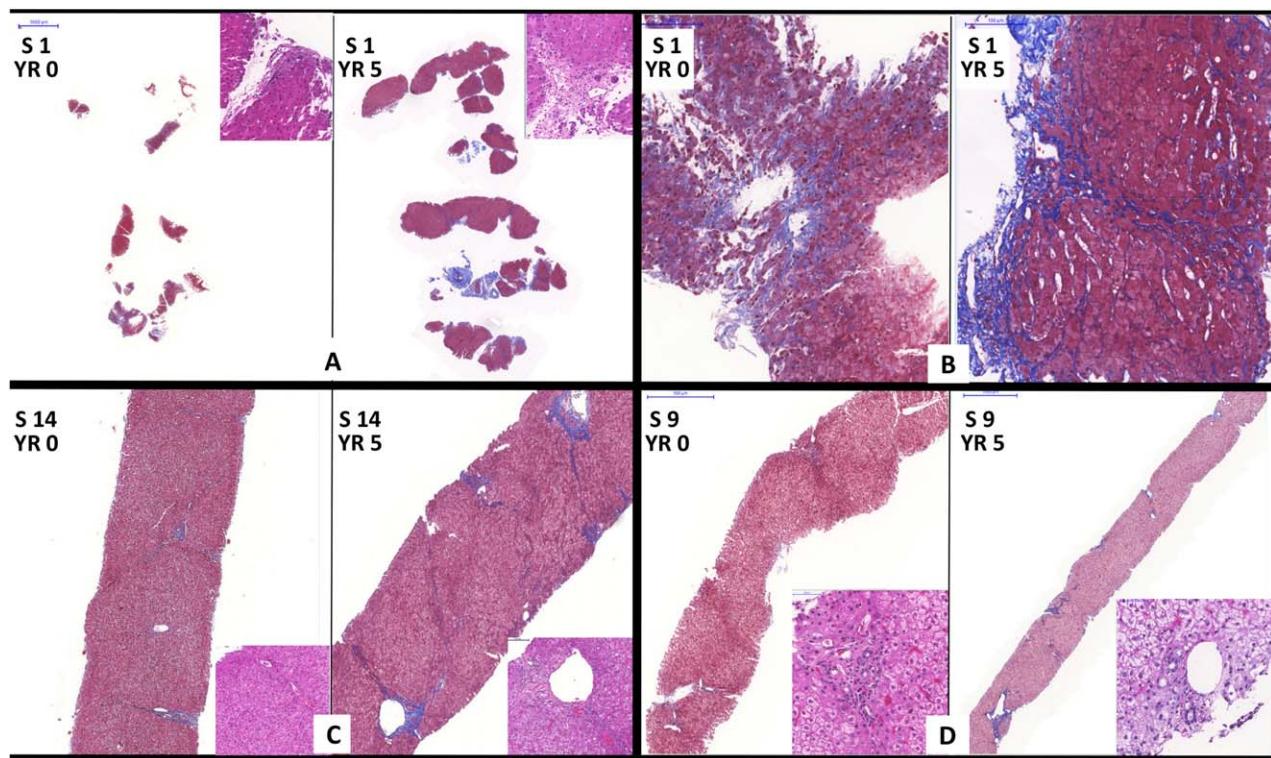
summed over four compartments increased in 7 of 8 subjects by a median (range) score of 2.25 (1.0-4.25), primarily from increased sinusoidal staining. The expression of MHC Class II antigen, the putative target of Class II DSA, was quantified by compartment; portal-based dendritic cells served as internal positive controls. In contrast to kidney<sup>(18)</sup> and heart<sup>(19)</sup> allografts that display constitutive expression of MHC Class II on all interstitial capillaries, liver allografts displayed low-to-modest MHC class II expression, limited predominantly to occasional portal capillaries and focally on sinusoidal endothelium. MHC Class II staining scores also increased modestly, again predominantly in the sinusoidal compartment (Fig. 6). Paired biopsies over 5 years showed increased sinusoidal staining for 6 of 10 subjects; portal capillary staining remained unchanged in 9 and decreased in 1 subject.

