CLINICAL TRIALS IN ORGAN TRANSPLANTATION IN CHILDREN
CTOTC-12

Safety of Donor Alloantigen Reactive Tregs to Facilitate Minimization and/or Discontinuation of Immunosuppression in Adult Liver Transplant Recipients

Version 7.0 / October 15, 2018

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Protocol CTOTC-12: darTregs for CNI Reduction	Version/Date: 7.0 / October 15, 2018
Title: *Safety of Donor Alloantigen Reactive Tregs (darTregs) to Facilitate Minimization and/or Discontinuation of Immunosuppression in Adult Liver Transplant Recipients (ARTEMIS)*	
Study Sponsor: *The National Institute of Allergy and Infectious Diseases (NIAID)*	
INSTRUCTIONS: The site Principal Investigator should print, sign, and date at the indicated location below. A copy should be kept for your records and the original signature page sent. After signature, please return the original of this form by surface mail to: DAIT Regulatory Management Center PPD, Inc. [redacted]	
I confirm that I have read the above protocol in the latest version. I understand it, and I will work according to the principles of Good Clinical Practice (GCP) as described in the United States Code of Federal Regulations (CFR) – 45 CFR part 46 and 21 CFR parts 50, 56, and 312, and in the International Conference on Harmonization (ICH) document *Guidance for Industry: E6 Good Clinical Practice: Consolidated Guidance* dated April 1996. Further, I will conduct the study in keeping with local legal and regulatory requirements. As the site Principal Investigator, I agree to carry out the study by the criteria written in the protocol and understand that no changes can be made to this protocol without the written permission of the IRB and NIAID. ______________________________ Site Principal Investigator (Print) ______________________________ _______________ Site Principal Investigator (Signature) Date	

Protocol Synopsis

Title	Safety of Donor **A**lloantigen **R**eactive **T**r**e**gs (darTregs) to Facilitate **M**inimization and/or Discontinuation of **I**mmuno**s**uppression in Adult Liver Transplant Recipients **(ARTEMIS)**
Short Title	darTregs for CNI Reduction
Protocol Number	CTOTC-12
ClinicalTrials.gov Identifier	NCT02474199
Clinical Phase	Phase I/II
Primary Safety Objective	This study will evaluate the safety and tolerability of a single IV dose of the darTregs product.
Secondary Safety Objective	This study will evaluate the safety of immunosuppression (IS) 1) minimization and 2) discontinuation after one IV dose of darTregs.
Primary Efficacy Objective	The study will evaluate the ability of a single IV dose of darTregs to reduce baseline, standard of care (SOC) calcineurin inhibitor (CNI) dosing by 75% along with discontinuation of either prednisone or mycophenolate mofetil (MMF), as applicable.
Secondary Efficacy Objective	This study will determine whether a single IV dose of darTregs can induce operational tolerance.
Mechanistic Objectives	We will assess the pharmacokinetic profile of darTregs by measuring the level of deuterium-labeled Tregs in circulation. Potential impact of darTregs therapy on immunological profiles will be assessed by comparing leukocyte phenotypes, tissue histology and gene expression in protocol and for-cause biopsies, and alloantibody before and after darTregs infusion.
Study Design	A multi-center, open-label clinical trial in adult liver transplant recipients with two primary interventions: • administration of a single IV dose of darTregs and • reduction of IS dosing with possible complete IS discontinuation. Adult living donor liver recipients two to six years after LT will initiate IS withdrawal. Twelve to 14 weeks later, they will receive a single dose of darTregs and continue IS withdrawal.
Primary Safety Endpoint	The safety and tolerability of a single infusion of darTregs administered to LT recipients will be assessed 24 weeks after darTregs by describing: 1. Occurrence of CTCAE Grade 3 or higher adverse events (AEs) attributable to the darTregs infusion including infusion reaction / cytokine release syndrome 2. Occurrence of study defined Grade 3 or higher infections 3. Occurrence of any malignancy, including PTLD
Secondary Safety Endpoint	The trial will assess the safety of IS withdrawal in the context of darTregs therapy by describing the following secondary safety endpoints: 1. Rate of composite outcome measure including refractory acute rejection, chronic rejection, re-transplantation, and death 2. Incidence of biopsy proven or clinical acute rejection and/or chronic rejection 3. Timing of biopsy proven or clinical acute rejection and/or chronic rejection 4. Severity of biopsy proven acute rejection and/or chronic rejection

Primary Efficacy Endpoint	The efficacy of a single IV dose of darTregs will be assessed by the number and proportion of LT subjects who are able to reduce CNI dosing by 75% and discontinue a 2^{nd} IS drug (if applicable) with stable liver tests for at least 12 weeks. The frequency of successful CNI minimization will be compared to historical cohorts of comparable adult LT recipients undergoing IS withdrawal.
Secondary Efficacy Endpoints	The efficacy of a single IV dose of darTregs infusion will be assessed by determining the number and percentage of subjects who have received darTregs and are identified as operationally tolerant, defined by maintaining stable allograft function (assessed by liver tests) and histology (determined by central pathologist reading in comparison to screening liver biopsy at study entry) in the absence of IS for one year. The frequency of tolerance will be compared to historical cohorts of adult liver transplant recipients undergoing IS withdrawal.
Primary Mechanistic Endpoints	The level and persistence of deuterium-labeled darTregs in the circulation will be determined by serial measurements of deuterium content in DNA from purified peripheral blood Tregs after darTregs infusion using gas chromatography-mass spectrometry (GC-MS) testing.
Secondary Mechanistic Endpoints	The overall increase of darTregs in circulation will be assessed using the alloreactive T cell frequency (ATF) assay
Exploratory Mechanistic Endpoints	The immunologic impact of infused darTregs will be determined by assessing the following: • Leukocyte phenotypes before and after darTregs infusion using multi-parameter flow cytometry (MFC). • Alloantibody responses before and after darTregs infusion during IS withdrawal. • Histology and multiplex immunohistochemistry of protocol and for cause biopsies • The composition of immune infiltrate in liver biopsies post Treg infusion and at the time of for-cause biopsies will be profiled using single-cell RNA+TCRseq
Accrual Objective	Up to 18 participants will be screened and enrolled to target up to 11 participants eligible for both IS withdrawal and darTregs infusion
Study Duration	The maximal length of trial participation for an individual subject is anticipated to be 2 years. The total trial duration will be 3 years. • One year period to accrue nine patients eligible for both IS withdrawal and darTregs infusion that will be given approximately 10-11 weeks after initiating IS withdrawal • Minimum of 52 weeks follow-up after any AR episode or or darTregs infusion • For all subjects able to discontinue IS, 52 weeks of follow-up after the last IS dose to assess for operational tolerance The duration of study participation will vary by subject depending on his/her duration of IS withdrawal. Accounting for a one year enrollment period, the primary endpoint for all participants will be assessed approximately 1.5 years after trial initiation.

Treatment Description	All eligible participants will initiate IS withdrawal. Approximately 10-12 weeks after initiating IS withdrawal, subjects will have autologous Tregs collected for darTregs manufacturing. During the last 2 weeks of IS withdrawal Step 2 (CNI reduced by 25%), **a single total dose of 400 x 10^6 ± 100 deuterium-labeled darTregs** will be infused intravenously. The subject will then, if eligible, resume IS withdrawal within 2 weeks after darTregs infusion (see Section entitled "Resumption of IS Withdrawal after darTregs Infusion" below). Only subjects who receive 300-500 x 10^6 darTregs and who meet the primary endpoint of 75% reduction in CNI from baseline after darTregs will be offered the opportunity to continue IS withdrawal until complete discontinuation of IS (secondary endpoint). Those who receive 100-300 x 10^6 darTregs will only be allowed to proceed to the primary endpoint but will not be eligible for complete IS withdrawal.
Study Enrollment/ IS Withdrawal Inclusion Criteria	Subjects who meet all of the following criteria are eligible for enrollment as study participants: 1. Able to understand and provide informed consent 2. Have received primary, solitary, living donor liver transplant more than 24 months but less than 84 months ago 3. Have a living donor who is willing to consent to one time phlebotomy of 100 mLs to enable manufacture of darTregs 4. Between 18 and 70 years of age at the time of study entry/consent 5. Have ALT consistently <60 U/L and either alkaline phosphatase consistently <150 U/L or GGT consistently <60 U/L 6. Currently receiving a calcineurin inhibitor (CNI) with 12 hour trough levels consistently <6.0ng/mL for tacrolimus; <150ng/mL for cyclosporine 7. Currently receiving CNI monotherapy or CNI and ONE of the following: a. Prednisone: maximum dose of 5mg / day b. Mycophenolate mofetil (MMF): maximum dose of 500mg bid for Cellcept or 360mg bid for Myfortic 8. Female and male subjects with reproductive potential must agree to use effective methods of birth control for the duration of the study 9. If history of HCC, LT recipients who have: a. AFP less than 100 µg/L at the time of transplant AND b. Explanted liver: i. with tumor burden within the Milan criteria and ii. without macro- or micro-vascular invasion and iii. without any lesions with poorly differentiated HCC and iv. without cholangiocarcinoma morphology c. Risk Estimation of Tumor Recurrence After Transplant (RETREAT) Score less than or equal to 3 10. If history of HCC, at the time of enrollment, subjects must also: a. Be 36 months or more post-transplant AND b. Without evidence of recurrent HCC defined as i. AFP within normal limits for performing laboratory ii. Confirmatory chest CT and iii. Confirmatory CT or MRI of the abdomen and pelvis 11. If history of HCV, recipients must be: a. Cured of HCV as defined by achieving SVR and be greater than or equal to six months after the end of treatment b. HCV RNA negative at time of study enrollment

Study Enrollment/ IS Withdrawal Exclusion Criteria	Subjects who meet any of these criteria are not eligible for study enrollment. 1. Transplant for liver disease secondary an autoimmune etiology (e.g. autoimmune hepatitis, primary sclerosing cholangitis, or primary biliary cirrhosis) 2. Matched at both HLA-DR loci to the donor 3. Organ, tissue or cell transplant prior to or after the primary solitary living donor liver transplant 4. For subjects with hepatitis B, detectible HBV DNA 5. History of malignancy within 5 years of enrollment. History of adequately treated in-situ cervical carcinoma and/or skin cancer (basal or squamous cell) will be permitted. 6. Serologic evidence of human immunodeficiency 1 or 2 infection 7. Epstein Barr Virus (EBV) sero-negativity (EBV naïve) if living donor is EBV sero-positive 8. Cytomegalovirus (CMV) sero-negativity (CMV naïve) if living donor is CMV sero-positive 9. Calculated GFR less than 50 mL/min/1.73m^2 at the time of enrollment 10. An episode of AR within one year of enrollment 11. Systemic illness requiring or likely to require recurrent or chronic IS drug use 12. Any chronic condition for which anti-coagulation cannot be safely interrupted for liver biopsy 13. Positive pregnancy test 14. Participation in any other studies that involved investigational drugs or regimens in the preceding year 15. Any other condition, in the investigator's judgment, that increases the risk of darTregs infusion or prevents safe trial participation 16. Unwilling or unable to adhere to study requirements and procedures 17. Screening liver biopsy with any of the following histological criteria, as determined by the reading of a central pathologist (Table 9)
darTregs Infusion Inclusion Criteria	Subjects will initiate IS withdrawal and, at the beginning of the 2nd step of the withdrawal algorithm (week 1- 2), undergo a final assessment to ensure eligibility for darTregs infusion. Subjects must meet the following criteria to receive darTregs infusion: 1. Stable liver tests, defined as ALT and either alkaline phosphatase or GGT either within normal limits OR <1.5 X baseline 2. No detectible circulating EBV or CMV DNA prior to darTregs infusion, assessed at the time of PBMC collection for manufacture 3. For subjects with HBV, no detectible circulating HBV DNA prior to darTreg infusion, assessed at the time of PBMC collection for manufacture 4. Able to understand and provide informed consent
darTregs Infusion Exclusion Criteria	Subjects who meet any of these criteria are not eligible for darTregs infusion: 1. Diagnosis of AR after initiation of IS withdrawal 2. Any vaccination given within 28 days prior to Treg collection for Treg production 3. Receipt of a vaccination within 14 days prior to Treg infusion 4. Unacceptable darTregs product 5. Positive pregnancy test 6. Clinical evidence of viral syndrome less than 7 days prior to darTregs infusion
Eligibility Criteria to Resume IS Withdrawal after darTregs infusion	Subjects are eligible to resume IS withdrawal after darTregs infusion if all criteria below are met: 1. Subject received at least 100 x 10^6 darTregs 2. ALT and either alkaline phosphatase or GGT remain within normal limits or ≤ 1.5 x baseline after darTregs infusion 3. For subjects with elevated liver tests as defined above, local pathology reading of liver biopsy 6-10 days after darTregs infusion is without acute rejection according to Banff criteria 4. IS withdrawal resumes no later than 14 days after darTregs infusion 5. Site principal investigator determines it is acceptable for the study subject to resume IS withdrawal

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Rutgers University
[redacted]
Rutgers University Cell Repository
[redacted]

Table of Contents

Glossary of Abbreviations

AE	Adverse Event
AFP	Alpha fetoprotein
ALT	Alanine Aminotransferase
ACR	Acute Cellular Rejection
AR	Acute Rejection
CFR	Code of Federal Regulations
CR	Chronic Rejection
CI	Confidence Interval
CMV	Cytomegalovirus
CNI	Calcineurin Inhibitor
CRF	Case Report Form
CT	X-ray computed tomography
CTCAE	Common Terminology Criteria for Adverse Events
DAIT	Division of Allergy, Immunology, and Transplantation
DSMB	Data Safety Monitoring Board
EBV	Epstein Barr Virus
FACS	Fluorescence Activated Cell Sorting
FDA	Food and Drug Administration
GCP	Good Clinical Practice
GFR	Glomerular Filtration Rate
GMP	Good Manufacturing Practice
GvHD	Graft Versus Host Disease
HBV	Hepatitis B Virus
HCC	Hepatocellular Carcinoma
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
ICH	International Conference on Harmonization
ICU	Intensive Care Unit
IgG	Immunoglobulin G
IND	Investigational New Drug
IRB	Institutional Review Board
IS	Immunosuppression
LT	Liver Transplantation or liver transplant
MELD	Model for End Stage Liver Disease
MFC	Multiparameter Flow Cytometry

MIHC	Multiplex Immunohistochemistry
MLR	Mixed Lymphocyte Reaction
MM	Medical Monitor
MMF	Mycophenolate Mofetil
mSAP	Mechanistic Statistical Analysis Plan
NIAID	National Institute of Allergy and Infectious Disease
PI	Principal Investigator
PTLD	Post -Transplant Lymphoproliferative Disorder
PBMC	Peripheral Blood Mononuclear Cell
RAI	Rejection Activity Index
RETREAT	Risk Estimation of Tumor Recurrence After Transplant
SACCC	Statistical and Clinical Coordinating Center
SAE	Serious Adverse Event
SAP	Statistical Analysis Plan
SAR	Suspected Adverse Reaction
sBc	Stimulated B cell
SOC	Standard of Care
SOP	Standard Operating Procedure
SVR	Sustained Virological Responses
SWFI	Sterile Water for Injection
Tac	Tacrolimus
Tconv	Conventional T Cell
Treg	Regulatory T Cell
TSDR	Treg-Specific Demethylation Region
ULN	Upper Limit of Normal
UNOS	United Network for Organ Sharing

Study Definitions Page

Acute Rejection, Severe (1997 Banff Criteria) (Demetris A. J., 1997)	Histopathologically, severe AR shows brisk portal inflammation that expands most of the triads and often extends into the periportal hepatic parenchyma, similar to moderate AR. More importantly, there is inflammation in and around the connective tissue sheath surrounding the terminal hepatic venules, which extends out into the perivenular parenchyma and is associated with perivenular hepatocyte necrosis. In fact, the combination of perivenular inflammation and associated necrosis is the critical lesion used to recognize severe AR. The associated mononuclear perivenular inflammation helps to distinguish ischemia-induced perivenular necrosis from that seen with severe AR. In some of these cases, the accompanying bile duct damage is severe, with disruption of the ductular basement membrane and even partial destruction of the duct, recognized by the presence of a few biliary epithelial cells. This can quickly lead to bile duct loss, or CR. It should be stressed the perivenular necrosis and inflammation of severe AR must occur in combination with the typical portal inflammation and duct damage characteristic of AR. Perivenular necrosis and inflammation alone can be seen with more mild forms of rejection, but these occur in association with none or mild portal inflammation and none or mild bile duct damage. In the vast majority of cases, the total score will range from 7 to 9 and the venular inflammation score is by definition a "3".
Acute Rejection, Steroid Refractory	Rejection failing to resolve with corticosteroid treatment, necessitating treatment with an antibody preparation such as Thymoglobulin®.
Allograft Dysfunction	ALT >120 U/L, alkaline phosphatase >300 U/L, or GGT >120 U/L.
Autoimmune hepatitis	Autoimmune hepatitis is defined by histopathological features of autoimmune hepatitis on liver biopsy described as moderate to severe, interface and/or perivenular necro-inflammatory activity mediated by an infiltrate that is comprised of >30% plasma cells, in more than an occasional foci. Histopathological features will be taken in context with one or more of the following clinical parameters: 1. Liver tests 2. ANA, ASMA, ALKMA, and quantitative IgG titers
Baseline Liver Tests (ALT, alkaline phosphatase, and GGT)	Baseline liver tests are defined as the average of two laboratory tests: those obtained just prior to and at the study entry screening visit.

<table>
<tr><td>Chronic Rejection (CR)

(2000 Banff Criteria)
(Demetris A. D., 2000)</td><td>Early and late chronic allograft rejection will be defined by the 2000 Banff update on histopathologic staging and reporting of CR. Features of early and late CR are listed below.
<table>
<tr><th>Structure</th><th>Early CR</th><th>Late CR</th></tr>
<tr><td>Small bile ducts (≤60 µm)</td><td>• Degenerative changes involving a majority of ducts: eosinophilic transformation of the cytoplasm; increased N:C ratio; nuclear hyperchromasia; uneven nuclear spacing; ducts only partially lined by biliary epithelial cells
• Bile duct loss in <50% of portal tracts</td><td>• Degenerative changes in remaining bile ducts
• Loss in >50% of portal tracts</td></tr>
<tr><td>Terminal hepatic venules and zone 3 hepatocytes</td><td>• Intimal/luminal inflammation
• Lytic zone 3 necrosis and inflammation
• Mild perivenular fibrosis</td><td>• Focal obliteration
• Variable inflammation
• Severe (bridging) fibrosis</td></tr>
<tr><td>Portal tract hepatic arterioles</td><td>• Occasional loss involving <25% of portal tracts</td><td>• Loss involving >25% of portal tracts</td></tr>
<tr><td>Other</td><td>• So-called ‘‘transition’’ hepatitis with spotty necrosis of hepatocytes</td><td>• Sinusoidal foam cell accumulation; marked cholestasis</td></tr>
<tr><td>Large perihilar hepatic artery branches</td><td>• Intimal inflammation, focal foam cell deposition without luminal compromise</td><td>• Lumenal narrowing by subintimal foam cells
• Fibrointimal proliferation</td></tr>
<tr><td>Large perihilar bile ducts</td><td>• Inflammation damage and focal foam cell deposition</td><td>• Mural fibrosis</td></tr>
</table></td></tr>
<tr><td>Investigational Agent</td><td>darTregs (Please see Section 6)</td></tr>
<tr><td>Liver pathology, explanted liver</td><td>Explanted liver for subjects with history of HCC must meet the following criteria:
• Tumor burden within Milan criteria AND
• Without macro- or micro-vascular invasion AND
• Without any lesions with poorly differentiated hepatocellular carcinoma AND
• Without cholangiocarcinoma morphology</td></tr>
<tr><td>Lost to Follow-up</td><td>Subject who cannot complete study visits due to inability to reach the subject, subject relocation, etc.</td></tr>
<tr><td>Medical Monitor (MM)</td><td>NIAID official who is responsible for the safety aspects of this trial. The MM is responsible for making the final assessment of each serious adverse event (SAE).</td></tr>
<tr><td>Milan Criteria</td><td>The Milan criteria state that a patient with HCC is eligible for transplantation if the tumor burden is:
• one lesion smaller than 5 cm or up to 3 lesions smaller than 3 cm with
• no extrahepatic manifestations and
• no vascular invasion</td></tr>
<tr><td>NIAID Project Manager</td><td>NIAID assigned project manager who is responsible for all day to day protocol-related operations, including version control, consent review, etc.</td></tr>
<tr><td>Principal Investigator</td><td>Investigator awarded NIH funding for the grant.</td></tr>
<tr><td>Program Officer</td><td>NIAID official who oversees the scientific and budgetary aspects of the grant.</td></tr>
<tr><td>Protocol Mandated Procedures</td><td>Any procedure performed solely for the purpose of this research study (not site-specific SOC).</td></tr>
<tr><td>Regulatory Affairs Officer</td><td>NIAID assigned officer responsible for regulatory aspects of study, communications with FDA, and GCP compliance, as applicable.</td></tr>
<tr><td>Resolution of Acute Rejection</td><td>ALT and either alkaline phosphatase or GGT are either ≤ 1.5 X ULN or ≤ 1.5 baseline or less than ≤ 1.5x ULN</td></tr>
</table>

Site Principal Investigator	Lead investigator listed on the FDA 1572 at a participating center who is responsible for the conduct of the study at that center.
Stable liver tests	ALT and alkaline phosphatase or GGT either ≤1.5 X upper limit of normal or ≤1.5 X baseline
Study Termination	Subjects who complete the study, are lost to follow up, withdraw consent, or die during the study. Data and specimens will no longer be expected from subjects who are terminated from the study.
Study Therapy	The investigational agents and all protocol required interventions and medications.
Sustained Virological Response	SVR is defined as undetectable HCV RNA 12 weeks after end of treatment
Tolerance	Tolerance will be adjudicated 1 year after the last dose of IS. ALT and GGT must be less than or equal to 1.5X baseline. Baseline ALT and GGT are defined as the average of two laboratory tests: those obtained just prior to and at the initial screening visit. In addition, liver biopsy will be read by central pathology and adjudicated according to strict, predetermined criteria. A tolerance adjudication committee will review and determine tolerance outcome for subjects who meet central pathology criteria but have elevated liver tests above 1.5X baseline.
Withdrawal from Therapy	Subject who stops study therapy prior to protocol described duration.

1. Background and Rationale

1.1 Mortality and morbidity of conventional IS medications

It is well known that conventional immunosuppression (IS) imposes substantial mortality risk for adult liver transplant (LT) recipients over their lifetime. The probability of death after LT has been reported to be trimodal: steepest in the first 6 months after LT, fairly flat between 6 months and 8 years, and then rises again (Watt KD, 2010). The late increase in the risk of death is thought to reflect the cumulative impact of IS exposure. Examining the causes of death for LT recipients strongly supports the notion that IS contributes substantially to increased mortality risk over time.

Standard IS medications suppress the immune system in a generalized, non-specific manner that leaves the transplant recipient vulnerable to infection and malignancy. Within the first 6 months to 1 year after LT, infection is the dominant cause of death (Figure 1) (Watt KD, 2010). While deaths secondary to infection continues to accumulate over time after LT, the slope is modest and steady over the decade plus follow-up period. However, the probability of death from hepatic causes (inclusive of recurrent disease) and malignancy increases steeply over time, representing the 1st and 2nd most common etiologies of death in the medium- and long-term after LT (Watt KD, 2010), (Chandok N, 2012), (Schoening WN, 2013), (Schoening WN, 2013), (Rodriguez-Peralvarez M, 2014). Among the drugs that are commonly employed in current IS regimens, calcineurin inhibitors (CNIs), the pillar of modern IS regimens, are most strongly associated with a pro-oncologic effect (Rodriguez-Peralvarez M, 2014). Minimization of lifetime exposure to CNIs may significantly mitigate the escalating risk of de novo malignancy for stable, long-term LT recipients.

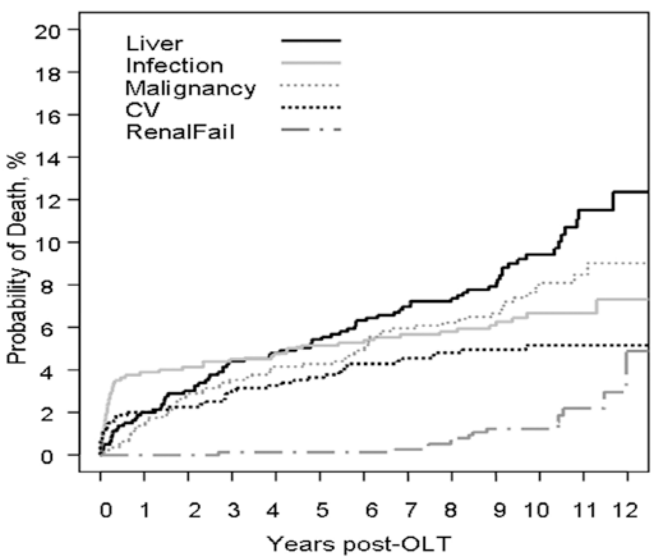


Figure 1. Cause specific probability of death after LT over time

In addition to increasing mortality risk secondary to immunologically-mediated mechanisms of weakening host defenses against infection and malignancy, CNIs are also well known to predispose to and/or exacerbate non-immunologically mediated medical co-morbidities that threaten the longevity of adult LT recipients. CNI exposure exerts a strong deleterious impact on renal function, particularly in the current climate of LT in the United States where deceased donor liver allocation is based on the Model for End Stage Liver Disease (MELD) score that has resulted in a steep increase in the proportion of LTs performed in candidates with acute kidney injury and/or chronic kidney disease (CKD) (Sharma P S. D., 2011) (Sharma P G. N., 2013). There is substantial literature that adult LT recipients are highly vulnerable to the development of severe chronic kidney disease (CKD) and even end stage renal disease (ESRD) over time (Ojo AO, 2003)(Sharma P G. N., 2013). Severe CKD in a LT recipient, defined as a GFR <30 ml/min/1.73m2, has been recently reported to increase the risk of death exponentially (Allen AM, 2014). As a result, renal failure emerges as an increasingly common cause of death late after LT (Watt KD, 2010) (Allen AM, 2014). There is interest in CNI minimization and/or withdrawal as a primary strategy to reduce CNI-mediated kidney injury (Farkas SA, 2009) (Saner FH, 2012)(Penninga L, 2012) (McKenna G, 2010; Trotter J, 2012). Although spontaneous operational tolerance may allow for IS/CNI withdrawal with reasonable success late after LT, the ability to induce tolerance with darTregs, and thereby minimize and/or completely discontinue CNIs early after LT is likely to better salvage and preserve renal function.

In addition to nephrotoxicity resulting in CKD/ESRD that predisposes to late mortality for LT recipients, CNIs are strongly associated with other potent atherosclerosis risk factors such hypertension, dyslipidemia (low high-density lipoproteins and high triglycerides), obesity, and diabetes / insulin resistance – the primary components of the metabolic syndrome (Berenson, 1992) (Davis, 2001) (Nair S. S., 2002) (Shalev, 2005) (Textor SC, 2000) (Varo E, 2002). These considerations are particularly important because the indication for LT is anticipated to, over the 5-10 decades, shift dramatically away from viral hepatitis (both hepatitis B and hepatitis C) to non-alcoholic fatty liver disease (NAFLD)/non-alcoholic steatohepatitis (NASH). The epidemiology of metabolic syndrome is regarded as not only a national but also a worldwide public health care concern with the hepatic component manifest as NAFLD/NASH (AJ., 2011). The influx of candidates undergoing LT with metabolic disease will undoubtedly translate into a heavy burden of metabolic syndrome post-transplant and focus intense attention on the negative impact of standard IS medications such as CNIs on these important medical co-morbidities (Pagadala M, 2009) (Watt KD, 2010) (Oliveira CP, 2013). Again, there is interest in reducing lifetime CNI exposure to avoid precipitating or exacerbating the components of metabolic syndrome that, alone or in combination with CKD/ESRD, predispose to cardiovascular morbidity and mortality for LT recipients.

