

SOP-ITN2-001 Procedures for anti-CD3+anti-IL-1 project

STANDARD OPERATING PROCEDURE

Pre-clinical Consortium on Combination Therapies for Type I Diabetes

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Title: Effects of subsequent and simultaneous anti-CD3 and anti-IL-1 therapy on reversal of diabetes in NOD mice

INTRODUCTION/PURPOSE

The goal of this study is to determine the efficacy of anti-CD3 monoclonal antibody therapy (alone), anti-IL-1b antibody (alone), or the combination of anti-CD3 plus anti-IL-1b administered simultaneously or sequentially, to reverse hyperglycemia in NOD mice with recent onset autoimmune diabetes.

IL-1 is a central pro-inflammatory cytokine that is expressed in islets under immunologic attack and is directly toxic to beta cells, particularly in combination with the cytokines TNF and IFN γ (1-3). IL-1 β has been shown to inhibit the generation of Tregs and its secretion has been associated with increased IL-17 and other inflammatory cytokines (4).

A combination of anti-CD3 mAb and IL-1 receptor antagonist (Anakinra) has proven to be highly effective in reversal of T1DM in NOD mice. However, IL-1RA is a human protein, and might have non-specific effects on the natural course of the mouse disease. Recently, we have obtained similar data using mouse anti-mouse IL-1 mAb in combination with anti-CD3 (5). Anti-CD3 mAb therapy with IL-1 blockade is associated with early induction of Th2/M2/Treg responses, which are beneficial in the mouse model and may be crucial for successful therapy in humans, however, it is not clear whether, for a better outcome, these two modalities have to be administered simultaneously, or one at a time. This study will determine whether anti-CD3+anti-IL-1 therapy given simultaneously has equivalent efficacy in reversing new onset diabetes to the same combination administered sequentially (anti-CD3 followed by anti-IL-1).

DEFINITIONS:

Diabetes: Type 1 diabetes with blood glucose \geq 250 mg/dl.

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

Reversal of diabetes: Blood glucose readings of $<$ 250 mg/dl on two consecutive measurements.

Cure: Reversal of diabetes with no relapse observed within days 31-60 (after the period of insulin release by insulin pellet).

NOD mice: Female NOD mice between 10 and 26 weeks of age (such as NOD/ShiLtJ from the Jackson Laboratory, or similar with cumulative incidence of T1D $>$ 60%).

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 DESIGN

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 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. Following allocation to a treatment group, blood glucose will be monitored two times per week in the morning. The study will run for 60-62 days at which point mice will be sacrificed for endpoint assessments. Mice may be removed from the study for humane reasons. Animals will be removed from the study and euthanized according to IACUC protocol once their weight drops below that permitted by each site's IACUC (or 20% weight loss if IACUC does not have a weight loss restriction) or 3 consecutive 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). 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. Blood glucose monitoring will be performed three times per week 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.

Following allocation to a treatment group, blood glucose will be monitored two times per week in the morning. Spacing between blood glucose measurements should be no more than four 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. Values above the upper limit of detection of the meter should be recorded as the upper limit of detection for that model of monitor as stated by the manufacturer. For example, the upper limit of detection for the Easy Check blood glucose monitor is 600 mg/dL, therefore, high readings for this monitor should be recorded as 600 mg/dL.

Blood Glucose Monitoring Equipment: Each site may use the model of blood glucose monitor of their preference. However, if a site wishes to purchase monitors specifically for this study, then the Easy Plus monitor (formerly known as Easy Check; Home Aide Diagnostics, Inc., Deerfield Beach, FL) is recommended for this study.

Body Weight Measurements

Body weights will be measured weekly and documented.

Implantation of Insulin Pellets

Diabetic mice in all groups, EXCEPT Y2, 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 (d 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.

Treatment with Antibodies

Anti-CD3 F(ab)₂ and control F(ab)₂ treatments: Hamster anti-mouse CD3 F(ab)₂ fragments of the 145-2C11 mAb at a dose 5 ug/mouse/day or control hamster F(ab)₂ fragments are injected intraperitoneally (i.p) for 4 days (days 1, 2, 3, and 4). Previous studies have demonstrated that this regimen is suboptimal at reversing T1D in NOD mice with only 50% of mice cured. The F(ab)₂ fragments are available from BioXcell, West Lebanon, NH.

Anti-IL-1b treatment simultaneous with anti-CD3 treatment: Anti-IL-1b (from "Novartis") is given at a dose of 75 ug /day i.p. on days 1, 3, and 5. This regimen proved to be 90% effective in reversal of T1D in NOD mice when combined with anti-CD3. On days 1, 3 and 5 both Abs are mixed in one syringe.

Anti-IL-1 treatment following anti-CD3 treatment: Anti-IL-1b (from "Novartis") is given at a dose of 75 ug/day i.p. on days 5, 7, & 9.

The total volume of injection is 150 uL per mouse; sterile PBS without Mg²⁺/Ca²⁺ (such as PBS, pH 7.4 Invitrogen/Life Technologies Catalog #10010-02) is used as diluent for all antibodies.