Finally, multiplex labeling for  $\alpha$ -SMA and CD34 was used to detect a shift toward a pathogenic phenotype in stellate<sup>(16)</sup> or sinusoidal endothelial cells.<sup>(15)</sup> Despite the slight increase of sinusoidal C4d and MHC Class II scores, there were no changes in CD34

or  $\alpha$ -SMA expression in periportal, sinusoidal, or perivenular regions (data not shown).

## EVOLUTION OF AUTOANTIBODIES, DSAS, DSA IgG SUBCLASS, AND C1q BINDING ACTIVITY OVER TIME FOR TOLERANT PEDIATRIC LIVER TRANSPLANT RECIPIENTS

Annual assessment of autoantibodies and quantitative IgG over 5 years did not identify any trends among the operationally tolerant subjects during and after IS withdrawal (Fig. 1; [Supporting Table S4](#)). The presence and strength of class I and II allo-antibodies in general and DSAs in particular were sequentially assessed. Eight of the 12 operationally tolerant subjects had no DSA at study entry. Three subjects (3, 7, and 14) never developed any detectable DSA, including subject 14 who suffered cholangitis and recurrent episodes of biliary obstruction ([Supporting Table S2](#)). At



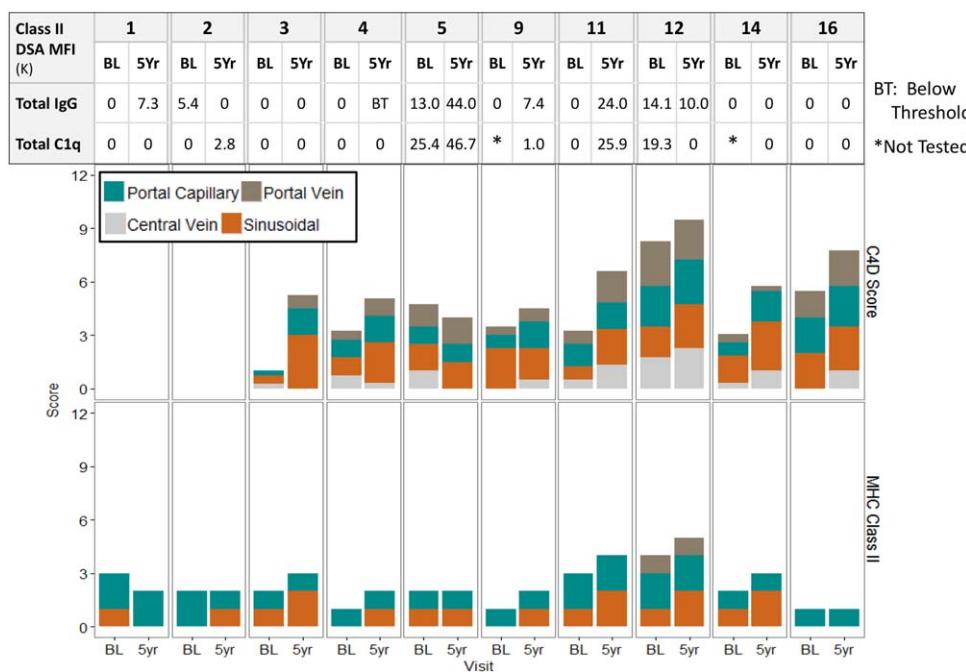
**FIG. 5.** Biopsies from three representative WISP-R subjects with abnormal liver test profiles as shown in Fig. 2B,C. Biopsies from 3 representative WISP-R participants are shown. The scale bar is shown in the upper left corner of the panels. Two participants (subjects 1 and 14; liver test profiles in Fig. 2B) who exhibited the most significant histopathological changes during the study had biliary obstruction secondary to anastomotic stricture that required intervention(s) during the 5-year follow-up period. (A,B) Subject 1: Trichrome-stained sections of year 0 and year 5 biopsies: Severe NRH changes and biopsy fragmentation were already evident at baseline, before IS withdrawal. The year 5 biopsy shows increased subsinusoidal fibrosis and architectural distortion. No portal inflammation was evident in either the year 0 or year 5 biopsy (A: hematoxylin-eosin-stained upper right insets). (C) Subject 14: year 0 and year 5 biopsies: This subject showed obvious obstructive cholangiopathic changes and increased portal/periportal fibrosis. The insets (40 $\times$  magnification) show noninflamed portal tracts. The third participant (subject 9; liver test profile in Fig. 2C) exhibited fluctuations in GGT. (D) Subject 9: Year 0 and year 5 biopsies showed neither inflammation nor noticeable change in fibrosis over 5 years.

