Evaluation of Donor Specific Immune Senescence and Exhaustion as Biomarkers of Operational Tolerance Following Liver Transplantation in Adults

Protocol ITN056ST

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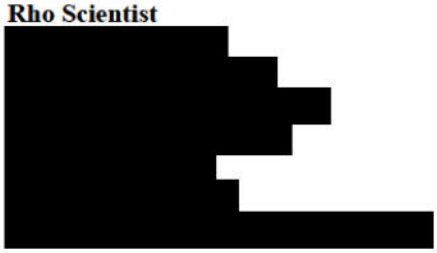

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Protocol Approval

Trial ID: ITN056ST	**Protocol Version:** 7.0
	Dated: June 15, 2020
IND #: N/A	**Protocol Chair:** [redacted]

Title: *Evaluation of Donor Specific Immune Senescence and Exhaustion as Biomarkers of Operational Tolerance Following Liver Transplantation in Adults*

I confirm that I have read the above protocol in the latest version. I understand it, and I will work according to the principles of Good Clinical Practice (GCP) as described in the United States Code of Federal Regulations (CFR) –21 CFR Parts 45, 50, 54, 56, and 312, and the International Conference on Harmonization (ICH) document "Guidance for Industry: E6 Good Clinical Practice: Consolidated Guidance". Further, I will conduct the study in keeping with local, legal, and regulatory requirements.

As the 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 written permission of the NIAID.

_____

Principal Investigator (***Print***)

_____ _____

Principal Investigator (***Sign***) **Date**

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Synopsis

Title	**Evaluation of Donor Specific Immune Senescence and Exhaustion as Biomarkers of Operational Tolerance Following Liver Transplantation in Adults**
Short Title	Immune Senescence and Exhaustion Biomarkers of Operational Tolerance in Adult Liver Transplantation
IND Sponsor	NIAID
Conducted by	Immune Tolerance Network
Protocol Chair	[redacted]
Accrual Objective	60 liver transplant recipients and their living donors, if applicable
Study Treatment	Immunosuppression Withdrawal
Study Design	This trial is a multi-center, prospective, open label, non-controlled, non-randomized, interventional cohort study in which 60 adult recipients of liver allografts will undergo gradual immunosuppression withdrawal. Liver recipients greater than 6 years post-transplant or greater than 3 years post-transplant and age greater than 50 years at time of screening will undergo liver biopsy and blood sampling before initiation of immunosuppression withdrawal. Participants may initiate withdrawal from calcineurin inhibitor (CNI) monotherapy or combination therapy with a CNI and prednisone or CNI and a mycophenolate compound. Eligible participants will undergo immunosuppressive withdrawal according to a pre-specified algorithm (see section 3.1.3) with the goal of achieving complete discontinuation of immunosuppressive medication between 24 and 45 weeks after initiation of withdrawal. Participants will undergo protocol biopsies at 1 and 3 years following drug discontinuation. Successfully weaned participants who remain rejection-free will undergo 3 years of follow-up after the last dose of immunosuppression. Participants who resume immunosuppression, due to biopsy-confirmed or presumed rejection, and will undergo 3-3.5 years of follow-up. Study investigators and participants will remain blinded to the results of tolerance biomarkers for individual participants until the end of the study. The tolerance biomarker results will be evaluated as a group once the primary endpoint is reached.

Participants will be enrolled by a consortium of US transplant sites. A parallel study with a harmonized clinical and mechanistic protocol is planned for a similar number of participants at EU sites.

Study Duration

Total study duration will be up to 391 weeks (7.5 years):

- The enrollment phase will be up to 156 weeks (3 years).
- The duration of the study for an individual participant may range from approximately 188 to 235 weeks, comprised of a screening phase of approximately 8 weeks, a withdrawal phase of approximately 24 to 45 weeks and a follow-up phase of approximately 156 to 182 weeks.
- The study primary endpoint, measured 52 weeks after the last participant's completion of immunosuppression withdrawal, could be achieved as early as 240 weeks (4.5 years), or as late as 265 weeks (5 years) after enrollment of the first participant.

Primary Objective

The primary objective is to determine whether a peripheral blood or graft lymphocyte phenotype of immune senescence or exhaustion is different between operationally tolerant and non-tolerant liver allograft recipients.

Primary Endpoint

The primary endpoint is the proportion of participants who achieve operational tolerance 52 weeks after completion of immunosuppression withdrawal defined by:

a. No evidence of rejection since enrollment in the study.

b. A liver biopsy at 52 weeks following discontinuation of all immunosuppression demonstrating absence of rejection per the Banff global assessment criteria[1-3]. The central pathology read will be used for this determination.

c. A liver biopsy at 52 weeks following discontinuation of all immunosuppression demonstrating histological stability consistent with operational tolerance per Banff 2012 criteria[4], defined as the *absence* of the histological findings in **Table 1**. The central pathology read will be used for this determination.

For the purposes of evaluating donor-specific exhaustion, operationally tolerant participants will be compared to those who fail immunosuppression withdrawal.

Secondary Endpoints

Safety

1. The proportion of participants who develop DSA or de novo anti-HLA antibodies after initiation of immunosuppression withdrawal.
2. The incidence, severity, and timing of acute rejection, steroid resistant rejection, and chronic rejection.
3. The incidence and progression of graft fibrosis in tolerant versus non-tolerant patients.
4. The incidence of graft loss.
5. The incidence of all–cause mortality.
6. The incidence of study-related SAEs.

Effectiveness

1. The proportion of operationally tolerant subjects who remain free of rejection at 3 years after completing immunosuppression withdrawal.
2. Changes in renal function (defined as estimated GFR calculated by CKD-EPI: http://www.qxmd.com/calculate-online/nephrology/ckd-epi-egfr) in tolerant versus non-tolerant participants at 1, 2 and 3 years after completing immunosuppression withdrawal.
3. Changes in Quality of Life in tolerant versus non-tolerant participants and in all participants at baseline versus the end of study participation, as measured by the NIDDK Liver Transplantation Database Quality of Life Form (see Appendix 7).
4. Changes in SF-36 (see Appendix 6) in tolerant versus non-tolerant participants and in all participants at baseline versus the end of study participation.
5. Predictive value and the correlative value of the following parameters with regard to operational tolerance:
    a. Time post-transplant
    b. Recipient age

Mechanistic

1. Mechanistic endpoints may assess both the predictive value and the correlative value of the following parameters with regard to operational tolerance:

    a. Phenotypic or molecular markers of immune senescence and/or exhaustion in T cells recovered from peripheral blood or liver tissue

    b. Donor-specific antibody

    c. Intra-allograft C4d

    d. Recipient anti-donor reactivity in vitro

    e. mRNA transcripts in blood and in liver allograft biopsies

    f. Thymic T cell output

    g. Peripheral blood and tissue miRNA expression

    h. Iron metabolism gene and serum proteins.

    i. Microchimerism by STR genotyping

    j. Gut microbiome profile

Inclusion Criteria

Recipient

Recipient participants *must meet all* of the following criteria to be eligible for this study:

1. At the time of screening:
    - 18 to 50 years old and more than 6 years post-transplant OR
    - Greater than 50 years old and more than 3 years post-transplant
2. Recipient of either deceased or living donor liver transplant.
3. Recipient of single organ transplant only
4. Must have a screening liver biopsy that fulfills the criteria in **Table 3** based on the central pathology reading
5. Liver function tests (Direct bilirubin, ALT) less than twice the upper limit of normal (ULN). ULN values for liver function tests will be defined by ranges from Harrison's Principles of Internal Medicine, 18th edition.
6. Receiving calcineurin inhibitor (CNI) based maintenance immunosuppression. Participants may also concurrently receive:

- low dose mycophenolate mofetil (MMF ≤ 1500 mg daily) or mycophenolic acid (≤ 1080 mg daily) OR
- prednisone ≤ 7.5 mg daily or equivalent corticosteroid.

7. Ability to sign informed consent

Living Donor

Living donor participants *must meet all* of the following criteria to be eligible for this study:

1. At the time of screening: ≥18 years old
2. Living donor of the liver allograft of an enrolled recipient participant
3. Ability to sign informed consent
4. Willingness to donate appropriate biologic samples

Exclusion Criteria

Recipient

Recipient participants who meet any of the following criteria will not be eligible for this study:

1. History of HCV infection (defined as a positive HCV antibody test)
2. Positive antigen-antibody immunoassay for HIV-1/2
3. Serum positivity for HBV surface antigen or HBV-DNA
4. History of immune-mediated liver disease in which immunosuppression discontinuation is inadvisable (autoimmune hepatitis, primary sclerosing cholangitis, primary biliary cirrhosis)
5. Any medical condition associated with a likely need for systemic corticosteroid administration, e.g., reactive airways disease
6. Prospective baseline liver biopsy showing any of the following: a) acute rejection according to the Banff global assessment criteria[1-3]; b) early or late chronic rejection according to the Banff global assessment criteria[1-3]; c) inflammatory activity and/or fibrosis in excess of permissive criteria according to Banff 2012 criteria[4] (see **Table 3**); d) any other histological findings that might make participation in the trial unsafe. Eligibility will be determined by the findings on the central biopsy reading.
7. Rejection within the 52 weeks prior to screening

8. Estimated GFR <40 ml/min as calculated by CKD-EPI method (to mitigate the risk of worsening renal failure should rejection occur and high level of CNI be required)

9. The need for chronic anti-coagulation that cannot be safely discontinued for a minimum of 1 week to safely perform a liver biopsy

10. Pregnant females and females of childbearing potential who are not using an effective method of birth control

11. Current drug or alcohol dependency

12. Inability to comply with the study visit schedule and required assessments, including frequent liver function monitoring and protocol biopsies

13. Inability to comply with study directed treatment

14. Any medical condition that in the opinion of the principal investigator would interfere with safe completion of the trial

15. Participation in another interventional clinical trial within the 4 weeks prior to screening

Living Donor

Living donor participants who meet any of the following criteria will not be eligible for this study:

1. Any medical condition, such as anemia, coagulopathy, etc., that in the opinion of the principal investigator would interfere with safe participation in the trial.

Abbreviations

AE	adverse event
ALT	alanine aminotransferase
AR	acute rejection
AST	aspartate aminotransferase
ATG	antithymocyte globlin
CBC	complete blood count
CFR	Code of Federal Regulations
CI	confidence interval
CKD	chronic kidney disease
CMV	cytomegalovirus
CNI	calcineurin inhibitor
COVID-19	coronavirus disease 2019
CR	chronic rejection
CRF	case report form
CRO	contract research organization
CsA	cyclosporine A
DAIT	Division of Allergy, Immunology and Transplantation
DSA	donor-specific alloAbs
DSMB	Data and Safety Monitoring Board
EBV	Epstein-Barr virus
ECG	electrocardiogram
EDC	electronic data capture
FCB	for-cause biopsy
FDA	US Food and Drug Administration
GCP	good clinical practice
GFR	glomerular filtration rate
GGT	gamma-glutamyl transferase
HBV	hepatitis B virus
HCV	hepatitis C virus
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HTLV1	Human T Cell Leukemia Virus Type 1
ICH	International Conference on Harmonisation

IRB	institutional review board
ISW	immunosuppression withdrawal
ITN	Immune Tolerance Network
ITT	intent to treat
LFT	Liver function test
MSAP	Mechanistic statistical analysis plan
MFC	multi-parameter flow cytometry
MedDRA	Medical Dictionary for Regulatory Activities
MHC	Major Histocompatibility Complex
MMF	mycophenolate mofetil
MOP	manual of operations
NIAID	National Institute of Allergy and Infectious Disease
NCI	National Cancer Institute
NIH	National Institute of Health
NCI-CTCAE	National Cancer Institute Common Terminology Criteria for Adverse Events
PAM	prediction analysis of microarrays
PBL	peripheral blood lymphocytes
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PP	per protocol
PRA	panel-reactive antibody
PTLD	Post-transplant lymphoproliferative disorder
QOL	Quality of Life
SAE	serious adverse event
SAM	significance analysis of microarrays
SAP	statistical analysis plan
SAR	suspected adverse event
SDCC	Statistical and data coordinating center
SOE	schedule of events
SRTR	Scientific Registry of Transplant Recipients
SSR	steroid resistant rejection
STR	short tandem repeats
TaC	Tacrolimus
ULN	upper limit of normal
WHO	World Health Organization

Study Definitions Page

Acute Rejection	Acute allograft rejection will be defined in accordance with Banff global assessment criteria. See Section 6.7.1 for details.
Acute Rejection Resolution	An episode of acute allograft rejection involving elevated ALT (with or without elevated GGT) will be considered as resolved when ALT is ≤ 1.5 times baseline levels. For these cases, it is recognized that GGT levels decline very slowly following rejection and therefore will not be used to define resolution. If acute allograft rejection with dysfunction involves elevated GGT alone, it will be considered resolved when GGT is ≤ 1.5 times baseline levels. (Section 6.8)
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 (Section 8.2.1)
Allograft Dysfunction	An unexplained elevation in ALT or GGT tests relative to baseline; ≥ 2-fold the ULN (based on Harrison's Principles of Internal Medicine, 18th edition) if the baseline value was < than the ULN; or, ≥ 2-fold the baseline value if the baseline value was ≥ than the ULN (Section 6.6.1)
Baseline Liver Function Test	The average of two LFTs taken at the following times: LFT taken at screening; and LFT taken 7 (+/- 2 days) before initiation of immunosuppression withdrawal. See Section 6.6.1 for details.
Enrollment	The signing of informed consent. (Section 3.1.1)
Failed immunosuppression withdrawal	Participants with a biopsy that is positive or suspicious for rejection, participants with presumed rejection who are unable to undergo a biopsy, participants who exceed 45 weeks to complete immunosuppression withdrawal, or participants who demonstrate any of the histological findings in **Table 1** on their biopsy performed 52 weeks following discontinuation of all immunosuppression (Sections 3.1.3.5 and 6.9).
Hepatitis C Virus	Presence of HCV confirmed by a positive HCV antibody test (Section 4.2.1)
Intent-to-Treat Sample	All participants who provide informed consent for study participation and begin immunosuppression withdrawal (Section 9.1)
Liver Function Tests	Includes direct bilirubin, ALT and GGT.
Liver Function Test ULN values	Value ranges are according to the Harrison's Principles of Internal Medicine, 18th edition (ALT: 41 U/L; GGT 58 U/L; Direct Bilirubin: 0.4 mg/dL) (Section 4.1)
Operational Tolerance	No evidence of rejection since enrollment in the study, a liver biopsy at 52 weeks following discontinuation of all immunosuppression demonstrating *absence* of rejection per the Banff global assessment criteria and histological stability consistent with operational tolerance per Banff 2012, defined as the *absence* of histological findings in **Table 1** (Section 3.3.1)
Per Protocol Sample	All participants who attempt immunosuppression withdrawal and do not have any unacceptable major protocol deviations (Section 9.1)
Screen Failure	Enrolled participants who do not fulfill eligibility criteria or who do not initiate immunosuppression withdrawal for any reason (Section 3.1.1)

1. BACKGROUND AND RATIONALE

1.1 BACKGROUND

Life-long immunosuppression is typically regarded as obligatory for solid-organ recipients to avoid graft loss from allo-immune attack. Evidence that not all patients require perpetual immunosuppression is found in a small subset of patients who successfully discontinue immunosuppression through non-compliance or out of medical necessity (e.g. PTLD), and yet sustain normal graft function, apparently indicating a state of operational tolerance. For kidney recipients, this fraction is estimated at 5% or less, but for liver it has traditionally been estimated at ~20%[5-8]. Precise prospective identification of individuals operationally tolerant to their donor organ would not only allow personalized medical patient care by safe drug elimination in select subjects, but also might provide clues to mechanisms accounting for tolerance generation. It is also conceivable that the knowledge gained will facilitate its prospective replication in those not predisposed to development of tolerance to their donor after staged drug withdrawal.

Recent studies suggest that peripheral blood lymphocyte gene expression analysis may characterize the immunological relationship of the recipient with the foreign graft, predicting rejection in heart recipients[9-11] and a state of functional tolerance liver and kidney patients[12-14]. Newell et al made the unexpected observation in a cross-sectional analysis of operationally tolerant renal transplant recipients that tolerance was associated with a gene signature dominated by B cell transcripts[12]. A three gene set validated by PCR was found to have high positive and negative predictive value. Interestingly, each of the three genes was involved in B cell maturation and development. These molecular signals were confirmed by PBL flow analysis which revealed parallel B cell subset differences with expansion of immature B cells that correlated with tolerance. Of note, the kidney signature identified by Newell has not yet been validated by a prospective weaning trial.

Martinez-Llordella et al studied 16 operationally tolerant liver recipients by PBL transcriptional profiling and detailed flow subset characterization and compared them to 16 patients on immunosuppression and 10 normal controls. A gene expression signature indicative of tolerance was identified that included genes encoding γδ T cells and NK cells. By flow there was an increase in the Treg subset[13]. An attempt was recently undertaken to validate these findings prospectively in a multicenter trial by weaning from immunosuppression[15] that is reviewed in greater detail below. This effort was headed by Dr. Sanchez-Fueyo, who will serve as a consultant for the accompanying proposal. The trial's molecular signature analysis, published by Bohne et al in JCI in 2012, revealed a robust signal in liver biopsy tissue unexpectedly dominated by transcripts associated with iron metabolism[8].

Also potentially relevant is the recent pilot study by Feng et al examining immunosuppression withdrawal in 20 pediatric liver transplant recipients[16]. Applying careful selection criteria, 60% of this group achieved operational tolerance defined as an absence of rejection and normal graft function 1 year after discontinuation of immunosuppression. Like the adult cohort of Bohne[8], successful withdrawal was associated with initiating withdrawal at a later time post-transplant. Other novel findings included a correlation of operational tolerance with pre-wean biopsies exhibiting less portal inflammation and lower C4d scores.

1.2 SCIENTIFIC RATIONALE

Perhaps the most revealing finding in the multi-center trial conducted by Sanchez-Fueyo in adult HCV+ and HCV negative liver recipients is the strong correlation of operational tolerance with both the duration of time from transplant and independently with recipient age. Of 98 patients undergoing prospective weaning, 42% achieved the primary endpoint of successful immunosuppression withdrawal, a rate of success markedly higher than the historic mark of ~20%. The likely cause of this difference is evident upon stratification of the cohort based on the duration of time from transplant. For patients between three and 5.7 years post-transplant at the time of initiating weaning, only 12% achieved the primary endpoint. In contrast, patients between 5.7 and 10.6 years post-transplant experienced a success rate of 38%, and in those >10.6 years post-transplant, the success rate was a striking 80%. Also of interest was the observation that within the cohort of patients <5.7 years post-transplant, no one under the age of 49.6 achieved operational tolerance, whereas 30% of those greater than 49.6 years were successful[15]. Thus, operational tolerance may be more prevalent than previously appreciated, thereby making it a more attainable clinical opportunity. However, before these results can be applied to modify standard of care clinical practice, it is prudent to conduct validation studies in adequately sized independent cohorts in the US.

These novel findings have a number of important implications in general, and also directly influence the design of the proposed trial. If these findings are correct, 30-40% of patients more than 5.7 years post-transplant and patients 3-5.7 years post-transplant who are >50 years old can successfully be weaned from immunosuppression. Targeting this demographic subset will significantly reduce the required sample size of weaned patients needed to achieve an operationally tolerant cohort of sufficient size for meaningful mechanistic analysis (compared with the cohort size needed if the tolerance rate was only 20%).

Second and perhaps more importantly, the higher rate of likely success also substantially improves the benefit to risk ratio of immunosuppression weaning when compared with traditional estimate of 20% anticipated success. This improvement provides better equipoise for a weaning attempt and will likely improve enrollment efficiency by engendering greater enthusiasm for participation by both the patients and their transplant physicians.

Finally, the correlation of successful weaning with both recipient age and time from transplant may suggest a clue to the mechanistic underpinnings of operational tolerance. We propose a unifying hypothesis for these two findings by implicating the related processes of T cell exhaustion and immune senescence in operational tolerance development. Specifically, we suggest that operational tolerance occurs through a process of donor-specific T cell exhaustion in the presence of chronic donor antigen exposure that leads to a gradual loss of donor reactivity over time. Furthermore, this process is fostered by an age-dependent decrease in the genesis of new donor-reactive T cell clones due to thymic atrophy-associated immune system senescence. This thesis will be tested as part of the multi-parameter analysis detailed below.

Varied data suggest that a time-dependent development of immune dysregulation can occur in the setting of chronic pathogen exposure, such as a chronic viral infection, especially for the CD8 T cell subset; this process has been termed ‘immune exhaustion’[17]. It is theorized that chronic antigen exposure and T cell stimulation can lead to acquired T cell incapacity and apoptosis. This is been noted to occur with chronic

infection by HIV, HCV, CMV and HTLV1[18-26]. Functionally, these changes are manifest by a sequential loss of IL-2 production, proliferation and cytotoxicity.

We theorize that the chronic residence of the foreign graft in a transplant recipient may recapitulate the effect of the chronic exposure to a viral pathogen leading to tonic stimulation of donor-reactive T cells and their resultant elimination or functional silencing over time. Further we suggest that this process may be rendered more efficient by the age-dependent reduction in thymic output, thereby reducing or eliminating a constant source of new naïve donor reactive cells with the potential to differentiate into T effector cells and explaining the parallel associations of weaning success with time from transplant and recipient age. Based on these concepts, we will explore whether the association of operational tolerance with greater recipient age is a consequence of global immune senescence. Measures of immune senescence will be incorporated into the immunophenotyping flow analysis described below. This will include the relative proportion of memory T cell subsets which accumulate with age (Naïve, T_{EM}, T_{CM}, T_{EMRA}, T_{EX}, T_{SEN}) and assessment of ongoing thymic output of nascent T cells with markers of recent emigrants. The degree of T cell replicative senescence will also be measured by telomerase activity/telomere length in PBL T cells and B cells via qPCR and/or Flow-FISH and by KLRG1 expression[27-31].

A number of cell surface markers have been found to correlate with T cell exhaustion including upregulation of PD-1,CTLA4, TIM-3, LAG-3, BIM, BLIMP-1, TIGIT and reduced expression of L-selectin, CCR7, IL-7R, IL-15R, and CD28[17].The appropriate flow panels to monitor immune senescence and exhaustion will be designed with the ITN. One valuable marker may be expression of PD-1, a key inhibitory co-receptor on CD4 and CD8 T cells[32]. Also of interest is the ratio of activated T cells noted by MHC class II or CD38 to exhausted T cells expressing PD-1 (CD57-, PD-1+), or senescent (CD57+, PD-1+) markers, and also markers of terminal differentiation (T_{EMRA} -- CD45RA+, CCR7-)[33-35]. In preliminary data Dr. Sanchez-Fueyo has demonstrated a significant increase in the percent PD-1+ CD8+ T cells in operationally tolerant versus non-tolerant liver recipients (A. Sanchez Fueyo, personal communication). In the sample size section below, data provided by Dr. Sanchez-Fueyo demonstrates a correlation between exhausted HCV-specific T cells (CD8+ +IFNg+CTLA4+PD-1+2B4+ T cells) and the development of operational tolerance in HCV+ liver recipients[36]. We hypothesize a similar correlation with allo-specific T cells and operational tolerance. Therefore for participants for whom donor material is available, we will evaluate the allo-specificity of CD4+ and CD8+ T cells with the exhausted phenotype. When donor MHC antigen alleles permit, we will utilize HLA tetramers to evaluate phenotypic changes associated with donor HLA antigen specific T cells.