1.2 Spontaneous / operational tolerance after LT

1.2.1 Previous experiences with IS withdrawal for adult and pediatric LT recipients

Historically, several different single centers have reported their experiences with transplant recipients who were no longer taking IS medications. Transplant recipients were electively, incidentally (non-adherence), or obligatorily (major contraindication to ongoing IS) weaned from IS. In sum, these series suggest that approximately 19% of selected adult and/or pediatric LT recipients are operationally tolerant (Mazariegos GV, 1997) (Oike, 2002) (Ramos, 1995) (Takatsuki, 2001) (Devlin, 1998) (Assy, 2007) (Tisone, 2006) (Eason JD, 2005) (Tryphonopoulos, 2005).

1.2.2 Recent trials of IS withdrawal for pediatric LT recipients

A pilot trial of IS withdrawal in 20 pediatric recipients of parental living donor transplants 4 or more years after LT (ITN029ST; WISP-R; NCT900320606) identified 12 tolerant subjects who have been off drug for 70.5-91.0 months as of September 30, 2014 (Table 1) (Feng S, 2012). Predictors of successful IS withdrawal were increased time after LT (tolerant versus non-tolerant subjects were 99.46±29.97 versus 69.70±13.67 months p=0.025), absent or minimal portal inflammation (p=0.03), and decreased C4d score (p=0.04) on screening liver biopsy were predictors of successful IS withdrawal.

Table 1. 12 Operationally Tolerant Pediatric LT Recipients from WISP-R *(as of September 30, 2014)*

Pt	Liver Disease	Age (years)		CNI Dose at Study Start	Months off IS
		Tx	Study Start		
1	BA	0.32	8.2	Tac 1.0 mg bid	107
2	BA	0.57	8.2	Tac 0.35 mg bid	107
3	BA	0.56	8.8	Tac 0.25 mg bid	106.5
4	BA	0.58	12.1	Tac 0.5 mg bid	91.5
5	BA	0.42	10.3	CsA 45 mg bid	91
7	BA	0.42	11.7	CsA 38 mg bid	45.3*
9	BA	0.75	5.2	Tac 0.5 mg bid	86.5
11	BA	0.61	6.4	CsA 50 mg bid	101
12	BA	0.56	11.2	CsA 50 mg qd	105
14	AIAT	0.32	10.2	CsA 35 mg qd	92
16	PFIC	2.5	8.9	Tac 2.0 mg qd	97
17	NSC	1.5	7.0	Tac 1.0 mg qd	91

*Subject withdrew consent and was terminated from the study 45.3 months after the last IS dose.

The data from the pilot IS withdrawal trial (WISP-R) for pediatric recipients of parental living donor LTs, led by Dr. Feng, suggest that IS withdrawal is safe and feasible. Twelve of the 20 enrollees (60%) have achieved the primary endpoint of one year off IS with normal graft function assessed by liver tests. Protocol biopsies performed more than two years and again more than four years after the last dose of IS have not shown significant histological change compared with the screening biopsies performed more than three or five years earlier, respectively. Eight patients failed to achieve the primary endpoint: one for violation of an exclusion criterion and seven for biopsy proven (n=2) or clinical (n=5) rejection. Five of the seven participants developed elevated liver tests during withdrawal, between 43 and 169 days after initiating IS withdrawal; 1 participant developed elevated liver tests after cessation of withdrawal, 333 days after initiating withdrawal. The seven participants were all treated with increased IS. Three received bolus corticosteroids (intravenous and/or oral) plus an increase in CNI dose beyond their dose at study start (escalation). Among the 4 that were not treated with corticosteroids, 1 was treated with escalation of IS while 3 were treated simply with return to their IS dose (baseline) at the start of the study (Table 2).

Table 2. Seven Non-Tolerant Participants from WISP-R

Pt	**Liver Disease**	**Age** (years)		**CNI Dose at Study Start**	**Peak ALT/GGT**	**Biopsy** (Central Pathology)	**IS Management**	**Elevated ALT/GGT**		
		Tx	**Study Start**					**Start** (D)*	**End** (D)*	**Duration** (Days)
8	OTC	7.5	15.3	Tac4.5 mg bid	121/244	ACR indeterminate	Baseline	43	162	119
15	BA	1.1	6.2	Tac1mg qam / 0.5 mg qpm	318/98	ACR moderate	IV+PO steroids Escalation	88	190	102
6	BA	0.75	8.0	Tac1.0 mg bid	562/127	ACR indeterminate	Escalation	155	515	360
20	BA	0.63	5.0	CsA50 mg bid	312/197	ACR indeterminate / biliary stricture	PO steroids Escalation	169	222	53
10	BA	0.44	6.6	CsA25 mg bid	134/25	ACR indeterminate	Baseline	333	402	69
19^	BA	0.50	5.2	Tac0.4 mg bid	NA	ACR mild	Baseline	NA		
18^	BA	0.48	6.6	Tac1.0 mg qd	NA	ACR indeterminate	PO steroids Escalation	NA		

Lastly, there is an ongoing, large prospective multicenter trial involving 12 pediatric transplant centers in North American (United States and Canada) (iWITH; RTB001; NCT01638559) that is fully enrolled. Compared to the pilot trial just described, this trial includes both deceased and living donor LT recipients. The primary objective of the trial will be to determine the frequency of operational tolerance in a well-defined population of pediatric LT recipients. Preliminarily, based on the observed incidence of AR, the prevalence of tolerance in this trial will be very comparable to the pilot study, in spite of including deceased donor LT recipients. This is harmonious with the literature that suggests minimal immunologic benefit of HLA matching in the liver transplant setting (Balan V, 2008) (Lan X, 2010) (Yosry A, 2012).

1.2.3 Recent trials of IS withdrawal for adult LT recipients

A European, multi-center, adult IS withdrawal trial enrolled 102 adult deceased donor LT recipients of which 41 (40%) were operationally tolerant (V-2005-CE512090-O; NCT00647283 (Benitez, 2013). Clinical parameters that differentiated tolerant from non-tolerant patients included increased time after transplantation (p<0.0001), increased age (p<0.0005), and male gender (p=0.016). Specifically, with respect to time after LT, among subjects who were 3.0-5.7 years after LT, only 3 of 24 (12.5%) succeeded with IS withdrawal, compared to 19 of 24 (79.2%) among subjects who were >10.6 years after LT (Table 3).

Table 3. Relationship of time after transplantation with proportion of tolerant subjects in NCT00647283		
Years since transplant	**Tolerant**	
	N	**%**
3.0 - 5.7	**3 / 24**	**12.5**
5.7 - 10	19 / 50	38.0
>10.6	19 / 24	79.2

Table 4. Prevalence of tolerance among subjects enrolled in AWISH		
Etiology of Liver Disease	**Tolerant**	
	N	%
HCV+	5 / 30	16.7
Non-immune, non-viral	**6 / 46**	**13.0**

Finally, a trial entitled "Gradual Withdrawal of Immune System Suppressing Drugs in Patients Receiving a Liver Transplant" (AWISH/ITN030ST/NCT00135694) that enrolled adult de novo LT recipients receiving standard IS who initiate IS withdrawal between one and two years after deceased donor LT. In total, 76 subjects attempted IS withdrawal; 5/30 (16.7%) subjects with HCV and 6/46 (13.0%) subjects with non-immune, non-viral liver disease were identified as tolerant (Table 4).

Taken together, the historical experiences and the recent adult and pediatric withdrawal trials convincingly show that operational tolerance indeed occurs in both adult and pediatric LT recipients, and perhaps more often in the latter (Mazariegos GV, 1997) (Oike, 2002) (Ramos, 1995) (Takatsuki, 2001) (Feng, 2012). AR episodes occurring during controlled, highly supervised IS withdrawal were consistently mild to moderate in histologic severity, almost never requiring antibody treatment, and resolved without permanent allograft damage or dysfunction (Mazariegos GV, 1997) (Ramos, 1995) (Takatsuki, 2001). Graft loss related to IS withdrawal has not been observed. Presumably, close surveillance ensures expeditious detection, diagnosis, and treatment of allograft rejection. The literature supports the intuition that delayed diagnosis and treatment leads to the varied outcomes of rejection occurring outside of the close surveillance characteristic of IS withdrawal protocols (Neil, 2001). Time after LT has emerged as a consistent factor relative to the prevalence of operational tolerance. Among adults 2-6 years after LT, the prevalence of tolerance is low, approximating 13%.

1.3 Rationale for Accelerating Successful IS Minimization / Withdrawal

One of the primary findings of the large, European, prospective multi-center trial of IS withdrawal for adult recipients of deceased donor LTs is that the frequency of operational tolerance is highly dependent on time after transplantation (Benitez, 2013)(Table 3). The success rate of IS withdrawal increased from 12.5% (3 of 24) to 38.0% (19 of 50) to 79.2% (19 of 24) for patients who were 3.0-5.7, 5.7–10.6, and >10.6 years after LT (Table 3). Moreover, in AWISH, where de novo adult deceased donor LT recipients who received standard IS underwent IS withdrawal that was initiated 1-2 years after LT, the frequency of operational tolerance among recipients with non-immune or non-viral liver disease was 13.0% (6/46). Finally, WISP-R, a multi-center prospective pilot trial of IS withdrawal for pediatric recipients of living parental donor LTs, increased time after transplantation was identified as significantly associated with operational tolerance (Feng, 2012). Therefore, spontaneous operational tolerance does occur among adult and pediatric LT recipients but only at a low frequency early after LT (<6 years). Since the toxicities of conventional IS as delineated above (Section 1.1) are cumulative, LT recipients could derive substantial benefit if standard IS in general and CNIs in specific could be minimized and/or discontinued earlier, within the first 2-6 years after LT. darTregs may therefore represent a strategy to actively control the allo-immune response and substantially increase the success rate of IS minimization or discontinuation in the early post-transplant timeframe.

1.4 Induction of Transplantation Tolerance

Acquisition of immune tolerance to self-antigens or allo-antigens is an active process that requires antigen exposure. Tolerogenic antigen exposure leads to inactivation of antigen-reactive T cells through apoptosis, injury and induction of immune regulatory mechanisms that maintain tolerance. The current approach of IS for transplant recipients blindfolds the immune system to prevent rejection, but also impedes tolerance induction. This may explain why spontaneous transplant tolerance is rare and the best predictor of tolerance is time after transplant, likely through the cumulative effect of low level of donor antigen exposure over a long period of time. Ideally, transplant patients should receive immunoregulatory regimens blocking rejection while allowing donor antigen recognition and tolerance induction. Such immunoregulatory regimens, including Treg therapy, have promoted tolerance and IS-independent graft survival in animal models of transplantation. While most of these experimental regimens are given in the peri-transplant period, we think a similar approach can be rationally applied to patient undergoing IS withdrawal at a later time point after transplantation. In our previous pilot trial of IS withdrawal in pediatric LT recipients (Feng, 2012) many years after transplantation, along with AWISH, a trial of IS withdrawal in adults starting 1-2 years after LT, we observed *de novo* production of donor-specific antibodies, demonstrating new immune recognition of donor antigen during IS withdrawal. Thus, IS withdrawal may present an important opportunity to induce tolerance using an immunoregulatory regimen.

1.5 Rationale for Selection of Investigational Product or Intervention

1.5.1 Rationale for darTregs Therapy

The recent elucidation of Tregs and their importance in suppressing autoimmunity and alloimmunity has inspired new thinking in managing alloresponses. Emerging data suggests designing IS regimens with a "Treg-centric" approach to promote regulation may favor induction of graft tolerance and improve long-term graft outcomes (Wood, 2003) (Bluestone, 2004) (Walsh, 2004) (Kang, 2007) (Sagoo P. G., 2008) (Waldmann, 2008) (Long, 2009). Unlike generalized IS regimens, Tregs are long-lived and function in a dominant and antigen-specific manner. Thus, therapeutic infusion of Tregs has potential to induce long-term donor-specific tolerance without impeding desired immune responses to pathogens and tumors in transplant patients. Research in animal models has demonstrated Tregs can be used to treat many auto-inflammatory diseases such as type 1 diabetes, inflammatory bowel disease, systemic lupus erythematosus, and multiple sclerosis. In addition, Treg therapies are efficacious in controlling alloimmune responses in graft-versus-host disease (GvHD) as well as organ and cell transplantation in animal models.

1.5.2 Rationale for darTregs Dosing

The Treg dose in protocol CTOTC-12 was selected based on three primary factors: prior human experience, estimated effective dose, and manufacturing capacity at the UCSF facility.

1.5.2.1 <u>Dosing precedents in humans</u>

Currently, there are five published reports of Treg therapy in humans, four studies in adults for GvHD (Di lanni, 2011) (Trzonkowski, 2009) (Brunstein, 2010) (Martelli MF D. I., 2014) and one study in children with new onset type 1 diabetes (Marek-Trzonkowska N., 2012). All these studies administered polyclonal Tregs, and currently no prior experiences with darTregs have been reported. Since polyclonal Tregs are most similar to darTregs when compared to other cellular therapies, it is nonetheless helpful to review the dosing and safety information from these studies. The results of these protocols, particularly with respect to the safety of Treg administration, are detailed in Section 1.6. The discussion below provides the rationale for selection of Treg doses for administration to human subjects participating in protocol CTOTC-12.

The highest dose used in the referenced GvHD studies was 2 infusions of 3 x 10^6 Tregs/kg each on day 1 and day 15 after cord blood transplantation. No infusion reactions or severe adverse events (AEs) was observed with this dose. In fact, the investigator reported that maximal tolerated dose could not be assessed due to limitation in manufacturing capacity (Brunstein, 2010).

In the type 1 diabetes pediatric study (Marek-Trzonkowska N., 2012), children age 8 to 16 were given 10 x 10^6 Tregs/kg (n=4) or 20 x 10^6 Tregs/kg (n=6), corresponding to 700 – 1,400 x 10^6 Tregs for the average 70kg adult human. It is worth noting that the Treg expansion protocol used for the Marek-Trzonkowska study is comparable to that currently used at UCSF for polyclonal Treg manufacture and many manufacturing processes are shared with the darTregs manufacturing process for CTOTC-12.

In an adult, UCSF-sponsored ongoing type 1 diabetes trial at UCSF, subjects in four escalating dose cohorts have been infused with 5, 40, 320, and 2,600 x 10^6 Tregs total dose, corresponding to approximately 0.07, 0.6, 4.6, and 38 x 10^6 Tregs/kg. All 14 patients in four dosing levels have been administered. As of September 2014, all 14 subjects were active in the study. Cohorts 1-4 are 3, 2.5, 1.5, and 1 year after administration, respectively. No infusion reaction of any grade was observed in any patient. Four serious adverse events (SAEs) deemed unrelated to Treg infusion were reported, as of August 2014.

Thus, based on published data and UCSF's clinical experience, infusions of up to 2,600 x 10^6 (38 x 10^6/kg) polyclonal Tregs were well tolerated.

1.5.2.2 Estimated efficacy dose for darTregs in organ transplantation

Results from preclinical studies in mouse models of transplantation in the past 30 years provide strong rationale for the use of Tregs to induce transplant tolerance (Tang Q, 2013). In these models, a high percentage of Tregs, as many as 30-50% of $CD4^+$ cells, is needed to prevent transplant rejection (Hara, 2001) (Graca, 2002). To achieve this high percentage of Tregs in a 70kg adult human, an infusion of 4,500 x 10^6 (642 x 10^6 Tregs/kg) Tregs would be needed. This dose can be reduced by 80 to 90% (to 500-1,000 x 10^6 total dose) if darTregs are used (Lee K, 2014). While these estimates provide useful guidelines for dose selection in humans, these should not be directly applied because of the many differences between mouse models and human trial design such as the use of concurrent IS and the timing of Treg infusion. Typically, Tregs are given around the time of transplantation in mouse models without additional IS when strong activation signals and highly inflammatory conditions increase the resistance of effector T cells to Treg-mediated suppression. Thus, it is very likely that a lower percentage/ fewer Tregs would be required to induce tolerance when IS is present to control T cell activation and when Tregs are given years after transplantation without acute tissue injury and inflammation of the surgery.

Recently, an ongoing clinical trial of Treg therapy in LT conducted in Japan has been reported, providing valuable clues (Yamashita, 2013) (Yamashita, 2014) (Todo S, 2016). This trial was conducted in de novo adult, living donor LT recipients on a standard triple IS regimen comprised of corticosteroids, tacrolimus, and MMF. Recipient peripheral blood mononuclear cells (PBMCs) expanded with irradiated donor PBMCs in the presence of anti-CD80 and anti-CD86 antibodies to block co-stimulation were administered 13 days after LT and 8 days after a single dose of cyclophosphamide (40mg/kg). In total, 610 - 2,590 x 10^6 expanded autologous PBMCs expanded in the presence of donor cells and containing an average of 28% Foxp3+ cells (31 - 466 x 10^6 $Foxp3^+$ Tregs) were infused (Table 5). IS was withdrawn gradually starting 6 months after LT. As of July, 2014 (personal communication; presentation at the World Transplant Congress, July 2014, San Francisco, CA; manuscript in preparation), 7 of 10 treated patients have been weaned off IS for 6-23 months with five recipients off IS for greater than 12 months (13 to 23 months), Three of 10 have failed IS withdrawal, two secondary to AR and one secondary to IS re-initiation for brachial plexus neuritis who later developed AR while on IS. The cell manufacturing protocol has been shown to inactivate effector T cells and favor the outgrowth of Tregs (Davies JK, 2009). Similarly manufactured cells have also been used therapeutically to control GvHD (Guinan EC, 1999) (Davies JK G. J., 2008), and kidney transplant rejection in non-human primates (Bashuda H, 2005).

To determine appropriate Treg dosing, the outcome relative to the dose of $Foxp3^+$ Tregs administered was considered. Three patients received only 590 - 630 x 10^6 PBMCs, corresponding to 31 to 43 x 10^6 $Foxp3^+$ Tregs. Two of these three subjects developed AR while 1 successfully discontinued IS and has remained off for 23 months. The other 6 subjects who have successfully discontinued IS received 790 to 2,590 x 10^6 PBMCs, corresponding to 94 to 466 x 10^6 $Foxp3^+$ Tregs. The other patient who failed IS withdrawal received 272 x 10^6 $Foxp3^+$ Tregs failed IS withdrawal due initially to brachial plexus neuritis and later AR.

This study suggests that Treg doses of <50 x 10^6 Tregs are not sufficient to induce operational tolerance (1 success out of 3). However, doses between 100 and 300 x 10^6 Tregs have some efficacy (2 successes out of 3) and doses >300 x 10^6 Tregs appear to be reliably adequate (4 successes out of 4) to support successful IS withdrawal.

The product used in this Japanese trial is enriched for donor alloantigen specificity as is the darTregs product we plan to use. However, there are several important differences regarding the context of Treg administration between their trial design and CTOTC-12. The depletional therapy that they administered prior to Treg infusion (cyclophosphamide) is likely to support Treg expansion, engraftment, and function; depletion is perceived to lower the Treg dose needed for efficacy. However, they administer Tregs 13 days after LT, during the highly inflamed peri-transplant period to subjects who are receiving a high IS burden in general and high CNI dosing in specific. Both of these factors may hamper the survival and function of their infused Tregs. CTOTC-12 study design administers darTregs to LT recipients with stable liver function on modest doses of CNIs long after transplantation, an immunologically quiescent timeframe but without depletion. We believe that these are compensatory differences and therefore can reasonably extrapolate their dosing regimen in this clinical trial.

Table 5. Treg infusions and IS withdrawal outcomes for 10 adult LT recipients: Hokkaido University.

Pt #	Total Cells Infused $\times10^6$	$CD4^+CD25^+Foxp3^+$ Cells Infused ($\times10^6$)	Days after LDLT*	Current IS	DSA	Months off IS*
1	**610**	**31**	**1620**	**none**	**Neg**	**33**
2	**2,540**	**466**	**1543**	**none**	**Class II+**	**31**
3	**790**	**94**	**1515**	**none**	**Class II+**	**32**
4	**2,450**	**441**	**1410**	**none**	**Neg**	**29**
7	**2,590**	**318**	**1263**	**none**	**Neg**	**13**
8	**700**	**304**	**1186**	**none**	**Neg**	**18**
10	**1,200**	**289**	**1018**	**none**	**Neg**	**16**
5	630	43	1326	FK 4 mg/d	ND	ACR; POD 394
9	590	33	1284	FK 4 mg/d	ND	ACR; POD 365
6	1,180	272	1123	MMF 500 mg/d PSL 5 mg/d	ND	Brachial plexus neuritis POD 206; ACR POD 311

*As of April 30, 2015

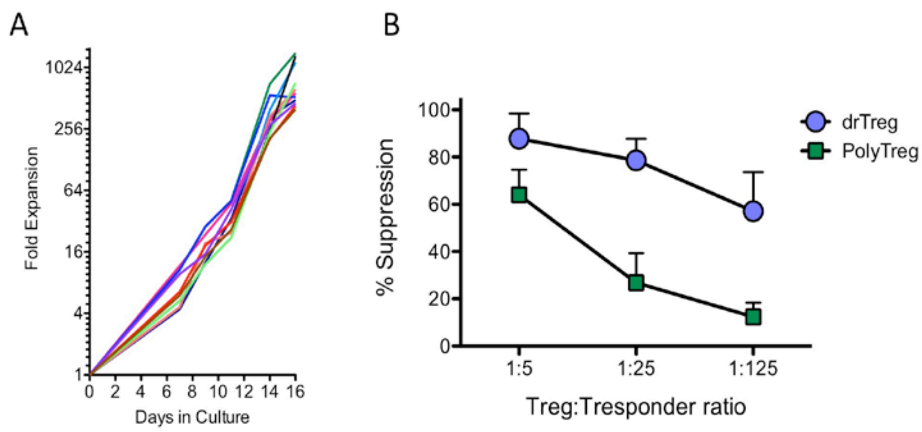
Although the Japanese study has many differences to our proposed study, this is currently the only Treg cell therapy trial in solid organ transplantation and the closest to our clinical scenario of adult LT. In sum total, we estimate that the requisite dose of darTregs to facilitate IS withdrawal in stable, long-term LT recipients may be similar to that reported in the Japanese trial. We estimate that a dose of <100 x 10^6 darTregs will have low efficacy; that a dose of 100 – 300 x 10^6 will have moderate efficacy; and that a dose of >300 x 10^6 will have high efficacy for successful IS minimization / discontinuation.

1.5.2.3 <u>Dose limitations imposed by darTregs manufacturing capacity</u>

The UCSF Treg manufacturing facility can routinely produce more than 600 x 10^6 darTregs from 1 unit of blood (450 ml) or 500 x 10^6 leukapheresis products, a quantity that is approximately double the dose estimated to be effective based on the LT Treg trial conducted in Japan. On average, 6.6 x 10^6 (range 3 to 11.9 x 10^6) Tregs can be purified from one unit of blood and expanded >200 fold (range 200 to 4000) during the 16-day ex vivo culture (Figure 2A) to produce more than 600 x 10^6 Tregs (Figure 2B). The expanded darTregs retain their phenotypes and are highly suppressive of donor antigen stimulated proliferation in vitro (Figure 2B).

Taken together, we plan to infuse a targeted dose of 400 x 10^6 ± 100 darTregs in this trial. A range of +/- 25% change from this targeted dose is allowed such that the range of darTregs to be infused is 300 - 500 x 10^6. Subjects receiving this dose will have the option, if eligible, to proceed to complete IS withdrawal. If less than 100 x 10^6 darTregs are produced for a patient, the product will not be infused because of the low likelihood of efficacy based on data from the Japanese LT Treg trial. If 100 - 300 x 10^6 darTregs are produced, the product will be infused and the patient will be allowed to start IS withdrawal because a favorable probability of efficacy with this dose compared to no treatment (Table 7 and Table 8). Participants receiving 100 – 300 x 10^6 darTregs will only be allowed to progress to the primary endpoint: 75% reduction of CNI dosing along with Prednisone or MMF discontinuation (if they entered the trial on Prednisone or MMF).

Figure 2. Expansion and suppressive function of darTregs.



A. Fold expansion of darTregs using 8 distinct donor-recipient combinations.

B. In vitro suppression of donor antigen stimulated proliferation comparing darTregs and polyclonal Tregs from the same individual. The graph is a summary of 5 different pairs of darTregs and polyclonal products.

1.5.3 Rationale for Proposed IS Withdrawal Algorithm

The IS withdrawal as proposed (Section 5.1; Table 11. CNI Withdrawal Algorithm, Table 12. MMF Withdrawal Algorithm) is based on recently completed or currently ongoing clinical trials for pediatric or adult liver transplant recipients (Table 6. Completed and ongoing IS withdrawal clinical trials). For comparison purposes, the currently proposed trial is listed as the last two entries: “ARTEMIS Minimization” and “ARTEMIS Complete Withdrawal”. The AWISH (ITN030ST/NCT00135694) and the European multi-center (Benitez, 2013) trials which provide historical controls for the proposed trial are also listed.

Essentially all trials withdraw IS completely over approximately 9 – 12 months, reducing IS doses in 6 – 10 steps, each of 4 – 8 week duration. The European multi-center trial (Benitez, 2013) has a less regimented withdrawal algorithm, with less clearly delineated dose reduction algorithm and a “target” withdrawal timeframe of 6 – 9 months. We designed the ARTEMIS withdrawal algorithm to fit well within the parameters of these tested approaches. There is no published data that speaks to the impact of either a significantly shorter or much longer weaning protocol on the safety or efficacy of withdrawal.

The currently proposed trial seeks to enroll subjects who are “early” after liver transplantation (2 – 6 years) who have a low likelihood, in the absence of darTregs, of successful IS minimization or complete discontinuation. Such subjects, when managed according to standard of care, are typically on a calcineurin-inhibitor (CNI) based regimen but often with an additional medication, either Prednisone or mycophenolate. The other adult withdrawal trials (de la Garza RG, 2013) (Benitez, 2013) similarly allow subjects on one- or two-drug therapy to attempt withdrawal. AWISH is unique, only allowing subjects on monotherapy even though withdrawal is initiated early after transplantation, between 1 – 2 years after transplant. This trial, however, enrolled subjects prior to liver transplantation and the protocol, rather than standard of care, determined the IS regimen from the time of transplantation.

We have decided to withdraw both CNI and either prednisone or MMF simultaneously rather than sequentially (Section 5.1) to allow gradual reduction of drugs, while maintaining the overall timeframe of withdrawal at approximately one year (48-50 weeks). We believe that gradual reduction of prednisone, from the maximal allowable entry dose of 5 mgs daily to none over 16 weeks (Section 5.1.2), is important for subjects who have been maintained on corticosteroids for as many as six years. There is little precedent to suggest that there is a difference in efficacy or safety with sequential versus simultaneous withdrawal of two IS medications.

Table 6. Completed and ongoing IS withdrawal clinical trials

STUDY	Adult / Pediatric	Time after Transplantation at Initiation of IS Withdrawal: Inclusion Criterion; *[Actual Data]*	# IS Medications at Baseline	Number of Dose Reductions	Duration at Each Dose	Minimum Duration of IS Withdrawal*
ITN029/WISP-R	Pediatric	> 4 yrs; *[median (range): 102.0 (60.0 – 183.8) mos]*	1	8	4 – 6 weeks	36 weeks
iWITH		> 4 yrs; *[mean ± SD: 104.4 ± 39.8 mos]*	1	8	4 – 6 weeks	36 weeks
ITN030/AWISH	Adult	1 – 2 yrs	1	8	8 weeks	56 weeks
Benitez et al., Hepatology 2013[1]		> 3 yrs; *[mean ± SD: 103 ± 47 mos]*	1 or 2; Sequential withdrawal if 2 drugs	Manuscript states: ". . . doses were gradually reduced by ¼ to ½ until the minimum feasible daily dose"	3 weeks	Manuscript states: "Target completion time for drug discontinuation was 6 to 9 months."
De la Garza et al., Liver Transplantation 2013[2]		> 3 yrs; *[median (range): 112 (72-160) mos]*	1 or 2; Unspecified whether simultaneous or sequential withdrawal if 2 drugs	6 - 10	one month	6 - 10 months
ARTEMIS Minimization		2 – 6 yrs	1 or 2; Simultaneous withdrawal if 2 drugs	5	6 - 8 weeks	24 – 26 weeks
ARTEMIS Complete Withdrawal				8	6 – 12 weeks	48 – 50 weeks

*The last dose reduction is complete discontinuation of IS. This "step" does not contribute to the total duration of IS withdrawal. "Pauses" for additional monitoring of liver tests can lengthen the duration of withdrawal. Hence, the minimum duration of withdrawal is shown.