Treatment groups:

Following the second consecutive confirmatory hyperglycemic reading, mice will be serially allocated to the following groups:

Treatment Group	Category	Description
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Y1	Test Group (Simultaneous anti-IL-1)	Insulin pellet day 1 Anti-CD3 at 5 micrograms/day on days 1, 2, 3, and 4 Anti-IL-1 at 75 micrograms /day on days 1, 3, & 5 (n=15)
Y2	Test Group (Simultaneous anti-IL-1)	No insulin pellet (control for early reversals) Anti-CD3 at 5 micrograms/day on days 1, 2, 3, and 4 Anti-IL-1 at 75 micrograms /day on days 1, 3, & 5 (n=5)
Y3	Test Group (Delayed anti-IL-1)	Insulin pellet day 1 Anti-CD3 at 5 micrograms/day on days 1, 2, 3, and 4 Anti-IL-1 at 75 micrograms /day on days 5, 7, & 9 (n=15)
Y4	Control	Insulin pellet day 1 Anti-CD3 at 5 micrograms/day on days 1, 2, 3, and 4 Normal mouse IgG2a at 75 micrograms /day on days 1, 3, & 5 (n=15)
Y5	Control	Insulin pellet day 1 (n=15) Anti-CD3 at 5 micrograms/day on days 1, 2, 3, and 4 Normal mouse IgG2a at 75 micrograms /day on days 5, 7, & 9 (n=15)
Y6	Control	Insulin pellet day 1 Control hamster F(ab) ₂ at 5 micrograms/day on days 1, 2, 3, and 4 Normal mouse IgG2a at 75 micrograms /day on days 1, 3, & 5 (n=15)
Y7	Control	Insulin pellet day 1 Control hamster F(ab) ₂ at 5 micrograms/day on days 1, 2, 3, and 4 Normal mouse IgG2a at 75 micrograms/day on days 5, 7, & 9 (n=5)
Y8	Control	Insulin pellet day 1

		Control hamster F(ab) ₂ at 5 micrograms/day on days 1, 2, 3, and 4 Anti-IL-1 at 75 micrograms/day on days 1, 3, & 5 (n=5)
Y9	Control	Insulin pellet day 1 Control hamster F(ab) ₂ at 5 micrograms/day on days 1, 2, 3, and 4 Anti-IL-1 at 75 micrograms/day on days 5, 7, & 9 (n=5)

Blood Sampling

Blood Collection: Approximately 150 microliters of blood should be collected for measurement of cytokine levels on day 2 and at study end (day 60-62). Blood should be collected via the route approved by each individual site's IACUC. The preferred route for blood collection is via the retro-orbital sinus, however, it is understood that each site's veterinary staff and IACUC may require blood be collected via a different method.

Sample Processing and Storage: Blood samples should be centrifuged at high speed (8000-17000 xg; 10,000 to 14,000 rpm on most centrifuges), the serum collected, and the sample stored at -80°C until assayed. A minimum of 50 microliters of serum is required for measurement of cytokine levels.

Mouse Necropsy

At the end of the study (d 60-62), mice should be sacrificed according to approved IACUC protocol and the following tissues should be harvested for endpoint assessments:

1. The maximum attainable volume of blood for measurement of cytokine levels (see *Blood Sampling*).
2. The pancreas for measurement of histology (See *Pancreas Histology*), total pancreatic insulin content (see *Measurement of Total Pancreatic Insulin Content*) and for generation of RNA for gene expression analysis (see *RNA Preparation*).
3. The spleen for flow cytometric analysis of autoantigen-specific CD8+ T cells (see *Quantitation of Autoantigen-specific CD8+ T Cells*), for in vitro cytokine secretion studies (see *Cytokines in Circulation and In Vitro*), and for generation of RNA from unsorted spleen cells for gene expression analysis.

Allocation of Tissues to Mechanistic Studies

At end of study, pancreas will be harvested and allocated serially to mechanistic studies in this order: 1. Histology, 2. Insulin content, and 3. RNA. Thus, for the treatment groups containing an n = 15, there will be an n of 5 in each mechanistic study; hopefully evenly distributed between responders and non-responders.

At end of study, spleen will be harvested and allocated serially to mechanistic studies in this order: 1. Flow cytometry, 2. In vitro cytokine secretion studies, 3. RNA (whole spleen). Thus, for the treatment groups containing an n = 15, there will be an n of 5 in each mechanistic study; hopefully evenly distributed between responders and non-responders.

Pancreas Histology

Harvesting of Pancreas for Histology

Anesthetize and euthanize mouse according to approved IACUC protocol. Open the abdomen and identify the pancreas by gently lifting the spleen. Recover the entire pancreas including the head near the spleen and the tail near the GI tract. Pancreas and pancreatic lymph nodes are pale and soft tissues that are difficult to distinguish grossly, but present very distinct histology. Place all together. Weigh the pancreas for subsequent beta cell mass calculations. Average pancreatic wet weight is about 120 mg.