Assessment of immune senescence and exhaustion in transplantation tolerance are very limited and provide only preliminary evidence of a role in graft survival. Gelson et al found evidence for exhaustion and replicative senescence in lymphocytes from liver recipients compared to matched controls, but tolerance was not directly examined[37]. Trzonkowski et al studied telomere length and senescence markers in young versus old (>60 years) kidney recipients and found them to correlate with an absence of rejection, though again tolerance was not studied[38]. In an experimental mouse model, Steger et al compared the response of donor specific CD8 T cells to spontaneously accepting allo-livers, and to rejecting heart or kidney grafts and found evidence for exhaustion in the former[39]. The most direct assessment of the association of T cell exhaustion with tolerance is found in the studies by Dr. Sanchez-Fueyo cited above.

In addition to searching for an association of operational tolerance with immune senescence, the trial proposed offers a number of other opportunities. Other novel aspects of the proposal include our plan to explore micro RNA correlates with operational liver transplant tolerance in samples of blood, serum and graft tissue. Recent data underscore the central role of micro RNA in regulating gene expression[40-47], and their relevance to the development and function of immune cells is now well established[48-52] including evidence that specific micro RNAs can be correlated with the immunological state of a kidney allograft – specifically rejection versus non-rejection[52,53]. Assessment of micro RNA in operational tolerance has not yet been reported. Because of their more limited repertoire and their stability in serum, a micro RNA tolerance signature may be more readily defined and could have greater practical utility than if graft tissue is needed. In parallel to micro RNA and transcriptome assessment, we will also examine: 1) liver graft biopsy specimens to characterize infiltrating cells and pathological structural changes, 2) flow cytometric analysis of PBL and 3) serologic assessment for donor specific antibody for tolerance-specific associations. When possible, such as in the case of living donor transplants in which donor samples are readily available, we will conduct evocative testing of recipient lymphocytes for reactivity to the donor as well as assessment for regulatory activity in both the B cell and T cell compartments.

Collectively, the proposed analyses have the potential to extend significantly existing reports attempting to define a composite molecular-biomarker signature of tolerance. An added attribute of the proposal is that it parallels a planned trial in pediatric patients with similar intent and will be harmonized clinically and mechanistically with a planned trial in the European Union. This will facilitate comparative analyses and cross-fertilization of mechanistic insights between the two age groups. Finally, the trial may offer the opportunity to validate the recently described liver tissue signature of tolerance in the US population. This would be valuable in promoting prospective withdrawal trials based on this signature in the US population.

1.3 CLINICAL EXPERIENCE

1.3.1 Safety

In the proposed trial, there is no investigational medication and the therapeutic intervention is staged immunosuppression withdrawal. The primary risk to participants is the possibility of experiencing graft rejection. Although we expect that more than 50% of subjects will experience rejection, liver withdrawal studies to date indicate that with appropriate patient selection and diligent serial monitoring, that the rejection episodes encountered can be treated successfully without long-term compromise of graft function or patient survival. The scientific evidence establishing that withdrawal can be performed with an acceptable risk profile lies in a number of recent withdrawal trials: first is the recently completed Sanchez Fueyo trial mentioned above[15]. Of 98 patients who attempted withdrawal, 57 experienced rejection (58%). In 21 cases rejection was reversed with reinstitution of basal immunosuppression in addition to higher CNI levels. In the remaining patients reinstitution of baseline immunosuppression was combined with low-dose steroids (20mg over 4-6 weeks; 30 patients), moderate-dose steroids (40-60 mg over 4-6 weeks; 4 patients), and steroid boluses (1 patient). Ultimately, normal graft function was restored in all[15].

Also pertinent to assessing the risks of immunosuppression withdrawal are the results of an ongoing weaning trial in liver transplant recipients headed by Dr. Avi Shaked (ITN030ST). This trial includes both a hepatitis C and a non-immune, non-viral (NINV) arm. In the former, of 30 HCV participants

randomized to immunosuppression withdrawal, five were found to be tolerant (17%) and 15 non-tolerant. In the NINV arm, of 47 participants randomized to immunosuppression withdrawal, 9 successfully completed withdrawal. However 4 of these 9 have resumed immunosuppression due to rejection. Five (11%) NINV participants remain off immunosuppression as of January 2015 (A. Shaked, personal communication). Of note, in this trial, withdrawal of immunosuppression began as early as one year post transplant, likely explaining the comparatively low rate of tolerance compared to that observed in the European trials[15]. These data further support our hypothesis that immunosuppression withdrawal may be more successful with increased time from transplant, perhaps due to immune exhaustion of alloreactive T cells. Nevertheless the consequences of rejection episodes in this study were not severe. All rejections were successfully treated and in only 5 cases was a steroid bolus required (A. Shaked, personal communication). Together, these two studies suggest that immunosuppression withdrawal can be attempted with a low likelihood of irreversible graft injury or graft loss when rejection is encountered.

1.3.2 Effectiveness

The structure of the current trial closely parallels the recently reported trial of Benitez et al in which 98 carefully selected liver transplant recipients underwent prospective weaning[15]. As noted above, overall, 42% achieved the primary endpoint of successful immunosuppression withdrawal. Patients between 5.7 and 10.6 years post-transplant experienced a success rate of 38%, and in those >10.6 years post-transplant, the success rate was a striking 80%. Also of interest was the observation that within the cohort of patients > 3 years and <5.7 years post-transplant, no one under the age of 49.6 achieved operational tolerance, whereas 30% of those greater than 49.6 years were successful. Based on the use of similar inclusion-exclusion selection criteria, we can expect an overall rate of successful weaning of approximately 30-40% depending on the age distribution and time post-transplant of the cohort enrolled.

1.4 SUMMARY OF KNOWN AND POTENTIAL RISKS AND BENEFITS FOR HUMAN PARTICIPANTS

Chronic immunosuppression is associated with a variety of life threatening side effects including infection, malignancy, hypertension, diabetes and increased risk of cardiovascular disease. Chronic CNI use is also associated with nephrotoxicity which is responsible for a significant rate of chronic renal failure (CRF) at 5 years post OLT. CRF confers a significant mortality risk and many patients ultimately require renal replacement therapy[54-59]. Patients demonstrating progressive loss in functional nephron mass are at greatest risk of progressing to CKD which is known to confer a more than 4 fold increase in mortality risk. Chronic renal injury may be aborted or prevented by the preemptive intervention of immunosuppression withdrawal. Elimination of CNI may preserve waning renal function and avoid the associated morbidity and mortality risk and the need for institution of renal replacement therapy. Furthermore, prednisone withdrawal may be associated with an improvement in metabolic functions, such as lipid profiles[60,61]. Identification of a reproducible and reliable tolerance signature will allow tailoring of immunosuppression to individual patient characteristics. It may also identify critical pathways responsible for a tolerant state that can be modified in those not achieving tolerance to their donor organ.

1.4.1 Risks

1.4.1.1 Risks associated with Immunosuppression Withdrawal

The primary risk of immunosuppression withdrawal is that of graft rejection. Based on the studies detailed above, we anticipate that 50% of enrolled subjects will experience a rejection episode. Graft rejection carries three attendant risks: risk of irreversible graft injury or failure, the need for liver biopsy to verify the diagnosis, and the risk accompanying treatment(s) needed to reverse the rejection once the diagnosis has been secured.

a. *Rejection associated graft injury or graft loss:* Given the high rate of expected rejection, the trial is specifically designed to allow early detection of rejection through frequent monitoring during the period of drug withdrawal and in the period early after immunosuppression cessation. Based on the experience of other withdrawal trials, with this approach it is expected that the majority of rejection episodes will be detected early and readily reverse. While theoretically possible, a severe rejection leading to graft loss, re-transplantation or patient death is very unlikely and has been only rarely observed with immunosuppression withdrawal in liver allograft recipients. In 4 separate studies of immunosuppression withdrawal on a total of 267 adult liver allograft recipients published between 1997 and 2013[15,62-64] only a single graft loss was reported. [62,63].

b. *For-cause liver biopsy risks;* Liver biopsy to permit histopathological assessment remains the gold standard in diagnosis of rejection and will be employed in all suspected cases of rejection in this trial unless clinically contraindicated or logistically infeasible. The procedure is usually performed percutaneously under ultrasound guidance and local anesthetic. It is usually associated with mild pain lasting only a few hours (rarely a few days). The risk of significant bleeding requiring transfusion is 0.5-1% and the risk of bile leak or injury to adjacent organs (pneumothorax, bowel perforation etc.) is even rarer and the risk of one of these complications leading to death is estimated at 0.1-0.01%[65].

c. *Risk associated with treatment of rejection:* The experience reported to date in liver immunosuppression withdrawal trials suggests that rejection episodes that occur during staged weaning and serial monitoring every 2-3 weeks are readily reversed by reinstitution of CNI-based immunosuppression, alone or in combination with low-dose steroids. Treatment of rejection may be associated with transient worsening of certain co-morbidities (diabetes, hypertension, hyperlipidemia, etc.), but is very unlikely to result in irreversible damage. The need to use strong immunosuppressive regimens to reverse rejection (e.g. repeated steroid boluses, T cell depleting antibodies) may lead to increased risk of infection (e.g. CMV reactivation), malignancy and renal dysfunction. Within a carefully monitored clinical trial the development of rejection episodes of such severity is extremely unlikely. In 4 separate studies of immunosuppression withdrawal on a total of 267 adult liver allograft recipients published between 1997 and 2013[15,62-64] only 2 episodes of severe rejection were reported[62,63]. One episode resulted in graft loss but the second reversed with steroids alone. In these 267 patients all other rejection episodes were graded as mild or moderate and were treated successfully with reinstitution of maintenance immunosuppression with or without steroids.

1.4.1.2 Risks associated with Blood Draws

Frequent blood draws to allow close monitoring of liver function during and after immunosuppression withdrawal is essential for the trial's safe conduct. Peripheral blood draws typically incur mild temporary discomfort. Rare but more serious risks include ecchymosis, thrombophlebitis and infection.

2. OBJECTIVES

2.1 PRIMARY OBJECTIVE

1. The primary objective is to determine whether a peripheral blood or graft lymphocyte phenotype of immune senescence or exhaustion is different between operationally tolerant and non-tolerant liver allograft recipients.

2.2 SECONDARY OBJECTIVES

1. To determine the frequency of operational tolerance after liver transplantation.
2. To determine the safety of staged immunosuppression withdrawal in selected liver transplant recipients.
3. To determine the impact of immunosuppression withdrawal on:
    a. Renal function
    b. Changes in Quality of Life (QOL)
4. To describe the relationship between operational tolerance and recipient age.
5. To describe the relationship between operational tolerance and time post-transplant.
6. To describe the relationship between operational tolerance and miRNA expression profile in blood and liver tissue prior to withdrawal of immunosuppression.
7. To describe the relationship between operational tolerance and mRNA expression profile in blood and liver tissue prior to withdrawal of immunosuppression.
8. To evaluate the relationship between operational tolerance and serologic and molecular markers of iron metabolism.
9. To describe the relationship between operational tolerance and multi-parameter flow analysis for lymphocyte subsets.
10. To validate a previously defined liver tissue-based transcriptional signature of operational tolerance.
11. To describe the relationship between operational tolerance and donor specific T-cell reactivity in cases where donor or donor-type material is available.
12. To describe the relationship between operational tolerance and the gut microbiome.

3. STUDY DESIGN

3.1 DESCRIPTION

This trial is a multi-center, prospective, open label, non-controlled, non-randomized, interventional cohort study in which 60 adult recipients of liver allografts will undergo gradual immunosuppression withdrawal. Liver recipients greater than 6 years post-transplant or greater than 3 years post-transplant and age greater than 50 years at time of screening will undergo liver biopsy and blood sampling before initiation of immunosuppression withdrawal. Participants may initiate withdrawal from calcineurin inhibitor (CNI) monotherapy or combination therapy with a CNI and prednisone or CNI and a mycophenolate compound. Eligible participants will undergo immunosuppressive withdrawal according to a pre-specified algorithm (see section 3.1.3) with the goal of achieving complete discontinuation of immunosuppressive medication between 24 and 45 weeks after initiation of withdrawal. Participants will undergo protocol biopsies at 1 and 3 years following drug discontinuation. Successfully weaned participants who remain rejection-free will undergo 3 years of follow-up after the last dose of immunosuppression. Participants who resume immunosuppression, due to biopsy-confirmed or presumed rejection, and will undergo 3-3.5 years of follow-up.

Study investigators and participants will remain blinded to the results of tolerance biomarkers for individual participants until the end of the study. The tolerance biomarker results will be evaluated as a group once the primary endpoint is reached.

Participants will be enrolled by a consortium of US transplant sites. A parallel study with a harmonized clinical and mechanistic protocol is planned for a similar number of participants at EU sites.

3.1.1 Enrollment and Accrual

The accrual goal for this study is 60 adult liver allograft recipients. For cases of adult living donor liver recipients the corresponding allograft donor will also be enrolled where feasible. Enrollment is defined as the signing of informed consent and will be obtained prior to the initiation of any screening or study mandated procedures. Enrolled participants who do not fulfill eligibility criteria or who do not initiate immunosuppression withdrawal for any reason will be considered screen failures and will not count towards the accrual goal. There is no upper limit on screen failures.

3.1.2 Screen Failures

Screening data will be collected on all participants who have signed informed consent and undergone screening but who fail to meet eligibility criteria. The number of participants identified as clinically suitable but who do not qualify for immunosuppression withdrawal will be assessed. The reasons for ineligibility will be tabulated. This analysis will clarify the study's relevance to the broader adult liver transplant population.

3.1.3 Immunosuppression Withdrawal

Participants who successfully complete all other screening assessments will undergo a baseline liver biopsy prior to initiation of immunosuppression withdrawal. The baseline liver biopsy will be used for biomarker studies and will also be used to rule out rejection and other pathological conditions that might

make it unsafe for the patient to withdrawal immunosuppression as specified in the eligibility criteria (see section 4).

Eligible participants will undergo gradual immunosuppression withdrawal according to the following algorithm(s). Dose reductions can occur within a +5 day window for each taper level.

3.1.3.1 Withdrawal from CNI

Participants will initiate CNI withdrawal after demonstrating stable liver function as documented by liver function tests (see Study Definitions) completed at Visit -1 and Visit 0. Both sets of liver function tests must meet eligibility requirements.

Withdrawal will occur in eight 3 week (21 day) intervals with each subsequent reduction based on LFT stability over the prior 3 week interval. No single reduction should exceed 50% of the daily dose except the final reduction. Withdrawal will proceed as indicated in Figure 1.

3.1.3.2 Concurrent Withdrawal from Prednisone or Equivalent Corticosteroid

Participants on CNI and prednisone will undergo withdrawal from the two therapies concurrently. Participants must meet criteria to initiate CNI withdrawal (section 3.1.3.1) to withdraw from prednisone. The exact schedule for prednisone withdrawal may be varied at the discretion of the investigator based on the clinical and immunologic status of the participant, but must be discontinued by the completion of CNI taper level 8[66]. Figure 1 provides an example of concurrent CNI and prednisone withdrawal, where the total daily dose of prednisone is reduced by 1 mg every 3 weeks until discontinued.

Figure 1. Flowchart of CNI, and if Applicable, Concurrent Prednisone Withdrawal

Visit	CNI Withdrawal		Prednisone Withdrawal *(for participants on CNI and prednisone only)*	
	Withdrawal Schedule	Example	Withdrawal Schedule	Example
0	Baseline Dose	3 mg daily	Baseline Dose	7 5 mg daily
101	Reduce total daily dose to 75% current total dose x 3 weeks	2 5 mg daily	Reduce total daily dose by 1 mg x 3 weeks	6 5 mg daily
102	Reduce total daily dose to 75% current total dose x 3 weeks	2 mg daily	Reduce total daily dose by 1 mg x 3 weeks	5 5 mg daily
103	Reduce total daily dose to 50-75% current total dose x 3 weeks	1 0-1 5 mg daily (select dose)	Reduce total daily dose by 1 mg x 3 weeks	4 5 mg daily
104	Give above dose 5x weekly x 3 weeks	1 5 mg 5x weekly	Reduce total daily dose by 1 mg x 3 weeks	3 5 mg daily
105	Give current dose 4x weekly x 3 weeks	1 5 mg 4x weekly	Reduce total daily dose by 1 mg x 3 weeks	2 5 mg daily
106	Give current dose 3x weekly x 3 weeks	1 5 mg 3x weekly	Reduce total daily dose by 1 mg x 3 weeks	1 5 mg daily
107	Give current dose 2x weekly x 3 weeks	1 5 mg 2x weekly	Reduce total daily dose by 1 mg x 3 weeks and discontinue	0 5 mg daily
108	Give current dose 1x weekly x 3 weeks and discontinue	1 5 mg 1x weekly	Off drug	----------

3.1.3.3 Withdrawal from a Mycophenolate Compound

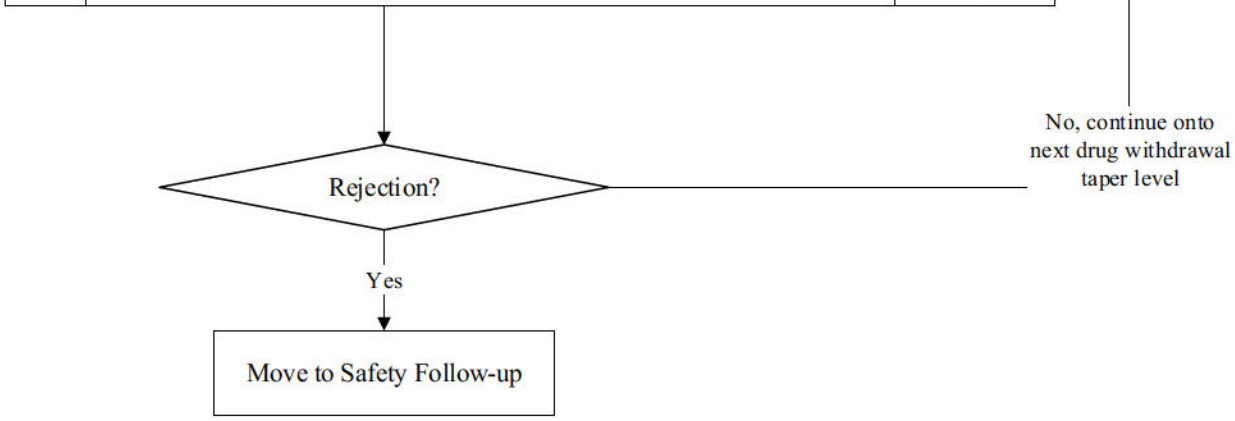
Participants on CNI and a mycophenolate compound will first undergo withdrawal from the CNI. Once the participant has completed CNI withdrawal, the participant must demonstrate at least 3 weeks of stable liver function, as documented by 2 sets of LFTs (see Study Definitions) separated by at least 1 week, before initiating withdrawal of the mycophenolate compound. If either of the two sets of LFTs meets the

definition of Allograft Dysfunction (section 6.6.1) the participant will not be eligible for further immunosuppression withdrawal and will move to safety follow-up (Appendix 3). Withdrawal of the mycophenolate compound must occur within 5 weeks of CNI discontinuation, and complete immunosuppression withdrawal may not exceed 45 weeks (see section 3.1.3.5).

Participants will be weaned from mycophenolate compound monotherapy in two dose reductions of approximately 50% each, which will occur over a total 6 week period in the absence of a pause, after which the drug will be discontinued as indicated in Figure 2.

Figure 2. Flowchart of MMF Withdrawal

Visit	MMF Withdrawal Schedule	Example
109	Baseline Dose	1500 mg
110	Reduce total daily dose to 50% current total dose x 3 weeks	1000 mg
111	Reduce total daily dose to 50% current total dose x 3 weeks and discontinue	500 mg



3.1.3.4 Pausing of Immunosuppression Withdrawal

Immunosuppressive drug withdrawal may be temporarily paused for up to 4 weeks. During this time interval the participant will remain at the current dose. If the participant cannot proceed to the next dose reduction after 4 weeks they will move to safety follow-up per Appendix 3. Participants are allowed up to 3 non-consecutive pauses or a cumulative total of 12 weeks.

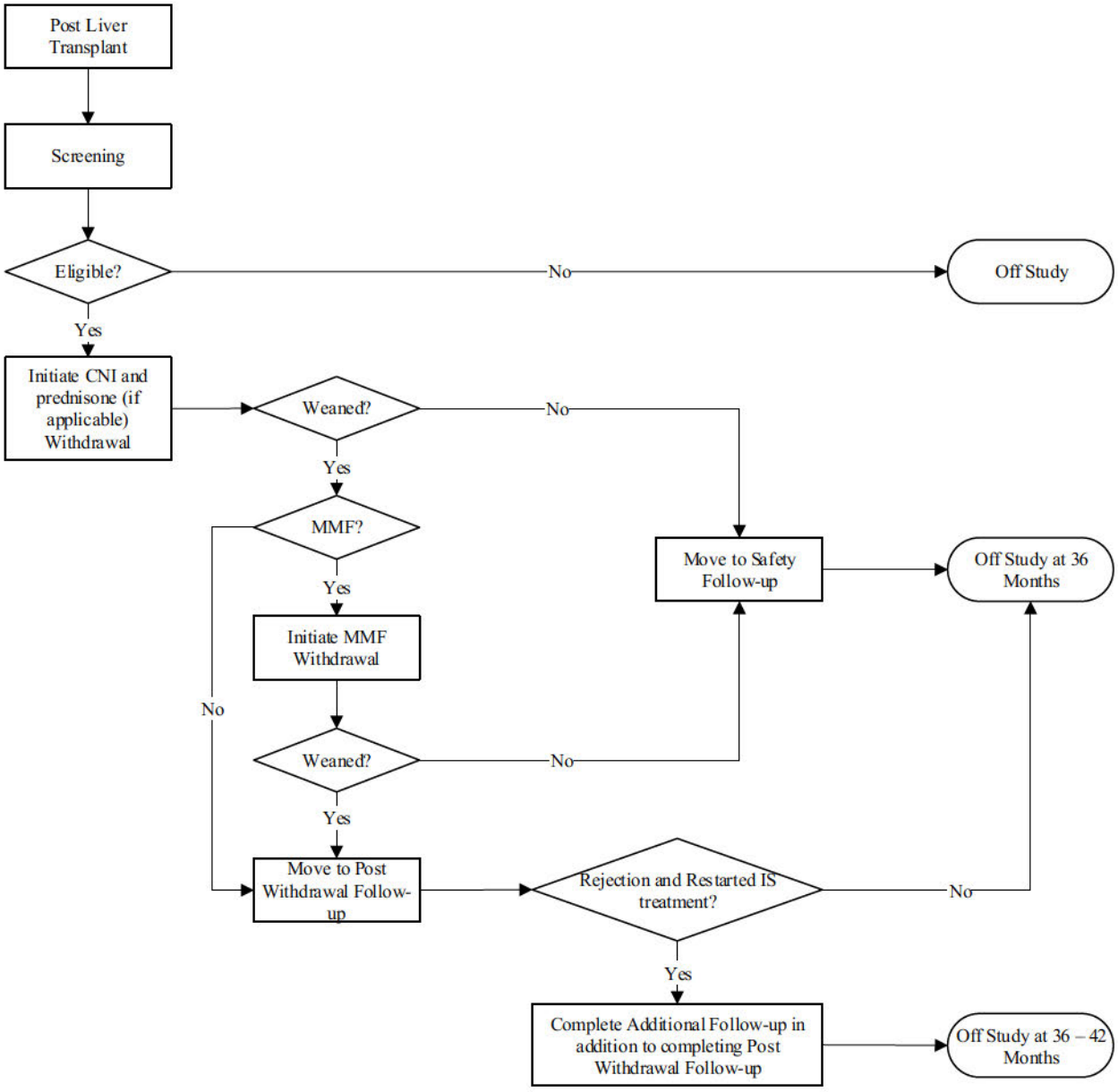
Cases in which extenuating circumstances lead to longer pauses will be considered by a committee consisting of the NIAID Medical Monitor, the ITN Clinical Trial Physician and the Protocol Chair. This committee will adjudicate whether the participant may proceed with immunosuppression withdrawal. If the committee determines that the participant may proceed with immunosuppression withdrawal despite exceeding the 12 week maximum, this will be considered and recorded as a protocol deviation.

