Donor-Alloantigen-Reactive Regulatory T Cell Therapy in Liver Transplantation

Version 7.0/ August 1, 2018

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Title: *Donor-Alloantigen-Reactive Regulatory T Cell Therapy in Liver Transplantation*	
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. 3900 Paramount Parkway Morrisville, NC 27560 Phone: (919) 456-4229	
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 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	Donor-Alloantigen-Reactive Regulatory T Cell (darTreg) Therapy in Liver Transplantation
Short Title	darTregs in Liver Transplantation (deLTa)
Protocol Number	RTB-002
ClinicalTrials.gov Identifier	NCT02188719
Clinical Phase	Phase I
Safety Objective	This study will evaluate the safety, tolerability, and dose limiting toxicities (DLTs) of a Treg-supportive Immunosuppression (IS) regimen and darTreg infusion for adult, de novo, liver transplant recipients.
Mechanistic Objectives	This study will evaluate 1) the effects of a Treg-supportive IS regimen and Treg infusion on immunological profiles of liver transplant recipients, and 2) explore the hypothesis that ex vivo-expanded darTregs will be biologically active.
Study Design	A multi-center, open-label, dose escalation, pilot trial with four cohorts (Treg-supportive IS only group + three darTreg dosing groups) conducted in a phased schema.
Safety Outcome	The safety, tolerability, and dose-limiting toxicities (DLTs) of darTreg therapy given within the context of a Treg-supportive IS regimen will be evaluated with the rate of the following events within 40 weeks of transplantation. Clinical outcomes that will be described are: 1. Incidence and severity of biopsy proven acute rejection (AR) and/or chronic rejection (CR) 2. Incidence of ≥ Grade 3 infections as defined in Section 14.3.1 3. Incidence of wound complications (≥ CTCAE Version 4.0, Grade 3) 4. Incidence of anemia, neutropenia, and/or thrombocytopenia (according to study defined reporting requirements, Section 14.3.1 Grading Criteria) 5. Incidence of adverse events (AEs) attributable to the darTreg infusion including infusion reaction / cytokine release syndrome (CRS) (≥ CTCAE Grade 3), and malignant cellular transformation.
Mechanistic Outcomes	1. Treg frequency in peripheral blood using Treg TruCount assay 2. Persistence of infused darTregs in blood and biopsies using deuterium labeling and T cell repertoire analysis 3. darTreg and darTconv frequency in peripheral blood using an in vitro allofrequency assay 4. Multicolor flow cytometry (MFC) to assess effect on immune profiles 5. Functional assessment of Treg in peripheral blood using a mixed lymphocyte reaction (MLR) suppression assay 6. Histology and immunophenotype of liver biopsies
Accrual Objective	18 - 24 subjects: • Cohort 1: 3 subjects from Center 1 (Cohort 1a) + 3 subjects from Center 2 (Cohort 1b) • Cohort 2: At least 3, and up to 6 subjects (Dose A) • Cohort 3: At least 3, and up to 6 subjects from either Center (Dose B) • Cohort 4: 6 subjects from either Center (Dose C).
Study Duration	4 year accrual period, 40 weeks of follow-up after transplantation

Treatment Description	<u>Treg-supportive IS regimen:</u> • Rabbit anti-thymocyte globulin (Thymoglobulin®) induction (3-4.5 mg/kg started by day 3) • Corticosteroids (500 mg intra-operatively, taper to off by week 5) • Mycophenolate mofetil (1000 mg total daily dose) • TAC, with target trough level of 6-8 µg/dL Between 30 and 44 days following transplant: Subjects who are not eliminated by Exclusion Criteria C1 (Section 4.3.3) will proceed in the study and receive either TAC-based or EVR-based IS, based on eligibility Criteria C2 (Section 4.3.4) • TAC-based IS: reduce TAC trough level to 3-8 µg/dL; continue MMF • EVR-based IS: reduce TAC trough level to 3-5 µg/dL; EVR target trough level of 6-8 µg/dL; decrease then discontinue MMF <u>Escalating darTregs therapy:</u> Eligible and consented patients in Cohorts 2, 3, or 4 will have peripheral blood mononuclear cells (PBMCs) collected, which will be cryopreserved for subsequent induction, purification, and expansion of darTregs. At the time of transplant and after verification of the participant's ongoing eligibility, donor cells will be collected and banked for production of stimulated donor B cells. Donor B cells will be used to generate darTregs for infusion at 11-20 weeks after transplant. Cohort 1 – Treg-supportive IS only Cohort 2 – darTreg infusion, 50 million (range 25 to 60 million) Cohort 3 – darTreg infusion, 200 million (range 100-240 million) Cohort 4 – darTreg infusion, 800 million (range 400-960 million)
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. End-stage liver disease and listed for deceased-donor primary solitary liver transplant 3. Between 21 and 70 years of age at the time of study entry/consent 4. Have a calculated MELD score ≤ 25 at the time of study entry/consent 5. Female and male subjects with reproductive potential must agree to use effective methods of birth control for the duration of the study 6. If history of HCV, have completed or in current treatment for HCV AND have no detectable HCV RNA 7. Subjects with HCC meeting Milan criteria

Exclusion Criteria	**Exclusion criteria A** (assessed at study enrollment, prior to study procedures). Subjects with any of the following are ineligible for study participation: 1. End stage liver disease secondary to autoimmune etiology (autoimmune hepatitis, primary biliary cirrhosis, or primary sclerosing cholangitis) 2. History of less than 5 years remission of malignancy, except for 1) HCC or 2) history of adequately treated in-situ cervical carcinoma, or adequately treated basal or squamous cell carcinoma of the skin 3. History of previous organ, tissue or cell transplant 4. Serologic evidence of human immunodeficiency 1 or 2 infection 5. Epstein Barr Virus (EBV) sero-negativity (EBV naïve candidates) 6. Cytomegalovirus (CMV) sero-negativity (CMV naïve candidates) if donor is positive 7. Chronic use of systemic glucocorticoids or other IS, or biologic immunomodulators 8. Chronic condition requiring anti-coagulation after liver transplantation 9. Any chronic illness or prior treatment which, in the opinion of the investigator, precludes study participation 10. Participation in any other studies that involved investigational drugs or regimens in the preceding year 11. Received any vaccination within 28 days prior to leukapheresis or blood collection for Treg manufacture 12. Hemoglobin <9.0 g/dL within 10 days prior to screening 13. Neutrophils <1,500/µL within 10 days prior to screening 14. Platelets <40,000/µL within 10 days prior to screening
Exclusion Criteria (continued)	**Exclusion criteria B** (assessed prior to administration of Thymoglobulin®)**:** 1. Calculated MELD score greater than 25 at the time of deceased donor liver transplant 2. Last AFP obtained prior to liver transplantation >400 µg/L for candidates with hepatocellular carcinoma (HCC) 3. Unacceptable PBMC product for participants enrolled in Cohorts 2, 3, or 4, per UCSF HICTF manufacturing specifications 4. Absence of donor cells for manufacturing for any participant 5. HLA-DR matched to donor at both loci 6. Subject is <21 or > 70 years of age at the time of transplantation 7. Located in the intensive care unit (ICU) 72 hours after transplantation 8. Hemoglobin <8.0 g/dL 9. Absolute neutrophil count <1,200/µL 10. Platelets <40,000/µL 11. Positive pregnancy test for females of child bearing potential 12. Unexpected histopathology on back table liver biopsy that contraindicates the initiation of Treg-supportive IS regimen. 13. Development of a condition requiring chronic anti-coagulation 14. Hypersensitivity to rabbit proteins or any excipient in Thymoglobulin® 15. Detectable HCV RNA (if history of HCV (e.g. HCV serology positive at screen A)) or less than six months after end of treatment for HCV at the time of transplantation (i.e., does not meet criteria for SVR)

Exclusion Criteria (continued)	**Exclusion criteria C1** (assessed at day 30-44 after transplantation for continuation in the trial) All subjects regardless of eligibility for EVR conversion, with any of the following will not receive darTregs and will move into safety follow up. 1. Explanted liver with evidence of increased risk of recurrent cancer risk (HCC tumor burden exceeding the Milan criteria; presence of vascular invasion; cholangiocarcinoma morphology 2. Insufficient depletion of recipient T cells, defined as a nadir CD3 count ≥50 cells/µL (50 cells/mcL) or total lymphocyte count ≥ 0.10 x 10^9/L if CD3 count is unavailable 3. Development of a condition requiring chronic anti-coagulation 4. Clinical evidence of untreated biliary obstruction 5. ALT >2.0 x upper limit of normal (ULN) 6. Inability to taper off corticosteroids by 44 days (+/- 2 days) after transplant 7. Detectable circulating HCV RNA at any time since study entry **Everolimus Conversion criteria C2** (assessed prior to conversion to EVR-based IS regimen; EVR cannot be initiated prior to 30 days after liver transplantation). Subjects with any of the following will remain on TAC-based IS regimen. 1. Evidence of hepatic artery stenosis or thrombosis by Doppler examination or angiography within 7 days prior to conversion 2. Urine protein/creatinine ratio >1.0 mg/mg within 7 days prior to conversion 3. Calculated GFR less than 30 ml/min per MDRD4 equation within 7 days prior to conversion 4. Physical examination documentation of abnormal wound healing or uncontrolled wound infection 5. Hemoglobin <8.0 g/dL within 7 days prior to conversion 6. Absolute neutrophil count <1,200/µL within 7 days prior to conversion 7. Platelets <50,000/µL within 7 days prior to conversion
Exclusion Criteria (continued)	**Exclusion criteria D** (assessed prior to darTreg-infusion): 1. Inability or unwillingness of participant to give written informed consent 2. Unacceptable darTreg product. 3. Detectable circulating EBV or CMV DNA within 10 days prior to darTreg infusion 4. Detectable circulating HBV DNA within 10 days prior to darTreg infusion 5. Detectable circulating HCV RNA within 10 days prior to darTreg infusion 6. ALT >1.5x ULN within 10 days prior to darTreg infusion 7. Most recent, but not greater than 10 days prior to darTreg infusion, 12 hour TAC trough levels of > 8 µg/L for all subjects 8. Most recent, but not greater than 10 days prior to darTreg infusion, 12 hour EVR trough levels of < 5 µg/L for subjects on EVR 9. For subjects on EVR-based IS, received MMF within 10 days prior to Treg infusion 10. Evidence of AR or CR according to Banff criteria on allograft biopsy on local assessment 11. Received any vaccination within 14 days prior to darTreg infusion 12. Positive pregnancy test for females of child bearing potential 13. Inability or unwillingness to comply with study protocol or procedures 14. Calculated eGFR less than 40 ml/min per MDRD4 equation within 10 days prior to infusion.

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Table of Contents

Glossary of Abbreviations

AE	Adverse Event
ALT	Alanine Aminotransferase
AR	Acute Rejection
CFR	Code of Federal Regulations
CI	Confidence Interval
CMV	Cytomegalovirus
CNI	Calcineurin Inhibitor
CR	Chronic Rejection
CRF	Case Report Form
CRS	Cytokine Release Syndrome
CTCAE	Common Terminology Criteria for Adverse Events
DAIT	Division of Allergy, Immunology, and Transplantation
dar	Donor Alloantigen Reactive
darTregs	Donor-Alloantigen-Reactive T Regulatory Cells
DSMB	Data Safety Monitoring Board
EBV	Epstein Barr Virus
EVR	Everolimus
FACS	Fluorescence Activated Cell Sorting
FDA	Food and Drug Administration
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
MELD	Model for End Stage Liver Disease
MFC	Multiparameter Flow Cytometry
MIHC	Multiplex Immunohistochemistry

MLR	Mixed Lymphocyte Reaction
MMF	Mycophenolate Mofetil
mSAP	Mechanistic Statistical Analysis Plan
mToR	Mammalian Target of Rapamycin
NIAID	National Institute of Allergy and Infectious Disease
PI	Principal Investigator
polyTregs	Polyclonally expanded Tregs
PTLD	Post-Transplant Lymphoproliferative Disorder
PBMC	Peripheral Blood Mononuclear Cell
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 Response (HCV aviremia: 24 weeks after completion of antiviral therapy)
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

<table>
<tr><td>Acute Rejection (AR)

(1997 Banff Criteria)
(Demetris A. J., 1997)</td><td>AR occurs as defined by Banff histopathologic criteria.</td></tr>
<tr><td>Clinical Rejection</td><td>Allograft dysfunction or elevated LTs where a biopsy is either not performed or is inconclusive for AR that results in an increase in IS dosing or reinstitution of IS</td></tr>
<tr><td>Chronic Rejection (CR)

(2000 Banff Criteria)
(Demetris A. D., 2000)</td><td>Early and late CR will be defined by the 2000 Banff update on histopathologic staging and reporting of CR. Features of early and late chronic rejection 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>• Luminal 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>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</td><td>NIAID official who is responsible for the safety aspects of this trial. The MM is responsible for making the final causal relationship assessment of each serious adverse eventAE.</td></tr>
<tr><td>Milan Criteria</td><td>The Milan criteria state that a patient with HCC is selected for transplantation when he or she has:
• one lesion smaller than 5 cm.
• up to 3 lesions smaller than 3 cm.
• no extrahepatic manifestations
• no vascular invasion</td></tr>
</table>

NIAID Project Manager	NIAID assigned project manager who is responsible for all day to day protocol related issues, including version control, consent review, etc.
Principal Investigator	Investigator awarded NIH funding for the grant.
Program Officer	NIAID official who oversees the scientific and budgetary aspects of the grant.
Protocol Mandated Procedures	Any procedure performed solely for the purpose of this research study (not site-specific standard of care).
Regulatory Affairs Officer	NIAID assigned officer responsible for regulatory aspects of study, including IND submissions and other communications with FDA, as applicable.
Rejection Activity Index	A numeric score that is part of the 1997 Banff schema for liver rejection. The RAI is based on adding 3 scores related to portal inflammation, bile duct inflammation, and venous endothelial inflammation.
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.
Severe Rejection (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 that 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 AR, but these occur in association with none or mild portal 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".
Steroid Refractory Rejection	Rejection that has failed to resolve with corticosteroid treatment, necessitating treatment with an antibody preparation such as Thymoglobulin®.
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 (darTregs and Thymoglobulin®) and all protocol required medications.
Withdrawal from Therapy	Subject who stops study therapy prior to protocol described duration.

1. Background and Rationale

1.1 Background and Scientific Rationale

Although ongoing refinement of immunosuppression (IS) regimens has substantially reduced the incidence of AR after solid organ transplant, long-term outcomes have stagnated partly due to morbidity and mortality associated with generalized, lifelong IS. The traditional approach to IS has emphasized non-specific suppression of T cell responses. The more recent elucidation of regulatory T cells (Tregs) and their importance in regulating immune responses has encouraged the reconfiguration of IS regimens to favor Treg development and function with the ultimate goal of inducing graft tolerance (Waldmann, 2008) (Kang, 2007) (Walsh, 2004) (Yeung, 2009) (Sanchez-Fueyo A. S., 2006) (Sagoo P. G., 2008) (Long, 2009). Multiple animal models have shown that adoptive transfer of Tregs can mitigate graft rejection and, in combination with "Treg-supportive" IS regimens, can induce long-term tolerance (Kang, 2007) (Riley, 2009) (Issa, 2010) (Nadig, 2010). Treg-supportive IS regimens have included the initial de-bulking of donor-specific T cells. Thymoglobulin®, a commonly used T-cell depleting agent in transplantation, appears to relatively spare Tregs (Sewgobind, 2009), thereby increasing Treg: T conventional cell (Tconv) ratio. Additionally, mammalian Target of Rapamycin (mTOR) inhibitors, a class of drug to which everolimus (EVR) belongs, suppress effector T-cells while fostering Treg development (Demirkiran A. T., 2008) (Demirkiran A. V., 2009). We aim to translate these basic and clinical findings into a viable clinical protocol. We propose to test the use of donor alloantigen-reactive Tregs (darTregs) in the context of T cell depletion, followed by either an EVR-based or TAC-based maintenance IS regimen as an approach to induce liver transplant tolerance. For several reasons, the liver transplant setting is ideal to evaluate the safety of Treg therapy as a strategy to either increase the likelihood of and/or accelerate the development of tolerance. First, liver allografts appear to be more tolerogenic than other allografts. Compared to recipients of other organs, liver transplant recipients require less IS and are relatively spared from both humoral and CR. Second, AR, albeit common, is readily treated without long-term sequelae, a key advantage for testing novel immunomodulatory agents and tolerance induction strategies. Third, emerging data from IS withdrawal trials in liver transplant recipients indicate that the rate of spontaneous tolerance increases over time after transplantation, from less than 10% within 3 years to as high as 80% after 10 years (Bohne, 2012) (Sanchez-Fueyo A. , 2011) (Feng S. U., 2012). As the first step toward a long-term goal of Treg immunotherapy, we propose to determine the safety of darTregs in combination with T cell depletion followed by EVR- or TAC-based Treg-supportive in adult, de novo liver transplant recipients. *We hypothesize that ex vivo-expanded darTregs administered to adult, de novo liver transplant recipients in combination with T cell depletion followed by Treg-supportive maintenance IS will be safe. Our study also aims to describe for the first time the persistence of administered darTregs in humans, as well as to describe the effects of T cell depletion and Treg-supportive IS on darTreg and darTconv after transplantation.* If successful, our study will define an approach for the therapeutic administration of Tregs, establish a new paradigm for the design of IS regimens, and set the stage for subsequent efficacy trials.

1.2 Rationale for Selection of Investigational Product or Intervention

1.2.1 Rationale for Treg Therapy

Liver transplantation can be life-saving therapy for liver failure. However, the maintenance of the transplanted liver requires continuous IS to prevent rejection by the host immune system. Although IS regimens have been refined over the last 20 years, with significant improvement in the prevention of AR, long-term outcomes have remained stagnant and the side effects of non-specific IS continue to cause morbidity and mortality (Feng S. , 2008). Therefore, a main focus of research has been to promote tolerance to transplanted livers so that IS can be minimized or completely withdrawn (Bohne, 2012) (Sanchez-Fueyo A. , 2011) (Feng S. U., 2012) (Londono, 2012).

In the past decade, we have learned that tolerance in organ transplantation is linked to the development and persistence of Tregs (Waldmann, 2008) (Kang, 2007) (Walsh, 2004) (Yeung, 2009) (Sanchez-Fueyo A. S., 2006) (Sagoo P. G., 2008) (Long, 2009). Therapeutic administration of Tregs has proven efficacy to control allograft rejection in multiple animal models through bystander suppression and infectious tolerance (Kang, 2007) (Riley, 2009) (Issa, 2010) (Nadig, 2010). In the setting of transplantation, most studies have utilized darTregs. These Tregs have been either isolated from tolerant animals that have higher frequencies of darTregs, or enriched or expanded in vitro using donor-type stimulator cells. On a cell for cell basis, our laboratory has demonstrated that darTregs are 10 to 100 times more potent at inhibiting alloreactive Tconvs in vitro than polyclonal Tregs (Brennan, 2011), and are approximately 5-6 times more potent on a cell

for cell basis than polyclonal Tregs in preventing rejection in a murine islet transplant model (Lee K, 2014). Moreover, darTregs are potentially safer than non-specific Tregs by offering targeted therapy instead of polyclonal, indiscriminate regulation that could theoretically lead to more general IS (Golshayan, 2007) (Raimondi, 2010). A GMP-compliant protocol to expand polyclonal Tregs 320 to 1250 fold *ex vivo* has been developed by our group and is currently being utilized for a trial in Type I diabetic patients (Putnam, 2009). Our group has subsequently developed a modified protocol to expand darTregs, allowing 200 to 1500 fold expansion with >70% of the Treg product demonstrating donor reactivity (Putnam AL, 2013). This protocol presents the opportunity to test the utility of darTregs in clinical organ transplantation.

Studies in mouse models of transplantation show that a high ratio of Tregs to conventional T (Tconv) cells such as 1:1 to 1:2, i.e. 33 to 50% of Tregs, is needed to prevent transplant rejection (Hara, 2001) (Graca, 2002), and tolerogenic treatment leads to early accumulation of 30% Tregs in the grafts (Fan, 2010). Tregs exist in equilibrium with Tconvs in the steady state and the percentages of Tregs remain constant in an individual for years. To alter this equilibrium in favor of Tregs, a combination of lymphodepleting the host and infusing expanded Tregs is needed.

A 70 Kg young adult human has 460 x 10^9 lymphocytes, 165.5 x 10^9 CD4+ T cells, and 13.1 x10^9 Tregs [4]. Using the immunosuppressive regimen described in this protocol, we estimate that at 10-12 week after transplant, CD4+ T cell count will be reduced to 25%, 41 x 10^9, and 10%, 4 x 10^9, will be Tregs. Thus, infusion of 14.5 x 10^9 polyclonal Tregs will increase the overall Treg frequency to 33%. darTregs are reported to be 10 to 100 times more effective at suppressing Tconv proliferation to alloantigens than polyclonal Tregs in in vitro suppression assays (Peters, 2008) (Chen, 2009) (Sagoo P. e., 2011) (Veerapathran, 2011). This means only 1/100th to 1/10th darTregs, 0.14 x $10^9$to 1.4 x 10^9, are needed to achieve the same efficacy as polyclonal Tregs.

It should be noted that the number of Tregs needed to prevent rejection in humans is unknown. The human immunological makeup is far more complex than that of laboratory mice born and maintained in specific pathogen-free cages. The presence of memory alloreactive T cells due to heterologous immunity in humans makes transplant rejection more difficult to control (Adams, 2003) (Selin, 2006). Therefore, even higher presence of Tregs may be needed to achieve efficacy in humans. On the other hand, most of mouse Treg studies do not use immunosuppressive drugs, which is certainly not an option in early clinical trials. Effective Treg dose in humans will likely vary depending concurrent immunosuppressive drugs. Thus, the numbers we estimate above is an approximation that can be used to guide the design of initial dose-seeking Treg therapy clinical trials, through which range of effective doses can be defined.

