SUPPLEMENTAL MATERIALS

Supplemental Table 1. AUC C-peptide following ATG/G-CSF versus Placebo based on duration of T1D at Baseline

	ATG/G-CSF AUC C-Peptide, nmol/L/min, Mean (SD)		Placebo AUC C-Peptide nmol/L/min, Mean (SD)	
	< 1 year	> 1 year	< 1 year	> 1 year
Baseline	0.59 (0.48)	0.80 (0.48)	0.92 (0.35)	0.69 (0.12)
3 months	0.64 (0.44)	0.72 (0.51)	0.77 (0.42)	0.22 (0.11)
6 months	0.74 (0.49)	0.82 (0.60)	0.70 (0.45)	0.15 (0.06)
12 months	0.70 (0.44)	0.77 (0.51)	0.58 (0.31)	0.19 (0.15)

Supplemental Table 2. Mean Change in Autoantibody Titers at 12 Months Compared to Baseline Between ATG/G-CSF and Placebo Treated Subjects

	Mean 2-hr AUC change from baseline: ATG/G-CSF vs placebo (SD)	95% Confidence Interval	P-value by t-test
GAD (units/ml)	11.75 (187.7)	(-156.8, 180.3)	0.89
IA2 (units/ml)	-24.13 (45.3)	(-64.8, 16.7)	0.23
IAA (units/ml)	0.28 (0.48)	(-0.15, 0.70)	0.20
ZnT8 (units/ml)	0.037(0.23)	(-0.18, 0.23)	0.79

Supplemental Table 3. CD3 Counts in ATG/G-CSF versus Placebo Treated Subjects

	ATG/G-CSF	Placebo	Mean 2-hr AUC change from baseline: ATG/G-CSF vs placebo (SD)	95% Confidence Interval	P-value by t-test
Baseline (17,7)	72.5	71.8	0.69 (6.5)	--	--
1 week (17,7)	46.0	69.3	-29.7 (18.4)	(-46.8,-12.6)	0.002
2 weeks (17,7)	51.0	64.9	-19.6 (16.3)	(-34.8,-4.4)	0.014
4 weeks (17,7)	55.6	63.1	-13.6 (14.1)	(-26.7,-3.8)	0.043
3 months (17,7)	58.5	64.2	-12.7 (13)	(-24.8,-0.6)	0.040
6 months (17,7)	60.2	70.5	-11.8 (8.3)	(-17.3,-6.3)	0.005
12 months (16,7)	62.6	64.7	-8.9 (7.8)	(-16.3,-1.5)	0.020

Supplemental Table 4. CD4 Counts in ATG/G-CSF versus Placebo Treated Subjects

	ATG/G-CSF	Placebo	Mean 2-hr AUC change from baseline: ATG/G-CSF vs placebo (SD)	95% Confidence Interval	P-value by t-test
Baseline (17,7)	67.0	66.8	0.1 (7.1)	--	--
1 week (17,7)	28.8	63.3	-34.5 (13.2)	(-46.8,-22.8)	0.001
2 weeks (17,7)	41.6	63.3	-21.5 (9.9)	(-30.8, -12.3)	<0.001
4 weeks (17,7)	44.3	66.7	-22.5 (8.2)	(-30.2, -14.9)	<0.001
3 months (17,7)	46.1	63.8	-18.3 (7.3)	(-25.1, -11.4)	<0.001
6 months (17,7)	47.6	64.5	-17.5 (7.5)	(-24.5, -10.5)	<0.001
12 months (16,7)	49.6	64.6	-15.5 (7.3)	(-22.3, -8.6)	<0.001

Supplemental Table 5. CD8 Counts in ATG/G-CSF versus Placebo Treated Subjects

	ATG/G-CSF	Placebo	Mean 2-hr AUC change from baseline: ATG/G-CSF vs placebo (SD)	95% Confidence Interval	P-value by t-test
Baseline (17,7)	26.6	27.9	-1.2 (6.0)	--	--
1 week (17,7)	54.5	31.7	23.7 (12.6)	(12.0, 35.5)	<0.001
2 weeks (17,7)	41.5	31.1	11.5 (13.5)	(-1.1, 24.0)	0.071
4 weeks (17,7)	43.5	27.5	17.3 (6.5)	(11.2, 23.4)	<0.001
3 months (17,7)	42.1	29.2	14.0 (5.8)	(8.6, 19.4)	<0.001
6 months (17,7)	41.0	29.2	13.4 (5.7)	(8.0, 18.7)	<0.001
12 months (16,7)	38.5	20.0	12.1 (5.3)	(7.1, 17.1)	<0.001

Supplemental Table 6. CD4:CD8 Ratio in ATG/G-CSF versus Placebo Treated Subjects

	ATG/G-CSF	Placebo	Mean 2-hr AUC change from baseline: ATG/G-CSF vs placebo (SD)	95% Confidence Interval	P-value by t-test
Baseline (17,7)	2.66	2.52	0.14 (0.97)	--	--
1 week (17,7)	0.54	2.45	-2.09 (1.0)	(-3.05,-1.17)	<0.001
2 weeks (17,7)	1.04	2.41	-1.55 (0.82)	(-2.31,-0.78)	<0.001
4 weeks (17,7)	1.08	2.56	-1.65 (0.71)	(-2.31,-0.98)	<0.001
3 months (17,7)	1.13	2.49	-1.56 (0.87)	(-2.41,-0.78)	0.001
6 months (17,7)	1.19	2.37	-1.38 (0.74)	(-2.08,-0.69)	<0.001
12 months (16,7)	1.30	2.61	-1.54 (0.99)	(-2.47,-0.61)	0.003

Supplemental Table 7. Treg:CD3 (%) by methylation analysis of the TSDR following ATG/G-CSF versus placebo

	ATG/G-CSF	Placebo	Mean 2-hr AUC change from baseline: ATG/G-CSF vs placebo (SD)	95% Confidence Interval	P-value by t-test
	Treg:CD3 Percent (SD)				
Baseline (17,8)	3.86 (1.2)	3.47 (1.0)	0.39 (1.14)	--	--
2 weeks (17,8)	6.74 (7.4)	3.26 (1.2)	0.73 (1.44)	-0.55, 2.00	0.25
6 months (17,8)	3.96 (1.2)	3.42 (0.9)	0.05 (0.24)	-0.16, 0.26	0.65
12 months (15,8)	4.03 (1.3)	3.85 (0.9)	-0.08 (0.24)	-0.30, 0.13	0.43

Supplemental Table 8. FOXP3^{+}Helios^{+} Treg (%) in ATG/G-CSF versus Placebo Treated Subjects

	ATG/G-CSF	Placebo	Mean 2-hr AUC change from baseline: ATG/G-CSF vs placebo (SD)	95% Confidence Interval	P-value by t-test
Baseline (16,6)	5.81	5.80	-0.008 (1.80)	--	--
1 week (16,7)	8.70	4.60	4.30 (4.4)	(-0.01, 8.70)	0.044
2 weeks (17,7)	10.8	5.88	4.59 (3.4)	(1.18, 8.0)	0.016
4 weeks (16,7)	8.92	5.31	3.50 (3.1)	(0.38, 6.6)	0.028
3 months (15,7)	9.29	5.61	3.13 (3.7)	(-0.88,7.15)	0.043
6 months (15,7)	8.74	6.32	2.02 (1.9)	(-0.07,4.11)	0.060
12 months (15,7)	7.70	5.82	1.45 (2.2)	(-0.85,3.8)	0.037