3.1.3.5 Discontinuation of Immunosuppression Withdrawal/Resumption of Immunosuppression

- Participants undergoing immunosuppression withdrawal who experience biopsy-confirmed rejection will discontinue further withdrawal and be re-started on immunosuppression.

- Participants who have successfully completed immunosuppression withdrawal who subsequently experience biopsy-proven rejection will be re-started on immunosuppression.
- Participants with presumed rejection who are treated with increased immunosuppression without a confirmatory biopsy may undergo a biopsy within a week after treatment. If the biopsy is negative for rejection the participants may continue with immunosuppression withdrawal. If the biopsy is positive or suspicious for rejection, or if the participant is unable to undergo a biopsy, they will be considered to have failed immunosuppression withdrawal and will be resumed on immunosuppression.
- Participants who exceed 45 weeks (see section 3.1) to complete immunosuppression withdrawal will remain in the study but will be considered to have failed immunosuppression withdrawal.
- Participants who fail immunosuppression withdrawal will not be allowed a second attempt.
- Participants who cannot complete immunosuppression withdrawal and who do not experience rejection will remain in the study but will be considered as 'failures'.
- Any participant who has initiated immunosuppression withdrawal and subsequently terminates from this study prematurely **will not be** replaced.

Figure 3. Flowchart of Study Protocol



3.2 STUDY DURATION

Total study duration will be up to 391 weeks (7.5 years):

- The enrollment phase will be up to 156 weeks (3 years).
- The duration of the study for an individual participant may range from approximately 188 to 235 weeks, comprised of a screening phase of approximately 8 weeks, a withdrawal phase of approximately 24 to 45 weeks and a follow-up phase of approximately 156 to 182 weeks.

- The study primary endpoint, measured 52 weeks after the last participant's completion of immunosuppression withdrawal, could be achieved as early as 240 weeks (4.5 years), or as late as 265 weeks (5 years) after enrollment of the first participant.

3.3 STUDY ENDPOINTS

3.3.1 Primary Endpoint

The primary endpoint is the proportion of participants who achieve operational tolerance 52 weeks after completion of immunosuppression withdrawal defined by:

a. No evidence of rejection since enrollment in the study.
b. A liver biopsy at 52 weeks following discontinuation of all immunosuppression demonstrating absence of rejection per the Banff global assessment criteria[1-3]. The central pathology read will be used for this determination.
c. A liver biopsy at 52 weeks following discontinuation of all immunosuppression demonstrating histological stability consistent with operational tolerance per Banff 2012 criteria[4], defined as the *absence* of the histological findings in **Table 1**. The central pathology read will be used for this determination.

For the purposes of evaluating donor-specific exhaustion, operationally tolerant participants will be compared to those who fail immunosuppression withdrawal.

Table 1. Follow-Up Biopsy Findings Suggesting that Patients are Unlikely to Benefit from Minimal or No IS*

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

***Patients with underlying AIH, HCV, PBC, or PSC are excluded[4].**
****Fibrosis should be graded as follows[67]:**
- **Portal/periportal: 0 – 3**
- **Peri-sinusoidal: 0 – 3**
- **Perivenular: 0 – 3**

3.3.2 Secondary Endpoints

Safety

1. The proportion of participants who develop DSA or de novo anti-HLA antibodies after initiation of immunosuppression withdrawal.
2. The incidence, severity, and timing of acute rejection, steroid resistant rejection, and chronic rejection.
3. The incidence and progression of graft fibrosis in tolerant versus non-tolerant patients.
4. The incidence of graft loss.
5. The incidence of all–cause mortality.
6. The incidence of study-related SAEs.

Effectiveness

1. The proportion of operationally tolerant subjects who remain free of rejection at 3 years after completing immunosuppression withdrawal.
2. Changes in renal function (defined as estimated GFR calculated by CKD-EPI: http://www.qxmd.com/calculate-online/nephrology/ckd-epi-egfr) in tolerant versus non-tolerant participants at 1, 2 and 3 years after completing immunosuppression withdrawal.
3. Changes in Quality of Life in tolerant versus non-tolerant participants and in all participants at baseline versus the end of study participation, as measured by the NIDDK Liver Transplantation Database Quality of Life Form (see Appendix 7).
4. Changes in SF-36 (see Appendix 6) in tolerant versus non-tolerant participants and in all participants at baseline versus the end of study participation.
5. Predictive value and the correlative value of the following parameters with regard to operational tolerance:
    a. Time post-transplant
    b. Recipient age

Mechanistic

1. Mechanistic endpoints may assess both the predictive value and the correlative value of the following parameters with regard to operational tolerance:
    a. Phenotypic or molecular markers of immune senescence and/or exhaustion in T cells recovered from peripheral blood or liver tissue

b. Donor-specific antibody

c. Intra-allograft C4d

d. Recipient anti-donor reactivity in vitro

e. mRNA transcripts in blood and in liver allograft biopsies

f. Thymic T cell output

g. Peripheral blood and tissue miRNA expression

h. Iron metabolism gene and serum proteins

i. Microchimerism by STR genotyping

j. Gut microbiome profile

3.4 RATIONALE FOR IMMUNOSUPPRESSION WITHDRAWAL

Long-term allograft survival generally requires lifelong immunosuppression which exposes patients at risk of infections, metabolic complications, malignancies, and drug-related toxicity[68-72]. The avoidance of these morbid side effects of immunosuppression has made the minimization or elimination of immunosuppression one of the most sought after goals of the transplant community. Achieving full drug elimination by operational tolerance or by protocols designed to achieve induced tolerance are now well-described in the literature[8,12,73,74]. Clinical experience shows that a liver allograft can resist and/or recover from acute or chronic rejection much better than other transplanted organs and may exhibit a higher rate of operational tolerance. The post-liver transplant setting therefore is ideal for attempts at immunosuppression minimization or withdrawal in selected recipients and can be considered ethically justified if risk is low and a clinical benefit anticipated.

Lifelong maintenance of immunosuppression is the current standard therapeutic approach for the majority of transplant patients in the long term because current diagnostic technology does not enable to identify recipients who are immunologically tolerant of their liver transplant from those who are not. Indeed, no immunological assay for donor-specific tolerance assessment has yet been validated.

In 1993, Starzl[75] reported a series of 11 liver transplant recipients maintaining normal liver function following the discontinuation of all immunosuppression drugs as a consequence of either noncompliance or lymphoproliferative disorders. On the basis of this observation, the authors designed a prospective trial to withdraw immunosuppression in patients with clinical complications of immunosuppression drugs. They enrolled 95 liver transplant recipients and in 28 of them immunosuppression was successfully weaned. Since then, a series of studies have been performed in which prospective withdrawal of immunosuppression was attempted in small cohorts of liver recipients.

Collectively, these studies suggest that operational tolerance develops in approximately 20% of cases[6,76-80]. Favorable clinical markers for successful immunosuppression withdrawal in these studies were at least 2 years after transplantation, low incidence of previous AR episodes, non-autoimmune liver disease and minimized immunosuppression in the immediate post-transplant period[74,75,79,80]. However, none of these

markers could be employed to accurately predict the outcome of weaning strategy. Although this strategy is associated with a high rate of acute rejection (AR), these episodes are mild in the majority of cases and can be easily solved without the need to administer high dose steroids. Frequent monitoring of liver function tests is however required to prevent liver damage from becoming irreversible. Given that rejection often develops in an atypical manner, both for-cause and protocol biopsies should be considered as mandatory because increased blood markers of liver dysfunction (liver enzymes and bilirubin) can occur in the absence of rejection[7,67,71], and subclinical rejection with normal liver function tests has also been reported in some patients.

In one study intended to evaluate the long-term histological outcome of immunosuppression withdrawal in liver transplant recipients, protocol liver biopsies were performed in 29 operationally tolerant living liver transplant recipients at an average of 121.2 months after liver transplantation[81]. Results were compared to those of recipients under maintained on immunosuppression (52 months after transplantation). The authors found that grafts from operationally tolerant recipients exhibited more fibrosis, ductular reactions and decreased luminal diameter of bile ducts as compared to patients receiving immunosuppression, and that these abnormalities improved after reintroduction of low dose immunosuppression. However, these data should be cautiously interpreted given the substantial difference in the post-transplant interval between the two groups. In addition, in six out of seven patients, improvement in fibrosis following immunosuppression reintroduction was restricted to one point of Ishak's fibrosis score, which lies within the range of potential sampling error. Of potential relevance to the current trial are the recent results of Feng et al. These investigators found that in carefully monitored studies[16,82] no histological damage has been observed after at least 3-year follow-up post withdrawal.

Given the shortage of histological data on operational tolerance patients, it is clear that more studies are needed in order to determine the consequences of chronic absence of immunosuppression therapy. It should therefore be considered mandatory to perform liver biopsies before immunosuppression withdrawal is attempted in order to detect the development of histological abnormality, in the presence or absence of new liver function test abnormalities.

3.5 STOPPING GUIDELINES

The Protocol Chair, the NIAID Medical Monitor and the Data and Safety Monitoring Board (DSMB) will periodically review safety data. If any one of the criteria listed below is met, study enrollment will be suspended and active participants will be maintained on their current immunosuppressive treatment regimens (i.e., withdrawal or discontinuation of immunosuppressive agents will be suspended) pending expedited review of all pertinent data by the NIAID Transplant DSMB, NIAID DAIT and the ITN. Participants who have completed immunosuppression withdrawal may be required to restart immunosuppression depending on the findings of the expedited review.

The criteria described below provide guidance for suspending trial enrollment based on the occurrence of selected AEs. Selected AEs of particular concern and their thresholds in this study are described below. A stopping guideline will be met if either of the following occurs:

1. Rejection resulting in death, re-transplantation, or listing for re-transplantation in any study participant.

2. If the lower bound of the one-sided 90% exact binomial confidence limit for the composite incidence of severe acute rejection and chronic rejection per the Banff global assessment criteria[1-3] and steroid-unresponsive (i.e.- 'refractory') rejection is greater than 5%.

3. Any grade 4 or higher adverse event attributed to the treatment of rejection or suspected rejection, as assessed by the NIAID Medical Monitor.

For the evaluation of stopping guidelines the rate of the composite outcome will be monitored continuously by the SDCC by estimating the incidence rate and its exact one-tailed lower 90% CI. If the lower CI limit exceeds the threshold, enrollment and further weaning will be suspended pending DSMB review. **Table 2** below gives the minimum numbers of participants with events of each type at which the stopping rules would be met for various numbers which are undergoing immunosuppression withdrawal or have withdrawn.

Table 2. Minimum Numbers of Participants with Events that Trigger DSMB Review and Study Interruption

Number of Participants Undergoing ISW or Have Withdrawn	Minimum Number of Participants with Severe AR, Refractory AR, or CR	Estimated Percentage of Participants Meeting the Stopping Rule	Associated Lower Bound of the Exact One-sided 90% Confidence Interval
10	2	20.0	5.45
20	3	15.0	5.64
30	4	13.3	5.94
40	5	12.5	6.21
50	6	12.0	6.43
60	6	10.0	5.34

AR= acute rejection; CR=chronic rejection

3.5.1 Ongoing Review

The protocol chair, the ITN clinical trial physician, the NIAID medical monitor and the NIAID Transplant Data and Safety Monitoring Board (DSMB) will periodically review safety data. Enrollment of participants in the trial and further immunosuppression withdrawal for current trial participants will be suspended at any time if any of these reviews concludes that there are significant safety concerns.

4. ELIGIBILITY

4.1 INCLUSION CRITERIA

4.1.1 Recipient

Recipient participants *must meet all* of the following criteria to be eligible for this study:

1. At the time of screening:
   - 18 to 50 years old and more than 6 years post-transplant OR
   - Greater than 50 years old and more than 3 years post-transplant
2. Recipient of either deceased or living donor liver transplant.
3. Recipient of single organ transplant only
4. Must have a screening liver biopsy that fulfills the criteria in **Table 3** based on the central pathology reading.
5. Liver function tests (Direct bilirubin, ALT) less than twice the upper limit of normal (ULN). ULN values for liver function tests will be defined by ranges from Harrison's Principles of Internal Medicine, 18th edition.
6. Receiving calcineurin inhibitor (CNI) based maintenance immunosuppression. Participants may also concurrently receive:
   - low dose mycophenolate mofetil (MMF ≤ 1500 mg daily) or mycophenolic acid (≤ 1080 mg daily) OR
   - prednisone ≤ 7.5 mg daily or equivalent corticosteroid.
7. Ability to sign informed consent

4.1.2 Living Donor

Living donor participants *must meet all* of the following criteria to be eligible for this study:

1. At the time of screening: ≥18 years old
2. Living donor of the liver allograft of an enrolled recipient participant
3. Ability to sign informed consent
4. Willingness to donate appropriate biologic samples

4.2 EXCLUSION CRITERIA

4.2.1 Recipient

Recipient participants who meet any of the following criteria will not be eligible for this study:

1. History of HCV infection (defined as a positive HCV antibody test)

2. Positive antigen-antibody immunoassay for HIV-1/2
3. Serum positivity for HBV surface antigen or HBV-DNA
4. History of immune-mediated liver disease in which immunosuppression discontinuation is inadvisable (autoimmune hepatitis, primary sclerosing cholangitis, primary biliary cirrhosis)
5. Any medical condition associated with a likely need for systemic corticosteroid administration, e.g., reactive airways disease
6. Prospective baseline liver biopsy showing any of the following: a) acute rejection according to the Banff global assessment criteria[1-3]; b) early or late chronic rejection according to the Banff global assessment criteria[1-3]; c) inflammatory activity and/or fibrosis in excess of permissive criteria according to Banff 2012 criteria[4] (see **Table 3**); d) any other histological findings that might make participation in the trial unsafe. Eligibility will be determined by the findings on the central biopsy reading.
7. Rejection within the 52 weeks prior to screening
8. Estimated GFR <40 ml/min as calculated by CKD-EPI method (to mitigate the risk of worsening renal failure should rejection occur and high level of CNI be required)
9. The need for chronic anti-coagulation that cannot be safely discontinued for a minimum of 1 week to safely perform a liver biopsy
10. Pregnant females and females of childbearing potential who are not using an effective method of birth control
11. Current drug or alcohol dependency
12. Inability to comply with the study visit schedule and required assessments, including frequent liver function monitoring and protocol biopsies
13. Inability to comply with study directed treatment
14. Any medical condition that in the opinion of the principal investigator would interfere with safe completion of the trial
15. Participation in another interventional clinical trial within the 4 weeks prior to screening

Table 3. Screening Biopsy Inclusion Criteria: Baseline Pre-weaning Biopsy findings Conducive to the Minimization of IS*

COMPARTMENT	FINDINGS
Portal inflammation and interface activity	This is preferably absent, but minimal to focal mild portal mononuclear inflammation may be present. Interface necro-inflammatory activity is absent or equivocal/minimal and, if present, involves a minority of portal tracts and not generally associated with fibrosis.
Centrizonal/perivenular inflammation	Negative for perivenular inflammation.
Bile duct changes	Lymphocytic bile duct damage, ductopenia, and biliary epithelial senescence changes are absent unless there is an alternative, non-immunological explanation (e.g. biliary strictures).
Fibrosis**	Fibrosis (if present) should be mild overall, and portal-to-portal bridging should not be more than rare. Perivenular and peri-sinusoidal fibrosis should not be more than mild according to the Banff criteria.
Arteries	Findings for obliterative or foam cell arteriopathy are negative.

***Patients with underlying AIH, HCV, PBC, or PSC are excluded[4].**

****Fibrosis should be graded as follows[67]:**

- **Portal/periportal: 0 – 3**
- **Peri-sinusoidal: 0 – 3**
- **Perivenular: 0 – 3**

4.2.2 Living Donor

Living donor participants who meet any of the following criteria will not be eligible for this study:

1. Any medical condition, such as anemia, coagulopathy, etc., that in the opinion of the principal investigator would interfere with safe participation in the trial.

4.3 PREMATURE TERMINATION OF A PARTICIPANT FROM THE STUDY

Participants may be prematurely terminated from the study 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 re-establish contact with the participant have failed).
3. The participant dies.
4. Any participant who fails screening and is deemed ineligible to initiate immunosuppression withdrawal; such a participant will be considered a screen failure.

4.3.1 Follow–up for Participants Prematurely Terminated from the Study

Participants who wish to withdraw consent prior to completing immunosuppression withdrawal will be asked to complete the safety follow-up schedule specified in Appendix 3. If they decline they will be asked to undergo a study termination visit containing the assessments listed in Appendix 3, Visit 301. If

they decline they will be terminated with no further study data collection. Participants who fail screening and never initiate immunosuppression withdrawal will have no further follow up.

Participants who wish to withdraw consent after successfully completing immunosuppression withdrawal will be asked to complete the post withdrawal follow-up schedule specified in Appendix 2. If they decline they will be asked to undergo a study termination visit containing the assessments listed in Appendix 2, Visit 201.

5. STUDY MEDICATIONS

5.1 INVESTIGATIONAL MEDICATION

This section left intentionally blank.

5.2 CONCOMITANT MEDICATIONS

5.2.1 Prophylactic Medications

Prophylaxis against fungal and Pneumocystis pneumonia infection should be considered for participants who receive high dose corticosteroids for treatment of rejection according to each center's standard of care.

Participants who receive depletional antibody therapy such as ATG or alemtuzumab for the treatment of severe rejection must receive prophylaxis against fungal, pneumocystis, and cytomegalovirus infection for a minimum of 12 weeks after completion of antibody therapy with medications prescribed according to each center's standard of care.

5.2.2 Other Medications

All immunosuppressive medications and any antibiotics, antimicrobials, and antifungals taken by or administered to study participants 30 days before their enrollment and throughout their study participation will be collected.

5.2.3 Prohibited Medications

- Short (≤7 days) courses of oral corticosteroids are permitted only for the treatment of unrelated illnesses such as asthma, allergy, etc. These should be avoided unless absolutely necessary.
- Otherwise, all immunosuppressive medications other than those used as specified in the protocol are prohibited during immunosuppression withdrawal unless rejection is suspected or diagnosed.
- Topical or inhaled corticosteroids or steroid mouthwashes will not be considered immunosuppressive medications.
- All live vaccines are prohibited for the duration of the trial.

6. STUDY PROCEDURES

6.1 VISIT WINDOWS

6.1.1 Scheduled Visits

Appendices 1 through 5-C present the schedule of events for this trial. See Section 6.2 to determine which schedules of visits to follow during the COVID-19 pandemic.

Visit 0 must occur within 56 days of Visit –1. Visit -1 may be done over multiple days following informed consent. The eligibility biopsy should be performed within 10 days of initiating Visit -1.

All other scheduled study visits must occur within the time limits specified below:

- Visit 101: within 7 days of Visit 0.
- Visit 201: Participants who initiate and successfully complete immunosuppression withdrawal should begin post withdrawal follow-up within 2 weeks of the date of completion of the participant's last taper level (see section 3.1.3).
- Visit 301: Participants who initiate but fail to successfully complete immunosuppression withdrawal (see Section 3.1.3.5) should begin safety follow-up within 2 weeks of the date of immunosuppression withdrawal failure.
- Visit RIS6: Participants who successfully complete immunosuppression withdrawal but then restart immunosuppression treatment will complete one additional follow-up visit as outlined in Appendix 4, in addition to post withdrawal follow-up, 26 weeks after immunosuppression is restarted. Appendix 2 visits should continue to occur according the participant's post withdrawal follow-up schedule. For the duration of the COVID-19 pandemic, do not conduct Visit RIS-6.
- Telephone consultations and laboratory evaluations done locally, outside of transplant center study visits, should be completed within:
    - ± 5 days of the scheduled time points during immunosuppression withdrawal;
    - ± 5 days of the scheduled time points within the first 26 weeks after completing immunosuppression withdrawal;
    - ± 7 days of the scheduled time points during follow-up weeks 26-52;
    - ± 14 days of the scheduled time points after 52 follow-up weeks.
- Up to 3 nonconsecutive pauses (up to 4 weeks each) may occur during Visits 101-111 (if needed) as outlined in section 3.1.3.4.
- All non-biopsy visits to the transplant center throughout the study should be completed within ± 2 weeks of the scheduled time points listed in Appendices 1 through 5-C. Biopsy Visits 204 and 208 should be completed within ± 4 weeks of the scheduled time points listed in Appendix 2. For

the duration of the COVID-19 pandemic, Biopsy Visit 208 should be completed -4 weeks to +6 months of the scheduled time point listed in Appendix 2-C.

- If a participant needs a for-cause biopsy within 8 weeks prior to a scheduled protocol visit, complete all clinical and mechanistic assessments listed in Appendix 5 at the time of the for-cause biopsy.
    - If the scheduled protocol visit occurs during Appendix 1A, complete all clinical assessments noted for the Appendix 1A visit but do not complete any mechanistic collections.
    - If the scheduled protocol visit is listed in Appendices 2-4, the for-cause biopsy visit will take the place of the scheduled protocol visit.
- If the Appendix 4 visit occurs within 1 month (4 weeks) of a scheduled protocol Appendix 2 visit, complete the Appendix 2 visit while collecting all mechanistic samples listed in the Appendix 4 visit in lieu of completing an additional visit.

Table 4. Frequency of Liver Function Tests

Population	**Timing**	**Frequency**
For all participants who initiate immunosuppression withdrawal	During immunosuppression withdrawal	Every 3 weeks*
	≤ 26 weeks post immunosuppression withdrawal	Every 4 weeks
	26-52 weeks post immunosuppression withdrawal	Every 8 weeks
	> 52 weeks post immunosuppression withdrawal	Every 12 weeks
	During Rejection	Liver function tests after the rejection episode per standard of care until resolution as determined by the investigator
Additional assessment for participants who successfully complete immunosuppression withdrawal and then restart immunosuppression therapy	26 weeks post immunosuppression restart	Once

*Every 2 weeks between Visits 103-105.

6.2 COVID-19 PANDEMIC SCHEDULE OF EVENTS

During the pandemic, participants should follow the COVID-19 schedule of events (Appendices 2-C, 3-C, and 5-C) to prioritize critical safety follow-up visits and assessments following completion or discontinuation of immunosuppression withdrawal. Site investigators should ensure that all research activities are conducted in accordance with state, local, and institutional guidance. Each institution will

ensure that standard COVID-19 screening and precautions are in place before any on-site study visits or when participants resume the regular schedule of events.

6.3 GENERAL ASSESSMENTS

6.3.1 Recipient

- Informed consent
- Medical and demographic history including liver transplant specifics
- Complete physical examination including height
- Limited physical examination (to include: respiratory, cardiovascular, gastrointestinal, skin and neurologic systems)
- Vital signs – Weight, temperature, blood pressure, respiration, and pulse
- Quality of Life Questionnaires (SF-36 and NIDDK Liver Transplant Database QOL questionnaires)
- Screening for status change since prior visit including assessment of adverse events and concomitant medications
- Telephone consultation
- Assess banked donor specimen availability (for recipients of deceased-donor allografts only)
- Electrocardiogram (ECG)

6.3.2 Living Donor (for recipients of living-donor allografts only)

- Informed Consent
- Medical and demographic history
- Concomitant medication review

6.4 CLINICAL LABORATORY ASSESSMENTS

6.4.1 Recipient

These laboratory assessments may be performed at study sites or at local laboratories:

- Hematology – CBC with differential and platelets
- Comprehensive Metabolic Panel (Na, K, Cl, HCO3, BUN, creatinine, glucose, albumin, total bilirubin, direct bilirubin, GGT, AST, ALT, alkaline phosphatase)
- Urine or blood pregnancy test

- Liver Function Tests (Direct bilirubin, ALT, GGT)
- Autoantibody panel (ANA, AMA, SMA, LKM, quantitative IgG)
- CMV (IgG) and EBV (IgG and IgM) serologies
- HIV-1/2 antigen-antibody immunoassay
- Hepatitis C antibody test
- HBV surface antigen and HBV DNA PCR
- Estimated GFR as determined by calculated CKD-EPI
- CNI levels
- Liver biopsy
    - Participants who are screen failures (see Section 3.1.1) may be rescreened. If a rescreened participant completed a protocol biopsy that met eligibility (see Section 4) during their initial screening window (6.1.1), an investigator may request that the candidate's data is reviewed by a committee consisting of the NIAID Medical Monitor, the ITN Clinical Trial Physician and the Protocol Chair. This committee will adjudicate whether the participant may use the biopsy previously completed in place of a new screening biopsy.