Based on all above considerations and the current manufacturing capacity, we selected to test three doses of darTregs. The initial dose in Cohort 2, 50 x 10^6, is below the range of estimated effective dose and is designed mainly to obtain safety profile of this novel therapy. The second dose in Cohort 3, 200 x 10^6, is in the lower range of estimated effective dose. The last Cohort 4 will receive the highest dose, 800 x 10^6, which is what we can currently reliably manufacture. We believe that this dose will most likely have an impact on alloimmune responses in patients.

1.2.2 Rationale for Lymphocyte Depletion using Thymoglobulin®

darTreg therapy has the potential to induce tolerance to the transplanted organ without impeding conventional immune responses. However, because of the exceptionally high frequency of donor-reactive T cells, "debulking" of the host alloreactive repertoire and adjunct IS are needed to create a more favorable setting for Tregs to control alloimmunity and to ensure long-term graft tolerance.

In the majority of animal transplant models of Treg therapy, efficacy appears to be optimal in a lymphodepleted host. This is likely due to the extremely high precursor frequency of alloreactive T cells, which can be a barrier to significantly altering the darTreg/darTconv ratio with darTreg administration. Lymphodepletion may favor the engraftment of the administered Tregs. Importantly, Thymoglobulin® has also been shown to favor the growth of Tregs in vitro (Boenisch, 2012), and to durably increase Treg percentages in humans after transplantation (Tang Q. J., 2012).

Tregs exist in equilibrium with Tconvs in the steady state. The most effective way of altering this equilibrium is by lymphodepleting the host and infusing expanded Tregs. We have estimated that an average 70Kg human has 165.5 x10^9

$CD4^+$ T cells and 13.1 $x10^9$ Tregs (~8%) (Tang Q. K., 2012). Thymoglobulin® treatment can deplete 95-99% of T cells from peripheral blood within one week after the initiation of the therapy. Assuming the depletion efficiency in lymphoid tissues is similar to that in the peripheral blood as seen in non-human primates (Preville, 2001), the total $CD4^+$ Tconvs will be reduced to about 4.6 x 10^9. Depletion of Tregs by Thymoglobulin® is slightly less efficient (Lopez, 2006) (Morelon, 2010), 90-98%, therefore approximately 0.6 x 10^9 Tregs, 13% of $CD4^+$ cells, will remain after Thymoglobulin® depletion. T cell counts recover from the nadir at one week, reaching an average plateau of 33% pre-transplant level for Tconvs and 41% pre-transplant level for Tregs at one month post-therapy (Tang Q. J., 2012). Thus, at the time of darTreg infusion between 11-20 weeks post-transplant, we expect that each patient will have a total of 50x10^9 $CD4^+$ T cells and 5.4 x 10^9 Tregs. Infusion of expanded darTregs at the higher doses of 0.2 x 10^9 and 0.8 x10^9 may allow us to see an increase in darTreg numbers in circulation, at least for a short period of time post infusion. Moreover, because endogenous darTregs are likely to be approximately 1-10% of the overall Tregs, the darTreg infusions are expected to have a correspondingly larger impact on the frequency of darTregs.

In this trial, participants who are medically stable and progressing well after liver transplantation, without the need for ICU-level care by 72 hours after transplantation, will receive a short course of Thymoglobulin®, to achieve lymphocyte depletion. The use of induction IS in liver transplantation setting in general and polyclonal Abs such as Thymoglobulin® in specific has markedly increased over the past decade *(http://optn.transplant.hrsa.gov/ar2009/906a_li.htm)*. This trend likely resulted from implementation of the Model for End Stage Liver Disease (MELD) (Kamath, 2001) allocation policy that has advantaged candidates with renal dysfunction, prompting interest in strategies that reduce early post-transplant exposure to calcineurin inhibitors (CNIs) that have known nephrotoxicity. Thymoglobulin® administration has therefore been used to delay introduction of CNIs (NCT00117689; NCT00564538; NCT00970073) (Soliman, 2007). As of 2008, nearly 30% of liver transplant recipients received induction therapy with approximately 14% receiving a polyclonal, T cell depleting agent (Thymoglobulin® or ATGAM). Thus Thymoglobulin® use in liver transplantation is common and appears to be safe (Nair, 2008) (Boillot, 2009) (Soliman, 2007).

1.2.3 Rationale for Everolimus Conversion

In subjects for whom it is considered safe, Treg-supportive IS will be implemented by reduction in TAC and conversion to mTOR inhibitor-based IS at least 30 days after liver transplantation. We will utilize EVR in this study due to its favorable pharmacokinetic profile compared to sirolimus, allowing more rapid adjustment of drug levels. In contrast to CNIs, mTOR inhibitors have been shown in animal and human models to foster the survival and proliferation of Tregs (Raimondi, 2010) (Battaglia M. A., 2005) (Battaglia M. A., 2006) (Pothoven, 2010) (Bocian, 2010) (Delgoffe, 2009). Moreover, recently, in February, 2013, EVR has received an indication as a maintenance IS agent in combination with reduced dose TAC if initiated 30 days or more after liver transplantation.

From a clinical perspective, mTOR inhibitors, as a class of drugs, have had efficacy in several different arenas, including the prevention of in-stent stenosis, the treatment of various cancers, and the prevention of rejection in the setting of solid organ transplantation. With respect to oncologic indications, EVR has been approved for the treatment of advanced renal cell carcinoma, subependymal glial cell astrocytomas in tuberous sclerosis patients, angiomyolipoma associated with tuberous sclerosis complex, breast cancer, and pancreatic neuroendocrine tumors and is being studied for efficacy against gastric cancer, hepatocellular carcinoma (HCC), and lymphoma. In transplantation, EVR has been approved for the prophylaxis of AR in adult kidney transplant recipients. A registration trial entitled "A 24 month, multicenter, open-label, randomized, controlled study to evaluate the efficacy and safety of concentration-controlled EVR to eliminate or to reduce TAC compared to TAC in *de novo* liver transplant recipients" has been conducted (Novartis Trial CRAD001H2304, 2007-001821-85; NCT00622869) (Di Simone, 2012). Based on the results of this worldwide clinical trial, the United States Food and Drug Administration has given EVR an indication as a maintenance IS agent for liver transplant recipients.

In this protocol, eligible participants will be treated with a regimen of low dose TAC (target trough levels of 3 – 5μg/L) and EVR (target trough levels of 6 – 8 μg/L) four to six weeks (not prior to day 30) after liver transplantation. This closely mirrors the dosing and conversion approach used in the trial referenced above which has had outstanding efficacy along with excellent safety and tolerability (Di Simone, 2012) compared to the control arm of TAC alone. At one year after transplantation, patients in the EVR + reduced TAC arm (comparable to the proposed Treg supportive IS regimen) had a

combined incidence of 6.7% for the primary efficacy endpoint of death, graft loss, and treated biopsy-proven AR which was non-inferior to that observed for the control arm (9.7%) (Di Simone, 2012). The one year incidence of treated biopsy proven AR alone was 2.9% compared to 7.0% in the control arm. The estimated GFR however, was superior in the EVR + reduced TAC arm with a difference of 8.50 mL/min/1.73m2, 97.5% CI 3.74, 13.27 mL/min/1.73 m2, p<0.001 for superiority). With delayed introduction of EVR, there was no increase in the frequency of wound healing complications that has been a concern with the use of mTOR inhibitors compared to the control arm which did not include EVR (Zuckermann, 2011) (Pengel, 2011) (Patel, 2009) (Flechner, 2009) (Di Simone, 2012). Therefore, Thymoglobulin® followed by delayed conversion to EVR, has been carefully designed to maintain adequate IS and support Tregs (both endogenous and infused) while avoiding excessive IS.

1.2.4 Rationale for a TAC-based IS Regimen

Although mTOR-based IS may be ideal for Treg survival, the compelling results of a recent study suggest that Treg infusion to subjects maintained on a regimen of TAC/MMF/prednisone is not only safe but also efficacious (Todo S, 2016) in adults who underwent living donor liver transplantation (with splenectomy). Subjects received a single dose of cyclophosphamide (40 mg/kg) on post-transplant day 8 to cytoreduce conventional Tcells (analogous to thymoglobulin in this trial) followed by a single infusion of a variable number of Tregs. Remarkably, 7 of 10 patients were successfully weaned off of IS, beginning at 6 months post-transplant and now are 16 to 33 months off of all IS. The three patients who failed IS withdrawal were reported to have autoimmune etiologies of liver disease. Thus, despite theoretical concerns of the survival and efficacy of Treg administration in the setting of TAC and MMF therapy, there is clinical evidence to suggest efficacy. It should also be noted that the Treg infusions using a variety of Treg products in a multi-national renal transplant trial (ONEStudy) have been administered in the setting of TAC/MMF/prednisone IS.

This trial importantly enrolls subjects undergoing deceased donor liver transplantation. As a consequence of increased cold ischemia time and reduced donor quality that results in substantial ischemia / reperfusion injury, a significant proportion of deceased donor liver recipients incur severe acute kidney injury in the immediate peri-transplant timeframe. Since trajectory of renal recovery is unpredictable, use of mTOR might not be appropriate because of low GFR and/or proteinuria. These subjects will be maintained on a regimen of TAC and MMF, albeit at TAC trough levels and MMF doses lower than standard of care to optimize renal function. We believe that this will be sufficient IS to prevent rejection since they have received thymoglobulin, which depletes T cells for months. Finally, it is also possible that subjects who successfully convert to EVR-based IS may experience toxicities that mandate EVR discontinuation concomitant with re-initiation of MMF. In light of the results of the recent Japanese trial, allowing darTreg administration in subjects maintained on either TAC-EVR or TAC-MMF IS is a justifiable approach. Obtaining safety and mechanistic data from subjects on either IS regimen in the context of darTreg administration will be valuable in proceeding with subsequent efficacy trials.

1.2.5 Rationale for Timing of Treg Administration

Most animal studies of Treg administration have administered Treg at the time of transplantation, in contrast to our study which proposes to give Tregs approximately 12-20 weeks after liver transplant. There are no published studies to our knowledge that have examined the optimal timing for Treg administration. In addition, modeling such studies in animals is difficult due to the variable responses of animals to conventional IS. In addition, the administration of T cell depleting agents often results in long term graft acceptance without further manipulation. Liver transplantation in particular is difficult to model due to the natural tolerogenicity of liver allografts which often require no IS for long-term survival.

We have chosen to delay Treg administration for several reasons. First, in the setting of liver transplantation, the potential for complications is very high, especially early after transplantation. In the context of a safety trial, administering Tregs at the time of transplant would be prohibitively risky. In addition, we expect that the window of T cell depletion after Thymoglobulin® will last at least until the time of Treg administration, 11-20 weeks after liver transplant. Thymoglobulin induction in renal transplant patients induced CD4 depletion out to at least 28 weeks, with no increase in CD4 counts between weeks 12-28 (Tang Q. J., 2012)(See Figure 1). This timing will also allow us to transition eligible subjects to an mTOR-based regimen, which is difficult immediately after liver transplantation for reasons discussed in *Section 1.2.2*. Waiting 11-20 weeks is also beneficial to a safety trial because the incidence of rejection is expected to be lower at that

time point, allowing a cleaner evaluation of the safety of Treg administration. Delayed administration will also enable us to manufacture darTregs as donor cells are required.

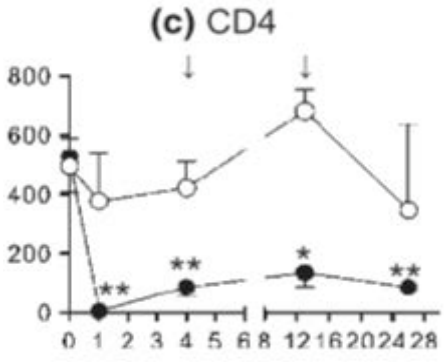


Figure 1. *Repopulation of CD4+ T cells after Thymoglobuin induction in renal transplant patients. X axis denotes weeks after transplant and Y axis represents Total cell number/ml blood (x 10^6). Open circles, control arm. Filled circles, thymoglobulin induction arm.*

Finally, theoretically, delaying Treg administration may have some benefit because it is well established that Tregs work better in a relatively quiescent milieu, rather than a highly pro-inflammatory environment as would be expected after a liver transplant. Overall the timing of Treg administration in this study is designed to maximize safety while potentially optimizing efficacy.

1.3 Preclinical Experience

1.3.1 Reactivity of darTregs

To determine the reactivity of the expanded darTregs toward the allogeneic stimulated B cells (sBc) used for primary expansion, we restimulated darTregs harvested on day 16 with the same sBcs used for darTreg expansion. The proliferation of the darTregs was monitored using CFSE dilution (*Figure 2. Reactivity of darTregs in vitro).* On average, 87.5% (range 72.5 to 95.2%) of the expanded darTregs proliferated in response to sBc stimulation. This essentially matched the proliferation achieved using anti-CD3 and anti-CD28 bead stimulation (average 88.8%, range 73.6 to 96%), suggesting that vast majority of the Tregs are reactive to the sBc allotype (*Figure 2. Reactivity of darTregs in vitro*).

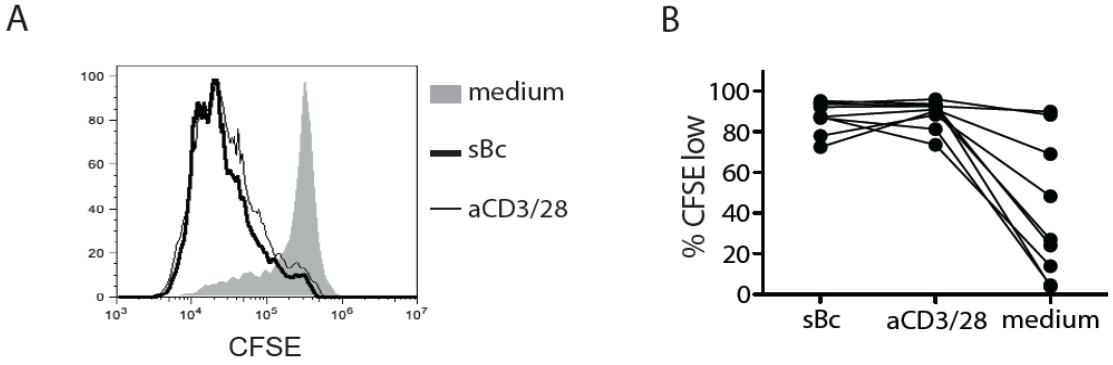


Figure 2. Reactivity of darTregs in vitro

1.3.2 Phenotype of darTregs

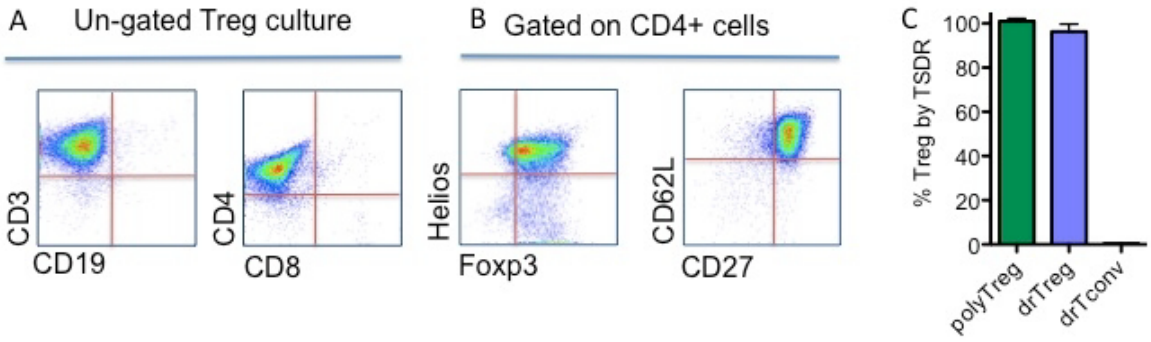
Expanded darTregs were found to be $CD3^+CD4^+$ with minimal $CD8^+$ T cell and $CD19^+$ and contamination (*Table* 1. *Phenotype of expanded darTregs* and *Figure 2. Phenotype* of darTregs*)*. The majority of the $CD4^+$ T cells were $FOXP3^+HELIOS^+$. In contrast, similarly expanded Tconvs were mostly $FOXP3^-HELIOS^+$ (data not shown). Previous reports have shown that co-expression of CD27 and CD62L correlated with suppressive activity of the expanded Tregs and could be used to distinguish between expanded Tregs and contaminating effector T cells. We found that Tregs expanded using this protocol were mostly $CD27^+CD62L^+$. Lastly, the expanded Tregs have > 80% demethylated TSDR (*Table 1. Phenotype of expanded darTregs* and

Figure 3. Phenotype of darTregs*)*. Collectively, the phenotype of Tregs expanded using allogeneic sBcs suggests that they are stable and committed Tregs.

Table 1. Phenotype of expanded darTregs

	CD3+	CD19+	CD4+	CD8+	FOXP3+	HELIOS+	CD62L+CD27+	TSDR
Mean	97.1	0.2	97.1	0.5	83.0	88.2	85.4	94.0
SD	2.6	0.2	1.9	0.2	10.8	6.6	6.4	15.5
n	14	14	14	14	14	14	10	10

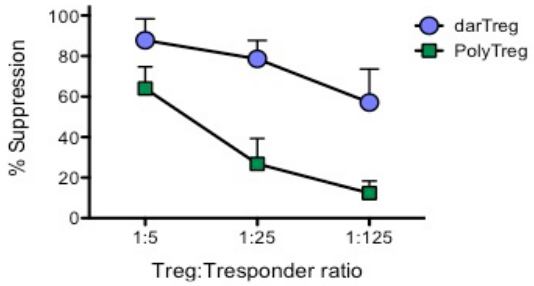
Figure 3. Phenotype of darTregs



1.3.3 In Vitro Function of darTregs

To determine the suppressive activity of darTregs in vitro, we measured the ability of darTregs to suppress one way MLRs. In this assay, PBMCs from the same person as the darTregs were stimulated with irradiated PBMCs from the same person from whom the allogeneic sBcs were generated. darTregs and polyclonally expanded Tregs (polyTregs) were added to the MLR at a ratio of 1 Treg per 5 PBMCs to 1 Treg per 125 PBMCs. Both polyTregs and darTregs suppressed the MLR (*Figure 4. In vitro suppression of MLR by* polyclonally *expanded Tregs and darTregs*). Side-by-side comparison show that the darTregs are 5 to 25 fold more potent at suppressing MLR than polyTregs.

Figure 4. In vitro suppression of MLR by* p*olyclonally expanded Tregs and darTregs

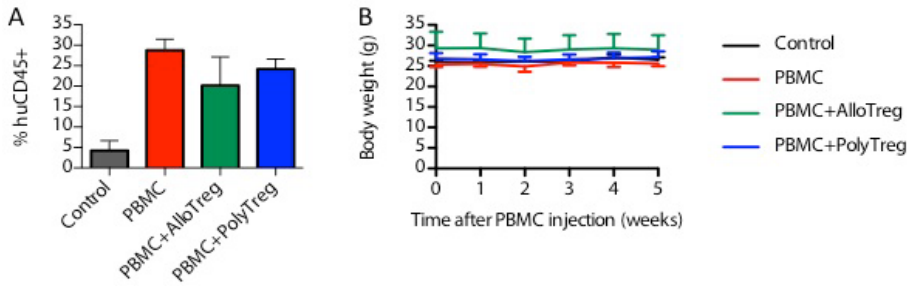


1.3.4 In vivo Function of darTregs in a Humanized Mouse Model of Transplantation

Using a model of alloimmune-mediated injury of human skin allografts (Sagoo P. N., 2011) we compared the protective function of darTregs and PolyTregs. We transplanted BALB/c.Rag2$^{-/-}\gamma c^{-/-}$ mice with human skin from a HLA-DR0401^{+} donor and allow the grafts to heal for 6 weeks. Then we isolated effector cells by depleting CD25^{+} cells from PBMC. These effector cells were transferred to the mice with healed in skin grafts alone or in combination with different preparations of syngeneic Tregs at a ratio 5:1 effector cells:Treg cells. PBMC donors were HLA-DR0401^{-} and alloantigen-reactive Tregs from these donors were expanded using HLA-DR0401^{+} CD40L-sBc. We then monitored the skin grafts in these mice until rejection or until up to a maximum of 6 weeks after PBMC reconstitution when the grafts were collected for histological

analysis. Levels of human leukocyte engraftment in spleens were similar in the three groups of mice that received human PBMC alone or in combination with Tregs (*Figure 5A*). No animal developed xenogeneic graft-versus-host disease symptoms confirmed by the maintenance of stable body weight *(Figure 5B*).

Figure 5. Characteristics of Characteristics of the humanized mouse model of skin transplantation. *A. PBMC reconstitution was determined at the end of the experiment, demonstrating that co-infusion of Tregs did not significantly alter the extent of PBMC reconstitution. B. Body weight of the BALB/c.Rag2-/-γc-/- mice in four experimental groups was assessed to determine general health status, demonstrating that PBMC infusion did not induce graft-versus-host disease*



We then analyzed the skin grafts using immunofluorescent microscopy *(Figure 6*). Compared to the skin grafts in control animals that did not receive PBMCs (Figure 6A), skin grafts in the PBMC alone group showed intense human CD45+ mononuclear cell infiltrates at the dermo-epidermal junctions with concomitant increase in keratinocyte proliferation, loss of involucrin, and decreased vascularization as indicated by the reduction in clustered CD31+ cells in the dermis *(Figure 6*B). These changes revealed active skin inflammation and loss of dermo-epidermal integrity mediated by the allogeneic human leukocytes. All these inflammatory parameters in the grafts were reduced by co-injection of PolyTregs, correlating with an increase in FOXP3+ cells *(Figure 6*C). Strikingly, skin grafts in mice that received alloantigen-reactive Tregs were nearly completely protected from histological features of graft injury and were indistinguishable from those in control grafts except for the infiltration of FOXP3+ cells at the dermo-epidermal junctions (*Figure 6D*). Quantitative analysis of these histological findings demonstrated significant reduction in Ki67+ keratinocytes, increase in CD31+ vascular endothelial cells, correlating with significantly higher FOXP3+ to CD3+ cell ratios in grafts of mice injected with alloantigen-reactive Tregs when compared to those in mice treated with polyTregs *(Figure 6E*). At a ratio of 5:1 effector:Tregs, darTregs, but not polyTregs, completely protected the skin grafts from pathological changes induced by the effectors cells.