Supplemental Table 9. $CD4^+$ CD45RA/CD45RO ratio following ATG/GCSF versus Placebo

	ATG/G-CSF	Placebo	Mean 2-hr AUC change from baseline: ATG/G-CSF vs placebo (SD)	95% Confidence Interval	P-value by t-test
Baseline (16,6)	1.31	1.03	-0.28 (0.68)	(-0.88,0.32)	0.34
1 week (16,7)	1.06	0.97	-0.09 (0.86)	(-0.84,0.67)	0.819
2 weeks (17,7)	1.18	0.93	-0.24 (0.93)	(-1.1,0.58)	0.546
4 weeks (16,7)	1.34	0.95	-0.39 (0.48)	(-1.4,0.6)	0.422
3 months (15,7)	1.07	1.02	-0.06 (0.74)	(-0.7,0.6)	0.857
6 months (15,7)	1.08	0.55	-0.01 (0.76)	(-0.68,0.66)	0.981
12 months (15,7)	1.11	0.87	-0.24 (0.68)	(-0.85,0.38)	0.436

Supplemental Table 10. CD19 (B cells) following ATG/GCSF versus Placebo

	ATG/G-CSF	Placebo	Mean 2-hr AUC change from baseline: ATG/G-CSF vs placebo (SD)	95% Confidence Interval	P-value by t-test
Baseline (16,6)	9.82	10.4	0.58 (5.0)	(-4.1,5.3)	0.799
1 week (16,7)	21.9	7.41	-14.5 (13.4)	(-26.4,-2.6)	0.019
2 weeks (17,7)	22.5	7.97	-14.6 (11.8)	(-25.0,-4.2)	0.008
4 weeks (16,7)	12.1	6.98	-5.08 (6.6)	(-10.9,0.8)	0.087
3 months (15,7)	10.5	6.60	-3.88 (5.5)	(-8.7,0.96)	0.111
6 months (15,7)	14.8	7.56	-7.22 (6.8)	(-13.3,1.17)	0.021
12 months (15,7)	13.6	7.90	-5.75 (5.3)	(-10.5,0.95)	0.021

Supplemental Table 11. WBC following ATG/GCSF versus Placebo

	ATG/G-CSF	Placebo	Mean 2-hr AUC change from baseline: ATG/G-CSF vs placebo (SD)	95% Confidence Interval	P-value by t-test
Baseline (17,8)	5.82	5.38	0.45 (1.35)	--	--
2 weeks (17,8)	16.3	5.91	9.96 (9.61)	(1.43,18.47)	0.024
4 weeks (17,8)	6.37	5.24	0.68 (2.01)	(-1.10,2.46)	0.438
6 weeks (17,8)	7.76	5.39	1.92 (2.30)	(-0.12,3.97)	0.064
8 weeks (17,8)	6.87	5.09	1.34 (1.51)	(0.37,2.30)	0.009
10 weeks (17,8)	7.36	5.74	1.17 (2.38)	(-0.94,3.28)	0.262
3 months (17,6)	7.09	5.23	1.42 (2.34)	(-0.66,3.49)	0.171
6 months (17,8)	4.84	5.44	-1.05 (1.24)	(-2.14,0.05)	0.061
12 months (16,8)	5.38	5.78	-0.91 (0.97)	(-1.78, -0.03)	0.042

Supplemental Table 12. Neutrophils (%) following ATG/GCSF versus Placebo

	ATG/G-CSF	Placebo	Mean 2-hr AUC change from baseline: ATG/G-CSF vs placebo (SD)	95% Confidence Interval	P-value by t-test
Baseline (17,8)	3.11	2.91	0.2 (1.13)	--	--
2 weeks (17,8)	15.0	3.11	11.71 (9.45)	(3.33, 20.1)	0.008
4 weeks (17,8)	4.58	3.08	1.30 (1.92)	(-0.41, 3.0)	0.129
6 weeks (17,8)	5.94	3.39	2.34 (1.91)	(0.65, 4.04)	0.009
8 weeks (17,8)	5.05	2.76	2.09 (1.49)	(0.76, 3.41)	0.003
10 weeks (17,8)	5.33	3.45	1.67 (1.99)	(-0.09, 3.44)	0.062
3 months (17,6)	5.12	2.93	1.98 (1.92)	(0.27, 3.69)	0.025
6 months (17,8)	2.99	3.12	-0.34 (0.92)	(-1.16,0.48)	0.399
12 months (16,8)	3.38	10.8	-7.67 (12.3)	(-18..7,3.35)	0.163

Supplemental Table 13. Lymphocytes (%) following ATG/GCSF versus Placebo

	ATG/G-CSF	Placebo	Mean 2-hr AUC change from baseline: ATG/G-CSF vs placebo (SD)	95% Confidence Interval	P-value by t-test
Baseline (17,8)	2.03	1.83	0.23 (0.61)	--	--
2 weeks (17,8)	0.96	2.19	-1.43 (0.96)	(-2.28,-0.58)	0.002 (<0.001)
4 weeks (17,8)	1.16	1.56	-0.60 (0.57)	(-1.11,-0.09)	0.022 (0.015)
6 weeks (17,8)	1.15	1.43	-0.48 (0.76)	(-1.16,0.20)	0.154 (0.154)
8 weeks (17,8)	1.21	1.76	-0.76 (0.49)	(-1.19,-0.33)	0.001 (0.001)
10 weeks (17,8)	1.26	1.71	-0.65 (0.58)	(-1.16,-0.14)	0.015 (0.014)
3 months (17,6)	1.25	1.70	-0.65 (0.63)	(-1.20,-0.09)	0.025 (0.023)
6 months (17,8)	1.26	1.76	-0.71 (0.51)	(-1.16,-0.26)	0.004 (0.002)
12 months (16,8)	1.34	4.57	-3.43 (4.49)	(-7.46, 0.60)	0.091 (<0.001)

Reversing Type 1 Diabetes After it is Established: A Pilot Safety and Feasibility Study of Anti-Thymocyte Globulin (Thymoglobulin®) and Pegylated GCSF (Neulasta®) in Established Type 1 Diabetes

UF IRB Protocol Number: 41-2010

UF GCRC Protocol Number: 797

ClinicalTrials.gov Identifier: NCT01106157

Sponsored by: The Helmsley Trust

Pharmaceutical Support Provided by: Genzyme

Study Principal Investigator: Michael Haller,MD

IND Sponsor: Michael Haller, MD

IND Number: 107185

Version Number: 4.0
12 July 2013

Site Principal Investigators:

Michael J. Haller, MD: University of Florida*

Stephen Gitelman, MD: University of California, San Francisco

Peter Gottlieb, MD, PhD: University of Colorado

The University of Florida will serve as the Coordinating Center for this protocol

Confidentiality Statement

This document is confidential and is to be distributed for review only to investigators, potential investigators, consultants, study staff, and applicable independent ethics committees or institutional review boards. The contents of this document shall not be disclosed to others without written authorization, unless it is necessary to obtain informed consent from potential study participants.

Signature Page

The signature below constitutes the approval of this protocol and the attachments, and provides the necessary assurances that this trial will be conducted according to all stipulations of the protocol, including all statements regarding confidentiality, and according to local legal and regulatory requirements and applicable U.S. federal regulations and ICH guidelines.

The Lead Principal Investigator (Protocol Chair) should sign Signature Page 1. A copy of this Signature Page 1 should be filed with the holder of the Regulatory documents and a copy should be maintained at the site.