study entry, none of the 12 operationally tolerant subjects had detectable class I DSA. Three subjects (5, 16, and 17) each developed a single class I DSA of low MFI (2,000-5,000) during either IS withdrawal or follow-up (Table 2A). Five subjects (1, 4, 9, 11, and 16) developed *de novo* class II DSA during IS withdrawal (Table 2B). Two developed a single anti-DR DSA and three developed a single anti-DQ DSA; *de novo* class II DSA was often transient, occurring at a single time point for 2 of the 5 subjects (4 and 16). Notably, all seven class II DSAs (four anti-DR and three anti-DQ) identified in the remaining 4 subjects before IS withdrawal, persisted during withdrawal and follow-up, without consistent or durable change in MFI over time (Table 2C).

The IgG subclass composition of class II DSAs harbored by tolerant pediatric liver transplant recipients

was determined to better delineate their functional nature. The four IgG subclasses, defined by their heavy-chain gene usage, are well known to differ in their ability to fix complement and affinity for Fc receptors. IgG3 is widely considered to be the most potent, followed closely by IgG1, whereas both IgG2 and IgG4 are considered weak.<sup>(20)</sup> The prevalence of each subclass is also known to diminish sequentially with IgG1 being the most common and IgG4 the least common.

Baseline, year 3, and year 5 specimens with class II DSA were subject to determination of IgG subclass. Seven of the 12 tolerant subjects exhibited at least one class II DSA at one or more of these three time points. IgG subclass MFIs are shown, along with those from standard single-antigen and C1q binding assays (Fig. 3). The IgG1 subclass was indeed the most frequently identified, found in all subjects for all class II



**FIG. 6.** Sum of class II DSA MFIs and biopsy C4d and MHC class II scores for operationally tolerant WISP-R subjects. For each operationally tolerant subject, data are shown at two time points: baseline (BL), before study entry and IS withdrawal, and year 5, 5 years after study entry, corresponding to 4+ years after last dose of IS. The sum of class II DSA single antigen and C1q binding MFIs at the specified time point is shown, aligned with the C4d and MHC class II scores by compartment.

DSAs for all time points except one (year 5; subject 12; DRB1\*1501). IgG2 and IgG4 class II DSAs were frequently found, in 6 and 4 of the 7 participants, respectively. In 3 subjects (5, 9, and 11), IgG2 and IgG4 MFI far exceeded that of IgG1. In contrast, IgG3 DSA was rarely identified, found in a single participant at a single time point for a single DSA (DQB1\*0602; subject 5; year 3).

Finally, all available baseline, year 3, and year 5 serum samples were tested, regardless of class II DSA presence, for C1q binding activity. C1q binding is an early step in the classical complement cascade, which culminates in the formation of the membrane attack complex and target cell destruction. A C1q binding MFI greater than 1,000 was considered positive. Of 10 baseline sera tested, three specimens (from subjects 5, 12, and 17), all with class II DSA of MFI >10,000 fixed C1q, two baseline sera were unavailable (subjects

9 and 14), but had no detectable alloantibody (Fig. 3). Over the course of 5 years, 7 of the 12 tolerant subjects had serum that fixed C1q at one or more time point tested. C1q binding activity was never detected in the complete absence of class II DSA. However, two class II DSAs of MFI <2,000 did exhibit C1q binding activity (subject 2, DQB1\*05 01, MFI 1,300, year 5; subject 9, DQB1\*03 01, MFI 1,900, year 3; Fig. 3). In contrast, several class II DSAs of variable MFIs (4,500 to >20,000) did not exhibit C1q binding activity. Neither the presence nor strength of C1q binding activity appeared to be associated with progressive inflammation or fibrosis over time (Fig. 3).