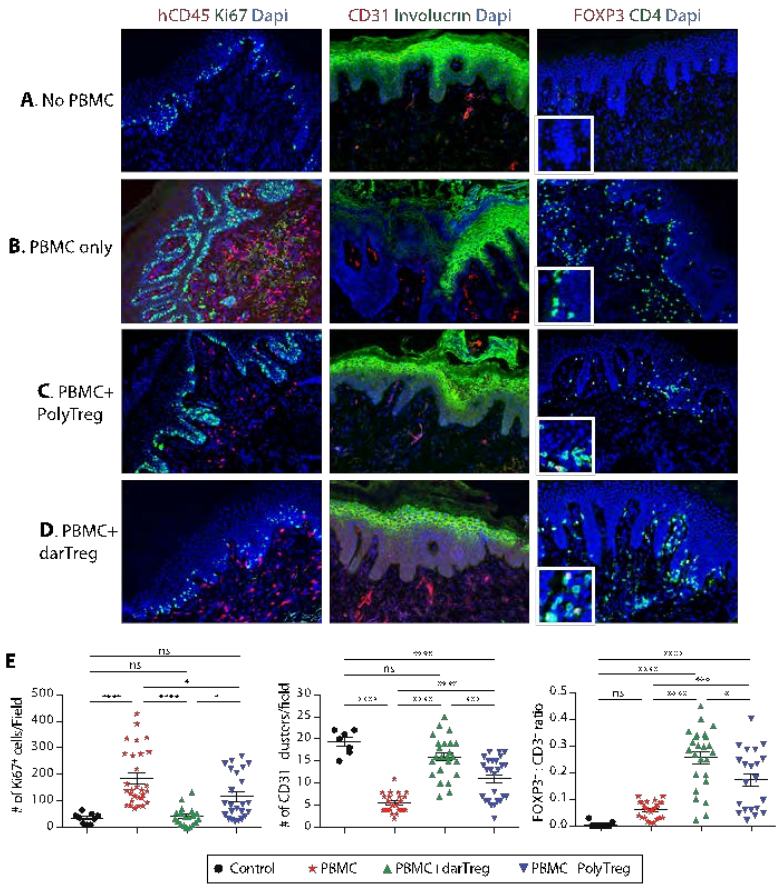


Figure 6. Suppression of skin allograft injury by PolyTregs and darTregs in vivo in a humanized mouse model. *A to D. Skin graft integrity and human leukocyte infiltration were analyzed using immunofluorescent microscopy and representative results for each experimental group is shown. E. immunofluorescence micrograph images were analyzed by counting 4 to 6 high-powered visual fields per stain for each graft. Quantitative results from four experimental groups were then compared. One-way ANOVA with Bonferroni multiple comparison test was used to determine the statistical significance of the differences.*

1.4 Clinical Experience with Treg Therapy

The cellular product to be utilized in this clinical trial has not been previously used in humans. Other relevant clinical experience with Treg infusion is in quite distinct settings. Three trials of Treg therapy for graft versus host disease (GvHD) in patients have been reported (Trzonkowski, 2009) (Brunstein, 2010) (Di Ianni, 2011). The first-in-man cases by Trzonkowski et al involved two patients. The first patient had chronic GvHD two years after 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 an accumulative dose of 3 × 10^6/kg expanded donor Tregs. A larger scale phase I trial by Brunstein et al. was reported in 2010 (Brunstein, 2010). Twenty-three patients with advanced hematologic malignancy 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 samples that had 4 to 6 HLA antigen match 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 detectable in circulation for up to 7 days. During the one-year period after Treg infusion, the investigators observed no DLTs or increase in adverse events (AEs) when compared to historical controls. Incidences of severe acute GvHD were significantly reduced in patients who received Treg therapy. The median time to development of GvHD in patients who received Treg infusion was longer compared to normal disease course although the delay was not significant. The third trial enrolled 28 patients with high-risk hematological malignancies (Di Ianni, 2011). Patients received anti-CD25 immunomagnetic bead-enriched donor Tregs without *ex vivo* expansion four days before receiving one haplo-mismatched hematopoietic stem cell and Tconv transplants 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. Patients demonstrated accelerated immune reconstitution, reduced CMV reactivation, and a lower incidence of tumor relapse and GvHD when compared to historical controls. Similar GvHD trials are being conducted with non-expanded Tregs ($CD4^+CD25^{hi}$) (P. Hoffmann and M. Edinger in Regensburg, Germany), Tregs grown in IL-10 and sirolimus (M. Grazia-Roncarolo, Milan), and $CD4^+CD127^-$ Tregs sorted from $CD4^+CD25^+$ CliniMACS selected population (R. Negrin, Stanford).

Recently, a clinical trial infusing Tregs in diabetic children was reported (Marek-Trzonkowska N., 2012). The investigators administered Tregs in 10 type 1 diabetic children (aged 8-16 years) within 2 months after diagnosis. In total, 4 patients received 10 × 10^6 Tregs/kg body wt, and the remaining 6 patients received 20 × 10^6 Tregs/kg body wt. The preparation consisted of sorted autologous $CD3^+CD4^+CD127^{lo/-}CD25^+$ Tregs expanded under good manufacturing practice conditions. No toxicity of the therapy was noted. A significant increase in the percentage of Tregs 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 UI/kg body wt of insulin daily, with 2 patients out of insulin completely, whereas in the untreated group 6 out 10 required >0.5UI/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.

The prior experience with polyclonal $CD4^+$ T cells has not suggested significant safety concerns with the administration of *ex vivo* expanded autologous cells. Over 400 infusions administered to subjects for treatment of HIV and myeloma have been performed by Bruce Levine and Carl June at the University of Pennsylvania (Levine B. , 2008). The cellular products administered in these clinical trials were activated $CD4^+$ and $CD4^+/CD8^+$ T cell polyclonal populations, which inherently have a high associated risk due the presence of T effector cells. The proven safety of these potentially high risk populations in conjunction with the low risk of the presence of contaminating effector cells in the proposed product advocates a low probability of toxicity of *ex vivo* expanded autologous $CD4^+127^{lo/-}25^+$ regulatory T cells. Notably, the inclusion of CD127 marker in our sorting procedure allows us to discriminate between T effector cells and true Tregs (Liu W., 2006).

Our manufacturing group is producing polyclonal Tregs for a phase I trial of ex-vivo expanded, polyclonal Tregs in recent onset type I diabetics. The trial completed enrollment in October 2013. A total of 26 potential participants signed consent and underwent screening assessments. Sixteen subjects were determined to be eligible and were enrolled. Two of these sixteen subjects were withdrawn from the study due to failure of Treg product to meet release criteria. Fourteen subjects (age range 18-43, 6 females and 8 males) were treated with a single dose of Ex Vivo Expanded Autologous $CD4^+CD127^{lo/-}CD25^+$ Polyclonal Tregs. Three subjects were treated in the first dosing cohort with 0.05x10^8 cells, three subjects were treated in the second dosing cohort with 0.4x10^8 cells, 4 subjects were treated in the third dosing cohort with a target dose of 3.2 x 10^8 cells, and 4 subjects were treated in the fourth dosing cohort with a target dose 26 x 10^8 cells. As of February 2014, all 14 subjects were active in the study. The first treated subject has completed the scheduled 2.5-year follow-up visit, and the last treated subject has completed the scheduled 3 month follow-up visit.

As of December 2013, 91 AEs have been reported in 15 subjects since the beginning of the trial. All 14 treated subjects reported at least 1 AE. One subject who underwent phlebotomy but was withdrawn before treatment reported 1 AE before withdrawal from the trial. Fifty-eight events were judged as grade 1 in severity, 25 were judged as grade 2, and 8 were judged as grade 3. There were no grade 4 or 5 AEs. Thirty events were judged to be possibly related, 15 unlikely related, and 46 unrelated to study therapy. Three SAEs have been reported. One judged unlikely and two judged unrelated to study participation.

The most common system organ class affected was Infections and Infestations followed by Gastrointestinal Disorders and General Disorders and Administration Site Conditions. Of 22 infections recorded, 13 were upper respiratory infections. Of those, nine were judged grade 1 and 4 were judge grade 2 in severity. One infection, reported as grade 2 pharyngitis, was subsequently demonstrated to reflect a new CMV infection that occurred prior to treatment with Tregs.

Two of the patients in cohorts 3 and one of the patients in cohort 4 are at Yale. Whole blood collected from the Yale patients were shipped to UCSF for expansion. After 14 days of expansion, the cellular products were shipped back to Yale for infusion, demonstrating the feasibility of the UCSF manufacturing team and facility to support multi-site studies. In addition, three of the cohort 3 patients and all 4 cohort 4 patients received Tregs that were labeled with deuterium during expansion, which allowed longitudinal monitoring of the infused cells in circulation. Six-month follow-up of cohort 3 and 3-month follow-up of cohort 4 patients show deuterium among circulating Tregs shortly after infusion. The signals peaked in the first two weeks and then decayed with a half-life of approximately 1 month (Figure 7). The signals from cohort 3 of $3.2x10^8$ cells remained detectable at 6 months. Together, these results demonstrate that the technology can detect $3.2x10^8$ cells infused into lymphoreplete hosts.

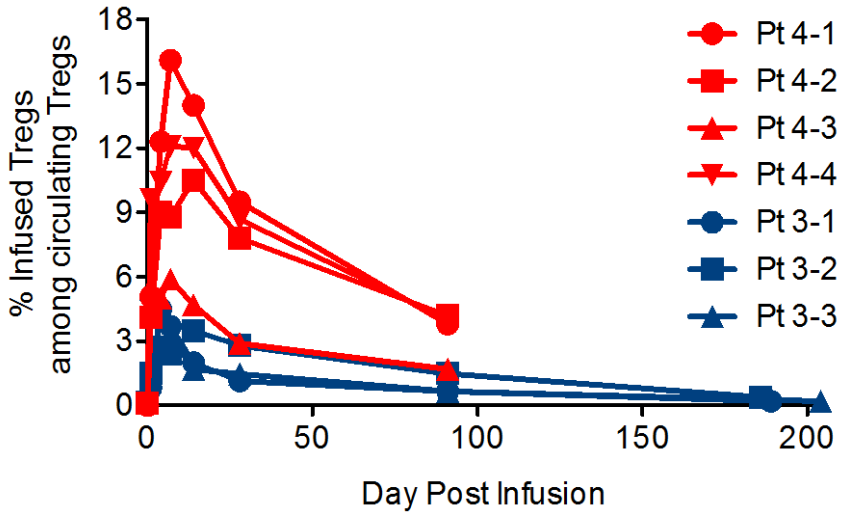


Figure 7 Detection of infused deuterium-labeled Tregs.

Peripheral blood Tregs were purified from study subjects in the T1D trial at indicated time after Treg infusion. Levels of deuterium in the purified Tregs were analyzed using gas chromatography-mass spectrometry. Blue symbols in the graph represent the 3 subjects in cohort 3 that received 3.2 x 10^8 Tregs and red symbols represent the 4 subjects in cohort 4 that received 26 x 10^8 Tregs.

Our expanded, darTreg product has a theoretical safety advantage compared to polyclonal Tregs in that darTregs will not have as many specificities as polyclonal Tregs, lessening the chance for non-specific IS. This first-in-class effort will generate some of the first clinical safety data in this area, provide information on kinetics of persistence, and possibly provide the first clues on how darTregs affect the alloimmune response in the setting of liver transplantation.

2. Study Hypotheses/Objectives

2.1 Safety Objective

This study will evaluate the safety, tolerability, and dose limiting toxicities (DLTs) of a darTreg infusion for adult, de novo, liver transplant recipients who have received a T cell depleting agent and are maintained on TAC- or EVR-based Treg-supportive IS regimen.

2.2 Mechanistic Objectives

This study will evaluate the effects of the Treg-supportive IS regimens on the immunological profiles of liver transplant patients. This study will also describe for the first time the persistence of administered darTregs in humans.

3. Study Design

3.1 Description of Study Design

This is a multi- center, open-label, dose- escalation, pilot study in which adult subjects undergoing primary solitary liver transplant will receive a T cell depletion agent (Thymoglobulin®) followed by Treg-supportive IS regimen based on either EVR or reduced-dose TAC (Cohorts 1a and 1b). Subjects in subsequent cohorts will also receive a single infusion of autologous, darTregs (Cohort 2: 50 million darTregs; Cohort 3: 200 million darTregs: or Cohort 4: 800 million darTregs). For individual participants, there are up to three successive stages in the study with specific eligibility criteria for each stage. As shown in *Figure* 8, the three stages are:

- Screening and Enrollment
- Eligibility assessment for EVR conversion and proceeding to T-reg infusion
- darTreg Infusion

Subjects will be followed for 40 weeks after transplant, during which clinical data along with peripheral blood (PBMCs and serum) and liver biopsy samples will be collected and analyzed (Appendix 1, 2 3).

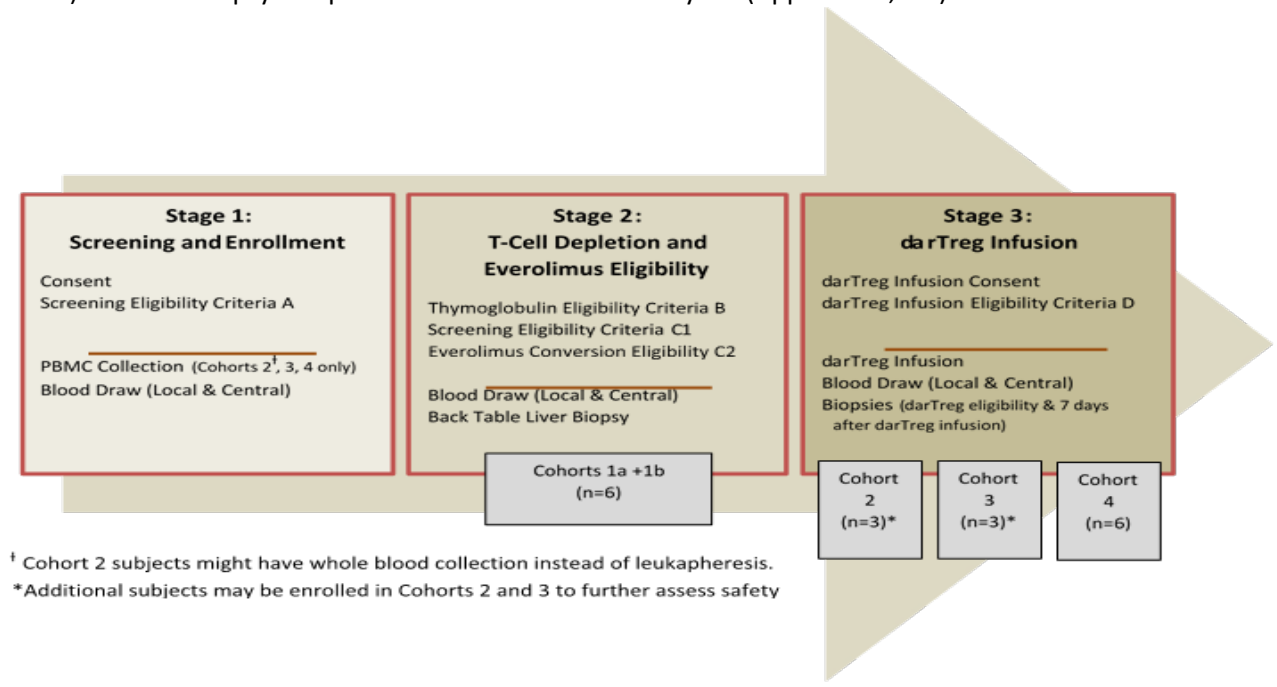


Figure 8. Three Stages of Trial

The four cohorts are summarized below, with details about the dose escalation plan in *Section 5.4*.

- Cohort 1: 3 subjects from Center 1 (Cohort 1a) + 3 subjects from Center 2 (Cohort 1b); these subjects will undergo T cell depletion followed by TAC- or EVR-based IS (Stage 1 and 2; they will not receive darTreg infusion (Stage 3)).
- Cohort 2: 3 subjects meeting eligibility criteria for all 3 Stages will receive 50 million darTregs (Dose A range 25-60 million).
- Cohort 3: 3 subjects meeting eligibility criteria for all 3 Stages will receive 200 million darTregs (Dose B range 100-240 million).
- Cohort 4: 6 subjects meeting eligibility criteria for all 3 Stages will receive 800 million darTregs (Dose C range 400-960 million).

3.2 Safety Outcomes

The safety, tolerability, and ties of darTreg therapy given within the context of either a TAC- or EVR-based IS regimen will be evaluated with the rate of the following events within 40 weeks of transplantation:

Clinical outcomes that will be described are:

1. Incidence and severity of biopsy-proven AR and/or CR (Demetris A. J., 1997) (Demetris A. D., 2000)
2. Incidence of ≥ Grade 3 infections as defined in *Section 14.3.1*
3. Incidence of wound complications (≥ CTCAE Grade 3)

4. Incidence of anemia, neutropenia, and/or thrombocytopenia (according to study defined reporting requirements, *Section 14.3.1*)
5. Incidence of AEs attributable to the darTreg infusion including infusion reaction / cytokine release syndrome (CRS) (≥ CTCAE Grade 3), and malignant cellular transformation.

3.3 Mechanistic Outcomes

Blood and liver biopsy samples from all patients enrolled in the study will be analyzed using MFC, T cell functional assays, and/or immunohistochemical techniques to describe the effect of TAC- vs EVR-based IS with or without darTreg administration on immune cell profiles over time. We will also describe the persistence of administered darTregs after infusion using TCR repertoire analysis as well as deuterium labeling of administered darTregs.

Mechanistic outcomes that will be assessed are:

1. Treg frequency in peripheral blood using Treg TruCount assay
2. Persistence of infused darTregs in peripheral blood and biopsies using deuterium labeling and T cell repertoire analysis
3. darTreg and darTconv frequency in peripheral blood using an in vitro allofrequency assay
4. MFC to assess effect on immune profiles
5. Functional assessment of Tregs in peripheral blood using a MLR suppression assay
6. Histology and immunophenotype of liver biopsies

4. Selection of Participants and Clinical Sites/Laboratories

4.1 Rationale for Study Population

The liver transplant setting was chosen for multiple reasons. First, the liver is considered an organ of immune privilege. As such, it is the only solid organ transplant associated with an appreciable rate of spontaneous operational tolerance (Demetris A. J., 2009) (Mazariegos G. , 2011). Although historical evidence was based on sporadic case reports and single center experiences (Assy, 2007) (Eason, 2001) (Girlanda, 2005) (Mazariegos G. , 2011) (Mazariegos G. J., 1997) (Oike, 2002) (Orlando, 2008) (Pons, 2003) (Ramos, 1995) (Takatsuki, 2001) (Tisone, 2006) (Tryphonopoulos, 2005) (Devlin, 1998), several ongoing or recently completed clinical trials have provided strong corroborating evidence (NCT00105235; NCT00135694; NCT00320606; NCT00436722; NCT00647283; NCT00668369; NCT01198314) (Martinez-Llordella, 2008) (Bohne, 2012) (Feng S. U., 2012) (Benitez C, 2013). Emerging data from these experiences and trials indicate that the likelihood of spontaneous tolerance increases with time after transplantation with estimated frequency of <10% for patients <3 years after transplant, 10% for patients between 3–6 years after transplant, 35% for those between 6–10 years after transplant, and >80% for patients >10 years after transplant (Martinez-Llordella, 2008) (Bohne, 2012) (Benitez C, 2013). This data suggests a substantial opportunity to increase the likelihood and/or accelerate the development of tolerance through novel approaches based on rigorous biological mechanisms. Second, it is known that, among solid organ allografts, the liver is uniquely resilient to rejection processes. AR, particularly for the non-hepatitis C virus (HCV), non-autoimmune population that we aim to enroll, has not exerted a detrimental event on patient or graft survival (Wiesner, 1998). Similarly, in the modern IS era, CR not only occurs rarely (Jain, 2001) but is also amenable to treatment (Inomata, 2001) (Blakolmer K. A., 2000) (Blakolmer K. E., 1999). Third, as a corollary of the above, a basic premise underlying all of the IS withdrawal trials is that AR occurring in a highly supervised setting, such as a clinical trial, will be diagnosed and treated expeditiously, leading to rapid resolution without any long-term sequelae. Therefore, determining an optimal dose of darTregs in this pilot dose escalation trial will set the stage for a Phase II safety and efficacy trial that includes attempted IS withdrawal.

<u>MELD Score</u>

We have chosen MELD score of ≤25 somewhat arbitrarily as the MELD score is a continuous metric of liver disease severity. As such, there is no obvious discrete threshold. A MELD score of 25 corresponds to an approximate 20% risk of death from liver disease in 90 days. This threshold will exclude severely / critically ill patients.

Historically, the median laboratory MELD score of UCSF liver transplant recipients is 19.3 while that of Emory is significantly lower. So a threshold of 25 will exclude less than one third of all deceased donor liver transplant candidates, ensuring the feasibility of our study design.

<u>Minimum Circulating Tregs</u>

We are requiring a minimum threshold of circulating Tregs at the time of recruitment for a practical reason. In order to be able to manufacture a darTreg product, we will need to be able to isolate a sufficient starting population of Tregs from the liver transplant candidate through phlebotomy or leukapheresis. Patients with very low concentrations of Tregs may not yield sufficient Treg numbers to support darTreg production.

After transplantation, we do believe that we need to achieve a dominance of Tregs over Tconvs. We aim to achieve this goal by the combination of depletion and administration of increasing doses of darTregs.

<u>HLA-DR Matching</u>

We are excluding liver transplant recipients who are matched to their donor at both HLA-DR as this will compromise the generation of the darTreg product.

4.2 Inclusion Criteria

Subjects who meet all of the following criteria are eligible for enrollment as study participants. Inclusion criteria should be re-affirmed as appropriate at each stage of the study.