Principal Investigator: Michael J. Haller, MD
Print/Type

Signed:  Date: 7/12/2013

Name/Title

List of Abbreviations

AE	Adverse Event/Adverse Experience
ATG	Anti-Thymocyte Globulin (Thymoglobulin®)
CMP	Complete Metabolic Panel
CBC	Complete Blood Count
CFR	Code of Federal Regulations
CRF	Case Report Form
DSMB	Data Safety Monitoring Board
GADA	Glutamic Acid Decarboxylase Antibody
GCP	Good Clinical Practice
GCRC	General Clinical Research Center
GCSF	Granulocyte Colony Stimulating Factor (Neulasta®)
HLA	Human Leukocyte Antigen
IA	Insulin Antibody
IA-2	Insulinoma Associated Antibody 2
ICA	Islet Cell Autoantibody
ICF	Informed Consent Form
IRB	Institutional Review Board
ITN	Immune Tolerance Network
MMTT	Mixed Meal Tolerance Test
NOD	Non Obese Diabetic
PI	Principal Investigator
PHI	Protected Human Information
SAE	Serious Adverse Event/Serious Adverse Experience
T1D	Type 1 Diabetes
Teff	Effector T-cells
Treg	Regulatory T-cells
ZnT8A	Zinc Transporter 8 Antibody

Protocol Summary

Full Title	Reversing Type 1 Diabetes After it is Established: A Pilot Safety and Feasibility Study of Anti-Thymocyte Globulin (Thymoglobulin®) and Pegylated GCSF(Neulasta®) in Established Type 1 Diabetes:
Short Title	Thymoglobulin® and Neulasta® for Type 1 Diabetes.
Clinical Trial Phase	Phase I/II
IND Sponsor	Michael Haller, MD
IND Number	107185
Conducted By	The University of Florida, the University of Colorado, and the University of California, San Francisco.
Principal Investigator	Michael J Haller, MD
Sample Size	25 treated subjects (17 will receive Thymoglobulin® and Neulasta®, while 8 will receive placebo).
Study Population	Both males and females and members of all racial and ethnic populations with type 1 diabetes (T1D) will be screened for potential study inclusion. An attempt will be made to enroll subjects to reflect the known difference in frequency of T1D amongst different racial and ethnic groups. Given the potential toxicities for this combination drug therapy, only subjects ≥12 years and < 45 years of age with a diagnosis of T1D for at least 4 months but no more than two years will be enrolled. No more than 12 subjects with diabetes duration <1 year will be randomized. This will ensure that the interests of the Helmsley Trust in performing studies in patients with established T1D are maintained.
Accrual Period	2 years
Study Design	Two-arm, 2:1 randomized, placebo controlled, single- blinded phase I/II trial. Potential subjects will be screened via a 4 hour MMTT to assess residual beta cell (C-peptide) function. C-peptide will be measured at -10, 0, 15, 30, 60, 90, 120, 150, 180, 210, and 240 minutes after 6cc/kg po Boost®. If the C-peptide level at any time is ≥ 0.1 pmol/ml, and the subject meets the additional inclusion and exclusion criteria, they will be eligible for randomization and enrollment in the study. The study will be randomized 2:1 such that 17 subjects will receive active therapy and 8 will receive placebo. Subjects must receive Thymoglobulin®/ Neulasta® or placebo within 8 weeks of randomization. Thymoglobulin® (2.5mg/kg)/placebo will be given as 0.5 mg/kg IV on day 1 and 2 mg/kg on day 2. Six doses of Neulasta® (6mg/dose)/placebo will be given SC every 2 weeks, with the first dose given prior to discharge following the Thymoglobulin® infusion. Complete metabolic panel (CMP) and CBC will be obtained at the screening visit, immediately prior to study drug initiation, daily during the Thymoglobulin® infusion admission, and at each follow up visit. Following discharge, daily phone calls will be made to the subjects during the first 5 days of therapy and weekly thereafter. In addition, weekly phone calls for the month following completion of therapy will be used to document adverse reactions.
Study Duration	Five Years. Subjects will have a screening visit, infusion admission (time 0), and up to 18 follow-up visits: 1 week, 2 weeks, 4 weeks, 6 weeks, 8 weeks, 10 weeks, 12 weeks, 6 months, 9 months, 12 months, 18 months, 24 months, 30 months, 36 months, 42 months, 48 months, 54 months, and 60 months after receiving study drug. Visits for individual subjects will continue between 24 and 60 months as long as the subject is producing C-peptide.

Study Agent/Intervention Description	Thymoglobulin® (Anti-Thymocyte Globulin or ATG) will be administered at a dose of 2.5mg/kg as 0.5mg/kg IV on day 1 and 2mg/kg on day 2. Neulasta ® (Pegylated GCSF) will be administered at a dose of 6mg SC every two weeks for 10 weeks for a total of 6 doses.
Primary Objective	To determine the ability of combination therapy (Thymoglobulin® + Neulasta®) to retain/enhance C-peptide production in established T1D patients demonstrating residual beta cell function. This notion will be assessed by longitudinal comparison of metabolic function (i.e., blood glucose control, insulin usage, mixed-meal stimulated C-peptide production) in subjects receiving Thymoglobulin® + Neulasta® versus placebo. The primary statistical hypothesis to be assessed in the study is whether the target population of change in the area under the curve (baseline to 12 months) in residual beta cell function (C-peptide) is the same for Thymoglobulin® + Neulasta® and placebo.
Secondary Objectives	Determine the influence of Thymoglobulin® + Neulasta® therapy on immune responsiveness including tolerance induction. To improve our mechanistic understanding of the potential of Thymoglobulin® + Neulasta® to modulate immune reactivities and induce tolerance in an autoimmune setting, with focus on the role for regulatory T (Treg) cells in these processes.
Exploratory Objectives	Additional immunologic studies may be attempted to further investigate the mechanisms involved in Thymoglobulin® + Neulasta® therapy
Key Endpoints	• Δ AUC – C-peptide • Δ A1c • Δ Insulin requirements (units/kg/d) • Δ Frequency of severe hypoglycemia • Δ Autoantibody/Antibody levels (GADA, ICA, IA, IA-2A, ZnT8A) • Δ Absolute Neutrophil Count • Δ Total White Cell Count • Δ Treg Frequency • HLA Type • Frequency of Adverse Events

Table of Contents

1. Background and Rationale

1.1 Introduction

Recent advances in our understanding of the pathogenesis of T1D have been somewhat overshadowed by our continued inability to develop a long lasting means to either reverse or prevent the disease (i.e., identify a cure), using a variety of interventions (e.g., cyclosporine, anti-CD3, anti-CD20, oral insulin) (1-3). We believe our inability to identify a method to prevent and/or reverse T1D in humans may have resulted, to a large extent, from deficiencies in our understanding of the mechanisms of autoimmunity that underlie beta cell destruction, a failure to understand the immunologic factors that contribute to T1D, and the absence of tools to monitor mechanisms underlying disease development or the efficacy of therapeutic interventions. A limited number of potential therapeutic agents have usage profiles affirming issues of equipoise. Nonetheless, we must use our expanding, yet limited understanding of T1D pathophysiology, in both animal models and man, along with the lessons learned from our previous interventional failures, to guide the development of novel approaches for the cure of this disease.