6.4.2 Living Donor

These laboratory assessments may be performed at study sites or at local laboratories:

- Hematology – CBC with differential and platelets
- Comprehensive Metabolic Panel (Na, K, Cl, HCO3, BUN, creatinine, glucose, albumin, total bilirubin, direct bilirubin, GGT, AST, ALT, alkaline phosphatase)

6.5 MECHANISTIC ASSESSMENTS

6.5.1 Recipient

- Medium and/or high resolution molecular HLA typing and DNA (HLA-A, HLA-B, HLA-C, HLA-DRB1, HLA-DRB3-5, HLA-DQA1, HLA-DQB1 and HLA-DPB1)
- Frozen PBMC – flow cytometry panel staining
- Frozen PBMC – gene expression profiling
- Frozen PBMC – cellular assays
- Serum – HLA alloantibodies, includes DSA, flow PRA
- Serum – miRNA/gene expression

- Serum – iron metabolism
- Serum – cytokine assays
- Serum – autoantibody panel
- Whole Blood – gene expression profiling
- Whole blood – DNA methylation studies
- Urine pellet – gene expression profiling
- Fecal – microbiome profiling
- Liver Biopsy – RNA gene expression profiling
- Liver Biopsy – histology
- Banked donor specimen procurement (for recipients of deceased-donor allografts only)

6.5.2 Living Donor (for recipients of living donor allografts only)

- Medium and/or high resolution molecular HLA typing and DNA (HLA-A, HLA-B, HLA-C, HLA-DRB1, HLA-DRB3-5, HLA-DQA1, HLA-DQB1 and HLA-DPB1)
- Frozen PBMCs – cellular assays

6.6 ALLOGRAFT DYSFUNCTION

6.6.1 Definition of Allograft Dysfunction and Indication for Allograft Biopsy

Allograft dysfunction is an unexplained elevation in ALT or GGT tests relative to baseline. The baseline LFT value for each participant is defined as the average of two LFTs taken at the following time points:

- The LFT taken at the screening visit
- An LFT taken 7 (+/- 2 days) before initiation of immunosuppression withdrawal

Allograft dysfunction is defined with regard to the following baseline values:

If the baseline value was less than the upper limit of normal range (ULN) and the current value is ≥ 2-fold the ULN

Or,

If the baseline value was ≥ the ULN and the current value is ≥ 2-fold the baseline value.

All assessments are based on local laboratory values. All ULN values will be defined by ranges from Harrison's Principles of Internal Medicine, 18th edition.

When allograft dysfunction occurs, participants should be thoroughly assessed for any concurrent illness or alternative diagnosis according to standard of care. Liver function tests may be repeated for both

verification and re-assessment. If allograft dysfunction persists for greater than 72 hours and no other etiology can be identified, then a for-cause biopsy must be performed. If a for-cause biopsy has been performed and is non-diagnostic, further biopsies may be performed at the discretion of the investigator. Mechanistic blood samples will be drawn per Appendix 5 with the initial for-cause biopsy that is performed for each episode.

6.7 DIAGNOSIS OF REJECTION

Unless medically contraindicated, all episodes of acute rejection must be confirmed by liver biopsy.

6.7.1 Acute Rejection

Acute allograft rejection will be defined in accordance with Banff global assessment criteria[1-3,65].

6.7.2 Chronic rejection

Chronic allograft rejection will be defined in accordance with Banff global assessment criteria[1-3].

6.7.3 Presumed Rejection

Every effort should be made to confirm all episodes of suspected rejection with a biopsy. However in cases where this is medically contraindicated, participants may be treated empirically based on clinical suspicion. Participants with allograft dysfunction who are treated with an increase in immunosuppression without a confirmatory biopsy will be considered to have **presumed rejection**.

Participants with presumed rejection must undergo a biopsy within a week after initiation of treatment for allograft dysfunction, unless medically contraindicated. If the biopsy is negative for rejection the participants may continue with immunosuppression withdrawal. If the biopsy is positive for rejection or if the participant is unable to undergo a biopsy they will be considered to have failed immunosuppression withdrawal.

6.8 MANAGEMENT OF ACUTE REJECTION

Immunosuppression will be reinstituted for all participants who experience biopsy-confirmed rejection or presumed rejection. Investigators may treat rejection according to institutional standard. The following however are recommended guidelines based on previous experience in liver allograft immunosuppression withdrawal trials:

- Allograft dysfunction with *no acute rejection or indeterminate for acute rejection*, and without other explanatory diagnosis should be treated at the discretion of the site investigator with reinstitution of the baseline IS (regimen employed before initiation of IS withdrawal). If liver tests do not improve within 4 weeks, repeat biopsy should be considered prior to further escalation of treatment.

- *Mild acute rejection* should be treated initially with reinstitution of baseline IS. If liver tests do not improve within 2 weeks, dose increase or addition of 20 mg oral prednisolone (or equivalent) should be considered. Corticosteroids will be rapidly tapered down over a 4 week period. A second biopsy can be performed at any time at the investigator's discretion.

- *Moderate acute rejection without jaundice and with mild biochemical abnormalities* should be treated with reinstitution of baseline IS and 20 mg oral prednisolone (or equivalent) with rapid taper down of steroid doses over a 4 week period. If liver tests do not improve within 2 weeks, conversion or addition of another agent should be considered prior to corticosteroids. A second biopsy can be performed at any time at the investigator's discretion.

- *Moderate acute rejection with marked biochemical abnormalities and/or jaundice*, *severe acute rejection*, or *chronic rejection*, should be treated according to site standard of care. Antibody treatment should be reserved for steroid-resistant acute rejection proven by repeat liver biopsy.

Participants who experience biopsy-confirmed rejection or presumed rejection prior to completing immunosuppression withdrawal will undergo safety follow-up per Appendix 3. If the episode of biopsy-confirmed rejection or presumed rejection occurs after the completion of immunosuppression withdrawal, the participant will continue on post withdrawal follow-up per Appendix 2. If the participant is restarted on immunosuppression therapy, the subject will be followed concurrently on Appendix 4 (except during the duration of the COVID-19 pandemic).

For participants who have completed CNI withdrawal and need to restart tacrolimus for biopsy confirmed or presumed acute rejection, an ECG will be performed prior to restarting tacrolimus. A follow-up ECG will be performed within 7 days after tacrolimus has been restarted. ECG findings will be monitored per the site's standard of care until the participant is taking a stable dose of tacrolimus. Evidence of a prolonged QTc interval will be appropriately evaluated and treated.

An episode of acute allograft rejection involving elevated ALT (with or without elevated GGT), will be considered as resolved when ALT is $\leq$ 1.5 times baseline levels. For these cases, it is recognized that GGT levels decline very slowly following rejection and therefore will not be used to define resolution. If acute allograft rejection with dysfunction involves elevated GGT alone, it will be considered resolved when GGT is $\leq$ 1.5 times baseline levels.

6.9 UNFAVORABLE BIOPSY FINDINGS REQUIRING REINSTITUTION OF IMMUNOSUPPRESSION

The protocol biopsy performed 52 weeks following discontinuation of all immunosuppression will be assessed by **Table 1** by central pathology. The presence of any or all of the histological findings described in **Table 1** on a follow-up biopsy after initiation of immunosuppression withdrawal suggests that participants are unlikely to benefit from minimal or no immunosuppression[4]. Participants who demonstrate any of the histological findings in **Table 1** will not be required to restart immunosuppression by this protocol; clinical management will be determined by the local pathology read and discussion with the participant (see section 6.10.2). Such participants will still be considered to have failed immunosuppression withdrawal.

6.10 LIVER BIOPSY

6.10.1 Types of Biopsies

- Protocol Biopsies

- Participants will undergo liver biopsies at times specified in the schedule of events (see Appendices 1 and 2). These biopsies will be used to monitor liver function, to screen for subclinical rejection, and for mechanistic analyses.

- For–cause Biopsies
  - For-cause biopsies (FCB) will be obtained to confirm suspected rejection following unexplained allograft dysfunction (see Section 6.6 for definition) and all mechanistic assessments listed in Appendix 5 should be performed.

6.10.2 Use and Interpretation of Liver Biopsies

All liver biopsies will be analyzed per the Banff global assessment criteria[1-3] by the central pathology laboratory. All protocol and for-cause biopsies will be analyzed by the local pathology laboratory. The guidelines for the use of these pathology reports are outlined below:

6.10.2.1 Protocol Biopsies

All protocol biopsies will be read by both the central and local pathologist. The central pathology read will be used for the purposes of analysis and determination of eligibility for immunosuppression withdrawal, and for assessment of the primary endpoint. The local pathology read will be used for the purposes of clinical management. The central pathology read will be available to the investigators for consideration at their discretion.

6.10.2.2 For-Cause Biopsies

The local pathology read will be used for clinical management for all for-cause biopsies. The central pathology read will be used for the purposes of analysis in conjunction with any clinical treatment to determine study outcomes such as reported rates of rejection.

Central pathology reads will also be collected for for-cause biopsies, but not used for treatment or determining rejection due to the time lag in reporting.

6.10.3 Liver biopsy assessments

All biopsies read by the central pathology core will be scored for 40 histopathological features. Biopsies will be assessed for adequacy, length, and the total number of portal tracts, with and without bile ducts. Necro-inflammatory activity, fibrosis, and architectural distortion will be graded according to the Ishak scale. AR- and CR-related activity will be graded and staged according to Banff criteria[1,3,83]. Patterns of fibrosis not directly attributable to hepatitis, AR, or CR will be scored on a scale of minimal (barely detectable), mild, moderate, and severe (bridging). Architectural distortion will be assessed by variability of lobular size evidenced by measured portal-to-central distance. Changes potentially associated with chronic immunosuppression including 1) hepatic artery branch hyalinosis; and 2) nodular regenerative hyperplasia will also be assessed. Since consensus scales for these parameters do not exist, they will be scored according to a semi-quantitative scale.

7. MECHANISTIC ASSAYS

7.1 RATIONALE FOR IMMUNE STUDIES

A key objective of this investigation is discovery of a multiparameter biomarker composed of demographic, cellular or molecular markers that would prospectively identify liver transplant recipients who can withdrawal all immunosuppression successfully with little or no risk of experiencing allograft rejection. Many of these analyses will be exploratory in nature. The recent demonstration that the likelihood of immunosuppression withdrawal success depends on time post-transplant and age suggests that operational tolerance is a dynamic process that evolves with time. We hypothesize that identification of combinations of molecular and cellular biomarkers in conjunction with demographic information may accurately characterize the subset of operationally tolerant patients thereby allowing withdrawal of immunosuppression in those most likely to succeed. Specifically, in collaboration with the ITN mechanistic core and the following investigators [redacted] we may investigate the following areas:

1. Multi-parameter flow cytometry of peripheral blood lymphocyte subsets including markers of senescence/exhaustion.
2. Signature of miRNA in blood, PBMC, serum and/or graft biopsy specimens.
3. Histological characterization of graft specimens from tolerant and non-tolerant patients.
4. Transcription signature in blood and/or PBMC, graft biopsy, and/or urine pellet mRNA.
5. Serum and molecular markers of iron metabolism
6. HLA typing and alloantibody monitoring
7. Assessment of the extent of hepatopoeitic microchimerism using STR
8. Microbiome composition
9. Recipient cell reactivity to donor cell/material

7.2 MULTI-PARAMETER FLOW CYTOMETRY (MFC) AND TETRAMER ANALYSIS

We have hypothesized that the dynamic development of operational tolerance in liver recipients occurs at least in part through a process of immunosenescence and lymphocyte exhaustion. Specifically, that thymic atrophy-associated reduction in the production of new alloreactive clones in conjunction with the chronic exposure of recipient T cells to high levels of donor antigen expressed by the graft leads to a gradual attrition or functional exhaustion of donor reactivity. With this thesis in mind, MFC panels may be designed with the ITN flow cytometry core and [redacted] to detect recent thymic emigrants (e.g., CD103, CCR5), T cells with evidence of replicative senescence (e.g., identified by telomere shortening), and markers of lymphocyte exhaustion (e.g., CD57-, PD-1+). These studies may be

performed in parallel with investigation of general B and T cell subsets along with other reported flow based markers of tolerance in transplant patients, for example γδ-T cells, NK T cells, Tregs and transitional and regulatory B cell subsets.

Flow cytometry may be performed on banked, frozen PBMC in batches at (an) appropriate timepoint(s). These experiments may be used to identify cell populations, cytokine production, and activation states. MFC data would be interpreted in conjunction with the other cellular, molecular, and immunologic assays that will be done in this trial as well as clinical data such as participant age, immunosuppression, rejection history, alloAb and DSA profiles, allograft histology, gene expression data, and longitudinal progress and outcome of immunosuppression withdrawal.

A central tenet to the argument that immunosenescence and lymphocyte exhaustion are responsible for development of operational tolerance in liver transplant recipients is that changes in the T cell repertoire or phenotype should be manifest in the donor specific subset of T cells. Inspection of donor specific T cell should provide greater sensitivity of analysis and will allow an internal control of non-donor specific T cells to account for global population changes dues to age and pathogen exposure. For example, by using either donor type stimulation and/or specific tetramers we may be able to examine donor specific changes in T cells with focus on markers of exhaustion and replicative senescence. If tetramers are used, we anticipate availability for approximately 70% of the patients in the study based on common HLA types.

Table 5. Potential markers for discriminating exhausted vs. senescent cells via flow cytometry

Exhaustion	Senescence
+ PD-1	shortened telomere length
+ CTLA-4	↑ KLRG-1
+ TIM-3	↑ p38 MAP kinase
+ LAG-3	- CD28
+ BIM	+ CD57
+ BLIMP-1	↓ CD31
- CD62L	
- CCR7	
- IL-7R	
- IL-15R	
- CD28	

7.3 DISCOVERY OF A MIRNA SIGNATURE OF OPERATIONAL TOLERANCE

A novel aspect of our proposal is to explore micro RNA correlates with operational liver transplant tolerance in samples of blood serum and graft tissue.

miRNA's, which are small 20-25 nucleotide single stranded molecules, have recently gained prominence as master regulators of gene expression through translational repression and/or mRNA degradation[41-44]. More than 700 miRNA's have been identified and their critical role in immune system development[45-51] the regulation of innate and adaptive immunity and may be key in T cell, B cell and Treg differentiation and function[44]. That the miRNA might also be involved in the regulation of tolerance versus rejection seems almost certain but this remains an area with little data[52]. In a recently reported analysis by

Suthanthiran's team, miRNAs were differentially expressed in rejecting kidney biopsies compared with biopsies showing normal histology. miRNA were selected by hierarchical clustering for assessment in a biopsy validation set. Two of the miRNA (miR-142-5p and miR-155) accurately predicted rejection with 100% sensitivity and 95% specificity. A second study with similar objectives using slightly different methodology also identified a panel of miRNA associated with rejection however there was no overlap between miRNA found significant in the two studies[41,52].

Despite their discordance as to which miRNA correlated with rejection, these studies reveal the enormous potential for miRNA as a diagnostic tool in the transplant setting. An important and novel component of the proposed mechanistic studies is we may perform miRNA analysis of serum, blood and liver tissue to identify a miRNA signature of operational liver graft tolerance. Two approaches may be applied. First, a PCR-based approach may be used to evaluate known miRNAs, and second, next generation sequencing may be used to capture both known and currently unknown miRNAs that may be associated with operational liver tolerance. As far as we are aware, such an analysis has not yet been performed in prospectively weaned liver transplant recipients. This approach holds some theoretical advantages over a transcriptome microarray survey based on the more limited number of miRNAs as well as the practical advantage that miRNAs are stable in serum. The studies represent a new and potentially powerful addition to studies reported to date searching for a molecular signature of tolerance.

7.4 SERUM AND MOLECULAR MARKERS OF IRON METABOLISM AND OPERATIONAL TOLERANCE

An unexpected observation in the recent immunosuppression withdrawal studies of Dr. Sanchez-Fueyo was that tolerance was associated with serum and molecular markers associated with iron homeostasis[8]. By microarray analysis, the transcriptional profile of operationally tolerant recipients was dominated by genes involved in iron metabolism including HAMP, TFRC (the genes with the greatest fold change), FTHL12, FTHL8, EPHX1, A2M, CP, FTHL3, FTHL11, ABAT and SFXN4. In the serum, collected prospectively from participants successfully weaned from immunosuppression the levels of hepcidin, the master regulator of iron metabolism, and ferritin were elevated and there was greater iron deposition in graft hepatocytes. We may follow up on these findings using samples from this new participant cohort and measure serum and/or graft iron makers and use Q-PCR to analyze previously detected genes in iron metabolism pathways prior to withdrawal and at the 1 and 2 year time point post successful weaning or rejection.

7.5 HISTOLOGICAL ASSESSMENTS OF TOLERANCE MECHANISMS

Liver allograft biopsy remains the gold standard diagnostic modality for detection of allograft rejection. Whether careful histological assessment may also provide a reliable measure of tolerance has been recently examined by Feng et al in pediatric patients[16] and is to be studied further in an NIH sponsored immunosuppression withdrawal trial in pediatric patients. Dr. Feng has generously shared their investigative plan using multiplex antigen labeling[84], to evaluate intragraft structural and phenotypic markers prior to immunosuppression withdrawal. The proposed staining panels are shown below (**Table 6**). Panels will be finalized at the time of experimentation to reflect the most current markers based on literature and state-of-the-art. A detailed focus on intragraft events is consistent with the notion that graft induced immune exhaustion is central to tolerogenic events.

We may test the hypothesis that the liver allograft itself plays a critical role in the development and maintenance of tolerance. To do so, we would rely on a histological analysis that parallels that in Dr.

Feng's planned trial, and/or the literature and state-of-the-art at the time of assessment. This has the advantage of permitting comparison of the pediatric and adult withdrawal cohorts. The findings would be interpreted in conjunction with the other cellular, molecular, and immunologic assays that will be done in this trial.

Table 6. Potential Multiplex Staining Panels for Liver Biopsy Specimens

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

7.6 GENE EXPRESSION

We propose to study gene expression in study participants, potentially using blood and/or PBMC (potentially whole PBMC and/or separated cell populations), urine pellet, and/or graft specimens collected prior to initiation of immunosuppression weaning. These experiments may also include DNA methylation assays using whole PBMC and/or enriched cell populations. This approach permits an unbiased assessment and avoids the confounding influences encountered when comparing already successfully weaned with those who failed weaning and remain on immunosuppression. These experiments may also be done on samples obtained during weaning, and following weaning success/failure. Comparison of pre, during, and post weaning samples in operationally tolerant patients may also be performed to determine whether any tolerance signal observed persists during and after immunosuppression discontinuation. Samples from tolerant participants may also be compared to appropriate samples from participants that have failed to be successfully withdrawn from immunosuppression during tapering (non-tolerant), and samples from participants that successfully withdrew from all immunosuppression during tapering, but were subsequently re-started on immunosuppression for clinical cause (returned to immunosuppression).

We will potentially analyze blood and/or PBMC, urine pellet, and/or liver biopsy specimens. Recent analysis of liver biopsies by microarray, followed by validation of gene changes by RT-PCR[8] has identified a molecular signature of tolerance that requires further assessment in the more diverse US population. Dr. Sanchez-Fueyo's liver tissue gene signature discriminated, with high specificity and sensitivity, at baseline those patients that could successfully withdraw immunosuppression from those that could not. This predictive signature was different from those previously reported from PBMCs or whole blood and appears more robust. Therefore, we may use Q-PCR to test for previously reported signatures in samples, as well as query for a novel signature(s) using microarray technology in samples.

Whole-genome microarray gene expression profiling may be performed first to comprehensively define the transcriptional patterns associated with operational tolerance and rejection in liver transplant recipients. RT-PCR experiments would then be conducted to: i) validate the most promising targets identified in the microarray experiments; ii) determine whether gene expression markers associated with operational tolerance in adult liver transplant recipients parallel those found to be relevant in the pediatric population in Dr. Feng's trial; and iii) to define gene expression predictive classifiers.

RNA may be extracted from liver biopsy tissue, urine pellet, peripheral blood, and/or PBMC collected at baseline and longitudinally, per the SOE. Results from biopsy microarray experiments may be compared with those obtained from peripheral blood samples and correlated with the clinical outcome of immunosuppression withdrawal. Differential gene expression between tolerant, non-tolerant, and/or returned to immunosuppression recipients may be assessed employing a variety of different bioinformatic approaches as outlined in the mechanistic statistical analysis plan (MSAP) (e.g. SAM, significance analysis of microarrays[85]) PAM (prediction analysis of microarrays)[86], misclassification penalized posterior (MiPP) [13] as well as applying machine learning approaches. Functional analysis of differential gene expression would be computed utilizing Ingenuity Pathway Analysis to identify molecular pathways significantly associated with operational tolerance and/or rejection in the peripheral blood and/or PBMC, urine pellet, and/or in the liver microenvironment.

The ITN core has extensive published experience with RT-PCR (Applied Biosystems, TaqMan, and Fluidigm Biomark platforms) in transplant settings[87,88]. Specimens could potentially be used for RT-PCR experiments from time-points such as baseline and annually post-immunosuppression withdrawal (for tolerant participants), and baseline, time of allograft dysfunction / AR, and subsequent years post-allograft dysfunction/AR (for non-tolerant and returned to immunosuppression patients). Depending on experiment design, cell subset populations (e.g., CD4+, CD8+, etc.) may be isolated prior to RNA extraction. Total RNA would be reverse transcribed to cDNA and amplified using primer/probes corresponding to the most highly discriminative genes identified in adult operationally tolerant liver transplant recipients. A representative list with 22 potential genes identified in RT-PCR experiments conducted employing PBMCs and 15 genes from previous liver biopsy studies is detailed in **Table 7**; the final list of genes to be assessed will be dependent on ongoing RT-PCR validation studies on whole-blood derived RNA and the current literature at the time of the experiment. Other potential genes include the 2 gene signature identified by the ITN507ST (FACTOR) study[12]. Genes relating to senescence, exhaustion, and/or anergy may also be assessed. Genes of interest identified in the microarray studies detailed below may also be validated by RT-PCR. Relative expression will be calculated according to the ΔΔCT method employing as a reference RNA sample pooled blood RNA from the 60 participants in the study. Similar experiments will be conducted employing primer/probes selected to match the most informative targets identified in the micro-array experiments. RT-PCR data will be used to verify microarray results and predictive modeling to develop RT-PCR-based expression classifiers of tolerance status and will be employed as described in the MSAP.