1. Able to understand and provide informed consent
2. End-stage liver disease and listed for deceased-donor primary solitary liver transplant
3. Between 21 and 70 years of age at the time of study entry /consent
4. Have a calculated MELD score ≤ 25 at the time of study entry /consent
5. Female and male subjects with reproductive potential must agree to use FDA approved methods of birth control for the duration of the study.
6. If history of HCV, have completed or are in current treatment for HCV AND have no detectable HCV RNA
7. Subjects with HCC meeting Milan criteria

4.3 Exclusion Criteria

4.3.1 Screening and Enrollment Exclusion Criteria A (Stage 1)

Below are exclusion criteria to be assessed at study enrollment, prior to **Stage 1 study procedures**. Subjects who meet any of these criteria are ineligible for study participation.

1. End stage liver disease secondary to autoimmune etiology (autoimmune hepatitis, primary biliary cirrhosis, or primary sclerosing cholangitis)
2. History of less than 5 years remission of malignancy, except for 1) HCC or 2), history of adequately treated in-situ cervical carcinoma, or adequately treated basal or squamous cell carcinoma of the skin
3. History of previous organ, tissue or cell transplant
4. Serologic evidence of human immunodeficiency 1 or 2 infection
5. Epstein Barr Virus (EBV) sero-negativity (EBV naïve candidates)
6. Cytomegalovirus (CMV) sero-negativity (CMV naïve candidates) if donor is positive
7. Chronic use of systemic glucocorticoids or other IS, or biologic immunomodulators
8. Chronic condition requiring anti-coagulation after liver transplantation
9. Any chronic illness or prior treatment which, in the opinion of the investigator, precludes study participation
10. Participation in any other studies that involved investigational drugs or regimens in the preceding year
11. Received any vaccination within 28 days prior to leukapheresis or blood collection for Treg manufacture
12. Hemoglobin <9.0 g/dL within 10 days prior to screening
13. Neutrophils <1,500/µL within 10 days prior to screening
14. Platelets <40,000/µL within 10 days prior to screening

4.3.2 Thymoglobulin® Exclusion Criteria B (Stage 2)

Below are exclusion criteria to be assessed prior to **administration of Thymoglobulin®**. Subjects who meet any of these criteria should not receive Thymoglobulin®.

1. Calculated MELD score greater than 25 at the time of deceased donor liver transplant
2. Last AFP obtained prior to liver transplantation >400 µg/L for candidates with HCC
3. Unacceptable PBMC product for participants enrolled in Cohorts 2, 3, or 4, per UCSF HICTF manufacturing specifications
4. Absence of donor cells for manufacturing for any participant
5. HLA-DR matched to donor at both loci
6. Subject is < 21 or > 70 years of age at the time of transplantation
7. Located in the ICU 72 hours after transplantation
8. Hemoglobin <8.0 g/dL
9. Absolute neutrophil count <1,200/µL
10. Platelets <40,000/µL
11. Positive pregnancy test for females of child bearing potential
12. Unexpected histopathology on back table liver biopsy that contraindicates the initiation of Treg supportive IS regimen.
13. Development of a condition requiring chronic anti-coagulation

14. Hypersensitivity to rabbit proteins or any excipient in Thymoglobulin®
15. Detectable HCV RNA (if history of HCV (e.g. serology positive at screen A)) or less than six months after end of treatment for HCV at the time of transplantation (i.e. does not meet criteria for SVR)

4.3.3 Exclusion Criteria C1

Below are exclusion criteria to be assessed at day 30-44 after transplantation for continuation in the trial. All subjects regardless of eligibility for EVR conversion, with any of the following will not receive darTregs and will move into safety follow up.

1. Explanted liver with evidence of increased risk of recurrent cancer (HCC tumor burden exceeding the Milan criteria; presence of vascular invasion; cholangiocarcinoma morphology)
2. Insufficient depletion of recipient T cells, defined as a nadir CD3 count ≥50 cells/µL (50 cells/mcL) or total lymphocyte count ≥ 0.1 x 10^9/L if CD3 count is unavailable
3. Development of a condition requiring chronic anti-coagulation
4. Clinical evidence of untreated biliary obstruction
5. ALT >2.0 x upper limit of normal (ULN)
6. Inability to taper off corticosteroids by 44 days (+/- 2 days) after transplant
7. Detectable circulating HCV

4.3.4 Everolimus Conversion Criteria C2 (Stage 2)

Below are exclusion criteria to be assessed prior to conversion to **EVR-based IS regimen.** Subjects who meet any of these criteria should not be converted to EVR-based IS regimen. EVR cannot be initiated prior to 30 days after liver transplantation. Subjects with any of the following will remain on TAC-based IS regimen.

1. Evidence of hepatic artery stenosis or thrombosis by Doppler examination or angiography within 7 days prior to conversion
2. Urine protein/creatinine ratio >1.0 mg/mg within 7 days prior to conversion
3. Calculated GFR less than 30 ml/min per MDRD4 equation within 7 days prior to conversion
4. Physical examination documentation of abnormal wound healing or uncontrolled wound infection
5. Hemoglobin <8.0 g/dL within 7 days prior to conversion
6. Absolute neutrophil count <1,200/µL within 7 days prior to conversion
7. Platelets <50,000/µL. within 7 days prior to conversion

4.3.5 darTreg Infusion Exclusion Criteria D (Stage 3)

Below are exclusion criteria to be assessed prior to darTreg infusion for subjects in Cohorts 2, 3, and 4 only. Subjects in Cohort 2, 3, or 4 who meet any of these criteria should not receive a **darTreg-infusion**:

1. Inability or unwillingness of participant to give additional written informed consent
2. Unacceptable darTreg product
3. Detectable circulating EBV or CMV DNA within 10 days prior to darTreg infusion
4. Detectable HBV DNA within 10 days prior to darTreg infusion
5. Detectable circulating HCV RNA within 10 days prior to darTreg infusion
6. ALT >1.5x ULN within 10 days of darTreg infusion
7. Most recent, but not greater than 10 days prior to darTreg infusion, 12 hour TAC trough levels of > 8 µg/L for all subjects
8. Most recent, but not greater than 10 days prior to darTreg infusion, 24 hour EVR trough levels of < 5 µg/L for all subjects on EVR
9. For subjects on EVR-based IS, received MMF within 10 days prior to darTreg infusion
10. Evidence of AR or CR according to Banff criteria on protocol allograft biopsy based on local assessment
11. Received any vaccination within 14 days prior to darTreg infusion
12. Positive pregnancy test for females of child bearing potential
13. Inability or unwillingness of participant to comply with study protocol or procedures
14. Calculated GFR less than 40 ml/min per MDRD4 equation within 10 days prior to infusion

4.4 Selection of Clinical Sites

UCSF performs approximately 130-140 adult liver transplants for chronic liver disease each year and has had outstanding outcomes based on SRTR data. At the time of protocol design, the median calculated MELD score at transplant is 19.3 (Washburn, 2009) such that approximately 65% would have been eligible based on a calculated MELD score of ≤25 at the time of transplant. However, multiple changes in US liver allocation policy has substantially changed the landscape of liver transplantation. Moreover, throughout the country, the median MELD score at transplant has increased significantly. At UCSF, nearly all subjects who undergo deceased donor liver transplantation across all blood groups with a laboratory MELD score of ≤25 are those who have been granted MELD exception points. The vast majority of these are candidates with HCC which has, historically, accounted for approximately 30-35% of the annual deceased donor liver transplant volume. Exclusion of candidates with active HCV in conjunction with HCC leaves approximately 15% or 20-22 recipients eligible for trial participation annually. Because this trial has additional eligibility assessment steps after enrollment, including donor eligibility for use of cells for a cellular product, we anticipate some attrition at each phase. Therefore, we aim to enroll a minimum of 6-8 patients per year to ensure that 4-5 will progress to darTreg infusion. The pace of enrollment was on par with this plan until October 2015, when the "Wait and Cap" allocation policy for HCC was implemented. The aim of this policy change is to better equalize waitlist mortality of patients with and without HCC. Since the HCC MELD exception score is now capped at 34 and donor livers are regionally shared for candidates with MELD scores ≥35, HCC candidates in Region 5 (UCSF), a region notorious for transplanting at high MELD scores, have much reduced access to transplantation. Our partnership with large transplant centers outside of our region is therefore critical to achieve the enrollment goals for this trial. The UCSF investigators have selected to partner with Mayo Clinic Rochester, Rochester, Minnesota and Northwestern Memorial Hospital, Chicago, Illinois, for many reasons. Mayo Clinic Rochester and Northwestern perform approximately 75-80 and 80-95, respectively, adult liver transplants for chronic liver disease on an annual basis. Both centers are located in United Network for Organ Sharing Region 7 while UCSF is located in Region 5. These two regions differ significantly in the MELD score required to access a deceased donor organ. The lower disease severity at Mayo Clinic Rochester will increase the proportion of transplant recipients eligible to participate in this trial.

In addition to the complementary clinical profile, the most compelling reason for us to partner with both Mayo Clinic Rochester and Northwestern is the breadth and depth of its infrastructure and expertise to support this highly complex trial of a novel therapy. Investigators at Northwestern produce a polyclonal regulatory T cell product which has been administered to living donor kidney recipients to prevent rejection in a Phase I clinical trial (NCT02145325). Both centers are also participating in another clinical trial administering darTreg therapy to facilitate IS withdrawal among adult living donor liver transplant recipients (CTOTC-12/ NCT02474199). Participation in two trials involving the same product in a similar patient population will substantially increase their familiarity with the study procedures and processes and enhance their ability to conduct the proposed trial with high fidelity. The combined infrastructure supporting both trials will escalate the commitment to the proper collection, processing, and shipment of samples for both darTreg manufacturing and for the accompanying mechanistic assays.

5. Investigational Agents

5.1 Deuterium-labelled darTreg Product

The darTreg product will be manufactured at Human Islet and Cellular Transplantation Facility (HICTF) and GMP Facility:
UCSF Mission Center Building
Rooms M640-650
1855 Folsom Street
San Francisco, CA 94103

5.1.1 Manufacturing, Formulation, Packaging, and Labeling

Recipient T cell Banking

Peripheral blood leukocytes will be collected via leukapheresis from participants enrolled into Cohorts 3 and 4 after successful screening and prior to transplantation. Cohort 2 subjects might have leukapheresis or whole blood collection for PBMC isolation. The whole blood or leukapheresis product will be transported to the manufacturing facility. PBMCs will be isolated using Ficoll density gradient centrifugation and cryopreserved in liquid nitrogen until the patient is eligible for darTreg infusion. If HBV DNA is greater than 1,000 IU/mL at the time of PBMC collection, then the resulting PBMC product will not be utilized to manufacture darTregs. At least $2x10^9$ PBMCs (leukapheresis) or $2x10^8$ PBMC (whole blood) are expected to be collected and banked from each subject. If the whole blood or leukapheresis product does not contain sufficient numbers of Tregs, phlebotomy or leukapheresis can be repeated to ensure an adequate cell number for product manufacture before transplant.

Donor B cell (sBcs) Banking and Activation

On the day of transplantation, donor spleen and/or whole blood will be collected and transported to the manufacturing facility for processing into a single cell suspension followed by stimulation and expansion with irradiated KT64-CD40L.HLADR0401 cells and expanded for 10 days in the presence of recombinant human IL-4, cyclosporine A and ganciclovir. The CD40L-stimulated donor sBcs will be sorted, irradiated, and cryopreserved until needed for darTreg expansion. Failure to collect donor cells will disqualify a subject from further trial participation for participants in Cohorts 2, 3, or 4. Participants in Cohorts 1a and 1b will be allowed to continue in the trial since they are not scheduled to undergo darTreg infusion.

Treg Expansion

The banked recipient PBMCs will be thawed, washed, stained and sorted for $CD4^+127^{lo/-}25^+$ Tregs. Purified Tregs will be cultured with irradiated sBcs at a ratio of 4:1 in deuterated media. On day 11, cells will be further stimulated with beads conjugated with anti-CD3 and anti-CD28 mAbs-conjugated beads at a 1:1 ratio. Beads will be removed and the appropriate number of cells will be counted and re-suspended in 100 mL of infusion media (see below) at the appropriate concentration, and aseptically filled in infusion bags. The final product will be quarantined at 4°C until release.

Each lot will be released for infusion if it meets the pre-defined specifications criteria, including ≥95% $CD4^+$, ≥60% $FOXP3^+$, ≥75% $TSDR^+$, ≤5% $CD8^+$, ≤1% $CD19^+$.

5.1.1.1 darTreg Formulation, Packaging and Labeling

The deuterium-labelled darTreg product is fresh, non-cryopreserved, sterile cell suspension of ≥85% viable, ≥95% CD4+, ≥60% $FoxP3^+$-, cells for IV administration of a single dose containing 50 (range: 25-60 x 10^6 cells), 200 (range: 100-240 x 10^6 cells) or 800 x 10^6 (range: 400-960 x 10^6 cells) darTregs and formulated in 100 mL of sterile infusion solution containing 49.02% (v/v) PlasmaLyte-A USP, 49.02% (v/v) Dextrose 5%-USP, 0.45% NaCl-USP, and 1.96% (v/v) 25% human serum albumin. The product is supplied in a sterile, PVC, 150-mL infusion bag (Charter Medical catalog #T3000). The final product should be stored and maintained at 2-10°C until ready to use.

The product will be released with an interim Certificate of Analysis (COA). The final COA will be provided once the sterility results become available.

5.1.2 Dosage, Preparation, and Administration

The deuterium-labelled darTreg product will be transported within UCSF and between UCSF and remote clinical centers in a shipping container with a temperature monitor and a coolant pack(s) to maintain the temperature between 2°C and 10°C. The product should be maintained in the shipping container until its administration. The infusion of the product must occur within 30 hours after final formulation. The identity of the donor, recipient and the product will be verified by two individuals prior to infusion.

Subjects meeting eligibility criteria for darTreg infusion will receive a single IV dose containing 50 (range 25-60), 200 (range 100-240), or 800 (range 400-960) million darTregs administered in approximately 20-30 minutes by gravity. The line will be primed with saline prior to infusion and the product bag, tubing, and peripheral IV line will be flushed with up to 250ml normal saline to ensure the complete dose is infused.

Pre-medications will be administered prior to infusion (see *Section 6.2.2*).

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 CRS. 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 which will allow ongoing monitoring for any infusion-related signs and symptoms.

5.1.3 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. Except for liver biopsy, the laboratory components of Screen D must be repeated within 10 days of planned infusion to ensure eligibility. In any case, infusion of darTregs must take place no later than 20 weeks after transplantation. If the second attempt at manufacturing fails, the subject will go into safety follow up (Appendix 4).

5.2 Thymoglobulin® (Anti-Thymocyte Globulin – Rabbit)

Thymoglobulin® (ATG) is manufactured by Genzyme Corporation (Cambridge, MA). Generic forms of the medication are also available but, for this trial, generic formulations should not be used. Thymoglobulin® is licensed for the treatment of renal transplant AR in conjunction with concomitant IS. Currently, there is no indication for the use of Thymoglobulin® as an induction therapy in the context of liver transplantation. Data from the Scientific Registry of Transplant Recipients, however, shows that there has been an increasing trend for liver transplant recipients to be treated with induction therapy over the past 10 – 15 years. In 1999, approximately 13% of liver transplant recipients received induction therapy with less than 2% receiving a polyclonal agent (Thymoglobulin® or ATGAM). By 2008, nearly 30% of liver transplant recipients received induction therapy with approximately 14% receiving a polyclonal, T cell depleting agent (Thymoglobulin® or ATGAM). Interest in using Thymoglobulin® has been driven by multiple motivations including the desire to reduce or even eliminate exposure to corticosteroids and to delay and / or minimize exposure to CNIs in an attempt to reduce nephrotoxicity and optimize short and long-term renal function. Hence, T cell depletion is already an accepted approach to IS in liver transplant patients.

For this trial, we have chosen to administer a course of Thymoglobulin® within 72 hours after transplantation with the goal of achieving adequate lymphocyte depletion. We have chosen a dose range, 3.0 – 4.5 mg/kg that balances the desire for depletion with hematologic side effects of Thymoglobulin® administration. This dose range has been shown to be as effective at depleting T cells as compared to the more common 6 mg/kg dosing (Wong, 2008). Moreover, immunologic outcomes appear to be indistinguishable between dose ranges (Wong, 2008). Dose dependent leukopenia and thrombocytopenia will be minimized by using lower doses, which will be particularly important for liver transplant patients who have depressed leukocyte and platelet counts at baseline.

5.2.1 Formulation, Packaging, and Labeling

Thymoglobulin® is a purified, preservative-free, pasteurized, gamma immune globulin, obtained by immunization of rabbits with human thymocytes. Thymoglobulin® is available as sterile, lyophilized powder to be reconstituted with sterile water for Injection (SWFI). Each package contains a 10 mL vial of freeze-dried Thymoglobulin® (25 mg) NDC# 58468-

0080-1. Each 10 mL vial contains 25 mg anti-thymocyte globulin (rabbit) as well as 50 mg glycine, 50 mg mannitol, and 10 mg sodium chloride. After reconstitution with 5 mL SWFI, each vial of reconstituted product contains approximately 5 mg/mL of Thymoglobulin®, of which >90% is rabbit gamma immune globulin (IgG). The reconstituted solution has a pH of 6.5 - 7.2.

Vials should be stored in a refrigerator at 2°C to 8°C (36°F to 46°F) and protected from light. The vials should not be frozen and should not be used after the expiration date indicated on the label. Reconstituted Thymoglobulin® is physically and chemically stable for up to 24 hours at room temperature; however, room temperature storage is not recommended. As Thymoglobulin® contains no preservatives; the reconstituted product should be used immediately. Any unused drug remaining after infusion must be discarded.

5.2.2 Dosage, Preparation, and Administration (Package Insert)

Reconstitution

After calculating the number of vials needed, using aseptic technique, reconstitute each vial of Thymoglobulin® with 5 mL of SWFI.

1. Allow Thymoglobulin® vials to reach room temperature before reconstituting the lyophilized product.
2. Aseptically remove caps to expose rubber stoppers.
3. Clean stoppers with germicidal or alcohol swab.
4. Aseptically reconstitute each vial of Thymoglobulin® lyophilized powder with the 5 mL of SWFI.
5. Rotate vial gently until powder is completely dissolved. Each reconstituted vial contains 25 mg or 5 mg/mL of Thymoglobulin®.
6. Inspect solution for particulate matter after reconstitution. Should some particulate matter remain, continue to gently rotate the vial until no particulate matter is visible. If particulate matter persists, discard this vial.

Dilution

1. Transfer the contents of the calculated number of Thymoglobulin® vials into the bag of infusion solution (saline or dextrose). Recommended volume: per one vial of Thymoglobulin® use 50 mL of infusion solution (total volume usually between 50 to 500 mL).
2. Mix the solution by inverting the bag gently only once or twice

Dosage and Administration

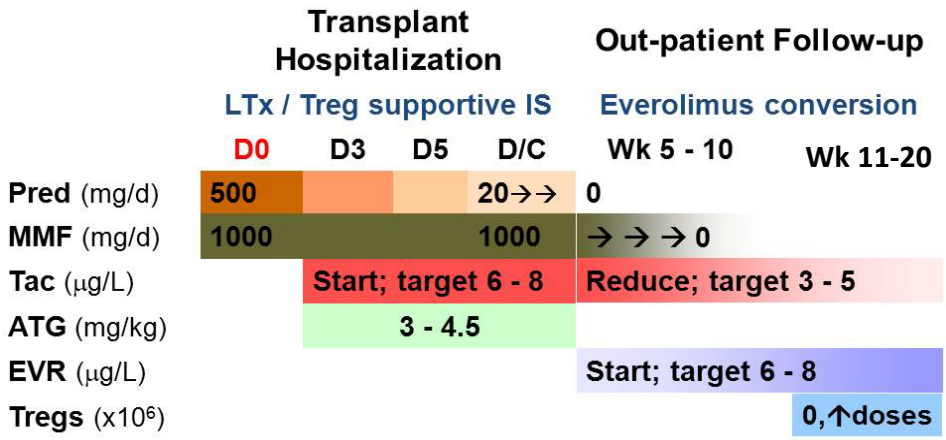
The recommended dosage of Thymoglobulin® for treatment of acute renal graft rejection is 1.5 mg/kg of body weight administered daily for 7 to 14 days.

The recommended route of administration is intravenous infusion using a high-flow vein. Thymoglobulin® should be infused over a minimum of 6 hours for the first infusion and over at least 4 hours on subsequent days of therapy. Thymoglobulin® should be administered through an in-line 0.22 micrometer filter.

5.2.3 Thymoglobulin® Study Dosing and Administration

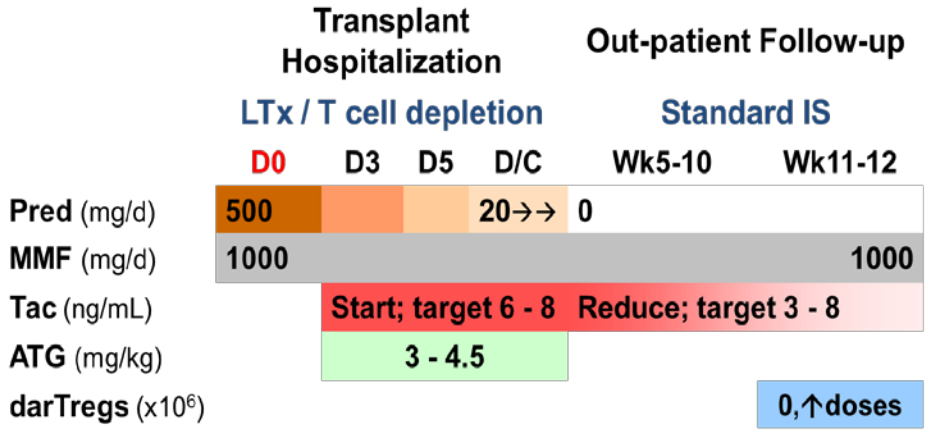
For this study, subjects will be given a dose range of 3.0-4.5 mg/kg total. Subjects who meet eligibility criteria for Thymoglobulin® will be given the first dose no later than 72 hours after transplantation. For transplants occurring during overnight hours, the first dose might begin on Day 2.