## Discussion

We have completed a prospective, multicenter, single-arm cohort study of IS withdrawal for stable,

**TABLE 2A. Evolution of Class I DSA During Long-Term Follow-up of Operationally Tolerant Subjects\***

| Patient ID | Baseline       | Year 1                   | Year 2           | Year 3                   | Year 4           | Year 5         |
|------------|----------------|--------------------------|------------------|--------------------------|------------------|----------------|
| 5          | No class I DSA | <b>B*07:02<br/>3,000</b> | No class I DSA   | No class I DSA           | No class I DSA   | No class I DSA |
| 16         | No class I DSA | <b>A*02:01<br/>3,000</b> | A*02:01<br>2,000 | No class I DSA           | No class I DSA   | No class I DSA |
| 17         | No class I DSA | No class I DSA           | No class I DSA   | <b>C*03:04<br/>2,200</b> | C*03:04<br>2,000 | No class I DSA |

\*Bold italicized entry denotes *de novo* class I DSA.

**TABLE 2B. Evolution of Class II DSA During Long-Term Follow-up of Operationally Tolerant Subjects Without Class II DSA at Baseline\***

| Patient ID | Baseline <sup>†</sup> | Year 1                                  | Year 2                          | Year 3 <sup>†</sup>        | Year 4                          | Year 5 <sup>†</sup>            |
|------------|-----------------------|---|---------------------------------|----------------------------|---------------------------------|--------------------------------|
| 1          | No class II DSA       | No class II DSA                         | No class II DSA                 | <b>DRB3*0101<br/>5,000</b> | DRB3*0101<br>12,000             | DRB3*0101<br>7,300             |
| 4          | No class II DSA       | <b>DQB1*0302<br/>2,300</b>              | No class II DSA                 | No class II DSA            | No class II DSA                 | No class II DSA                |
| 9          | No class II DSA       | <b>DQA1*05/<br/>DQB1*0301<br/>9,000</b> | DQA1*05/<br>DQB1*03:01<br>4,400 | No class II DSA            | DQA1*05/<br>DQB1*03:01<br>3,800 | DQA1*05/<br>DQB1*0301<br>7,400 |
| 11         | No class II DSA       | <b>DQB1*0202<br/>16,000</b>             | DQB1*0202<br>17,000             | DQB1*0202<br>>20,000       | DQB1*0202<br>>20,000            | DQB1*0202<br>>20,000           |
| 16         | No class II DSA       | <b>DRB1*1501<br/>4,000</b>              | No class II DSA                 | No class II DSA            | No class II DSA                 | No class II DSA                |

\*Bold italicized entry denotes *de novo* class II DSA.

<sup>†</sup>Baseline, year 3, and year 5 specimens were tested for IgG subclass as shown in Fig. 3.

long-term pediatric recipients of parental living donor liver grafts. The strength of our trial lies in its prospective nature and the longitudinal collection of simultaneous protocol biopsies and peripheral blood, allowing us to assess whether immunological events in the periphery are reflected as allograft damage. Previously, we have reported on the trial's primary endpoint—the proportion of participants who are operationally tolerant, defined as those who remain off IS for at least 1 year.<sup>(14)</sup> We now report on the detailed histological and serological characterization of the operationally tolerant subjects over a 5-year period.