[1]Benitez C. (2013). Prospective multi-center clinical trial of immunosuppressive drug withdrawal in stable adult liver transplant recipients. *Hepatology, 58*(5), 1824-35.
[2]de la Garza RG. (2013). Trial of Complete Weaning From Immunosuppression for Liver Transplant Recipients: Factors Predictive of Tolerance. *Liver Transplantation,* 19:937–44

1.5.4 Rationale for Timing of Treg Administration during IS Minimization/Withdrawal

Protocol CTOTC-12 is designed to administer darTregs at a time that will maximize clinical efficacy and thereby increase the likelihood that participants will derive the benefit of successful IS minimization or discontinuation.

To enter the trial, subjects must be between two and six years after living donor LT and stable on low to moderate dose CNI, as defined by 12 hour trough CNI levels. As patients during this post-LT timeframe may still be maintained on a 2nd drug, subjects can enter the trial if they are similarly maintained on low to moderate dose of a 2nd IS medication. There is precedent for allowing adult subjects on more than a single drug to enter IS withdrawal trial (Benitez, 2013).

We have imposed specific limits for 12 hour CNI trough levels to ensure that the infused darTregs are not exposed to high circulating levels of CNIs. Moreover, the darTregs infusion will occur after a 25% reduction of the study entry CNI dose. Treg function depends on activation by the cognate interaction between T cell receptors and the antigen-presenting cells. High CNI doses are often used early after transplantation to block Treg activation and suppress IL-2 production by conventional T cells. Tregs are thus deprived of this essential survival and growth factor in the early post-transplant period. However, reports suggest that low to moderate CNI levels, as would be expected for the population enrolled in this study who are not recently transplanted, may be less harmful for Tregs because 1) Tregs have more active Ca++ signaling machinery than conventional T cells requiring a higher CNI concentration to be blocked; 2) IL-2 receptor on Tregs have higher binding affinity than those expressed on conventional T cells and effectively compete when IL-2 production is restricted. Therefore, the function or persistence of the infused Tregs should not be critically impacted by the ongoing, moderate CNI doses subjects will be taking in this protocol (Brandt C, 2009) (Calvo-Turrubiartes M, 2009). Tregs also function by conferring regulatory activities to conventional T cells, termed “infectious tolerance.” This activity depends on antigen activation of conventional T cells and Tregs (Qin S., 1993), (Dieckmann, 2002), (Jonuleit, 2002), (Kendal, 2011)) that may also be inhibited by exposure to high levels of CNIs. Finally, subjects may enter the trial if they are on a modest dose of a 2nd IS medication, either Prednisone or MMF. At the doses typically given to patients 2-6 years after liver transplantation, neither of these two drugs have a substantial impact on Treg function or longevity (Lim DG1, 2010).

We have deliberately selected to administer darTregs at the end of the 2nd IS withdrawal step for several reasons. First, as mentioned above, darTregs will be infused after 25% reduction of CNI dosing. Second, delaying darTregs infusion will eliminate subjects who develop abnormal liver tests/AR very early in the IS withdrawal algorithm, perhaps those who are the farthest from tolerance. The 1st step (Table 7) stipulates that the morning and evening doses be combined into a single dose, taken in the morning. As such, there is no dose reduction. The 2nd step entails a 25% dose reduction. In AWISH (ITN030ST/NCT00135694), out of 46 subjects on CNI monotherapy who initiated IS withdrawal, a single subject each failed at the 1st and at the 2nd step. Third, administration at the end of the 2nd step will also deliver darTregs more proximal to the time when immune responses are escalating, as reflected by the timing of AR in AWISH (ITN030ST/NCT00135694).

Table 7. Stepwise Outcome of IS Withdrawal for AWISH Subjects Maintained on CNI Monotherapy

STEP	IS REDUCTION: % OF STARTING DOSE	# SUBJECTS ENTERING STEP	# SUBJECTS FAILING DURING STEP	% FAILURE PER STEP	CUMULATIVE FAILURE RATE
1	0%*	46	1	2.2%	2.2%
2	25%	45	1	2.2%	4.3%
3	50%	44	11	25.0%	28.3%
4	**75%**	**33**	**18**	**54.5%**	**67.4%**
5	85.7%	15	4	26.7%	76.1%
6	92.9%	11	4	36.4%	84.8%
7	96.4%	7	1	14.3%	87.0%
8	***100%***	***6***	***0***	***0%***	***87%***

*First step combines twice daily dosing into a single daily dose and therefore does not involve dose reduction.

1.5.5 Rationale for Timing of IS Withdrawal Resumption after darTregs Administration

The protocol mandates that IS withdrawal be initiated soon, within 14 days after darTregs infusion to maximize the therapeutic effects and potential benefit of darTregs administration. Initiation of IS withdrawal soon after darTregs infusion will favor tolerance induction for the following reasons. First, CNI is known to be detrimental to darTregs function and homeostasis (Baan, 2005) (Rudensky, 2006) (Pascual J, 2008). Although CTOTC-12 patients will receive lower doses of CNIs compared with typical transplant patients, further reduction in CNI exposure will be beneficial for the infused Tregs. Second, it is well established that Treg induction of transplantation tolerance depends on a process called infectious tolerance (Kendal AR, 2010). The infused darTregs create a tolerogenic microenvironment in the tissue, allowing host T cells activated in this environment to acquire regulatory properties and thereby propagating tolerance. Infectious tolerance requires therapeutic Tregs and host T cells to be activated by their T cell receptors, which is blocked by CNI. Therefore it is proposed that reduction of CNI doses will favor development of infectious tolerance. Third, our experience with the ex-vivo expanded autologous $CD4^{+}CD127^{lo/-}CD25^{+}$ Tregs thus far show that the infused Tregs peaked in the circulation within two weeks of infusion and persisted at a relatively low level for at least 3 months (data shown in UCSF IB). We think it is best to reduce CNI exposure when the infused Tregs are at the highest circulating levels to maximize the effect of Treg therapy.

1.6 Clinical Experience with Treg Therapy

There have been and continue to be multiple clinical experiences with Treg infusion in distinct settings that provide relevant information to this protocol with respect to both the safety and the efficacy of Tregs. Tregs have been administered to adults and children, to patients on and off generalized IS, for wide-ranging indications including the prevention or treatment of GvHD, the stabilization and/or reversal of type I diabetes mellitus, and the induction of tolerance after LT (Table 8).

Table 8. Summary of Treg cell therapy in humans										
Study		Patients		Treg		Adjunct IS	Follow-up	Major findings		Status
Lead	Disease	N	Age	Type*	Dose			Safety	Efficacy	
Trzonkowski	GvHD	2+	40, 44, NR	A	0.1-3 x 10^6/kg 2 - 4 x 10^6/kg	Steroid, ATG, MMF, Tac	2-3 mos	No relapse	Reverse cGvHD, ineffective in aGvHD	Published 2009; ongoing
Brunstein	GvHD	23	12-70	B	0.1-6 x 10^6/kg	MMF, CsA or Sirolimus	2-24 mos	No increase in infections or relapse	No cGvHD	Published 2010
Di Ianni	GvHD	28	21-60	C	2-4 x 10^6/kg	none	12 mos	No increase in relapse, positive response to flu vaccine	Reduced aGvHD	Published 2011
Edinger	GvHD	9	NR	C	5 x 10^6/kg	none	NR	No severe infections, no relapse	No GvHD	Reported 2011
Marek-Trzonkowski	T1D	10	8-16	A	10-20 x 10^6/kg	none	4 mos	No safety concerns	C-peptide stabilization	Published 2012
Yamashita	LT	10	39-59	D	0.6 - 2.6x10^9 (8.6 – 37.1 x 10^6/kg)	CYA, Steroid, MMF, CNI	16-36 mos	No infusion AE	7 pts off IS	Abstract, ongoing
Bluestone	T1D	14	18-43	A	0.05 - 2.6x10^9 (0.7 – 37.1 x 10^6/kg)	none	3-27 mos	No SAEs related to therapy	Stable c-peptide	Published 2015

Treg type:
A: FACS purified polyclonally expanded nTreg, most similar to UCSF protocol
B: MACS-enriched polyclonally expanded nTregs
C: MACS-enriched non-expanded nTregs
D: PBMC expanded with irradiated allogeneic PBMC with costimulation blockade

Abbreviations:
GvHD: graft-versus-host disease; cGvHD: chronic GvHD; aGvHD: acute GvHD
IS: immunosuppression
T1D: type 1 diabetes CsA: cyclosporine
Tac: Tacrolimus
CNI: calcineurin inhibitor of either tacrolimus or cyclosporine
MMF: mycophenolate mofetil
ATG: anti-thymocyte globulin
CYA: Cyclophosphamide
NR: not reported

1.6.1 Treg Therapy for Treatment or Prevention of GvHD

Initial human experiences in Treg therapy were all for GvHD. The first-in-man use of Tregs was by Trzonkowski et al. to treat established GvHD (Trzonkowski, 2009). As such, these two patients were also receiving IS. The first patient had chronic GvHD two years after bone marrow transplantation. After receiving 0.1 × 10^6/kg fluorescence-activated cell sorting (FACS) purified *ex vivo* expanded Tregs from the donor, the symptoms subsided and the patient was successfully withdrawn from IS. The second patient had acute disease that progressed despite three infusions with a cumulative dose of 3 × 10^6/kg expanded donor Tregs. After the initial report in 2009, the investigators continued to enroll patients who are taking conventional IS with chronic and acute GvHD for Treg therapy. Their recent review suggests that Treg therapy is effective at reversing chronic GvHD and allowing patients to be weaned off IS, but ineffective at controlling acute GvHD (Trzonkowski P, 2013)

In all of the other GvHD trials reported thus far, Tregs were given at the time of stem cell transplant to prevent the development of GvHD. A phase I trial by Brunstein et al. was reported in 2010 (Brunstein, 2010). Twenty-three patients with advanced hematologic malignancies were enrolled and treated with two units of umbilical cord blood as source of stem cells and effector T cells. Tregs were isolated using anti-CD25 immunomagnetic bead selection from third-party cord blood donors who were matched at least 4 of 6 HLA loci with the recipient. Up to 6 × 10^6/kg Tregs, expanded *ex vivo* using anti-CD3 and anti-CD28 conjugated beads, were infused. The infused Tregs were detectible in circulation for up to 7 days. During the one-year period after Treg infusion, the investigators observed no dose-limiting toxicities or increase in AEs when compared to historical controls. Incidences of severe acute GvHD were significantly reduced in patients who received Treg therapy. For those who developed GvHD after Treg therapy, the median time to disease onset was longer. The investigator monitored opportunistic infections and tumor relapse rates to determine if Treg therapy led to over-IS in these severely immunocompromised individuals. They reported that Treg treatment did not increase opportunistic infection or tumor relapse rates when compared to historical control patients who underwent the same regimen without Treg infusion.

Another published GvHD prophylaxis trial enrolled 28 patients with high-risk hematological malignancies (Di lanni, 2011). Patients received anti-CD25 immunomagnetic bead-enriched donor Tregs without *ex vivo* expansion four days before receiving one haplo-mismatched hematopoietic stem cell transplant (average 9.4 x 10^6 stem cells/kg) and CD19-depleted donor PBMC as source of conventional T cells (Tconvs) from the same donors. The majority of the patients received 2 × 10^6/kg Tregs with 1 × 10^6/kg Tconvs. No adjunct IS was given after transplant due to the lower risk of GvHD secondary to the infusion of a lower dose of donor T cells. Patients demonstrated accelerated immune reconstitution, reduced cytomegalovirus (CMV) reactivation, a lower incidence of tumor relapse, and less GvHD when compared to historical controls. More recently, the same group published their phase II trial using a similar protocol in 43 high-risk leukemia patients. The results were consistent with their previous report that Tregs given concomitantly to stem cells and Tconv cells enabled infusion of higher dose of Tconv cells for the prevention of cancer relapse without increasing GvHD (Martelli MF D. I., 2014).

Similar GvHD trials are being conducted with non-expanded Tregs (CD4+CD25hi) (P. Hoffmann and M. Edinger in Regensburg, Germany), Tregs grown in IL-10 and sirolimus (M. Grazia-Roncarolo, Milan), CD4+CD25+ Tregs immunoselected via CliniMACS (M.F. Martelli, Italy), and $CD4^+CD127^-$ Tregs sorted from a $CD34^+CD25^+$ CliniMACS selected population (R. Negrin, Stanford).

1.6.2 Treg Therapy in Type 1 Diabetes

1.6.2.1 Treg Therapy in Children with Type 1 Diabetes

A clinical trial infusing Tregs in diabetic children has been reported (Marek-Trzonkowska N., 2012). The investigators administered Tregs to 10 type 1 diabetic children (aged 8-16 years) within 2 months after diagnosis. In total, 4 patients received 10 × 10^6 Tregs/kg, and the remaining 6 patients received 20 × 10^6 Tregs/kg. The preparation consisted of sorted autologous $CD3^+CD4^+CD127^{lo/-}CD25^+$ Tregs expanded under good manufacturing practice (GMP) conditions. The children were not receiving IS. For safety, the investigators monitored infusion reactions, episode of hyper- or hypo-glycemia episodes, and infections. No toxicity of the Treg therapy was noted. To assess the efficacy of the treatment, the investigators monitored percentages of circulating Tregs in patients after infusion and metabolic parameters including fasting C-peptide levels, HgbA1c, and daily insulin requirements. A significant increase in Treg percentages in the peripheral blood was observed on the day of infusion. These patients were followed along with matched type 1 diabetic patients not treated with Tregs. Half a year after type 1 diabetes onset (4-5 months after Tregs infusion), 8 patients treated with Tregs still required <0.5 IU/kg of insulin daily, with 2 patients no longer requiring insulin. In the untreated group, 6 out of 10 required > 0.5 IU/kg. In addition, plasma C-peptide levels were significantly higher in the treated group as compared with those not treated. The investigators concluded that the administration of Tregs was safe and tolerable in children with recent-onset type 1 diabetes.

Since the publication of this trial, the lead investigator has presented updates at international meetings that the metabolic improvements in Treg-treated children did not persist beyond 6 months after treatment. Given the promising profile of the treatment, the team has reported treatment of additional children with *repeated Treg infusions* resulting in more sustained effects.

1.6.2.2 Pharmacokinetics and Product Metabolism in Humans

Stable isotope labeling has been employed to track CD4+CD127lo/-CD25+ polyclonal Tregs in the peripheral vascular space in the study "A Phase 1 Safety Trial of CD4+CD127lo/-CD25+ Polyclonal Treg Adoptive Immunotherapy for the Treatment of Type 1 Diabetes" conducted under IND 14462 and in the study "A Pilot Trial of CD4+CD127lo/-CD25+ Polyclonal Treg Adoptive Immunotherapy in Renal Transplant Recipients (TASKp)" conducted under IND 15711. During ex vivo expansion, the ^{2}H label from deuterated glucose (^{2}H2-glucose) contained in the cell culture medium is incorporated into the deoxyribose moiety in replicating DNA through the de novo purine nucleotide synthesis pathway. Following infusion of stable isotope-labeled Tregs, the total number of Tregs in peripheral blood can be measured by flow cytometry and stable-isotope enrichment in purified Tregs can be determined. Following isolation and hydrolysis of genomic DNA, the isotopic enrichment of the purine deoxyribonucleosides in Tregs sorted from whole blood can be assessed by gas chromatography/mass spectrometry. The change in ^{2}H enrichment in the total Treg pool can be assessed at specified time points post-infusion.

In the T1D Phase 1 study, three subjects in cohort 3 and four subjects in cohort 4 were treated with 2H-labeled Tregs. The 2H2 (deuterium) label was incorporated into replicating DNA such that 59.8% +/- 1.03% (SD) of the deoxyribose in purine deoxyribonucleosides isolated from cellular DNA was labeled in the expanded Tregs (range 58.2% to 60.9%). The labeled cells were transferred into the patients and blood samples were harvested at various time points after administration. Tregs sorted from purified peripheral blood mononuclear cells (PBMCs), were analyzed for deuterium enrichment of in the DNA Figure 3. As seen in Figure 8 (inset), at day 1, there was a significant percentage of deuterium label in the DNA of circulating Tregs in each individual. The maximal percentage of the adoptively transferred PolyTregs occurred by 7-14 days, after which point there was a decline in the percentage of labeled Tregs in the circulation. Thus, by ~ 90 days post infusion about 25% of the peak labeling in cells was still observed in the circulation. Interestingly, this percentage stabilized over the next 9 months resulting in the prolonged presence of labeled Tregs in the circulation at least one year after transfer. Our pharmacokinetic analysis of the survival of the Tregs indicated a two-phase decay curve, with the average half-life of the fast decay phase of about 19.6 days (range 4.7 to 32.5 days) and a second slow decay phase with a half-life of a year or more in 4 of 7 patients studied.

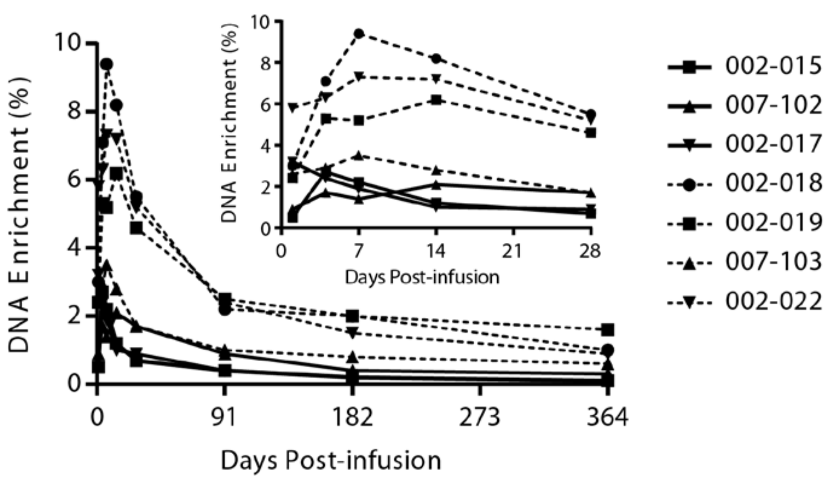


Figure 3. Treg Tracking by Stable Isotope Labeling.

Three subjects (002-015, 007-102, and 002-017) were treated with a single dose of ^{2}H-labeled Tregs at a target dose of 3.2 x10^8 cells, and four subjects (002-018, 002-019, 007-103, and 002-022) were treated with a target dose of 26 x10^8 cells that were approximately 60% enriched for the ^{2}H-label. Peripheral blood was collected on days 1, 4, 7, 14, 28, 91, 182, and 364 days post infusion, and Tregs were sorted from the peripheral blood. Following isolation and hydrolysis of genomic DNA, the ^{2}H isotopic enrichment of the purine deoxyribonucleosides in Tregs sorted from whole blood was assessed by gas chromatography/mass spectrometry. Background enrichment of unlabeled Tregs was ≤0.1% for each of the seven subjects.

Stable isotope labeling has also been utilized to track CD4+CD127lo/-CD25+ Polyclonal Tregs in the peripheral blood in the two subjects in the TASKp trial using the same procedure. Data has been obtained for the first 6 months for subject 1 and for the first month for subject 2. The pharmacokinetic profile of Tregs in these two patients was similar to that obtained from T1D patients who received the same dose of Tregs in spite of the fact that these patients were being treated with immunosuppressive drugs (Figure 4).

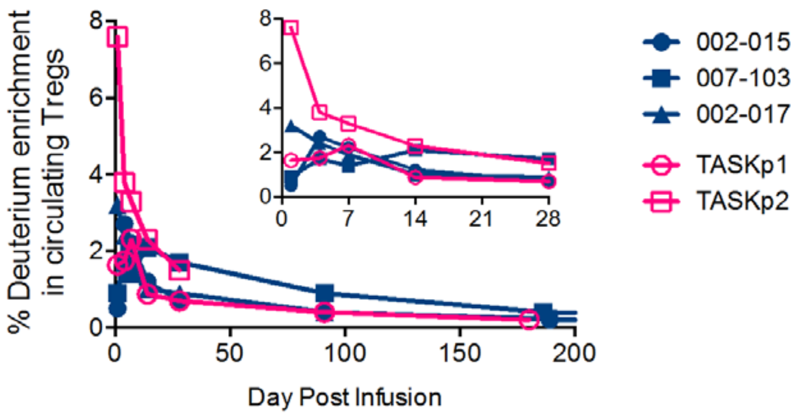


Figure 4. Survival of infused polyclonal Tregs in transplant patients.

Two kidney transplant recipients (TASKp1 and TASKp2) and three type 1 diabetes patients (002-015, 007-102, and 002-017) were treated with a single dose of ^{2}H-labeled Tregs at a target dose of 3.2 x10^{8} cells. Peripheral blood was collected on days 1, 4, 7, 14, 28, 91, and 182 days post infusion, and Tregs were sorted. Following isolation and hydrolysis of genomic DNA, the ^{2}H isotopic enrichment of the purine deoxyribonucleosides in Tregs sorted from whole blood was assessed by gas chromatography/mass spectrometry.

1.6.2.3 Treg Therapy in Adults with Type 1 Diabetes

The UCSF Diabetes Center group completed enrollment and infusion of expanded, polyclonal Tregs in recent onset adult type I diabetics in a phase 1 trial in October of 2013. Tregs were manufactured at UCSF GMP facility by a team that will also be responsible for producing Tregs for this trial. The UCSF T1D trial included 4 escalating dosing cohorts – 3 patients each at 5 x 10^{6} and 40 x 10^{6} Tregs and 4 patients each at 320 x 10^{6} and 2,600 x 10^{6} Tregs (Bluestone JA, 2015).

1.6.2.4 Safety of Treg Therapy in Adults with Type 1 Diabetes

As of May 2015, 145 AEs have been reported in 15 subjects since the beginning of the trial. All 14 treated subjects have reported at least 1 AE. One subject who underwent phlebotomy but was withdrawn before treated reported 1 AE before withdrawal from the trial. Ninety-two events were judged as mild in severity, 42 were judged as moderate, 9 were judged as severe, and 2 were judged as life-threatening. Thirty-one events were judged to be possibly related, 33 unlikely related and 81 unrelated to study therapy. The most common SOC affected was “Infections and Infestations” followed by “Gastrointestinal Disorders” and “General Disorders and Administration Site Conditions.” Of 36 infections recorded, 24 were upper respiratory infections. Of those, 19 were judged grade 1 (CTCAE category: Infections and Infestations Other, Specify) and 5 were judge grade 2 in severity (CTCAE designation; Upper Respiratory Infection). One infection, initially reported as grade 2 pharyngitis, was subsequently demonstrated to reflect a new cytomegalovirus (CMV) infection that occurred prior to treatment with Tregs.

Of the nine AEs judged as severe (grade 3), all were judged unlikely related or unrelated to the investigational agent. Two occurred prior to Treg infusion and were judged unrelated to the investigational agent. Four events were hypoglycemia, of which three were judged unrelated and one judged unlikely related to the investigational agent. One event was syncope occurring 35 weeks after Treg infusion. One event was hyperglycemia occurring 10 weeks after Treg infusion. One was depressed level of consciousness due to inebriation.

Two events were life-threatening (grade 4) events of hypoglycemia in one subject occurring 59 and 62 weeks after Treg infusion. Grade 4 hypoglycemia is defined with a glucose < 30 mg/dL. Both of these events were judged unrelated to the investigational product.

In the 24 hours following infusion, only 4 AEs in 4 subjects were reported. Two were mild headache, one was mild nausea and one was mild abdominal pain. Thus infusion reactions have been limited.

Among the AEs, four were serious adverse events (SAE). Three severe (grade 3) hypoglycemic SAEs, one judged unlikely related and two judged unrelated to the investigational product have been reported. One severe (grade 3) hyperglycemic SAE judged unrelated to the investigational product has been reported.

A review of laboratory parameters demonstrates a pattern of an approximately 0.5-1 g/dL drop in hemoglobin after protocol-specified phlebotomy performed day -16 to -14, which recovers by day 28 to 91 post-infusion. One subject was noted to have an elevated LDH, which was judged to be due to a CMV infection acquired prior to Treg infusion as discussed above. This same individual had detectable CMV on days 7, 14 and 21. CMV was undetectable by day 28, and the infection resolved without antiviral treatment. Otherwise neither EBV nor CMV has been detected in the trial.

1.6.2.5 Feasibility of Multi-Site Trials

Two of the enrolled patients in cohort 3 and one of four patients in cohort 4 were at Yale University. Whole blood collected from Yale was shipped to UCSF for expansion. After 14 days of expansion, the cellular products were shipped back to Yale for infusion. These experiences demonstrate that raw materials and Treg products are sufficiently stable for overnight shipment to the UCSF manufacturing team and that the manufacturing facility can support multi-site studies.

2. Study Objectives

2.1 Primary Safety Objective: darTregs Infusion

This study will evaluate the safety and tolerability of a single infusion of donor alloantigen reactive regulatory T cells (darTregs) in adult LT recipients.

2.1.1 Secondary Safety Objective: IS Withdrawal

This study will evaluate the safety of IS 1) reduction and 2) discontinuation after one IV dose of darTregs.

2.2 Primary Efficacy Objective: IS Minimization

The study will evaluate the ability of a single IV dose of darTregs to reduce baseline, standard of care (SOC) CNI dose by 75% along with discontinuation of either prednisone or mycophenolate mofetil (MMF), as applicable.

2.3 Secondary Efficacy Objective: Tolerance

This study will determine the number and proportion of LT recipients who become operationally tolerant with darTregs infusion. We hypothesize that a single dose of darTregs in a well-defined cohort of LT recipients will induce operational tolerance as revealed by subsequent IS withdrawal.

2.4 Mechanistic Objectives

We will assess the pharmacokinetic profile of darTregs by measuring the level of deuterium-labeled darTregs in circulation. Potential impact of darTregs therapy on immunological profiles will be assessed by comparing leukocyte phenotypes and tissue histology in protocol and for-cause biopsies, and alloantibody before and after darTregs infusion.

3. Study Design

3.1 Description of Study Design

This is a multi-center, open-label clinical trial that aims to enroll at least 9 and up to 11 adult LT recipients to receive 300-500 million darTregs 12–26 weeks after initiating IS withdrawal. Up to 18 subjects will be enrolled to target 9-11 subjects eligible for IS withdrawal and darTregs infusion. Subjects who have had a study eligibility biopsy alone and those who have begun IS withdrawal will receive darTregs if the subject continues to be eligible. After darTregs infusion, subjects will resume IS withdrawal. Subjects will be assessed for their ability to reduce IS by 75% while maintaining normal liver tests. Subjects willing to proceed with IS withdrawal will then attempt to discontinue IS altogether. Those able to maintain normal liver tests and demonstrate stable allograft histology for one year in the complete absence of IS will be identified as operationally tolerant.

Up to 18 subjects will be enrolled in the study to account for potential dropout prior to darTregs infusion. Dropout can occur based on screening liver biopsy findings (estimated to occur at 20-33% frequency; 3–5 subjects) in Screen 1; or IS withdrawal failure during the early steps of IS withdrawal (10%; 1-2 subjects) in Screen 2. Subject participation is anticipated to be 24 months. Therefore, the total study duration is projected to be 42 months.