The first and second doses of Thymoglobulin® must be 0.75 mg/kg administered over 12 hours. Additional doses of 0.75 -1.5 mg/kg IV will be administered over 6-12 hours, or at the site investigator's discretion, until CD3 count is less than or equal to 50 cells/µL (50 cells/mcL) or total lymphocyte count is less than or equal to 0.10 x 10^9/L when CD3 count is unavailable; or when the maximal dose of 4.5/mg/kg has been given.



Note: If Myfortic is used, dosing should be 360 mg BID.

Figure 9. EVR-based Regimen after Thymoglobulin®



Note: If Myfortic is used, dosing should be 360 mg BID.

Figure 10. TAC-based Regimen after Thymoglobulin®

5.3 Drug Accountability

The investigator will maintain adequate records of the disposition of the darTreg products and Thymoglobulin®, including the date, lot ID, and dose of the product, dose received, name of the recipient (participant-by-participant accounting), and a detailed accounting of any product that is accidentally or deliberately destroyed. Used vials of Thymoglobulin will be maintained on site until drug reconciliation. No Thymoglobulin vial may be destroyed without the expressed approval of study sponsor.

Records for receipt, storage, use, and disposition will be maintained by the study site. A dispensing log will be kept current for each participant. All records regarding the disposition of the investigational product will be available for inspection.

5.4 Toxicity Prevention and Management (Dose Escalation Plan)

5.4.1 Overview

Eligible patients will receive either no darTreg infusion (a total of 6 subjects, 3 at each clinical site; Cohorts 1a and 1b) or a single infusion of darTregs at one of 3 dose levels: 50, 200, and 800 million (Cohorts 2, 3, and 4). Three patients will be planned for each darTreg dose, with the possibility of expanding to 6 patients for Cohorts 2 and 3 as required; the last dosing group will have 6 patients.

Progression from one group to the next will be based on the cumulative frequency and severity of specific AEs as described in 14.8.

5.4.2 Enrollment Pace and Dynamics

5.4.2.1. Cohort 1

Subject enrollment will take place in a staggered fashion (Figure 11. Staggered Enrollment). Center 1 will enroll the first group of 3 participants (Cohort 1a) that will receive Thymoglobulin®+EVR IS but will not receive darTregs. Upon completion of enrollment and at least 12 weeks of observation for the last participant in Cohort 1a, and in the absence of any safety signals, enrollment will proceed in Cohort 2 (darTreg Dose A). Enrollment into Cohort 2 from participating centers will not begin until enrollment in cohort 1b is complete.

5.4.2.2. Cohorts 2, 3, and 4

For Cohorts 2, 3, and 4 that will receive escalating darTreg doses, the first subject at each dose will be monitored for 4 weeks after darTreg infusion to ensure that there are no safety signals *(Section 14.8*). Subsequently, a 4-week observation period between darTreg infusions for the second and third subjects in the same cohort and between the last subject in one cohort and the first darTreg infusion in the next cohort is recommended but not mandatory. If the expected time interval between darTreg infusions is less than two weeks, the PI and NIAID medical monitor will review the status of the study and determine whether Thymoglobulin® should be initiated, Progression from one cohort to the next will depend on the cumulative incidence of sentinel AEs as described in *Section 14.8*.

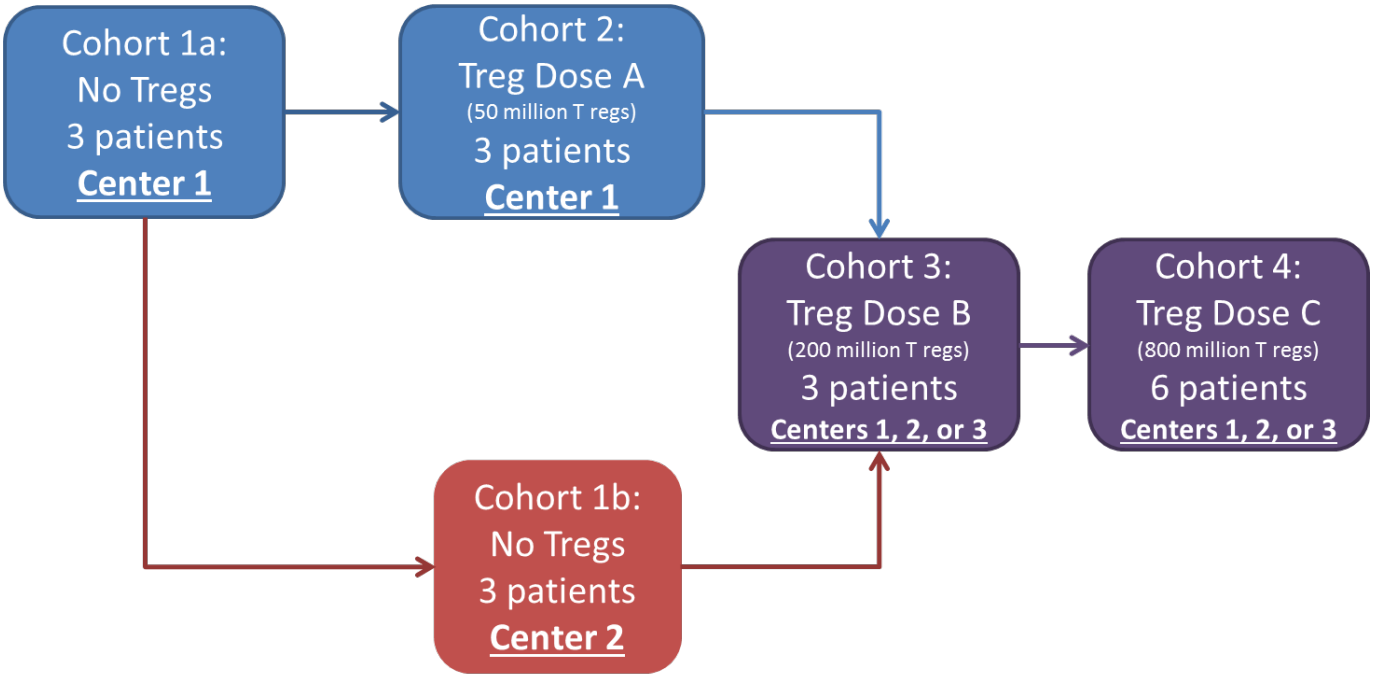


Figure 11. Staggered Enrollment

5.4.3 Rationale for Staggering Intervals

All participants in Cohort 1a – those who receive Treg supportive IS alone - must be monitored for 12 weeks prior to enrollment of Cohort 2 and Cohort 1B by the second clinical site. The rationale for selecting 12 weeks is that this interval covers the time period that has the highest incidence of important post-liver transplant complications such as rejection and infection, particularly bacterial and fungal infections related to the transplant operation including those directly related to the transplant incision / wound.

Within each darTreg dosing cohort, there is a mandatory four-week observation period after the administration of darTregs to the 1st patient of each cohort before the 2nd patient can receive darTregs. We have selected a shorter observation time interval for several reasons. First, darTregs will be administered sometime between Week 11-20 after transplant. This is a period of relative clinical quiescence that will readily permit optimal identification of AEs that may be precipitated by the darTreg infusion. Liver transplant recipients have largely recovered from the transplant operation as well as from the baseline liver disease. They continue on anti-viral (until 6 months after transplantation) and anti-

pneumocystis prophylaxis (until at least 12 months after transplantation) such that the frequency of these infections is very low. Second, we anticipate that the majority of complications associated with darTreg administration (infusion reaction, AR, or infection) are most likely to occur early, soon after administration while the beneficial effect of darTreg administration may well evolve and/or intensify over time. Infection may be precipitated by contamination of the darTreg preparation with infectious agents or with non-donor-alloantigen-reactive Tregs that lead to generalized over-immunosuppression. Similarly, rejection may be precipitated by contamination of the darTreg preparation with T effector cells. In these cases, the deleterious impact of darTreg infusion is expected to manifest soon – within 4 weeks – after infusion. While malignant transformation of infused cells is a concern, the timeframe of this complication as well as its manner of presentation is certainly difficult if not impossible to predict and therefore, this safety concern cannot be mitigated by any sort of mandatory waiting period.

5.4.4 Management Plan for Out of Range darTreg Doses

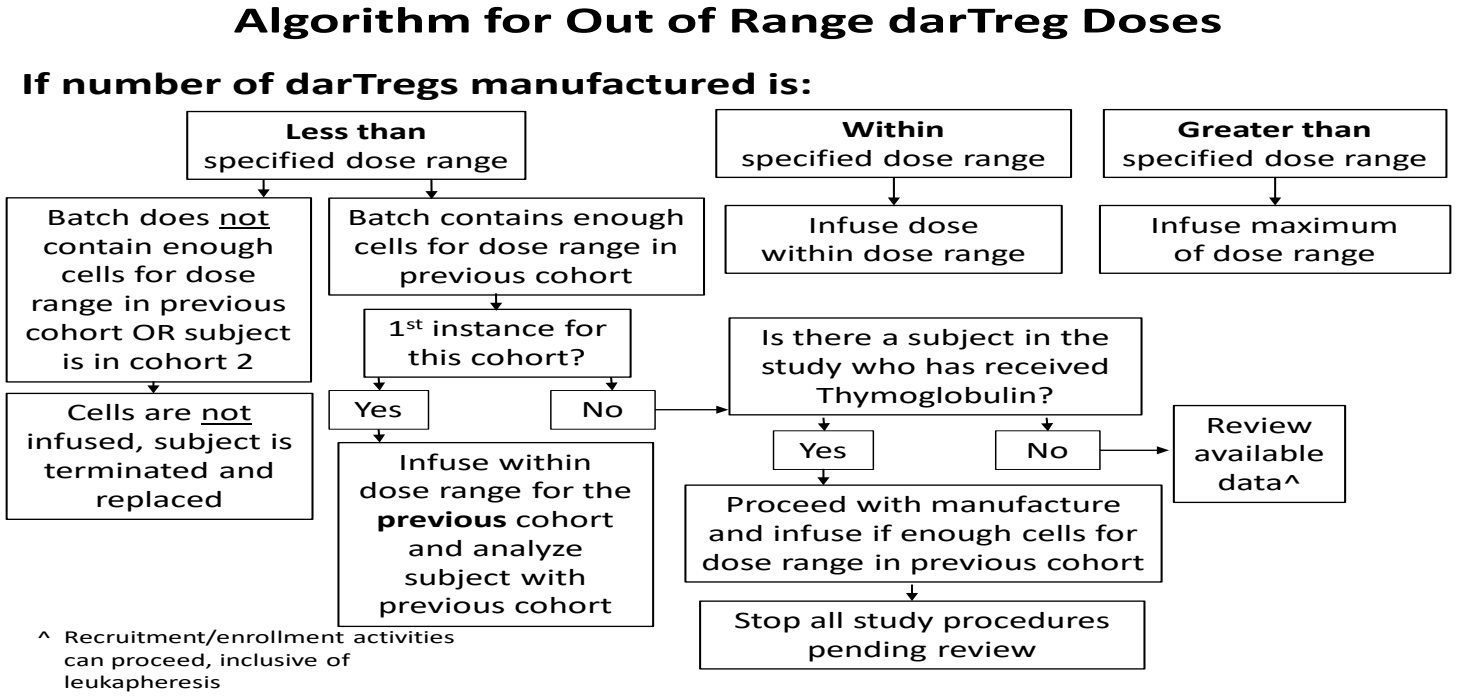
For subjects in cohort 2, any product with less than 25 million darTregs will not be infused and subject will be replaced. In cohort 3, products falling short of defined dose range for the cohort will be infused up to maximum of range for cohort 2 in up to 2 subjects, with the subject(s) contributing to cohort 2. The UCSF HICTF, NIAID MM, and study PI will review available information after the first instance of the dose falling short of target range, and again in the event of a second product not meeting the specified dose. One subject will be allowed to receive study directed Thymoglobulin under circumstances described. Recruitment and enrollment activities can continue during this time. If a third instance of lower-than-expected dose is manufactured, the study will be paused and DSMB will review available data prior to the trial proceeding.

If a higher dose of darTregs is produced than specified for a cohort, the maximum defined dose for that cohort will be packaged in the product and infused. Cells not infused will be used for research.

Table 2. RTB-002/delta Dose Ranges

	Target Dose for Cohort	Dose Range
Cohort 2	50 million cells	25-60 million cells
Cohort 3	200 million cells	100-240 million cells
Cohort 4	800 million cells	400-960 million cells

Figure 12. Algorithm for Out of Range darTreg Doses



6. Other Medications

6.1 Immunosuppressive Medications

6.1.1 Zortress® (Everolimus)

For this study, EVR will be used in combination with TAC following taper and cessation of corticosteroids (Figure 9. EVR-based Regimen after Thymoglobulin®). The suggested starting dose for this trial is 1.0–1.5 mg bid since our target trough level is 6 to 8 µg/mL. This is based on data from the de novo liver transplant EVR trial. Participants who had a trough level of 6-8 µg/mL required a mean (median, standard deviation) daily dose of 4.4 (4.0, 1.75) mg. Subjects meeting eligibility criteria for EVR conversion (Screen C2) will begin EVR no sooner than 30 days after liver transplantation and no later than 44 days after transplantation; with target trough levels of 6-8 µg/L. EVR target trough levels will be further reduced to 4-6 µg/L 24 – 26 weeks after transplantation.

6.1.2 Tacrolimus

Tacrolimus will be initiated within 72 hours of liver transplantation, targeting 12 hour trough levels of 6-8 µg/L. At 5-6 weeks after transplantation, after EVR is initiated in eligible subjects, the TAC target 12 hour trough levels will be decreased to 3-5 µg/L (Figure 9. EVR-based Regimen after Thymoglobulin®). Subjects ineligible for EVR conversion will remain on TAC with target levels of 3-8 µg/L with MMF (Figure 10. TAC-based Regimen after Thymoglobulin®).

Inability to tolerate TAC leading to discontinuation or conversion to cyclosporine will result in early termination from the clinical trial and reversion to standard of care IS.

6.1.3 MMF

MMF will be initiated within 24 hours of transplantation. If Cellcept is used, then the dosing will be 500 mg twice daily intravenously or by mouth. If Myfortic is used, then the dosing should be 360 mg twice daily by mouth. For subjects who convert to EVR, MMF must be discontinued as soon as target EVR trough levels have been achieved. Subjects ineligible for EVR conversion will remain on Cellcept or Myfortic.

6.1.4 Prednisone

Solumedrol 500 mg will be given intravenously on the day of transplantation. Additional Solumedrol will be prescribed according to center standard of care. Oral prednisone should be initiated once oral medication is tolerated. Steroid doses may be further tapered on an individualized basis depending on the clinical status of patient and function of graft.
Corticosteroids must be discontinued by day 44 after transplant. For subjects eligible for EVR, corticosteroids must be discontinued prior to initiating EVR.

6.1.5 Other permitted concomitant medications

Other non-immunosuppressive concomitant medications used as standard of care in the management of the liver transplant subject (e.g. cholesterol lowering medications, blood pressure lowering medications, etc.) not specifically described in *Section 6.3. Prohibited Medications* are acceptable in the study.

6.2 Prophylactic Medications

6.2.1 Recommended Pre-Medications for Thymoglobulin®

Solumedrol 500 mg IV is recommended prior to first dose of Thymoglobulin®; and Solumedrol 250 mg IV prior to the second dose of Thymoglobulin®. Corticosteroid dosing prior to subsequent doses of Thymoglobulin® will be at the site investigator's discretion. Acetaminophen 500 mg PO and diphenhydramine 50 mg IV 1 hour prior to first and second doses of Thymoglobulin® is also recommended. At the site investigator's discretion, acetaminophen and diphenhydramine can be continued every 6 hours around the clock to cover the timeframe of the first two infusions.

6.2.2 Pre-Medications for darTreg Infusion

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

6.2.3 Anti-Infective Prophylaxis Post-Transplantation

Intravenous gancyclovir and/or oral Valcyte will be administered for the prophylaxis of CMV and EBV for at least six months after liver transplantation. Participants should receive center standard of care prophylaxis for candida infection until discontinuation of corticosteroids and pneumocystis infection for the duration of the study.

6.3 Vaccinations

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

6.4 Prohibited Medications

Cyclosporine cannot be used as an alternative to tacrolimus in this trial. The indication for use of EVR in liver transplantation for the primary prophylaxis of AR is in combination with tacrolimus.

IS medications other than those specified in the protocol are similarly disallowed. These include, but are not limited to azathioprine, basiliximab, alemtuzumab, sirolimus, rituximab, intravenous immune globulin, and belatacept.

The use of live vaccines will be proscribed during the follow up period, as per standard of care for liver transplant recipients. Examples include (but are not limited to) the following: intranasal influenza, measles, mumps, rubella, oral polio, BCG, yellow fever, varicella, and TY21a typhoid vaccines.

Grapefruit and grapefruit juice inhibit cytochrome P450 3A4 and P-gp activity and should therefore be avoided with concomitant use of EVR. Patients with rare hereditary problems of galactose intolerance, the Lapp lactase deficiency or glucose-galactose mal absorption should not take EVR as this may result in diarrhea and malabsorption.

Administration of medications known to interact with tacrolimus and/or EVR is allowed but tacrolimus and EVR levels should be carefully monitored and dosing titrated to maintain the target levels to minimize toxicity while maintaining efficacy.

7. Premature Discontinuation of Investigational Therapy

7.1 Premature Discontinuation of Thymoglobulin and/or Treg Supportive IS Regimen

Study IS regimen may be prematurely discontinued for any participant for any of the following reasons:

1. Inability to tolerate at least 3 mg/kg Thymoglobulin® or inability to deplete CD3 count to less than or equal to 50µ/L (50/mcL) or total lymphocyte count to less than equal to 0.1 x 109/L after study Thymoglobulin® administration
2. Inability to tolerate TAC- or EVR-based maintenance IS
3. The diagnosis of severe AR (Banff criteria) at any time after transplantation
4. The diagnosis of early or late CR (Banff criteria) at any time after transplantation
5. The occurrence of steroid refractory rejection at any time after transplantation
6. Poor compliance with study procedures.
7. Investigator decision that the study IS regimen and/or future study treatment is no longer in the best interest of the participant.
8. Failure to manufacture and supply the cellular product two times for the same subject

There are a myriad of diagnoses that might lead an investigator to determine that the study IS regimen and/or darTreg infusion is no longer in the best interest of the participant. While there are likely an infinite number of specific reasons, general reasons include severe, life-threatening infections, malignancy (excluding skin cancer), GvHD, pneumonitis, severe renal dysfunction / proteinuria, serious medical co-morbidity such as myocardial infarction, and logistical issues including patient relocation and record of non-compliance.

The (Data Safety Monitoring Board) DSMB will be provided with information about cases of premature discontinuation occurring in any stage. Subjects who have prematurely discontinued the study regimen will enter into a schedule of events (SOE) of reduced monitoring intensity for purpose of safety (Please refer to *Section 13.4 and Safety Follow-Up SOE*)

7.2 Premature Discontinuation of darTreg Infusion

darTreg infusion will be stopped and will not be restarted if there is a hypersensitivity reaction, a CTCAE Grade ≥3 infusion-related reaction, a CTCAE Grade ≥3 CRS, or any other infusion related serious adverse event.

8. Study Mandated Procedures

8.1 Leukapheresis or Whole Blood Draw for PBMC Collection

Leukapheresis may be necessary to ensure collection of adequate numbers of autologous Tregs to support ex vivo expansion of darTregs for infusion after liver transplantation, particularly for those enrolled in Cohorts 3 and 4. The study team will make a decision in the best interest of a particular subject based on the current clinical picture including assigned study cohort, peripheral Tregs in circulation, and hemoglobin level. If a subject has a hemoglobin level greater than 10 gm/dL, he or she will have the option to undergo phlebotomy. If the patient has a hemoglobin level less than or equal to 10 gm/dL and remains eligible for the study, the patient will undergo leukapheresis. Number of Tregs in peripheral circulation will also guide decision for method of PBMC collection (leukapheresis or whole blood) where subjects should have at least 30/µL peripheral blood Tregs for whole blood collection. For subjects to have whole blood draw, 450-500ml whole blood will be collected.

8.2 Blood draws

Blood draws are necessary to carefully and frequently evaluate allograft function after liver transplantation as well as after darTreg infusion. Liver tests are the most reliable marker for post-transplant complications including AR. Finally, blood draws will also be done to enable the planned mechanistic studies aimed at delineating the presence and impact of infused darTregs.

8.3 Liver biopsies

For all subjects, local pathology information will be obtained for assessment of eligibility criteria B (no central pathology assessment). If a pathology report is unavailable from donor records, a liver biopsy will be obtained on the back table.

For patients enrolled in Cohort 1 (either 1a or 1b), there will be a second protocol liver biopsy performed 12 weeks after transplantation for central laboratory assessments.

For subjects enrolled in Cohorts 2, 3, or 4, there are two additional protocol biopsies after the initial, back table biopsy. The second protocol liver biopsy will be obtained approximately 10-14 weeks after transplantation, and approximately 7 – 10 days prior to darTreg infusion to ensure that darTregs will only be infused in the context of a normal allograft with essentially normal allograft histology and the absence of any diagnostic criteria for AR or CR. Eligibility criteria D will be assessed with local pathology information. However, slides and additional tissue will be collected for a central reading and mechanistic assays. The darTregs eligibility biopsy does not have to be repeated if a second preparation of darTregs is manufactured and infusion of the product is delayed. The third and last protocol liver biopsy for Cohorts 2, 3, or 4 will be one week after darTreg infusion for central laboratory assessments.

There will be up to two passes for each protocol biopsy. Up to one additional pass might be required during clinically indicated (standard of care) biopsies to obtain adequate tissue for the planned mechanistic studies. The same set of specimens will be collected for each 16g biopsy (protocol driven or clinically indicated/for cause) in the following priority: 1) 1.5 cm fixed in formalin for histopathology; 2) 1.5-2.0 cm in buffered saline for detection of deuterium labeled cells; 3) 0.5-1cm in RNALater for gene expression and other mechanistic studies.