Place the pancreas on a dry sponge in a histology cassette. Spread it out gently to flatten it in as compact an area as possible. There should not be any major 'mounds' of pancreatic tissue, nor should there be excessive stretching. Use only one sponge. Close the cassette lid securely, label and place in fixative (10% buffered formalin). Allow pancreas to fix overnight at room temperature. To minimize over-fixation, do not fix tissue for longer than 24 hours.

End fixation by transferring cassettes to either PBS or 70% ethanol and store at 4 °C. The samples are now ready for processing and embedding.

Scoring Insulinitis

Insulinitis can be scored on H&E stained slides using the following scale:

0 = no infiltrates

1 = peri-islet infiltrates

2 = intra-islet infiltrates $\leq 50\%$

3 = intra-islet infiltrates $> 50\%$

4 = non-islet infiltrates (optional, does not refer to islet-associated inflammation)

The scores from different levels can be averaged for each animal and expressed as percentage of 100 or by number of islets with each score

Measurement of Total Pancreatic Insulin Content

Frozen pancreata will be submitted to the Yale site for analysis.

Whole pancreata are removed, snap-frozen in a cryovial in liquid nitrogen, and stored at -80°C until 5-6 samples from each treatment group are accumulated. Frozen pancreata are transferred into 600 μL of cold (-20°C) acid-ethanol (0.54% HCL in 70% ethanol with 0.1% Triton X100) and is chopped with scissors inside a 1.5 mL eppendorf test tube. The samples are incubated for 24h at -20°C , homogenized with a plastic pestle, after which another 600 μL of acid-ethanol is added, and the samples are extracted for additional 24h at -20°C . Samples are centrifuged; supernatants are collected, and stored at -20°C until assayed for insulin. For insulin determination, samples are diluted 50-500 times with PBS. Insulin ELISA is performed using Ultra Sensitive Mouse Insulin ELISA kit (Crystal Chem Inc., Downers Grove, IL). Total pancreatic insulin content (ng/pancreas) is calculated by multiplying values from the assay (ng/mL) by dilution factor and by 1.2 (volume of acid-ethanol). Mean total insulin content on Day 30 in mice that reverse to euglycemia is about 800 ng/pancreas; in those that do not reverse is about 100 ng/pancreas.

Cytokines in Circulation and In Vitro

Cytokines in circulation are directly measured in serum samples collected on Day 2. Serum samples should be collected and stored at -80°C. Cytokine levels will be analyzed in serum samples at one site at the conclusion of the study.

For in vitro cytokine secretion studies, spleen cells from treated mice are cultured in AIM-V medium (Invitrogen) at 10^6 /mL, with PMA (5 ng/mL) and ionomycin (50 ng/mL) for 24 h. Supernatants (100 uL) are collected and kept frozen until assayed. Cytokines (IL-4, IL-5, IL-10, IFN γ , IL-12, IL-18, IL-17, TNF) are measured using Milliplex Map kit (Millipore Corporation, Billerica, MA) and Bioplex analyzer (Bio-Rad, Hercules, CA).

Quantitation of Autoantigen-specific CD8+ T Cells

NRP specific CD8+ lymphocytes are identified by staining with PE-conjugated H2K^d tetramer with NRP-V7 peptide KYNKANVFL, a mimotope of islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) (8). The tetramer will be obtained from Dr. P. Santamaria. A common stock of tetramer will be procured and distributed to all sites. Cells are co-stained with anti-CD8 (positive gating) and anti-CD19 (negative gating) and analyzed by flow cytometry using FACSCalibur (BD Immunocytometry Systems, CA). Data are analyzed with FlowJo software (TreeStar Inc, Ashland, OR).

RNA preparation

Small (5-10 mg) pieces of pancreata or spleen, or whole pellets of sorted cells are processed for RNA isolation using RNeasy Mini Kit from Qiagen Sciences (Germantown, MD) according to the manufacturer's protocol. RNA amount is measured on Nanodrop spectrophotometer. RNA samples are kept frozen at -80°C until used for gene expression analysis by qPCR.

Data Analysis

Interim data analysis is performed each time by adding new data to GraphPad Prism5 (GraphPad Software Inc., San Diego, CA) files for each parameter assessed. ANOVA is used for comparison between the groups with Dunnett's or Dunn's multiple comparison post tests. All groups are compared to double-placebo-treated mice (Group Y7). Survival curves are compared using log rank test.

Final data analysis will occur when all groups are filled and reached their endpoints. $P < 0.05$ will be considered significant. With $0.05 > P < 0.06$, we will add 2-3 mice to the group under the question in an attempt to reach $P < 0.05$.

DOCUMENTATION TO BE MAINTAINED

Data sheets with blood glucose values.

Body weights measured weekly.

Files reflecting measurements of cytokines, flow cytometry data, pancreatic insulin content, and data on pancreatic and splenic gene expression.

Statistical analyses of experimental data.

Adverse effects of treatment, if any.

REFERENCES TO OTHER APPLICABLE SOPS

SOP-NOD-001: Naming Schema

SOP-NOD-0011: Mouse Necropsy

REFERENCES

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FORMS/ATTACHMENTS

None

REVISION HISTORY

Effective Date	Revision	Author	Description of Changes