Everolimus

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Biomarkers Predicting Successful Tacrolimus Withdrawal and Everolimus (Zortress®) Monotherapy Early After Liver Transplantation

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List of abbreviations

AASLD	American Association for the Study of Liver Diseases
AE	Adverse event
ALT	Alanine aminotransferase
ANC	Absoulte neutrophil count
AR	Acute rejection
AST	Aspartate aminotransferase
AUROC	Area Under Receiver Operating Characteristic (Curve)
CFSE	Carboxyfluorescein succinimidyl ester
CKD	Chronic kidney disease
CNI	Calcineurin inhibitor
CBC	Complete blood count
Cr	Creatinine
CRF	Case Report/Record Form
CRD	Clinical Research and Development
CPO	Country Pharma Organization
CRO	Contract Research Organization
CSR	Clinical Study Report
CTC	Comprehensive Transplant Center
DR	Donor
DSA	Donor specific antibodies
DSMB	Data Safety Monitoring Board
ECG	Electrocardiogram
eCRF	Electronic case report form
eGFR	Estimated glomerular filtration rate
EVL	Everolimus
FDA	Food and Drug Administration
GCP	Good Clinical Practice
GFR	Glomerular filtration rate
GGT	Gamma-Glutamyl Transpeptidase
HBV	Hepatitis B virus
HCV	Hepatitis C virus

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HCV-R	Hepatitis C virus recurrence
HgbA1C	Hemoglobin A1C
HIPAA	Health Insurance Portability and Accountability Act
HIV	Human Immunodeficiency Virus
HLA	Human leukocyte antigen
HLA-DR	Human leukocyte antigen-donor
ICH	International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use
ICH	International Conference on Harmonization
I/E	Inclusion/exclusion
ICH	Immunohistochemical
IL-2	Interleukin 2
IM	Immune Monitoring
IMS	Immune Monitoring Signals
IRB	Institutional Review Board
IS	Immunosuppression
IVRS	Interactive Voice Response System
LFT	Liver function tests
LT	Liver transplantation
MAP	Multi-Analyte Profiling
MFI	Mean Fluorescence Intensity
miRNA	Micro ribonucleic acid
MLR	Mixed lymphocyte reaction
MPA	Mycophenolic acid
mRNA	Messenger ribonucleic acid
NK cells	Natural killer cells
NU	Northwestern University
NU IRB	Northwestern University Institutional Review Board
OLT	Orthotopic Liver Transplant
PBMC	Peripheral blood mononuclear cell
PHI	Protected health information
PI	Principal Investigator

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PTLD	Post-transplant lymphoproliferative disorder
PRED	Prednisone
RM-ANOVA	Repeated Measures of Analysis of Variance
RN	Registered nurse
SAE	serious adverse event
SOC	Standard of Care
SRL	Sirolimus
SI	Stimulation indices
TAC	Tacrolimus
TAC-WD	Tacrolimus withdrawal
Tregs	T-regulatory cells
WBC	White blood cell

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Glossary of terms

Assessment	A procedure used to generate data required by the study
Control drug	A study drug used as a comparator to reduce assessment bias, preserve blinding of investigational drug, assess internal study validity, and/or evaluate comparative effects of the investigational drug
Enrollment	Point/time of patient entry into the study; the point at which informed consent must be obtained (i.e. prior to starting any of the procedures described in the protocol)
Investigational drug	The study drug whose properties are being tested in the study; this definition is consistent with US CFR 21 Section 312.3 and is synonymous with “investigational new drug.”
Medication number	A unique identifier on the label of each medication package in studies that dispense medication using an IVR system
Patient number	A number assigned to each patient who enrolls in the study. When combined with the center number, a unique identifier is created for each patient in the study.
Stage	A major subdivision of the study timeline; begins and ends with major study milestones such as enrollment, randomization, completion of treatment, etc.
Period	A minor subdivision of the study timeline; divides phases into smaller functional segments such as screening, baseline, titration, washout, etc.
Premature patient withdrawal	Point/time when the patient exits from the study prior to the planned completion of all study drug administration and assessments; at this time all study drug administration is discontinued and no further assessments are planned
Randomization number	A unique identifier assigned to each randomized patient, corresponding to a specific treatment arm assignment
Stop study participation	Point/time at which the patient came in for a final evaluation visit or when study drug was discontinued whichever is later
Study drug	Any drug administered to the patient as part of the required study procedures; includes investigational drug and any control drugs
Study drug discontinuation	Point/time when patient permanently stops taking study drug for any reason; may or may not also be the point/time of premature patient withdrawal
Variable	Information used in the data analysis; derived directly or indirectly from data collected using specified assessments at specified time points

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Protocol synopsis

Title of study: Biomarkers Predicting Successful Tacrolimus Withdrawal and Everolimus (Zortress®) Monotherapy Early After Liver Transplantation

Purpose and rationale:

The purpose of this study is to develop a biomarker strategy testing transplant immunoregulation to guide safe elimination of calcineurin inhibitor (CNI) therapy in favor of everolimus (EVL) early after liver transplantation (LT). We plan to analyze a set of biomarkers testing genomic and immunophenotypic measures of immune reactivity and regulation, expected to be enhanced by EVL and predictive of successful conversion.

Objectives:

To identify cellular and genomic metrics of immune regulation that may predict successful TAC withdrawal in favor of EVL early after LT.

Our *overall objective* is to utilize biomarkers that will clarify the decision to consider tacrolimus withdrawal/EVL (TAC-WD/EVL) and maintain EVL without TAC. Proving the hypothesis that EVL promotes early immunoregulation in those patients not developing rejection with TAC-WD would provide:

1) rationale for early CNI to EVL conversion when the benefit and risk are highest

2) bioassays to guide selection of appropriate candidates for TAC-WD/EVL vs. continued TAC in patients likely to develop rejection with CNI withdrawal.

Primary endpoint:

The primary endpoint is the peripheral blood Treg% (or other immunoregulatory cells) and messenger ribonucleic acid (mRNA) acute rejection (AR) signatures seen prior to and during either successful (no rejection) vs. failed (rejection) TAC-WD/EVL.

Secondary endpoints:

Clinical endpoints as correlates for the success and failure of TAC-WD/EVL will serve as secondary endpoints. This will include but not be limited to the following at 6 months (± 1 month) post-LT: liver allograft and renal function, EVL & TAC side effects, infectious/malignant complications, and patient/graft survival. At study end, all secondary endpoints will be statistically compared between those who succeed or fail TAC-WD/EVL.

Population:

We will select adult (≥ 18 yrs. of age) LT recipients without immune disease, active viral disease, and previous or combined organ transplantation to avoid the confounding of coexistent inflammatory/infectious processes and prior transplants on the biomarker analysis.

Inclusion/Exclusion criteria:

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Inclusion Criteria:

- Adult LT candidates ≥ 18 years of age
- Listed for or recent (within 2 months) recipient of deceased or living donor liver transplantation

Exclusion Criteria:

- Combined or previous organ transplantation
- Human immunodeficiency virus (HIV) infection
- Inability to provide informed consent or comply with the protocol.
- Transplantation for autoimmune liver disease (Autoimmune hepatitis, Primary Biliary Cirrhosis, Primary Sclerosing Cholangitis)

Investigational and reference therapy:

Zortress® (everolimus) at advised doses and trough levels.

Study design:

Overall: Prospective, open-label pilot study comparing immunoregulatory assays in non-viremic LT patients undergoing TAC-WD/EVL. The patients will serve as their own control for assays, clinical, biochemical, and histological measures, and the groups will be divided and compared by the presence or absence of rejection before, during and after the TAC-WD/EVL phase (months 0-6 (± 1 month)). As this is a pilot study, we hope to generate these preliminary data to power a larger trial testing the specific biomarker(s) at more specific time-points identified here in more definitively determining who may or may not be successful with undergoing CNI withdrawal in favor of EVL.

Efficacy assessments:

- The proportion of patients who are successfully withdrawn from TAC and continued on EVL without rejection or other complications at six months (± 1 month) post-LT.
- The following assessments following addition of EVL at one to two months and attempted withdrawal of TAC between 3-6 months (± 1 month) in favor of EVL maintenance: liver allograft and renal function, EVL & TAC side effects and discontinuations, infectious/malignant complications, and patient/graft survival.

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- Biomarker assessments: Peripheral blood Treg% (or other immunoregulatory cells) and messenger ribonucleic acid (mRNA) AR signatures seen prior to and during either successful (no rejection) vs. failed (rejection) TAC-WD/EVL.

Other assessments:

- Other samples collected for future studies (see exploratory objectives below)

Data analysis:

The primary objective is to test the working hypothesis that, in a prospective study, cellular and genomic markers of immunoregulation become more pronounced after early rejection-free TAC-WD/EVL compared to failed (rejection) TAC-WD. Conversely, if these markers do not signify immunoregulation, we hypothesize that TAC-WD/EVL will not be successful (i.e. rejection will ensue requiring reinstatement of TAC). Our hypothesis is based on our previous observations that late mTOR-I conversion increased % of Treg/DCreg in blood/tissue sites and generated immunoregulatory proteogenomic signatures and that AR can be diagnosed in the peripheral blood by genomic microarrays. Therefore, the plan is to perform sequential flow cytometry immunophenotyping and genomic signature analysis in the blood before, during and after attempted TAC-WD/EVL.

Studies Planned and Methods:

i) Blood Immunophenotyping. The approach will be to conduct flow cytometry immunophenotyping of peripheral blood before TAC-WD initiation (3 months (± 1 month)), right at total TAC-WD (4 months (± 1 month)), 2 weeks after full TAC-WD (4.5 months (± 1 month)) and study end (6 months (± 1 month)) post-LT to analyze cellular changes. Methods: The Immune Monitoring Laboratory at NU will perform the PBMC immunophenotyping. For statistical analysis, we perform within subject comparisons on PBMC for both successful and failed TAC-WD/EVL groups using appropriate paired analysis (paired t-test or Wilcoxon Signed Rank test). For the main hypothesis testing, repeated measures of analysis of variance (RM-ANOVA) are used to test the trajectory variations between the groups on PBMC over time. Two-sided F-test statistics are applied; α=0.05 (stats by NU Transplant Statistician).

ii) Gene Expression Microarrays: The approach will be to conduct parallel serial genomic bioassays of peripheral blood before and after attempted TAC-WD/EVL (same time points as immunophenotyping). Methods: The Genomics Core at Scripps will perform the microarray analysis. Blood will be collected in PaxGene tubes and sent to Scripps for the gene microarrays. For statistical analysis, we report for each mRNA and microRNA comparison the T-test p-value, its non-parametric analog (Kruskal Wallis test), the fold change, and for multi-analyte signatures, the area under the receiver operating curve (concordance index/C-index) and q-values by member genes/proteins. We then calculate the predictive accuracy, sensitivity and specificity of each signature (statistics by Dr. Daniel Salomon's informatics group at Scripps Clinic)

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1 Background

It has been assumed that standard IS agents, the calcineurin inhibitors (CNI) cyclosporine and tacrolimus (TAC), are indefinitely required to prevent allograft rejection following liver transplantation (LT). However, this has occurred at the expense of chronic CNI toxicity, e.g. chronic kidney disease (CKD), metabolic complications, infections and malignancy[1-3]. The mortality related to CKD is only remediated by a curative kidney transplant, usurping costs and valuable organ resources[3]. While CNI therapy is universally given immediately after LT, subsequent conversion from CNI to non-CNI therapy could reduce these adverse effects. Agents such as mammalian target of rapamycin inhibitors (mTOR-I) sirolimus (SRL) and everolimus (EVL) have a different mechanism of action and may stabilize or improve renal function in patients with CKD related to CNI therapy[4-13]. However, most of the studies have involved late CNI to mTOR-I conversion when CKD is already "set in" and likely irreversible. Thus, early full conversion (<1 year post-op), i.e. CNI discontinuation in favor of mTOR-I therapy) before the onset of irreversible CKD, would be the most optimal approach. However, higher SRL doses and troughs (≥8 ng/mL) are required in this time period given the lower immunosuppressive potency of SRL vs. CNI, but this can result in intolerable SRL side effects and discontinuation in ~30% and still lead to rejection [14-16]. In addition, SRL has known risks of hepatic artery thrombosis in the early post-operative period and cause mortality in liver recipients, thus leading to a "black box warning" and lack of indication in this population. Taking these considerations into account, safer, more tolerable mTOR-I therapy (such as EVL) would be preferable for use in the LT population.