9. Known and Potential Risks and Benefits to Participants

9.1 Risks of Thymoglobulin® (Anti-Thymocyte Globulin – Rabbit)

Risks associated with Thymoglobulin® include immune-mediated reactions, infections, reactivation of infection, malaise, dizziness, sepsis, thrombocytopenia, neutropenia, and increased incidence of malignancies. Occasional reactions are observed at the infusion site including pain, swelling and erythema.

Immune-mediated reactions

In rare instances, serious immune-mediated reactions have been reported with the use of Thymoglobulin and consist of anaphylaxis or severe cytokine release syndrome (CRS). Very rarely, fatal anaphylaxis has been reported. If an anaphylactic reaction occurs, the infusion should be terminated immediately. Medical personnel should be available to treat patients who experience anaphylaxis. Emergency treatment such as 0.3 mL to 0.5 mL aqueous epinephrine (1:1000 dilution) subcutaneously and other resuscitative measures including oxygen, intravenous fluids, antihistamines, corticosteroids, pressor amines, and airway management, as clinically indicated, should be provided. Thymoglobulin or other rabbit immunoglobulins should not be administered again for such patients. Severe, acute infusion-associated reactions (IARs) are consistent with CRS attributed to the release of cytokines by activated monocytes and lymphocytes. In rare instances, these reported reactions are associated with serious cardiorespiratory events and/or death. Thymoglobulin contains a mixture primarily of antibodies to T cell antigens, but it is largely unknown which specificities mediate the alteration in immunoregulation. Thymoglobulin may potentially contain or promote undesired or harmful antibody specificities, but which may be difficult to predict, identify or to exclude. Live vaccines should not be administered to patients about to receive, receiving, or after treatment with Thymoglobulin. Concomitant administration of Thymoglobulin with live virus vaccines carries a potential of uncontrolled viral replication in the immunosuppressed patient. There is insufficient information to fully define the extent of the risk, or the period of time during which the risk exists. If administered, live viruses may interfere with Thymoglobulin treatment. Skin testing is not advised prior to Thymoglobulin administration.

Infection

Thymoglobulin® is routinely used in combination with other IS agents. Infections (bacterial, fungal, viral and protozoal), reactivation of infection (particularly CMV) and sepsis have been reported after Thymoglobulin® administration in combination with multiple IS agents. Severe acute infections can be fatal.

Malignancy

Use of IS agents, including Thymoglobulin®, may increase the incidence of malignancies, including lymphoma or post-transplant lymphoproliferative disease (PTLD).

Carcinogenesis, Mutagenesis, Impairment of Fertility

The carcinogenic and mutagenic potential of Thymoglobulin® and its potential to impair fertility have not been studied.

Hematologic Effects

Thrombocytopenia and/or leukopenia (including lymphopenia and neutropenia) have been identified and are reversible following dose adjustments. When thrombocytopenia and/or leukopenia are not part of the underlying disease or associated with the condition for which Thymoglobulin is being administered, the following dose reductions are suggested:

- A reduction in dosage must be considered if the platelet count is between 50,000 and 75,000 cells/mm3 or if the white blood cell count is between 2,000 and 3,000 cells/mm3;
- Stopping Thymoglobulin treatment should be considered if persistent and severe thrombocytopenia (< 50,000 cells/mm3) occurs or leukopenia (< 2,000 cells/mm3) develops.

White blood cell and platelet counts should be monitored during and after Thymoglobulin therapy

Pregnancy: Pregnancy Category C

Animal reproduction studies have not been conducted with Thymoglobulin®. It is also not known whether Thymoglobulin® can cause fetal harm or can affect reproduction capacity. Thymoglobulin® should be given to a pregnant woman only if

clearly needed. Pregnancy testing will be routinely performed in female patients pre-transplant. Although extremely unlikely, any pregnant patient will be excluded from the study.

9.2 Risks of Everolimus, TAC, MMF, and Prednisone

TAC, MMF, and prednisone are considered standard of care maintenance IS medications in liver transplantation. EVR is not as widely used in liver transplantation but recently received an FDA-approved indication for liver transplantation. The risks for dose related toxicities and side effects from these immunosuppressive medications are not different for subjects in this trial. However, the reduced dosing or target trough levels in conjunction with other agents in the Treg-supportive IS regimen may expose subjects to an increased risk of rejection.

9.3 Risks of darTreg Infusion

9.3.1 Risks of autologous ex vivo expanded polyclonal Treg infusion

Although polyclonal Treg infusion has been innocuous in animal models, there is currently scant experience in humans. Three Treg therapy trials in GvHD have been reported. The first-in-man trial by Trzonkowski et al involved two patients (Trzonkowski, 2009). The first patient had chronic GvHD two years after transplantation. After receiving 0.1 x 10^6/Kg flow sorted ex vivo expanded Tregs from the donor, the symptoms subsided and the patient was successfully withdrawn from IS. The second patient had acute GvHD disease that progressed despite three infusions with an accumulative dose of 3 x 10^6/Kg expanded donor Tregs. A larger scale phase I trial led by Brunstein et al (Brunstein, 2010) enrolled twenty three patients with advanced hematologic malignancy. The patients were 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 samples that had 4 to 6 HLA match with the recipient. Up to 6 x 10^6/Kg Tregs were infused after ex vivo expansion using anti-CD3 and anti-CD28 conjugated beads. The infused Tregs were detectable in circulation for up to 7 days. During the one-year period after Treg infusion, the investigators observed no DLTs and AEs when compared to historical controls. Incidences of acute and chronic GvHD were reduced in patients received Treg therapy. The third trial enrolled 28 patients with high-risk hematological malignancies (Di Ianni, 2011). Patients received anti-CD25 immunomagnetic bead enriched donor Tregs without ex vivo expansion four days before receiving one haplo-mismatched hematopoietic stem cell and Tconv transplants from the same donors. A majority of the patients received 2 x 10^6/Kg Tregs with 1 x 10^6/Kg Tconvs and no adjunct IS was given after transplant. Patients demonstrated accelerated immune reconstitution, reduced CMV reactivation, low incidence of tumor relapse and GvHD. Collectively, these studies show that Treg therapy has minimal toxicity in the setting of GvHD.

Recently, a phase I/II study applying polyclonally expanded FACS purified Tregs to type diabetic patients has been reported by the Trzonkowski group (Marek-Trzonkowska N, 2012). The study enrolled 10 type 1 diabetic children (aged 8-16 years) within 2 months after diagnosis. Four patients received 10 × 10^6 Tregs/kg body wt and the remaining 6 patients received 20 × 10^6 Tregs/kg body wt. The patients were followed for 4-5 months after Treg infusion and no toxicity of the therapy was noted. The authors concluded that Treg therapy was safe and well tolerated in children.

At UCSF, a phase I Treg dose-escalating trial evaluating safety of Treg therapy in type 1 diabetic patients is ongoing. As of January 23, 2013, 10 enrolled subjects have received a single infusion of Tregs. Three subjects were treated in the first dosing cohort with 0.05x10^8 cells, three subjects were treated in the second dosing cohort with 0.4x10^8 cells, and four subjects were treated in the third dosing cohort with 3.2x10^8 cells. Notably, two of these 4 subjects were enrolled at treated at Yale (New Haven, Connecticut). Thirty-four AEs have been reported in these subjects. All 6 subjects in Cohorts 1 and 2 had at least one adverse event. Twenty-eight events were judged as mild in severity, three were judged as moderate, and three were judged as severe. Sixteen were judged to be possibly related, four unlikely related and 14 unrelated to study therapy. No SAEs have been reported. No events that would disallow dose escalation have occurred. The Data Safety and Monitoring Board will be reviewing the data in accordance with the protocol plan to determine if the study can proceed to enroll the final cohort that will receive a cell dose of 26 x10^8 cells in four subjects.

9.3.2 Risks of autologous ex vivo expanded darTregs infusion

There is limited experience in humans using the darTreg product. Because infusion of *ex vivo* explanded effector T cells has been reported to be associated with infusion reactions, recipients of arTregs should be closely monitored after infusion

for possible infusion reactions including nausea, hypotension, fevers, chills, and pain. Since the arTreg product is immunosuppressive, possible side effects include over-immunosuppression with increased risks of infections and difficulty clearing infections.

A safety concern of Treg infusion is potential impairment of immune responses to pathogens and/or malignancies. darTregs utilized in this trial are unlikely to possess the full breadth of specificities offered by non-specific, polyclonal Treg pool. Moreover, animal studies suggest that adoptive transfer of alloantigen-specific Tregs does not alter immune responses to viral pathogens (Bushell, 2005). The infusion of Tregs could theoretically impair anti-tumor immunity, which could be of particular concern in transplant recipients at increased risk of PTLD and/or recurrent HCC. With the infusion of darTregs, there is a theoretical possibility that donor reactive T effector cells may be infused and contribute to rejection.

Lastly, an ancillary reagent used in the manufacturing of darTreg is a CD40L-expressing cell line, KT64.CD40L.HLADR0401, which is derived from the human myelogenous leukemia cell line K562. These cells have been irradiated 10,000 rads (5 times the lethal dose); they are therefore not expected to persist in darTreg cultures. To further prevent the infusion of replication competent K562 cells along with the darTregs, the final darTreg product must meet a release criterion of a negative assay for the BCR-ABL translocation, a genetic marker identifying K562 cells. The risk of transmission of myelogenous leukemia in the Treg product is considered to be negligible.

Possible adverse reactions include, but are not limited to, abdominal cramps, anaphylaxis, back pain, chest tightness/pain, and/or chills at the time of infusion, fever ≤38.5^{o}C, flashing, heat/pain at the IV site, hemoglobinuria, hives, mild rigors, nausea, headache, oliguria, pruritus, sever rigors, syncope, tachycardia, vomiting, and wheezing.

9.4 Risks of Study Mandated Procedures

9.4.1 Risks of Leukapheresis

The common risks of leukapheresis include bruising and discomfort at the site of needle placement, typically in the antecubital 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. Our inclusion/exclusion criteria specify a minimum acceptable platelet count for participants. Thrombosis and bleeding could theoretically occur, although they are rarely if ever observed.

9.4.2 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. The amount of blood that may be drawn from adult subjects for research purposes will not be more than 168 mL over an eight-week period. The additional amount of blood could contribute to the development of anemia. The subject's clinical condition will be taken into consideration to determine if research blood tests can be performed.

9.4.3 Risks of Liver Biopsy

Mild AEs resulting from 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 adverse event 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.

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).

9.5 Potential Benefits

This study provides no direct or immediate benefit to the participants. However, the results of the study could influence future care of transplant patients.

10. Study Visits

10.1 Stage 1: Screening, Enrollment and PBMC Collection

The first stage of the study encompasses screening A and enrollment. Participants will be selected from the liver transplant waiting lists according to the study eligibility criteria (Screen A). 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. The potential participant will sign an informed consent form before undergoing any screening or study procedures. 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 meets inclusion criteria and exclusion criteria A (*Section 4.2 and 4.3.1*).

Eligible and consented participants enrolled in Cohort 2, 3, or 4 will undergo leukapheresis (or blood draw option for Cohort 2) at any time prior to transplantation to isolate PBMCs, which will be cryopreserved for subsequent Treg purification and expansion. Participants who have detectable HBV DNA at the time of PBMC collection will be terminated from the trial. At the time of transplantation, donor material for these participants will be collected to make donor sBcs and banked to support future expansion of darTregs.

At the time of transplantation, participants will receive Solumedrol, 500 mg intravenously. Within 24 hours after transplantation, participants will be started on MMF in the form of either Cellcept 500mg bid intravenously or orally or Myfortic 360 mg bid by mouth. Within 72 hours after transplantation, participants will be prescribed tacrolimus to target 12-hour trough levels of 6 – 8 μg/L. Please refer to *Appendix 1. Recipient Schedule of Events (Stages 1 and 2)*. Note that collection of AEs and SAEs in Stage 1 are limited to events occurring within 72 hours after leukapheresis.

10.2 Stage 2: Thymoglobulin® and TAC- or EVR-based Maintenance IS

The second stage of the study begins after liver transplantation with administration of Thymoglobulin®. The first dose must be initiated within 72 hours of transplantation (*Appendix 1. Recipient Schedule of Events (Stages 1 and 2)*. Prior to Thymoglobulin® administration, enrollment inclusion criteria *(Section 4.2)* should be re-reviewed to ensure eligibility. In addition, exclusion criteria B *(Section 4.3.2)* should also be reviewed prior to administration of Thymoglobulin®. Note that collection of AEs and SAEs in Stage 2 begins with Thymoglobulin® administration.

Between 5-6 weeks after transplantation, participants who continue to meet study eligibility Screen C1 (Section 4.3.3) will be assessed by everolimus conversion criteria C2 (*Section 4.3.4*) will initiate conversion to an EVR-based IS regimen. EVR should be initiated at a dose of 1.0-1.5 mg bid, targeting 12 hour trough levels of 6-8 μg/L no earlier than 30 days after liver transplantation. As EVR is introduced, both MMF and tacrolimus dosing should be reduced. MMF should be completely discontinued when target EVR levels are achieved. Tacrolimus should be reduced to target 12-hour trough levels of 3-5 μg/L.

During Stage 2, study assessments will take place daily during administration of Thymoglobulin®. Starting at week 2, study assessments will occur weekly until 12 weeks after transplantation. Participants will have assessments at weeks 2, 3, 4, 6, 8, and 10 after transplantation. Participants enrolled in Cohorts 2, 3, and 4 must undergo an allograft biopsy between week 10-14 after transplantation as part of the eligibility determination for darTreg infusion (*Appendix 1. Recipient Schedule of Events (Stages 1 and 2)and Appendix 3)*.

Participants in Cohort 1 (1a and 1b) should undergo an allograft biopsy 12 weeks after transplantation.

10.3 Follow-up for Cohorts 1a and 1b

Participants enrolled in Cohorts 1a and 1b who will not receive darTreg infusion will follow Appendix 2. Recipient Schedule of Events (Cohort 1a and 1b) starting at week 12 after transplantation. Visits to the transplant center will take place at week 12, 16, 24 and 40 after transplantation. Laboratory assessments will take place every 4 weeks until the end of the study, 40 weeks after transplantation. Please refer to *Appendix 2. Recipient Schedule of Events (Cohort 1a and 1b Follow Up)*

10.4 Stage 3: darTreg Infusion for Cohorts 2, 3, and 4

Participants enrolled in Cohorts 2, 3, and 4 must undergo the following study procedures and visits. darTreg infusion will take place between weeks 11-20 after liver transplantation and will require an overnight stay. Screen D eligibility must be met. Blood for central laboratory assessments will be drawn prior to the infusion and 24 hours after the infusion is complete. Visits to the transplant center will take place 1 week and 4 weeks after darTreg infusion as well as 12 and 28 weeks after darTreg infusion (approximately 24 and 40 weeks after transplantation, respectively). A liver biopsy will be done in conjunction with the visit 1 week after darTreg infusion. Thereafter, laboratory assessments must take place every two weeks for 10 weeks, followed by every 4 weeks until the end of the study, 28 weeks after darTreg infusion, or 40 weeks after transplantation. Details regarding study procedures including monitoring, laboratory assessment, and blood draws for mechanistic studies are specified in ***Appendix 1. Recipient Schedule of Events (Stage 3: Cohorts 2, 3, and 4).***

10.5 Unscheduled Visits

Unscheduled or additional visits will typically occur secondary to standard of care management for liver transplant recipients or secondary to concern for allograft dysfunction when there are liver tests that are elevated above baseline. Abnormal liver tests should be assessed according to center standard of care protocols. A liver biopsy should be performed to confirm any clinical suspicion of rejection prior to the administration of any treatment for rejection. 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 LFT's). Blood for mechanistic assays are not required unless the unscheduled visit coincides with a scheduled visit with central laboratory assessments.

10.6 Safety Follow Up

Subjects who are in Stage 2 or 3 and discontinue the study prematurely will be followed according to the safety follow up SOE (*See Section 13.4*, *Appendix 4. Recipient Safety Follow Up (Early Termination from Stage 2)* and *Appendix 5. Recipient Safety Follow Up (Early Termination from Stage 3)*.

10.7 Visit Windows

Study visits should take place within the following time limits shown in *Table 2. Study Visit Windows*

Table 3. Study Visit Windows

Stage 1 and 2 Study Visits	Visit Window
Screen A	-6 Months
Visit 1: PBMC Collection for darTreg manufacturing	Prior to transplant
Visit 2: Transplantation	-24 hours Central labs can be drawn up to 1 year before solumedrol dose in OR
Screen B	-24 hours before first dose
Visits 3a, 3b, 4, 5a, 5b: Days 3, 4, and 5 after transplantation	None
Screen C and Visits 6-15, 15a, 15b: Weeks 2-13 after transplantation	± 2 days
Cohort 1 Follow Up Study Visits	Visit Window
Visits 16: Week 12 after transplantation	-14/+10 days
Visits 17-23: Months 4-9 after transplantation	± 5 days
Stage 3 Study Visits	Visit Window
Screen D	-10 days
Visit T0: Day of darTreg Infusion	None
Visit T2: Day 1 after darTreg Infusion	± 1 hour
Visit T3: Day 7 after darTreg Infusion	± 1 day
Visit T4-T5: Day 14 and 28 after darTreg Infusion	± 2 days
Visits T6-T13: Weeks 6, 8, 10, 12, 16, 20, 24 and 28 after darTreg Infusion	± 5 days
Safety Follow Up (Early Termination from Stage 2 or 3)	Visit Window
All visits	± 7 days

11. Mechanistic Assays

11.1 HLA Typing

DNA will be isolated from whole blood collected in a K2EDTA tube from both recipients and donors using the QIAmp Blood Mini Kit™ (Qiagen, Inc.). Automated DNA sequencing using both commercial (Abbott Molecular Inc., Illinois and QIAGEN Inc., CA) and in house reagents will be used to HLA type the samples. Loci sequenced will included HLA Class I (HLA-A, -B and -C) and HLA Class II (HLA-DRB1/3/4/5, -DQA1 and - DQB1). For Class I, exons 2 and 3 containing the defining polymorphic regions for most alleles will be amplified and sequenced. For Class II, typically only exon 2 will be sequenced. Any sequencing ambiguities will be resolved by sequencing additional exon(s).

11.2 T Cell Phenotype and Function

We will use MFC to profile leukocyte subpopulations, determine frequencies of donor-reactive T cells, assess donor-specific suppression by darTregs, and use deuterium labeling and TCR sequencing to track infused darTregs. Together, these assays allow us to assess the impact of darTregs therapy and/or Treg-supportive IS on the alloimmune profile of the patients.

Treg TruCount analysis: One ml of whole blood will be collected at the time points indicated in the SOE. The samples will be used directly for analysis without cryopreservation. Fifty microliters of the blood will be aliquoted into a TruCount tube, stained with fluorochrome-conjugated antibodies to CD4, 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 determine if the counts are altered by darTreg therapy.

Frequency of donor-reactive T cells: We will use the assay described in *Figure 13* to 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, especially in the 200 million and 800 million dose cohorts.

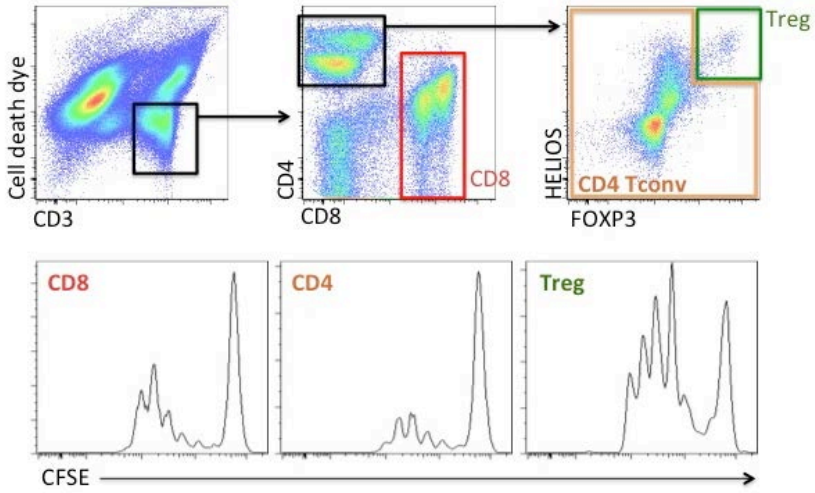


Figure 13. Assay for measuring frequency of donor-reactive T cells.

In vitro suppression: We will assess suppression by Tregs isolated from patients at time points indicated in the SOE. Pre-transplant 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 $CD4^+CD127^{lo/-}CD25^+$. PBMC for this assay will be obtained from whole blood on the day of transplantation. The cultures will be stimulated with irradiated donor splenocytes to assess donor-specific suppression and with anti-CD3 and anti-CD28 to assess nonspecific suppression.

MFC panels: We will use MFC to determine the percentages of leukocyte subsets in peripheral blood using panels of antibodies developed in our lab. Panels and markers are summarized in *Table 3. MFC Panel Markers.* Adjustments might be made if future data from other trials are able to better inform the panel selection.

Table 4. MFC Panel Markers

Panel	Cell #	Markers								
Leukocyte panel	0.25m	CD45	CD14	CD3	CD19	CD56	CD16	CD4	CD8	CD7
Effector/Memory/ Naïve T Cells	0.5m	CD3	CD4	CD8	CD45RA	CD27	CD28	CCR7		
Treg Panel	1m	CD3	CD4	CD25	CD127	FOXP3	HELIOS			
B cells	0.5m	DR	CD19	CD20	CD27	CD38	IgD			

11.3 Donor HLA Antibody

We will assess for the development of donor-specific antibodies as they have been implicated as a risk factor for acute rejection, chronic rejection, and the development of fibrosis (Musat, 2011) (Paterno, 2012) (Kaneku, 2012).

Serum samples collected from participants at time points after liver transplantation as specified in the SOE and will be evaluated for the presence or absence of donor-specific alloantibodies. Assessments will include donor-specific cross-matching with cryopreserved cells, testing against a panel of cryopreserved lymphocytes and/or HLA antigen-coated microparticles.

