STANDARD OPERATING PROCEDURE

Pre-clinical Consortium on Combination Therapies for Type I Diabetes

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Title: Determination of optimal IL-2 dosing for maintaining antigen-specific Tregs in new onset diabetic NOD mice.

INTRODUCTION/PURPOSE

Previous pre-clinical and clinical data have suggested that Tregs may provide an effective therapy for the treatment of type 1 diabetes (T1D) (1,2). Similarly, low dose IL-2 has had significant impact on Tregs and has been shown to be efficacious in treating NOD mice (3). Low dose IL-2 is currently being examined in individuals with new onset T1D (4). Clinical studies have suggested that *ex vivo* expanded Tregs can have a short half-life *in vivo*, perhaps due to a shortage of IL-2 (5). Current results suggest that infused Tregs in humans have a multiphase decay pattern. The infused Tregs take a few days to enter into circulation, after which they exhibit a precipitous drop (loss of ~75% over 2-3 weeks). Following this initial drop the remaining cells seem to stably persist for a year. The mechanisms behind these dynamics are uncertain, and in question is whether co-administration with IL-2 might be able to prevent or reduce the early decline in cell numbers. If IL-2 does not promote or stabilize the antigen-specific portion of the response, this combination might not be worth pursuing in people.

The pilot study described here is a short-term dosing study to determine, based on mechanistic analyses, the optimal dose of IL-2/anti-IL-2 complex that will be combined with one of two BDC 2.5 Treg doses in a therapeutic study. The goal of the larger therapeutic study is to ascertain whether the combination of polyclonal regulatory T cells (Tregs) plus IL-2 is additive/synergistic with Treg therapy for reversing new onset disease in non-obese diabetic (NOD) mice. The outcome of these studies will determine the applicability of the approach to human clinical intervention. The dosing and pharmacodynamics of this therapeutic combination also will be evaluated by the Consortium.

ENDPOINTS

The primary endpoint of this study is to determine whether IL-2 treatment enables the ex vivo expanded Tregs to persist in a recipient. Histologic and flow cytometric analyses will seek to determine whether the Tregs are still there, whether they have expanded, and whether they have retained their Treg (FoxP3+) phenotype.

The secondary endpoint of this study is to determine whether Treg plus IL-2 therapy results in disease reversal in new onset NODs. Blood glucose data will be collected to address this question.

The third question to be examined in this study is whether these Tregs downregulate Teffectors. To answer this question, flow cytometric analyses will seek to determine whether the number of Teffectors present and/or their function (IFN-gamma production) are altered by the presence of islet antigen-specific Tregs.

Finally, histologic analyses will seek to determine whether Tregs home to the pancreas.

DEFINITIONS

Diabetes: Type 1 diabetes with blood glucose >= 250 mg/dl.

New onset diabetic: Blood glucose readings of >= 250 mg/dl on two consecutive days.

Reversal of diabetes: Blood glucose readings of < 250 mg/dl for 3 regularly scheduled readings following entry into the study. This definition may need to be revised if it does not permit us to clearly distinguish between spontaneous reversions in the untreated control group versus treated groups.

NOD mice: Female NOD mice between 10 and 26 weeks of age.

Day 1: The day of initiation of treatment. Study enrollment and treatment initiation will begin the same day as the second consecutive confirmatory hyperglycemic reading.

OVERVIEW OF STUDY

The pilot study described here is a short-term dosing study to determine, based on mechanistic analyses, the optimal dose of IL-2/anti-IL-2 complex that will be combined with BDC 2.5 Tregs in a therapeutic study. In this study, NOD mice at disease onset (day 1) will be dosed with 3×10^6 BDC2.5 expanded Thy1.1+ Tregs in the presence of IL-2 or IL-2/anti-IL-2 complexes. Spleen and pancreatic lymph nodes will be evaluated for Treg expansion, activation state and survival of the transferred and endogenous Tregs at 1 week and 1 month. Additional analyses will assess pathogenic immunity, including overall changes in effector T cells (Teff) versus naïve T cells (Tnaive) by flow cytometry. Not all studies will be performed at each site depending on capabilities.