Table 7. Potential gene list for RT-PCR experiments: Target genes depend source of RNA

Whole Blood Specimens				**Liver Biopsy Specimens**		
CTBP2	CXC3R1	SLAMF7	PTCH1	MIF	IFNG	ACO1
CLIC3	PTGDR	GNPTAB	KLRB1	TFRC	MCOLN1	CDHR2
KLRF1	C10orf119	FLJ14213	RGS3	SOCS1	HMOX1	SLC5A12
IL2RB	CD9	CD160	CD244	ADORA3	HFE2	PEBP1
OSBPL5	ERBB2	NKG7	GEMIN7	HAMP	SLC11A2	DAB2
FEZ1	NCALD					

7.7 OLIGONUCLEOTIDE MICROARRAY

Microarray experiments may be performed on all baselines samples (blood, PBMC, urine pellet and/or liver biopsy) after all participants have a clinical phenotype based on the outcome of ISW. Since the primary endpoint for each participant will likely be reached within 2 years of enrollment and since complete trial enrollment is anticipated to take one year, the earliest that this experiment could be done is during the third year of the trial. We may test the specific hypothesis that biomarkers predict tolerance versus rejection/non-tolerance. Therefore, the array results will only be interpretable with knowledge of

patient's clinical outcome (weaning success or failure). Potential comparison groups may be participants that are tolerant vs. non-tolerant vs. returned to immunosuppression, or tolerant vs. non-tolerant and/or returned to immunosuppression. Based on these results, additional microarrays may be performed after year 5, once all possible blood and biopsy specimens have been collected. Biopsy and blood samples to monitor changes in gene expression from baseline are drawn at regular intervals, as shown in **Table 7**.

The results from these experiments would be analyzed as described in the MSAP to: 1) identify differential gene expression between operationally tolerant recipients and those failing ISW and how these differences evolve over time; 2) define the molecular pathways associated with operational tolerance; 3) identify gene markers to be incorporated into RT-PCR-based gene expression predictive classifiers; and/or 4) establish the effect of immunosuppression withdrawal on blood and biopsy-derived transcriptional patterns. Gene expression results are also expected to contribute to a predictive cross-platform biomarker signature, whereby all mechanistic and clinical information is merged together and interrogated using protocols described in the MSAP to identify those measures that are indicative of tolerance and when assayed in combination increase the predictive power of the signature.

Global transcriptional profiling may be performed using the Affymetrix GeneChip oligonucleotide microarray platform employing whole-genome last generation human chips (Affymetrix Gene Titan Platform and Human Genome U219 arrays). With current pre-hybridization sample processing protocols, these experiments can be conducted employing just 100 ngs of RNA per sample. Affymetrix Gene Titan Platform can process 96 samples simultaneously, thus reducing the risks of batch effects. To further reduce this potential problem, baseline and follow-up tolerant vs. returned to immunosuppression and/or non-tolerant RNA samples will be equally distributed across experimental batches/array plates, but group designations will not be available to the technician performing the assay. Additionally, a selected RNA reference sample will be run in every single batch (pooled RNA from all participants in the study). We will potentially establish baseline transcriptional profiles comparing pre-weaning samples from tolerant and returned to immunosuppression/non-tolerant recipients. Next, changes in gene expression patterns over time would be analyzed in paired samples to identify markers of rejection (returned to immunosuppression/non-tolerant patients) and to study the stability of tolerance-related patterns (tolerant patients) as described in the MSAP.

7.8 HLA ALLOAB AND FLOW CYTOMETRY CROSSMATCHING

HLA Abs have not been strongly implicated as a risk factor for AR, CR, or graft loss for liver transplant recipients, in contrast to kidney transplant recipients[89]. Nevertheless, their presence at baseline may preclude successful immunosuppression weaning. Moreover, after initiation of weaning, monitoring the increasing breadth and/or strength of alloAbs, particularly if they are directed against donor antigens, may be important as this may signal the presence of an immune response that will be deleterious to long-term allograft histology.

Serum samples collected from participants may therefore be evaluated for alloAbs, including donor-specific alloAbs (DSA). Assessments will be performed prior to immunosuppression weaning (baseline) and at multiple time points during and after immunosuppression withdrawal. An initial screen will determine if the participant has detectible alloAbs. If the screening assay is positive, Ab specificities will then be determined using beads coated with single HLA-antigens.

HLA typing of donor and recipient will be necessary to assign donor specificity. Whole blood, cells, or buccal swabs, will be collected from all recipients for HLA typing. For recipients of a living donor allograft, every effort will be made to collect blood, cells, or buccal swab from the living donors. For recipients of deceased donor allografts, donor HLA typing information will be retrieved from the United Network for Organ Sharing (UNOS) database (www.unos.org) by the site investigator. When and if possible, cells/samples such as splenocytes, lymph node cells, PBMC, and/or whole blood from deceased donors will be used for HLA typing. Data will be provided to the ITN for analysis.

Autoantibodies may also impact or signify graft function and pathology[90]. We may quantify autoantibodies such as total IgG, ANA (anti-nuclear antibody), and LKM (anti-liver-kidney microsomal antibody), both in participants that succeed and fail immunosuppression withdrawal. These data may also be compared to and analyzed in conjunction with other parameters, such as alloantibody status and histology. Autoantibody panels and comparison groups will be identified before experimentation, based on the literature and state-of-the-art.

7.9 MICROBIOME COMPOSITION

The composition of the gut microbiome and its impact on the immune system is an evolving area of study[91]. There are not currently markers that correlate with a tolerant vs. non-tolerant outcome. We will bank and store microbiome samples, and use current literature and expert opinion to guide our experimental design and analysis.

7.10 IDENTIFICATION/CHARACTERIZATION OF DONOR-REACTIVE CELLS AND RESPONSES

One hypothesis of this study is that in tolerant recipients, donor-reactive cells become either exhausted and/or senescent. We may be able to identify and characterize donor-reactive cells that are exhausted, senescent, and/or anergic. For example, banked recipient PBMC may be included in an MLR/cell culture with either donor-derived cells and/or donor type material. When and if possible, cells such as splenocytes, lymph node cells, and/or PBMC from deceased donors may be used. Potential assay read-outs may include proliferation (e.g., CFSE dilution) and/or cytokine production (as detected by flow cytometry). Individual recipient PBMC populations (e.g., CD4+, CD8+) and/or unseparated cells may be used as responder cells. To identify donor-reactive recipient cells that are exhausted, an anti-exhaustion agent may be included in the experiment (e.g., addition of anti-PD-1 to culture). Controls for these experiments may include assessing responses of donor cells to self and/or third party cells/ type material and/or recall antigens (e.g., flu vaccine). To reduce variability between experiments, assays will be batched as appropriate. Assay specifics will be finalized based on literature available at the time of experimentation. Serum from recipients and/or supernatants from cell culture may also be assessed for cytokine profiles (e.g., by Luminex, etc.).

7.11 QUALITY CHECK/PRELIMINARY STUDIES

Samples acquired from this trial may be used for either quality checks (if deemed necessary) or preliminary studies that could potentially be applied to the entire cohort of participants. Examples of when a quality check may be requested include issues that may arise from shipping/storage, to ensure sites are collecting samples correctly, or to compare data generated longitudinally within a core lab.

Preliminary studies may include a pilot experiment when changing to a new core or piloting an assay/technique that may be of interest to this study or tolerance, in general.

7.12 FUTURE/ UNPLANNED STUDIES

Specimens stored during the trial may be used in future assays to reevaluate biological responses as research tests are developed over time. Additionally, samples may be used for assays/ experiments outside the scope of this proposal, such investigation of miRNA expression, differences in the TCR repertoire as evaluated by sequencing, proteomics or other explorations that may emerge and be compelling during the trial period. Reevaluations or new assays will only be performed on samples of participants who have consented for future research. Blood samples will be collected and saved to allow for the possible use in later gene association studies. Specific consent will be obtained for the storage and use of participant DNA. The ITN sample sharing policy will apply for the provision of samples to study or outside investigators.

7.13 SPECIMEN LOGISTICS

Sites will be trained in the collection, processing, shipment, and tracking of mechanistic research specimens. The ITN will monitor specimen quality, shipping compliance, etc., and retrain any clinical site that is not producing optimum quality mechanistic samples. Sites will process all mechanistic samples according to the ITN standard procedures and use the ITN Specimen Tracking System (STS) software to identify and track all mechanistic specimens. The sites will be required to have certain laboratory equipment for use in following ITN procedures, such as a centrifuge for spinning primary blood tubes, a micropipettor to aliquot specimens, and freezer to store frozen specimens until they can be shipped. Sites will use appropriate courier service for shipping specimens to repositories/ core labs, per ITN standard procedures. All shipping will conform to Department of Transportation regulations (49 CFR 173.199) for Diagnostic Specimens.

7.13.1 Specimen Tracking Procedures

The ITN will track all mechanistic specimens until the final disposition of all material is known. Samples will remain in the ITN repository until used for assays or destroyed.

7.13.2 Sample Storage

Samples sent to the ITN repository will be 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.immunetolerance.org). Any research conducted using stored samples for future use may also need appropriate regulatory approval, such as Institutional Review Board per the study consent.

8. SAFETY MONITORING

8.1 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 that data. Adverse events that are classified as serious according to the definition of health authorities must be reported promptly (per Section 8.5, Reporting of Serious Adverse Events) to DAIT/NIAID. Appropriate notifications must also be made to site principal investigators and Institutional Review Boards (IRBs), as applicable.

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), Common Terminology Criteria for Adverse Events (CTCAE), Version 4.03 (June 14, 2010): http://ctep.cancer.gov/reporting/ctc.html.

8.2 DEFINITIONS

8.2.1 Adverse Event

An adverse event (AE) is defined as 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 (ICH E-6 Guidelines for GCP).

8.2.2 Allograft Dysfunction and/or Acute Rejection as Adverse Events

Allograft dysfunction and/or acute rejection, including clinical rejection (as defined in Section 6.6.1) are considered adverse events in this study. As with other adverse events, allograft dysfunction and rejection events should be triaged and entered on the appropriate CRFs within defined timelines for AE and SAE reporting (see Sections 8.4 and 8.5).

8.2.3 Suspected Adverse Reaction (SAR)

A suspected adverse reaction (SAR) is any adverse event for which there is a reasonable possibility that the investigational study therapy or procedure caused the adverse event. For the purposes of safety reporting,

'reasonable possibility' means there is evidence to suggest a causal relationship between the study therapy or procedure and the adverse event. A suspected adverse reaction implies a lesser degree of certainty about causality than adverse reaction, which means any adverse event caused by a study therapy or procedure.

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

8.2.4 Unexpected Adverse Event

An adverse or suspected adverse reaction is considered "unexpected" if it is not consistent with the risk information described in the protocol or other experience pertaining to ISW or study procedures in this population.

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

8.2.5 Serious Adverse Event

An adverse event or suspected adverse reaction is considered "serious" if, in the view of either the investigator or DAIT/NIAID, it results in any of the following:

1) Death.

2) A life-threatening event. An AE or SAR 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 SAR that, had it occurred in a more severe form, might have caused death.

3) Inpatient hospitalization or prolongation of existing hospitalization.

4) Persistent or significant incapacity or substantial disruption of the ability to conduct normal life functions.

5) Congenital anomaly or birth defect.

6) An important medical event that may not result in death, be life threatening, or require hospitalization may be considered an SAE when, based on appropriate medical judgment, it may jeopardize the participant and may require medical or surgical intervention to prevent one of the outcomes listed above. This would also include confirmed cases of COVID-19.

8.3 GRADING AND ATTRIBUTION OF ADVERSE EVENTS

8.3.1 Grading Criteria

The study site will grade the severity of all AEs experienced by study participants according to the *National Cancer Institute (NCI), Common Terminology Criteria for Adverse Events, Version 4.03 (June 14, 2010).*

This document (referred to herein as the NCI-CTCAE manual) provides a common language to describe levels of severity, to analyze and interpret data, and to articulate the clinical significance of all adverse events.

Elevated liver tests (GGT or ALT) will not be reported as adverse events unless, relative to baseline, they exceed ≥ 2-fold the ULN (based on Harrison's Principles of Internal Medicine, 18th edition) if the baseline value was < than the ULN; or, ≥ 2-fold the baseline value if the baseline value was ≥ than the ULN.

AEs not included in the NCI CTCAE, should be recorded and graded 1 to 5 according to the General Grade Definition provided below.

Where the CTCAE relies on site normal ranges to assess grades, the normal ranges listed in Harrison's Principles of Internal Medicine, 18th edition (McGraw-Hill, 2011) will be used. Guidance for grading AEs for AR and elevated LFT(s) is provided in the MOP.

AEs will be recorded and graded whether or not they are related to disease progression or study protocol.

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.

8.3.2 Attribution Definition

Adverse events will be categorized for their relation to each of the following:

- Study therapy: Immunosuppression withdrawal
- Study procedures (liver biopsy, blood draw)

The relationship, or attribution, of an AE to immunosuppression withdrawal or other study procedures will initially be determined by the site investigator and recorded on the appropriate case report form. Final determination of attribution will be determined by DAIT/NIAID. The relationship of an AE to the study therapy will be defined by using the descriptors provided in **Table 8**.

Table 8. Attribution of AEs

Code	Descriptor	Definition
UNRELATED CATEGORY		
1	Unrelated	The adverse event is definitely not related to the study treatment.
RELATED CATEGORIES		
2	Possible	The adverse event might or might not be related to the study treatment. (This grade is assigned when uncertainty exists)
3	Definite	The adverse event is definitely related to the study treatment.

8.4 COLLECTION AND RECORDING OF ADVERSE EVENTS

8.4.1 Collection Period

AEs/SAEs will be collected from enrollment until 30 days after study completion, or until 30 days after the participant prematurely withdraws from the study.

8.4.2 Collecting Adverse Events

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

- Observing the subject.
- Questioning the subject in an objective manner.
- Receiving an unsolicited complaint from the subject.
- In addition, an abnormal value or result from a clinical or laboratory evaluation can also indicate an adverse event, as defined in Section 8.3, Grading and Attribution of Adverse Events.

8.4.3 Exceptions to Collection

Prior to initiating immunosuppression withdrawal, only AEs/SAEs associated with protocol-mandated blood draws or the screening biopsy will be collected from study enrollment until initiation of immunosuppression withdrawal. Adverse event collection for allograft living donors will be limited to those events that the investigator determines are associated with protocol-mandated blood draws.

8.4.4 Recording Adverse Events

Throughout the study, the investigator will record adverse events and serious adverse events as described previously (Section 8.2, Definitions) on the appropriate case report form.

Only Grade 2 and higher adverse events (see Section 8.3.1) will be recorded, with the following exceptions: all episodes of allograft dysfunction and rejection will be recorded regardless of grade. Adverse events must be recorded by the site on the appropriate AE/SAE CRF within 5 business days of the site learning of the adverse event(s). Please refer to Section 8.5 for reporting of events meeting serious criteria.

Once recorded, an AE/SAE will be followed until it resolves with or without sequelae, or 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.

8.5 REPORTING OF SERIOUS ADVERSE EVENTS

8.5.1 Reporting of Serious Adverse Events to Sponsor

This section describes the responsibilities of the site investigator to report serious adverse events to the sponsor via the SDCC eCRF. Timely reporting of adverse events is required by ICH E6 guidelines.

Site investigators must report all serious adverse events (see Section 8.2.5, Serious Adverse Event), regardless of relationship or expectedness to blood draw, protocol biopsy, or immunosuppression withdrawal within 24 hours of discovering the event, except as noted in Section 8.4.3. All confirmed cases of COVID-19 will also be reported as SAEs, regardless of severity.

For serious adverse events, all requested information on the AE/SAE eCRF should be provided to the SDCC. However, unavailable details of the event should not delay submission of the known information. As additional details become available, the AE/SAE eCRF should be updated and submitted.

8.5.2 Reporting of Adverse Events to IRBs

All investigators must report adverse events in a timely fashion to their respective IRBs in accordance with applicable regulations and guidelines.

8.6 REPORTING PREGNANCY

The investigator should be informed immediately of any pregnancy in a study subject. Monitoring of the pregnant subject should continue until the conclusion of the pregnancy.

The investigator should report to the SDCC all pregnancies within one business day of becoming aware of the event using the Pregnancy eCRF. All pregnancies identified during the study must be followed to conclusion and the outcome of each must be reported. The Pregnancy eCRF should be updated and submitted to the SDCC when details about the outcome are available.

Information requested about the delivery will 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 must be submitted to the SDCC using the SAE reporting procedures described above.

8.7 REPORTING OF OTHER SAFETY INFORMATION

An investigator should promptly notify the Sponsor via the SDCC when an “unanticipated problem involving risks to subjects or others” is identified, which is not otherwise reportable as an adverse event.

8.7.1 Discontinuation of Immunosuppression Withdrawal

For this study, an unanticipated problem will include discontinuation of ISW for any reason. Discontinuation of ISW should be recorded on the discontinuation eCRF within 5 business days of the action.

8.8 REVIEW OF SAFETY INFORMATION

8.8.1 Medical Monitor Review

The DAIT/NIAID Medical Monitor will receive monthly reports compiling new and accumulating information on AEs, SAEs, and pregnancies recorded by the sites on appropriate eCRFs.

In addition, the Medical Monitor will review and triage SAE and pregnancy reports received from the SDCC (See Sections 8.5.1, Reporting of Serious Adverse Events to Sponsor, and 8.6, Pregnancy Reporting).

8.8.2 DSMB Review

The Data and Safety Monitoring Board (DSMB) will 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 AEs and SAEs. The DSMB will be informed of significant safety events in a timely manner (See Section 3.5).

In addition to the pre-scheduled data reviews and planned safety monitoring, the DSMB may be called upon for ad hoc reviews. The DSMB will review any event that potentially impacts safety at the request of the principal investigator and/or the DAIT/NIAID medical officer. In addition, the study stopping rules described in Section 3.5 will trigger an ad hoc comprehensive DSMB Safety Review.

After careful review of the data, the DSMB will make recommendations regarding study conduct and/or continuation.

9. STATISTICAL CONSIDERATIONS AND ANALYTICAL PLAN

9.1 ANALYSIS SAMPLES

Intent to treat (ITT) sample: All participants who provide informed consent for study participation and begin immunosuppression withdrawal.

Per protocol (PP) sample: All participants who attempt immunosuppression withdrawal and do not have any unacceptable major protocol deviations. Subjects experiencing major protocol deviations may be excluded at the discretion of the study team during a blinded review of deviations.

9.2 ANALYSIS OF ENDPOINTS

The principal features of the plan for statistical data analysis are outlined in this protocol and will be described in greater detail in the statistical analysis plan (SAP) and mechanistic statistical analysis plan (MSAP). Analysis of study data will be conducted to address all objectives of the trial and other

interrelationships among all data elements of interest to the Investigators and of relevance to the objects of the study.

9.2.1 Primary Endpoint

The primary endpoint is defined in section 3.3.1.

The primary endpoint will be analyzed using the ITT sample and descriptively summarized with a point estimate and two-sided, 95% confidence interval. All participants who failed to complete immunosuppression withdrawal, regardless of reason, will be considered to have failed the primary endpoint. Participants without a biopsy 52 weeks following completion of immunosuppression withdrawal or who resume immunosuppression prior to 52 weeks will be considered to have failed the primary endpoint. This endpoint will also be analyzed using the PP sample.

9.2.2 Secondary Endpoints

The secondary endpoints are defined in section 3.3.2. These will be analyzed using the ITT and PP samples.

- Proportion endpoints will be descriptively summarized using frequency tables with frequencies, percentages, and 95% confidence intervals. Chi-squared tests will be used for comparisons between operationally tolerant and non-tolerant participants.
- Time-to-event endpoints will be assessed using Kaplan-Meier survival estimates and associated two-sided 95% confidence intervals. Subjects lost to competing risks or lost to follow-up will be censored at the time of occurrence of these events.
- Continuous endpoints will be summarized using descriptive statistics (n, mean, standard deviation, median, minimum, maximum). T-tests will be used for comparisons between operationally tolerant and non-tolerant participants.
- Categorical endpoints will be summarized using counts (n) and percentages (%).Chi-squared tests will be used for comparisons between operationally tolerant and non-tolerant participants.

9.2.3 Safety Analysis

All participants in the ITT sample will be included in the safety analyses. Screen failures will be presented separately. All adverse events will be classified by body system and preferred term according to the MedDRA dictionary. Frequency tables by category of event (e.g. serious, related) and by NCI-CTCAE v. 4.03 grade will be presented. Select laboratory values will be presented graphically.

9.2.4 Medical History

Medical history within the past 12 months—including the existence of current signs and symptoms—will be collected for each body system.

9.2.5 Use of Medications

Prophylactic and other medications as specified in sections 5.2.1 and 5.2.2 taken by or administered to study participants beginning 30 days before enrollment and continuing throughout the study will be collected.

9.2.6 Study Completion

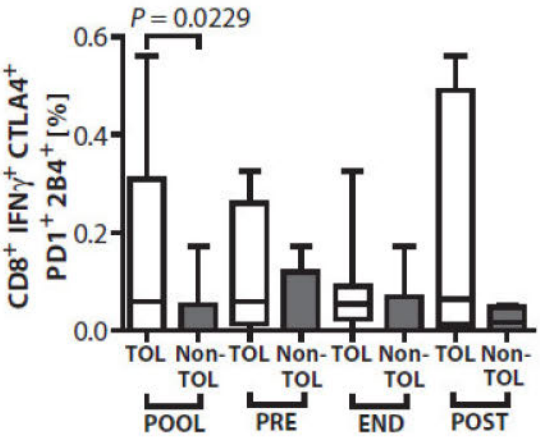
The percentage of participants who complete the study, losses to follow-up, time to lost to follow-up, and reason for termination will be presented.

9.3 SAMPLE SIZE

In order to estimate the proportion of operational tolerance with the desired level of precision, enrollment will continue until 60 participants who meet eligibility criteria are enrolled and initiate immunosuppression withdrawal. If the expected proportion of participants who exhibit operational tolerance is 40%, as demonstrated in a study of adult liver recipients by Alberto Sánchez-Fueyo[15], the exact 95% confidence interval on the proportion of operationally tolerant participants is (27.6%, 53.5%). If the expected proportion of participants who exhibit operational tolerance is 60%, the associated exact 95% confidence interval would be (46.5%, 72.4%). Thus, 60 subjects provide the ability to estimate the proportion of operational tolerance within plus or minus approximately 13%.

To ensure secondary endpoints have sufficient power, additional sample size calculations were performed based on published data from Dr. Sanchez Fueyo[36] in HCV positive liver recipients in which immunosuppression withdrawal was attempted based on the presence of at least one previously reported signature of operational tolerance (by flow cytometry, peripheral blood Vδγ1+/Vδγ2+ T cell ratio with 82% sensitivity, 53% specificity, 67% positive predictive value, 73% negative predictive value; by real-time PCR, PBMC with 93.33% sensitivity, 93.79% specificity, 93.33% positive predictive value, 93.75% negative predictive value in a retrospective cohort of 28 tolerant, 33 non-tolerant recipients). Of 34 patients in whom immunosuppression was attempted, 17 were successfully withdrawn. Tolerance was significantly associated with an expansion of exhausted PD1/CTLA4/2B4 positive, HCV reactive (IFNγ+ following HCV peptide stimulation) circulating CD8+ T cells.

Correlation of CD8+IFNγ+CTLA4+PD1+2B4+ marker with tolerance



Based on these data, members of the ITN performed the following sample size estimate analysis for this trial based on the following assumptions: 80% and 90% power, 5% level of significance.

For CD8+IFNγ+CTLA4+PD1+2B4+

The mean difference expected in the two groups is -0.1155. The pooled standard deviation is 0.152.

Power	Tolerant Proportion	Non-Tolerant Proportion	Total N	Tolerant N
0.808	0.6	0.4	60	36
0.907	0.6	0.4	80	48
0.796	0.5	0.5	56	28
0.904	0.5	0.5	76	38
0.808	0.4	0.6	60	24
0.907	0.4	0.6	80	32
0.818	0.3	0.7	70	21
0.903	0.3	0.7	90	27

Analyses involving all other biomarkers will be exploratory in nature.

9.4 INTERIM ANALYSIS

No formal interim analysis of effectiveness is planned for this study. The DSMB will receive periodic safety reports on participants. The DSMB may request modifications to the protocol based on its review of the findings.