Nevertheless, faced with CKD progression, clinicians are commonly confronted with difficult decisions, none of which are clearly considered the most optimal: 1) maintain CNI therapy and accept the nephrotoxicity; 2) reduce CNI to sub-therapeutic levels and risk acute rejection; 3) reduce CNI doses and add other IS (mycophenolic acid or mTOR-I) to decrease the rejection risk, but accept the potential for CKD progression given the continued CNI therapy and the combined toxicity/immunosuppression of the second agent; 4) stop CNI and convert to a non-nephrotoxic agent (mTOR-I like EVL) to minimize CNI effects and over-immunosuppression. While some studies have not shown an increased risk of rejection with the last approach[12,13], a recent major randomized trial did demonstrate a higher risk compared to either TAC monotherapy or combination low dose TAC and EVL[17]. Despite this arm (TAC-withdrawal and continued EVL; TAC-WD/EVL) being stopped early because of this, the remaining patients had the most preserved glomerular filtration rate (GFR) at one-year post-LT. Thus, it would represent a major advance to be able to determine more specifically which patients would fare well (improved GFR without AR) or poorly (rejection) with early TAC-WD/EVL. However, the absence of serial, specific biomarkers of immunoregulation available to guide this approach is a significant roadblock to achieving this endpoint.

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Therefore, specific to this proposal and supported by our unique data in LT recipients[18-20], an unexplored advantage of mTOR-I therapy lies in its potential to promote an immunoregulatory state. This could facilitate success with early CNI withdrawal in favor of EVL under current target doses/trough levels. As the most immunoregulatory organ transplanted, the liver houses numerous extramedullary immunoregulatory hematopoietic cells and secretes several immunoregulatory proteins[21-24]. The percentage of LT recipients able to undergo IS withdrawal directly from CNI therapy appears to be the highest of all organ recipients, although still only ~20% successful [24]. This clinically unacceptable rate may be due to CNI mechanisms inhibiting immunoregulation and the lack of predictive assays to detect immunoregulation. A key difference between mTOR-I and CNIs is their effect on regulatory T cells (Tregs) important in the suppression of alloimmunity. As an inhibitor of downstream IL-2 signaling after T cell activation, mTOR-I blocks alloreactive T cell proliferation but appears to promote Tregs ($CD4^+CD25^{high}FOXP3^+$), regulatory cytokines (TGF-β1), and tolerogenic dendritic cells (DC2, $ILT3^+/4^+$) [25-31]. In contrast, CNI therapy inhibits IL-2 transcription and has been thought to negatively impact Treg generation[32-35].

We have also uniquely demonstrated robust donor-specific regulatory effects of SRL in MLR cultures[18]. In these studies, TAC, SRL or media without agents were added separately to MLRs using HLA 2 DR matched and mismatched volunteers. Stimulation indices (SI) of ^3H-TDR uptake, cell proliferation, and the generation of carboxyfluoroscein succinimidyl ester (CFSE) labeled $CD4^+CD25^{High}FOXP3^+$ cells by flow cytometry were initially compared. Each group of MLR-generated cells were then added as third components to CFSE labeled responding cells in freshly prepared primary MLRs, to determine allo-specific vs. non-specific inhibitory and Treg "recruitment" effects (*in vitro* "infectious tolerance"). Tacrolimus inhibited SI and $CD4^+CD25^{High}FOXP3^+$ cell generation in both HLA-DR matched and mismatched pairs, particularly at therapeutic levels (≥ 5 ng/ml). SRL had an equivalent lymphoproliferative inhibitory effect in matched pairs but was associated with a significantly higher % generation of $CD4^+CD25^{High}FOXP3^+$ cells. SRL-MLR-generated Tregs added as third components allo-specifically inhibited MLR proliferation and "recruited" additional autologous Tregs compared with addition of TAC- or media-MLR-generated cell preps [Fig. 1, ref[18]]. These results can explain the differing *in vitro* effects of these agents, now with donor-specific regulatory implications for mTOR-I. The immunoregulatory effects of EVL appear to be even more profound than SRL. In a Novartis supported laboratory study (submitted to the American Transplant Congress; manuscript in preparation), we analyzed and compared the *in vitro* immunoregulatory effects of EVL vs. SRL vs. TAC and found the greatest augmentation of Tregs with EVL in culture, particularly in combination with mycophenolate.

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Clinically, we demonstrated in another study that LT recipients previously converted to SRL had higher percentages of PBMC Tregs compared to recipients on other IS therapies [19]. This led to a prospective TAC to SRL conversion trial supported by the American Association for the Study of Liver Disease Career Development Award and Wyeth/Pfizer (Levitsky) [20]. Our aim was to determine if systemic phenotypic Tregs ($CD4^+CD25^{high}FOXP3^+$), regulatory dendritic cells (DCregs: $ILT3^+/4^+$), and immunoregulatory proteogenomic signatures increase in LT recipients converted directly from TAC to SRL monotherapy. Twenty non-immune non-viremic LT recipients were successfully converted for renal dysfunction. The mean eGFR increased from 45.9±7.9 to 49.6±10.8 ($p=0.003$). Tregs increased significantly in PBMC, marrow [Fig. 2, from ref [20]] and LT biopsy cultures. Biopsy immunohistology FOXP3:CD3 and CD4:CD8 ratios became significantly higher after conversion [Fig. 3, from ref [20]]. Peripheral blood DCregs increased significantly (Fig. 2, $p<0.01$). Both pre- (TAC) and post- (SRL) conversion sera inhibited MLR proliferation, although only TAC sera suppressed Treg generation. 289 genes and 22 proteins, many of which are important in immunoregulatory pathways, were newly expressed after conversion [Fig. 4: from ref [20]]. In conclusion, these studies demonstrated that TAC to SRL conversion increases systemic (blood, allograft, marrow) Tregs, blood DCregs and regulatory proteogenomic signatures in LT recipients. However, it is not known if this profile correlates with and predicts successful *early* mTOR-I use (i.e. TAC-WD/EVL) as in this proposal.

Equally important as the chosen IS regimen is identifying predictive biomarkers, such as those we have tested above. Various reports demonstrate a higher percentage of $CD4^+CD25^{high}FOXP3^+$ cells, DC2, γδ T cells and specific genomic signatures in tolerant LT recipients versus those on maintenance IS [36-45]. Our collaborative group has previously performed proteogenomic assays (mRNA profiles, proteomic analytes) to identify markers of acute rejection (AR) and chronic allograft nephropathy in kidney recipients, as well as CKD post-LT[46-48]. In addition, we recently conducted a study profiling (mRNA gene expression) 114 blood and 57 liver biopsy samples of patients with different clinical phenotypes, and have identified both blood and tissue mRNA signatures that can distinguish AR from other major causes of graft injury (hepatitis C recurrence, mixed HCV-R and AR, other causes) in LT recipients with high predictive accuracy (AUC 0.89 – 0.9; Figure 5)[49]. These biomarkers have the potential to be useful to enhance the specificity of diagnosis, particularly in managing patients with contrasting etiologies (e.g., AR vs. HCV-R). In addition, the immune/inflammatory nature of these AR profiles may help inform decisions to perform liver biopsies and in immune monitoring during immunosuppression reduction, as proposed herein. Yet, the presence of 'snap-shot' non-specific cell populations and differential gene/protein arrays only provides circumstantial evidence for their role in immunoregulation[50,51]. Prospective sequential assays would provide a more accurate measure of the effects of mTOR-I on immunoregulation. We anticipate that these expression patterns will be useful as regulatory signatures that may predict successful CNI withdrawal, as suggested by our recent trial and other reports[20,39,42].

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2 Purpose and rationale

We propose to develop a biomarker strategy testing transplant immunoregulation to guide safe elimination of CNI therapy in favor of EVL early after LT. We plan to analyze a set of biomarkers testing genomic and immunophenotypic measures of immune reactivity and regulation, expected to be enhanced by EVL and predictive of successful conversion. These bioassays will be compared in this initial pilot study prospectively between non-viremic LT recipients on TAC + EVL combination therapy (EVL added at 1 to 2 months) who are either successfully (no rejection) or unsuccessfully (rejection) withdrawn from TAC at month 3-4 (± 1 month) from LT. Our overall objective is to utilize biomarkers that will clarify the decision to consider TAC-WD/EVL and maintain EVL without TAC. Proving the hypothesis that EVL promotes early immunoregulation in those patients not developing rejection with TAC-WD would provide 1) rationale for early CNI to EVL conversion when the benefit and risk are highest and 2) bioassays to guide selection of appropriate candidates for TAC-WD/EVL vs. continued TAC in patients likely to develop rejection with CNI withdrawal.

3 Objectives

Primary Objective: To identify cellular and genomic metrics of immune regulation that may predict successful TAC withdrawal in favor of EVL early after LT.

It is our hypothesis that these markers of immunoregulation are more pronounced with successful (no rejection) vs. unsuccessful (rejection) TAC-WD/EVL. The approach will be to analyze the peripheral blood for phenotypic Tregs correlating with mRNA footprints of regulation. These analyses will be performed in a prospective pilot clinical trial of LT recipients on standard combination TAC and EVL at 1 to 2 months post-LT selected to undergo TAC-WD/continued EVL at 3-4 months (± 1 month). Thus, based on the clinical trials, we expect rejection to occur in 20% in the 1-2 month period following TAC-WD/EVL and that this will be predicted by bioassays analyzed prior to and/or immediately after full TAC-WD. Alternatively, we expect 80% to not experience rejection with TAC-WD/EVL and that this will be predicted by bioassays analyzed prior to and/or right after full TAC-WD. Therefore, the use of these assays would eventually serve to detect and predict appropriate candidates for successful early TAC-WD/EVL, enhancing the use of EVL in LT and allowing patients to be spared of CNI toxicity past the first 3-4 months following LT.

3.1 Primary endpoints

The primary endpoint is the peripheral blood Treg% (or other immunoregulatory cells) and mRNA AR signatures seen prior to and during either successful (no rejection) vs. failed (rejection) TAC-WD/EVL.

3.2 Secondary endpoints

Clinical endpoints as correlates for the success and failure of TAC-WD/EVL will serve as clinical secondary endpoints. This will include but not be limited to the following at 6 months (± 1 month) post-LT: liver allograft and renal function, EVL & TAC side effects, infectious/malignant complications, and patient/graft survival. At study end, all secondary endpoints will be statistically compared between those who succeed or fail TAC-WD/EVL.

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The other mechanistic biomarker endpoints tested potentially in the future (unfunded exploratory objectives) will focus on comparing biopsy and peripheral blood microarray signatures at the time of a rejection biopsy, comparing these biopsy signatures in rejections that occur before and after TAC-WD, and other predictive biomarkers (miRNA, DSA, proteomic, donor specific assays- see exploratory objectives below).