Study Design

This pilot study will establish the IL -2 dosing demonstrating selective, but suboptimal activation of adoptively transferred and endogenous Tregs in new onset NOD mice, based on mechanistic analyses.

Beginning at 10 weeks of age, blood glucose will be monitored three times per week in the morning. Once a mouse registers a blood glucose reading ≥250 mg/dL, diabetes onset will be confirmed by re-measurement of blood glucose levels the next day (day 1). Mice with blood glucose levels greater than or equal to 250 mg/dL for two consecutive days will be considered diabetic and will be entered into a treatment group the same day. Following allocation to a treatment group, blood glucose will be monitored three times per week in the morning. The study will run for 28 days post-T1D onset. Mice will be sacrificed at 6-8 and 28-32 days for endpoint assessments.

This study will use 30 mice in groups of six. Two control groups (saline +/- Tregs) and four experimental groups treated with 0, 12,500 IU Proleukin®, or one of two doses of IL-2/anti-IL-2 complexes. All groups will receive insulin pellet implants on day 1 of study. Serum will be collected on days 1, 7, and 28. Three mice will be sacrificed at one week and three mice will be sacrificed at four weeks for flow cytometry and histology.

Group	Group	n	Insulin Pellet (SC)	BDC2.5 Tregs (RO)	IL-2 (IP)	Day of Sacrifice
1A	No Treg Control	3	Day 1	None	None; control saline only	7
1B	No Treg Control	3	Day 1	None	None; control saline only	28
2A	Tregs	3	Day 1	Day 1 **(3 x 10^6/dose)**	None; control saline only	7
2B	Tregs	3	Day 1	Day 1 **(3 x 10^6/dose)**	None; control saline only	28
3A	Tregs + IL-2	3	Day 1	Day 1 **(3 x 10^6/dose)**	Days 1, 2, 3, 4, & 5 (12,500 IU per day)	7
3B	Tregs + IL-2	3	Day 1	Day 1 **(3 x 10^6/dose)**	Days 1, 2, 3, 4, & 5 (12,500 IU per day)	28
4A	Tregs +IL-2/anti-IL-2 complex I	3	Day 1	Day 1 **(3 x 10^6/dose)**	Days 1, 3, & 5 (5 µg IL-2 + 0.5 µg anti-IL-2 per Tx)	7
4B	Tregs +IL-2/anti-IL-2 complex I	3	Day 1	Day 1 **(3 x 10^6/dose)**	Days 1, 3, & 5 (5 µg IL-2 + 0.5 µg anti-IL-2 per Tx)	28
5A	Tregs +IL-2/anti-IL-2 complex II	3	Day 1	Day 1 **(3 x 10^6/dose)**	Days 1, 3, & 5 (2.5 µg IL-2 + 0.25 µg anti-IL-2 per Tx)	7
5B	Tregs +IL-2/anti-IL-2 complex II	3	Day 1	Day 1 **(3 x 10^6/dose)**	Days 1, 3, & 5 (2.5 µg IL-2 + 0.25 µg anti-IL-2 per Tx)	28

Enrollment Schedule

Following the second consecutive confirmatory hyperglycemic reading, mice will enrolled in the study according to a staggered and fixed enrollment schedule (1st diabetic animal into Group 1A, 2nd diabetic animal into Group 1B, etc.) Data will not be collected randomly, but according to a predetermined (protocol-specific) schedule. Please refer to the attached allocation spreadsheet for order of enrollment.

Criteria for Removal from Study

Mice may be removed from the study for humane reasons. Animals will be removed from the studies and euthanized according to IACUC protocol under the following conditions:

- At any time, if a mouse's weight drops below that permitted by each site's IACUC (or 20% weight loss if IACUC does not have a weight loss restriction).
- Before day 30 of study, if there are 3 consecutive regularly scheduled maximum blood glucose measurements as permitted by each site's IACUC (or 3 consecutive blood glucose measurements

exceeding the upper limit of detection for a meter if IACUC does not have a guideline for blood glucose measurements).
- The mouse exhibits signs of significant illness related distress (shaking, hunched, non-ambulatory, cool to the touch, etc.)

If possible, endpoint assessments should be performed at time of sacrifice.