9.5 REPORTING DEVIATIONS FROM THE ORIGINAL STATISTICAL PLAN

The principal features of both the study design and the plan for statistical data analysis are outlined in this protocol and in the statistical analysis plan (SAP). Any change in these features requires either a protocol or an SAP amendment, which is subject to review by the DSMB, the study sponsor(s), and the health authorities. These changes will be described in the final study report as appropriate.

10. ACCESS TO SOURCE DATA/DOCUMENTS

The investigational sites participating in this study will maintain the highest degree of confidentiality permitted for the clinical and research information obtained from participants in this clinical trial. Medical and research records should be maintained at each site in the strictest confidence. However, as a part of the quality assurance and legal responsibilities of an investigation, the investigational sites must permit authorized representatives of the ITN, sponsor, and health authorities to examine (and to copy when required by applicable law) clinical records for the purposes of quality assurance reviews, audits, and evaluation of the study safety and progress. Unless required by the laws permitting copying of records, only the coded identity associated with documents or other participant data may be copied (and any personally identifying information must be obscured). Authorized representatives as noted above are bound to maintain the strict confidentiality of medical and research information that may be linked to identified individuals. The investigational sites will normally be notified in advance of auditing visits.

11. QUALITY CONTROL AND QUALITY ASSURANCE

The investigator is required to keep accurate records to ensure that the conduct of the study is fully documented. The investigator is required to ensure that all CRFs are completed for every participant entered in the trial.

The sponsor is responsible for regular inspection of the conduct of the trial, for verifying adherence to the protocol, and for confirming the completeness, consistency, and accuracy of all documented data.

The CRFs will be completed online via a web-based electronic data capture (EDC) system that has been validated and is compliant with Part 11 Title 21 of the Code of Federal Regulations. Study staff at the site will enter information into the electronic CRFs, and the data will be stored remotely at a central database. Data quality will be ensured through the EDC system's continuous monitoring of data and real-time detection and correction of errors. All elements of data entry (i.e., time, date, verbatim text, and the name of the person performing the data entry) will be recorded in an electronic audit trail to allow all changes in the database to be monitored and maintained in accordance with federal regulations.

12. ETHICAL CONSIDERATIONS AND COMPLIANCE WITH GOOD CLINICAL PRACTICE

12.1 STATEMENT OF COMPLIANCE

This trial will be conducted in compliance with the protocol, current Good Clinical Practice (GCP) guidelines—adopting the principles of the Declaration of Helsinki—and all applicable regulatory requirements.

Prior to study initiation, the protocol and the informed consent documents will be reviewed and approved by the sponsor and an appropriate ethics review committee or institutional review board (IRB). Any amendments to the protocol or consent materials must also be approved by the Sponsor and the IRB before they are implemented.

12.2 INFORMED CONSENT

The informed consent form is a means of providing information about the trial to a prospective participant and allows for an informed decision about participation in the study. All participants (or their legally acceptable representative) must read, sign, and date a consent form before participating in the study, taking the study drug, and/or undergoing any study-specific procedures. If a participant does not speak and read English, the consent materials must be translated into the appropriate language.

The informed consent form must be updated or revised whenever important new safety information is available, whenever the protocol is amended, and/or whenever any new information becomes available that may affect participation in the trial.

A copy of the informed consent will be given to a prospective participant for review. The attending physician, in the presence of a witness, will review the consent and answer questions. The participant will be informed that participation is voluntary and that he/she may withdraw from the study at any time, for any reason.

12.3 PRIVACY AND CONFIDENTIALITY

A participant's privacy and confidentiality will be respected throughout the study. Each participant will be assigned a sequential identification number. This number, rather than the participant's name, will be used to collect, store, and report participant information.

13. PUBLICATION POLICY

The ITN policy on publication of study results will apply to this study. [redacted]

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APPENDIX 1A. SCHEDULE OF EVENTS – IMMUNOSUPPRESSION WITHDRAWAL

	CNI Withdrawal										MMF Withdrawal (if applicable)		
	Prednisone Withdrawal (if applicable)												
Taper Level	**N/A**	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**0**	**1**	**2**
Visit Number[1,2]	**-1**	**0**	**101[3]**	**102**	**103**	**104**	**105**	**106**	**107**	**108[4]**	**109[5]**	**110[6]**	**111**
General Assessments													
Informed consent for ISW	X												
Medical and demographic history including liver transplant specifics	X												
Complete physical exam including height	X												
Vital signs including weight	X	X			X		X				X		
Limited physical exam		X			X		X				X		
Quality of Life Questionnaires		X									X		
Status Change since prior visit[7]	X----------									X	X----------		X
Telephone consultation			X	X		X		X	X	X		X	X
Banked donor sample availability[8]	X												
ECG		X[9]											
Local Clinical Laboratory Assessments													
LFTs	X	Every 3 weeks			Every 2 weeks		Every 3 weeks				Every 3 weeks		
CBC w/differential	X	X			X		X				X		
Comprehensive metabolic panel, includes LFTs	X	X			X		X				X		
Urine or blood hCG	X												
Autoantibody panel[10]	X												
Viral Serology/Antibody/Immunoassay tests[11,12]	X												

[1] Up to 3 nonconsecutive pauses may occur during visits 101-111 (if needed) as outlined in section 3.1.3.4.

[2] If a for-cause liver biopsy is needed, follow all assessments specified on Appendix 5. If the for-cause liver biopsy occurs within 8 weeks of a scheduled visit, complete all clinical and mechanistic assessments listed in Appendix 5 at the time of the for-cause liver biopsy. Complete all clinical assessments during the scheduled visit but do not complete any mechanistic collections.

[3] CNI withdrawal must begin within 7 days of Visit 0 and after demonstrating stable liver function (see section 3.1.3.1).

[4] Move to visit 109 if the participant is on a mycophenolate compound therapy or to visit 201 (Appendix 2) if the participant was on CNI alone or CNI and prednisone and successfully completed withdrawal. Move to visit 301 (Appendix 3) if the participant failed to successfully withdraw from immunosuppressive treatment.

[5] Complete within 2 weeks of the date of completion of the participant's last CNI taper level (see section 3.1.3) if the participant is on a mycophenolate compound therapy.

[6] Mycophenolate compound immunosuppression withdrawal to be initiated once the participant has completed CNI withdrawal and upon confirmation of at least 3 weeks of stable liver function (see section 3.1.3.3).

[7] Includes assessment of change in adverse events and concomitant medications. Changes should be recorded in eCRFs as appropriate.

[8] For recipients of deceased-donor allografts only.

[9] For participants who have not completed a baseline ECG (i.e. Visit 0 occurred prior to protocol version 5.0) and are either 1) undergoing immunosuppression withdrawal or 2) off tacrolimus, an ECG will be performed at their next transplant center study visit.

[10] Includes ANA, AMA, SMA, LKM and quantitative IgG.

[11] Includes CMV (IgG) and EBV (IgG and IgM) serologies, HIV-1/2 antigen-antibody immunoassay, HCV antibody test, HBV surface antigen and HBV DNA PCR.

[12] No need to repeat the HIV-1/2 antigen-antibody immunoassay if the test was previously performed within the last year (≤ 12 months) prior to initiating immunosuppression withdrawal.

	CNI Withdrawal										MMF Withdrawal (if applicable)		
	Prednisone Withdrawal (if applicable)												
Taper Level	**N/A**	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**0**	**1**	**2**
Visit Number[1,2]	**-1**	**0**	**101[3]**	**102**	**103**	**104**	**105**	**106**	**107**	**108[4]**	**109[5]**	**110[6]**	**111**
CNI levels		X											
Liver biopsy[13]	X												
Mechanistic Laboratory Assessments													
HLA Typing[14]	X												
Frozen PBMC collection		X			X		X				X		
Serum collection		X			X		X				X		
Whole blood collection		X			X		X				X		
Urine pellet collection		X			X		X				X		
Fecal collection		X[15]			X						X[16]		
Liver biopsy collection	X[17]												
Banked donor sample procurement[18]		X											
Immunosuppression Medication													
Tacrolimus or cyclosporine A (CNI)	X	X	X	X	X	X	X	X	X	X			
Prednisone or equivalent[19]	X	X	X	X[20]	X[20]	X[20]	X[20]	X[20]	X[20]	X[20]			
MMF or equivalent[21]	X	X	X	X	X	X	X	X	X	X	X	X	X

[13] Liver biopsy should be performed within 10 days of initiating Visit -1 and once the participant has met all other eligibility criteria. Liver function tests obtained within the 60 days prior to informed consent may be used to verify whether the subject's liver function meets study criteria prior to the screening biopsy.

[14] Includes medium and/or high resolution molecular HLA typing and DNA (HLA-A, HLA-B, HLA-C, HLA-DRB1, HLA-DRB3-5, HLA-DQA1, HLA-DQB1 and HLA-DPB1).

[15] Participant must bring fecal sample to Visit 0 in specimen collection container provided at Visit -1.

[16] Participant must bring fecal sample to Visit 109 in specimen collection container provided at Visit 108.

[17] For participants who are rescreened, if the participant completed a protocol biopsy that met eligibility (see Section 4) during their initial screen, an investigator may request that the candidate's data is reviewed by an adjudication committee to determine whether the previously completed biopsy may be used in place of a new screening biopsy (see Section 6.4.1).

[18] Only for recipients of deceased-donor allografts: banked specimen, if available, may be collected from immunosuppression withdrawal initiation to end of study. Banked specimen include cells/samples such as splenocytes, lymph node cells, PBMC, and/or whole blood.

[19] Applicable for participants on CNI and prednisone only. Follow section 3.1.3.2 for withdrawal from prednisone or equivalent.

[20] Complete as applicable. Exact schedule for prednisone withdrawal may be varied at the discretion of the investigator.

[21] Applicable for participants on CNI and a mycophenolate compound only. Follow section 3.1.3.3 for withdrawal from a mycophenolate compound.

APPENDIX 1B. SCHEDULE OF EVENTS – LIVING DONOR (FOR RECIPIENTS OF LIVING DONOR ALLOGRAFTS ONLY)

Visit Number	-1	0
General Assessments		
Informed consent	X	
Medical and demographic history	X	
Concomitant medication review	X	
Local Clinical Laboratory Assessments		
CBC w/differential	X	X[1]
Comprehensive metabolic panel	X	
Mechanistic Laboratory Assessments[2]		
HLA Typing[3]	X	
Frozen PBMC collection		X

[1] CBC w/differential does not need to be repeated if mechanistic laboratory assessments are collected on the same day as the Visit -1 CBC w/differential assessment.

[2] Mechanistic laboratory assessments may occur on Visit -1 if all other visit assessments are completed and the resulted clinical laboratory assessments confirm the donor participant's eligibility (section 4.1.2 and 4.2.2).

[3] Includes medium and/or high resolution molecular HLA typing and DNA (HLA-A, HLA-B, HLA-C, HLA-DRB1, HLA-DRB3-5, HLA-DQA1, HLA-DQB1 and HLA-DPB1).

APPENDIX 2. SCHEDULE OF EVENTS – POST WITHDRAWAL FOLLOW-UP

IMPORTANT NOTE: For the duration of the COVID-19 pandemic, follow the COVID-19 Modified Appendix 2-C instead (per Section 6.2).

IS Withdrawal Follow-up Weeks[1]	0	13	26	52	78	104	130	156
Visit Number[2,3]	201[4]	202	203	204	205	206	207	208
General Assessments								
Limited physical exam	X	X	X	X	X	X	X	X
Vital signs including weight	X	X	X	X	X	X	X	X
Quality of Life Questionnaires	X		X	X	X	X	X	X
Status Change since prior visit[5]	X	X	X	X	X	X	X	X
Telephone consultation[6]	X----------							----------X
ECG	Prior to restarting tacrolimus[7]							
Local Clinical Laboratory Assessments								
LFTs[8]	Every 4 weeks		Every 8 weeks		Every 12 weeks			
CBC w/differential	X	X	X	X	X	X	X	X
Comprehensive metabolic panel, includes LFTs	X	X	X	X	X	X	X	X
Liver biopsy				X				X
Mechanistic Laboratory Assessments								
Frozen PBMC collection	X		X	X		X		X
Serum collection	X		X	X		X		X
Whole blood collection	X		X	X		X		X
Urine pellet collection	X		X	X		X		X
Fecal collection[9]	X		X	X		X		X
Liver biopsy collection				X				X

[1] The assessments outlined in Appendix 2 should be completed for all participants who initiated and successfully completed immunosuppression withdrawal. Follow-up should begin within 2 weeks of the date of completion of the participant's last taper level (see section 3.1.3).

[2] If a for-cause liver biopsy is needed, follow all assessments specified in Appendix 5. If the for-cause biopsy occurs within 8 weeks prior to a scheduled protocol visit, complete all clinical and mechanistic assessments listed in Appendix 5 at the time of the for-cause biopsy. The for-cause biopsy visit will take the place of the scheduled protocol visit.

[3] If a participant restarts immunosuppression after successfully completing immunosuppression withdrawal, follow the assessments listed in Appendix 4. **Appendix 2 visits to be completed in addition and concurrently with Appendix 4.**

[4] For any participant requesting early termination from the study, follow all assessments specified here, Visit 201.

[5] Includes assessment of change in adverse events and concomitant medications. Changes should be recorded in eCRFs as appropriate.

[6] Complete following the performance of each liver function test assessment done outside of a transplant center clinical visit; AEs and concomitant medications to be reviewed.

[7] For participants who need to restart tacrolimus for biopsy-confirmed or presumed acute rejection (see section 6.8). A follow-up ECG will be performed within 7 days after tacrolimus has been restarted. ECG findings will be monitored per the site's standard of care until the participant is taking a stable dose of tacrolimus. Evidence of a prolonged QTc interval will be appropriately evaluated and treated.

[8] If participant experiences a rejection episode, complete liver function tests per standard of care until resolution as determined by the investigator. Record all liver function tests performed during a rejection episode in EDC.

[9] Participant must bring fecal sample in a specimen collection container provided at the previous protocol visit.

APPENDIX 2-C. (COVID-19 MODIFIED) SCHEDULE OF EVENTS – POST WITHDRAWAL FOLLOW-UP

IS Withdrawal Follow-up Weeks[1]	0	13	26	52	78	104	130	156
Visit Number[2]	201[3]	202	203	204	205	206	207	208
General Assessments								
Limited physical exam								X
Vital signs including weight								X
Quality of Life Questionnaires[4]				X	X	X	X	X
Status Change since prior visit								X
Telephone consultation[5]	X----------							----------X
ECG	Prior to restarting tacrolimus[6]							
Local Clinical Laboratory Assessments								
LFTs[7]			Every 8 weeks		Every 12 weeks			
CBC w/differential			X	X	X	X	X	X
Comprehensive metabolic panel, includes LFTs			X	X	X	X	X	X
Liver biopsy								X
Mechanistic Laboratory Assessments								
Frozen PBMC collection								X
Serum collection								X
Whole blood collection								X
Urine pellet collection								X
Fecal collection								X
Liver biopsy collection								X

[1] The assessments outlined in Appendix 2 should be completed for all participants who initiated and successfully completed immunosuppression withdrawal. Follow-up should begin within 2 weeks of the date of completion of the participant's last taper level (see section 3.1.3).

[2] If a for-cause liver biopsy is needed, follow Appendix 5.

[3] For any participant requesting early termination from the study, collect the following assessments if possible: telephone consultation with AE and concomitant medication update, CBC with diff, and comprehensive metabolic panel including LFTs.

[4] Complete Quality of Life assessment by mail.

[5] Complete following the performance of each liver function test assessment done outside of a transplant center clinical visit; AEs and concomitant medications to be reviewed.

[6] For participants who need to restart tacrolimus for biopsy-confirmed or presumed acute rejection (see section 6.8). A follow-up ECG will be performed within 7 days after tacrolimus has been restarted. ECG findings will be monitored per the site's standard of care until the participant is taking a stable dose of tacrolimus. Evidence of a prolonged QTc interval will be appropriately evaluated and treated.

[7] If participant experiences a rejection episode, complete liver function tests per standard of care until resolution as determined by the investigator. Record all liver function tests performed during a rejection episode in EDC.

APPENDIX 3. SCHEDULE OF EVENTS – SAFETY FOLLOW-UP

IMPORTANT NOTE: For the duration of the COVID-19 pandemic, follow the COVID-19 Modified Appendix 3-C instead.

Safety Follow-up Weeks[1]	0	13	26	52	104	156
Visit Number[2]	**301[3,4]**	**302**	**303**	**304**	**305**	**306**
General Assessments						
Limited Physical Exam	X	X	X	X	X	X
Vital signed including weight	X	X	X	X	X	X
Quality of Life Questionnaires	X		X	X	X	X
Status Change since prior visit[5]	X	X	X	X	X	X
Telephone consultation[6]	X----------					----------X
ECG	Prior to restarting tacrolimus if on MMF monotherapy[7]					
Local Clinical Laboratory Assessments						
LFTs[8]	Every 4 weeks		Every 8 weeks		Every 12 weeks	
CBC w/differential	X	X	X	X	X	X
Comprehensive metabolic panel, includes LFTs	X	X	X	X	X	X
Mechanistic Laboratory Assessments						
Frozen PBMC collection	X		X	X	X	X
Serum collection	X		X	X	X	X
Whole blood collection	X		X	X	X	X
Urine pellet collection	X		X	X	X	X
Fecal collection	X		X	X	X	X

[1] The assessments outlined in Appendix 3 should be completed for all participants who initiated but did not complete immunosuppression withdrawal. Follow-up should begin within 2 weeks of the date of immunosuppression withdrawal failure (see section 3.1.3.5).

[2] If a for-cause liver biopsy is needed, follow all assessments specified in Appendix 5. If the for-cause biopsy occurs within 8 weeks prior to a scheduled protocol visit, complete all clinical and mechanistic assessments listed in Appendix 5 at the time of the for-cause biopsy. The for-cause biopsy visit will take the place of the scheduled protocol visit.

[3] If a for-cause liver biopsy visit took place within 8 weeks of visit 301, all mechanistic assessments listed in Appendix 5 at the time of the for-cause biopsy visit should be completed in lieu of mechanistic assessments listed for visit 301.

[4] For any participant requesting early termination from the study, follow all assessments specified here, Visit 301.

[5] Includes assessment of change in adverse events and concomitant medications. Changes should be recorded in eCRFs as appropriate.

[6] Complete following the performance of each liver function test assessment done outside of a transplant center clinical visit; AEs and concomitant medications to be reviewed.

[7] For participants on MMF monotherapy who need to restart tacrolimus for biopsy-confirmed rejection or presumed acute rejection (see section 6.8). A follow-up ECG will be performed within 7 days after tacrolimus has been restarted. ECG findings will be monitored per the site's standard of care until the subject is taking a stable dose of tacrolimus. Evidence of a prolonged QTc interval will be appropriately evaluated and treated.

[8] If participant experiences a rejection episode, complete liver function tests per standard of care until resolution as determined by the investigator. Record all liver function tests performed during a rejection episode in EDC.

APPENDIX 3-C. (COVID-19 MODIFIED) SCHEDULE OF EVENTS – SAFETY FOLLOW-UP

Safety Follow-up Weeks[1]	0	13	26	52	104	156
Visit Number[2]	301[3]	302	303	304	305	306
General Assessments						
Quality of Life Questionnaires[4]					X	X
Telephone consultation[5]	X----------					----------X
ECG	Prior to restarting tacrolimus if on MMF monotherapy[6,7]					
Local Clinical Laboratory Assessments						
LFTs[8]					Every 12 weeks	
CBC w/differential					X	X
Comprehensive metabolic panel, includes LFTs					X	X

[1] The assessments outlined in Appendix 3 should be completed for all participants who initiated but did not complete immunosuppression withdrawal. Follow-up should begin within 2 weeks of the date of immunosuppression withdrawal failure (see section 3.1.3.5).

[2] If a for-cause liver biopsy is needed, follow Appendix 5.

[3] For any participant requesting early termination from the study, collect the following assessments if possible: telephone consultation with AE and concomitant medication update, CBC with diff, and comprehensive metabolic panel including LFTs.

[4] Complete Quality of Life assessment by mail.

[5] Complete following the performance of each liver function test assessment done outside of a transplant center clinical visit; AEs and concomitant medications to be reviewed.

[6] Perform locally or at study site, as necessary.

[7] For participants on MMF monotherapy who need to restart tacrolimus for biopsy-confirmed rejection or presumed acute rejection (see section 6.8). A follow-up ECG will be performed within 7 days after tacrolimus has been restarted. ECG findings will be monitored per the site's standard of care until the subject is taking a stable dose of tacrolimus. Evidence of a prolonged QTc interval will be appropriately evaluated and treated.

[8] If participant experiences a rejection episode, complete liver function tests per standard of care until resolution as determined by the investigator. Record all liver function tests performed during a rejection episode in EDC.

APPENDIX 4. SCHEDULE OF EVENTS—ADDITIONAL, CONCURRENT FOLLOW-UP FOR PARTICIPANTS WHO RESTART IMMUNOSUPPRESSION AFTER SUCCESSFUL COMPLETION OF IMMUNOSUPPRESSION WITHDRAWAL - *THESE VISITS ARE TO OCCUR CONCURRENTLY WITH THE VISITS OUTLINED IN APPENDIX 2*

IMPORTANT NOTE: For the duration of the COVID-19 pandemic, **do not follow** this Appendix.

Additional Follow-Up Weeks[1]	26
Visit Number[2,3]	RIS6[4]
General Assessments	
Limited physical exam	X
Vital signs including weight	X
Status Change since prior visit[5]	X
Local Clinical Laboratory Assessments	
CBC w/differential	X
Comprehensive metabolic panel, includes LFTs	X
Mechanistic Laboratory Assessments	
Frozen PBMC collection	X
Serum collection	X
Whole blood collection	X
Urine pellet collection	X
Fecal collection	X

[1] The assessments outlined in Appendix 4 should be completed for all participants who restart immunosuppression after successfully completing immunosuppression withdrawal. **Appendix 2 visits to be completed in addition and concurrently with Appendix 4.**

[2] If the visit occurs within 1 month (4 weeks) of a scheduled Appendix 2 visit, complete the Appendix 2 visit but complete all mechanistic laboratory assessments required by the Appendix 4 visit in lieu of conducting a separate Appendix 4 visit.

[3] If a for-cause liver biopsy is needed, follow all assessments specified in Appendix 5. If the for-cause biopsy occurs within 8 weeks prior to a scheduled protocol visit, complete all clinical and mechanistic assessments listed in Appendix 5 at the time of the for-cause biopsy. The for-cause biopsy visit will take the place of the scheduled protocol visit.

[4] Visit to be completed 26 weeks after immunosuppression restart date.

[5] Includes assessment of change in adverse events and concomitant medications. Changes should be recorded in eCRFs as appropriate.

APPENDIX 5. SCHEDULE OF EVENTS – FOR-CAUSE BIOPSY

IMPORTANT NOTE: For the duration of the COVID-19 pandemic, follow the COVID-19 Modified Appendix 5-C instead.

Visit Number	FCB[1]	PBV[2]
General Assessments		
Limited physical exam	X[3]	X
Vital signs including weight	X	X
Status Change since prior visit[4]	X	X
ECG	Prior to restarting tacrolimus[5]	
Local Clinical Laboratory Assessments		
CBC w/differential	X	X
Comprehensive metabolic panel, includes LFTs	X	X
Liver biopsy	X	
Mechanistic Laboratory Assessments		
Frozen PBMC collection	X	
Serum collection	X	
Whole blood collection	X	
Urine pellet collection	X	
Fecal collection	X	
Liver biopsy collection	X	

[2] Participants in post withdrawal follow-up (Appendix 2) or safety follow-up (Appendix 3) who experience a rejection episode will have transplant center visits every 13 weeks until liver function test normalization. If post rejection visits are needed outside of the scheduled Appendix 2 or Appendix 3 visits, follow all assessments specified here, Visit PBV, for those visits.