The primary safety endpoints are the development of rejection with TAC-WD and any graft failure due to rejection, the latter of which is not expected to happen based on the registration studies. We expect that 20% of patients will experience rejection that is reversible with resumption of TAC +/- corticosteroids, without any graft loss, all based on the registration studies. However, if any patient experiences a graft loss due to the protocol (TAC-WD) or ≥30% rejection is seen, the study will be terminated due to safety concerns.

3.3 Exploratory objectives

As this is a pilot exploratory study, a number of possibile directions may emanate from these initial results:

1) If the immunophenotypic and/or genomic biomarkers are preliminarily predictive (primary endpoint met) of AR, we would then imminently request funding to adequately power a larger study focusing in on those specific time points and biomarkers identified from the initial pilot study. This could include expansion of our single center study or perhaps a multicenter trial utilizing the AR biomarkers identified to guide patient management - e.g. continued TAC WD/EVL in patients without serial AR signals vs. no TAC WD or TAC resumption in patients with these predictive AR signals to avoid AR. This type of personalized approach would be transformational if it could be instituted in clinical practice - to avoid CNI toxicity in those predicted not reject and to avoid rejection in those predicted to reject with CNI withdrawal.

2) If the immunophenotypic and genomic biomarkers are not predictive (primary endpoint not met) of AR, we would then request funding for other biomarkers testing from the blood samples we collected and stored pre/during/post-TAC WD. There may be alternative biomarkers that are more predictive of AR that will need to be explored, and we will have these samples available. These would include, but not limited to, the following assays which we have experience in performing:

i) *MicroRNA:* miRNA measures can be performed from whole blood collected at each time point using the Illumina Truseq protocol. In brief, Truseq small RNA library preparations are done on RNA from clinical samples that have been extracted using the Trizol protocol and purified using the RNEasy RNA purification method that has been standardized for small RNA preservation.

ii) *Donor Specific Antibodies (DSA):* The adverse effects of DSA in clinical LT have been increasing recognized as shown by recent studies linking the development of DSA to rejection episodes [52,53]. Therefore, we can test for the development of DSA in the current cohort of patients at the same time points. Since estimation of DSA is expensive, we can monitor for the presence of panel reactive antibodies in the sera serially at the various time points, and only test those with high PRA for DSA. The NU Comprehensive Transplant Center Lab can perform these assays.

iii) *Cellular and Plasma Proteomics:* Our recent studies of TAC to SRL conversion in LT had revealed protein profiling of the serum/plasma can yield important information on the

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immunoregulatory status of the patients[20]. Therefore, we will collect and store sera for future serial protein analysis (same time points), either as an antibody approach contracted out to Myriad RBM®, Austin, TX or performed at NU via a collaboration with Dr. Neil Kelleher whose laboratory performs top down/bottom up proteomics (both cells and plasma) by mass spectrometry.

iv) Donor-specific Immunoregulation. Regulatory T cells with donor-specificity are critical in maintaining transplant immunoregulation and may be even more sensitive than immunophenotyping or proteogenomic arrays. In future studies, pending funding, the approach will be to perform the Treg-MLR, assessing both donor-specific immunoregulation and hyporesponsiveness serially before and after EVL addition and TAC-WD/EVL or at rejection as a comparator - all compared with non-specific measures collected at the same time points [18,54,55]. For this, donor spleen/lymph nodes at organ retrieval and recipient lymph nodes (collected from the native explant liver hilum) are cryopreserved at the time of transplantation. Recipient pre-transplant cryopreserved CFSE-labeled lymph node cells or PBMC ($1\text{-}5x10^5$) are stimulated with cryopreserved donor or third party irradiated cells ($1\text{-}5x10^5$) in the presence of recipient PKH26 labeled *pre-* vs. *post-* transplant PBMC as third component modulators (as low as $5x10^2$ cell dilutions). After 7 days, standard 18-hour ^{3}H-TdR incorporation assays and $CD4^+$ CFSE quantitation (lymphoproliferation) are performed and the percentage inhibition calculated. The %Treg ($CD4^+CD25^{high}FoxP3^+$ cells) are calculated in the CFSE-labeled responder population by flow cytometric analyses after gating out the PKH26 labeled modulators. RM-ANOVA is performed to test the hypothesis that recipients' EVL-influenced post-operative cells "recruit" Tregs and inhibit proliferation *in vitro* in a donor-specific manner vs. TAC-influenced cells, particularly in non-rejecting vs. rejecting subjects.

3) Finally, in either scenario, there may be interest from our group or Novartis in comparing <u>diagnostic</u> (not predictive) peripheral blood and biopsy genomic biomarkers <u>at the time of AR</u> occuring before and after TAC WD/EVL. We have already identified genomic AR signatures in blood and biopsy from our prior work (all TAC-treated recipients), which can serve as comparative data for this trial[49]. There may be differences in AR signals in TAC-treated patients vs. those who have TAC withdrawn and are on EVL monotherapy. With additional future funding, we can perform the same mRNA as above at the time of any AR and compare rejections that occur pre- and post-TAC withdrawal. This will help identify signals that may be more specific of EVL (CNI-free) rejection to be able to clinically utilize these signals to guide diagnosis and patient management. Also, we can determine if biopsy vs. peripheral blood biomarker AR signals are similar or different, so as to ascertain if blood instead of biopsies (which are risky & costly) can be utilized for rejection diagnosis specific to EVL (CNI-free) therapy. For now, we will store blood (sera/plasma, PBMC, RNA) and biopsy tissue (RNAlater) at the time of AR if these analyses are of future interest.

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4 Study design

Overall: Prospective, open-label pilot study comparing immunoregulatory assays in non-viremic LT patients undergoing TAC-WD/EVL. The patients will serve as their own control for assays, clinical, biochemical, and histological measures, and the groups will be divided and compared by the presence or absence of rejection before, during and after the TAC-WD/EVL phase (months 0-6 (± 1 month)). We expect to find assay measures (primary objective) consistent with immunoregulation in patients who are successfully withdrawn from TAC compared to signs of immunoreactivity/lack of immunoregulation in those who fail TAC-WD due to rejection and require TAC reinstitution.

Clinical Trial & Timelines (Figure 6 in Appendices): Patients will be approached for study enrollment within six months before LT but can be enrolled up until 60 days post-LT. At transplant if possible, patients consented pre-LT will have donor spleen/lymph node cells and recipient lymph node/blood cells/sera cryopreserved. This will be collected for potential future studies of donor-specific immunoregulation and donor specific antibodies (funds not requested – see Exploratory Objectives). Post-transplant, patients will be treated as standard of care with the following protocol (no antibody induction): TAC therapy (target trough 7-10 ng/mL) +/- MPA between 1-3 g/d (Cellcept®, Myfortic® or generic alternate), corticosteroids (intravenous solumedrol 500 mg daily day 2, prednisone taper from 200 mg down to 20 mg daily at week 1, then slow taper to 5 mg daily by month 3). Patients not consent pre-LT will be approached within the first 60 days post-LT. At month 1 to 2 post-LT , study continuation criteria will be determined (see inclusion criteria). If met, EVL will be started (MPA discontinued if patient treated with this) and the EVL goal troughs will be 5-8 ng/ml and TAC 3-5 ng/ml [17]. At 3 months (± 1 month) post-LT, patients on stable TAC + EVL meeting continuation criteria will undergo the TAC-WD/EVL phase. TAC will be reduced by 50% of daily dose. After two weeks, if liver enzymes are stable, TAC will be reduced 50% each week and discontinued at month 4 (± 1 month) post transplant, if criteria are met. From month 4-6 (± 1 month), the patients will be on EVL therapy alone (trough 5-8 ng/ml) with stable prednisone 5 mg/day until study end (6 months (± 1 month)) or rejection (expected in ~20%).

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Peripheral blood bioassays will be performed pre-TAC-WD (1 to 2 months and 3 months (± 1 month)), immediately pre-TAC-WD (4 months (± 1 month)), 2 weeks post-TAC-WD (4.5 months (± 1 month) post-LT), at biopsy-proven rejection in any phase of the study (before or after TAC-WD phase), and at 6 months (± 1 month) post-LT (Timeline Figure 6). We will also store extra blood samples (funds not requested – See Exploratory Objectives). Liver biopsies for standard histology will be performed only for cause at the time of rejection and not required by the protocol – extra tissue placed in RNAlater will be collected (funds not requested). The blood biomarkers will be compared between the groups (AR vs. no AR). Additionally, the patients will be monitored as standard of care with clinical laboratory tests (CBC, liver/renal function tests, IS trough levels) for other respective secondary endpoints (hematologic, liver/renal function) every 1-2 weeks. Clinical laboratory tests for other endpoints (hyperlipidemia- lipid profiles, diabetes- HbA1C, proteinuria- urine protein/creatinine) will be performed pre-EVL addition and at 6 months (± 1 month) post-LT, all per our standard of care. Routine clinic visits will occur per standard of care (1, 2, 3, 4 & 6 months) to monitor for side effects and document any adverse events, in addition to our research coordinators maintaining close communication with the patients. The study will be completed at 6 months (± 1 month) post-LT, which will include final assessments of clinical and laboratory outcomes. Case report forms and electronic databases documenting all immune assay results and clinical data/outcomes will be utilized for all endpoint comparisons.

5 Population

This is a single-center (Northwestern University) study. We intend to enroll 25 subjects of which 20 are expected to undergo the TAC-WD/EVL phase of the study.

We will select adult LT recipients without immune disease, active viral disease, and previous or combined organ transplantation to avoid the confounding of coexistent inflammatory/infectious processes and prior transplants on the biomarker analysis. We will also have two 'checks' for study continuation after initial enrollment. The first is at one to two months post-LT to ensure there is no contraindication to starting EVL, that renal/liver function is reasonably preserved and stable, and that there is a low predicted risk of future TAC WD. The second check is to determine if TAC-WD at 3 months (± 1 month) is safe - similar criteria (no rejections, stable liver/renal function, etc) will be used.

5.1 Inclusion criteria

Patients must give written informed consent 90 days prior to transplant or up to 60 days after transplant) before any assessment is performed.

Patients eligible for inclusion in this study have to fulfill all of the following criteria:

Inclusion/Exclusion criteria:

Inclusion Criteria:

- Adult LT candidates ≥ 18 years of age

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- Listed for or recent (within 2 months) recipient of deceased or living donor liver transplantation

5.2 Exclusion criteria

Exclusion Criteria:

- Combined or previous organ transplantation
- HIV infection
- Inability to provide informed consent or comply with the protocol
- Transplantation for autoimmune liver disease (Autoimmune hepatitis, Primary Biliary Cirrhosis, Primary Sclerosing Cholangitis)

Criteria for Study Continuation and EVL addition (1 to 2 months post-LT):

1. Currently on TAC + prednisone +/- MPA therapy with no contraindication to starting EVL (i.e. wound infection; significant proteinuria (urine prot/cr ratio ≥1.0), hepatic artery thrombosis/stenosis, cytopenias (WBC <1.5, ANC<1.0, Platelet< 30, Hemoglobin <7), other deemed by investigator)

2. No more than 2 rejection episodes or 1 steroid-resistant rejection requiring lymphodepletion therapy

3. No active biliary, infectious, or malignant process

4. eGFR ≥30 ml/min/1.73m^2

5. No significant liver function abnormalities (Direct bilirubin, AST/ALT, Alkphos ≥ 2x elevated)

6. If on current or prior antiviral therapy, HBV and HCV viral load should be undetectable

7. No combined transplantation (e.g. should be primary liver transplant alone)

Criteria for Study Continuation and TAC-WD initiation (3 months (± 1 month) post-LT):

1. Tolerating TAC + EVL combination

2. Same other criteria as 1 to 2 months criteria above

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For all time points above, a ±1 month window is allowable except that EVL must not be started until ≥1 month post-LT and Visit 6 must occur ≥2 weeks after Visit 5

6 Treatment

6.1 Investigational and reference therapy

Zortress (Everolimus) is a macrolide immunosuppressant. Zortress® (Everolimus) is a macrolide immunosuppressant. The chemical name of everolimus is (1R, 9S, 12S, 15R, 16E, 18R, 19R, 21R, 23S, 24E, 26E, 28E, 30S, 32S, 35R)-1, 18-dihydroxy-12-{(1R)-2-[(1S,3R,4R)-4-(2hydroxyethoxy)-3-methoxycyclohexyl-1-methylethyl}-19,30-dimethoxy-15, 17, 21,23, 29, 35-hexamethyl-11, 36-dioxa-4-aza-tricyclo[30.3.1.$0^{4,9}$] hexatriaconta-16,24,26,28-tetraene-2, 3, 10,14,20-pentaone.