11.4 Histology and Multiplex Immunohistochemistry

In collaboration with Dr. A. Jake Demetris, we will perform extensive histology and mIHC analysis of protocol biopsy samples obtained prior to darTreg infusion and 1 week after darTreg infusion. The cohort that does not receive darTreg will get a single biopsy at 12 weeks after transplantation, the approximate time of darTreg infusion for the other groups. The design of these analyses will be guided by histology and mIHC data from tolerant patients in the Feng Immunosuppression Withdrawal trial and our current understanding of liver transplant tolerance. Histological analyses will evaluate 40 histopathological features to determine tissue integrity and degree of inflammation. mIHC analyses are summarized in *Table 4. mIHC Markers*.

Table 5. mIHC Markers

mIHC Panel	Rationale
C4d/CD31	Decrease in C4d deposits on the hepatic microvasculature is associated with liver transplant tolerance. Determine if Treg therapy leads to decrease in C4d deposits
CD3/γδ -1/ γδ -2	Portal tract ratio of γδ -1/ γδ -2 >1.0 is associated with operational tolerance. Determine if Treg therapy promotes this signature
CD3/CD45RO/CD45RA	Monitor the relative ratio of naïve to memory T cells; test the hypothesis that Treg therapy leads to a reduction in portal-based CD3+/CD45RO+ (memory) T cells
CD4/Tbet/GATA-3/IL-17/FoxP3	Monitor the polarization of CD4+ lymphocytes within the allograft to determine whether an increase of putative Tregs contributes to tolerance.
IL10/TGFβ/HLADR	Monitor expression of immunomodulatory cytokines by HLA-DR expressing cells in the liver such as Kupffer and B cells.
CK19/CD31/HLADR	Up-regulation of HLA-DR on biliary epithelium (CK19+) and vascular endothelium (CD31+) makes these cells targets of immune rejection. Determine if Treg therapy prevents DR induction.

11.5 Deuterium Labeling and Detection

Our clinical trial relies on transferring darTregs that have been expanded hundreds-of-fold *in vitro*. These cells will be indistinguishable from endogenous Tregs by standard surface markers; therefore we will not be able to distinguish administered darTregs from endogenous Tregs. Because the cells are being expanded prior to infusion, it provides a unique opportunity to label the cells prior to infusion to monitor engraftment and persistence of the administered darTregs. 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 (Klein PD, 1986) (Koletzko B1, 1997), and they have been safely used as cellular, molecular, and metabolic markers in patients and healthy controls for more than 6 decades (Klein PD, 1986) (Koletzko B1, 1997).

In Protocol RTB-002, Tregs from transplant patients will be labeled 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 14) prior to infusion in the patient. Based on data from *in vitro* labeling studies, we expect the cells to be ~60% enriched for ^{2}H. After infusion, PBMCs will be isolated and stored at specified time points as described in the SOE. DNA prepared from these samples will be analyzed by gas chromatography-mass spectrometry. Based on prior studies, we expect sensitivities in the range of approximately ±0.05 to 0.10% enrichment (which refers to the fraction of labeled molecules) (Busch, 2007) (Macallan, 1998). As detailed in *Section 1.2.2*, we expect the patients to have about 5 x 10^{9} endogenous Tregs 10 to 13 weeks after Thymoglobulin® treatment. Therefore, an infusion of 200 and 800 x 10^{6} darTreg in the higher dose groups will likely result in a detectable increase in overall Treg numbers, detectable by TruCount (BD Biosciences) and MFC assays (Section 10.2) at early time points. Deuterium labeling will allow investigators to distinguish between infused darTregs and endogenous Tregs, thus unequivocally demonstrate the presence of infused darTregs in the periphery. Similarly, presence of deuterium-labeled Tregs in the graft will be determined by analyzing genomic DNA extracted from FACS-purified $CD4^{+}$ cells isolated from biopsy tissues collected at 1 week after darTreg infusion and at the time of rejection. Deuterium labeling will be detectable for at least 4 cell divisions of the administered darTregs. We expect this method of detection to be especially advantageous at early time points (up to 28 days) post infusion, prior to significant division, exit into tissues, and/or loss of darTregs.

11.6 TCR Sequencing

Recent advances in sequencing technologies have led to PCR based strategies to detect rare T cell clones based on a unique TCR sequence. Sensitivities of up to 1 in 100,000 cells have been reported (Robins, 2012) with this technique. Using this technology, we have analyzed TCR usage of expanded darTregs. Our results demonstrate that the repertoire of darTregs is narrower than the repertoire of Tregs freshly isolated from peripheral blood, but remained highly diverse. The highest frequency of a single T cell clone in the darTreg product was around 1% and the top 20 clones collectively represented 5 to 10% of the total population. Interestingly, by comparing the repertoire before and after expansion, we have found that the top 20 clones mostly undetectable in the pre-expansion Treg samples. This suggests that we will be able to identify darTregs after infusion using TCR analysis. We will determine the top clones in darTregs using sequencing and detect the top clones after infusion using PCR. Although this technology may allow us to track cells early after infusion, this technique has the potential to be complementary to the deuterium labeling approach. Deuterium labeling may be advantageous early after infusion as the percentage of labeled cells will be high. However, with cell division, signal will be difficult to detect after approximately 4 divisions. The deuterium labeling approach has already proved successful in the Type I diabetes trial with polyclonally expanded Treg. TCR sequencing may potentially be less sensitive early, depending on the degree of oligoclonality; however, if successful clones persist and divide over time in patients, these clones will give a stronger signal over time. PBMCs will be sorted for Treg from frozen, banked specimens and cell pellets will be used for TCR sequencing by Adaptive Biotechnologies.

12. Biospecimen Storage

Whole blood or leukapheresis samples for Treg manufacturing will be collected and cryopreserved in aliquots according to established SOPs and stored in UCSF GMP cell manufacturing facility. Some of the aliquots will be sent to ITN repository for mechanistic studies.

Samples collected solely for mechanistic studies will be sent to the ITN repository and stored under specific conditions to maintain long-term sample integrity, as well as specimen tracking from receipt to shipment to alternate locations. A 21 CFR Part 11 validated database system can be used to track shipment date, location shipped to, carrier, items shipped, amount shipped, barcode numbers, protocol number, and associated comments about each individual specimen. Storage temperature, location, processing and aliquoting, and freeze/thaw events may also be recorded.

If the study subject allows storage, the subject's specimens will be stored indefinitely. The subject can change their mind at any time and have their stored specimens destroyed by notifying the study physician in writing. In such cases, the site coordinator would send all requests for sample destruction to the ITN. The site will receive confirmation that the specimen was destroyed as requested. If the subject's samples have already been analyzed, then the data will be used as part of the overall analysis. The subject can only request to have samples destroyed if they still exist, i.e. have not already been used in an experiment.

Specimens at the ITN core or repository can only be transferred to another destination with appropriate authorization per ITN standard procedures. Purpose for accessing/transferring the specimen (within study assay as defined by the protocol or future studies), evaluation of subject consent for the purpose provided, verification of specimen identifiers, and quality and quantity of the specimen are some of the items checked prior to authorization.

If the purpose is for future studies, and the subject consents for storage for future use, the subject's sample may be made available to the scientific research community per the ITN Sample Sharing Policy (www.immunetolerancenetwork.org). Any research conducted using stored samples for future use may also need appropriate regulatory approval, such as Institutional Review Board (IRB) per the study consent.

Exceptions to the above banking condition apply to the samples used for Treg TruCount, and deuterium detection in blood and biopsies because the assays need to be performed on fresh samples. Donor specimens will be stored until the end of study analyses and then be destroyed or anonymized.

13. Criteria for Participant Completion and Premature Study Termination

13.1 Participant Completion

Subjects in Cohorts 1a and 1b who have completed assessments at all study visits outlined in the SOE after lymphocyte depletion with Thymoglobulin° and TAC- or EVR-based Treg supportive IS regimen will have completed the study. Subjects in Cohorts 2, 3, and 4 who have completed assessments at all study visits outlined in the schedule of events after receiving Thymoglobulin°, Treg supportive IS, and darTreg infusion will have completed the study. The site PI will determine whether these participants should continue with the Treg supportive IS regimen or whether these participants should be converted to the site's standard of care IS regimen.

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, because the investigator no longer believes participation is in the best interest of the participant or because of non-compliance with follow-up and/or study procedures, follow-up should be according to the safety follow-up schedules of events (*Section 13.4*).

13.3 Participant Replacement

Any participant who is deemed ineligible to initiate Thymoglobulin° (Stage 2, Screening B) will be replaced. Any participant in Cohorts 2, 3, or 4 in whom Thymoglobulin° has been administered but is then deemed ineligible to receive darTreg infusion (Stage 3, Screening D) will be replaced if deemed appropriate after review by the NIAID MM, PI, and two transplant physicians independent of this study. This does not replace or change the stopping rules detailed in *Section 14.8.1* . All decisions made related to participant replacement in this stage will be reviewed during the standard DSMB review as described in *Section 14.8.1* Any participant who has received any part of a darTreg infusion (Stage 3) and subsequently terminates from this study prematurely will not be replaced.

13.4 Follow-up after Early Study Withdrawal

13.4.1 Follow-up after Premature Discontinuation from Stage 1

Subjects who are prematurely withdrawn from Stage 1 of the study will be terminated from the study and will not be followed. Subjects who move to Stage 2 but discontinue from the trial prior to receiving study directed Thymoglobulin will not be followed.

13.4.2 Follow-up after Premature Discontinuation from Stage 2 or Stage 3

All subjects who are prematurely withdrawn from Stage 2 and have received any study directed Thymoglobulin®(without receiving darTregs) will have a safety follow up visit 4 weeks after stopping study therapy (Appendix 4). In addition, these subjects will continue the safety follow up schedule (Appendix 4) until 40 weeks after transplantation. Subjects prematurely discontinuing study therapy from Stage 3 after receiving any part of a darTreg infusion will be followed according to Appendix 5 starting with a follow up visit within 4 weeks after the decision to stop study therapy.

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 the procedures for appropriately collecting, grading, recording, and reporting those data. AEs that are classified as serious according to the definition of health authorities must be reported promptly to the sponsor DAIT/NIAID or other Sponsor. Appropriate notifications will also be made to site PIs, 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 Event

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 Adverse Events (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 agents (*Section 5*) caused the AE. For the purposes of safety reporting, 'reasonable possibility' means there is evidence to suggest a causal relationship between the drug 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)).

14.2.1.2 Unexpected Adverse Event

An AE or suspected adverse reaction is considered "unexpected" if it is not listed in the package insert 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 IND.

"Unexpected" also refers to AEs or suspected adverse reactions that are mentioned in the package insert 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 (21 CFR 312.32(a).

14.2.2 Serious Adverse Event

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 0* 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.

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 NCI-CTCAE – Version 4.0 for all AEs with the exception of Infection. 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 with the exception of infection and hematologic events described below.
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

Other than those described below, any events grade 2 or higher (including study defined grading of infections) will be recorded on the appropriate AE CRF for this study.

For hematologic events listed below, grade 3 or higher will be recorded on the appropriate AE case report form (CRF) for this study:

- Neutrophil count decreased
- Anemia (Hemoglobin decreased)
- Platelet count decreased
- Total lymphocyte count decreased
- Lymphocyte subset count decreased (including CD4)

Decreased CD4 lymphocytes and lymphocyte counts within 6 months after last study dose of Thymoglobulin® do not require reporting.

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. Changes in grade from screening to baseline will also be recorded as AEs, but are not treatment-emergent. 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 5. Attribution of Adverse Events. 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 6. 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	Definite	The AE is clearly related.

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

- Lymphocyte depletion with Thymoglobulin®
- Treg-supportive IS regimen – reduced dose tacrolimus, reduced dose MMF, reduced dose methylprednisolone, and EVR (if applicable)
- darTreg
- PBMC collection (Leukapheresis or 450-500 ml blood draw)
- Liver biopsy

Other than 450-500 ml blood draw for PBMC collection, AEs associated with blood draws do not have to be reported unless the investigator deems an event reportable.

14.4 Collection and Recording of Adverse Events

14.4.1 Collection Period

During Stage 1, AEs will be collected from the time of first study procedure (leukapheresis or 450-500 ml blood draw), until 72 hours after completion of the leukapheresis procedure or 450-500 ml blood draw. AEs occurring outside the 72 hour window should be reported if the investigator deems a possible association with the protocol mandated leukapheresis or blood draw.

During Stages 2 and 3 (including Cohort 1 follow up), AEs will be collected from the time of first Thymoglobulin® administration, until a subject completes study participation or until 30 days after he/she prematurely withdraws (without withdrawing consent) or is withdrawn from the study.

Additionally, elevated liver tests (i.e. ALT, AST, ALP, GGT, and bilirubin) must be reported starting 7 days after transplant. Events involving unexpected elevations of liver tests in the first week should be reported according the site investigator's judgment.

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 14.3 Grading and Attribution of AEs.*

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.

14.4.4 Recording Adverse Events

Throughout the study, the investigator will record AEs and SAEs as described previously (*Section 14.2, Definitions)* 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, 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 Serious Adverse Events to Sponsor

This section describes the responsibilities of the site investigator to report SAEs to the sponsor via the Statistical and Clinical Coordinating Center (SACCC) eCRF. Timely reporting of AEs is required by 21 CFR and ICH E6 guidelines.

Site investigators will report all SAEs to the sponsor, regardless of relationship or expectedness 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

After an AE requiring 24 hour reporting (13.5.1, *Reporting of SAEs to Sponsor*) is submitted by the site investigator and assessed by DAIT/NIAID, there are two options for DAIT/NIAID to report the AE to the health authority:

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 (*Section14.2.2*, *Suspected Adverse Reaction,* and *Unexpected AE*)
- Serious and not a suspected adverse reaction (Section 14.2.2, Suspected Adverse Reaction)
- Pregnancies not reported as SAEs

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

This option, 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, Suspected Adverse Reaction* and Section 14.2.2, *Unexpected AE* and 21 CFR 312.32(c)(1)i).

The sponsor shall report any suspected adverse reaction that is both serious and unexpected. 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 protocols, 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.

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.

14.5.3 Reporting of Adverse Events to IRBs/IECs

All investigators shall report AEs, including expedited reports, in a timely fashion to their respective IRBs/IECs in accordance with applicable institutional 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/IEC 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 as expedited events 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 medical monitor, and the NIAID 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 that there are significant safety concerns.

In addition to the dose escalation plan (Section 5.4), the SACCC will continuously monitor accumulating safety data to determine if any of the following criteria are satisfied. If a criterion is satisfied, the trial will be placed on hold pending DSMB review, during which event rates and other clinical data will be evaluated. During this holding period, screening, enrollment, and initiation of any new study treatment or procedure will cease. Specifically, participants who are in Stage 1 will not be allowed to initiate Stage 2 and participants in Stage 2 will not be allowed to initiate Stage 3. However, participants who have received Thymoglobulin® will be allowed to proceed to EVR conversion unless specifically disallowed by the Medical Monitor.

14.8.1 Study Stopping Rules

The decision to pause enrollment pending DSMB review will be based on the occurrence of selected SAEs described below:

1. Death or graft loss

2. Infections of Grade ≥3 as defined in Section 14.3.1
3. Severe AR
    a. Histological: according to 1997 Banff criteria (Demetris A. D., 2000)
    b. Clinical: steroid refractory rejection (see Study Definitions)
4. CR (early or late) according to 2000 Banff criteria (Demetris A. J., 1997)
5. PTLD
6. Malignancy excluding recurrent HCC and skin cancer
7. CTCAE ≥ 3 Infusion reaction
8. Failure to manufacture and supply the cellular product for 2 subjects.

Since the trial is small and these events are of particular concern, and the risks are expected to be different in the two stages of the trial, absolute thresholds for the numbers of such events have been established for each event in each stage that would satisfy the stopping rule.

Table 7. Minimum Numbers of Selected AEs in each Stage that would Satisfy the Stopping Rule

Selected SAE	Lymphocyte Depletion/Treg Supportive IS Stage 2	darTreg Infusion Stage 3
Death or graft loss	1	1
Grade ≥3 Infection (Section 14.3.1)	1	1
Severe AR (histological or steroid refractory rejection)	2	1
CR	1	1
PTLD	1	1
Malignancy excluding recurrent HCC and in situ skin cancer	1	1
CTCAE ≥ 4 Infusion reaction	N/A	1

Every patient who initiates Stage 2, defined as receiving any amount of a Thymoglobulin® infusion, will contribute to the assessment of the safety of Treg supportive IS. At the point in time when the patient initiates Stage 3, defined as receiving any amount of a darTreg infusion, he/she will contribute to the safety assessment for darTreg infusion. Any death or Grade ≥3 infection (defined in Section 14.3.1) occurring in subjects prior to initiation of Thymoglobulin® infusion (prior to Stage 2) will not stop the trial.

The UCSF HICTF, NIAID MM, and study PI will review available information after the first instance of the dose falling short of target range, and again in the event of a second product not meeting the specified dose. If a pause is required due to manufacturing failures, one subject will be allowed to receive study directed Thymoglobulin during the review process. Recruitment and enrollment activities can continue during this time. If a third instance of lower-than-expected dose is manufactured, the study will be paused and DSMB will review available data prior to the trial proceeding.

14.8.2 Medical Monitor Review

The DAIT/NIAID Medical Monitor shall receive at least monthly reports from the SACCC compiling new and accumulating information on AEs, SAEs, and pregnancies recorded by the study site(s) on appropriate eCRFs.

In addition, the Medical Monitor shall review and make decisions on the disposition of the SAE and pregnancy reports received from the site investigator via the SACCC (See *Sections 14.5 and 14.6*).

14.8.3 DSMB Review

The DSMB shall review safety data at least yearly during planned DSMB Data Review Meetings. Data for the planned safety reviews will include, at a minimum, a listing of all reported adverse 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 PI or DAIT/NIAID. Please see *Section 5.4 Dose Escalation Plan/Toxicity Management*.

15. Statistical Considerations and Analytical Plan

15.1 Overview

The objectives of this multi-center, open-label, pilot dose-escalation study are described in *Section 3. Study Design*. Briefly, they are to evaluate the safety, tolerability, DLTs and biological activity of ex vivo-expanded darTregs in combination with lymphocyte depletion using Thymoglobulin° and TAC- or EVR-based Treg-supportive IS in adult liver transplant recipients.

15.2 Outcomes

The study design as well as the safety, clinical, and mechanistic outcomes are described in *Section 3*.

15.3 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. Subjects who do not receive Thymoglobulin® or subjects who receive Thymoglobulin®, but do not subsequently receive darTregs will be replaced according section 13.3.

15.4 Analysis Plan

Statistical analyses of the safety and clinical outcomes will be descriptive, 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).

15.4.1 Analysis Samples

The Safety Sample will be comprised of all study subjects who are consented and receive any study therapy, including leukapheresis, T cell depletion followed by TAC- or EVR-based Treg-supportive IS and darTreg infusion. This excludes consented subjects who were terminated before receiving any study treatment.

Safety and clinical outcomes will be analyzed within the Safety Sample as a whole and within the following two treatment-related analysis periods during which:

- Subjects receive any Thymoglobulin°, followed by TAC- or EVR-based darTreg supportive IS
- Subjects receive darTreg infusions at any dose.

Subjects will contribute AEs and weeks at risk to each analysis period for as long as they are on the corresponding study treatment in that analysis period. Thus, if they progress from T cell depletion to darTreg infusion at any dose, they will contribute subsequent AEs and weeks at risk to the darTreg infusion analysis period when they initiate any darTreg infusion and that will continue until the end of their follow-up time on study.

In addition, a third subset of the safety sample will include any enrolled subject who has PBMCs collected for darTreg manufacturing (phlebotomy or leukapheresis) and may or may not receive subsequent Thymoglobulin®, darTreg supportive IS or darTreg infusions.

15.4.2 Analysis of Safety Outcomes

As the safety outcomes are all person-week incidence rates of selected AEs as described in *Section 3.2*, they will be estimated using proportions and exact two-sided 95% confidence intervals in the Safety Sample as a whole and within the two analysis periods defined above.

15.4.3 Supportive Analyses

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

15.4.4 Analyses of Exploratory Outcomes

Mechanistic assays are designed to monitor various Treg cell subset levels over time before and immediately after the initiation of any darTreg infusion. These Treg cell subset measures 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.

15.4.5 Descriptive Analyses

Disposition of subjects will be summarized in the Safety Sample. Reasons for termination of subjects who did not receive whole blood draw or leukapheresis for manufacturing, those who underwent whole blood draw or leukapheresis for manufacturing but did not receive liver transplantation, and those who underwent whole blood draw or leukapheresis for manufacturing and liver transplantation but did not receive any Thymoglobulin or Treg supportive IS will be tabulated.

Standard descriptive statistics for continuous and categorical variables will be used to summarize the following on all subjects in the Safety Sample and for the two analysis periods defined above:

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

15.5 Interim Analyses

No formal interim analyses of this study are planned.

15.6 Sample Size Considerations

This pilot study is designed to evaluate 18 subjects through Stage 3 in a modified 3+3 dose escalation design. Therefore, no formal power and sample size analyses have been performed. However, with 18 evaluable subjects undergoing darTreg supportive IS for 12 weeks of follow-up (6 subjects for 40 weeks of follow-up) and up to 12 evaluable subjects undergoing darTreg infusions for 28 weeks of follow-up after infusion, exact 95% confidence intervals on person-week incidence rates will vary in width by analysis period. For example, the following table (*Table 8. Incidence Rates and Confidence Intervals for Selected Numbers of Events and Person-Weeks)* shows incidence rates and confidence intervals for selected numbers of events and person-weeks of 216 (18 subjects x 12 weeks), 336 (12 subjects x 28 weeks), and 384 (6 subjects x 40 weeks + 12 subjects x 12 weeks) in the two analysis periods. It is not known at this time how many subjects may be expected to comprise the entire Safety Sample, which includes subjects who only received leukapheresis.