The only articles (two in total) that have assessed the long-term impact of IS withdrawal were in adults.<sup>(2,21)</sup> IS withdrawal, among recipients with hepatitis C virus, was associated with reduced prevalence of hyperglycemia, cardiovascular disease, and infection episodes.<sup>(2)</sup> IS withdrawal, however, did not yield similar benefits in a cohort without hepatitis C.<sup>(21)</sup> Similarly, in our pediatric cohort, IS withdrawal did not mitigate components of metabolic syndrome.<sup>(22)</sup> Given that the benefit of remaining off IS has not been convincingly demonstrated for either adults or children, assessment of its safety over time is paramount.

The issue of greatest concern for operationally tolerant pediatric liver transplant recipients is that of allograft deterioration. Insufficient IS has been hypothesized as a possible etiology for the inflammation and/or fibrosis observed in long-surviving pediatric liver allograft recipients who have been maintained on standard-of-care IS<sup>(3-8)</sup> as well as those who have undergone IS withdrawal.<sup>(9,13)</sup> We have shown that, over 5 years—approximately 1 year of IS reduction plus 4 years of no IS, there has been no systematic or progressive increase in either inflammation or fibrosis by light microscopy. Overall, the absence of detectable allograft inflammation and progressive architectural distortion suggests operational tolerance. However, the frequent presence of DSA, commonly interpreted as an ongoing allo-immune response, contrasts with the apparent lack of damaging effector responses within the allograft. In order to further explore this conundrum, we interrogated both the antibody response and the allograft tissue in detail.

The majority of our tolerant subjects (8 of 12) initiated IS withdrawal without any DSA, consistent with reports that patients without DSA are more likely to be tolerant.<sup>(23,24)</sup> Notably, 4 tolerant subjects did have

**TABLE 2C. Evolution of Class II DSA During Long-Term Follow-up of Operationally Tolerant Subjects With Class II DSA at Baseline**

| Patient ID | Class II DSA | Baseline* | Yr 1    | Yr 2    | Yr 3*        | Yr 4    | Yr 5*        |
|------------|--------------|-----------|---------|---------|--------------|---------|--------------|
| 2          | DQB1*0501    | 5,400     | 16,000  | 2,000   | >20,000      | >20,000 | Not detected |
| 5          | DQB1*0602    | 11,000    | >20,000 | 19,000  | >20,000      | 13,000  | >20,000      |
|            | DRB5*0101    | 2,000     | 5,000   | 8,000   | 13,000       | 13,000  | >20,000      |
| 12         | DRB1*1501    | 2,600     | 5,000   | 2,200   | Not detected | 3,300   | Not detected |
|            | DRB5*0101    | 11,500    | 18,000  | 9,300   | 9,200        | 14,000  | 10,000       |
| 17         | DQB1*0602    | >20,000   | 11,000  | 14,000  | 14,500       | 15,000  | 16,000       |
|            | DRB5*0101    | 17,000    | 17,500  | >20,000 | >20,000      | >20,000 | 16,000       |

\*Baseline, year 3, and year 5 specimens were tested for IgG subclass as shown in Fig. 3.

DSA at study entry, indicating that the presence and persistence of DSA was not prohibitive of operational tolerance. Moreover, 7 tolerant subjects developed *de novo* DSA (with either class I or class II specificity) that were often of high MFI and occasionally persistent. The DSA response of these operationally tolerant subjects, both preexisting and *de novo*, exhibited a clear dominance of class II over class I specificity. Intriguingly, the emergence of *de novo* class II DSA was similarly observed in a recent pilot trial of tolerance induction in adult liver transplant recipients utilizing a regulatory T-cell-enriched product.<sup>(25)</sup> The preponderance of class II DSA may be explained by the pattern of antigen expression and antigen clearance within the liver. In the quiescent, noninjured, noninflamed liver, class I antigens are constitutively expressed by all cells. The liver secretes class I HLA antigens that can bind to and neutralize circulating class I DSA to form immune complexes that are cleared by Kupffer cells (reviewed in Demetris et al.<sup>(26)</sup>). Preferential clearance of class I compared to class II DSA from the circulation has been reported after both liver transplantation alone and simultaneous liver and kidney transplantation.<sup>(27-29)</sup> Class II expression within the quiescent, noninjured, and noninflamed liver is largely restricted to hematolymphoid cells with only weak and focal microvascular endothelial expression.<sup>(26,30)</sup> The down-regulation of class II expression has been attributed to lipopolysaccharide-induced production of interleukin (IL)-10 by Kupffer cells.<sup>(31,32)</sup> Notably, original descriptions of rodent liver allograft “tolerance” includes persistence of anti-class II DSA and paucity of donor class II antigen expression in the liver.<sup>(33)</sup>