PROCEDURES

Blood Glucose Monitoring

Female NOD mice between 10 and 26 weeks of age will be monitored for diabetes onset and the readings documented. Blood glucose monitoring will be performed every two to three days in the morning. The spacing between monitoring days should be such that there is never more than two days that pass without blood glucose measurements. For example, monitoring on Friday and Monday is acceptable, but monitoring on Friday and Tuesday would result in too many days between blood glucose measurements.

Day 0 of treatment is defined as the day of the first of the two consecutive blood glucose readings.

Following allocation to a treatment group, blood glucose will be monitored every two to three days in the morning. Spacing between blood glucose measurements should be no more than three days.

Procedure for Blood Glucose Monitoring: The tip of the tail is cut with scissors and a droplet of blood is placed into the slot on the end of a test strip inserted into a portable blood glucose monitor. The value is recorded.

Blood Glucose Monitoring Equipment: Each site should use the same model of blood glucose monitor during this study. The One Touch® Ultra 2® blood glucose meter and One Touch® Ultra® Blue test strips (LifeScan, Inc.) should be used for this study. Prior to blood glucose testing, proper function of the meter and strips should be confirmed by testing One Touch® Ultra® Control Solution according to manufacturer's instructions. Proper meter and strip function should be documented and linked to each episode of blood glucose monitoring.

Body Weight Measurements: Body weights will be measured weekly and documented.

Identification of Animals Enrolled in Study

In order to prevent duplication in animal identifiers between sites, new onset diabetics should be assigned a unique identifier upon allocation to a study group. This unique identifier should have the following format: Site – ITN Project # - Treatment Group – Animal ID. The variables that may be used in each field are as follows:

- Site = F (Florida), Y (Yale), or C (Colorado)
- ITN Project # = 5P (Tregs plus IL-2 pilot study)
- Treatment Group = 1, 2, 3, etc
- Animal ID = animal # (microchip #, ear tag, tattoo, etc)

For example, animal F-5P-1-1585 is from the Florida site during the project 5 pilot, received treatment 1, and its ID is 1585.

Implantation of Insulin Pellets

Diabetic mice in all groups, will receive one LinBit sustained release insulin pellet implant (Linshin Canada, Inc., Toronto, Ontario, Canada) the same day as the second consecutive confirmatory hyperglycemic reading (day 1). Insulin pellet implantation should be performed according to IACUC approved protocol. The recommended procedure for the implantation of an insulin pellet (LinShin Canada, Inc.) is described in the following paragraphs, however, it is understood that each site's veterinary staff and IACUC may require that this method be modified to their specifications.

Procedure for Implantation of Insulin Pellets

Anesthesia: Mice should be anesthetized according to approved IACUC protocol. LinShin Canada, Inc., the manufacturer of LinBits, recommends the use of volatile halohydrocarbon liquids or any short-acting anesthetic agents of a few minutes duration, such as isoflurane (6).

Implantation Site: Insulin pellets should be placed subcutaneously under the mid dorsal skin or any other convenient site on the back.

Implantation: Once surgical plane anesthesia is achieved, the surgical site should be prepared according to surgical best practices (7). In brief, the fur over the implantation site is shaved and the surgical site is scrubbed with povidone-iodine (possible option, Povidone Iodine Prep Pads, Fisher Scientific catalog # NC9959102) followed by 70% alcohol (possible option, Fisherbrand Alcohol Prep Pads, Fisher Scientific catalog #06-669-62). The skin over the implantation site is pinched between thumb and index finger. An opening is created in the skin by using a 16 gauge disposable hypodermic needle to pierce the pinched skin. The needle is withdrawn. A 12-gauge trocar (Linshin catalog # G12-SS) is briefly immersed in a diluted (~2%) solution of povidone-iodine and pushed through the opening to a length of at least 1.5 cm. The pellet (Bio-erodible LinBit, Linshin Canada, Inc., Toronto, ON) is briefly immersed (20 sec) in the ~2% solution of povidone-iodine and inserted into the proximal end of the trocar. The stylet is immersed in the ~2% povidone-iodine solution and then it is used to push the implant through the trocar until it exits from the distal end. The wound is closed with a single metal wound clip and a drop of 10% povidone-iodine solution is placed over the skin opening. The wound clip is removed 8-10 days after surgery.