Everolimus inhibits antigenic and interleukin (IL-2 and IL-15) stimulated activation and proliferation of T and B lymphocytes. In cells, everolimus binds to a cytoplasmic protein, the FK506 Binding Protein-12 (FKBP-12), to form an immunosuppressive complex (everolimus: FKBP-12) that binds to and inhibits the mammalian Target Of Rapamycin (mTOR), a key regulatory kinase. In the presence of everolimus phosphorylation of p70 S6 ribosomal protein kinase (p70S6K), a substrate of mTOR, is inhibited. Consequently, phosphorylation of the ribosomal S6 protein and subsequent protein synthesis and cell proliferation are inhibited. The everolimus: FKBP-12 complex has no effect on calcineurin activity.

In rats and nonhuman primate models, everolimus effectively reduces kidney allograft rejection resulting in prolonged graft survival.

Everolimus pharmacokinetics has been characterized after oral administration of single and multiple doses to adult kidney transplant patients, hepatically-impaired patients, and healthy subjects.

6.2 Treatment arms

All patients will be assigned to Zortress® (everolimus) at advised doses and trough levels. After enrollment criteria met, EVL will be added (goal trough level 5-8 ng/ml) and TAC reduced (goal trough level 3-5 ng/ml). When TAC is discontinued, EVL will be maintained at goal trough of 5-8 ng/ml and prednisone kept for at least the study time period (6 months (± 1 month)) at 5 mg/day. See study design (Figure 6 in Appendices).

6.3 Treating the patient

6.3.1 Patient numbering

Subjects entering the study will be given sequential numbers in the order they are screened, e.g.; 01, 02, 03….

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6.3.2 Dispensing the study drug

"Study drug" is obtained via a prescription from the physician and filled at any pharmacy (local walk-in/mail-order).

6.3.3 Study drug supply, storage and tracking

Pharmacies should be storing the drug at room temperature: 25°C (77°C); excursions permitted to 15-30°C (59-86°F). Medication should be protected from light and moisture.

6.3.4 Instructions for prescribing and taking the study drug

"Study" drug will be obtained via a prescription. Everolimus is taken orally twice daily with doses approximately 12 hours apart. Everolimus can be taken with/without food.

This is an approved drug for the following:
Kidney transplant in low-moderate immunologic risk. It is to be used in combination with basiliximab, cyclosporine (reduced dose) and corticosteroids.

It is also approved for use in liver transplantation. It is not to be administered earlier than 30 days post-transplant. It is to be used in combination with tacrolimus (reduced doses) and corticosteroids.

6.3.5 Permitted study drug dose adjustments and interruptions

Per protocol to maintain the required trough levels of tacrolimus and everolimus therapy (Figure 6)

6.3.6 Other concomitant treatment

Any medication (prescribed or over-the-counter), alternative/complimentary medication, any medical or surgical procedure that the subject takes or undergoes during the study must be reported to the study team and will be reported in the CRF. This is to ensure that any elevation in liver function tests or rejection episode is most likely related to immunosuppression withdrawal and does not have another cause outside of the study.

It is important to note that medications that are normally used in post-transplant patients (antivirals, antibacterials, and other prophylactic meds, along with other prescribed meds) are allowed in this protocol.

Medications with strong-moderate CYP3A4 inhibitors (cyclosporine, detoconazole, erythromycin, verapamil) and CYP3A4 inducers (e.g., rifampin) may affect everolimus concentrations. In this case Zortress dose adjustments should be considered.

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6.3.7 Study drug discontinuation and premature patient withdrawal

Subjects may be withdrawn from the trial at any time for any reason, either by choice or due to medical indications. Subjects who are non-compliant with study guidelines, medications and clinic examinations will be withdrawn from the study. The NU IRB may also discontinue the study at any point. The subjects will be fully informed of these actions**.** If patients are withdrawn prematurely, they will still be followed through the follow-up time period of the study as per standard medical care. These data are important to collect to determine differences in this group compared to those who completed the study.

6.3.8 Study completion and post-study treatment

Study will be completed at the six month (± 1 month) time point post-liver transplantation

7 Visit schedule and assessments

Table 7-1 Assessment schedule

Visit**	1	2	3	4	5	6	7	At rejection***	Early Term./Study Comp.
Day	-90 to +30	0 (Tx)	30	90	120	135	180		
Inc./Exc. Criteria	X		X	X	X				
Obtain informed consent	X								
Physical Exam			X	X	X		X		
Donor/Recipient Collections		X*							
Blood (Flow, PBMC, RNA, sera)			X*	X	X	X	X	X	
Tissue RNA								X	
Study Completion Form							X	X	X

*unfunded and not mandatory for this protocol

** ± 1 monthwindow allowable

Visit 1 = Patient enrollment SOC visit (I/E criteria, informed consent)

Visit 2= Transplant SOC- if possible, donor spleen/lymph nodes and recipient lymph node/blood

Visit 3= Day 30 SOC visit- if Inc./Exc. criteria met, add everolimus and stop MPA if being used. Collect blood if possible (unfunded for this study).

Visit 4= Day 90 SOC visit- if Inc./Exc. criteria met, start tacrolimus withdrawal. Collect blood.

Visit 5= Day 120 SOC visit- if Inc./Exc. criteria met, stop tacrolimus. Collect blood.

Visit 6 = Day 135 visit- collect blood.

Visit 7= Day 180 SOC visit- Study termination. Collect blood.

***At any biopsy concerning for rejection: Collect blood and tissue in RNAlater. If rejection, study termination.

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7.1 Patient demographics/other baseline characteristics

Basic demographics (age, sex, race, cause of liver disease, past medical history) and laboratory values will be determined prior to enrollment. To determine continuation criteria at one to two months and three months (± 1 month), the medical charts will be reviewed to determine laboratory values and ensure inclusion/exclusion criteria are met. Throughout the trial, history and physicals per standard of care will be performed at most of the study time points (see schedule above) as well as laboratory values.

7.2 Treatment exposure and compliance

All immunosuppressant drug levels will be documented at the study time points, and compliance with the dosing will be assessed at each study time point by the PI and coordinator. Side effects of tacrolimus (renal, neurologic, metabolic, etc) and everolimus (hyperlipidemia, edema, proteinuria, etc) will be documented in the case report forms

7.3 Efficacy

The major efficacy assessment is the percentage of patients successfully withdrawn from TAC therapy in favor of EVL therapy without rejection or major complications. Rejection will be evaluated by liver biopsy when indicated (typically 2-3 fold increase in liver aminotransferases). If rejection occurs during any point of the trial following EVL addition, the study will be terminated for that patient.

Other efficacy endpoints will be the improvement in TAC side effects (renal, neurologic, metabolic, etc.) and development of EVL side effects (hyperlipidemia, edema, proteinuria, etc) in those successfully withdrawn from TAC without rejection. For those unable to withdraw from TAC due to rejection, we will assess for TAC side effects and EVL side effects if this drug is continued following rejection. Liver and renal function, and hematological parameters will be followed per SOC throughout this biomarker study

The PI and research coordinator will perform these assessments at the study time points at Northwestern and at any intervening time point if the patient needs a liver biopsy to exclude rejection or other complication (hospitalization, infection, etc.). Patients enrolled also will be contacted by phone for any changes in their immunosuppression dosing or other medications in between visits.

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7.4 Safety/Potential Adverse Events/Protection Against Risk

The risks to the patient are limited to the minor risk of performing venipuncture and the risk of rejection with TAC-WD. The National Cancer Institute Common Terminology Criteria for Adverse Events v3.0 (CTCAE), dated December 12, 2003 will be used to grade all adverse (either serious or non-serious) events. It is the responsibility of the Principal Investigator to oversee the safety of the study. This safety monitoring will include careful assessment and appropriate reporting of adverse events as noted above. Medical monitoring will include a regular assessment of the number and type of serious adverse events. Information on all adverse events (signs, symptoms, abnormal diagnostic procedures, treatments) will be recorded in the appropriate adverse event module of the case report form (CRF). The clinical course of each event will be followed until resolution, stabilization, or until it has been determined that the study participation is not the cause. Serious adverse events that are still ongoing at the end of the study period will be followed to determine the final outcome.

Reports of all serious adverse events (including follow-up) will be submitted to the IRB within 24 hours of the investigator's knowledge of the event. Follow-up reports will be submitted to the IRB within 10 working days. The IRB/Office for the Protection of Research Subjects at NU is under the direction of Lewis Smith, MD- (312) 503-9338. As this is a prospective study using primary data collection with a drug of interest all adverse events (AEs) – including serious adverse events (SAEs) and safety endpoints (where relevant) – will be collected and recorded in the study and safety database, irrespective of causal association. All adverse reactions identified will be reported to the local Health Authority in accordance with national regulatory requirements.

All of the risks and benefits will be discussed with the patient at the time of informed consent. The patient will undergo informed consent prior to all liver biopsies and suspected rejection will be managed as per our standard of care (SOC). The biopsies will be performed using a 16-18 Gauge BioPince® biopsy instrument, which utilizes a Tri-axial "Cut and Capture" cannula system. We will remove a 0.5-1 cm biopsy piece from the main 3 cm sample for genomic analysis. Thus, only one pass liver biopsy is required per SOC and not extra for the study. The patient will be monitored for 2 hours after each liver biopsy in the Procedure Unit at Northwestern. Vital signs will be performed before and every 15 minutes x 1 hour, every 30 minutes x 1 hour per standard protocol. Any atypical symptom after biopsy, i.e. shortness of breath, hypotension, tachycardia, fever, severe abdominal pain, will be investigated immediately by the physician performing the biopsy.

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The patient will be monitored for any complication of this study as he/she would as part of our standard management, i.e. monitoring of graft function, kidney function, trough IS levels, cholesterol levels, urine protein/creatinine ratio. This follows the clinical practice that has been occurring at our center over the last 20 years. There is a risk of rejection with TAC-WD (~20%) which will be discussed with the patient. If a graft loss occurs due to the study protocol or ≥30% rejection occurs with TAC-WD, the study will be terminated for safety reasons. This is counterbalanced by the equipoise of the potential for preservation of renal function and other TAC side effects by TAC-WD. The blood collection will occur at the time of the standard of care blood tests after transplantation and thus will not add additional venipuncture risk. The allowed amount of blood per venipuncture/day will not be exceeded according to our IRB rules. Overall, the patient will be well informed of our standard of care approaches and research protocols, with appropriate discussion and consent.