[3] Complete only if for-cause biopsy visit occurs within 8 weeks prior to a scheduled protocol visit and replaces that scheduled visit.

[4] Includes assessment of change in adverse events and concomitant medications. Changes should be recorded in eCRFs as appropriate.

[5] For participants who completed CNI withdrawal and need to restart tacrolimus for biopsy confirmed or presumed acute rejection, complete the ECG assessment (also noted in Appendix 2 & Appendix 3) prior to restarting tacrolimus (section 6.8). A follow-up ECG will be performed within 7 days after tacrolimus has been restarted. ECG findings will be monitored per the site's standard of care until the subject is taking a stable dose of tacrolimus. Evidence of a prolonged QTc interval will be appropriately evaluated and treated.

APPENDIX 5-C. (COVID-19 MODIFIED) SCHEDULE OF EVENTS – FOR-CAUSE BIOPSY

Visit Number	FCB[1]	PBV[1]
General Assessments		
Limited physical exam	X[2]	X[2]
Vital signs including weight	X	X
Status Change since prior visit[3]	X	X
ECG	Prior to restarting tacrolimus[4,5]	
Local Clinical Laboratory Assessments		
CBC w/differential	X	X
Comprehensive metabolic panel, includes LFTs	X	X
Liver biopsy	X	

[1] Participants in post withdrawal follow-up (Appendix 2) or safety follow-up (Appendix 3) who experience a rejection episode will have transplant center visits per local standard of care.

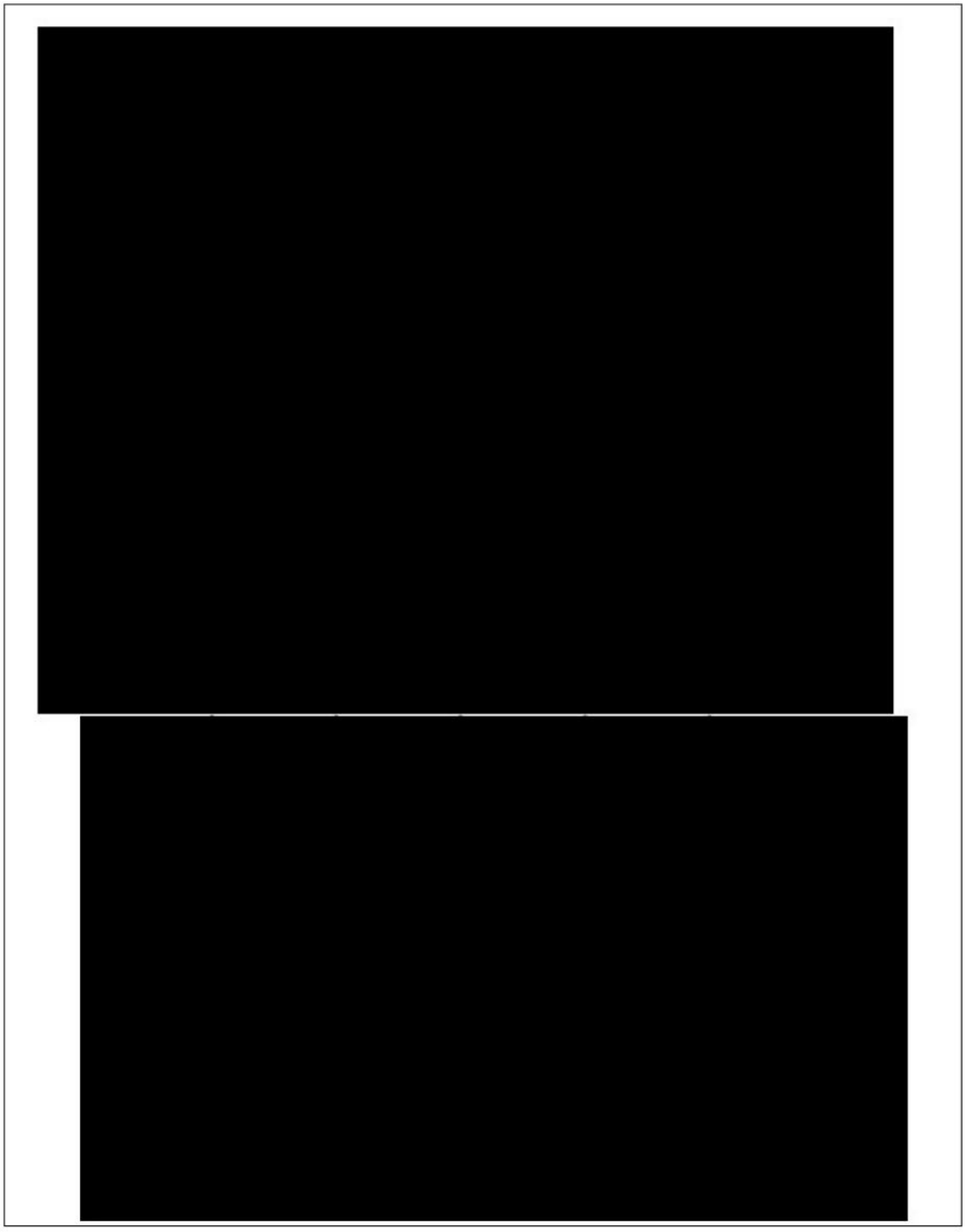
[2] If subject is seen locally, a limited physical exam will be performed per standard of care, but results will not be recorded.

[3] Includes in person or telephone assessment of change in adverse events and concomitant medications. Changes should be recorded in eCRFs as appropriate.

[4] Perform locally or at study site, as necessary.

[5] For participants who completed CNI withdrawal and need to restart tacrolimus for biopsy confirmed or presumed acute rejection, complete the ECG assessment (also noted in Appendix 2 & Appendix 3) prior to restarting tacrolimus (section 6.8). A follow-up ECG will be performed within 7 days after tacrolimus has been restarted. ECG findings will be monitored per the site's standard of care until the subject is taking a stable dose of tacrolimus. Evidence of a prolonged QTc interval will be appropriately evaluated and treated.

APPENDIX 6. SF-36 HEALTH SURVEY



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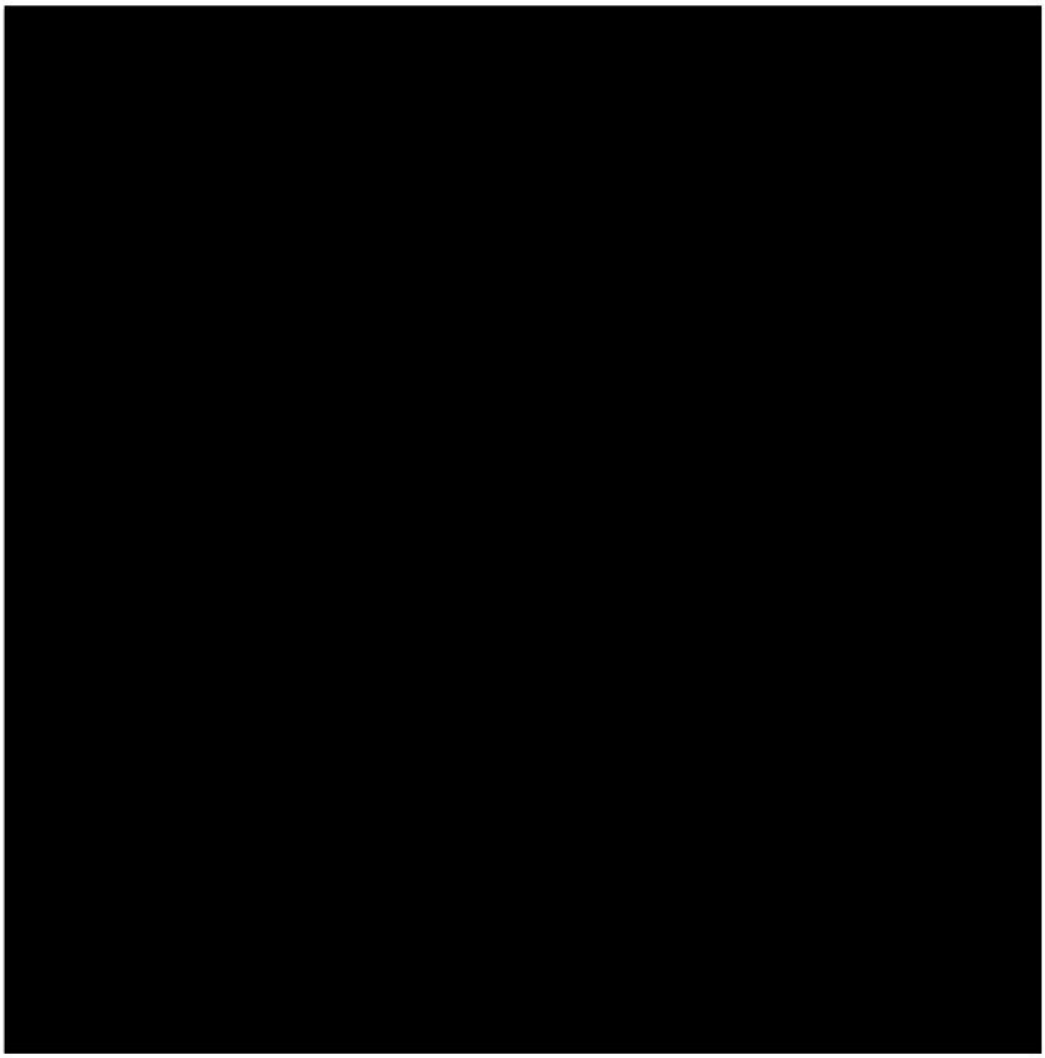

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THANK YOU FOR COMPLETING THESE QUESTIONS!

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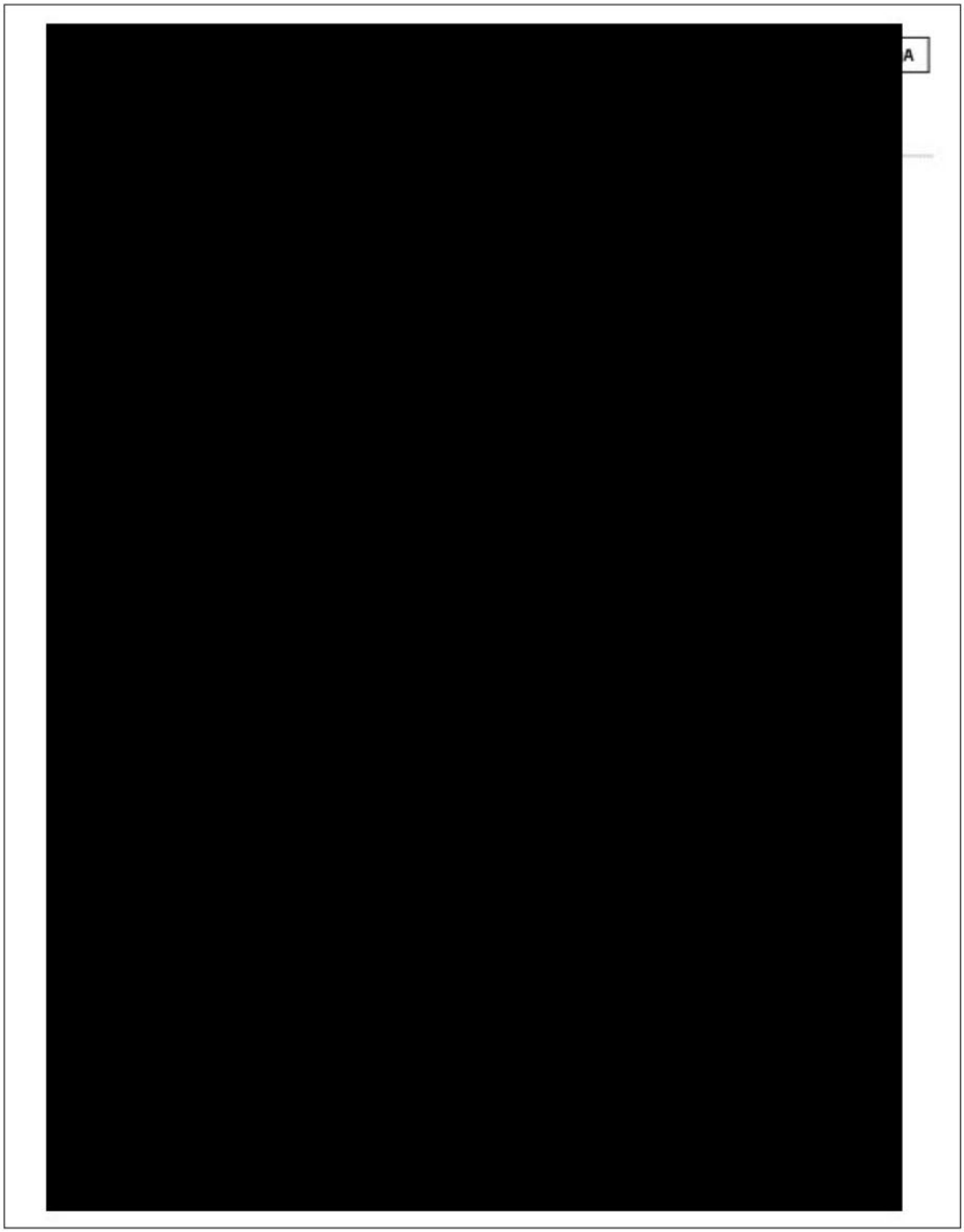
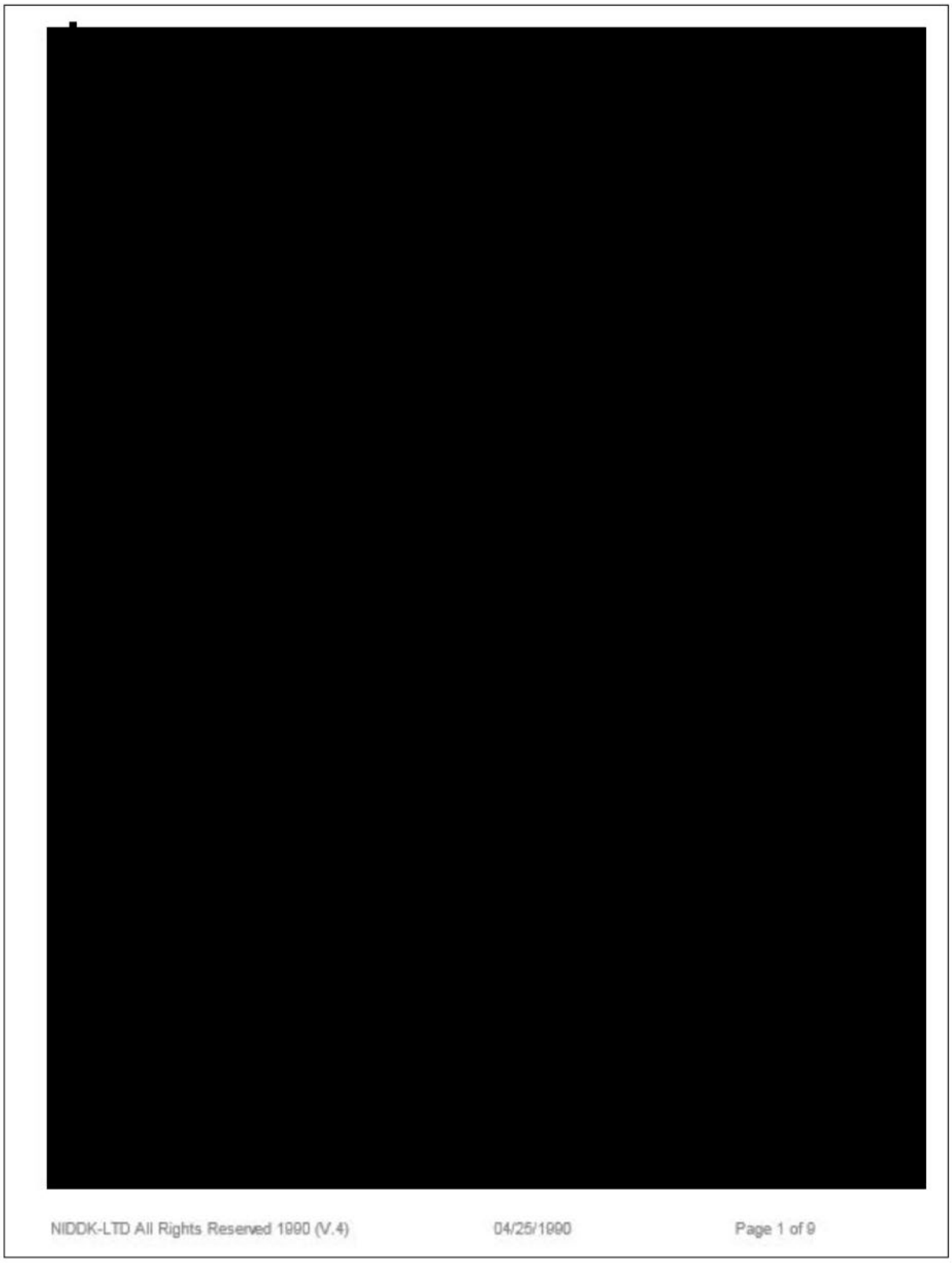
APPENDIX 7. NIDDK LIVER TRANSPLANTATION DATABASE: QUALITY OF LIFE FORM (ADULTS)

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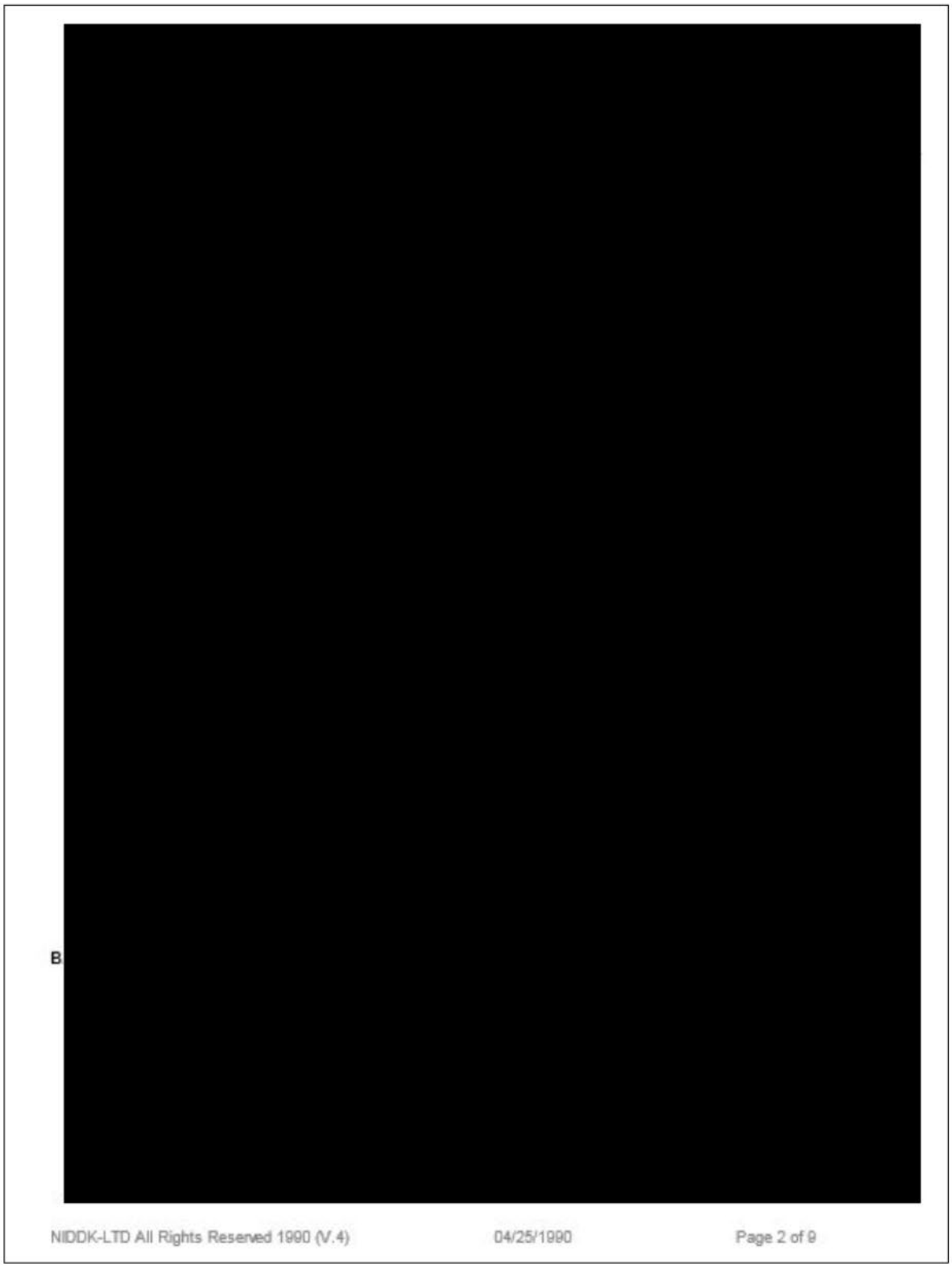
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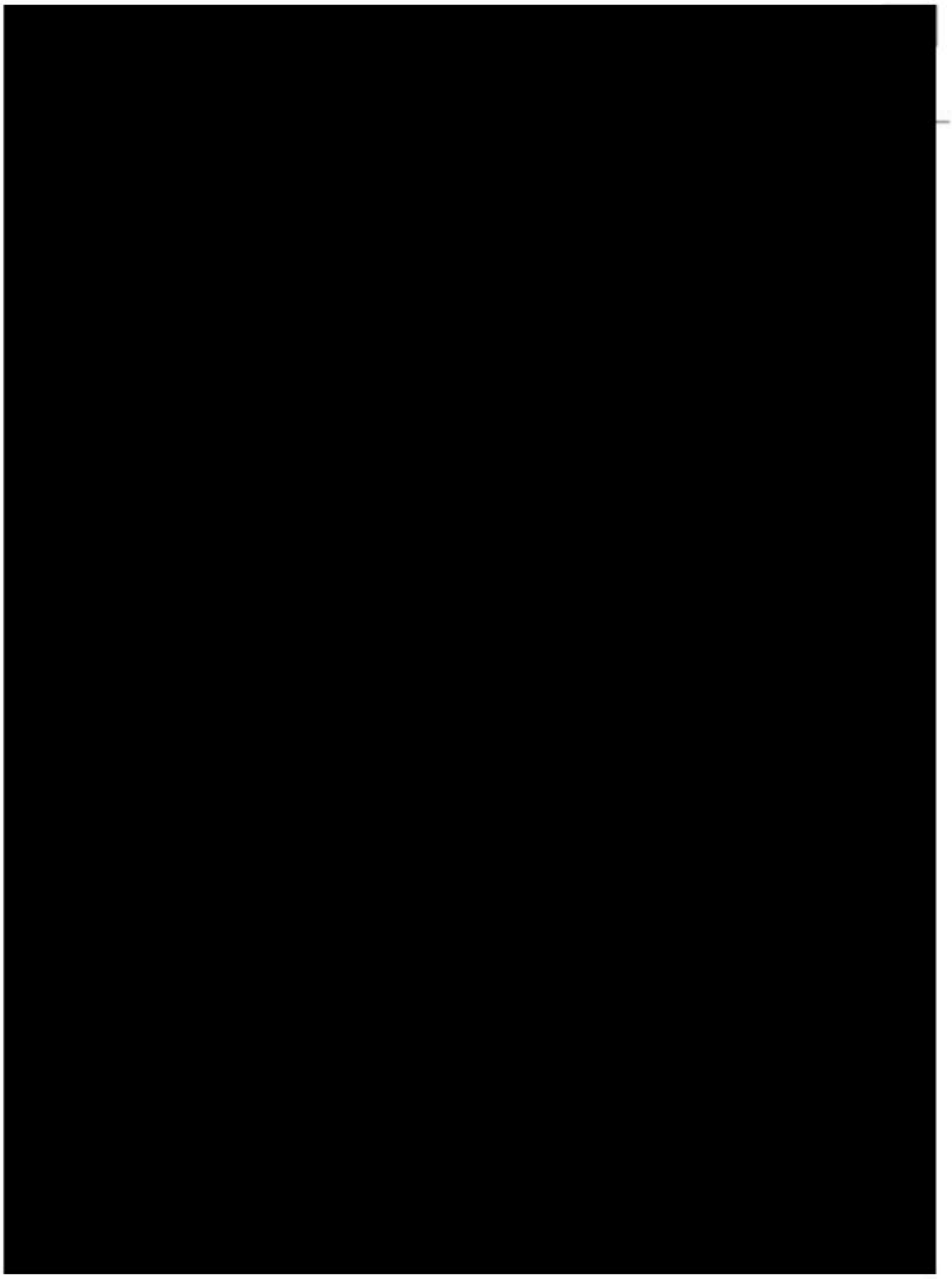