The liver’s unique ability to clear anti-class I antibodies, as discussed above, may explain the absence of circulating class I DSA and the presence of class II antibodies in liver transplant recipients. However, essentially all organ transplant recipients, not just liver recipients, exhibit a dominance of class II DSA. Moreover, the presence of  $\alpha$ -DQ DSA has been consistently associated with poor allograft outcomes after renal, cardiac, and liver transplantation.<sup>(11,34-38)</sup> The biological rationale for why class II antigens in general and DQ antigens in particular elicit the strongest antibody responses is unknown. Poor donor-recipient DQ matching may also be an explanation for kidney transplant recipients, given that the DR, but not DQ, loci are considered in kidney allocation. However, this is an unlikely explanation for liver transplant recipients given that HLA matching does not enter into either organ allocation or acceptance. Among our

operationally tolerant pediatric liver transplant recipients, class II DSA, including  $\alpha$ -DQ DSA, was common.

To further explore the DSA response of our operationally tolerant subjects, we characterized DSA by IgG subclass and C1q binding activity. In our cohort, IgG1 was pervasive, IgG2 was common, IgG4 was frequent, but IgG3 was rare, identified only in a single subject at a single time point. The relative stability of allograft histopathology may be partially attributable to the rarity of IgG3 DSA, which has been associated with inferior outcomes for both kidney and liver transplant recipients.<sup>(39,40)</sup> Among adult, non-HLA identical, primary kidney transplant recipients, IgG3 DSA was associated with increased rates of rejection and graft loss as well as lower glomerular filtration rates at last follow-up.<sup>(39)</sup> Among adult, primary liver transplant recipients, preformed IgG3 DSA independently predicted death (hazard ratio [HR], 2.4;  $P < 0.001$ ); among those without preformed DSA who survived for at least 1 year, *de novo* IgG3 DSA also independently predicted death (HR, 2.1;  $P = 0.004$ ).<sup>(40)</sup>

In our operationally tolerant subjects, the identified class II DSA frequently exhibited C1q binding activity. The clinical importance of complement binding has been reported for various allografts.<sup>(11,35,40-42)</sup> In the kidney, C1q<sup>+</sup> DSA has been associated with unfavorable histopathological features, including microvascular inflammation, C4d deposition, transplant glomerulopathy, interstitial and tubular inflammation, and interstitial fibrosis and tubular atrophy. Moreover, those with C1q<sup>+</sup> DSA suffered poor 5-year graft survival (54%), compared to those without DSA (94%) and those with noncomplement binding DSA (93%;  $P < 0.001$  for both comparisons).<sup>(41)</sup> In contrast, the impact of C1q<sup>+</sup> DSA on liver transplant outcomes is less clear with conflicting reports in adult recipients.<sup>(40,43)</sup> A single report regarding pediatric liver transplant recipients found that C1q binding activity often coincided with high MFI and correlated with a nontolerant phenotype.<sup>(11)</sup>

Despite the presence of high MFI DSA with C1q positivity, the “signature” lesion of antibody-mediated rejection in all allografts, microvascular inflammation,<sup>(44)</sup> was not observed in our withdrawal trial or in the tolerance induction trial.<sup>(25)</sup> We did however note an increased number of Kupffer cells after IS withdrawal, which, along with LSECs, exhibit known scavenger functions. Efficient clearance of immune complexes, activated complement components, and platelet aggregates by these scavenger cells may protect

the liver allograft from antibody-mediated injury (reviewed in Demetris et al.<sup>(26)</sup>). Furthermore, we did not observe phenotypic changes in LSECs characteristic of their response to injury or fibrogenesis.<sup>(45-48)</sup> This finding is wholly consistent with the lack of progressive fibrosis observed by light microscopy. We did observe a modest increase in sinusoidal C4d deposits. Whether undetectable arterial intimal LSEC injury or microvascular endothelial cell alterations (e.g., up-regulation of complement regulatory proteins or cytoprotective molecules) are occurring is currently being investigated.