Another risk involves breach of patient confidentiality during the study. To avoid this, all informed consents, medical records, and electronic databases will be stored in a locked location at Northwestern, with access limited to study personnel. Patient samples utilized for research will be de-identified by a coding system only accessed by the Co-PIs. Special procedures for ensuring patient confidentiality will be implemented in accordance with the HIPAA. Data transmission and the distributed data systems will have multiple layers of security. Each study subject will be assigned a unique identification number. Only this number will be used to identify subjects in any individual tabulation. It is expected that only group data will be published. If individual subject data are to be published, no identifying information will be included. The study files will be maintained in a secure location. Access to computerized data will be restricted to study personnel. Password authentication will be enforced. These passwords will be changed on a quarterly basis and whenever the Database Administrator makes a determination for a security change.

7.4.1 Physical examination

On visits 3, 4, 5, 7 and early termination an exam will be performed.

7.4.2 Vital signs

Vital signs including blood pressure, pulse, respiratory rate and temperature will be taken at every SOC visit and study-related visit when the subjects come to clinic.

7.4.3 Height and weight

Subjects will be weighed at every visit and their height will be taken at the first study visit.

7.4.4 Laboratory evaluations

At liver transplant donor/recipient collection of lymph nodes and liver biopsy tissue will take place if the patient is consented prior to transplantation. Liver biopsy tissue will also be collected at the time of a suspected rejection.

Blood samples for flow cytometry, PBMCs, RNA and sera will be collected at visits 3, 4, 5, 6, 7 and rejection.

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Standard SOC laboratory tests will be performed per our transplant center post-LT protocol: CBC, CMP, trough IS levels three times weekly (0-1 month post-LT), two times weekly (1-3 months post-LT), one time weekly (3-6 months post-LT).

7.4.5 Pregnancy and assessments of fertility

Pregnancy

There are no adequate and well-controlled studies of Zortress in pregnant women. In rats and rabbits, everolimus crossed the placenta and was toxic to the conceptus. The potential risk for humans in unknown. Zortress should be given to pregnant women only if the potential benefit to the mother justifies the potential risk to the fetus. Women of childbearing potential should be advised to use effective contraception methods while they are receiving Zortress and up to 8 weeks after treatment has been stopped.

Nursing Mothers

It is not known whether everolimus is excreted in human milk. Everolimus and/or its metabolites readily transferred into milk of lactating rats at a concentration 3.5 times higher than in maternal serum. Because many drugs are excreted in human milk and because of the potential for serious adverse reactions in nursing infants from everolimus, women should avoid breast-feeding during treatment with Zortress.

7.4.6 Appropriateness of safety measurements

All safety measurements are appropriate as standard of care for patient management and this protocol.

7.4.7 Other biomarkers

Please see Exploratory Objectives above

8 Safety monitoring

It is the responsibility of the Principal Investigator and sub-investigators to oversee the safety of the study at their site. This safety monitoring will include careful assessment and appropriate reporting of adverse events as noted above. Medical monitoring will include a regular assessment of the number and type of serious adverse events.

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8.1 Adverse events

Definition of an AE: Any untoward medical occurrence in a subject administered a pharmaceutical product that does not necessarily have a causal relationship with the treatment. An AE can therefore be any unfavorable and unintended sign (including an abnormal laboratory finding), symptom, or disease temporally associated with the use of a medicinal (investigational) product, whether or not considered related to the investigational medicinal product. Investigational Medicinal Product (IMP) includes the drug under evaluation and the comparator drug(s) if specified as part of the research objective, given at any time during the study. Medical conditions/diseases present before starting the drug of interest are only considered adverse events if they worsen after starting the drug of interest.

The occurrence of adverse events will be sought by non-directive questioning of the patient at each visit during the study. Adverse events also may be detected when they are volunteered by the patient during or between visits or through physical examination, laboratory test, or other assessments. All adverse events will be recorded in the study database including the following information:

1. the severity grade (mild, moderate, severe) or (grade 1-4)
2. its relationship to the drug(s) of interest (suspected/not suspected)
3. its duration (start and end dates or if continuing at final exam)
4. whether it constitutes a serious adverse event (SAE)

8.2 Serious adverse event reporting

An SAE: is any untoward medical occurrence that at any dose:

- results in death,
- is life-threatening,
- requires inpatient hospitalization or prolongation of existing hospitalization,
- results in persistent or significant disability/incapacity,
- is a congenital anomaly/birth defect,
- is otherwise a significant medical event.

This includes any SAEs likely to arise from the trial indication or progression of underlying/concomitant illness (es) (e.g. progression of cancer in oncology trials), unless specified in the protocol as study specific exemptions.

Any SAE, irrespective of causality, occurring after the subject has provided informed consent and until four weeks after the subject has stopped study participation must be reported unless otherwise stated in the protocol. SAEs occurring after four weeks from ending study participation should only be reported if considered by the Investigator attributable to the exposure to the investigational drug(s) during the trial period. This includes the period in which the study protocol interferes with the standard medical treatment given to a subject, even if study treatment has not yet started (e.g. withdrawal of previous treatment during washout period, change in treatment to a fixed dose of concomitant medication).

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Timelines: All serious adverse events (SAEs) from interventional clinical trials must be reported by the sites to Sponsor within 24 hours of occurrence of the SAE. The timelines for investigator initiated trials reporting to Novartis will be done as per Third Party Study/Investigator Initiated Trial Agreement.

Follow-up reports:

SAEs will be followed until resolution or until it is judged to be permanent, and an assessment will be made at each visit (or more frequently, if necessary) of any changes in severity, the suspected relationship to the drug of interest, the interventions required to treat it, and the outcome.

The Sponsor shall support Novartis in the following-up of all SAEs so that complete information is available to maintain patient safety and also as part of any commitments by Novartis to any Health authority OR specific Health authority follow-up requests for the product under investigation.

8.3 Pregnancies

To ensure patient safety, each pregnancy in a patient (or a patient's partner) on study drug must be reported to Novartis within 24 hours of learning of its occurrence. The pregnancy will be followed up to determine outcome, including spontaneous or voluntary termination, details of the birth, and the presence or absence of any birth defects, congenital abnormalities, or maternal and/or newborn complications.

9 Data review and database management

9.1 Data collection (data handling and record keeping)

This study is to be conducted according to US and international standards of Good Clinical Practice (FDA Title 21 part 312 and International Conference on Harmonization guidelines), applicable government regulations and Institutional research policies and procedures. This protocol and any amendments will be submitted to the Northwestern University Institutional Review Board (NU IRB), for formal approval of the study conduct.

Information about study subjects will be kept confidential and managed according to the requirements of the Health Insurance Portability and Accountability Act of 1996 (HIPAA).

Consent:

All subjects for this study will be provided with a printed consent form describing this study and providing sufficient information for subjects to make an informed decision about their participation in this study. This consent form will be submitted with the protocol for review and approval by the NU IRB for the study. The formal consent of a subject, using the NU IRB-approved consent form, will be obtained before the subject is submitted to any study procedures. This consent form will be signed by the subject and the investigator-designated research professional obtaining the consent. The subject will be given a signed copy of the informed consent for their records.

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Source Documents:
Source data is all information, original records of clinical findings, observations, or other activities in a clinical trial necessary for the reconstruction and evaluation of the trial. Source data are contained in source documents. Examples of these original documents, and data records include, but are not limited to: hospital records, clinical and office charts, laboratory notes, memoranda, subjects' diaries or evaluation checklists, pharmacy dispensing records, recorded data from automated instruments, copies or transcriptions certified after verification as being accurate and complete, microfiches, photographic negatives, microfilm or magnetic media, x-rays, subject files, and records kept at the pharmacy, at the laboratories, and at medico-technical departments involved in the clinical trial.

Confidentiality:
Information about study subjects will be kept confidential and managed according to the requirements of the Health Insurance Portability and Accountability Act of 1996 (HIPAA). Those regulations require a signed subject authorization (which is incorporated into a single document which includes the Northwestern University Informed Consent Form). This combination document must inform the subject of the following:

- What protected health information (PHI) will be collected from subjects in this study
- Who will have access to that information and why
- Who will use or disclose that information
- The rights of a research subject to revoke their authorization for use of their PHI.

In the event that a subject revokes authorization to collect or use PHI, the investigator, by regulation, retains the ability to use all information collected prior to the revocation of subject authorization. For subjects that have revoked authorization to collect or use PHI, attempts should be made to obtain permission to collect at least vital status (i.e. that the subject is alive) at the end of their scheduled study period.

9.2 Database management and quality control

Case Report Forms:

After study initiation, the electronic case report form (eCRF) will be the primary data collection instrument for the study. Only the Investigators will have access to this password protected file. No subject identifiers will be included in the eCRF and electronic databases that are used to record clinical data on patients. All clinical data will be kept completely confidential.

Auditing and Inspecting:
The investigator will permit study-related monitoring, audits, and inspections by the NU IRB, the sponsor, government regulatory bodies, and University compliance and quality assurance groups of all study related documents (e.g. source documents, regulatory documents, data collection instruments, study data etc.). The investigator will ensure the capability for inspections of applicable study-related facilities (e.g. pharmacy, diagnostic laboratory, etc.).

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Participation as an investigator in this study implies acceptance of potential inspection by government regulatory authorities and applicable University compliance and quality assurance offices.

Record Retention:
It is the investigator's responsibility to retain study essential documents on site for at least 2 years after the study has been formally terminated at the NU IRB. After this time, it is up to the investigator to determine whether the documents should be stored off-site at O'Hare Records, located in Rosemont, IL for up to 13 more years. Once the investigator determines how long to keep the records, when that time frame is up it is the responsibility of the investigator to ensure that all study records are properly disposed of by shredding.

10 Data analysis

10.1 Populations for analysis

Since there is no randomization in the study, the groups that are analyzed are those that have rejection before TAC-WD, those who develop rejection during/after TAC-WD, and those who never have rejection before or after TAC-WD.

10.2 Analysis of the primary objective(s)

Primary objective: To identify cellular and molecular metrics of immune regulation that may predict successful TAC withdrawal in favor of EVL early after LT.

Overall Strategy and Rationale: The objective is to test the working hypothesis that, in a prospective study, cellular and genomic markers of immunoregulation become more pronounced after early rejection-free TAC-WD/EVL compared to failed (rejection) TAC-WD. Conversely, if these markers do not signify immunoregulation, we hypothesize that TAC-WD/EVL will not be successful (i.e. rejection will ensue requiring reinstatement of TAC). The rationale is that the immunoregulatory mechanisms of IS therapy (EVL vs. CNI) can be more adequately clarified and that a higher % of patients can undergo successful TAC-WD/EVL by utilizing these serial, safe blood biomarkers to predict rejection, before it occurs, i.e. by comparing these signals right before and after TAC-WD. Our hypothesis is based on our previous observations that late mTOR-I conversion increased % of Treg/DCreg in blood/tissue sites and generated immunoregulatory proteogenomic signatures and that AR can be diagnosed in the peripheral blood by genomic microarrays. Therefore, the plan is to perform sequential flow cytometry immunophenotyping and genomic signature analysis in the blood before, during and after attempted TAC-WD/EVL.