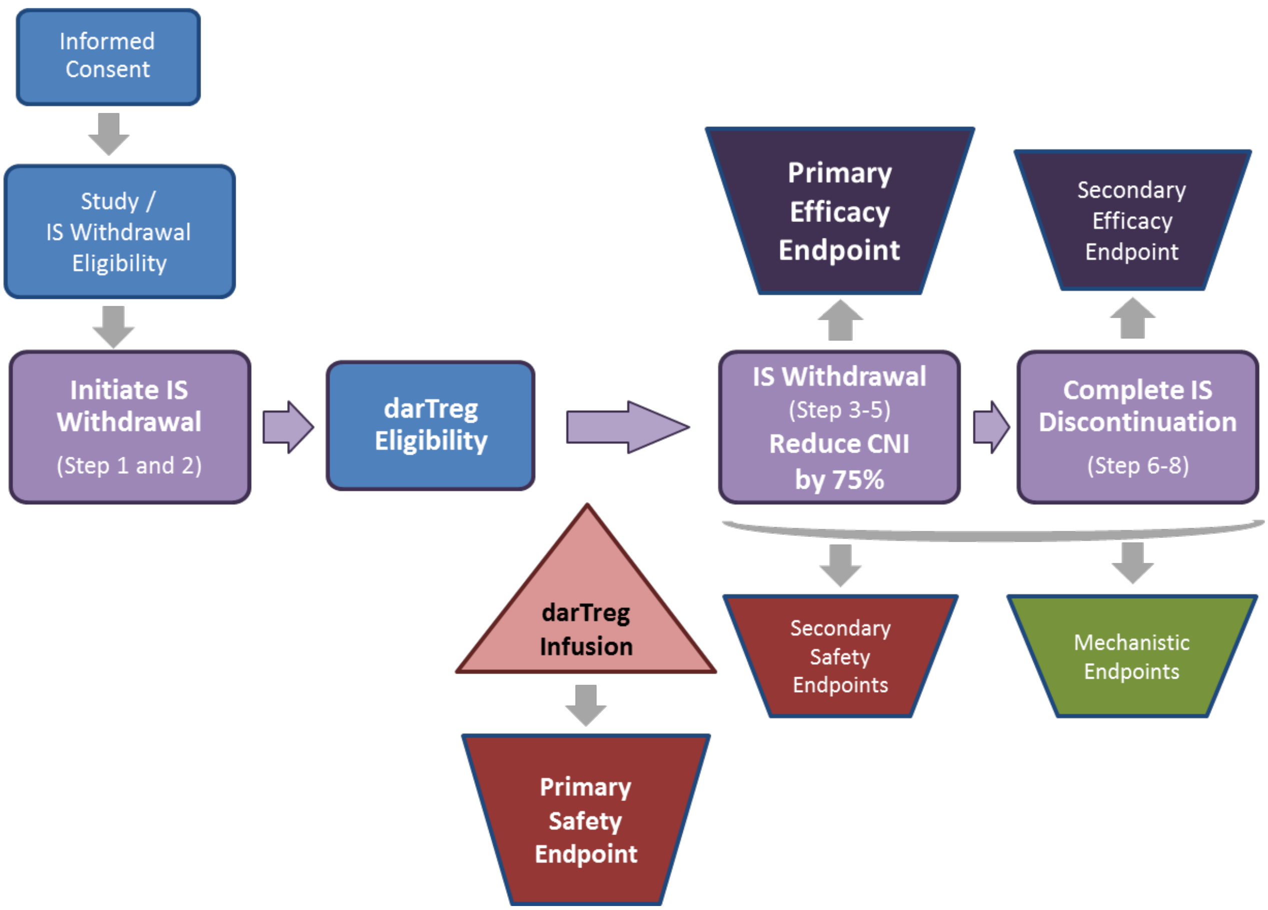


Figure 5. Study Design

3.2 Primary Safety Endpoint

The safety and tolerability of a single infusion of darTregs administered to adult living donor LT recipients will be assessed 24 weeks after darTregs by describing:

1. Occurrence of CTCAE Grade 3 or higher AEs attributable to the darTregs infusion including infusion reaction / cytokine release syndrome
2. Occurrence of study defined Grade 3 or higher infections
3. Occurrence of any malignancy, including PTLD

3.3 Secondary Safety Endpoints

The trial will assess the safety of IS withdrawal in the context of darTregs therapy by describing the following secondary safety endpoints:

1. Rate of composite outcome measure including refractory acute rejection, chronic rejection, re-transplantation, and death
2. Incidence of biopsy proven or clinical acute rejection and/or chronic rejection
3. Timing of biopsy proven or clinical acute rejection and/or chronic rejection
4. Severity of biopsy proven acute rejection and/or chronic rejection

3.4 Primary Efficacy Endpoint

The efficacy of a single IV dose of darTregs will be assessed by the number and proportion of LT subjects who are able to reduce CNI dosing by 75% and discontinue a 2nd IS drug (if applicable) with stable liver tests for at least 12 weeks. The frequency of successful CNI minimization will be compared to historical cohorts of comparable adult LT recipients undergoing IS withdrawal.

3.5 Secondary Efficacy Endpoint

The efficacy of a single IV dose of darTregs infusion will be assessed by determining the number and percentage of subjects who have received darTregs and are identified as operationally tolerant, defined by maintaining stable allograft function (assessed by liver tests) and histology (determined by central pathologist reading in comparison to screening liver biopsy at study entry) in the absence of IS for one year. The frequency of tolerance will be compared to historical cohorts of adult liver transplant recipients undergoing IS withdrawal.

3.6 Primary Mechanistic Endpoint

The level and persistence of deuterium-labeled darTregs in the circulation will be determined by serial measurements of deuterium content in DNA from purified peripheral blood Tregs after darTregs infusion using gas chromatography-mass spectrometry (GC-MS) testing.

3.7 Secondary Mechanistic Endpoints

The overall increase of darTregs in circulation will be assessed using the alloreactive T cell frequency (ATF) assay

3.8 Exploratory Mechanistic Endpoints

The immunologic impact of infused darTregs will be determined by assessing the following:

- Leukocyte phenotypes before and after darTregs infusion using multi-parameter flow cytometry (MFC).
- Alloantibody responses before and after darTregs infusion during IS withdrawal.
- Histology and multiplex immunohistochemistry of protocol and for cause biopsies
- The composition of immune infiltrate in liver biopsies post Treg infusion and at the time of for-cause biopsies will be profiled using single-cell RNA+TCRseq

4. Selection of Participants and Clinical Sites/Laboratories

4.1 Rationale for Study Population

The study aims to prospectively identify a cohort of stable, adult, living donor LT recipients who are 24 months but less than 84 months after transplantation to attempt IS withdrawal. Subjects will initiate IS withdrawal, receive a single infusion of darTregs, and resume IS withdrawal, to reduce IS by 75% and possibly discontinue all IS while maintaining stable allograft function and stable allograft histology.

4.1.1 Rationale for Adult Living Donor LT Recipients

Adult living donor LT recipients are selected for multiple reasons. First, manufacturing of the darTregs product requires access to donor lymphocytes, either from the peripheral blood or from the spleen. Consent of living donors for HLA typing is required to ensure there is at least one mismatch at the DR locus and, pending full eligibility assessment of the recipient, 70 mLs of blood will be drawn to make stimulated B cells (sBcs) for darTregs manufacturing. Second, adult to adult living donor LT has matured over the past 15 years such that it is no longer perceived as an experimental or non-standard procedure. Patients 24-84 months after living donor LT with a good profile of liver tests and liver histology do not pose any contraindication to enrollment in a trial of IS withdrawal supported by darTregs.

4.1.2 Rationale for Adult Living Donor LT Recipients 2-6 Years after Transplantation

The decision to enroll subjects who are 24-84 months after LT is informed by the outcomes of previous IS withdrawal trials. As the primary endpoint for this trial is IS minimization, defined as successful reduction of SOC CNI dosing by 75% and, if applicable, discontinuation of a 2nd IS medication, we aimed to select a population with expected low success rates. Previous IS withdrawal trials in both adult and pediatric deceased and living donor LT recipients indicate that the success rate of tolerance increases with time after LT (Feng, 2012; Benitez, 2013).

Prospective IS withdrawal trials also provide data regarding failure rates for subjects who are early after LT. The prospective European multi-center trial (NCT00647283) allowed adult recipients more than 3 years after LT to enroll and withdraw IS. In that trial, as shown in Table 3, subjects who were 3.0-5.7 years after LT had a 12.5% (3 of 24) prevalence of operational tolerance. This frequency is strikingly similar to the 13% frequency (6 of 46) of tolerance observed in AWISH (ITN030ST/NCT00135694) for participants who were 1-2 years post LT for non-viral, non-immune liver disease. Therefore, the study cohort is expected to have a 12-13% prevalence of operational tolerance. The relatively low rate of success, as demonstrated by AWISH (ITN030ST/NCT00135694) and the European trial (Benitez, 2013), suggests that a contemporaneous control group for this safety study is not warranted as it would expose subjects to unnecessary risk.

The low frequency of tolerance – complete discontinuation of IS for one year with stable liver tests and allograft histology - also reflects the low frequency of success with reducing CNI dosing by 75%. As can be seen in Table 7, 31 of 46 (67.4%) AWISH (ITN030ST/NCT00135694) subjects did not tolerate a 75% CNI dose reduction, with 2 (4.3%) failing during steps 1 or 2 and 29 (63%.0%) subjects failing during steps 3 or 4.

Finally, it is important to note that these two trials enrolled adult deceased donor LT recipients while CTOTC-12 proposes to enroll adult living donor LT recipients. There are two primary lines of evidence to suggest that the historical cohort is an appropriate comparator group in that the success rate of IS minimization and discontinuation is not substantially affected by whether the donor was living or deceased. First, as presented in Section 1.2.2 , WISP-R, a pilot trial that enrolled pediatric parental living donor LT recipients, identified 12 (60%) tolerant subjects and 7 (35%) non-tolerant subjects; one subject has an indeterminate phenotype secondary to early study discontinuation. Currently, the iWITH trial has enrolled 88 subjects, of whom 57 (64.8%) were recipients from deceased donors. As of 10/31/2014, 10 subjects are still undergoing withdrawal. Among the 78 other subjects, 31 (35.2%) have experienced AR while 46 (60.5%) have successfully discontinued all IS medications from less than one month to 16 months. One subject returned to IS therapy after 13 months. Although only a few subjects off of IS currently meet the definition of operational tolerance, we anticipate that the vast majority of these subjects will ultimately meet tolerance criteria based on the fact that previous adult and pediatric withdrawal trials have observed a negligible rate of developing AR after discontinuing IS. Moreover, the observed rate of AR thus far strongly also strongly supports our contention that donor type – living versus deceased – does not strongly impact success / failure rates of IS withdrawal. Second, in clear contrast to kidney transplantation, the degree of HLA matching has not exerted a significant influence either on the risk of rejection or on the outcomes after LT (Balan V, 2008) (Lan X, 2010) (Yosry A, 2012)(A Shaked, 2009). We hypothesize that a single infusion of darTregs given towards the end of step 2 (25% CNI dose reduction) will enable subjects to reduce CNI dosing by 75% (and discontinue a 2nd IS medication, if applicable) while maintaining stable liver tests.

4.1.3 Rationale for Inclusion of Subjects with History of HCC

Eligibility criteria relative to hepatocellular carcinoma (HCC) in this trial has been strongly associated with low rates of recurrent HCC. The requirement for ALL of the criteria to be satisfied results in a cohort of HCC recipients with extremely low HCC recurrence risk, particularly as subjects must be more than three years after LT.

4.1.4 Rationale for Enrollment of Liver Transplant Recipients with History of HCV

Remarkable advances have recently developed in the treatment of chronic hepatitis C virus (HCV) infection before and after LT. What is unique about HCV is that the RNA replicon is cytoplasmic and does not establish latency or incorporate into the cellular genome, like HIV or other viruses (HSV, VZV). Thus, patients with HCV who achieve sustained virological responses (SVR = virus undetectable at least 3 months after antiviral therapy) are 'cured' and are not at risk of recurrence from the initial virus. It is well known that achievement of a SVR following HCV therapy is associated with significantly improved long term outcomes, such as reduced rates of fibrosis progression and liver decompensation (Morgan TR, 2010). This is also true in patients who are cured of HCV with antiviral therapy following LT; they have no risk of recurrence and have excellent long term graft function and outcomes, similar to LT recipients who never had HCV infection (Levitsky J, 2013). In the past, HCV therapy, however, included the use of interferon which was both difficult to tolerate following LT and was associated with rejection and immune-mediated graft dysfunction (Levitsky J F. M., 2012). Fortunately, in the past 2 years, direct-acting antiviral agents (DAAs) have been approved to treat patients with chronic HCV infection, including LT recipients, resulting in 90%+ cure rates with minimal side effects or complications (Brown RS, 2015) (Kwo PY, 2014). Liver transplant centers, including those in the ARTEMIS study, are actively treating recipients with HCV infection and have a growing population of patients achieving SVR. Given the virus does not relapse and is effectively cured with therapy, there is no reason non-viremic patients with excellent histology should be excluded from cellular therapy and tolerance trials, such as ARTEMIS, as they represent a similar immunological risk as non-HCV non-immune patients. In addition, immunosuppression withdrawal can be achieved in viremic patients, as HCV expands CD8+ Tcells that are less responsive (e.g. "exhausted), further supporting enrollment of non-viremic patients, an even lower risk group, into withdrawal studies (Bohne F, 2014).

4.2 Study Enrollment/ darTregs Infusion Eligibility Criteria

4.2.1 Study Enrollment / IS Withdrawal Inclusion Criteria

Subjects who meet all of the following criteria are eligible for enrollment as study participants.

1. Able to understand and provide informed consent
2. Have received primary, solitary, living donor LT more than 24 months but less than 84 months ago
3. Have a living donor who is willing to consent to one time phlebotomy of 100mLs to enable manufacture of darTregs
4. Between 18 and 70 years of age at the time of study entry / consent
5. Have ALT consistently <60 U/L and either alkaline phosphatase consistently <150 U/L or GGT consistently <60 U/L during the preceding 12 months
6. Currently receiving a calcineurin inhibitor (CNI) with 12 hour trough levels consistently <6.0ng/mL for tacrolimus; <150ng/mL for cyclosporine during the preceding 6 months
7. Currently receiving CNI monotherapy or CNI and ONE of the following:
    a. Prednisone: maximum dose of 5 mg / day
    b. Mycophenolate mofetil (MMF): maximum dose of 500 mg bid for Cellcept® or 360 mg bid for Myfortic®
8. Female and male subjects with reproductive potential must agree to use effective methods of birth control for the duration of the study
9. If history of HCC, recipients who have:
    a. AFP less than 100 µg/L at the time of transplant
    b. Explanted liver:

   i. With tumor burden within the Milan criteria and
   ii. Without macro- or micro-vascular invasion and
   iii. Without any lesions with poorly differentiated HCC and
   iv. Without cholangiocarcinoma morphology
  c. Risk Estimation of Tumor Recurrence After Transplant (RETREAT) Score less than or equal to 3
10. If history of HCC, at the time of enrollment, subjects must also:
  a. Be more than 36 months post-transplant AND
  b. Without evidence of recurrent HCC defined as
   i. AFP within normal limits for performing laboratory
   ii. Confirmatory chest CT and
   iii. Confirmatory CT or MRI of the abdomen and pelvis
11. If history of HCV, recipients must be:
  a. Cured of HCV (greater than or equal to six months after the end of treatment
  b. HCV RNA negative at time of study enrollment

4.2.2 Study Enrollment / IS Withdrawal Exclusion Criteria

Subjects who meet any of these criteria are not eligible for IS withdrawal.

1. Transplant for liver disease secondary to an autoimmune etiology (e.g. autoimmune hepatitis, primary sclerosing cholangitis, primary biliary cirrhosis)
2. Matched at both HLA-DR loci to the donor
3. Organ, tissue, or cell transplant prior to or after the primary solitary living donor LT
4. For subjects with HBV, detectible HBV DNA
5. History of malignancy within 5 years of enrollment. History of adequately treated in-situ cervical carcinoma and/or adequately treated skin cancer (basal or squamous cell) will be permitted
6. Serologic evidence of human immunodeficiency 1 or 2 infection
7. Epstein Barr Virus (EBV) sero-negativity (EBV naïve) if living donor is EBV sero-positive
8. Cytomegalovirus (CMV) sero-negativity if living donor is CMV sero-positive
9. Calculated glomerular filtration rate less than 50 mL /min/1.73m2 at time of enrollment
10. AR within one year of enrollment
11. Systemic illness requiring or likely to require recurrent or chronic IS drug use
12. Any chronic condition for which anti-coagulation cannot be safely interrupted for liver biopsy
13. Positive pregnancy test
14. Participation in any other studies that involved investigational drugs or regimens in the preceding year
15. Any other condition, in the investigator's judgment, that increases the risk of darTregs infusion or prevents safe trial participation
16. Unwilling or unable to adhere to study requirements and procedures
17. Screening liver biopsy with acute rejection, early or late chronic rejection according to Banff criteria, or any inflammatory activity and/or fibrosis in excess of histological criteria, as determined by the reading of a central pathologist (Table 9)

*Table 9. Central Pathology Histological Inflammatory and/or Fibrosis Criteria for Screening Biopsy**	
Compartment	**Findings**
Portal inflammation and interface activity	This is preferably absent, but minimal to focal mild portal mononuclear inflammation may be present. Interface necro-inflammatory activity is absent or equivocal/minimal and, if present, involves a minority of portal tracts and not generally associated with fibrosis.
Centrizonal/perivenular inflammation	Negative for perivenular inflammation.
Bile duct changes	Lymphocytic bile duct damage, ductopenia, and biliary epithelial senescence changes are absent unless there is an alternative, non-immunological explanation (e.g. biliary strictures).
Fibrosis**	Fibrosis should be graded according to Venturi C, et al., AJT 2012. Portal fibrosis: 0-3 but portal-to-portal bridging (Ishak3), if present, must be not more than rare. Peri-sinusoidal and peri-venular fibrosis:0-1 only.
Arteries	Findings for obliterative or foam cell arteriopathy and isolated lymphocytic arteritis (isolated v lesions) are negative.
*Patients with underlying AIH, PBC, or PSC are excluded (Demetris A. , 2012) Patients with severe architectural distortion for other reasons (e.g. nodular regression hyperplasia, outflow obstruction etc.) should also not be included. ** Fibrosis for both non-HCV subjects and HCV subjects with SVR should be graded as follows (Venturi C, 2012): Portal/periportal: 0 – 3 Peri-sinusoidal: 0 – 3. Perivenular: 0 – 3.	

4.3 darTregs Infusion Eligibility Criteria

4.3.1 darTregs Infusion Inclusion Criteria

Subjects will initiate IS withdrawal and, at the beginning of the 2nd step of the withdrawal algorithm (week 1-2), undergo a final assessment to ensure eligibility for darTregs infusion. Subjects must have, since initiating IS withdrawal:

1. Stable liver tests, defined as ALT and alkaline phosphatase or GGT either ≤1.5 X upper limit of normal or ≤1.5 X baseline.
2. No detectible circulating EBV or CMV DNA prior to Treg Infusion, assessed at the time of PBMC collection for manufacture
3. For subjects with HBV, no detectible circulating HBV DNA prior to Treg infusion, assessed at the time of PBMC collection for manufacture
4. Able to understand and provide informed consent

If liver tests are abnormal after time of initial darTregs Infusion screening and PBMC collection, final eligibility must be confirmed with the study team prior to darTregs infusion.

4.3.2 darTregs Infusion Exclusion Criteria

1. Diagnosis of AR after initiation of IS withdrawal
2. Any vaccination given within 28 days prior to Treg collection for darTregs production
3. Receipt of a vaccination within 14 days prior to darTregs infusion
4. Unacceptable darTregs product

5. Positive pregnancy test
6. Clinical evidence of viral syndrome less than 7 days prior to darTregs infusion

4.4 Eligibility Criteria to Resume IS Withdrawal after darTregs Infusion

Subjects are eligible to resume IS withdrawal after darTregs infusion if all criteria below are met:

1. Subject received at least 100 x 10^6 darTregs
2. ALT and either alkaline phosphatase or GGT remain within normal limits or ≤ 1.5 x baseline after darTregs infusion
3. For subjects with elevated liver tests as defined above, local pathology reading of liver biopsy 6-10 days after darTregs infusion is without acute rejection according to Banff criteria
4. IS withdrawal resumes no later than 14 days after darTregs infusion
5. Site principal investigator determines it is acceptable for the study subject to resume IS withdrawal

4.5 Clinical Sites

This trial will recruit adult living donor recipients 24-84 months after LT from three sites in the United States. All participating centers are consistently high volume adult to adult living donor LT centers with annual volumes shown below. Assuming a clinical trial start date in 2016 and an enrollment period extending to the end of 2017 (12-18 months), Table 10 shows that there were a total of 331 living donor LTs performed amongst the three sites during the period 1/1/2009 – 12/31/2015. This volume of adult living donor LTs should be adequate to support the estimated enrollment of 18 subjects, such that 12-15 subjects will be eligible to initiate IS withdrawal and 10-14 subjects will be eligible to receive darTregs and at least 9 (and up to 11) subjects will receive 300-500 X 10^6 darTregs. All three institutions have a strong track record of clinical trial investigation and are therefore accustomed to recruiting and enrolling patients for clinical trials.

Table 10. Number of Patients Eligible for ARTEMIS

Center	**ADULT LIVING DONOR TRANSPLANT VOLUMES**							
	2016	**2015**	**2014**	**2013**	**2012**	**2011**	**2010**	**2009**
Mayo	22	20	23	19	20	22	15	16
UCSF	29	31	18	13	9	9	8	5
Northwestern	10	8	19	16	8	21	17	19
TOTAL	**61**	**54**	**60**	**48**	**37**	**52**	**40**	**40**

4.5.1 Manufacturing Facility

darTregs will be manufactured at the Human Islet and Cellular Transplantation Facility, a GMP Facility which is an FDA-registered 4,500 sq. ft. laboratory at UCSF. Quality Assurance is independent of cell manufacturing and over-sees operations.

5. Investigational Intervention: IS Withdrawal

5.1 IS Withdrawal

Participants who fulfill all eligibility criteria will withdraw IS. Subjects may enter on CNI monotherapy or a CNI-based regimen with either Prednisone or MMF as a second IS medication. Subjects on two drugs will reduce dosing of both drugs simultaneously. The algorithm for dose reduction for each agent is delineated below.

5.1.1 CNI taper algorithm

Subjects on CNI inhibitor monotherapy with either tacrolimus or cyclosporine will change the CNI dosing according to the algorithm shown in Table 11. Step 2 has a variable duration to allow for flexibility in timing of darTregs infusion which should be administered during the last week of the 2nd step. Moreover, a ±7 day window is allowed for IS reduction at each step, including Step 2.

Finally, there is the option of invoking a "logistical pause" of up to 26 weeks (see Section 5.4) during Step 2 of the CNI taper algorithm in the event of manufacturing and/or patient scheduling/logistical conflicts, including manufacturing failure of the first darTreg lot and the requirements to manufacture a second production run. During such a logistical pause, tapering of Prednisone and/or MMF will also be suspended.

Subjects who attain a 75% reduction of CNI dosing will also, if applicable, have discontinued Prednisone or MMF (refer to Table 11 and Table 12 below). They will remain at this dose for a total of 12 weeks with ongoing monitoring of liver tests. If liver tests remain stable, they will have met the primary efficacy endpoint of the study. Subjects will then be asked whether they wish to continue with IS withdrawal, attempting complete discontinuation. Only subjects who receive 300 – 500 x 10^6 darTregs will have the option of complete IS withdrawal.

5.1.1.1. <u>Pause in CNI taper</u>

At any step, additional monitoring at a specific dose level may be undertaken prior to continuing withdrawal. Unless a logistical pause is implemented during Step 2, intentional "pauses" can last no longer than 4 weeks by which time withdrawal must resume or a biopsy must be performed. Unless a study stopping rule is applied or a stipulation approved by the NIAID MM and/or Data Safety Monitoring Board (DSMB), failure to resume withdrawal will be considered a failure of IS withdrawal. Aside from logistical pauses during Step 2, the total duration of pauses during withdrawal cannot exceed 8 weeks during the initial IS withdrawal attempt.
During Step 1 and Step 2 before darTregs is administered, a pause cannot exceed 4 weeks.

Table 11. CNI Withdrawal Algorithm

STEP	DOSE (mg)	FREQUENCY	% REDUCTION	DURATION
ENTRY	X	BID	n/a	
1	2X	QD	0	6 weeks
2	**1.5X**	**QD**	**25**	**6 – 8 weeks***
	Administer darTregs during the last 2 weeks of Step 2			
3	X	QD	50	6 weeks
4	X	5 D / week	63.5%	6 weeks
5	X	QOD	75	12 weeks
Continuation of CNI Withdrawal pending 2nd consent				
6	X	2X/WK	86	6 weeks
7	X	1X/WK	93	6 weeks
8	**OFF CNIs**			

5.1.2 Prednisone taper algorithm

If a subject enters the trial on prednisone, the maximal allowable dose is 5 mg/day. The dose will be reduced by 1mg increments every 4 weeks until the subject has discontinued Prednisone. As with CNI reduction, a decision may be made to undertake additional monitoring prior to continuing with dose reduction. Such intentional "pauses" can last no longer than 4 weeks each and the total duration of pauses during Prednisone withdrawal cannot exceed 8 weeks. Prednisone tapering will also be stopped as part of a logistical pause during Step 2 during the CNI taper algorithm (see Section 5.4) for a maximum of 26 weeks.

5.1.3 MMF taper algorithm

If a subject enters the trial on MMF, the maximal allowable dose is Cellcept® 500 mg bid or Myfortic® 360 mg bid. The algorithm for dose reduction is shown in Table 12. Depending on the dose at entry, MMF will be discontinued either 8 or 16 weeks after initiation of IS withdrawal. As with CNI reduction, a decision may be made to undertake additional monitoring prior to continuing with dose reduction. Such intentional "pauses" can last no longer than 4 weeks each. The total duration of pauses during MMF withdrawal cannot exceed 4 weeks if there is one step or 8 weeks if there are two steps. MMF tapering will also be stopped as part of a logistical pause during Step 2 during the CNI taper algorithm (see Section 5.4) for a maximum of 26 weeks.

Table 12. MMF Withdrawal Algorithm

STEP	STARTING DOSE: Cellcept 500 mg bid OR Myfortic 360 mg bid		
	AM Dose	PM Dose	DURATION
1	250 or 180	250 or 180	8 weeks
2	250 or 180	0	8 weeks
OFF MMF			
STEP	STARTING DOSE: Cellcept 500 mg am / 250 mg pm OR Myfortic 360 mg am / 180 mg pm		
1	250 or 180	250 or 180	8 weeks
2	250 or 180	0	8 weeks
OFF MMF			
STEP	STARTING DOSE: Cellcept 250 mg bid OR Myfortic 180 mg bid		
1	250 or 180	0	8 weeks
OFF MMF			

5.2 Windows During IS Withdrawal

Dose reductions for each drug can occur within a 7-day window at each dose level. The overall duration of the IS withdrawal algorithm will be determined by the duration of CNI withdrawal, varying with each subject's need to "pause".

5.3 Logistical Pause during Step 2 of CNI Withdrawal Algorithm

During Step 2, an IS withdrawal pause of up to 26 weeks + 7 days (CNI and Prednisone / MMF, as appropriate) can be invoked to accommodate logistical challenges. The logistical pause may be triggered by any number of constraints related to scheduling, collection, and shipping of donor or recipient manufacturing materials. Similarly, a logistical pause might be utilized when faced with limitations imposed by the darTreg manufacturing facility, at the clinical site for the infusion, and/or with subject's availability for an overnight hospital stay and post-infusion biopsy.

During the logistical pause, liver tests must be monitored every two weeks until 12 weeks after the last IS dose change. Thereafter, liver tests must be monitored at least every 4 weeks until the pause is terminated. If allograft dysfunction develops during the logistical pause, protocol guidelines must be followed (Section 5.5 Allograft Dysfunction).

At the end of the logistical pause, the subject must continue to meet all eligibility requirements to proceed with darTreg infusion.

5.4 Definition of Operational Tolerance

Tolerance will be adjudicated 1 year after completing complete IS withdrawal. Tolerance in this study is based on stable liver tests and central pathology liver biopsy reading. ALT and either alkaline phosphatase or GGT must be either ≤1.5X normal limits or ≤1.5X baseline. Baseline ALT, alkaline phosphatase, and GGT are defined as the average of two laboratory tests: those obtained just prior to study entry and at the study entry screening visit. A tolerance adjudication committee will convene to review cases, if warranted. A liver biopsy will be obtained and compared to the baseline biopsy. Pathologic criteria for tolerance are defined in Table 13 below.