In summary, 5-year follow-up of our tolerant pediatric cohort is notable for a consistent disparity between ongoing peripheral allo-antibody responses and evidence of allograft damage. The immunological mechanisms that underlie these provocative observations remain to be elucidated, but “tolerogenic” properties unique to the liver are likely contributors. Traditional explanations for the muting of T-cell responses within the liver include a bias of liver antigen-presenting cells toward eliciting regulatory, rather than effector, responses, the predisposition of activated T cells toward exhaustion and/or apoptosis attributed to the expression of negative costimulatory molecules and anti-inflammatory cytokines (IL-10 and transforming growth factor beta) by multiple intrahepatic cell populations, the production of soluble MHC class I, and the reduced expression of MHC class II.<sup>(49,50)</sup> Other possible explanations include the scarcity of IgG3 DSA subclass, activation of liver protective mechanisms (e.g., increasing the number of Kupffer cells to phagocytize the products of DSA reactions), or up-regulation of complement regulatory or cytoprotective molecules on the endothelium. Our observations suggest that some or all of these mechanisms explain the failure of the immune response to cause clinical or histopathological damage to operationally tolerant allografts. We, however, cannot exclude the possibility that damage to the allograft might be too subtle to detect during the 5-year interval examined.

Although we have comprehensively characterized a unique cohort of operationally tolerant pediatric liver transplant recipients, we recognize that our study does have important weaknesses. First and perhaps foremost, the cohort is modest in size because it derives from a pilot study of IS withdrawal. Unfortunately, it is impossible to enlarge the cohort because it is the operationally tolerant subset of subjects enrolled in WISP-R. However, the modest number of subjects allowed us to study and present each in great detail.

Second, our subjects are highly selected and relatively homogeneous, all pediatric recipients of parental living donor allografts. Our findings may therefore have limited generalizability. Relevance to pediatric recipients of deceased donor allografts who are identified as operationally tolerant may emerge once data are available from a currently ongoing trial of IS withdrawal for 88 pediatric deceased and living donor liver transplant recipients at 12 transplant centers in North America (iWITH; NCT01638559). Third, our study, which was focused on identifying and studying operationally tolerant subjects, did not have a control cohort. As such, it is impossible to determine whether the evolution of DSA, IgG subclass, and C1q binding profiles specifically reflect the withdrawal and/or absence of IS. Finally, as already mentioned above, although our detailed and comprehensive 5-year follow-up data of an operationally tolerant cohort are unique, even longer follow-up to delineate the clinical and histological impact of IS withdrawal is critically important to maximize the longevity of pediatric liver transplant recipients.

In conclusion, we have demonstrated that IS discontinuation has been safe in a closely monitored cohort of operationally tolerant pediatric recipients that has not been associated with histological deterioration of allografts over 5 years of follow-up. Despite the persistence or development of DSA and modest increases in sinusoidal C4d staining, the allografts did not evidence damage from either humoral or cellular effector mechanisms. Microvascular inflammation, progressive fibrosis, or changes in LSEC or stellate cell phenotype did not develop over more than 5 years of follow-up. Although it is possible that progressive pathology may emerge with longer follow-up, progressive histological deterioration of allografts has been well-described in patients maintained on IS. Regardless, limitations imposed by the size and homogeneity of this cohort—pediatric recipients of parental living donor liver grafts—are being addressed in a similar, but substantially larger study (iWITH; NCT01638559) inclusive of deceased donor transplant recipients with the expectation that a better understanding of the mechanistic underpinnings of “liver allograft acceptance” will be achieved.

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