Studies Planned and Methods:

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i) Blood Immunophenotyping. The approach will be to conduct flow cytometry immunophenotyping of peripheral blood before TAC-WD initiation (1 to 2 months and 3 months (± 1 month)), right at total TAC-WD (4 months (± 1 month)), 2 weeks after full TAC-WD (4.5 months (± 1 month)) and study end (6 months (± 1 month)) post-LT to analyze cellular changes. Methods: The Immune Monitoring Laboratory at NU (Co-Director James Mathew, PhD) will perform the immunophenotyping. PBMC are isolated from heparinized 30 cc samples on Ficoll-Hypaque gradients. Surface markers are detected with monoclonal antibodies for the T cell subsets CD3, CD4, CD8, CD25, CD45RA, CTLA4 and CD127 followed by fixation/permeabilization/incubation with human FOXP3 antibodies and final flow cytometric analysis. RBC-lysed peripheral blood is also labeled with monoclonal antibodies for T cell subsets, naïve/memory/regulatory B cells (CD19, CD27, IgD, IgM, IL-6), monocytes (CD14) and NK cells (CD56) to quantify the absolute cell numbers per μL. In addition, dendritic cell surface markers are analyzed as follows: monocytoid vs. plasmacytoid (CD11c; CD123) ratio; antigen processing markers (CD83; CD205); markers inducing Tregs (ILT3; ILT4). For statistical analysis, we perform within subject comparisons on PBMC for both successful and failed TAC-WD/EVL groups using appropriate paired analysis (paired t-test or Wilcoxon Signed Rank test). For the main hypothesis testing, repeated measures of analysis of variance (RM-ANOVA) are used to test the trajectory variations between the groups on PBMC over time. Two-sided F-test statistics are applied; α=0.05 (stats by NU Transplant Statistician).

ii) Gene Expression Microarrays: The approach will be to conduct parallel serial genomic bioassays of peripheral blood before and after attempted TAC-WD/EVL (same time points as immunophenotyping). Methods: The Genomics Core at Scripps Clinic (Director, Daniel Salomon, MD) will perform the microarray analysis. Blood will be collected in PaxGene tubes and sent to Scripps for the gene microarrays. Affymetrix® Hu133 Plus Peg microarrays will be used to test for differentially expressed genes between those developing AR vs. without AR during TAC WD. We will also perform biological function and canonical pathway mapping of several immune/inflammatory gene pathways. For statistical analysis, we report for each mRNA and microRNA comparison the T-test p-value, its non-parametric analog (Kruskal Wallis test), the fold change, and for multi-analyte signatures, the area under the receiver operating curve (concordance index/C-index) and q-values by member genes/proteins. We then calculate the predictive accuracy, sensitivity and specificity of each signature (statistics by Dr. Daniel Salomon's informatics group at Scripps Clinic)

10.2.1 Variable

The main variables are the peripheral blood Treg% (or other immunoregulatory cells) and mRNA AR signatures seen prior to and during either successful (no rejection) vs. failed (rejection) TAC-WD/EVL.

10.2.2 Statistical hypothesis, model, and method of analysis

The statistical analysis of the primary variables (immune assays) is described in detail above

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10.2.3 Handling of missing values/censoring/discontinuations

In this study, discontinuation will usually be due to rejection occurring during the study protocol (TAC-WD/EVL). While unlikely as this trial is a small pilot study, patients lost to follow-up will be removed from the trial and analysis.

10.2.4 Supportive analyses

None – see Exploratory Objectives above

10.3 Analysis of secondary objectives

Clinical endpoints as correlates for the success and failure of TAC-WD/EVL will serve as secondary endpoints. This will include but not be limited to the following at 6 months (± 1 month) post-LT: liver allograft and renal function, EVL & TAC side effects, infectious/malignant complications, and patient/graft survival. At study end, all secondary endpoints will be statistically compared between those who succeed or fail TAC-WD/EVL.

10.3.1 Efficacy (secondary)

All potential clinical endpoints will be recorded by case report form. Since this is a small pilot study, the clinical outcomes will be mainly descriptive in nature, e.g. rejection rate, medication side effect rate, liver/renal function outcomes, infectious complications, malignancy, and patient/graft survival. However, we will statistically compare these secondary endpoints between those who succeed or fail TAC-WD/EVL by standard continuous and categorical statistical tests.

10.3.2 Safety/Stopping Rules

If any patient experiences a graft loss due to the protocol (TAC-WD) or ≥30% rejection is seen, the study will be terminated due to safety concerns.

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10.3.3 Biomarkers/Studies Planned

i) Blood Immunophenotyping. The approach will be to conduct flow cytometry immunophenotyping of peripheral blood before TAC-WD initiation (3 months (± 1 month)), right at total TAC-WD (4 months (± 1 month)), 2 weeks after full TAC-WD (4.5 months (± 1 month)) and study end (6 months (± 1 month)) post-LT to analyze cellular changes. Methods [19,20]: The Immune Monitoring Laboratory at NU (Co-Director James Mathew, PhD) will perform the immunophenotyping. PBMC are isolated from heparinized 30 cc samples on Ficoll-Hypaque gradients. Surface markers are detected with monoclonal antibodies for the T cell subsets CD3, CD4, CD8, CD25, CD45RA, CTLA4 and CD127 followed by fixation/permeabilization/incubation with human FOXP3 antibodies and final flow cytometric analysis. RBC-lysed peripheral blood is also labeled with monoclonal antibodies for T cell subsets, naïve/memory/regulatory B cells (CD19, CD27, IgD, IgM, IL-6), monocytes (CD14) and NK cells (CD56) to quantify the absolute cell numbers per μL. In addition, dendritic cell surface markers are analyzed as follows: monocytoid vs. plasmacytoid (CD11c; CD123) ratio; antigen processing markers (CD83; CD205); markers inducing Tregs (ILT3; ILT4). For statistical analysis, we perform within subject comparisons on PBMC for both successful and failed TAC-WD/EVL groups using appropriate paired analysis (paired t-test or Wilcoxon Signed Rank test). For the main hypothesis testing, repeated measures of analysis of variance (RM-ANOVA) are used to test the trajectory variations between the groups on PBMC over time. Two-sided F-test statistics are applied; α=0.05 (stats by NU Transplant Statistician).

ii) Gene Expression Microarrays: The approach will be to conduct parallel serial genomic bioassays of peripheral blood before and after attempted TAC-WD/EVL (same time points as immunophenotyping). Methods: The Genomics Core at Scripps Clinic (Director, Daniel Salomon, MD) will perform the microarray analysis. Blood will be collected in PaxGene tubes and sent to Scripps for the gene microarrays [20,46,47,49]. Affymetrix® Hu133 Plus Peg microarrays will be used to test for differentially expressed genes between those developing AR vs. without AR during TAC WD. We will also perform biological function and canonical pathway mapping of several immune/inflammatory gene pathways. *For statistical analysis,* we report for each mRNA and microRNA comparison the T-test p-value, its non-parametric analog (Kruskal Wallis test), the fold change, and for multi-analyte signatures, the area under the receiver operating curve (concordance index/C-index) and q-values by member genes/proteins. We then calculate the predictive accuracy, sensitivity and specificity of each signature (statistics by Dr. Daniel Salomon's informatics group at Scripps Clinic).

10.4 Sample size calculation

As this is an exploratory pilot study, we will enroll an initial 25 patients, 20 of who are expect to undergo TAC-WD/EVL. Of these, we expect 14-15 will not have AR during TAC-WD and 4-5 are expected to have AR, based on the initial EVL clinical trial[17]. This will allow us to have preliminary information to proceed ahead to one of a few different directions, as outlined in Exploratory Objectives. Therefore, a power analysis was not needed for this pilot study.

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10.5 Power for analysis of critical secondary variables

Secondary clinical variables will be documented for descriptive reporting and to compare between those with and without AR. Given this is a small exploratory study, a power analysis for both primary and secondary variables were not considered necessary.

10.6 Interim analysis

Given the small size of this pilot study, an interim analysis will not be performed as clinical events or biomarker results will be limited at any interim time point. However, we will be continuously monitoring our acute rejection rates and stopping rules (e.g. any graft loss or rejection≥30% believed to be due to TAC-WD/EVL).

11 Ethical considerations

11.1 Regulatory and ethical compliance

This clinical study was designed and shall be implemented and reported in accordance with the ICH Harmonized Tripartite Guidelines for Good Clinical Practice, with applicable local regulations (including European Directive 2001/20/EC, US Code of Federal Regulations Title 21, and Japanese Ministry of Health, Labor, and Welfare), and with the ethical principles laid down in the Declaration of Helsinki.

11.2 Informed consent procedures

Eligible patients may only be included in the study after providing written (witnessed, where required by law or regulation), NU IRB-approved informed consent, or, if incapable of doing so, after such consent has been provided by a legally acceptable representative of the patient. In cases where the patient's representative gives consent, the patient should be informed about the study to the extent possible given his/her understanding. If the patient is capable of doing so, he/she should indicate assent by personally signing and dating the written informed consent document or a separate assent form. Informed consent must be obtained before conducting any study-specific procedures (i.e. all of the procedures described in the protocol). The process of obtaining informed consent should be documented in the patient source documents.

Women of child bearing potential should be informed that taking the study medication may involve unknown risks to the fetus if pregnancy were to occur during the study and agree that in order to participate in the study they must adhere to the contraception requirement for the duration of the study. If there is any question that the patient will not reliably comply, they should not be entered in the study.

11.3 Responsibilities of the investigator and NU IRB

This study is to be conducted according to US and international standards of Good Clinical Practice (FDA Title 21 part 312 and International Conference on Harmonization guidelines), applicable government regulations and Northwestern University Institutional research policies and procedures.

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This protocol and any amendments will be submitted to the Northwestern University Institutional Review Board (NU IRB), in agreement with local legal prescriptions, for formal approval of the study conduct. The decision of the NU IRB concerning the conduct of the study will be made in writing to the investigator before commencement of this study.

All subjects for this study will be provided a consent form describing this study and providing sufficient information for subjects to make an informed decision about their participation in this study. See Appendices for a copy of the Subject Informed Consent Form. This consent form will be submitted with the protocol for review and approval by the NU IRB for the study. The formal consent of a subject, using the NU IRB-approved consent form, must be obtained before that subject is submitted to any study procedure. This consent form must be signed by the subject or legally acceptable surrogate, and the investigator/ investigator-designated research professional obtaining the consent.

A signed and dated statement that the protocol and informed consent have been approved by the NU IRB must be given to Novartis before study initiation.

11.4 Publication of study protocol and results

The final study report is expected to be sent to Novartis no later than 9 months after the last patient visit.

The data abstracted and analyzed from this study will be submitted to scientific meetings. It is expected that this will be in the form of an abstract and presented as a poster or orally. In addition, the data will be sent to a professional peer reviewed journal for publication.

Subjects will not be identified in any way at the scientific meeting if this abstract is chosen for an oral or poster presentation. Subjects will not be identified in any written format of this data analysis.

Neither the complete nor any part of the results of the study carried out under this protocol, nor any of the information provided by the sponsor (Dr. Josh Levitsky) for the purposes of performing the study, will be published or passed on to any third party without the consent of the study sponsor.

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12 Protocol adherence

12.1 Protocol Amendments

Any change or addition to the protocol can only be made in a written protocol amendment that must be approved by Novartis, Health Authorities where required, and the NU IRB. Only amendments that are required for patient safety may be implemented prior to NU IRB approval. Notwithstanding the need for approval of formal protocol amendments, the investigator is expected to take any immediate action required for the safety of any patient included in this study, even if this action represents a deviation from the protocol.