Table 13. Tolerance Biopsy Criteria*

Compartment	Findings
Portal inflammation and interface activity	Increased portal inflammation (in comparison with a pre-weaning biopsy sample), especially in association with histopathological evidence of tissue damage manifest as: focally worsening or more prevalent lymphocytic bile duct damage, interface hepatitis, fibrosis, or the appearance of definite venous endotheliitis.
Centrizonal/perivenular inflammation	New onset perivenular inflammation (in comparison with a pre-weaning biopsy sample) associated with even mild perivenular necro-inflammatory activity. Note: these changes might be present in the absence of typical portal changes of rejection.
Bile duct changes	New-onset biliary epithelial cell senescence changes or ductopenia when sampling problems and/or an alternative, non-immunological explanation (e.g. biliary strictures) can be reasonably excluded
Fibrosis**	Greater than 1 grade increase in fibrosis in any one compartment: (a) portal/periportal; (b) peri-sinusoidal; or (c) perivenular fibrosis; or new onset bridging fibrosis without an alternative explanation (e.g. biliary strictures) that is reasonably prevalent and not readily explained by a possible sampling error.
Arteries	Any evidence of foam cell or obliterative arteriopathy

*Patients with underlying AIH, PBC, or PSC are excluded (Demetris A. , 2012).
** Fibrosis should be graded as follows (Venturi C, 2012):
Portal/periportal: 0 – 3
Peri-sinusoidal: 0 – 3.
Perivenular: 0 – 3.

5.5 Allograft Dysfunction

Allograft dysfunction occurs when ALT is >120 U/L, alkaline phosphatase is >300 U/L, or GGT is >120 U/L. If allograft dysfunction is unexplained, liver biopsy must be performed, although liver tests can be repeated once for confirmation prior to biopsy. At the discretion of the site principal investigator, allograft biopsy may be triggered by elevated liver tests below the threshold that defines allograft dysfunction but above the individual subject's baseline.

5.6 Acute Rejection (AR)

Clinically indicated biopsies will be read locally according to the Banff global assessment criteria (Demetris A. J., 1997) to guide prompt clinical decision-making. If the biopsy is non-diagnostic, other causes of liver dysfunction should be thoroughly considered. Liver biopsies will then be reviewed centrally and data analysis will be based on central pathology readings. In the case of a non-diagnostic biopsy performed for either allograft dysfunction or elevated liver tests (liver tests above baseline but not meeting criteria for allograft dysfunction), including biopsies read as “indeterminate” AR, a clinical management decision to increase or to reinitiate IS constitutes a clinical diagnosis (as opposed to a histologic diagnosis) of AR.

5.6.1 Treatment of AR

Treatment for AR will be determined by the site investigator. The therapeutic regimen can be comprised of one or more of the following treatment options:

- Steroid pulse: a defined course of steroids, prescribed either intravenously or orally or both
- Dose increase: resumption (if completely discontinued) or dose increase of CNI, MMF, or prednisone
  - For CNI and MMF: this terminology can only be used for the drug was part of the subject's maintenance IS regimen immediately prior to study entry
  - For Prednisone: this terminology does not include the "pulse" of steroids used to treat acute rejection but can be utilized if a subject entered the trial on prednisone and returns to a maintenance IS regimen that includes prednisone
- Initiation of an additional agent(s)
  - This terminology does not apply to a corticosteroid pulse (defined below) which represents a discrete course of treatment and does not apply if a new IS medication is initiated to substitute for another medication.
- Substitution: substituting one IS medication for another. Examples include:
  - Tacrolimus for cyclosporine, or vice versa
  - mTOR inhibitor for prednisone, CNI, or MMF
  - MMF for prednisone
- Antibody treatment: administration of a course of an antibody preparation, typically thymoglobulin

Antibody treatment should be reserved for AR refractory to intravenous corticosteroid therapy and given ideally after a second biopsy demonstrating the absence of treatment response.

5.6.2 Resolution of AR

ALT and alkaline phosphatase/GGT will be used to assess whether AR has resolved. AR is considered resolved when ALT and alkaline phosphatase or GGT are either ≤1.5 X ULN or ≤1.5 baseline.

5.6.3 Chronic Rejection (CR)

A diagnosis of CR requires abnormal total and direct bilirubin and liver histology that fulfills Banff criteria. CR will be treated according to center SOC. Any participant who develops CR will be considered to have failed IS withdrawal and will enter follow up according to *Appendix 7. Medium Frequency Schedule after Rejection*.

5.7 Premature Discontinuation of IS Withdrawal

Participants who fail IS withdrawal will be followed for 52 weeks from the date of rejection (*Appendix 7. Medium Frequency Schedule after Rejection*).

Subjects who require a pause beyond 26 weeks in Step 2 or otherwise do not receive darTregs will not continue IS withdrawal and will have 26 weeks of follow-up from the time of last dose change or rejection (Appendix 7). Subjects who experience rejection will have 52 weeks of follow up as previously described. Any further changes to IS will be determined by the site investigator.

6. Investigational Agent: darTregs Infusion

6.1 Formulation, Packaging, and Labeling

Collection of Recipient T cells

Peripheral blood leukocytes will be collected from eligible participants approximately 9-12 weeks after initiation of IS withdrawal. Collection will be by phlebotomy (450 mls whole blood) or leukapheresis. The whole blood or leukopheresed cells will be immediately transported to the manufacturing facility. PBMCs will be isolated by density gradient centrifugation using Ficoll. At least $1x10^9$ PBMCs are expected to be collected from each subject. If the whole blood or leukapheresis product does not contain sufficient numbers of Tregs, leukapheresis or phlebotomy can be repeated to ensure an adequate cell number for product manufacture.

Blood collected at off-site location will be shipped via next-day service to UCSF and processed as described above.

Donor B cell (sBcs) Production and Banking

Peripheral blood leukocytes will be collected from living donors by phlebotomy (70 mls whole blood) and transported to the UCSF manufacturing facility for processing and production of sBcs. sBcs will be generated by stimulation with irradiated KT64-CD40L.HLADR0401 cells and expanded for 10 days in the presence of commercially available recombinant human IL-4. The donor sBcs will be irradiated and cryopreserved until needed for darTregs expansion.

darTregs Expansion

Sorted $CD4^{+}CD25^{+}CD127^{lo/-}$ Treg obtained from the processed PBMC will be ex vivo expanded for a total of 16 days in medium supplemented with deuterated glucose in co-cultures with the donor sBcs followed by a second stimulation with commercially available anti-CD3 and anti-CD28 Mab-coated magnetic beads for secondary stimulation. darTregs will be expanded in medium supplemented with deuterated glucose to label the product for tracking after infusion. At the end of the 16-day expansion, the anti-CD3 and anti-CD28 beads will be removed by magnetic separation.

darTregs packaging

The darTregs product will be filled in sterile 150 ml PVC infusion bags. The filled product will be maintained at 2-10°C at all times until administration. A Certificate of Analysis documenting the lot release test results will accompany the final product during delivery to the bedside. Infusion must occur within 30 hr of final product formulation.

Prior to lot release, the product label will be affixed to the infusion bag and will include the product identifier, date of cell harvest, expiration date and time, and the name and unique identifier of the intended recipient.

The darTregs products manufactured for subjects at an off-site location will be shipped from UCSF to the clinical site via next-day service using validated conditions and containers. The remote site will test the received product according to UCSF established procedures. Qualified staff at the remote site will receive the darTregs and verify the recorded shipment temperatures were maintained within the validated range, perform a cell count, viability determination, to ensure product quality characteristics have been retained during shipping. The data collected will be reported to UCSF prior to infusion.

6.2 Dosage, Preparation, and Administration

The product is a sterile cell suspension of $400 \pm 100 \times 10^6$ darTregs (range $300\text{-}500 \times 10^6$) in 100 mL of 49.02% (v/v) PlasmaLyte-A, 49.02@ (v/v) Dextrose 5%, 0.45% NaCl, and 1.96% (v/v) 25% human serum albumin and filled in a sterile infusion bag.infused IV by gravity in approximately 20 to 30 minutes. The IV line should be primed with saline prior to administration of the product. Following administration of the product the bag, tubing and peripheral IV line are flushed with normal saline to ensure the complete dose is infused. The IV line will be maintained after the infusion and the subject will be asked to remain in the clinical research unit for a minimum of 24 hours, to allow ongoing monitoring for any infusion-related signs and symptoms.

Preparations less than 100×10^6 darTregs will not be infused. Cells not infused will be used for research (See Section 1.5.2.3 and 13.3).

Pre-medications (acetaminophen 650 mg and diphenhydramine 25-30 mg) will be administered prior to infusion (Section 7.2.1). Vitals signs will be monitored before, during, and after the infusion. Emergency medical equipment will be available during the infusion in case the subject has an allergic response or an infusion reaction that can result in a cytokine release syndrome.

6.3 Drug Accountability

Under Title 21 of the Code of Federal Regulations (21CFR §312.62), the investigator will maintain adequate records of the disposition of the darTregs products, including the date and quantity of the investigational product received, to whom the investigational product was dispensed (participant-by-participant accounting), and a detailed accounting of any investigational product that is accidentally or deliberately destroyed.

Records for receipt, storage, use, and disposition will be maintained by the study site. A dispensing log will be kept current for each participant. This log will contain the identification of each participant and the date and quantity of biologic product dispensed.

All records regarding the disposition of the investigational product will be available for inspection. Unused product will be de-identified and destroyed. They may be utilized for laboratory studies.

6.4 Intervals between darTregs infusions

Eligible subjects will receive a single infusion of darTregs at the end of the 2nd step of IS withdrawal. darTregs infusions will be staggered between subjects. There will be a mandatory observation interval of 4 weeks after each darTregs infusion for the first 3 subjects to ensure that there are no acute safety signals (Section 14.8.3) related to either the infusion and/or the resumption of IS withdrawal. Subsequently, for the remaining 6 subjects, a 4 week interval between darTregs infusions is preferred. However, if the expected time interval between darTregs infusions should be less than 4 weeks, the PI and NIAID medical monitor will determine together whether there are any safety concerns based on available study data to preclude administration of the darTregs infusion at a shorter interval than 4 weeks.

6.5 Repeated darTregs Manufacturing

The manufacturing process can be repeated once for a particular subject if there or technical or logistical issues with the first preparation. The study team will review the available manufacturing information to determine if a second manufacturing attempt is likely to be successful. The laboratory components of darTregs infusion eligibility criteria must be repeated within 10 days of planned infusion to ensure eligibility. In any case, infusion of darTregs must take place no later than 26 weeks during Step 2 of IS withdrawal. If the second attempt at manufacturing fails, the subject will be followed for at least 26 weeks from the time of last dose change (Section 5.7).

6.6 Premature Discontinuation of darTregs Infusion

A darTregs infusion will be stopped for an individual subject and will not be restarted if any of the following occur:

- hypersensitivity reaction
- CTCAE Grade 3 or higher infusion-related reaction, including cytokine release syndrome,
- any other infusion-related SAE.

A subject who receives any part of the Treg infusion will be followed for at least 52 weeks from the date of darTregs infusion.

No more than two manufacturing attempts will be undertaken for a particular subject.

7. Other Medications

7.1 mTOR inhibitors

Subjects cannot be on mTOR inhibitors (sirolimus or everolimus) at time of study entry. However, an mTOR inhibitor may be used to treat AR or CR.

7.2 Prophylactic Medications

7.2.1 Pre-Medications for darTregs Infusion

Pre-medications will be administered 30-60 minutes prior to the darTregs infusion. Pre-medications will include 650 mg acetaminophen and 25-50 mg diphenhydramine intravenously or by mouth.

7.2.2 Anti-Infective Prophylaxis after Corticosteroid or Antibody Treatment for Rejection

Participants should receive center SOC prophylaxis after oral or intravenous corticosteroids, with or without a course of rabbit thymoglobulin for treatment of rejection.

7.3 Vaccinations

Subjects should receive seasonal influenza and other vaccinations as SOC. However, subjects eligible for darTregs should not receive any vaccination within 28 days prior to blood collection or leukapheresis for darTregs manufacturing and/or within 14 days prior to the actual infusion (see Schedule of Events and Treg exclusion criteria). In addition, the subjects should not have any vaccination for 28 days after the date of darTregs infusion. These time parameters are in place to minimize the chance of having enrichment of influenza-reactive Tregs in the product and ensure that darTregs infusion does not negatively impact influenza immunity elicited by the vaccine.

7.4 Other permitted concomitant medications

Other non-IS concomitant medications used as SOC in the management of the LT subject not specifically described in Section 5.6.1 are acceptable in the study.

8. Study Mandated Procedures

8.1 Blood Draws

Laboratory blood draws are necessary to carefully and frequently evaluate allograft function during and after IS withdrawal. In this trial, darTregs infusion will be administered during the time course of IS withdrawal. Liver tests are the most reliable marker for all etiologies of allograft dysfunction, including AR and CR. Blood draws will also be done to enable the planned mechanistic studies. Please refer to Appendices 2-7.

8.2 Leukapheresis or Blood Draw for PBMC Collection

PBMCs will be collected via leukapheresis or phlebotomy. Leukapheresis will be performed for participants who are relatively anemic, defined as a hemoglobin ≤10.5 gm/dL. For subjects who are not anemic, whole blood collection (450 mls) is an option for PBMC collection.

Leukapheresis will take place in an apheresis center with qualified staff. The time requirement for the procedure is usually between 1 and 1.5 hours to process 6 liters of blood, which historically yields at least 1×10^9 PBMCs needed for manufacture. Use of anti-coagulant and specific processing procedures at participating clinical sites will be reviewed. Subjects will be monitored throughout the procedure and replacement therapy (e.g. calcium) will be provided, if required.

Donor and recipient PBMC collection for manufacturing can be repeated up to two times as long as blood volume limits for research are not exceeded for a given time period.

8.3 Liver biopsies

Liver biopsies both protocol and for cause, will be done during the trial. For each biopsy, a 16G needle will be used and a total of 4cm of tissue will be required. Therefore, two passes may be required for each liver biopsy performed during the trial. A separate consent that describes the procedure and its risks in detail, including the need for more than a single pass to obtain adequate tissue will be signed prior to each biopsy.

8.3.1 Protocol Mandated Liver Biopsies

Subjects will have two to three protocol mandated liver biopsies during study participation. All subjects will undergo a protocol biopsy to determine eligibility for study entry and IS withdrawal. A second liver biopsy will be performed 6-10 days after darTregs infusion. If a subject succeeds at withdrawing completely from IS, they will undergo a third protocol biopsy to adjudicate operational tolerance one year after the last IS dose.

8.3.2 Clinically Indicated (For Cause) Biopsies

Clinically indicated biopsies will be obtained when liver tests are elevated at the discretion of the site principal investigator or mandatorily, when allograft dysfunction thresholds as defined in Section 5.5, unless there is another explanation for allograft dysfunction. Local pathology reading will direct treatment of the subject. Both blood and tissue specimens, will be collected for central laboratory assessments at the time of a for cause biopsy.

9. Known and Potential Risks and Benefits to Participants

9.1 Risks of IS Withdrawal

The main risks of IS withdrawal are (1) AR and/or CR (2) silent / subclinical chronic allograft injury (3) graft loss and (4) potential immune and non-immune complications associated with increased dosing or reinstitution of IS. The data from recent pediatric as well as adult trials provides significant reassurance that these risks are minimal when IS withdrawal is conducted within the context of a clinical trial under highly supervised conditions (Benitez, 2013). In spite of low risk, for completeness of consideration, we will segment AR risks into mild, moderate and severe risk categories based on severity as determined by Banff criteria (Demetris A. A., 2006) (Demetris A. J., 1997) (Demetris A. D., 2000) and response to therapy. CR will be assigned to the severe risk category. We will also address the potential impact of AR on short and long-term graft function and on short and long term IS exposure. Although severe AR is a defined risk, we consider it to be unlikely given the (1) enrollment of carefully selected subjects who have undergone screening allograft biopsy (2) intense monitoring of allograft function during IS withdrawal and (3) preliminary results from multiple IS withdrawal trials in both adults and children.

<u>Mild</u>

IS withdrawal will trigger episodes of AR. AR episodes that occur under close monitoring inherent in IS withdrawal protocols have been mild to moderate in histological severity and easily reversed, almost never requiring treatment with potent antibodies. Allograft recovery from mild AR, diagnosed according to Banff criteria (Demetris A. A., 2006) (Demetris A. J., 1997) (Demetris A. D., 2000) is almost always complete, occurring without significant fibrosis, architectural distortion, or loss of function (Mazariegos GV, 1997) (Ramos, 1995) (Tisone, 2006) (Koshiba, 2007) (Feng, 2012). Although treatment of mild AR requires increased IS dosing relative to the dose at which AR occurred, it is expected that overall IS exposure during and after the trial would be comparable (Benitez, 2013).

<u>Moderate</u>

Moderate risks associated with IS withdrawal include the risk of moderate AR, diagnosed according to Banff criteria (Demetris A. A., 2006) (Demetris A. J., 1997) (Demetris A. D., 2000). Although it is expected that moderate AR will be effectively treated by brief exposure to corticosteroids and/or dose increase, substitution, or addition of IS medications. Allograft recovery from moderate AR should be complete, occurring without significant fibrosis, architectural distortion, or loss of function. Therefore, long-term and indefinite increased IS exposure is not anticipated.

A second moderate risk of IS withdrawal is related to progressive allograft changes that may occur silently without changes in liver tests. In the ITN029, a pilot trial of IS withdrawal for pediatric LT recipients, 11 of 12 participants have undergone late protocol biopsies more than 4 years after the last IS dose. Compared to screening biopsies, there was no systematic change in allograft histology. Moreover, in NCT00647283, a multi-center prospective trial of IS withdrawal for adult LT recipients, protocol biopsy 3 years after IS withdrawal have not exhibited any evidence of progressive allograft histopathology after AR associated with IS withdrawal. Therefore, while the limited nature of the currently available data urge caution, they do not robustly support the notion that IS withdrawal is inherently associated with substantial risk for histological deterioration.

<u>Severe</u>

Severe risks associated with IS withdrawal include steroid unresponsive or refractory AR or CR, as defined by Banff criteria (Demetris A. A., 2006) (Demetris A. J., 1997) (Demetris A. D., 2000). In each of these scenarios, there is increased risk of graft loss leading to a requirement for re-transplant along with increased short- and long-term IS exposure. Consequently, each scenario is associated with increased susceptibility to infection, elevated blood pressure, renal dysfunction, and/or diabetes. Among adult and pediatric LT recipients who have undergone IS withdrawal, severe rejection has been exceedingly rare.

9.1.1 Risk of Treatment for Rejection

All transplant recipients require IS as SOC and the well described risks of IS (Section 1.1) are no greater for study participants. However, rejection during the trial will require treatment with one or more of the following: corticosteroids, increase in CNI dosing, conversion to a different IS agent, addition of a new IS medication, and/or thymoglobulin. The additional exposure to IS agents and possible concomitant medications (i.e. prophylaxis for opportunistic infections) as a result of rejection during this trial is a risk that study participants might not otherwise have. Moreover, although the subjects will have had reduced IS exposure during IS withdrawal prior to failure, an episode of rejection will certainly necessitate increased IS exposure in the short-term but may also result in increased IS exposure cumulatively and/or in the long-term.

9.2 Risks of darTregs infusion

Transfusion/Infusion Reaction

Side effects reported from previous human trials involving T cell infusions include transient fever, chills, and/or nausea. Infusion reactions are, however, usually self-limited and resolve without any permanent sequelae. Pre-medications will be administered to mitigate the risk and severity of infusion reactions.

Immune Suppression

Tregs are known to suppress naïve T cell responses to a variety of antigens. Less is known about ongoing immune responses especially to viruses and bacteria. It is not known whether Tregs in general or darTregs in particular will alter protective immunity.

Infection

As with any therapy that suppresses the immune system, there is a risk of developing infections. It should be noted that on a theoretical basis, this risk is minimal, since the total input of darTregs is far below the resident Treg population. Moreover, the darTregs product is manufactured to be highly enriched for Tregs with donor-specificity. We will, however, take additional precautions and exclude subjects with detectible EBV, CMV, or HBV DNA prior to infusion.

Loss of Tumor Surveillance

T lymphocytes are one major component of tumor surveillance and it is possible that cells that inhibit T lymphocytes could impair this function. There has not been evidence of tumors in preclinical models. The impact of Tregs on tumor surveillance in the organ transplant recipient is unknown. Clearly, the long term follow up of all treated patients will determine whether there is evidence of an increase in the frequency of tumors. The population under study, adult LT recipients who are two to six years after LT is a generally low-risk population for malignancies, with the exception of skin cancer.

PTLD

Treg IS has been shown to enhance tumor growth in some small animal model systems. Thus, complications such as PTLD are possible on a theoretical basis. Clinical experience in transplant recipients suggests that the risk of PTLD is highest in those who develop a primary EBV infection while immunosuppressed. Therefore, we are excluding patients who are EBV naïve to minimize the risk. We will also check EBV DNA shortly prior to planned darTregs infusion and exclude any subjects with detectible EBV DNA.

Rejection

It is possible that darTregs could have anti-allograft effect and thus precipitate AR. This may occur secondary to contamination of the darTregs product by T effector cells or secondary to instability of the regulatory phenotype such that darTregs change from a regulatory to an effector phenotype after in vivo adoptive transfer. These risks may be mitigated by the IS that subjects will still be taking when darTregs are administered.

9.3 Risks of Study Mandated Procedures

9.3.1 Risks of Blood Draw

Risks of blood draw or venipuncture are typically minimal with temporary local discomfort. More serious risks would include ecchymosis and, rarely, localized infection. There is also the risk of anemia, particularly for the large volume blood draw (450 mls) for darTregs manufacturing. However, subjects are screened for study eligibility and are not expected to have significant baseline anemia.

9.3.2 Risks of Leukapheresis

The common risks of leukapheresis include bruising and discomfort at the site of needle placement, typically in the ante cubital fossae. Calcium level in blood may fall due the citrate anticoagulant used to prevent clotting in the leukapheresis instrument. Hypocalcemia can lead to perioral or digital numbness and tingling. Calcium replacement may be used during the procedure and is routinely used at the conclusion of the procedure. Platelet count may fall due to platelet loss during processing. Hemorrhagic complications due to thrombocytopenia have not been reported in normal donors. Thrombosis and bleeding could theoretically occur, although they are rarely if ever observed. The study population consists of relatively healthy subjects with excellent liver tests and liver function. In addition, subjects will have undergone rigorous assessment of general and cardiac health as part of the standard evaluation for liver transplant candidacy that is standard of care for both clinical sites. As a result, subjects are expected to tolerate the procedure with a low side effect profile.

9.3.3 Risks of Liver Biopsy

Participation in this clinical trial includes protocol biopsies and, potentially, additional for cause or clinically indicated biopsies.

Mild risks of a liver biopsy include local pain during and for a short period of time (hours or at most days) after the procedure that will be experienced to some degree by every participant. The second AE that is typically of mild to moderate severity is bleeding. Although some bleeding likely occurs with every biopsy, it typically does not result in any symptoms; the only sign might be a small decrement in hemoglobin / hematocrit. More serious bleeding after a liver biopsy is typically diagnosed by a significant drop in the hemoglobin / hematocrit that does not cause any symptoms. The risk of requiring a transfusion secondary to excessive bleeding is 0.5 to 1% (Rockey, 2009). Even rarer would be symptomatic hemorrhage and/or the requirement for operative or other procedural intervention to stop bleeding.

Another potential complication of liver biopsy is a cholangitis, a clinical syndrome characterized by fever, abdominal pain, and abnormal liver tests. The etiology of cholangitis occurring after liver biopsy is occult biliary stricture, a known long-term complication after LT (Porrett PM, 2009). Biliary stasis resulting from biliary stricture can lead to bacterial colonization of the biliary tree. Liver biopsy may precipitate biliary leak and/or bacteremia producing the signs and symptoms delineated above. Treatment of cholangitis typically requires in-patient hospitalization for intravenous antibiotics. While diagnostic procedures including radiographic imaging are often undertaken, intervention is typically undertaken electively after recovery from the acute episode unless necessitated by ongoing hemodynamic instability in spite of intravenous antibiotics and maximal supportive therapy.

Other potentially serious risks associated with liver biopsy include pneumothorax or colonic perforation. If either were to occur, hospitalization as well as procedural or operative intervention may be necessary. Finally, there is a very small risk of death after liver biopsy, estimated at 0.1 to 0.01% (Rockey, 2009).

9.4 Potential Benefits of darTregs Infusion to Facilitate IS Minimization and/or Complete Withdrawal

There might be no direct benefit to study participants undergoing IS withdrawal and/or receiving darTregs infusion.

Autologous, ex vivo expanded darTregs are administered to a population of adult LT recipients 2-6 years after LT in an effort to facilitate IS minimization and induce tolerance earlier after transplantation. It is anticipated that successful IS minimization and/or withdrawal may offer benefits by reducing the risk of both mortality and morbidity for adult LT recipients.

One of the primary findings of the prospective multi-center trial of IS withdrawal for adult and pediatric LT recipients is that the frequency of operational tolerance is highly dependent on time after transplantation (Feng, 2012) (Benitez, 2013). If autologous darTregs demonstrate efficacy, allowing subjects 2-6 years after LT to successfully minimize or completely discontinue IS, then study participants would reap the benefits of lower lifetime exposure to conventional, non-specific IS drugs which have been extensively described in the literature and summarized in Section 1.1.

Although successful CNI dose reduction of 75% with discontinuation of a 2nd IS agent or complete IS discontinuation of IS would likely offer the greatest benefit, attempted but failed withdrawal can provide valuable information as to the subject's required IS dosing to prevent AR. For example, if AR occurs late in the withdrawal algorithm, when a subject is taking 14.3% (Step 6) or less, then the subject's ultimate and long-term maintenance regimen, after treatment of AR and stabilization, may well be less than the dose at study entry. As result, participation in this clinical trial may reduce the long-term cumulative IS burden for a subject.

10. Study Visits

10.1 Living Donor

Prior to performing the screening liver biopsy in the liver recipient, the living donor will be consented and undergo a buccal swab for HLA typing. This will enable determination of the degree of HLA DR matching between the donor and the recipient. If the donor and recipient are not fully matched at DR, then the recipient can undergo screening liver biopsy. Once the recipient is deemed fully eligible to initiate IS withdrawal, then 100 mls of blood will be collected from the living donor for the manufacturing of darTregs and mechanistic assays. The blood must be collected within 6 weeks of the recipient initiating IS withdrawal. Additional blood will be collected at the same study visit to for infectious disease screening, as required by CFR 1271 (*Appendix 1. Living Donor Assessments*). This will complete study participation for the living donor.

10.2 LT Recipient

10.2.1 Screening, Enrollment, and Initiation of IS Withdrawal

Participants will be identified by reviewing the LT recipients cared for by the UCSF and Mayo Clinic transplant centers according to the study eligibility criteria (Section 4.2). The potential participant will sign an informed consent form before undergoing any screening or study procedures. The LT recipient will be asked to perform a buccal swab for HLA typing as part of screening for the study. If the subject continues to be eligible, the research study will be explained in lay terms to each potential research participant by the site PI or designee listed on FDA form 1572. Surrogate consent will not be permitted. Once the informed consent form has been signed, the participant will be assigned a unique participant number. All screening procedures will be completed to ensure that the subject eligibility criteria. Very importantly, the site PI or designee listed on the FDA form 1572 will need to determine if the living donor is willing to consent to study participation.

Consented and otherwise eligible participants will undergo a protocol liver biopsy that will be read by the central pathologist. If the central pathology reading is permissive of IS withdrawal, then IS withdrawal can be initiated. IS withdrawal must begin within 8 weeks of the screening biopsy.