Table 8. Incidence Rates and Confidence Intervals for Selected Numbers of Events and Person-Weeks

Number of Events	Total Person-Weeks	Person-Weeks Incidence Rate	Lower 95% Confidence Limit	Upper 95% Confidence Limit
2	216	0.0093	0.0011	0.0334
4	216	0.0185	0.0050	0.0474
6	216	0.0278	0.0102	0.0605
8	216	0.0370	0.0160	0.0730
10	216	0.0463	0.0222	0.0851
12	216	0.0556	0.0287	0.0970
14	216	0.0648	0.0354	0.1087
16	216	0.0741	0.0423	0.1203
18	216	0.0833	0.0494	0.1317
20	216	0.0926	0.0566	0.1430
2	336	0.0060	0.0007	0.0215
4	336	0.0119	0.0032	0.0305
6	336	0.0179	0.0066	0.0389
8	336	0.0238	0.0103	0.0469
10	336	0.0298	0.0143	0.0547
12	336	0.0357	0.0185	0.0624
14	336	0.0417	0.0228	0.0699
16	336	0.0476	0.0272	0.0773
18	336	0.0536	0.0317	0.0847
20	336	0.0595	0.0364	0.0919
2	384	0.0052	0.0006	0.0188
4	384	0.0104	0.0028	0.0267
6	384	0.0156	0.0057	0.0340
8	384	0.0208	0.0090	0.0410
10	384	0.0260	0.0125	0.0479
12	384	0.0313	0.0161	0.0546
14	384	0.0365	0.0199	0.0612
16	384	0.0417	0.0238	0.0677
18	384	0.0469	0.0278	0.0741
20	384	0.0521	0.0318	0.0804

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 well-being 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 PI 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, 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. The PI or designee listed on the FDA 1572 will review the consent and answer questions. 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. A copy of the signed consent form will be given to the participant.

The consent process will be ongoing; the consent form will be reviewed with each subject in Cohorts 2, 3, and 4 prior to darTreg infusion. 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) except for those lawfully required for manufacture and administration of cell therapies, and to ensure that the appropriate cell product is administered to the intended recipient.

19. Publication Policy

The publication guidelines and policies stipulated in the grant will apply to this trial.

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Appendix 1. Recipient Schedule of Events (Stages 1 and 2)

	STAGE 1				Stage 2																							Clinically Indicated Bx/ Rejection
	Screen A	PBMC Collection[1]	Day 0 (Tx)[11]	Screen B	D2	D3	D4	D5	D6	W2	W3	W4	Screen C1, C2[2]	W5	W6	W7	W8	W9	W10	W11	W12	W13	W14	W16	W18	W20		
Visit Number	**Screen A**	**1**	**2**	**Screen B**	**3a[10]**	**3b**	**4**	**5a**	**5b[10]**	**6**	**7**	**8**	**Screen C**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**15a[13]**	**15b[13]**	**15c[13]**	**15d[13]**	**15e[13]**	**15f[13]**		**CIB/ Rej.[14]**
Visit Window	- 6 Months	Prior to Tx	-3mons	-24 hrs	None					+/-2 days																		N/A
Study Assessments																												
Inclusion Criteria and Exclusion Criteria A	x	x																										
Inclusion Criteria and Exclusion Criteria B				x																								
Inclusion Criteria and Exclusion Criteria C													x[2]															
Informed Consent	x																											
Demographics (DOB, Gender, Ethnicity)	x																											
Medical History	x																											
Physical Examination	x					x				x	x	x							x[12]									x
HCC Assessment, Pathology, Treatment	x		x																									
Review/Collect Current Immunosuppressive and Anti-Infective Medications			→			→	→	→		→	→	→	→	→	→	→	→	→	→	→	→	→	→	→	→	→		x
Adverse Event/Serious Adverse Event Assessment		x[6]				→	→	→		→	→	→	→	→	→	→	→	→	→	→	→	→	→	→	→	→		x
Investigational Agents and Immunosuppressive Medications																												
PBMC Collection *(450ml Phlebotomy[1] or Leukapheresis for Cohort 2, Leukapheresis for Cohorts 3 and 4)*		x																										
Solumedrol 500 mg			x[7]																									
Prednisone Taper			→	→		→	→	→		→	→	→	→	→	Discontinue steroids by week 5													
MMF (Cellcept 500 mg BID or Myfortic 360 mg BID)			x	x		x	x	x		x	x	x	x	D/c MMF once EVR target trough is reached.[5]														
Tacrolimus					Initiate within 72 hrs of tx, target trough 6-8 µg/L[5]									TAC target trough 3-5 µg/L[5]															
Thymoglobulin® Infusion (3-4.5 mg/kg)					First dose within 72 hrs of tx[10]																							
EVR Conversion														EVR target trough 6-8 µg/L[2, 5]													
darTreg Expansion *(Cohorts 2, 3, and 4 Only)*																				x[11]								
Local Laboratory Assessments																												
Pregnancy Test (Urine or Serum hCG)				2																								
HLA Typing	x																											
Viral Serology: HIV Ab, CMV IgG, EBV IgG, HBV (HBsAg, HBcAb, HBeAb, HBeAg, HDVAb), HCVAb[3]	x																											
Viral Nucleic Acid Testing: HBV DNA (for HBV positive subjects)	x	x																										
Viral Nucleic Acid Testing: HCV NAT (Screen A: most current test within 6 months prior to txp, Screen C: all subjects)	x			x									x															
CBC (with differential and platelets)	x		x[8]	x		x	x	x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		x
CD3 Count					x[10]	x	x	x	x[10]																			
CD4 Count													x															
Basic Chemistry (Na, K, Cl, CO2, Creatinine, BUN)	x		x[8]	x		x	x	x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		x
Liver Function Tests (ALT, AST, Alk Phos, GGT, T Bilirubin, D Bilirubin)	x		x[8]	x		x	x	x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		x
INR/PTT	x		x[8]	x		x	x	x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		x
AFP[4]	x		x[4]																									
Tacrolimus Level										x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		x
EVR Level[5]															x	x	x	x	x	x	x	x	x	x	x	x		x
Ultrasound and Doppler Examination													x															
Urine Protein/Creatinine Ratio													x															
Local Pathology Results - Graft Routine Histology			x[9]																x[13]									x
Central Laboratory Assessments																												
Protocol Liver Biopsy																			x[13]									x
Treg TruCount (2ml Green Heparin Tube)	2																											Collect only when CIB visit coincides with scheduled visit with central labs.
Allofrequency Assay & MFC-Leukocyte, Treg, Tact/mem, B Cell Panels (10 ml Green Heparin Tube)			10^7										10															
MLR Suppression Responder Cells (10-10 ml Green Heparin Tubes)			100																									
MLR Suppression (2-10ml Green Heparin Tubes)			20^7										20															
Alloantibody Analysis-Donor HLA Ab (3ml Serum Separator Tube)			3^7																									
	2		133										30															

[1] PBMC Collection Visit does not apply to Cohort 1. Cohort 2 can have whole blood collection for PBMC isolation if hemoglobin is ≥10.5 mg/dl; leukapheresis if <10.5 mg/dl.

[2] Screening for eligibility C1 and C2 can be assessed between 5-6 weeks (30-44 days) after transplantation. Everolimus can be started on or after 30 days after liver transplantation if eligibility criteria are met (Screen C1).

[3] HIV/HBV/HCV serology can be within one year of screening.

[4] AFP is only required for subjects known to have HCC. At Screen A, AFP should be drawn if unavailable within 3 preceding months; AFP should be redrawn at time of transplant. After transplant, AFP should be recorded every 3 months.

[5] First EVR level should be obtained within one week of first dose (+/- 2 days). MMF discontinuation and TAC trough 3-5 µg/L for subjects converting to EVR only. Subjects continuing on TAC-based IS should have troughs 6-8 µg/L.

[6] Prior to Thymoglobulin, collect AE's/SAE's occurring within 72 hours after PBMC Collection only. Collect all AE's/SAE's grade 2 and higher from the time of first Thymoglobulin infusion.

[7] Central laboratory assessments may be drawn up to one year BEFORE solumedrol dose in OR. Once drawn, mechanistic labs do not need to be repeated.

[8] CBC, Basic Chemistry, Liver Function Tests, and PT/INR/PTT should be collected as close to, but prior to transplant as possible.

[9] Liver allograft biopsy should be obtained prior to transplantation (back table) for local pathology reading only; no specimen for central pathology

[10] Screening should take place within 24 hours prior to Thymoglobulin®. The first dose of Thymoglobulin® is to be given within 72 hours after transplantation. Additional doses of 0.75-1.5 mg/kg IV will be administered until CD3 count is ≤50 cells/µL (50 cells/mcL) or total lymphocyte count is ≤ 0.10 x 10^9/L; or when maximum dose of 4.5/mg/kg has been given. Day 2 and Day 6 visits are only needed if Thymoglobulin is given on these days.

[11] Treg expansion should be initiated 2-3 weeks prior to planned Treg infusion if subjects continue to meet eligibility criteria. Please refer to Stage 3 Schedule of Events. .

[12] Visits to the transplant center are required for physical exams. A transplant center visit and physical exam is not required for Cohort 1 at Week 10; physical exam and transplant center biopsy visit between Week 10-14 for Cohorts 2, 3, and 4 only.

[13] Subjects in Cohorts 2, 3, and 4 should have protocol biopsy between week 10 and 14 to assess eligibility D for Stage 3 (See Stage 3 SOE). Subjects in Cohort 1 should have a protocol biopsy at week 12 after transplant (See Stage 2 Follow Up). Visits 15a-15f should be conducted to collect weekly labs if subject has not started Stage 3 SOE.

[14] Local laboratory assessments collected (retrospective chart review) at the time of clinically indicated biopsy should reflect reason for biopsy (e.g. elevated LFT's).

Appendix 2. Recipient Schedule of Events (Cohort 1a and 1b Follow Up)

Time after Transplant	**W12**	**W20**	**W24**	**W28**	**W32**	**W36**	**W40**	**Clinically Indicated**
Time after darTregs**	**T0****	**T56****	**T84****	**Tw16****	**Tw20****	**Tw24****	**Tw28****	**Biopsy/ Rejection**
Visit Number	**16**	**18**	**19**	**20**	**21**	**22**	**23**	**CIB/ Rej.**
Visit Window	-14/+10 days							Within -14 days of study visit
Study Assessments								
Physical Examination	x						x	x
Review/Collect Current Immunosuppressive and Anti-Infective Medications	→	→	→	→	→	→	→	x
Concomitant Medications (inlcuding treatment for rejection and HBV)	→	→	→	→	→	→	→	x
Adverse Event/ Serious Adverse Assessment	→	→	→	→	→	→	→	x
Investigational Agents and Immunosuppressive Medications								
Tacrolimus Maintenance	TAC target trough 3-5 µg/L							
EVR Maintenance	EVR target trough 6-8 µg/L			EVR target trough 4-6 µg/L				
Local Laboratory Assessments								
HBV DNA and HBsAb Quantitative	x							
HCV RNA (for previously HCV positive subjects only)	x							
CBC (with differential and platelets)								x
CD4 Count	x		x				x	
Basic Chemistry (Na, K, Cl, CO2, BUN)	x	x	x	x	x	x	x	x
Liver Function Tests (ALT, AST, Alk Phos, GGT, T Bilirubin; D Bilirubin)	x	x	x	x	x	x	x	x
INR/PTT	x							
AFP[2]	x		x				x	
Tacrolimus Level	x	x	x	x	x	x	x	x
EVR Level	x	x	x	x	x	x	x	x
Local Pathology Results - Graft Routine Histology								x
Central Laboratory Assessments								
Protocol Liver Biopsy[1]	x							x
Allofrequency Assay & MFC-Leukocyte, Treg, Tact/mem, B Cell Panels (10 ml Green Heparin Tube)	10		10				10	Collect only when CIB visit coincides with scheduled visit with central
MLR Suppression (2-10ml Green Heparin Tubes)	20		20				20	
Treg Detection: TCR Sequencing (10ml Green Heparin Tube)	10		10				10	
Alloantibody Analysis-Donor HLA Ab (3ml Serum Separator Tube)	3		3				3	
	43.0		43.0				43.0	

** Corresponds with Cohorts 2, 3, and 4 time points (post drTreg infusion).

[1]For each protocol (W12) or clinically indicated biopsy, one specimen will be in formalin for unstained slides, one specimen in PBS for deuterium tracking, and one specimen in RNALater.

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

Appendix 3. Recipient Schedule of Events (Stage 3: Cohorts 2, 3, and 4)

	STAGE 3[1]													
Time after Transplant		**W12**		**W13**	**W14**	**W16**	**W18**	**W20**	**W22**	**W24**	**W28**	**W32**	**W36**	**W40**	
		darTreg Week 1 (Tw)[2]			**Tw2**	**Tw4**	**Tw6**	**Tw8**	**Tw10**	**Tw12**					
		darTreg Days (T)[2]									**Tw16**	**Tw20**	**Tw24**	**Tw28**	
Time Points (Time after darTregs)	**Screen D (-T1)**	**Treg Infusion T0[2]**	**h24**	**T7**	**T14**	**T28**	**T42**	**T56**	**T70**	**T84**					**Clinically Indicated Biopsy/Rej**
Visit Number	**Screen D**	**T0**	**T2**	**T3**	**T4**	**T5**	**T6**	**T7**	**T8**	**T9**	**T10**	**T11**	**T12**	**T13**	**CIB/ Rej.**
Visit Window	-10 days before Tregs	None	±1 hr	±1 day	±2 days		± 5 days								Within -14 days of study visit
Study Assessments															
Inclusion Criteria and Exclusion Criteria D	x														
Treg Informed Consent	x														
Physical Examination	x	x	x	x		x				x				x	
Review/Collect Current Immunosuppressive and Anti-Infective Medications	→	→	→	→	→	→	→	→	→	→	→	→	→	→	x
Concomitant Medications (including treatment for rejection and HBV)	→	→	→	→	→	→	→	→	→	→	→	→	→	→	x
Adverse Event/ Serious Adverse Assessment	→	→	→	→	→	→	→	→	→	→	→	→	→	→	x
Investigational Agents and Immunosuppressive Medications															
darTreg Infusion (50, 200, or 800) *(infuse between 11 and 20weeks after txp)*		x													
Tacrolimus Maintenance	TAC target trough 3-5 µg/L														
Everolimus Maintenance	EVR target trough 6-8 µg/L											EVR target trough 4-6 µg/L			
Local Laboratory Assessments															
Pregnancy Test (Urine or Serum hCG)	x														
HBV DNA	x														
HCV RNA (All Subjects)	x														
CMV and EBV DNA	x			x		x				x				x	
CBC (with differential and platelets)	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
CD4 Count		x	x	x		x				x				x	x
Basic Chemistry (Na, K, Cl, CO2, Creatinine, BUN)	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Liver Function Tests (ALT, AST, Alk Phos, GGT, T Bilirubin; D Bilirubin)	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
AFP[4]										x				x	
Tacrolimus Level	x			x	x	x	x	x	x	x	x	x	x	x	x
EVR Level	x			x	x	x	x	x	x	x	x	x	x	x	x
Local Pathology Results - Graft Routine Histology	x[3]														x
Central Laboratory Assessments															
Protocol Liver Biopsy[5]	x[3]			x											x
Allofrequency Assay & MFC-Leukocyte, Treg, Tact/mem, B Cell Panels (10 ml Green Heparin Tube)		10	10	10		10				10				10	Collect only when CIB visit coincides with scheduled visit with central labs.
MLR Suppression (2-10ml Green Heparin Tubes)		20		20		20				20				20	
Treg Detection: TCR Sequencing (10ml Green Tube)		10	10	10		10				10				10	
Treg Detection: Heavy Glucose Labeling (10ml Green Tube)			10	10		10				10				10	
Alloantibody Analysis-Donor HLA Ab (3 ml Serum Separator Tube)		3												3	
		43.0	30.0	50.0		50.0				50.0				53.0	

[1] Stage 3 begins with screen D for darTreg infusion. If a second attempt at Treg infusion is planned, laboratory assessments (except for liver biopsy) for Screen D must be repeated.

[2] T0 designates day of darTreg infusion. h = hour, T = day, Tw = week; where (x) indicates number of hours, days, or weeks after darTReg infusion.

[3] Subjects in Cohorts 2, 3, and 4 should have biopsy between week 10 and 12 to assess eligibility D for Stage 3.

[4] AFP is only required for subjects known to have HCC.

[5] For each protocol (Eligibility D and Day 7) or clinically indicated biopsy, one specimen will be in formalin for unstained slides, one specimen in PBS for deuterium tracking, and one specimen in RNALater.

Appendix 4. Recipient Safety Follow Up after Thymoglobulin (Early Termination from Stage 2)

* This schedule should be utilized for subjects who terminate from the study after receiving any study directed Thymoglobulin, but no darTregs.

Time Points	Wk 4 after Term[1]	W12[1]	W24[1]	W40[3]	Clinically Indicated Biopsy/Rej
Visit Number	**F1**	**F1a**	**F2**	**F3**	**CIB**
Visit Window	± 7 days				Within -14 days of study visit
Study Assessments					
Physical Examination	x			x	
Review/Collect Current Immunosuppressive & Anti-Infective Medications	x	x	x	x	x
Rejection Episodes	→	→	→	→	→
Adverse Event/ Serious Adverse Event Assessment	→	→	→	→	→
Local Laboratory Assessments					
CBC (with differential and platelets)	x	x	x	x	x
CD4 Count		x	x	x	x
Basic Chemistry (Na, K, Cl, CO2, BUN, creatinine)	x	x	x	x	x
Liver Function Tests (ALT, AST, Alk Phos, T Bilirubin; D Bilirubin, GGT)	x	x	x	x	x
Tacrolimus Level[2]	x	x	x	x	x
EVR Level[2]	x	x	x	x	x
Central Laboratory Assessments					
Allofrequency Assay & MFC-Leukocyte, Treg, Tact/mem, B Cell Panels (10 ml Green Heparin Tube)		10	10	10	10[3]
MLR Suppression (2-10ml Green Heparin Tubes)		20	20	20	20[3]
TCR Sequencing (10ml Green Heparin Tube)		10	10	10	10[3]
Alloantibody Analysis-Donor HLA Ab (3ml Serum Separator Tube)		3	3	3	3[3]

[1] Subjects who prematurely terminate from Stage 2 of the study after receiving a partial dose of study Thymoglobulin should have the first safety follow up visit 4 weeks after stopping protocol therapy. After the first follow up visit, the subject will use the next applicable study visit and continue this schedule until 40 weeks after transplant. Follow up visit F1 (4 weeks after termination) should be replaced with W12 (F1a) or W24 (F2) if the subject terminates within 4 weeks of that visit.

[2] Tacrolimus and EVR levels should be collected from standard of care labs, if available.

[3] Central laboratory assessments do not need to be collected for CIB if collected less than 6 weeks ago.

Appendix 5. Recipient Safety Follow Up (Early Termination from Stage 3)

* This schedule should be utilized for subjects who terminate from the study after receiving any darTregs.

Time Points (Weeks after darTregs)	**Wk 4 after Term[1]**	**Wk 12[1]**	**Wk 28**	**Clinically Indicated Biopsy/ Rejection**
Time Points (Weeks after Transplant)		**W24[1]**	**W40[2]**	
Visit Number	**F1**	**F2**	**F3**	**CIB**
Visit Window	± 7 days			Within -14 days of study visit
Study Assessments				
Physical Examination	x		x	
Review/Collect Current Immunosuppressive and Anti-Infective Medications	x	x	x	
Rejection Episodes	→	→	→	→
Adverse Event/ Serious Adverse Event Assessment	→	→	→	→
Local Laboratory Assessments				
CBC (with differential and platelets)	x	x	x	x
CD4 Count		x	x	x
Basic Chemistry (Na, K, Cl, CO2, BUN, creatinine)	x	x	x	x
Liver Function Tests (ALT, AST, Alk Phos, T Bilirubin; D Bilirubin, GGT)	x	x	x	x
Tacrolimus Level[3]	x	x	x	x
Everolimus Level[3]	x	x	x	x
Local Pathology Results - Graft Routine Histology				x
Central Laboratory Assessments				
Protocol Liver Biopsy				x
Allofrequency Assay & MFC-Leukocyte, Treg, Tact/mem, B Cell Panels (10 ml Green Heparin Tube)		10	10	Collect only when CIB visit coincides with scheduled visit with central labs.
MLR Suppression (2-10ml Green Heparin Tubes)[11]		20	20	
TCR Sequencing (10ml Green Heparin Tube)		10	10	
Heavy Glucose Labeling (10ml Green Heparin Tube)		10	10	
Alloantibody Analysis-Donor HLA Ab (3ml Serum Separator Tube)		3	3	
	0.0	53.0	53.0	

[1] Subjects who prematurely terminate from Stage 3 of the study should have the the first safety follow up visit 4 weeks after stopping protocol therapy. Follow up visit F1 (4 weeks after termination) should be replaced with visit F2 or visit F3 if the subject terminates within 4 weeks of that visit.

[2] The second follow-up visit takes place 10 months (week 40) after transplant.

[3] Tacrolimus and EVR levels should be collected from standard of care labs, if available.

[4] For each clinically indicated biopsy, one specimen will be in formalin for unstained slides, one specimen in PBS for deuterium tracking, and one specimen in RNALater.

Appendix 6. Donor Schedule of Events

Study Assessments/Data Collection[1]
Demographics (DOB, Gender, Ethnicity)
Medical and Social History
Physical Assessment
ABO Blood Type
HIV Ab, HCV Ab, HBsAg, HBcAb
EBV Ab
HBV, HCV, HIV, WNV[1] NAT
RPR
T.Cruzi Ab[1]
Manufacturing and Central Laboratory Assessments
Donor Eligibility per 21CFR 1271 including CMV Total Antibody, HTLV 1 and 2) (6ml Red Top) *(Cohort 2, 3, 4 only)*
Donor Blood for Banking or HLA Typing[2] (3ml Gold or Red Top Tube)*(Cohort 2, 3, 4 only)*
1cm^3 Spleen (B Cells and HLA Typing)
Donor Blood for Manufacturing (70ml Green Top Tubes) *(UCSF only)*

[1]Data should be collected from medical records or UNOS records retrospectively. Except for WNV and T. Cruzi, viral tests should be ~~but~~ ordered if not available. WNV NAT and T. Cruzi Ab should be collected for study/manufacutring records if available.

[2]An additional tube should be collected w henever available for subjects in Cohort 2, 3, and 4.