The majority of attempts to reverse T1D in humans have, to date, involved the delivery of agents as monotherapy. As a result, we and others have recently questioned whether the delivery of therapeutics in combination might produce a synergistic response that would allow for the successful reversal of T1D (2, 3). The success of combination therapy for advancing the treatment of patients with HIV and cancer demonstrates a model for multi-agent therapy in diseases involving multiple pathways required for their successful treatment and cure. As already mentioned, previous attempts to cure or reverse T1D have largely been focused on single agents designed to intervene in a singular aspect of diabetes pathogenesis (e.g., immunosuppression, immunomodulation, beta cell rest, islet regeneration). We believe "combination therapy" with the right "cocktail" of agents aimed at providing immunosuppression, immunomodulation, *and* islet regeneration may greatly improve our ability to cure T1D. To be clear, some individual agents have shown promise in animal models of T1D, only to fall short of producing a similar response in humans. Other agents, while efficacious as a monotherapy, may be limited by toxicities. Initial interventional studies with cyclosporine, azothioprine, oral insulin, subcutaneous insulin and nicotinamide have now been replaced by a new generation of agents with improved safety and potentially improved efficacy profiles. Immunosuppressive agents such as Thymoglobulin and anti-CD3, immunomodulators such as vitamin D and G-CSF, autoantigens (i.e., insulin, GAD), and even potential beta cell regenerative agents (e.g., Exendin-4) have all demonstrated at least some potential for ameliorating T1D (3-11). While NIH TrialNet, the NIH Immune Tolerance Network (ITN), and the Juvenile Diabetes Research Foundation (JDRF) have led support to ongoing studies to investigate many of these individual agents, few "combination therapy" intervention studies have been initiated to date.

Strong interest has been directed towards an improved understanding of the mechanisms that regulate the immune response, as well as those that form a state of immunological tolerance; not only in T1D, but also other autoimmune disorders (e.g., allergy, transplantation, and oncology, amongst others). We and others have recently reported findings related to the frequency and function of T reg cells, characterized by the expression of CD4+CD25+Foxp3+, and their role in maintaining tolerance in T1D. While this literature represents a somewhat conflicting body of observations, we believe the studies proposed in this application represent a novel opportunity to continue to address this issue, identify the therapeutic capacity of agents to reverse T1D, induce tolerance, and understand the contributions of Treg to both the process of tolerance induction as well as the pathogenic defects underlying the natural history of T1D.

Thus, based on strong preliminary data demonstrating efficacy and potential mechanisms leading to reversal of diabetes in NOD mice with this combination, as well as preliminary safety studies in humans with T1D with both Thymoglobulin® or Neulasta® as monotherapies, we will test the hypothesis that a short course of Thymoglobulin® + Neulasta® given in combination will lead to preservation of C-peptide in patients with established T1D.

1.2 Combination Therapy in T1D

Exogenous insulin therapy provides a remarkable, life-saving treatment for those with T1D, but ultimately, it is not a cure. Within the last decade, T1D has seen the design and implementation of a number of well-organized and controlled clinical trials. However, an objective account of previous therapeutic failures in a vast majority of those efforts suggests that novel approaches are needed to succeed in our quest to cure T1D. Indeed, as the incidence of T1D continues to rise, the necessity for novel approaches has never been clearer. As we attempt to design novel intervention strategies, we are confronted with the paradox that early intervention is more likely to be effective, but may require treatment of subjects who will ultimately never develop disease. While intervention (i.e., those with T1D) is less likely to be successful, it allows for more aggressive therapy as it targets only those already diagnosed with diabetes. Since patients with new onset T1D lose beta cell function over time, they provide an excellent group to study as any prolonged preservation of insulin production can be considered successful, within a relatively short time frame. In addition, the relative certainty that most patients with T1D will eventually lose the remainder of their endogenous insulin production provides some degree of comfort for investigators considering aggressive intervention strategies that could unintentionally speed the loss of C-peptide production. Admitting that we still do not have adequate understanding of T1D to expose large groups of children who may never develop diabetes to interventions with potential side effects, we believe that current efforts that are aggressive in their nature should be limited to those patients with T1D.

If one agrees that novel approaches are required to overcome the therapeutic failures that unfortunately describe past efforts in T1D prevention/reversal, the question turns to what interventions are novel? We have proposed the concept that novel approaches to the treatment of T1D may be developed from the successful therapeutic approaches used in other complex diseases such as HIV, cancer, and lupus. Specifically, "combination therapy" has demonstrated that multiple agents are often far superior to the use of any single drug. As we still lack insight into many of the precise mechanisms that induce T1D, combination therapy may also allow effective targeting of the more broadly understood areas of T1D pathogenesis. As the use of individual agents has largely failed to produce prolonged preservation of C-peptide, we believe agents that are potentially efficacious and acceptably safe should now be used in combination with the intent to alter the course of beta cell destruction (1, 2). It is conceivable that sufficient beta cell mass exists at or near the time of symptomatic onset to the extent that intervention with agents aimed at averting the ongoing autoimmune response (e.g., anti-CD3, anti-CD20, ATG, etc.), when combined with those intended at repair/regeneration of beta cells (e.g., glucagon-like-peptide-1 (GLP-1), exendin-4, etc.), might allow for a reversal of T1D (4-12). It should also be noted that an emerging body of experimental evidence in the NOD mouse model of T1D has suggested that therapeutic interventions targeting the development of Treg may also contribute to this goal (13-15). To this end, we have recently obtained data indicating the ability for the combination of ATG and G-CSF to induce Treg in vivo, as well as show marked effectiveness in preventing T1D in NOD mice when administered in the period immediately prior to hyperglycemic onset, as well as reversing T1D when administered at disease onset (see preliminary data and 16). Other investigators have reported long-term restoration to euglycema in NOD mice, previously identified as overtly hyperglycemic, through treatment with anti-lymphoycyte serum (ALS) in combination with exendin-4 (17).

With this concept, as additional single agents show potential to impart partial benefit for C-peptide preservation, successive pilot "combination therapy" studies can continually be established to determine if combination therapies can improve on previous successes. While testing successive cohorts with multiple different agents is admittedly a daunting process, there is no doubt that the advancement of HIV and cancer therapy was brought about by exactly such an approach. The real challenge, therefore, is not in deciding whether or not to proceed with combination therapy, but in deciding which combination of agents should initially be chosen. While many candidates for inclusion could be considered, we find strong basis for a combination of ATG and G-CSF.

1.3 ATG Monotherapy

1.3.1 Rationale for ATG Therapy in T1D

Preclinical studies have shown that ALS treatment of the NOD mouse (18) and BB rat (19) with recent onset T1D can induce remission, supporting proof of principle for such an approach in humans. Specifically, Maki and colleagues demonstrated that treatment of overtly diabetic NOD mice with ALS, a polyclonal anti–T-cell antibody (with some similarity to ATG), induced a long-term abrogation of autoimmunity and in 50% of treated mice, achieved a permanent clinical remission (18). Reversal of hyperglycemia, however, was a slow process, requiring 75–105 days. Studies employing both anti-CD3 and anti-CD4 depleting monoclonal antibody in NOD mice showed that a short course of these drugs, soon after T1D onset, induced remission for more than 100 days and also prevented destruction of syngeneic islet grafts (20, 21). ALS treatment of biobreeding rats induced remission in 36% of animals with recent onset T1D for at least 60 days, and also prevented development of the disease pre-diabetic rats (19).