10.2.2 Assessments during IS Withdrawal (High Frequency Schedules)

Participants undergoing withdrawal, both prior to and after darTregs infusion will be assessed with liver tests (ALT, alkaline phosphatase, GGT, and total and direct bilirubin) once every two weeks during withdrawal and for 12 weeks after achieving 75% CNI reduction and, finally, for 12 weeks after successful complete withdrawal, as applicable (*Appendix 2. Study Entry and IS Withdrawal (High Frequency)*; *Appendix 3. Logistical Pause in Step 2 SOE*, *Appendix 4. darTregs Infusion SOEAppendix 5. IS Withdrawal after darTregs Infusion to Step 5/75% CNI Reduction (High Frequency)* or *Appendix 6. Complete Immunosuppression Withdrawal (High Frequency)*). During high frequency follow up, telephone consultations will collect medical history, concomitant medications, and AEs between transplant center visits. Transplant center visits with phlebotomy for mechanistic studies will be required every 12 weeks.

All subjects will have leukapheresis or 450 mls of blood drawn for darTregs manufacturing approximately 3-5 weeks into the 2nd step of CNI withdrawal. Blood will be immediately processed to isolate circulating Tregs and placed into culture for darTregs manufacturing. If a logistical pause is used, blood draw or leukapheresis will be repeated (see Appendix 3).

The visit schedule will commence every two weeks from the start of IS withdrawal (*Appendix 2. Study Entry and IS Withdrawal (High Frequency)* but will be interrupted by the darTregs infusion. The high frequency visit schedule will resume 2 weeks after darTregs infusion and continue until 12 weeks after the subject starts the 5th CNI withdrawal step (75% CNI dose reduction).

If the subject consents to continue IS withdrawal after darTregs, the subject will resume the high frequency visit schedule until 12 weeks after the last CNI dose, unless the subject fails IS withdrawal.

10.2.3 darTregs Infusion and Resumption of IS Withdrawal

Participants will receive one IV infusion of darTregs during an overnight stay. darTregs will be infused through a peripheral intravenous catheter over 20-30 minutes. All participants will be closely monitored for infusion reactions as well as other AEs. Blood will be drawn for clinical and mechanistic assessments 1 day after infusion. Liver tests will be evaluated to determine if the subject is eligible to resume IS withdrawal according to the guidelines stipulated in Section 4.4. IS withdrawal can resume as early as the next day, and no later than 14 days after darTregs infusion. The final determination regarding the resumption of IS withdrawal after darTregs infusion should always be the clinical judgment of the site principal investigator.

Eligible subjects will be instructed on how to reduce their IS prior to discharge from the hospital after the darTregs infusion. Subjects will be asked to return to the transplant center for a protocol biopsy 6-10 days after darTregs infusion (*Appendix 3. Logistical Pause in Step 2 SOE*, *Appendix 4. darTregs Infusion SOE*).

Subjects with elevated liver tests after darTregs infusion are ineligible to continue IS withdrawal until further evaluation. No changes in IS, including Prednisone or MMF, if applicable, should be made until liver tests are rechecked OR the protocol liver biopsy performed 6-10 days after darTregs infusion has been evaluated by the local pathologist. If liver tests improve and meet eligibility criteria OR liver biopsy does not show rejection, IS withdrawal can resume.

All participants who receive darTregs will be followed for a minimum of 52 weeks after the infusion. The time on high or medium frequency assessment schedules will depend on whether IS withdrawal is continued (Step 6-8) and the duration of IS withdrawal (*Appendix 5. IS Withdrawal after darTregs Infusion to Step 5/75% CNI Reduction (High Frequency)* and *Appendix 6. Complete Immunosuppression Withdrawal (High Frequency)*). All subjects, irrespective of outcome, will continue to follow-up with their transplant center according to center SOC indefinitely.

10.2.3.1 <u>Adjudication of Discrepant Pathology Readings for the Post-darTreg infusion (Day 7) Biopsy</u>

As described previously (Section 5.5), the local pathology reading will guide clinical decision making. However, discrepant reads where the central pathologist finds rejection on the day 7 biopsy after darTregs will require a review of clinical and pathology data by the PI, site investigator, and NIAID MM within 14 days of awareness.

10.2.4 Medium Frequency Schedule

Medium frequency schedule entails laboratory assessments every 4 weeks, telephone consultations to collect ongoing changes in medical history and concomitant medications every 8 weeks. A transplant center visit will be required at the end of medium frequency follow-up.

The duration on medium frequency schedule of events will vary by subject and depend on the status of the subject entering the medium frequency schedule. Reasons for leaving high frequency and entering medium frequency include failing IS withdrawal secondary to AR (biopsy-proven or clinical AR), elevated liver tests without diagnosis of AR, ineligibility to proceed with IS withdrawal after darTregs infusion, or prolonged duration of pauses during IS withdrawal. Subjects who fail IS withdrawal (with or without receiving darTregs) will utilize the medium frequency follow up schedule. These subjects will enter the medium frequency schedule four weeks after failing IS withdrawal and continue on the schedule for 52 weeks. A transplant center visit is required at the end of medium frequency follow-up and marks the end of study participation.

Subjects will also enter medium frequency after completing the requisite high frequency schedule required after successful 75% CNI dose reduction or successful discontinuation of IS altogether. Subjects will continue with medium frequency visits (every 4 weeks) until 52 weeks after darTregs infusion or 52 weeks after the last dose of IS, whichever is longer. The visit at the end of medium frequency follow up will be at the transplant center and marks the end of study participation. For subjects who are completely off IS, a biopsy will be performed to adjudicate tolerance.

10.2.5 Unscheduled Visits

Additional visits for a liver biopsy will occur when there is allograft dysfunction or at the discretion of the site investigator (Section 5.5 and 8.3.2). In addition to research specimens, local pathology results generated with unscheduled visits will be collected for the study.

Local laboratory assessments recorded for the study at the time of clinically indicated biopsy should reflect reason for biopsy (e.g. elevated liver tests).

If a participant needs a clinically indicated/for-cause biopsy within 4 weeks prior to a scheduled protocol visit, then all scheduled blood collections can be collected at the same time as the for-cause biopsy in lieu of the scheduled visit. Similarly, a telephone visit and local laboratory assessments are not required for the study if a subject has a transplant center visit.

10.3 Visit Windows

Study visits should take place within the following time limits shown below:

Table 14. SOE Visit Windows	
High Frequency SOE Visit Windows	
Study Visit	**Visit Window**
Study Entry Eligibility Screening Visit (Screen 1)	Eligibility labs and biopsy should be within 8 weeks prior to initiation of IS withdrawal
Remote Visits (Telephone and Local Liver Tests)	± 3 days
Transplant Center Visits	± 14 days
Recipient PBMC Collection for Manufacturing	17 (UCSF) or 18 (Mayo) days before darTreg infusion
Medium Frequency SOE Visit Windows	
Study Visit	**Visit Window**
Entry into Medium Frequency SOE	Within 2 weeks after rejection (Appendix 7) Within 4 weeks after completing high frequency SOE (Appendix 8)
Week 2 (Appendix 7)	± 3 days
Remote Visits (Telephone and Local Liver Tests)	± 5 days
End of Study	± 14 days
darTregs Infusion SOE Visit Windows	
Study Visit	**Visit Window**
darTregs Eligibility Screening Visit	Between day 14-41 of the 2nd withdrawal step (10-13 weeks)
Treg Blood Draw for darTregs manufacturing	Between day 18-45 of the 2nd withdrawal step
Day 0 = darTregs Infusion	During the last 2 weeks of the 2nd withdrawal step
Day 1 after darTregs infusion	± 1 hour
Protocol biopsy visit after darTregs infusion	Between 6-10 days after darTregs infusion
Resumption of IS withdrawal	Between day 1-14 after darTregs infusion; corresponds to between day 36 – day 63 of the 2nd withdrawal step
Day 14 after darTregs infusion	± 2 days

11. Mechanistic Assays

The goal of mechanistic studies in ARTEMIS is to determine the pharmacokinetics of darTregs and their impact on the alloimmune status of subjects. We will define darTregs pharmacokinetics by measuring deuterium enrichment in circulation. We will evaluate alloimmunity using leukocyte phenotyping, mixed lymphocyte reaction, serum donor-specific antibody levels, subclass, and C1q activity, and multi-parameter immunohistochemical analysis of graft biopsies. Details on rationale, sample collection, processing, and storage, assay procedures, and data analysis for each of the assay can be found below. Since there is no established biomarker for graft tolerance and the number of patients to be enrolled in this trial is low, most of the mechanistic assessments described below are exploratory in nature.

11.1 darTregs pharmacokinetics

PK will be determined by assessment of deuterium labelled genomic DNA isolated from PBMC and biopsies of darTregs recipients. Since darTregs are autologous, they will be indistinguishable from endogenous Tregs by standard surface markers and administered darTregs will not be identifiable using conventional means. The T cells are expanded prior to infusion, providing a unique opportunity to label the cells during culture so that the presence and persistence of the administered darTregs can be traced. For over a decade, the Hellerstein group, and others, using their techniques have applied stable isotope labeling with mass spectrometric analysis to measure the replication of murine and human cells *in vitro* and *in vivo*. Importantly, stable isotopes are non-radioactive and non-toxic, and they have been safely used as cellular, molecular, and metabolic markers in patients and healthy controls for more than 6 decades.

We plan to label darTregs with deuterium by including the ^{2}H label in the culture medium during the entirety of the expansion phase *in vitro* (day 0 to day 16) prior to infusion in the patient. Based on data from *in vitro* labeling studies, we expect the cells to be ~60% enrichment for ^{2}H. After infusion of the deuterium-labelled investigational product, PBMCs will be isolated and cryopreserved at time points stipulated in the SOE. Tregs will be sorted from the thawed PBMCs in batches and genomic DNA will be prepared from these samples for gas chromatography-mass spectrometry analysis of ^{2}H enrichment as shown in Figure 2. In addition, genomic DNA from subsets of peripheral blood CD4$^+$ Tconv will be analyzed to determine if infused Tregs lose Treg markers after infusion. Based on prior studies, we expect sensitivities in the range of 0.05 to 0.10% enrichment (which refers to the fraction of labeled molecules) (Busch, 2007) (Macallan, 1998). Peripheral blood results from this analysis will be presented as number of infused darTregs per ml of blood and % of darTregs among all Tregs in circulation.

11.2 Treg TruCount Analysis

Samples collected for this assay will be used directly for analysis without cryopreservation. Blood will be aliquoted into a TruCount tube, stained with fluorochrome-conjugated antibodies to CD4, CD45, CD25 and CD127, and analyzed on a flow cytometer to enumerate the numbers of Tregs. This assay would allow us to obtain the absolute Treg counts in one microliter of blood and establish the counts at baseline; and later determine if counts are altered by darTregs therapy.

11.3 MFC Panels

The MFC analysis is focused on defining leukocyte subsets, T cell activation/exhaustion status, and CD4+ and CD8+ Treg frequencies. Extensively validated MFC panels will be used to quantify changes in leukocyte populations in blood (Table 15) collected prior to during and after IS withdrawal. Blood will be collected at time points specified on the SOE. The blood samples will be shipped to a central laboratory for processing into PBMC and then cryopreserved. Absolute counts of each cell subsets, percentages and percent changed over baseline will be calculated. Changes in the values will be correlated with the level of IS, stage of IS withdrawal, Treg therapy, and the final outcome of IS withdrawal.

Table 15. ARTEMIS MFC panels		
Panel Names	**Markers**	**Rationale**
Leuko	CD3, CD4, CD14, CD16, CD19, CD56, HLA-DR	To determine the numbers and percentages of T cells, B cells, subsets of monocytes, subsets of NK cells and dendritic cells
Treg	CD3, CD4, CD8, CD25, CD127, FOXP3, HELIOS	To determine the number of Tregs
Tact	CD3, CD4, CD8, CD27, CD28, CD45RA, CCR7	To determine the activation status of T cells
Texh	CD3, CD4, CD8, CD57, PD1, Tim3	To determine the percentage of T cells that express exhausted or inhibited phenotype that is implicated in transplant tolerance

11.4 Donor Specific Assays

11.4.1 Frequency of donor-reactive T cells

We will use the assay described in Figure 6to determine the frequency of donor-reactive CD4+ Tconvs, CD8+ T cells, and Tregs at time points indicated in the SOE. We expect to see an increase in darTregs shortly after infusion.

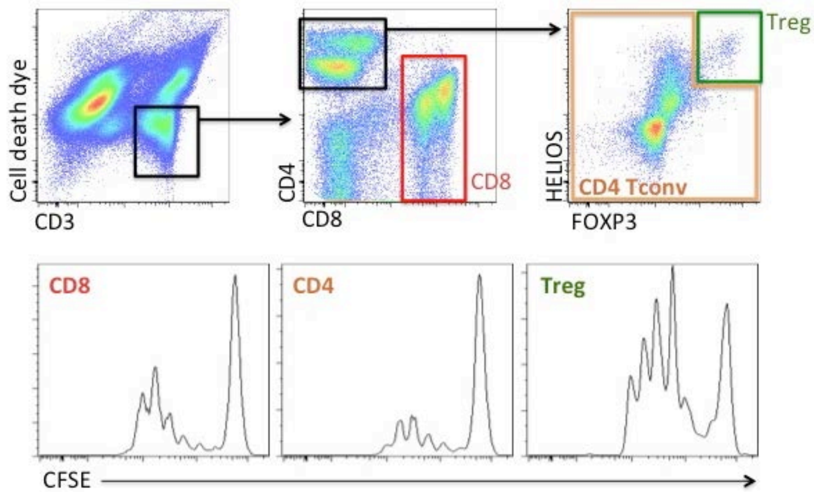


Figure 6. Assay for measuring frequency of donor-reactive T cells

11.4.2 In vitro suppression

We will assess suppression by Tregs isolated from patients at time points indicated in the SOE. Pre- infusion PBMCs will be used as responders mixed with Tregs from various time points. Tregs will be sorted from frozen PBMCs based on the cell surface phenotype of CD4+CD127lo/-CD25+. The cultures will be stimulated with irradiated donor PBMC to assess donor-specific suppression and with anti-CD3 and anti-CD28 to assess nonspecific suppression.

11.5 Alloantibodies

HLA antibodies in general and DSAs in specific have not been strongly implicated as a common risk factor for AR, CR, or graft loss in LT (Kaneku H. , 2010). Nevertheless, their presence at baseline may preclude successful IS withdrawal. Moreover, increasing breadth and/or strength of DSAs during IS withdrawal is evidence of an anti-donor immune response. The presence and strength of HLA antibodies/DSA at baseline and longitudinally, will also be analyzed for correlations with histological findings (C4d and MHC class II staining patterns and intensities on protocol and indication liver biopsies) (Feng, 2012). Finally, we will also plan to characterize the IgG subclass and the C1q activity of DSAs as these additional parameters are increasingly correlated to the functional importance of DSA in liver and/or kidney transplantation (Gao ZH, 2004) (Lobashevsky A, 2010) (Kaneku H, 2012) (Sutherland SM, 2012) (Freitas MC, 2013) (Loupy A, 2013).

11.5.1 HLA Typing

HLA typing will be performed for all donor and recipient pairs from buccal cells collected at the time of study screening. HLA typing data will be used to conduct alloantibody studies described above.

11.6 Histology and Multiplex Immunohistochemistry (mIHC)

In collaboration with Dr. A. Jake Demetris, we will perform extensive histology and mIHC analysis of protocol biopsy samples obtained at screening, 1 week after Treg infusion, all for-cause biopsy samples, and 12 months after last IS dose (if applicable). Banff criteria will be used to score histologic findings as described in Section 4 and 5. The design of these analyses will be guided by histology and mIHC data from tolerant patients in iWITH and our current understanding of LT tolerance. Histological analyses will evaluate 40 histopathological features to determine tissue integrity and degree of inflammation. mIHC analyses are summarized in Table 16.

Table 16. ARTEMIS Immunohistochemistry Panels

Panel	Rationale
C4d/CD31	Determine extent and intensity of C4d deposits on hepatic microvasculature as a barometer of anti-donor reactivity; test hypothesis that total C4d score ≥ 6 is associated with withdrawal failure
CD3/γδ-1/γδ-2	Test hypothesis that portal tract ratio of γδ-1/γδ-2 > 1.0 is associated with operational tolerance.
CK19/CD31/HLA-DR	Test hypothesis that inappropriate expression of HLA-DR on bile ducts is associated with failed withdrawal; monitor rejection targets, CK19+ biliary epithelium); CD31+ endothelium, for immune activation via up regulation of HLA-DR, which is not normally expressed.
CD3/CD45RO/CD45RA	Monitor relative ratio of naïve to memory T cells; test the hypothesis that an increase in portal-based CD3+/CD45RO+ (memory) T cells is associated with failed ISW
CD4/Tbet/GATA-3/IL-17/FoxP3	Monitor polarization of CD4+ lymphocytes within the allograft to determine whether an increase of putative regulatory T cells contributes to tolerance
IL10/TGFβ/HLADR	Monitor expression of immunomodulatory cytokines by HLA-DR expressing cells in the liver such as Kupffer's cells and B cells.
CD56/PD-1/CD3	Determine the relative number/ratio of CD3+, CD56+, and PD-1+ lymphocytes and whether changes in NKT cells in the liver is associated with operational tolerance
CD5/CD19/CD27/IgG	Determine ratio of naïve to memory B cells and B1:B2 intra-hepatic B cells and whether memory B cells or B-regulatory cells residing in the liver might contribute to allograft acceptance or rejection

11.7 Gene Expression Profiling

A piece of liver tissue from both protocol driven and clinically indicated liver biopsies will be preserved in RNALater and banked at -80°C. Future studies might include examining how levels of RNA expression of immunoregulatory genes such as IL-10, Tim3, PD1, etc., changes in the setting of IS withdrawal and donor antigen exposure.

11.8 Single Cell RNA+TCRseq of Infiltrates

Phenotype and specificity of graft–infiltrating T cells may provide important clues to their long-term impact on graft outcomes and the effect of the novel study regimen. While cells from biopsy offers most biologically relevant data obtained from the target tissue, the challenge in analyzing graft-infiltrating immune cells is their low number. With single cell RNAseq combined with TCRseq, rich mechanistic data can be generated with limited samples. Figure 7 shows that this approach can detect CD4 and CD8 T cells, CD3-NKG7+ NK cells, B cells, monocytes, and dendritic cells in kidney biopsies. From patients undergoing subclinical inflammation. In addition, close to 2000 T cells with paired TCR were identified (Figure 7B) and global gene expression profile of the expanded top clones can be obtained (Figure 7C). In the examples shown, the top clones in the first biopsy are CD8 cytotoxic cells (clone1, highly expressed XCL2, GZMB, and GNLY) and effector cytokine producing cells (clone 2 and 3 highly express HSPA1B, LTB, IL-7R, and MTE), whereas the #1 top clone in second biopsy are likely regulatory T cells that highly expressed IL10, CTLA-4, LAG3, PDCD1, HAVCR2 and low expression of all granzymes and top clones 2 and 3 are cytotoxic memory cells with higher expression of granzymes, GNLY, LTB, and CCL5. Thus, single-cell RNA+TCRseq from biopsy is feasible and provides high dimension data for dissecting graft infiltrating immune cells. In addition, tracking the infiltrating T cell clones using single cell TCRseq also digitizes the TCR so that the sequence may be used in follow-up analysis for specificities of the TCR if needed. To do this, paired TCRab chains can be expressed in reporter cell lines to test donor or common pathogen specificity using assay established in Tang lab (Spence, 2018).

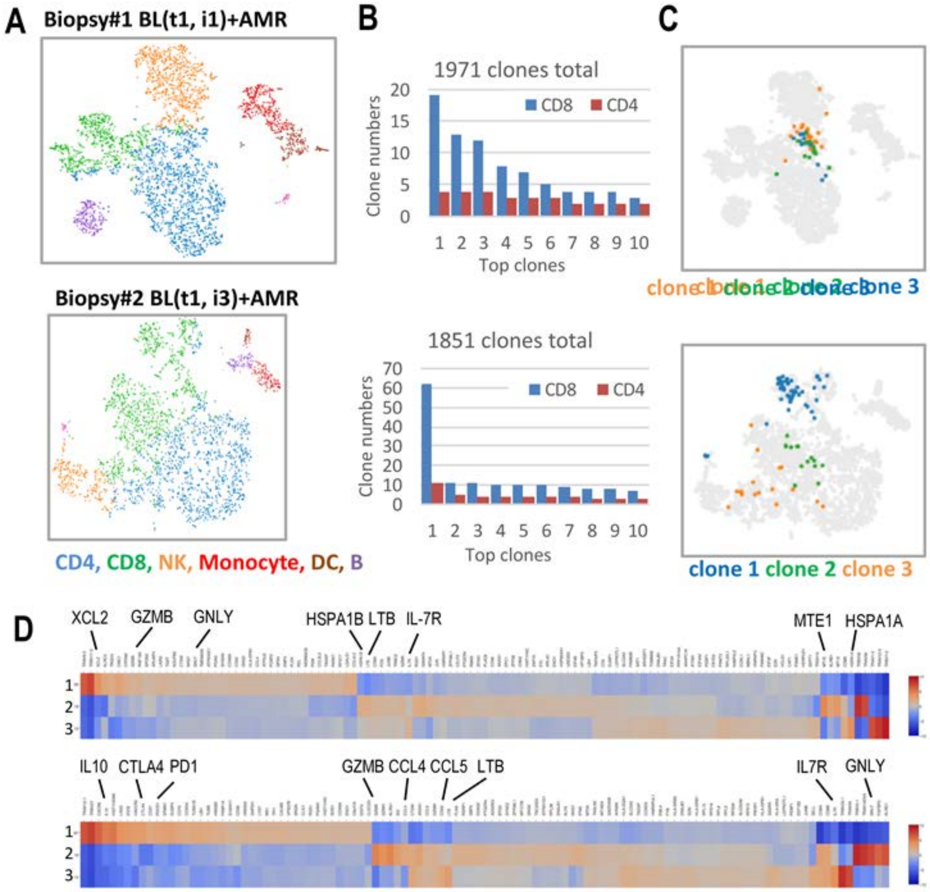


Figure 7. *Single-cell RNA+TCRseq analyses of kidney biopsies.*

12. Biospecimen Storage

Biological specimens obtained under this protocol may be used in future assays to reevaluate biological responses as additional research tests are developed over time. Blood for gene expression, PBMCs, and serum will be collected at time points already scheduled for the core mechanistic studies, in order to allow specimens to be stored for use in new assays that have yet to be optimized or conceived, or assays performed by other *CTOT or CTOTC* consortia members for cross-validation studies. Appropriate informed consent will be obtained for both the collection and storing of samples. The specimens from these evaluations may be stored beyond the funding period.

13. Criteria for Participant Completion and Premature Study Termination

13.1 Participant Completion

After study completion, all subjects, whether or not on IS, will revert to SOC follow-up at the site investigator's transplant center.

13.1.1 Study Completion

Subjects who do not initiate IS withdrawal will discontinue study participation without further study follow up. Subjects who fail IS withdrawal, whether prior to or after receiving darTregs, will be followed for a minimum of 52 weeks from the date of rejection for safety. Subjects who receive darTregs will be followed for a minimum of 52 weeks after darTregs infusion, irrespective of any other outcome. Subjects who consent to and succeed at complete IS withdrawal will be followed for 52 weeks after the last IS dose and, at the end of that follow-up, will be assessed for tolerance (blood and biopsy).

13.2 Participant Withdrawal Criteria

Participants may be prematurely terminated from the study without any further follow-up for the following reasons:

1. The participant elects to withdraw consent from all future study activities, including follow-up
2. The participant is “lost to follow-up” (i.e., no further follow-up is possible because attempts to reestablish contact with the participant have failed)
3. The participant dies
4. If a participant prematurely terminates from the study because of graft loss or because of non-compliance with follow-up and/or study procedures
5. If the principal investigator, site investigator, and/or medical monitor (MM) believe study intervention is no longer in the best interest of the participant.

If a subject prematurely terminates from the trial, the subject’s IS regimen will be determined by the site investigator based on the best interest of the subject.

13.3 Participant Replacement

Any subject who receives less than 100 x 10^6 darTregs will be replaced.

14. Safety Monitoring and Reporting

14.1 Overview

This section defines the types of safety data that will be collected under this protocol and outlines procedures for appropriately collecting, grading, recording, and reporting those data. AEs that are classified as serious according to the definition of health authorities as well as other events of interest must be reported promptly (Section 14.5) to the sponsor DAIT/NIAID. Appropriate notifications will also be made to site principal investigators, Institutional Review Boards (IRBs) and health authorities.

Information in this section complies with ICH Guideline E2A: Clinical Safety Data Management: Definitions and Standards for Expedited Reporting, ICH Guideline E-6: Guideline for Good Clinical Practice, 21CFR Parts 312 and 320, and applies the standards set forth in the National Cancer Institute (NCI) CTCAE, Version 4.0: http://ctep.cancer.gov/reporting/ctc.html.

14.2 Definitions

14.2.1 Adverse Events (AEs)

Any untoward or unfavorable medical occurrence associated with the subject's participation in the research, whether or not considered related to the subject's participation in the research (modified from the definition of AEs in the 1996 International Conference on Harmonization E-6 Guidelines for Good Clinical Practice) (from OHRP "Guidance on Reviewing and Reporting Unanticipated Problems Involving Risks to Subjects or Others and AEs (1/15/07)" http://www.hhs.gov/ohrp/policy/advevntguid.html#Q2)

14.2.1.1. Suspected Adverse Reaction

A suspected adverse reaction is any AE for which there is a reasonable possibility that the investigational agent (Section 6) caused the AE. For the purposes of IND safety reporting, 'reasonable possibility' means there is evidence to suggest a causal relationship between the drug (i.e. darTregs) and the AE. A suspected adverse reaction implies a lesser degree of certainty about causality than adverse reaction, which means any AE caused by a drug (21 CFR 312.32(a)).

Suspected adverse reactions associated with IS withdrawal, blood draw, or liver biopsy are collected and reported to the sponsor. The sponsor will relay any suspected adverse reactions to the DSMB, as appropriate.

14.2.2 Unexpected AEs

For events assessed in association with the investigational agent (darTregs), an AE or suspected adverse reaction is considered "unexpected" if it is not listed in the investigator's brochure or is not listed at the specificity, severity or rate of occurrence that has been observed; or is not consistent with the risk information described in the general investigational plan or elsewhere in the study protocol.

"Unexpected" also refers to AEs or suspected adverse reactions that are mentioned in the investigator's brochure as occurring with a class of drugs or as anticipated from the pharmacological properties of the drug, but are not specifically mentioned as occurring with the particular drug under investigation (darTregs) (21 CFR 312.32(a).

For events assessed in association with IS withdrawal or liver biopsy, an AE or suspected adverse reaction is considered "unexpected" if it is not listed in the protocol or is not listed at the specificity, severity or rate of occurrence that has been observed.

14.2.3 Serious Adverse Events

An AE or suspected adverse reaction is considered "serious" if, in the view of either the investigator or Sponsor, it results in any of the following outcomes (21 CFR 312.32(a)):

1. Death.
2. A life-threatening event: An AE or suspected adverse reaction is considered "life-threatening" if, in the view of either the investigator or DAIT/NIAID, its occurrence places the subject at immediate risk of death. It does not include an AE or suspected adverse reaction that, had it occurred in a more severe form, might have caused death.

3. Inpatient hospitalization or prolongation of existing hospitalization (Please see Section 13.4.3 for exceptions).
4. Persistent or significant incapacity or substantial disruption of the ability to conduct normal life functions.
5. Congenital anomaly or birth defect.
6. Important medical events that might not result in death, be life threatening, or require hospitalization may be considered serious when, based upon medical judgment, they might jeopardize the subject and might require medical or surgical intervention to prevent one of the outcomes listed above.

The events of interest below should be reported as a serious adverse event (within 24 hours of awareness) even if the event does not meet serious criteria:

- Biopsy Proven or Clinical (Treated) AR
- CR
- Infusion Reactions CTCAE grade 2 or higher
- Malignancy, PTLD
- Infections study defined grade 3 or higher

14.3 Grading and Attribution of Adverse Events

14.3.1 Grading Criteria

The study site will grade the severity of AEs experienced by the study subjects according to the criteria set forth in the National Cancer Institute's CTCAE – Version 4.0 for all AEs. This document (referred to herein as the NCI-CTCAE manual) provides a common language to describe levels of severity, to analyze and interpret data, and to articulate the clinical significance of all AEs. The NCI-CTCAE has been reviewed by the study investigators and has been deemed appropriate for the subject population to be studied in this protocol for all AEs except infection and elevated liver tests.