13 Figures and References

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Appendices:

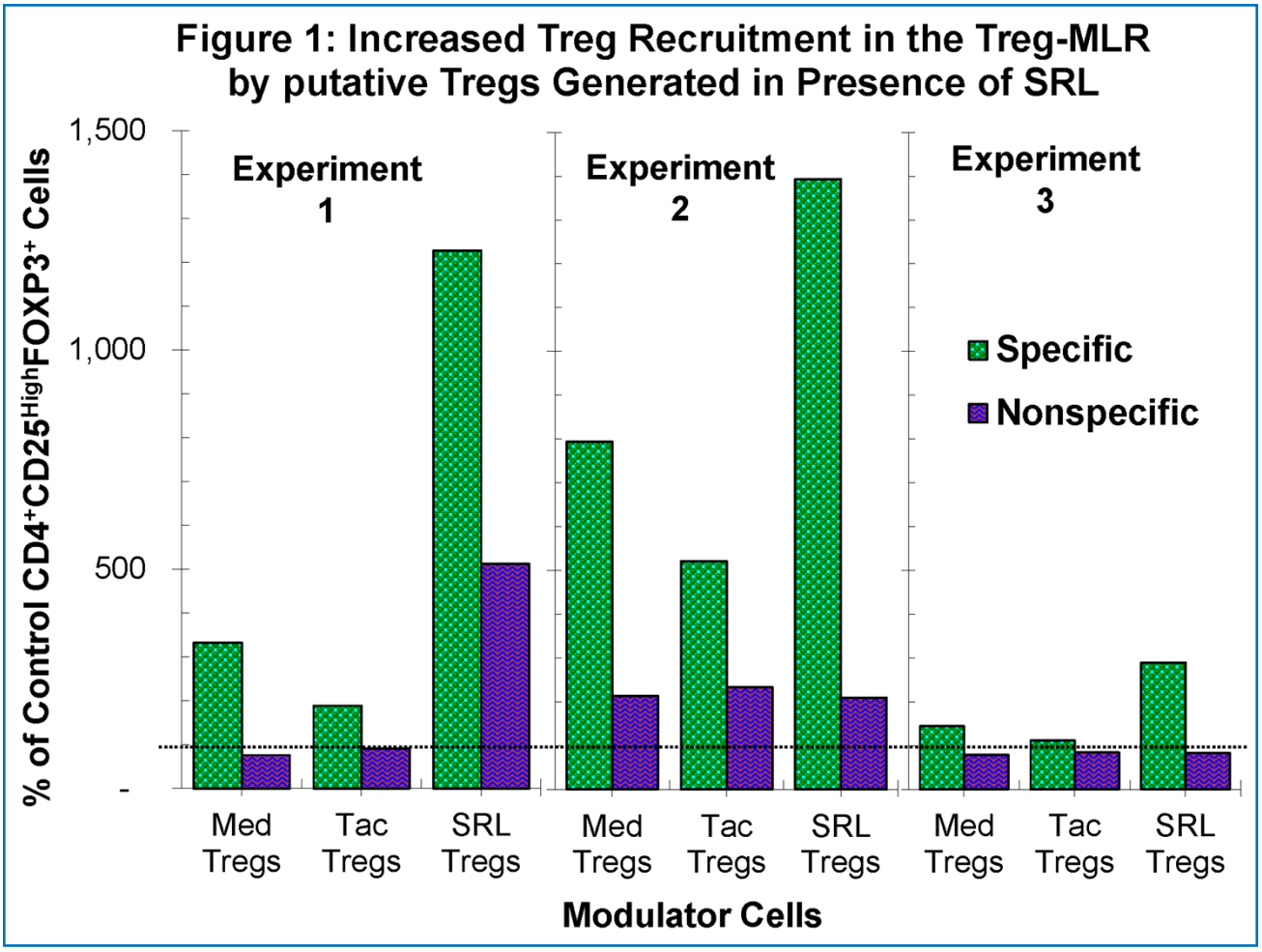

Figure 1: New $CD4^{+}CD25^{High}FOXP3^{+}$Treg "Recruitment" by Allo-specific Tregs Generated in the Presence of Media, TAC or SRL in MLR. Bulk cultures of volunteer responder cells were stimulated with 2 DR-matched x-irradiated cells in the presence of either no drugs, 5ng/ml TAC or SRL for 7 days and putative $CD4^{+}CD25^{High}CD127^{-}$ cells were enriched using the Regulatory T cell Isolation Kit and AutoMACS (Miltenyi Biotech), after negatively selecting out CD8, CD19 and CD127. Then, in 3 separate experiments, $5x10^{4}$ enriched Tregs or fresh autologous control PBMC were added as modulators to $5x10^{5}$ autologous fresh CFSE-labeled responding PBMC and x-irradiated (unlabeled) specific and non-specific stimulator PBMC. The modulator cells and x-irradiated stimulator cells were all labeled with PKH26 to differentiate these from the readout of CFSE labeled responding cells. After another 7 days, 5-color flow cytometric assays were performed to estimate $CD4^{+}CD25^{High}FOXP3^{+}$ cells in the CFSE-labeled responder PBMC. *Recruitment* data are calculated as percentage increase in $CD4^{+}CD25^{High}FOXP3^{+}$ cell generation with the Tregs over that observed with the control fresh PBMC modulators (dotted line). Note that a more marked and allospecific effect of SRL cultured modulators occurred (green diamond bars, SRL vs. TAC and media, p=0.01). A value below the dotted line or a change of ≤10% from control is considered to indicate the absence of recruitment.

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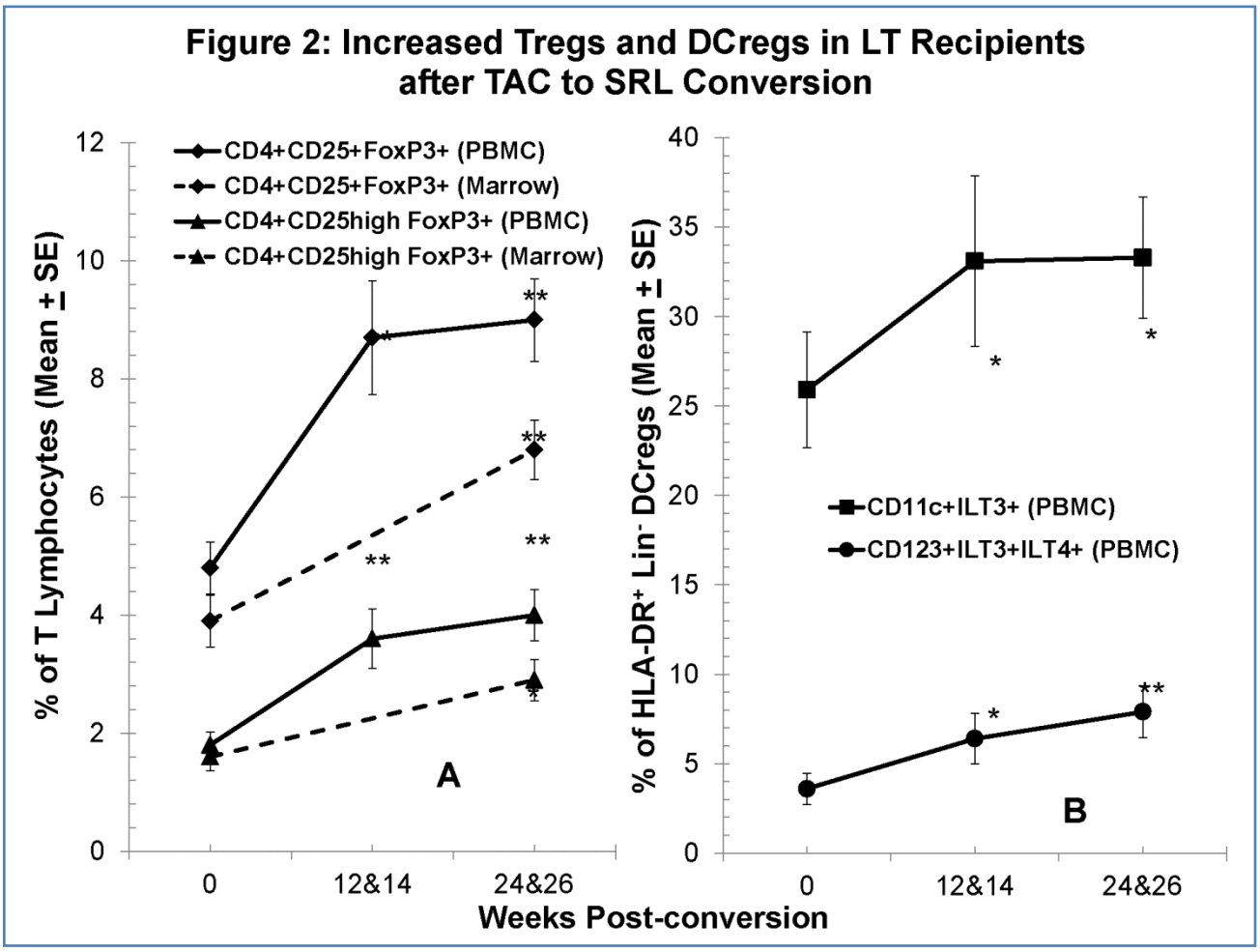

Figure 2: Increase in Phenotypic Regulatory T Cells and Regulatory Dendritic Cells with Tacrolimus to Sirolimus Conversion. Immunophenotyping flow cytometry was performed on peripheral blood and bone marrow aspirate specimens before (0, horizontal axis) and after (means of 12 and 14 weeks and of 24 and 26 weeks, horizontal axis) conversion from tacrolimus to sirolimus. Note the significant increase in the percentage of phenotypic Tregs ($CD4^+CD25^+FOXP3^+$, $CD4^+CD25^{high}FOXP3^+$) in the peripheral blood and marrow **(A)**, as well as the number of peripheral blood $CD123^+ILT3^+ILT4^+$ and $CD11c^+ILT3^+$ DCregs **(B)**, following sirolimus conversion. * $p<0.05$ and ** $p<0.01$ compared to time point 0 (pre-conversion on TAC).

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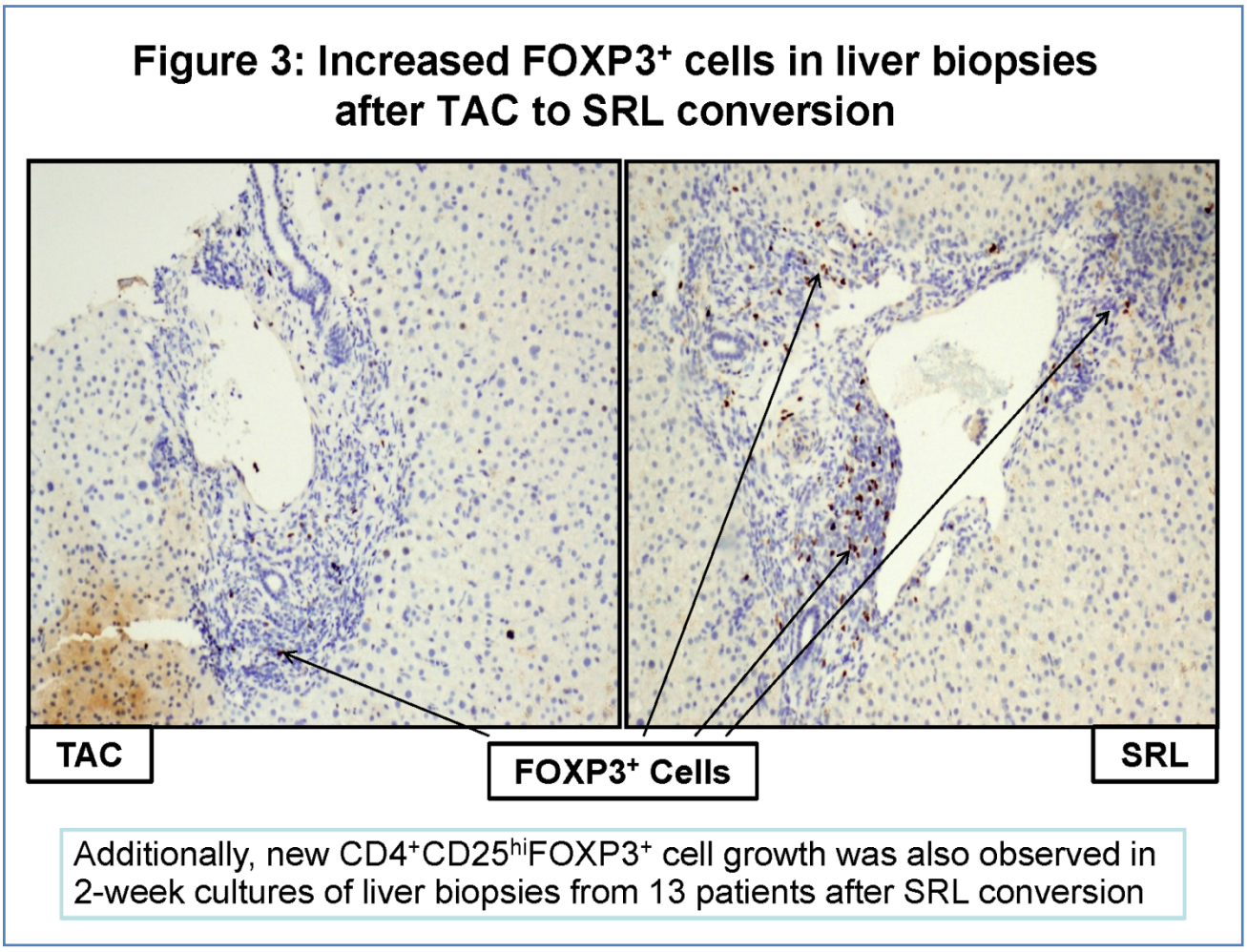