The case for combination of ATG with other agents received support from follow-up studies in new-onset NOD mice involving the addition of Exendin-4, a long-acting GLP-1 agonist shown to augment insulin secretion in rodents (22) as well as T1D (23) and T2D (24) subjects, where remissions were seen in 90% of combination treated mice. While our own NOD studies (Preliminary Data section) found ATG and G-CSF to be a superior combination (in comparison to ALS and Exendin-4) both sets of data provide additional support for the concept of combination therapy and it's superiority to relatively effective monotherapy in terms of reversing T1D.

As further proof of the potential for translation from mouse to man, studies in human transplantation and various autoimmune conditions suggest that ATG may indeed induce tolerance. ATG has been successfully used as combination therapy in preventing rejection following organ transplantation. After induction with ATG, transplant recipients can be managed successfully with only limited maintenance monotherapy (25, 26), with weaning of some to as little as a single dose of tacrolimus per week. ATG, either alone or in combination with other agents, has also been used to treat a variety of autoimmune conditions, including Wegner's granulomatosis, lupus, rheumatoid arthritis, multiple sclerosis, scleroderma, aplastic anemia and myelodysplastic syndrome; with greater efficacy observed when used in combination (27-38). Extensive studies with ATG in a non-human primate model demonstrates its effectiveness as a single agent in T-cell depletion intravascularly and in peripheral lymphoid organs, as well as in prolongation of allograft transplantation of skin and heart (39). Studies do, in fact, exist in support of ATG's possible efficacy in T1D. Specifically, early limited human studies with equine ATG and prednisone with new onset T1D suggested efficacy in prolonging the honeymoon phase (11, 40). As far as current studies, in an ongoing randomized, placebo-controlled, single-blinded trial with ATG (ATG-Fresenius, Germany), T1D participants aged 18-35 years receive a total dose of 18 mg/kg of ATG, administered in four infusions. Of the 17 study participants treated 11 received drug while 6 received placebo. In terms of efficacy, increased glucagon-stimulated C-peptide levels, a lower insulin requirement, and lower glycosylated hemoglobin levels have been seen in the ATG group (but not placebo group) 12 months into the study (41). Most promising, two ATG treated subjects achieved remission (i.e., off exogenous insulin for at least 1 month and a fasting glycemia below 126 mg%).

Currently, a phase 2 study in humans with new-onset T1D funded by the Immune Tolerance Network (ITN) is underway to determine if ATG will lead to preservation of C-peptide. The study will test the hypothesis that selective deletion of lymphocytes will reset the immunologic rheostat, effect dynamic immune regulation and perhaps induce and maintain tolerance in T1D. As this study will also help establish safety data for the use of ATG in humans in T1D, the background adverse event rate will be established in this population and will allow for the study of combination therapies that include ATG and additional tolerance inducing agents such as GSCF.

1.3.2 Potential Mechanism of Action

ATG appears to induce both generalized immunosuppression and immunoregulation, with affects on APC and immunoregulatory cell function (42). It prevents B-cell proliferation and differentiation, as well as mediates T-cell suppressive effects via inhibition of proliferative responses to mitogens (43, 44). A single "typical" or "standard" dose of ATG reduces the total lymphocyte count by more than 85%. T-cell depletion may result from complement-dependent opsonization and cellular lysis, Fc-dependent opsonization, or Fas-mediated apoptosis; particularly at lower ATG concentrations, at which ATG exhibits preferential effects on preactivated, as opposed to nonactivated, T-cells. T-cell depletion in peripheral blood persists for several days to several months following cessation of ATG administration. Recovery from treatment-induced lymphocyte depletion is gradual and total lymphocyte counts usually return to normal with 2 months after ATG administration.

Following ATG function, the CD4:CD8 T cell ratio remains significantly and persistently lower and the ratio may correlate with the outcome in transplantation studies (45-48). In the preliminary anti-CD3 study in humans with new onset T1D, this ratio was the best predictor of responders (4). In both NOD mouse and human studies, treatment with anti-CD3 led to an increase in the CD4+CD25+ T cell population (49, 50). An effect on CD8+ regulatory T-cell population may also be present when utilizing anti-CD3 (51). Following renal transplants with ATG induction, the CD8+ and CD57+ (and CD28-) T-cell population has been shown to increase from 1%-15% at baseline to 70%-80% (52). Similar findings have been noted following solid organ and bone marrow transplants (52,53). With this, it has been hypothesized that ATG administration may induce a regulatory population of CD8+, CD57+ and CD28- cells that is critical for selective downregulation of pathogenic self-reactive CD4+ Th1 cells and the induction of tolerance.

Other studies have suggested alternative mechanisms to underlie the beneficial effects of ATG in vivo. As with anti-CD3, ATG may induce partial T-cell activation, leading to an anergic state (39, 53, 54). In cynomolgus monkeys, ATG appears to coat T cells, leading to a downregulation in surface expression of CD2, CD3, CD4, and CD8 molecules, along with impaired immune responses in mixed lymphocyte reactions (39). In addition, antibodies to adhesion molecules may interfere with cellular adhesion and endothelial interactions, as well as T-cell migration to sites of inflammation. ATG may also prevent costimulation of T cells by binding directly to APC, and may induce complement-mediated lysis of these cells; particularly in more mature APC (55). The survival of immature DC thus may be more tolerogenic. Antigens recognized by ATG include CD86, CD32, CD4, CD11b, CD29, and CD51/61; some of which are shared by lymphoctyes and DC. In addition, ATG contains antibodies that cross-react with B-cell surface antigens, allowing for induction of activated B-cell (another source of APC) apoptosis in vitro (56-58).

1.4 Granulocyte colony-stimulating factor (G-CSF) monotherapy

1.4.1 Rationale for G-CSF Therapy in T1D

In a spontaneous diabetes model, G-CSF treatment of NOD mice at 4 weeks of age for 5 consecutive days, repeated every 4 weeks thereafter until 16 weeks of age, prevented disease onset and insulitis when compared to injection of excipient (Figure 1) (59). Protection from diabetes onset correlated with the recruitment of both DC and CD4+CD25+ Treg cells. Indeed, the G-CSF recipients showed peri-pancreatic lymph node accumulation of functional CD4+CD25+ Treg cells (60). Importantly, co-transfer of the functional Treg populations along with diabetogenic splenocytes into secondary NOD-SCID recipients actively suppressed diabetes. In addition, DC transferred from mice given a single 5-day-long G-CSF treatment to secondary NOD recipients triggered enhanced accumulation of CD4+CD25+ Treg cells compared to recipients given non-G-CSF treated DC. Thus, G-CSF likely restores a balance between immunogenic and tolerogenic DC, resulting in the recruitment of functional CD4+CD25+ Treg cells.