AEs will be graded on a scale from 1 to 5 according to the following standards in the NCI-CTCAE manual:

- Grade 1 = mild AE.
- Grade 2 = moderate AE.
- Grade 3 = severe and undesirable AE.
- Grade 4 = life-threatening or disabling AE.
- Grade 5 = death.

For any AE of Infection, the following grading system will be used for study participants:

- Grade 1 = asymptomatic; clinical or diagnostic observation only; intervention with oral antibiotic, antifungal, or antiviral agent only; no invasive intervention required
- Grade 2 = symptomatic; intervention with intravenous antibiotic, antifungal, or antiviral agent; invasive intervention may be required
- Grade 3 = any infection associated with hemodynamic compromise requiring pressors; any infection necessitating ICU level of care; any infection necessitating operative intervention; any infection involving the central nervous system; any infection with a positive fungal blood culture; any proven or probable aspergillus infection; any tissue invasive fungal infection; any pneumocystis jiroveci infection
- Grade 4 = life-threatening infection
- Grade 5 = death resulting from infection

Events, grade 2 or higher will be recorded on the appropriate AE case report form for this study.

For grading an abnormal value or result of a clinical or laboratory evaluation (including, but not limited to, a radiograph, an ultrasound, an electrocardiogram etc.), a treatment-emergent AE is defined as an increase in grade from baseline or from the last post-baseline value that doesn't meet grading criteria. If a specific event or result from a given clinical or laboratory evaluation is not included in the NCI-CTCAE manual, then an abnormal result would be considered an AE if changes in therapy or monitoring are implemented as a result of the event/result.

14.3.2 Attribution Definitions

The relationship, or attribution, of an AE to the study therapy regimen or study procedure(s) will initially be determined by the site investigator and recorded on the appropriate AE/SAE form. Final determination of attribution for safety reporting will be determined by DAIT/NIAID. The relationship of an AE to study therapy regimen or procedures will be determined using the descriptors and definitions provided in (Table 17). For additional information and a printable version of the NCI-CTCAE manual, consult the NCI-CTCAE web site: *http://ctep.cancer.gov/reporting/ctc.html*.

Table 17. Attribution of Adverse Events

Code	Descriptor	**Relationship** (to primary investigational product and/or other concurrent mandated study therapy or study procedure)
Unrelated Category		
1	Unrelated	The AE is clearly not related.
Related Categories		
2	Possible	The AE has a reasonable possibility to be related; there is evidence to suggest a causal relationship.
3	Related	The AE is clearly related.

Attribution assessment for the following study interventions and procedures will be made when a SAE is reported:

- IS withdrawal
- darTregs infusion
- Blood draw (Donor or Recipient)
- Leukapheresis
- Liver biopsy

14.4 Collection and Recording of Adverse Events

14.4.1 Collection Period

AEs will be collected from the time of study mandated liver biopsy procedure until a subject completes study participation or until 30 days after he/she prematurely withdraws.

14.4.2 Collecting Adverse Events

AEs (including SAEs) may be discovered through any of these methods:

- Observing the subject
- Interviewing the subject (e.g., using a checklist, structured questioning, diary, etc.)
- Receiving an unsolicited complaint from the subject

In addition, an abnormal value or result from a clinical or laboratory evaluation can also indicate an AE, as defined in Section 14.2.1 .

14.4.3 Exceptions to Collection

Elective hospitalizations, hospitalization solely for a diagnostic procedure, or hospital admissions to conduct protocol mandated procedures are not to be collected as an AE unless hospitalization is prolonged due to complications.

Only AEs associated with protocol mandated liver biopsy will be collected from the time of the study eligibility biopsy to the initiation of initial IS withdrawal.

A pause in IS withdrawal alone is not reportable as an AE.

14.4.4 Recording Adverse Events

Throughout the study, the investigator will record AEs and SAEs as described previously on the appropriate AE/SAE form regardless of the relationship to study therapy regimen or study procedure.

Once recorded, an AE/SAE will be followed until it resolves with or without sequelae. AE/SAE will be followed until the end of study participation, or until 30 days after the subject prematurely withdraws (without withdrawing consent) /or is withdrawn from the study, whichever occurs first.

14.5 Reporting of Serious Adverse Events and Adverse Events

14.5.1 Reporting of SAEs to Sponsor

This section describes the responsibilities of the site investigator to report serious AEs to the sponsor via the SACCC eCRF. Timely reporting of AEs is required by 21 CFR and ICH E6 guidelines.

Site investigators will report all SAEs, regardless of relationship or expectedness to blood draw or liver biopsy with research specimen collection, IS withdrawal, or Treg infusion within 24 hours of discovering the event.

For SAEs, all requested information on the AE/SAE form should be provided. However, unavailable details of the event will not delay submission of the known information. As additional details become available, the AE/SAE should be updated and submitted.

14.5.2 Reporting to Health Authority

SAEs (Section 14.2.3 14.4.2) submitted by the site investigator are assessed by the DAIT/NIAID medical monitor. DAIT/NIAID will report the SAE to the appropriate health authorities as follows:

14.5.2.1. Annual Reporting

DAIT/NIAID will include in the annual study report to health authorities all AEs classified as:

- Serious, expected, suspected adverse reactions
- Serious and not a suspected adverse reaction
- Serious, unexpected suspected adverse reactions occurring prior to Treg infusion.

Note that all AEs (not just those requiring 24-hour reporting) will be reported in the Annual IND Report.

14.5.2.2. Expedited Safety Reporting

DAIT/NIAID shall notify the FDA and all participating site investigators of expedited Safety Reports within 15 calendar days; unexpected fatal or immediately life-threatening suspected adverse reaction(s) shall be reported as soon as possible or within 7 calendar days.

Expedited reporting, with 2 possible categories, applies if the AE is classified as one of the following:

Category 1: **Serious and unexpected suspected adverse reaction [SUSAR]** (Section 14.2.1.1, 14.2.3 and 21 CFR 312.32(c)(1)i). The sponsor shall report any suspected adverse reaction that is both serious, unexpected and occurs after Treg infusion. The sponsor shall report an AE as a suspected adverse reaction only if there is evidence to suggest a causal relationship between the study drug and the AE, such as:

1. A single occurrence of an event that is uncommon and known to be strongly associated with drug exposure (e.g., angioedema, hepatic injury, or Stevens-Johnson Syndrome);
2. One or more occurrences of an event that is not commonly associated with drug exposure, but is otherwise uncommon in the population exposed to the drug (e.g., tendon rupture);
3. An aggregate analysis of specific events observed in a clinical trial (such as known consequences of the underlying disease or condition under investigation or other events that commonly occur in the study population independent of drug therapy) that indicates those events occur more frequently in the drug treatment group than in a concurrent or historical control group.

Category 2: Any findings from studies that suggests a significant human risk

The sponsor shall report any findings from other epidemiological studies, analyses of AEs within the current study or pooled analysis across clinical studies or animal or *in vitro* testing (e.g. mutagenicity, teratogenicity, carcinogenicity) that suggest a significant risk in humans exposed to the drug that would result in a safety-related change in the protocol, informed consent, investigator brochure or package insert or other aspects of the overall conduct of the study.

14.5.3 Reporting of AEs to IRBs
All investigators shall report AEs, including expedited reports, in a timely fashion to their respective IRBs/IECs in accordance with applicable regulations and guidelines. All IND Safety Reports to the FDA shall be distributed by DAIT/NIAID or designee to all participating institutions for site IRB submission.

14.6 Pregnancy Reporting
The investigator shall be informed immediately of any pregnancy in a study subject or a partner of a study subject. A pregnant subject shall be instructed to not stop taking IS study medication unless directed by his/her study physician. The investigator shall counsel the subject and discuss the risks of continuing with the pregnancy and the possible effects on the fetus. Monitoring of the pregnant subject shall continue until the conclusion of the pregnancy.

The investigator shall report to the SACCC all pregnancies within 1 business day of becoming aware of the event using the Pregnancy form. All pregnancies identified during the study shall be followed to conclusion and the outcome of each must be reported. The Pregnancy form shall be updated and submitted to the SACCC when details about the outcome are available. When possible, similar information shall be obtained for a pregnancy occurring in a partner of a study subject.

Information requested about the delivery shall include:
- Gestational age at delivery
- Birth weight, length, and head circumference
- Gender
- Appearance, pulse, grimace, activity, and respiration (APGAR) score at 1 minute, 5 minutes, and 24 hours after birth, if available
- Any abnormalities.

Should the pregnancy result in a congenital abnormality or birth defect, an SAE shall be submitted to the SACCC using the SAE reporting procedures described above. Pregnancies reported as SAE's will be reported to the FDA as described above.

14.7 Reporting of Other Safety Information
An investigator shall promptly notify the site IRB as well as the SACCC using the AE/SAE form when an "unanticipated problem involving risks to subjects or others" is identified, which is not otherwise reportable as an AE.

14.8 Review of Safety Information
The PI, the NIAID MM, and the NIAID/DAIT Transplant DSMB will review safety data on an ongoing basis. Enrollment and initiation of study treatment may be suspended at any time if any of these reviews conclude there are significant safety concerns. Study treatment includes first withdrawal attempt, darTregs infusion, and continuation of IS withdrawal.

14.8.1 MM Review
The NIAID MM shall receive monthly reports from the SACCC compiling new and accumulating information on AEs and SAEs recorded by the study site(s) on appropriate eCRFs.

In addition, the MM shall review and make decisions on the disposition of the SAE reports (including any infusion related events) received from the site investigator via the SACCC in a real time manner.

14.8.2 DSMB Review
The DSMB shall review safety data at least biannually during planned DSMB Data Review Meetings. Data for the planned safety reviews will include, at a minimum, a listing of all reported AEs and SAEs.

The DSMB will be informed of an Expedited Safety Report in a timely manner.

In addition to the pre-scheduled data reviews and planned safety monitoring, the DSMB may be called upon for *ad hoc* reviews. The DSMB will review any event that potentially impacts safety at the request of the principal investigator or DAIT/NIAID.

14.8.3 Study Stopping Rules

NIAID and the SACCC will continuously monitor accumulating safety data to determine if any stopping rule criterion is satisfied. The criteria for pausing enrollment pending DSMB review is based on any occurrence of selected AEs delineated in Table 18. Since the trial is small and the above events are of particular concern, a single occurrence of any event listed will require DSMB and/or MM review within 48 hours.

In addition to AE's described, failure to manufacture and supply the cellular product for 2 subjects will also require a pause in the trial.

If a study stopping rule is met, study enrollment will be paused, darTregs infusions will be held, and darTregs manufacturing should not begin. If the study is stopped due to darTregs infusion reactions, subjects who have already received darTregs will continue with the IS withdrawal algorithm. If any other stopping rule is met, subjects will remain at their current IS dose without further dose reduction until the DSMB and/or NIAID MM authorizes the study to continue. Any subject who has successfully completed IS withdrawal will remain off drug unless clinical condition mandates otherwise. In the event that darTregs manufacturing for an eligible subject was already initiated every attempt will be made to expedite review such that a prompt decision is made regarding the appropriate management for these subjects.

Table 18. Selected AEs that Constitute a Study Stopping Rule

Any time during the study	After darTregs Infusion
Death or graft loss	CTCAE Grade 3 or higher AEs attributable to the darTregs infusion including infusion reaction/cytokine release syndrome in 2 subjects
CR*	Any infections of Grade 3 or higher as defined in Section 14.3.1
Severe AR (histological or steroid refractory rejection)*	Any malignancy, including PTLD

* Banff criteria

Cessation of IS withdrawal mandated by the NIAID DSMB or MM review will not be considered a "pause".

15. Statistical Considerations and Analytical Plan

15.1 Statistical Analyses

Statistical analyses of the safety and clinical outcomes will be descriptive for the analysis samples defined below in section 15.1.1., employing standard methods for the estimation of person-week incidence rates and their exact two-sided 95% confidence intervals. Statistical analyses of the mechanistic outcomes will be exploratory in nature. The plans for statistical analyses of study data will be described in more detail in a Statistical Analysis Plan (SAP).

15.1.1 Analysis Populations

The summary descriptive analyses will be performed on the following subject populations:

1. All subjects who give informed consent and undergo the screening liver biopsy.
2. The Intent-to-treat I sample (ITT1) will be defined as all subjects who initiate IS withdrawal
3. The Intent-to-treat II sample (ITT2) will be defined as all subjects who receive darTregs.
4. The Per-Protocol sample (PP) will be defined as all subjects who receive at least 300 x 10^6 cells of darTregs.

15.2 Endpoint Assessments

15.2.1 Safety Endpoints

Table 19 describes the safety endpoints and their corresponding parameters to be estimated in the study. Safety endpoints will be listed or summarized, as appropriate, using standard descriptive statistics for continuous and categorical data.

Table 19. Analyses of Safety Endpoints

ENDPOINT	ANALYSIS PARAMETER	ANALYSIS POPULATION
Primary Endpoints - darTregs Infusion		
Incidence of AEs (CTCAE grade ≥3) attributable to darTregs infusion (including infusion reaction/cytokine release)	Number of AEs of CTCAE grade ≥ 3 Possibly or Definitely related to darTregs infusion	ITT2, PP
Incidence of infections ≥grade 3	Number of infectious AEs of grade ≥ 3 (defined in Section 14.3.1)	ITT2, PP
Incidence of any malignancy including PTLD	Number of participants with any malignancy including PTLD	ITT2, PP
Secondary Endpoints - IS withdrawal		
Rate of composite outcome measure including refractory AR, CR, re-transplantation, and death	Proportion of participants with at least one of the events with exact binomial 95% confidence limits	ITT1, PP
Incidence of biopsy proven or clinical AR and/or CR	Number of biopsy proven or clinical AR or CR AEs	ITT1, PP
Timing of biopsy proven or clinical AR and/or CR	Time from darTregs infusion to AR and/or CR	ITT2, PP
Severity of biopsy proven AR and/or CR	Banff criteria	ITT1, PP

15.2.2 Efficacy Endpoints

Table 20 describes efficacy endpoints and their corresponding parameters to be estimated in the study. Efficacy endpoints will be listed or summarized, as appropriate, using standard descriptive statistics for continuous and categorical data.

Table 20. Analyses of Efficacy Endpoints		
ENDPOINT	**ANALYSIS PARAMETER**	**ANALYSIS SAMPLE**
Primary Endpoint		
Number and proportion of subjects who reduce CNI by 75% and discontinue a 2nd IS drug, if applicable, with stable liver tests	Proportion with exact binomial 95% confidence limits	ITT1, ITT2, PP
Secondary Endpoint		
Number and proportion of subjects who successfully withdraw from all IS after darTregs infusion	Proportion with exact binomial 95% confidence limits	ITT2, PP
Time duration that subjects tolerate complete IS discontinuation	Time interval between the last IS dose and reinstitution of IS	ITT2, PP
Number and proportion of subjects who successfully withdraw from all IS after darTregs infusion and are operationally tolerant	Proportion with exact binomial 95% confidence limits	ITT2, PP

15.2.3 Measures to Minimize Bias

Although the study is open-label, the mechanistic analyses of recipient specimens at the central laboratories will be blinded with respect to the status of the recipient in the study.

15.2.4 Supportive Analyses

Since this is a small pilot study, no sub-group analyses, sensitivity analyses or covariate adjustments are planned.

15.2.5 Analyses of Exploratory Mechanistic Outcomes

Mechanistic assays are designed to monitor the impact of darTregs therapy on the immune system and the liver graft. These objectives are accomplished by performing measurement on the peripheral blood and the liver biopsy samples collected before and after darTregs infusion. The results will be summarized using standard statistical methods for continuous variables and will be displayed graphically by subject over time. The exploratory analyses of the mechanistic outcomes will be described in more detail in the SAP.

15.2.6 Descriptive Analyses

Disposition of subjects will be summarized in the all subjects receiving Tregs (ITT2). Standard descriptive statistics for continuous and categorical variables will be used to summarize the following on all subjects for the analysis periods defined above:

- baseline and demographic characteristics of the subjects
- use of concomitant medications
- reasons for early termination
- all reported AEs

15.3 Interim Analyses

No formal interim analyses of this study are planned.

15.4 Sample Size Considerations

The proposed trial is designed as a pilot trial to assess the safety of administering autologous, darTregs to stable adult living donor LT recipients. Currently, as of 11/1/2014, ex vivo expanded, autologous, darTregs have not yet been administered to solid organ transplant recipients taking IS medications. There are however two trials open to enrollment that plan to administer darTregs to adult, de novo, living donor kidney transplant recipients (NCT02244801) and adult, de novo, deceased donor liver transplant recipients (NCT02188719). As a pilot trial designed to assess safety, this trial design cannot be driven by specific sample size calculations based on previous data related to the safety of darTregs administration as none exists.

We however propose to use available data regarding the success, the failure, and the timing failure of IS withdrawal among stable adult liver transplant patients to demonstrate the prospect that darTregs will offer a benefit in terms of increasing the success rate of IS minimization, complete withdrawal, and/or operational tolerance.

Previous IS withdrawal trials in adult liver transplant recipients together demonstrate that patients who are 1 – 6 years after LT have an approximate operationally tolerant success rate of 13%. Moreover, in AWISH (NCT00135694), among the 44 subjects who tolerated a 25% decrease in their CNI dosing, 66% failed in that they were unable to tolerate 75% dose reduction. We propose that darTregs infusion will increase the proportion of subjects able to tolerate a 75% CNI dose reduction along with, if applicable, complete discontinuation of either Prednisone or MMF for 12 weeks while maintaining stable liver tests. We will examine the power to assess this as the primary endpoint in the following table. We have selected to administer darTregs to 9-11 subjects, who will comprise the ITT2 analysis sample. If the actual failure rate is 22% (2/9 subjects), then we will have 89% power at an alpha level of 0.05 to conclude that this failure rate differs from the baseline failure rate of 66% in the absence of darTregs using an exact one-sided binomial test. Similarly, with eleven subjects, if the actual failure rate is 27% (3/11 subjects), then we will have 85% power at an alpha level of 0.05 to conclude that this failure rate differs from the baseline failure rate of 66% in the absence of darTregs using an exact one-sided binomial test.

Table 21. Sample Size Power Calculation

Sample Size	Failure Rate	Power	Alpha
8	1/8	0.988	**0.047**
	2/8	**0.886**	
	3/8	0.658	
	4/8	0.385	
10	1/10	0.987	0.022
	2/10	**0.879**	
	3/10	0.650	
	4/10	0.382	
11	1/11	0.998	**0.043**
	2/11	0.965	
	3/11	**0.845**	
	4/11	0.631	
	5/11	0.384	

Subjects who are able to maintain stable liver tests for 12 weeks after 75% CNI reduction and discontinuation of Prednisone or MMF will be offered the opportunity to continue CNI withdrawal aiming to stop CNIs. Subjects will be considered to have successfully discontinue all IS medications if they maintain stable liver tests for 12 weeks after the last IS / CNI dose. Subjects will be considered to be operationally tolerant if they maintain stable liver tests and stable allograft histology 52 weeks after the last IS / CNI dose. While not the primary endpoint of the study, this is an important secondary endpoint and thus warrants discussion. Tables 22**Error! Reference source not found.** and 23 show that if 4 or more subjects either succeed at complete discontinuation or become tolerant after darTregs treatment, the one-sided 95% Confidence Limit is >13% which is the historical rate of complete IS discontinuation and operational tolerance.

Table 22. Incidence Rates and Confidence Intervals for 9 Subjects Attempting Complete IS Withdrawal after Treatment with darTregs		
Number of Subjects Able to Discontinue All IS or Achieve Operational Tolerance	**Incidence Rate** (%)	**One-Sided 95% Lower Confidence Limit** (%)
1	11.11	0.57
2	22.22	4.10
3	33.33	9.77
4	***44.44***	***16.88***
5	***55.56***	***25.14***
6	***66.67***	***34.49***
7	***77.78***	***45.04***
8	***88.89***	***57.09***
9	***100.00***	***71.69***

Table 23. Incidence Rates and Confidence Intervals for 11 Subjects Attempting Complete IS Withdrawal after Treatment with darTregs		
Number of Subjects Able to Discontinue All IS or Achieve Operational Tolerance	**Incidence Rate** (%)	**One-Sided 95% Lower Confidence Limit** (%)
1	9.09	0.47
2	18.18	3.33
3	27.27	7.88
4	***36.36***	***13.51***
5	***45.45***	***19.96***
6	***54.55***	***27.12***
7	***63.64***	***34.98***
8	***72.73***	***43.56***
9	***81.82***	***52.99***
10	***90.91***	***63.56***
11	***100.00***	***76.16***

16. Identification and Access to Source Data

16.1 Source Data

Source documents and source data are considered to be the original documentation where subject information, visits consultations, examinations and other information are recorded. Documentation of source data is necessary for the reconstruction, evaluation and validation of clinical findings, observations and other activities during a clinical trial.

16.2 Access to Source Data

The site investigators and site staff will make all source data available to the DAIT/NIAID, as well as to relevant health authorities. Authorized representatives as noted above are bound to maintain the strict confidentiality of medical and research information that may be linked to identified individuals.

17. Protocol Deviations

17.1 Protocol Deviation Definitions

Protocol Deviation – The investigators and site staff will conduct the study in accordance to the protocol; no deviations from the protocol are permitted. Any change, divergence, or departure from the study design or procedures constitutes a protocol deviation. As a result of any deviation, corrective actions will be developed by the site and implemented promptly.

Major Protocol Deviation (Protocol Violation) - A Protocol Violation is a deviation from the IRB approved protocol that may affect the subject's rights, safety, or wellbeing and/or the completeness, accuracy and reliability of the study data. In addition, protocol violations include willful or knowing breaches of human subject protection regulations, or policies, any action that is inconsistent with the NIH Human Research Protection Program's research, medical, and ethical principles, and a serious or continuing noncompliance with federal, state, local or institutional human subject protection regulations, policies, or procedures.

Non-Major Protocol Deviation - A non-major protocol deviation is any change, divergence, or departure from the study design or procedures of a research protocol that does not have a major impact on the subject's rights, safety or well-being, or the completeness, accuracy and reliability of the study data.

17.2 Reporting and Managing Protocol Deviations

The study site principal investigator has the responsibility to identify, document and report protocol deviations as directed by the study Sponsor. However, protocol deviations may also be identified during site monitoring visits or during other forms of study conduct review.

Deviations that impact the ability of the Sponsor and PI to assess the study outcomes will be collected for this study. When a deviation is identified, the site will record the deviation on the eCRF. In addition, the site will be responsible to report deviations to the applicable IRB, according to local guidelines.

The SACCC will compile monthly listings of deviations. NIAID, the study PI and DSMB will review deviations on a regular basis. Protocol deviations will also be included in the annual report to FDA.

18. Ethical Considerations and Compliance with Good Clinical Practice

18.1 Statement of Compliance

This clinical study will be conducted using good clinical practice (GCP), as delineated in *Guidance for Industry: E6 Good Clinical Practice Consolidated Guidance*, and according to the criteria specified in this study protocol. Before study initiation, the protocol and the informed consent documents will be reviewed and approved by each site's IRB, NIAID, and FDA. Any amendments to the protocol or to the consent materials will also be approved by the same entities before they are implemented.

18.2 Informed Consent Process

The consent process will provide information about the study to a prospective participant and will allow adequate time for review and discussion prior to his/her decision. Donor and recipient consents for buccal swab/HLA typing will be mailed. Once the LT recipient is determined to be eligible for the study, the principal investigator or designee listed on the FDA 1572 will review the consent and answer questions with both the donor and recipient prior to further study procedures. A physician listed on the 1572 must participate in the consent process. The prospective participant will be told that being in the trial is voluntary and that he or she may withdraw from the study at any time, for any reason. All participants will read, sign, and date a consent form before undergoing any study procedures. Consent materials will be presented in participants' primary language.

The consent process will be ongoing; the consent form will be reviewed with each subject again prior to proceeding with complete IS withdrawal. The consent forms will be revised when important new safety information is available, the protocol is amended, and/or new information becomes available that may affect participation in the study.

The consent process must be appropriately documented in the subject's records. A copy of the signed consent forms should be given to each subject.

18.3 Privacy and Confidentiality

A participant's privacy and confidentiality will be respected throughout the study. Each participant will be assigned a unique identification number and these numbers rather than names will be used to collect, store, and report participant information. Site personnel will not transmit documents containing personal health identifiers (PHI) to the study sponsor or their representatives.

19. Publication Policy

The CTOTC publication guidelines and policies will apply to this trial.

20. References

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Appendix 1. Living Donor Assessments

<table>
<tr><th></th><th>Screening</th><th>PBMC Collection</th></tr>
<tr><td>Visit Number</td><td>D1</td><td>D2</td></tr>
<tr><td>Visit Window</td><td></td><td>After Recipient Screen 1*</td></tr>
<tr><td colspan="3">Study Assessments</td></tr>
<tr><td>Donor Consent</td><td>x</td><td></td></tr>
<tr><td>Demographics (Age at Donation, Gender, Ethnicity)</td><td></td><td>x</td></tr>
<tr><td>Medical and Social History (Conduct prior to scheduling PBMC Collection)[1]</td><td></td><td>x</td></tr>
<tr><td>Physical Examination</td><td></td><td>x</td></tr>
<tr><td colspan="3">UCSF and NW Donor Screening Tests[2]</td></tr>
<tr><td>Donor HLA Typing (Buccal Swab)[3]</td><td colspan="2">x</td></tr>
<tr><td>HLA Typing Class I and II</td><td colspan="2">x[3]</td></tr>
<tr><td>CMV IgG[4]</td><td colspan="2">x</td></tr>
<tr><td>EBV IgG[4]</td><td colspan="2">x</td></tr>
<tr><td>Creative Testing Solutions Profile H without ABO/Rh (HBsAg, HBc, HCV, HIV/HCV/HBV NAT IDS, WNV NAT IDS, HIV 1/2, HTLV 1/2, Syphilis Screen MHA-TP, CMV, T. cruzi) (2 x 6 ml EDTA Lavender Top and 1 x 6 ml Serum Red Top)</td><td></td><td>x</td></tr>
<tr><td colspan="3">Mayo Donor Screening Tests[2]</td></tr>
<tr><td>Donor HLA Typing (Buccal Swab)[2]</td><td></td><td>x</td></tr>
<tr><td>HLA Typing Class I and II</td><td></td><td>x[3]</td></tr>
<tr><td>CMV IgG[4]</td><td></td><td>x</td></tr>
<tr><td>EBV IgG[4]</td><td></td><td>x</td></tr>
<tr><td>Mayo Test ID CTBMT (Secondary ID 89562) (HBsAg, HBc, HCV, HIV/HCV/HBV NAT IDS, WNV NAT IDS, HIV 1/2, HTLV 1/2, Syphilis Screen MHA-TP, CMV, T. cruzi)</td><td></td><td>x</td></tr>
<tr><td colspan="3">PBMC Collection for darTregs Manufacture</td></tr>
<tr><td>PBMC Collection for darTreg Manufacturing (70ml in Green Heparin Tubes)</td><td></td><td>x</td></tr>
<tr><td colspan="3">Central Laboratory Assessment</td></tr>
<tr><td>Donor Reactive T Cell Frequency & In Vitro Suppression (30 ml in Green Heparin Tubes)</td><td></td><td>x</td></tr>
</table>

* Donor blood collection can be drawn after recipient is deemed eligible for withdrawal, preferably after Step 1. Donor blood draw can be earlier but not less than 18 days prior to planned recipient PBMC collection.

[1] Donor medical and social history questionnaire can be conducted remotely and repeated, as needed, on the day of PBMC Collection.

[2] UCSF will send all donor testing to CTS. Mayo will have donor testing performed locally. Both sites will send results to manufacturing facility and enter results in study database. Please order specific tests/ item numbers for FDA approved tests.