Therapies

Insulin Pellet: Diabetic mice in all groups, will receive one LinBit sustained release insulin pellet implant (Linshin Canada, Inc., Toronto, Ontario, Canada) the same day as the second consecutive confirmatory hyperglycemic reading (day 1).

BDC2.5 Tregs: On day 1 of the study, mice from groups 2-5 will receive a retro-orbital injection of 3×10^6 live, Treg cells in a volume of 0.1 ml. Number of cells and viability of the injected Tregs should be documented.

BDC2.5 Tregs will be generated in the laboratory of Jeff Bluestone and distributed frozen to all sites involved in the study. BDC2.5 Tregs will be isolated from BDC2.5 transgenic NOD Thy1.1+ mice using

flow cytometric sorting (anti-CD4, anti-CD25 and anti-CD62L). The Tregs will be expanded *ex vivo* using anti-CD3 plus anti-CD28 coated dynal beads plus exogenous IL-2 as described in Tang et al. (1). The cells will be labeled in culture with deuterium as a means of monitoring cell survival and proliferation. After 10-12 days of expansion, the cells will be harvested, counted and frozen at 3.3-6.6 x 10^6 cells per vial.

At time of disease onset, the cells will be thawed by established procedures (see **SOP-ITN5P-002: Thawing Mouse Treg Cell Stocks**), counted and viability determined by the method preferred by each site, and 3 X 10^6 live cells injected via the retro-orbital plexus into mice. Previous studies have demonstrated that these cells (at a concentration of 10 x 10^6) reversed T1D in NOD mice (1). The doses chosen for this study are expected to be suboptimal at reversing diabetes in the NOD.

Saline Control: Mice in groups 1 and 2 will receive 0.1ml volume IP of 0.9% Sodium Chloride Injection, USP on day(s) 1, 3 & 5.

IL-2 Treatments

Proleukin® (aldesleukin): a human recombinant interleukin 2 (IL-2) product; aldesleukin is a highly purified protein with a molecular weight of approximately 15,300 daltons. The chemical name is desalanyl-1, serine-125 human interleukin. Proleukin, a lymphokine, is produced by recombinant DNA technology using a genetically engineered *E. coli* strain containing an analog of the human IL-2 gene. Genetic engineering techniques were used to modify the human IL-2 gene, and the resulting expression clone encodes a modified human IL-2. This recombinant form differs from native IL-2 in the following ways: a) Proleukin is not glycosylated because it is derived from *E. coli*; b) the molecule has no N-terminal alanine; the codon for this amino acid was deleted during the genetic engineering procedure; c) the molecule has serine substituted for cysteine at amino acid position 125; this was accomplished by site specific manipulation during the genetic engineering procedure; and d) the aggregation state of Proleukin is likely to be different from that of native IL-2.

Group 3 mice will receive 12,500 IU of Proleukin on days 1-5 in a volume of 0.1 ml. This dose is suboptimal at reversing diabetes in NOD mice (3). The diluent for IL-2 is sterile Phosphate Buffered Saline (PBS) without Mg2+/Ca2+ (such as PBS, pH 7.4 Invitrogen/Life Technologies Catalog #10010-02).

IL-2/anti-IL-2 complexes: On days 1, 3, and 5, mice in groups 4 and 5 will receive IP injections of different doses of IL-2/anti-IL-2 complexes comprised of recombinant mouse IL-2 (eBioscience) and the anti-IL-2 antibody (clone JES6-1A12, R&D Systems).

Group 4 mice will receive complexes consisting of 5.0 μg IL-2 and 0.5 μg anti-IL-2 IP in a volume of 100 ul.

Group 5 mice will receive complexes consisting of 2.5 μg IL-2 and 0.25 μg anti-IL-2 IP in a volume of 100 ul.

Handling and Storage of Reagents

Linβit® sustained release, bio-erodible, insulin implants: LinBits consist of insulin in micro-recrystallized palmitic acid. They are provided as non-sterile, white, 2 mm x 3 mm pellets. Recommended storage is at room temperature (25°C). Avoid excessive heat. Protect from freezing.