Based on supportive data from animal models, several groups have moved on to initiate clinical trials of G-CSF based therapies in human autoimmune disease. Perhaps the most extensive human data derives from studies of G-CSF therapy in patients with Crohn's disease. Several studies have now demonstrated improvement in

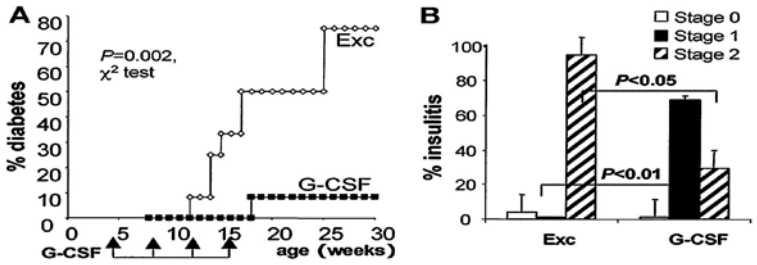


Figure 1. Treatment with G-CSF prevents spontaneous diabetes and destructive insulitis. *A*: NOD mice (*n* = 12 per group) were treated with G-CSF (200 µg/kg) or excipient (exc) for 5 consecutive days every 4 weeks starting at age 4 weeks. Data are from one representative experiment out of three. G-CSF significantly reduced diabetes incidence. *B*: Insulitis was scored at age 25 weeks (means ± SD of islet counts from three to six organs per group). (*Adapted from Kared et al. Diabetes 54:78-84, 2005).*

clinical disease scores in patients with Crohn's disease provided varying courses of G-CSF (61-62). Specifically, a 12 week course of 300mcg daily G-CSF, demonstrated marked reduction in the Crohns Disease Activity Index amongst 15 subjects with active disease (62). In a smaller study of Crohn's disease patients with a history of severe endoscopic ileitis, a similar 12-week course of G-CSF was associated with clinical remission of disease in all 5 subjects (61).

Nevertheless, the body of G-CSF animal data must be interpreted with some caution. G-CSF therapy in animal models of lupus and multiple sclerosis models failed to show efficacy in either setting. However, in the case of multiple sclerosis, a potential worsening of disease was observed when applied in pilot human studies (63,64). While it would be naïve to ignore the theoretical risk of speeding the expected destruction of remaining beta cells with G-CSF, there is a clear need to determine if animal data suggestive of a protective role for G-CSF in the NOD mouse can be translated into clinical benefit for patients with T1D.

1.4.2 Potential Mechanism of Action

G-CSF, an agent well known to help mobilize hematopoietic precursors from the bone marrow, has been used clinically for nearly 20 years to help repopulate peripheral cell counts in patients undergoing cancer therapy. However, a rapidly growing body of experimental and clinical evidence suggests that G-CSF has the potential to be used in the treatment of autoimmune diseases. G-CSF has been shown to favor the differentiation and mobilization of Treg cells, induce tolerogenic DC, and alter the balance between proinflammatory and anti-inflammatory soluble mediators both animals and in humans (Figure 2) (65-67). Specifically, G-CSF can mobilize functional bone marrow $CD4^+CD25^+FoxP3^+$ Treg cells. Preclinical models of G-CSF-induced inhibition of autoimmune and allogeneic T cell responses have demonstrated the potential efficacy of G-CSF in treating Crohn's disease, myasthenia gravis, and T1D.

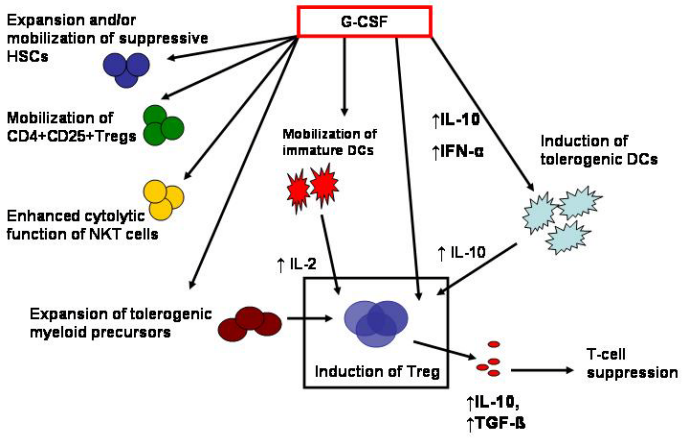


Figure 2. Effects of G-CSF on immune function. The G-CSF induced modulation of cytokine production, T cell polarization, and DC functional profiles is depicted schematically. G-CSF can alter the balance between proinflammatory and anti-inflammatory soluble mediators, favor the differentiation and mobilization of Treg cells, and induce tolerogenic DC both in animals and in humans. *(Adapted from Rutella et al, J. Immunology 2005, 175: 7085–7091,* ***(65)****.*

Wit

h this extensive experience of clinical use, G-CSF therapy is known to be both safe and well tolerated. The side effect profile of short course G-CSF therapy is limited; with frequency and severity of toxicities being dose dependent (greater with larger doses and longer duration) and minimal in comparison to many of the agents being used in current T1D intervention studies. Specifically, the most common side effect associated with G-CSF therapy is bone pain (occurring in 10-20% of patients) which occurs 2-3 days after initiation of therapy. This di scomfort i s generally c ontrollable with ac etaminophen. I nfrequent s ide e ffects i nclude r edness at injection site, fever, headache, chest pain and dizziness (occurring in less than 10% of patients). Since G-CSF is a well-tolerated, easily administered therapy, we initiated a pilot study using G-CSF monotherapy in humans with recent-onset T1D supported by the JDRF and NIAID in 2008.

1.5. Preliminary Studies (Using a combination of ATG and G-CSF)

1.5.1 G-CSF E nhances t he Long -Term Reversal of Diabetes Afforded by murine ATG in the NOD

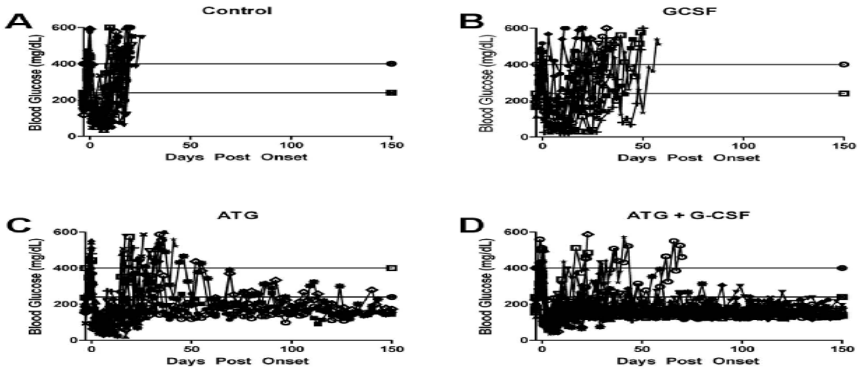


Figure 3. Blood glucose values were obtained for up to 150 days post-onset in NOD mice treated with (**a**) control, (**b**) G-CSF, (**c**) murine ATG, or (**d**) murine ATG + G-CSF.

To de termine the e fficacy of a c ombination of ATG and G-CSF in NOD mice with `new-onset' diabetes, female NOD mice were monitored 3 t imes per w eek f or hyperglycemia (defined as a blood glucose > 240mg/dL) by t ail bl eed for up to 150 day s as s hown (Figure 3) . Animals m easuring above t his t hreshold o n t wo c onsecutive days were considered diabetic. Murine ATG was administered via two intraperitoneal (IP) injections of 500µg murine ATG or, as a control, 500µg rIgG (Jackson ImmunoResearch) given 72 hours apart for a total dose of 1mg. These animals also received a subcutaneous LinBit insulin implant (LinShin Canada) providing sustained release of insulin for approximately 3 weeks. Failure of the therapy was defined as blood glucose levels above 400 mg/dl for two consecutive measurements. In the suboptimal dosing study, the dose of murine ATG was reduced to 290ug per animal, over two injections. Neupogen (Amgen, Inc.) was used for G-CSF therapy for both suboptimal and standard dosing studies. A dose of 6ug/animal was diluted in 100uL of 5% dextrose per manufacturer's recommendation and injected IP daily for a maximum of 8 weeks. Blood sugar was monitored 3 t imes per week until either failure occurred, or the end of the study period. Blood glucose levels were monitored in all mice treated at diabetes onset for up to 150 days. As shown (Figure 3), the administration of murine ATG alone to new-onset NOD mice resulted in durable (i.e., > 150 days post-onset) remissions from overt hyperglycemia in 33% (5/15) of treated animals, while neither control (0/16) nor G-CSF monotherapy (0/14 mice) pr ovided s uch r eversions H owever, t he c ombination of murine A TG and G -CSF therapy resulted in a remission rate of 75%, a significantly greater rate of remission than was seen with murine ATG monotherapy (12/16; P = 0.0000006 versus control, P = 0.013 versus murine ATG).