[3] HLA typing must be repeated if testing performed previously did not include DR, DP and DQ.

[4] CMV IgG and EBV IgG should be obtained only if donor tested negative at the time of donation. EBV IgG should be obtained if not previously available.

Appendix 2. Study Entry and IS Withdrawal (High Frequency)

This schedule of events should be used at the time of screening for study entry and initial IS withdrawal. IS withdrawal must begin no later than 8 weeks after date of screening biopsy. The duration on this SOE will vary by subject and depend on pauses during IS withdrawal. However, the maximum time allowed on this SOE is 18 weeks. *At step 2 of IS withdrawal, subjects should be screened for darTregs eligibility (Appendix 4, darTreg SOE)*. Subjects who do not meet eligibility for darTregs should switch to Appendix 6, Medium Frequency Follow Up.

	Study/ISW Eligibility[1]	Start ISW	Week 2	Week 4	Week 6	Week 8	Week[5] 10	PBMC	Week[5] 12	Week[5] 14	*Possible visits if ISW was paused* Week 16	Week 18	Clinically Indicated Biopsy
Visit Number	Screen 1	ISW 1	1	2	3	4	5	PBMC	6	7	8	9	CIB
Visit Window		Start ISW	± 3 days					17 or 18 days prior to infusion	± 14 days	± 3 days			Replaces closest study visit
Immunosuppression Withdrawal Algorithm													
	← Any single pause during Step 1 or 2 must be less than 4 weeks. The maximum time allowed on this SOE is18 weeks[5] →												
IS Withdrawal		x	*Step 1 (6 weeks)*			*Step 2 (6-8 weeks)*							
Study Assessments													
Study/ IS Withdrawal Eligibility	x												
Informed Consent	x												
Telephone Consultation		x[2]		x		x					x		
Transplant Center Visit		x[2]						x					x
Physical Examination/ Vital Signs	x							x					
Review/Collect Concomitant Medications	→	→	→	→	→	→	→	→	→	→	→	→	→
Adverse Event/Serious Adverse Event Assessment	→	→	→	→	→	→	→	→	→	→	→	→	→
HBV (HBsAg, HBcAb), HCV (HCVAb) serology (retrospective data from pre-transplant work up)	x												
Local Laboratory Assessments													
Recipient HLA Typing (Buccal Swab)	x												
Pregnancy Test	x												
CMV, EBV IgG[3]	x[3]												
HBV by PCR (for subjects with HBV)	x							x					
HCV RNA (for subjects with history of HCV)	x												
HIV 1 & 2 serology	x												
Hemoglobin A1C	x												
Basic Chemistry (Glucose, Na, K, Cl, CO2, Creatinine, BUN)	x								x				x
Liver Tests (ALT, Alk Phos, GGT, T Bilirubin, D Bilirubin)	x		x	x	x	x	x		x	x	x	x	x
CBC (with differential and platelets)	x							x[4]					x
CD4 Count													x
Tacrolimus Level	x												
AFP[8]	x							x					
Chest CT[8]	x												
Abdominal CT or MRI[8]	x												
Local Pathology Results - Graft Routine Histology													x
PBMC Collection for darTregs Manufacture													
Leukapheresis or 450 ml Blood Collection for PBMC Isolation/ Treg Manufacturing								x[5]					
Central Laboratory Assessments													
Protocol Biopsy	x[7]												x[7]
Treg TruCount (2ml Green Heparin Tube)	x												
MFC Panels -Leukocyte, Treg, Tact, Texh (2 x 10ml Green Heparin Tube)	x												x
Donor-reactive Tcell Frequency/In Vitro Suppression (10ml Green Heparin Tube)	x												x
Alloantibodies (3ml Red Top Tube)	x												x
PBMCs for Banking (4 x 10ml Green Heparin Tube)	x												
PBMCs for Banking (2 x 10ml Green Heparin Tube)								x[6]					x
Serum for Banking (7ml Red Top Tube)	x							x[6]					x
mRNA for Banking (2 x 2.5 Paxgene Tube)	x							x[6]					x

[1] Laboratory tests for Screen 1 should be dated within 8 weeks prior to start of IS withdrawal.
[2] Transplant center visit not required to start ISW.
[3] CMV and EBV IgG testing should be done only if recipient CMV and EBV IgG was negative at the time of transplant. EBV IgG should be obtained if not previously available. Donor EBV and CMV IgG must be also be negative for EBV and CMV negative
[4] CBC with differential can be obtained once between week 10-12. This test can be performed locally prior to transplant center visit.
[5] Leukapheresis or blood collection for Treg manufacturing should occur 17 (UCSF) to 18 (Mayo or NU) days prior to planned date of infusion, at the transplant center visit. HBV by PCR should be obtained on the day of PBMC collection for darTregs manufacture. Specimens for this visit will likely be collected at the week 10 or week 12 visit.This is also the time of Treg eligibility screening (Appendix 4). Subjects should continue with liver tests every 2 weeks until darTreg infusion. If logistical pause is used, please complete visits 8 and 9 on this SOE and move to Appendix 3 (Logistical Pause SOE). Both a safety pause and logistical pause can be used in Step 2. However, each pause cannot exceed the specified weeks.
[6] Specimens for banking should be collected at the time of PBMC collection OR just prior to darTreg infusion, not both.
[7] For Screen 1 biopsy and clinically indicated biopsies prior to darTreg, biopsy specimen should be prioritized: 1) 2.0cm formalin, 2) 1.0-1.5cm PBS for flow/deuterium tracking, and 3) 0.5-1.0cm RNALater.
[8] AFP, CT, MRI is only required for subjects known to have HCC. At Screen A, AFP, Chest CT and abdominal CT or MRI should be done if unavailable within 3 preceding months. After enrollment AFP should be collected every transplant center visit.

Appendix 3. Logistical Pause in Step 2 SOE

This schedule of events should be used if a logistical pause is being implemented during Step 2 of IS withdrawal. Subjects should have liver tests every two weeks for 12 weeks. Subjects must continue with liver tests every 2 weeks for 12 weeks, then every 4 weeks until darTregs infusion or study termination. Subjects who do not receive darTregs should follow this schedule for at least 26 weeks of follow up after last IS dose change. The duration on this SOE will vary by subject and depend on the length of the logistical pause. However, the maximum time allowed on this SOE is 26 weeks. *During this logistical pause, subjects will have PBMC's collected again for manufacturing and should be screened for darTregs eligibility (Appendix 4, darTreg SOE) around 2 weeks prior to anticipated infusion*. If a subject fails IS withdrawal at any time, the medium frequency schedule should be used (Appendix 7) until 52 weeks after date of rejection.

	Week 20	Week 22	Week 24	Week 28	Week 32	Week 36	PBMC[1] 2	Week 40	Week 44	End of Study	Clinically Indicated Biopsy
Visit Number	10	11	12	13	14	15	PBMC 2	16	17	E	CIB
Visit Window	± 3 days						17 or 18 days prior to infusion	± 5 days			Replaces closest study visit
Immunosuppression Withdrawal Algorithm											
	← Logistical pause during Step 2 must be less than 26 weeks (+ 7 day window). →										
IS Withdrawal	*Step 2*										
Study Assessments											
Telephone Consultation	x		x		x			x			
Review/Collect Concomitant Medications	→	→	→	→	→	→	→	→	→	→	→
Adverse Event/Serious Adverse Event Assessment	→	→	→	→	→	→	→	→	→	→	→
Local Laboratory Assessments											
HBV by PCR (for subjects with HBV)							x			x	
HCV RNA (for subjects with history of HCV)										x	
Basic Chemistry (Glucose, Na, K, Cl, CO2, Creatinine, BUN)							x			x	x
Liver Tests (ALT, Alk Phos, GGT, T Bilirubin, D Bilirubin)	x	x	x	x	x	x		x	x	x	x
CBC (with differential and platelets)										x	x
AFP[2]										x	
Chest CT[2]										x	
Pelvic CT or MRI[2]										x	
Local Pathology Results - Graft Routine Histology										x	x
PBMC Collection for darTregs Manufacture											
Leukapheresis or 450 ml Blood Collection for PBMC Isolation/ Treg Manufacturing							x[1]				
Central Laboratory Assessments											
Protocol Biopsy											x[3]
MFC Panels -Leukocyte, Treg, Tact, Texh (2 x 10ml Green Heparin Tube)											x
Donor-reactive Tcell Frequency/In Vitro Suppression (10ml Green Heparin Tube)											x
Alloantibodies (3ml Red Top Tube)											x
PBMCs for Banking (2 x 10ml Green Heparin Tube)											x
Serum for Banking (7ml Red Top Tube)											x
mRNA for Banking (2 x 2.5 Paxgene Tube)											x

[1]Leukapheresis or blood collection for Treg manufacturing should occur 17 (UCSF) to 18 (Mayo or NU) days prior to planned date of infusion, at the transplant center visit. Specimens for this visit will be collected after scheduling infusion date with UCSF HICTF; this is not a fixed time point in the SOE. This is also the time of Treg eligibility screening (Appendix 4).

[2]AFP, CT, MRI is only required for subjects known to have HCC.

[3]For Screen 1 biopsy and clinically indicated biopsies prior to darTreg, biopsy specimen should be prioritized: 1) 2.0cm formalin, 2) 1.0-1.5cm PBS for flow/deuterium tracking, and 3) 0.5-1.0cm RNALater.

Appendix 4. darTregs Infusion SOE

Screening (Screen 2) for Treg infusion should start at Step 2 of initial ISW. Tregs must be infused during the last 2 weeks of Step 2. ISW must resume within 14 days after darTreg infusion. Once ISW is resumed, Appendix 4, ISW after darTregs SOE should be followed two weeks after date of IS dose reduction (1 week after Day 7 visit).

	Treg Eligibility[1]	**Treg Infusion**	**Day 1**	**Resume ISW[4]**	**Day 7**
Visit Number	**Screen 2**	**T0**	**T1**	**ISW 2**	**T2**
Visit Window	During ISW Step 2	During last 2 weeks of Step 2	± 1 hour	≤ 14 days after Tregs	-1/+3 days
Study Assessments					
Treg Infusion Eligibility Criteria	x				
Physical Examination/ Vital Signs		x			
Review/Collect Concomitant Medications	→	→	→	→	→
Adverse Event/Serious Adverse Event Assessment	→	→	→	→	→
Study Interventions					
400 x 10^6 darTreg Infusion		x			
Resume IS withdrawal (Step 3) no later than day 14 after darTreg infusion				→	
Local Laboratory Assessments					
Pregnancy Test	x				
Basic Chemistry (Glucose, Na, K, Cl, CO2, Creatinine, BUN)	x[2]		x		
Liver Tests (ALT, Alk Phos, GGT, T Bilirubin, D Bilirubin)	x[2]	x	x		x
CBC (with differential and platelets)					x
CD4 Count		x	x		x
Tacrolimus Level					
Local Pathology Results - Graft Routine Histology					x
Central Laboratory Assessments					
Protocol Biopsy					x[5]
MFC Panels -Leukocyte, Treg, Tact, Texh (2x10ml Green Heparin Tube)		x[3]			
Donor-reactive Tcell Frequency/In Vitro Suppression (10ml Green Heparin Tube)		x[3]			
Alloantibodies (3ml Red Top Tube)		x[3]			
Treg PK: Heavy Glucose Labeling (10ml Green Tube)			x		x
PBMCs for Banking (2 x 10ml Green Heparin Tube)		x[3]	x		x
Serum for Banking (7ml Red Top Tube)		x[3]	x		x
mRNA for Banking (2 x 2.5 Paxgene Tube)		x[3]	x		x

[1] Treg Eligibility screening visit does not require a transplant center visit. High frequency visits should continue until darTreg infusion.

[2] Laboratory tests for Screen 2 (Treg eligibility) should be dated within 14 days prior to Treg infusion. Fresh draw is not required.

[3] Blood for central laboratory assessments should be collected prior to darTreg infusion at Visit T0. Specimens for banking should not be repeated at T0 if already collected at time of PBMC collection.

[4] IS withdrawal instructions should be given in person. This can be done at the infusion visit if eligibility is met. IS withdrawal can start as early as one day after Tregs but no later than 14 days after Treg infusion.

[5] For Visit T2/Day 7 after darTreg or clinically indicated biopsy after darTreg, biopsy specimen should be prioritized: 1) 2.0cm PBS for flow/deuterium tracking, 2) 1.0-1.5cm formalin, and 3) 0.5-1.0cm RNALate

Appendix 5. IS Withdrawal after darTregs Infusion to Step 5/75% CNI Reduction (High Frequency)

■ This schedule of events should be used after darTreg infusion, starting 2 weeks after resuming IS withdrawal. There will be a day 7 transplant center visit (shown on the Treg SOE) before starting this SOE. The duration on this SOE will vary by subject and depend on pauses during IS withdrawal. However, the maximum time allowed for withdrawal to Step 5 is 32 weeks, less if pauses were made previously.

■ When CNI has been reduced by 75% (step 5) for 12 weeks, subjects will either 1) continue IS withdrawal on high frequency visits using Appendix 6, or 2) switch to medium frequency (Appendix 8) until 52 weeks after darTregs.

■ If a subject fails IS withdrawal at any time, the medium frequency schedule should be used (Appendix 7) until 52 weeks after date of darTregs infusion or date of rejection.

	Biweekly visits after darTregs												*Possible visits if ISW was paused*				Step 5 End	Clinically Indicated Biopsy
Weeks after Resuming IS Withdrawal (darTreg Infusion)	**W2**	**W4**	**W6**	**W8**	**W10**	**W12[1]**	**W14**	**W16**	**W18**	**W20**	**W22**	**W24[1]**	**W26**	**W28**	**W30**	**W32**		
Visit Number	**T3**	**T4**	**T5**	**T6**	**T7**	**T8**	**T9**	**T10**	**T11**	**T12**	**T13**	**T14**	**T15**	**T16**	**T17**	**T18**	**E1**	**CIB**
Visit Window	± 3 days	± 3 days				± 14 days	± 3 days						± 3 days				± 14 days	Replaces closest study visit
Immunosuppression Withdrawal																		
IS Withdrawal: Maximum time allowed for withdrawal to Step 5 is 32 weeks	*← Any single pause during must be less than 4 weeks, 8 weeks cummulative. →*												*Additional visits if ISW was paused.*					
	Step 3 (6 weeks)			*Step 4 (6 weeks)*			*Step 5 (12 weeks)*											
Study Assessments																		
Telephone Consultation				x				x		x				x		x		
Complete Withdrawal Informed Consent																	x	
Transplant Center Visit		x				x											x	x
Physical Examination/ Vital Signs						x											x	
Review/Collect Concomitant Medications	→	→	→	→	→	→	→	→	→	→	→	→	→	→	→	→	→	→
Adverse Event/Serious Adverse Event Assessment	→	→	→	→	→	→	→	→	→	→	→	→	→	→	→	→	→	→
Local Laboratory Assessments																		
CMV, EBV by PCR		x				x											x	
Basic Chemistry (Glucose, Na, K, Cl, CO2, Creatinine, BUN)						x											x	x
Liver Tests (ALT, Alk Phos, GGT, T Bilirubin, D Bilirubin)	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
CBC (with differential and platelets)						x											x	x
CD4 Count	x	x				x											x	x
AFP						x											x	
Local Pathology Results - Graft Routine Histology																	x	x
Central Laboratory Assessments																		
Protocol Biopsy																		x[2]
MFC Panels -Leukocyte, Treg, Tact, Texh (2x10ml Green Heparin Tube)		x				x											x	x
Donor-reactive Tcell Frequency/In Vitro Suppression (10ml Green Heparin Tube)		x				x											x	x
Alloantibodies (3 ml Red Top Tube)		x				x											x	x
Treg PK: Heavy Glucose Labeling (10ml Green Tube)		x				x											x	x
PBMCs for Banking (2 x 10ml Green Heparin Tube)		x				x											x	x
Serum for Banking (7ml Red Top Tube)		x				x											x	x
mRNA for Banking (2 x 2.5 Paxgene Tube)		x				x											x	x

[1] A study visit should be done in person at the transplant center 12 weeks after resuming IS Withdrawal and at the completion of Step 5. The Step 5 End Visit should take place at week 24 or later (if there were pauses during ISW). If the subject consents to continue withdrawal, instructions for next dose change should be given and Appendix 5 should be followed with assessments every 2 weeks. If the subject is maintaining at 75% IS, medium frequency visits (Appendix 7) should start 4 weeks after W24.

[2] For Visit T2/Day 7 after darTreg or clinically indicated biopsy after darTreg, biopsy specimen should be prioritized: 1) 2.0cm PBS for flow/deuterium tracking, 2) 1.0-1.5cm formalin, and 3) 0.5-1.0cm RNALater.

Appendix 6. Complete Immunosuppression Withdrawal (High Frequency)

■ This schedule of events should be used if subject consents to continue IS withdrawal. The duration on this SOE will vary by subject and depend on pauses during IS withdrawal. However, the maximum time allowed for Steps 6 & 7 is 20 weeks; less if pauses were made previously.

■ If a subject successfully completes withdrawal, a transplant center visit with protocol biopsy should take place 12 weeks after starting Step 7. The subject will continue high frequency visits on this SOE for 12 weeks after the biopsy. If the subject has reached 52 weeks of follow up after receiving darTregs and resuming IS withdrawal, the last study visit will be after 12 weeks of follow up medium frequency schedule is not needed. If the subject has not reached 52 weeks, Appendix 8, Medium Frequency should be followed until 52 weeks after darTreg.

■ If a subject fails IS withdrawal at any time, the medium frequency schedule should be used (Appendix 7) until 52 weeks after rejection.

	Biweekly visits Step 6 & 7						*Possible visits if ISW is paused*				*12 week follow up after IS discontinuation (Step 8)*						End of Study[1]	Clinically Indicated Biopsy
Weeks after Resuming IS Withdrawal (darTreg Infusion)	**W26**	**W28**	**W30**	**W32**	**W34**	**W36**	**W38**	**W40**	**W42**	**W44**	**W2**	**W4**	**W6**	**W8**	**W10**	**W12**		
Visit Number	**T19**	**T20**	**T21**	**T22**	**T23**	**T24**	**T25**	**T26**	**T27**	**T28**	**C1**	**C2**	**C3**	**C4**	**C5**	**C6**	**E2**	**CIB**
Visit Window	± 3 days					± 14 days	± 3 days				± 3 days						± 14 days	Replaces closest study visit
Immunosuppression Withdrawal																		
IS Withdrawal	*←Any single pause during must be less than 4 weeks, 8 weeks cummulative.→*																	
	Step 6 (6 weeks)			*Step 7 (6 weeks)*			*Extension if ISW paused*											
Study Assessments																		
Telephone Consultation		x		x				x		x				x		x		
Transplant Center Visit						x						x					x	
Physical Examination/ Vital Signs						x												x
Review/Collect Concomitant Medications	→	→	→	→	→	→					→	→	→	→	→	→	→	→
Adverse Event/Serious Adverse Event Assessment	→	→	→	→	→	→					→	→	→	→	→	→	→	→
Local Laboratory Assessments																		
CMV, EBV by PCR						x						x					x	
HCV RNA (for subjects with history of HCV)																	x	
Basic Chemistry (Glucose, Na, K, Cl, CO2, Creatinine, BUN)						x						x					x	x
Liver Tests (ALT, Alk Phos, GGT, T Bilirubin, D Bilirubin)	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		x	x
CBC (with differential and platelets)						x						x					x	x
CD4 Count						x						x					x	x
AFP[2]						x						x					x	
Chest CT[2]																	x	
Abdominal CT or MRI[2]																	x	
Local Pathology Results - Graft Routine Histology																		x
Central Laboratory Assessments																		
Protocol Biopsy																	x[3]	x[4]
MFC Panels -Leukocyte, Treg, Tact, Texh (2x10ml Green Heparin Tube)						x						x					x	x
Donor-reactive Tcell Frequency/In Vitro Suppression (10ml Green Heparin Tube)						x						x					x	x
Alloantibodies (3ml Red Top Tube)						x						x					x	x
PBMCs for Banking (2 x 10ml Green Heparin Tube)						x						x					x	x
Serum for Banking (7ml Red Top Tube)						x						x					x	x
mRNA for Banking (2 x 2.5 Paxgene Tube)						x						x					x	x

[1]For some subjects, 52 w eeks of follow up can be completed using this schedule, w ithout moving to medium frequency. These subjects should have the End of Study visit after completing 12 w eeks of high frequency follow up after last dose of IS, even if longer than 52 w eeks

[2]AFP, CT, MRI is only required for subjects know n to have HCC.

[3] Only subjects w ho are completely off IS should have protocol biopsy to assess for tolerance. For tolerance biopsy (off IS after darTreg), biopsy specimen should be prioritized: 1) 2.0cm formalin, 2) 1.0-1.5cm PBS, and 3) 0.5-1.0cm RNALater.

[4] For Visit T2/Day 7 after darTreg or clinically indicated biopsy after darTreg, biopsy specimen should be prioritized: 1) 2.0cm PBS for flow /deuterium tracking, 2) 1.0-1.5cm formalin, and 3) 0.5-1.0cm RNALater.

Appendix 7. Medium Frequency Schedule after Rejection

■ All subjects must have at least 52 weeks of follow up after receiving darTregs and resuming IS withdrawal; this will be a combination of high and medium frequency visits.

■ Subjects failing IS withdrawal will enter into medium frequency follow up 2 weeks after rejection. Note the study visit weeks restart at the time the subject starts medium frequency follow up (e.g. two weeks after completing final 12 weeks of high frequency follow up). Subjects should be followed for 52 weeks total follow up after rejection.

■ Subjects who experience rejection on medium frequency follow up (partial or complete ISW after darTregs) will restart this medium frequency SOE until 52 weeks.

Weeks on Medium Frequency Follow Up	**W2[1]**	**W4**	**W8**	**W12**	**W16**	**W20**	**W24**	**W28**	**W32**	**W36**	**W40**	**W44**	**W48**	**End of Study**	**Clinically Indicated Biopsy**
Visit Number	**R[1]**	**M1R**	**M2R**	**M3R**	**M4R**	**M5R**	**M6R**	**M7R**	**M8R**	**M9R**	**M10R**	**M11R**	**M12R**	**E3**	**CIB**
Visit Window	± 3 days	± 5 days												± 14 days	- 6 weeks
Study Assessments															
Physical Examination/ Vital Signs														x	
Telephone Visit	x	x		x		x				x		x			
Transplant Center Visit								x						x	x
Review/Collect Current Immunosuppressive and Anti-Infective Medications	→	→	→	→	→	→	→	→	→	→	→	→	→	→	→
Review/Collect Concomitant Medications	→	→	→	→	→	→	→	→	→	→	→	→	→	→	→
Adverse Event/Serious Adverse Event Assessment	→	→	→	→	→	→	→	→	→	→	→	→	→	→	→
Local Laboratory Assessments															
CMV, EBV by PCR								x						x	
HCV RNA (for subjects with history of HCV)														x	
Basic Chemistry (Glucose, Na, K, Cl, CO2, Creatinine, BUN)								x						x	x
Liver Tests (ALT, Alk Phos, GGT, T Bilirubin, D Bilirubin)	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
CBC (with differential and platelets)								x						x	x
C4D Count								x						x	x
AFP[2]								x						x	
Chest CT[2]														x	
Pelvic CT or MRI[2]														x	
Local Pathology Results - Graft Routine Histology															x
Central Laboratory Assessments															
Protocol Biopsy															x[3]
MFC Panels -Leukocyte, Treg, Tact, Texh (2x10ml Green Heparin Tube)								x						x	x
Donor-reactive Tcell Frequency/In Vitro Suppression (10ml Green Heparin Tube)								x						x	x
Alloantibodies (3ml Red Top Tube)								x						x	x
Treg PK: Heavy Glucose Labeling (10ml Green Tube)								x						x	x
PBMCs for Banking (2 x 10ml Green Heparin Tube)								x						x	x
Serum for Banking (7ml Red Top Tube)								x						x	x
mRNA for Banking (2 x 2.5 Paxgene Tube)								x						x	x

[1]Subjects with rejection should have a follow up visit 2 weeks after CIB, then continue with medium frequency schedule.

[2]AFP, CT, MRI is only required for subjects known to have HCC.

[3]For Visit T2/Day 7 after darTreg or clinically indicated biopsy after darTreg, biopsy specimen should be prioritized: 1) 2.0cm PBS for flow/deuterium tracking, 2) 1.0-1.5cm formalin, and 3) 0.5-1.0cm RNALater.

Appendix 8. Medium Frequency Schedule after Partial or Complete IS Withdrawal

■ All subjects must have at least 52 weeks of follow up after receiving darTregs and resuming IS withdrawal; this might be accomplished without using the medium frequency schedule or will be a combination of high and medium frequency visits.

■ Subjects successful with partial or complete withdrawal should use this SOE after completing 12 weeks of high frequency follow up (Appendix 5 or 6). The duration on this SOE will vary by subject and depend on pauses during IS withdrawal.

■ Subjects who do not receive darTregs after starting IS withdrawal will have at least 26 weeks of follow up after last IS dose change. 52 weeks of follow up is required if rejection occurs.

Weeks on Medium Frequency Follow Up	**W4**	**W8**	**W12**	**W16**	**W20**	**W24**	**W28**	**End of Study[1]**	**Clinically Indicated Biopsy**
Weeks Continuing at Reduced CNI (from Appendix 4)	**W28**	**W32**	**W36**	**W40**	**W44**	**W48**	**W52**		
Weeks after Resuming IS Withdrawal (darTreg Infusion)	**W52**	**W56**	**W60**	**W64**	**W68**	**W72**	**W76**		
Visit Number for Follow Up (Partial ISW: a, Completed ISW: b)	**M1**	**M2**	**M3**	**M4**	**M5**	**M6**	**M7**	**E4**	**CIB**
Visit Window	± 5 days							± 14 days	- 6 weeks
Study Assessments									
Physical Examination/ Vital Signs									
Telephone Visit		x		x		x			
Transplant Center Visit								x	x
Review/Collect Current Immunosuppressive and Anti-Infective Medications	→	→	→	→	→	→	→	→	→
Review/Collect Concomitant Medications	→	→	→	→	→	→	→	→	→
Adverse Event/Serious Adverse Event Assessment	→	→	→	→	→	→	→	→	→
Local Laboratory Assessments									
CMV, EBV by PCR								x	
HCV RNA (for subjects with history of HCV)								x	
Basic Chemistry (Glucose, Na, K, Cl, CO2, Creatinine, BUN)								x	x
Liver Tests (ALT, Alk Phos, GGT, T Bilirubin, D Bilirubin)	x	x	x	x	x	x	x	x	x
CBC (with differential and platelets)								x	x
C4D Count								x	x
AFP[2]								x	
Chest CT[2]								x	
Pelvic CT or MRI[2]								x	
Local Pathology Results - Graft Routine Histology									x
Central Laboratory Assessments									
Protocol Biopsy								x[3]	x[4]
MFC Panels -Leukocyte, Treg, Tact, Texh (2x10ml Green Heparin Tube)								x	x
Donor-reactive Tcell Frequency/In Vitro Suppression (10ml Green Heparin Tube)								x	x
Alloantibodies (3ml Red Top Tube)								x	x
Treg PK: Heavy Glucose Labeling (10ml Green Tube)								x	x
PBMCs for Banking (2 x 10ml Green Heparin Tube)								x	x
Serum for Banking (7ml Red Top Tube)								x	x
mRNA for Banking (2 x 2.5 Paxgene Tube)								x	x

[1]The last study visit should take place when 52 weeks of follow up (after darTregs and resuming IS withdrawal) have been completed. The timing will vary by subject.

[2]AFP, CT, MRI is only required for subjects known to have HCC.

[3]Only subjects who are completely off IS should have protocol biopsy to assess for tolerance. For tolerance biopsy (off IS after darTreg), biopsy specimen should be prioritized: 1) 2.0cm formalin, 2) 1.0-1.5cm PBS, and 3) 0.5-1.0cm RNALater.

[4]For Visit T2/Day 7 after darTreg or clinically indicated biopsy after darTreg, biopsy specimen should be prioritized: 1) 2.0cm PBS for flow/deuterium tracking, 2)