BDC2.5 T regulatory cells: BDC2.5 Tregs will be generated in the laboratory of Jeff Bluestone and vials consisting of 3-6 x 10^6 cells will be distributed frozen to all sites involved in the study. Upon receipt of the cells, determine whether cells thawed during shipment and store in liquid nitrogen until needed.

To prepare the reagent for administration, thaw one vial of 3.3 x 10^6 cells per mouse to be treated according to the instructions detailed in **SOP-ITN5P-002: Thawing Mouse Treg Cell Stocks.** Cell counts and viability will be determined by the method preferred by each site. Centrifuge the cells at 350-500 x g, decant the supernatant, and dilute the cells to 30 x 10^6 cells/ml in sterile Phosphate Buffered Saline (PBS) without Mg2+/Ca2+ (such as PBS, pH 7.4 Invitrogen/Life Technologies Catalog #10010-02). Inject 100 µl of this cell suspension via the retro-orbital plexus according to your site's approved IACUC protocol.

0.9% Sodium Chloride Injection, USP: Sodium Chloride Injection, UPS is provided as a sterile, colorless, transparent, liquid [pH: 5.6 (4.5-7.0); Calculated Osmolarity: 308 mOsmol/liter]. Recommended Storage is at room temperature (25°C). Avoid excessive heat. Protect from freezing. Use only if solution is clear and container and seals are intact.

IL-2 (Proleukin®): Proleukin® is supplied as a sterile, white to off-white, lyophilized cake in single-use vials intended for intravenous administration. When reconstituted with 1.2 mL Sterile Water for Injection, USP, each mL contains 18 million International Units (1.1 mg) Proleukin, 50 mg mannitol, and 0.18 mg sodium dodecyl sulfate, buffered with approximately 0.17 mg monobasic and 0.89 mg dibasic sodium phosphate to a pH of 7.5 (range 7.2 to 7.8).

Proleukin® will be reconstituted and aliquots prepared at Benaroya Research Institute and distributed (ready to use) to participating laboratories according to the following directions:

1. Reconstitute one vial containing 22x 10^6 IU lyophilized Proleukin with 1.2mL sterile water for final concentration of 18x 10^6 IU/mL. During reconstitution, the sterile water should be directed at the side of the vial and the contents gently swirled to avoid excess foaming. DO NOT SHAKE.

2. Prepare 18x10µL (18,000 U/µL) tubes, freeze at -80°C. Each aliquot contains sufficient reagent for one mouse (five injections) + extra. Distribute 6 aliquots to each site.

Upon receipt by the site, store aliquots at -80°C.

Mice are dosed with 12,500 IU in a volume of 100 microliters (IP). To prepare the reagent for administration, dilute the stock with sterile PBS without Mg2+/Ca2+ (such as PBS, pH 7.4 Invitrogen/Life Technologies Catalog #10010-02). For each mouse, on the first day of treatment (Day 1), thaw one aliquot and add 1.43 mL PBS (for a final volume of 1.44 mL (125,000 U/mL). 100 µL contains 12,500 IU.

Store the remainder of Proleukin® aliquot at 4°C, and use for subsequent injections (Days 2, 3, 4, 5).

IL-2/anti-IL-2 complexes:

The diluent for both recombinant mouse IL-2 and anti-IL-2 monoclonal antibody is sterile Phosphate Buffered Saline (PBS) without Mg2+/Ca2+ (such as PBS, pH 7.4 Invitrogen/Life Technologies Catalog #10010-02).

Mouse IL-2 Recombinant Protein (eBioscience #34-8021; 0.5mg/mL) is provided as a sterile liquid. It will be apportioned at Benaroya Research Institute and distributed to participating laboratories according to the following directions:

1. For Group 4 (5 μg/treatment), dispense 36 μl of 500 μg/mL stock reagent into individual tubes. Dispense a total of 18 tubes. Freeze at -80°C. Ship six aliquots to each site. Each tube contains sufficient reagent (plus extra) for 3 injections of a single mouse.
2. For Group 5 (2.5 μg/treatment), dispense 15 μl of 500 μg/mL stock reagent into individual tubes. Dispense a total of 18 tubes. Freeze at -80°C. Ship six aliquots to each site. Each tube contains sufficient reagent (plus extra) for 3 injections of a single mouse.