Interestingly and very importantly, in terms of dosage in humans, G-CSF improved the therapeutic capacity for diabetes reversal even when in combination with a suboptimal dose (290ug) of murine ATG (Figure 4).

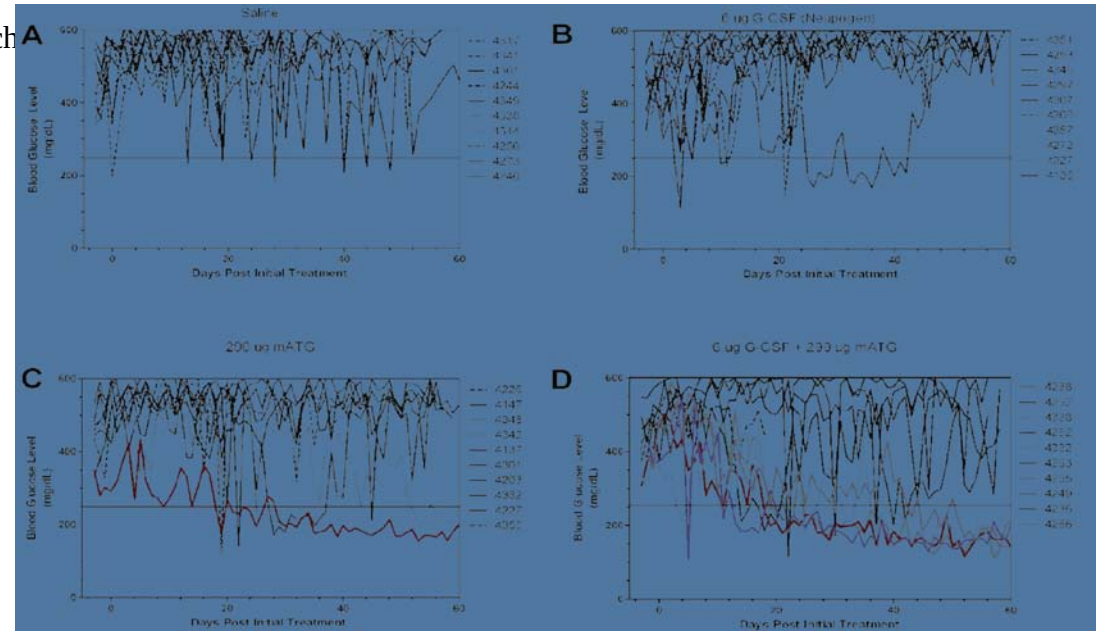


Figure 4. The addition of G-CSF improves the reversal rate of suboptimal murine ATG in new onset NOD mice. No long-term reversal was achieved by either (**a**) control-therapy (0/15) or (**b**) with G-CSF monotherapy (0/14). (**c**) Using only 290ug/mouse (compared with a standard murine ATG dose of 1000ug, remission occurred in only 20% (2/10) of NOD mice. (**d**) The addition of G-CSF, however, raised this reversal rate to 50% (5/10).

1.5.2 Combination Therapy Enables Reversal of Higher New-Onset Glycemia than Murine ATG Monotherapy

Given the higher rate of reversal observed with combination therapy, we examine how this higher rate correlated with the blood glucose at the time of diabetes onset. Indeed, at time course analysis following diabetes onset revealed pronounced differences in the ability of these therapies to remit based upon starting blood glucose values.

Successful treatment with murine ATG was largely limited to values of ≤380mg/dL (mean 317.2mg/dL; range 256-398mg/dL), whereas combination therapy of murine ATG and G-CSF significantly increased the therapeutic ceiling to ~500 mg/dL (mean 401.8 mg/dL; range 264-500mg/dL) (Figure 5).

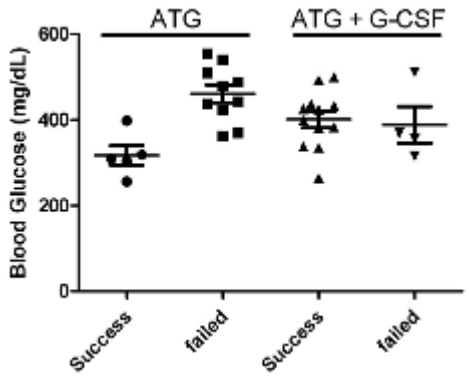


Figure 5. G-CSF enhances the ability of murine ATG to reverse mice with greater hyperglycemia at diabetes onset. Therapeutic success of murine ATG therapy was largely limited to starting blood glucose values of 380mg/dL and below with an average of 317.2mg/dL. The addition of G-CSF to murine ATG treatment significantly raised the starting average blood glucose of successful therapy to 401.8/mg/dL (P = 0.019).

1.5.3 Glucose Control Improves With Time in Reversed NOD Mice

Having observed substantial rates of diabetes remission, we sought to determine whether this return to euglycemia would be durable in the face of a glucose challenge. To do so, during the course of the reversal trial using the suboptimal dose of murine ATG, we measured glucose control via IP glucose tolerance tests (IPGTT) at 60, 90, and 120 days post-onset in reversed mice. An improvement in IPGTT, as measured by the area under the curve from 0 to 120 minutes, was observed from the 60 to 120 day time points (Figure 6). This improvement in glucose control occurred in spite of the cessation of both murine ATG and G-CSF therapies prior to the 60-day time point.