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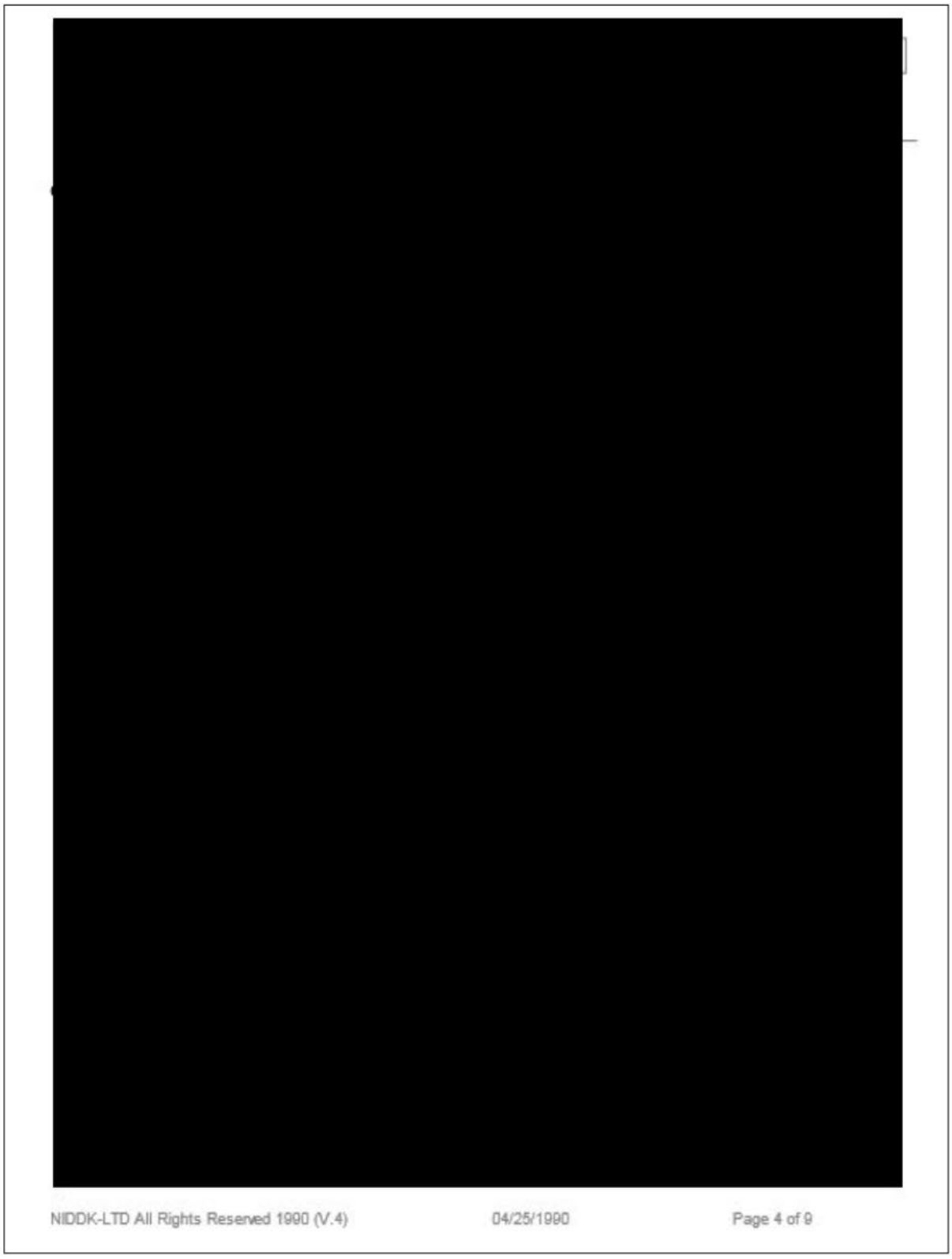
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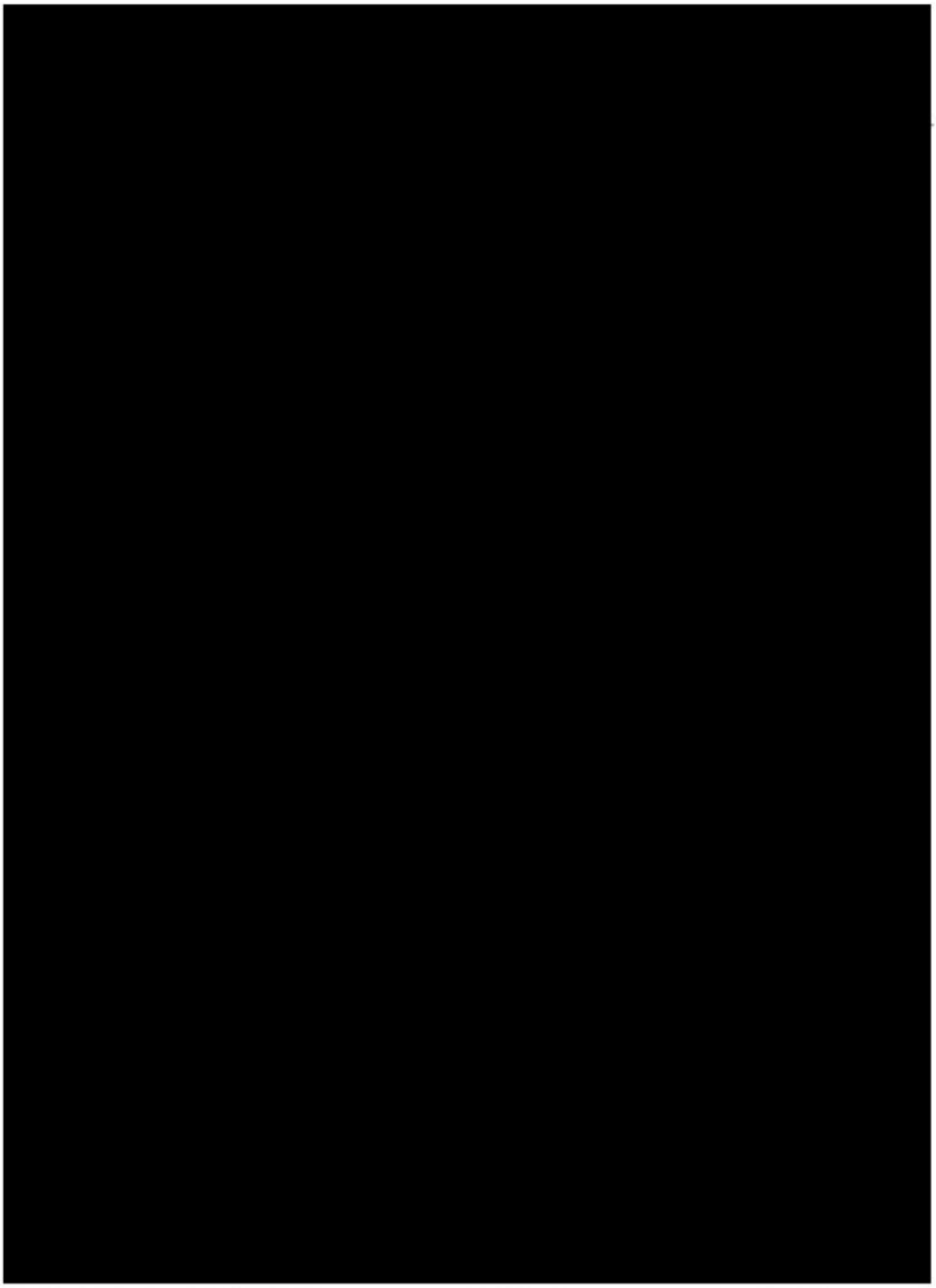

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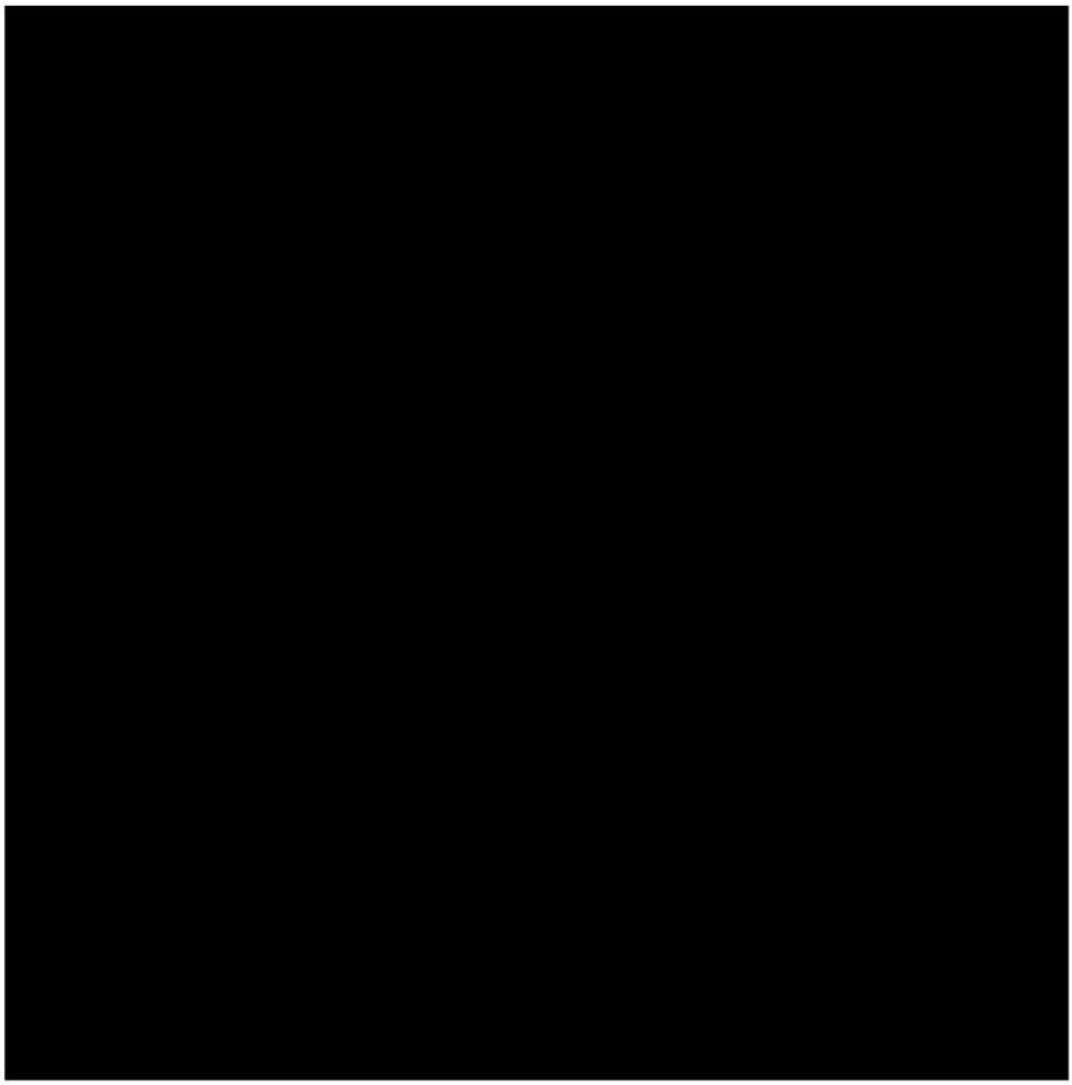

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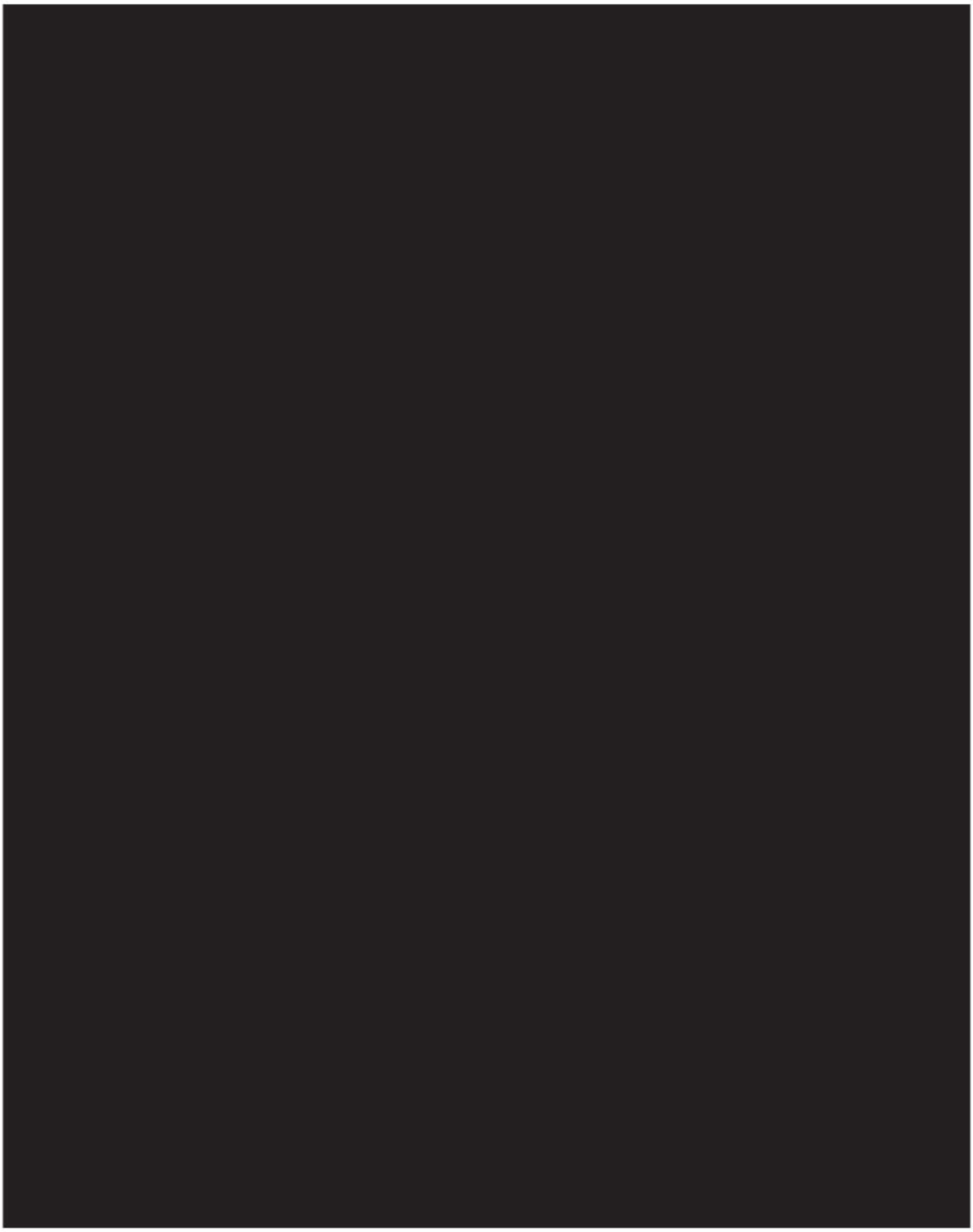

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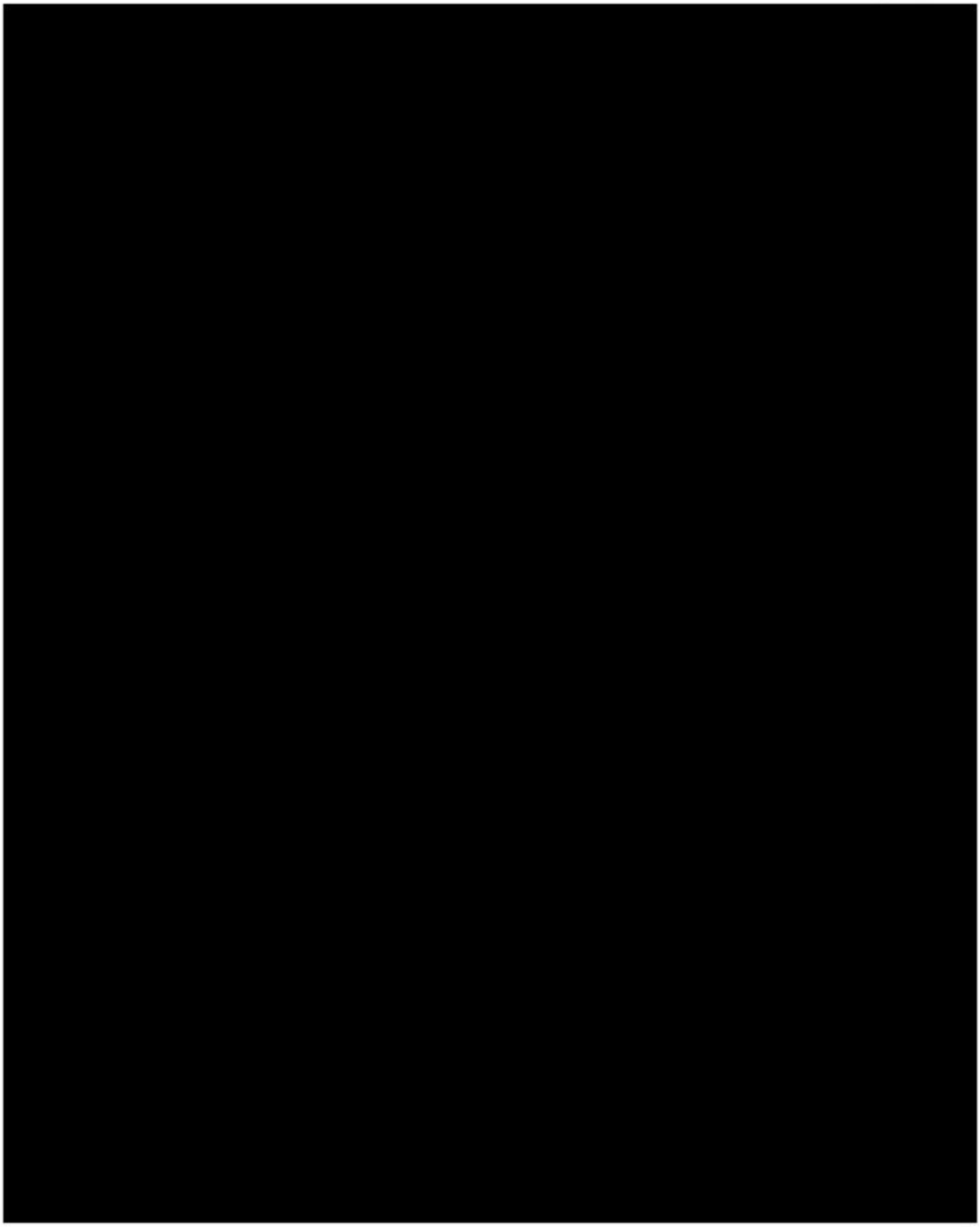

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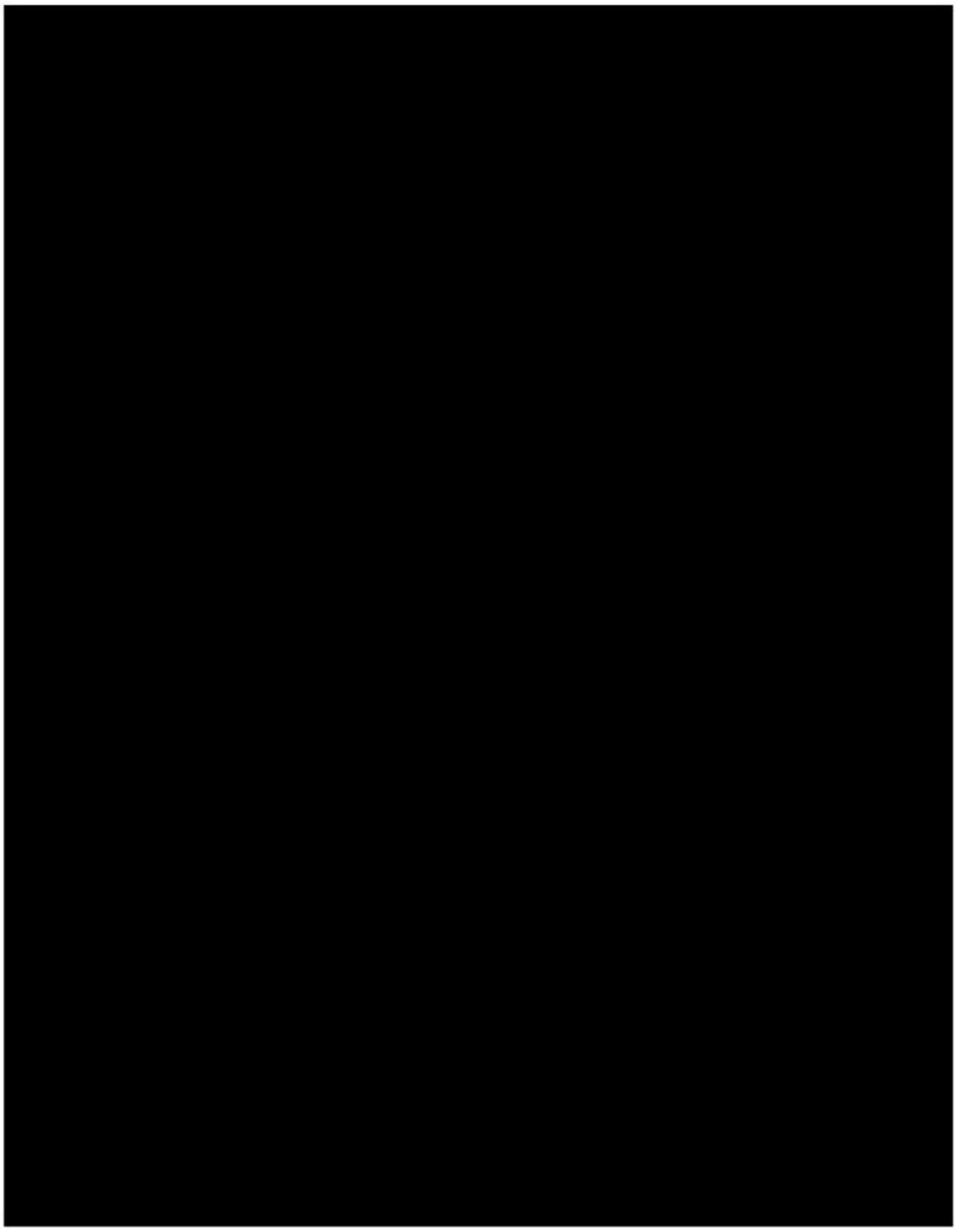

Thank you for spending the time to fill out this form.

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