Upon receipt by the site, store aliquots at -80°C.

Anti-Mouse IL-2, clone JES6-1A12 (eBioscience #16-7022) is provided as 50 μl of a 1 mg/mL sterile, aqueous solution. It will be apportioned at Benaroya Research Institute and distributed to participating laboratories according to the following directions:

1. Add 2450 μl of 1x PBS to the 50 μl stock solution to make 2500 μl of a 20 μg/mL anti-IL-2 solution.
2. For Group 4 (0.5 μg/treatment), dispense 90 μl of 20 μg/mL reagent into individual tubes. Dispense a total of 18 tubes. Freeze at -80°C. Ship six aliquots to each site. Each tube contains sufficient reagent (plus extra) for 3 injections of a single mouse.
3. For Group 5 (0.25 μg/treatment), dispense 45 μl of 20 μg/mL reagent into individual tubes. Dispense a total of 18 tubes. Freeze at -80°C. Ship six aliquots to each site. Each tube contains sufficient reagent (plus extra) for 3 injections of a single mouse.

Upon receipt by the site, store aliquots at 2-8°C.

<u>Preparing IL-2/Anti-IL-2 Complexes for Administration</u>

The IL-2/anti-IL-2 complexes should be prepared fresh on the day of injection. Mice are dosed with IL-2/anti-IL-2 complexes on days 1, 3, and 5. Each stock vial contains sufficient reagent (plus extra) for 3 injections of a single mouse.

Group 4

1. To prepare the complexes for administration, combine 10 μl of 500 μg/mL (5 μg) recombinant mIL-2 with 25 μl of 20 μg/mL (0.5 μg) Anti-Mouse-IL-2 and incubate for 15 min at 37°C
2. Store any unused reagent at 2-8°C for use in day 3 and 5 injections in the same mouse.
3. After the incubation period is complete, add 90 μl 1x PBS to the mixture to bring the final volume to 100 μl.
4. Inject IP immediately. Note: this is a very small volume to inject. To ensure that the entire volume of reagent is injected and not left behind in the needle, load the syringe with the 100 μl of reagent and a small cushion of air between the liquid and the plunger. Use this air to expel any extra reagent left in the needle during the IP injection.

Group 5

1. To prepare the complexes for administration, combine 5 μl of 500 μg/mL (2.5 μg) recombinant mIL-2 with 12.5 μl of 20 μg/mL (0.25 μg) Anti-Mouse-IL-2 and incubate for 15 min at 37°C.
2. Store any unused reagent at 2-8°C for use in day 3 and 5 injections in the same mouse.
3. After the incubation period is complete, add 95 μl 1x PBS to the mixture to bring the final volume to 100 μl.
4. Inject IP immediately. Note: this is a very small volume to inject. To ensure that the entire volume of reagent is injected and not left behind in the needle, load the syringe with the 100 μl of reagent and a small cushion of air between the liquid and the plunger. Use this air to expel any extra reagent left in the needle during the IP injection.

Sample Collection

Samples will be collected according to the schedule described in this protocol. The following samples should be collected:

All sites should collect a stool sample from each animal at 10 weeks of age for baseline evaluation of the gut microbiome. Please refer to **SOP-ITN5P-003 Mouse Stool Collection** for the collection and storage procedures.

All sites should collect 100 microliters of serum from each animal on study days 1, 7 (can occur on day 6-8 for flexibility), and 28 (can occur on day 28-32 for flexibility). Means of serum collection can be determined by site preference. Serum samples should be stored at -80°C.

On day 7 (can occur on day 6-8 for flexibility), animals from subgroup A of groups 1-5 will be sacrificed and spleen and pancreatic lymph nodes collected for flow cytometry (following supplemental **SOP-ITN5P-005: Analysis of Treg and Teffector Cell Populations by Flow Cytometry**). Pancreas should be collected for histology (see supplemental **SOP ITN5P-004 Collection and Freezing of Fresh NOD Pancreas Tissue in OCT for Cryostat Sectioning**).