Figure 3: Increased FOXP3$^+$ cells in Liver Biopsies after Tacrolimus to Sirolimus Conversion: Immunohistochemical staining was performed on liver biopsy specimens before and after conversion from tacrolimus to sirolimus (representative example shown). Note the increase in the number of FOXP3$^+$ Cells (arrows) following sirolimus conversion.

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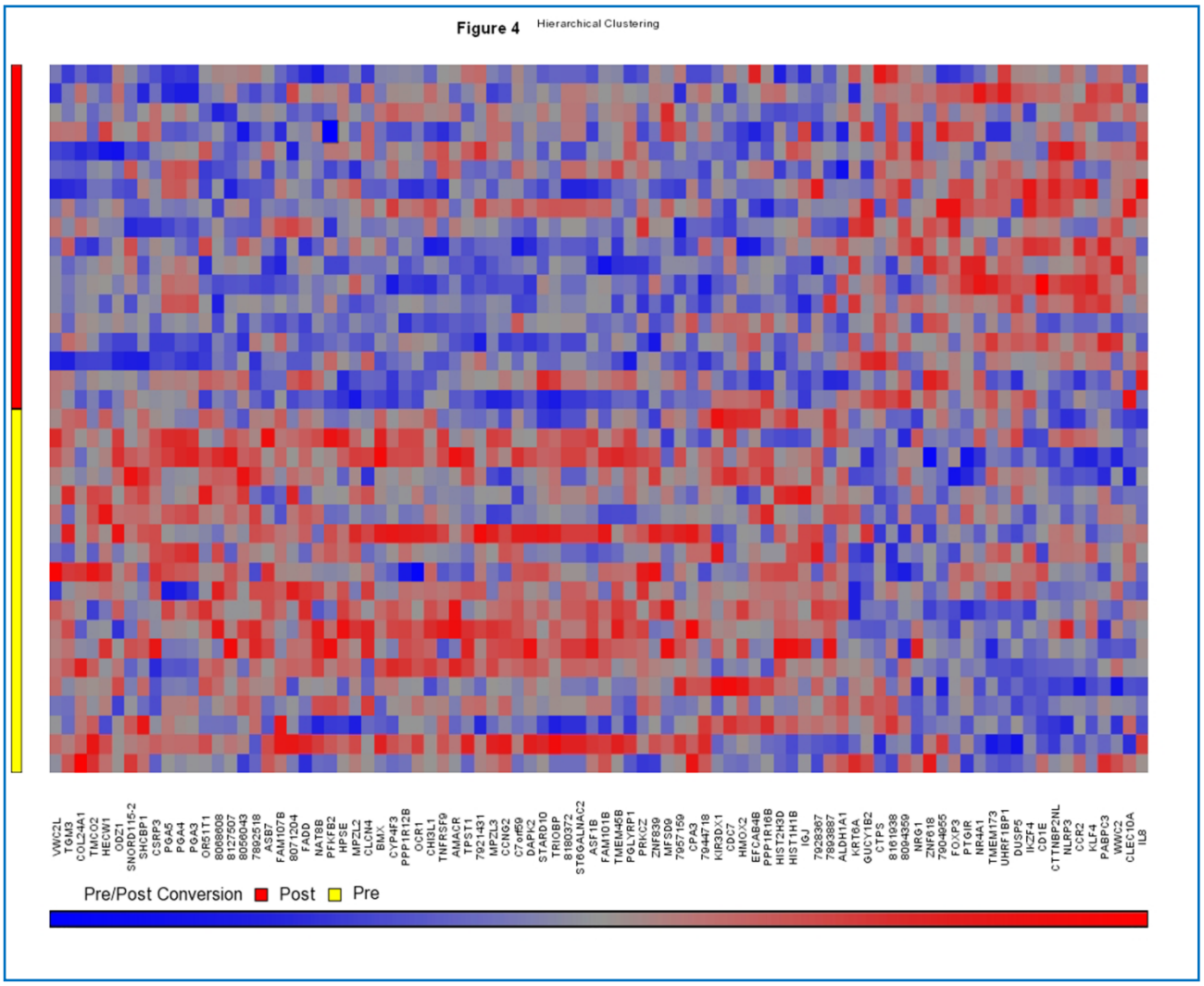

Figure 4: Heat Map of Immune/Inflammatory Gene Transcripts (n=93) Before and After Tacrolimus to Sirolimus Conversion. The bottom half of the heat map (yellow bar on the left) represents the peripheral blood gene transcripts present in patients pre-conversion (TAC), while the top half (red bar on the left) represents the transcripts post-conversion (SRL). Below the heat map is the color-coding for the various genes represented - red represents up-regulated genes, blue signifies down-regulated genes, and gray denotes unchanged transcripts. The gradation of color represents the intensity of the signal in log value.

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Figure 5: Blood and Biopsy mRNA differences in LT recipients with acute rejection, HCV recurrence and mixed AR/HCV-recurrence.

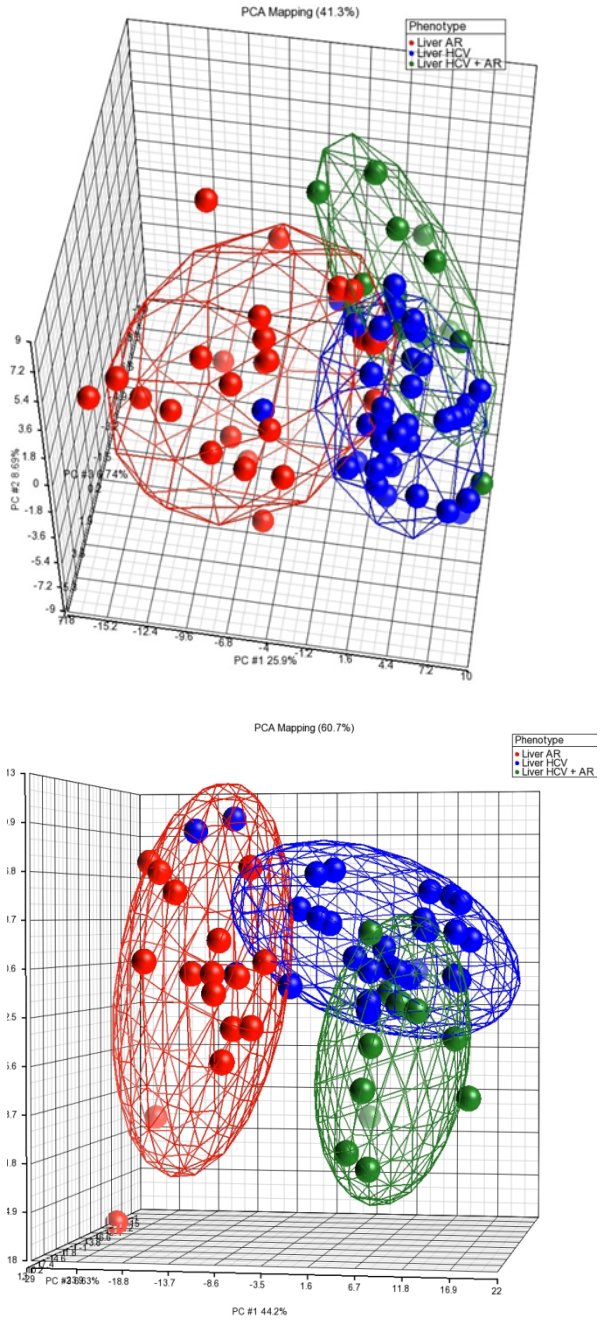

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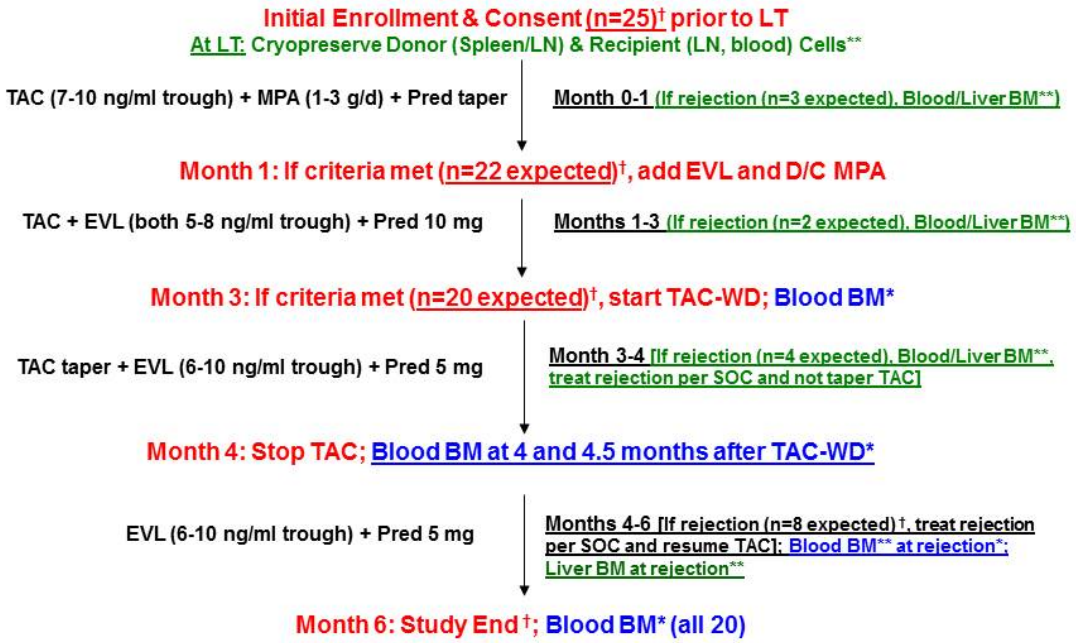
Figure 6: Time Table for Sample Collection & Assessment



† Although 25 patients are initially enrolled, we expect 20 will reach the Month 4 time point: 15 then expected TAC-WD success (no rejection) vs. 5 expected failures (rejection)

* Blood Biomarkers (BM): Flow (Subsets, Tregs & DCregs), Microarrays (whole blood)

** SAMPLES STORED - NO FUNDING REQUESTED:
- At LT: Donor/Recipient Cells as described
- At Rejection: Blood/Liver Graft Biomarkers (BM): PBMC, blood and tissue RNA, Sera/Plasma

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