On day 28 (can occur on day 28-32 for flexibility), animals from subgroup B of groups 1-5 will be sacrificed and spleen and pancreatic lymph nodes collected for flow cytometry (following supplemental **SOP-ITN5P-005: Analysis of Treg and Teffector Cell Populations by Flow Cytometry**). Pancreas should be collected for histology (following supplemental **SOP ITN5P-004Collection and Freezing of Fresh NOD Pancreas Tissue in OCT for Cryostat Sectioning**).

Mechanistic Analyses

Mechanistic analyses may not be performed by every site based on availability of equipment and technical expertise.

SOPs will be provided detailing the procedures for:

- Thawing Mouse Treg Cell Stocks
- Pancreas collection and fixation
- Collection and Freezing of Fresh NOD Pancreas Tissue in OCT for Cryostat Sectioning Flow cytometry staining panels for Tregs and Teffectors.

ADDITIONAL DATA COLLECTION

Blood glucose measurements (every 2-3 days)
Body weights (once per week)

DATA ANALYSIS

Blood glucose data will be analyzed using GraphPad Prism (GraphPad Software Inc., San Diego, CA). 'Survival' curves will be generated from blood glucose data and compared using log rank tests. Test groups 2 through 4 will be compared to the group that did not receive IL-2 (group 1). $P<0.05$ will be considered significant.

Tregs will be quantified using flow cytometry, and the absolute and percentage change in Tregs from baseline to week 1 and baseline to week 4 will be assessed for each group. The absolute and percentage change in Tregs at each time point in test groups 2-4 will be compared to group 1 (no IL-2) to assess whether the addition of IL-2 or IL-2/anti-IL2 complex is able to enhance the survival of Tregs.

DOCUMENTATION TO BE MAINTAINED

Data sheets containing blood glucose values (3 times per week) and body weights (once per week). Adverse effects of treatments, if any. Flow cytometric analyses will be documented.

REFERENCES TO OTHER APPLICABLE SOPS

SOP-ITN5P-002: Thawing Mouse Treg Cell Stocks
SOP-ITN5P-003: Mouse Stool Collection
SOP-ITN5P-004: Collection and Freezing of Fresh NOD Pancreas Tissue in OCT for Cryostat Sectioning
SOP-ITN5P-005: Analysis of Treg and Teffector Cell Populations by Flow Cytometry

In a follow-up therapeutic study, mice will be treated with the optimal amount of IL-2 or IL-2/anti-IL-2 complexes plus one of two doses of BDC2.5 Tregs. Beginning at disease onset, the blood glucose of NOD mice will be monitored three times per week in the morning. Once a mouse registers a blood glucose reading greater than or equal to 250 mg/dL, diabetes onset will be confirmed by re-measurement of blood glucose levels the next day. Mice with blood glucose levels greater than or equal to 250 mg/dL for two consecutive days will be considered diabetic and will be entered into a treatment group the same day (day 1). Following allocation to a treatment group, blood glucose will be monitored three times per week in the morning. The study will run for 90 days post-T1D onset. Mice should be sacrificed between 90-95 days for endpoint assessments.

REFERENCES

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FORMS/ATTACHMENTS

None

REVISION HISTORY

Effective Date	Revision Version	Author	Description of Changes
7/13/14	Version 1	Bluestone	Original Version
8/5/14	Version 2	Bluestone	Added details regarding study design
12/11/2014	Version 3	NSC members/ Straub	Incorporated changes discussed by steering committee on 11/19
12/30/14	Version 4	Bluestone	Edited, corrected and made changes based on study committee comments
1/13/15	Version 5	Bernstein	Insulin pellet added. Edited.
2/6/15	Version 6	Bernstein	Drug dosing added. Edited.
3/5/15	Version 7	Bernstein	Dilutions for anti-IL2 adjusted. Edited.
3/24/15	Version 8	T Kupfer	Updated title to reflect mechanistic goals of pilot project. Edited for logic flow. Corrected wrong citations. Added no Treg control group. Divided expt groups into A & B subgroups. Clarified study endpoints.
8/10/15	Version 9	L Straub & T Kupfer	Incorporated final SOP numbers and cleaned up tracking changes.
9/14/15	Version 9.1	C Le	Corrected mouse anti-IL2 volumes for the IL-2/anti-IL2 complex