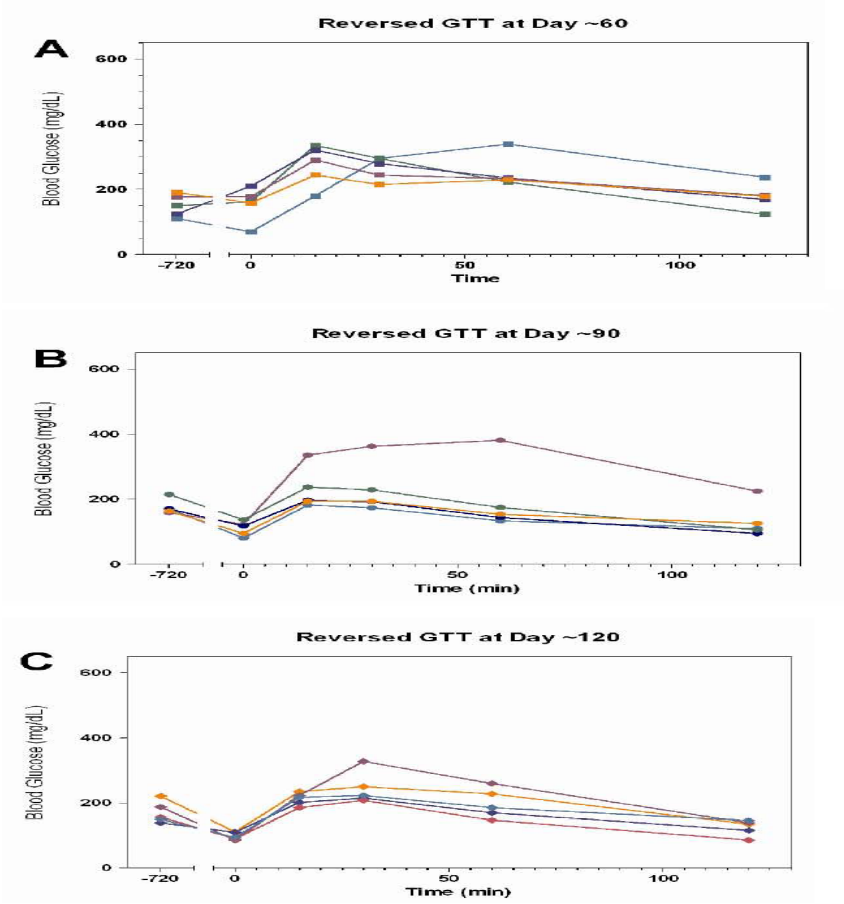


Figure 6. IPGTT indicates improving glucose control from 60 to 120 days post-onset in combination therapy-treated NOD mice. NOD mice remitted from diabetes using combination therapy were administered an IPGTT at (**a**) 60, (**b**) 90, and (**c**) 120 days post-onset. An area under the curve (AUC) analysis revealed a significant improvement at 120 days compared with 60 days (p=0.0235,

1.5.4 Murine ATG + G-CSF Combination Therapy Induces Immunomodulation

To address the question of whether G-CSF-mediated enhancement of diabetes reversal was due to induction of immunoregulation, murine ATG and G-CSF (as both mono- and combination-therapy) were administered to pre-diabetic 12-week-old female NOD mice for up to 8 weeks. Four groups were treated with control, murine ATG, G-CSF, or murine ATG + G-CSF. As with the T1D reversal studies, murine ATG was administered in two doses 72 hours apart. G-CSF was administered for up to 8 weeks. Timed sacrifices were performed at weeks 0, 2 , 4 , and 8 pos t-initiation o f t herapy (n=5/group/time poi nt) an d various m echanistic an alyses were performed. A nalysis of peripheral bl ood r evealed m arked l eukocyte de pletion i n m urine ATG-treated m ice versus all other groups at 2 week (Figure 7A), with movement back towards pre-treatment levels at 4 and 8 weeks pos t-induction. H owever, t he addi tion of G -CSF t o m urine A TG a fforded a s ignificant i ncrease i n leukocytes a t 2 w eeks v ersus murine A TG al one. I n par ticular, G-CSF i ncreased the per centage o f s plenic macrophages (Figure 7 B) and neut rophils (Figure 7C). B oth murine ATG as w ell as G -CSF hav e been reported t o i nduce a po pulation of Treg *in vivo*. P redictably, al l t reatments ut ilized in t hese e fforts her ein demonstrated a r educed per centage o f Tr eg at 2 weeks v ersus c ontrol ani mals (Figure 7D), d ue t o ei ther short-term depletion by murine ATG or mobilization of macrophages and neutrophils by G-CSF. G-CSF therapy led to an increase in Treg versus control as early as 4 weeks w hile c ombination therapy had the greatest i ncrease i n Tr eg v ersus al l ot her t reatments a t 8 w eeks. A s indicated by t he i ncrease i n T reg the immunomodulatory benefit afforded by G-CSF continued through 8 w eeks, despite the lack of mobilization of macrophages and neutrophils beyond 2 weeks. Thus the greatest percentage of Treg cells was observed in mice receiving combination therapy. This is not surprising given that both murine ATG and G-CSF individually have been shown to induce a population of Treg cells. The fact that by combining the two therapies results in a greater percentage of Treg after 8 weeks of therapy than either monotherapy lends more support to the use of combination therapy.

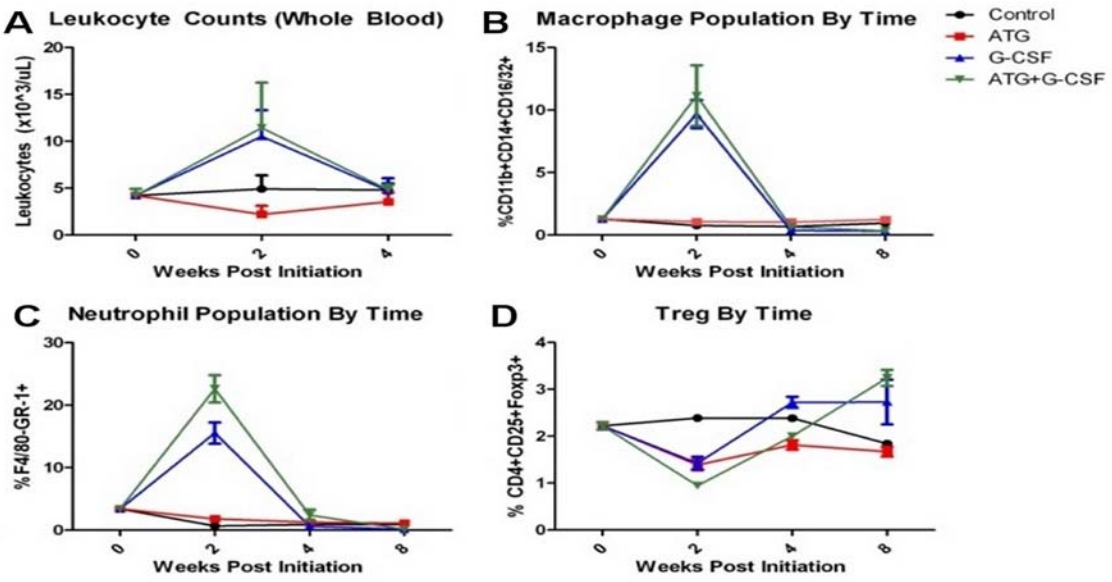


Figure. 7. Murine A TG and G -CSF c ombination therapy i n NOD m ice i nduces i mmunomodulation. (A) An alteration in per ipheral b lood l eukocyte c ounts w as obs erved onl y 2 w eeks af ter i nitiation of t herapy, w ith murine ATG + G-CSF treated mice exhibiting a significantly greater number (P = 0.0129, unpaired t test) than murine ATG monotherapy. No differences were seen at later time points. G-CSF and murine ATG + G-CSF treated mice exhibited significant increases in splenic (B) macrophages (P < 0.0001, P = 0.0027, respectively; unpaired t test) and (C) neutrophils (P < 0.0001, P < 0.0001, respectively; unpaired t test) 2 weeks after initiation of therapy with no differences observed thereafter. (D) The percentage of splenic T_{reg} was reduced in all treatments versus control at 2 weeks due to murine ATG-mediated depletion and/or the mobilization ef fect of G-CSF, but was significantly increased versus control at 4 weeks in G-CSF-treated mice (P = 0.003, unpaired t test) and at 8 weeks in murine ATG + G-CSF treated mice (P < 0.0001, unpaired t test).

1.5.5 Pancreatic Islets are Protected from Autoimmune Destruction by Murine ATG and G-CSF

The health of the islets at the endpoint of the pre-diabetic study was an important consideration. As such, insulitis scoring (Figure 8A) was performed to determine the degree of lymphocytic infiltration over the 8 weeks of therapy in pre-diabetic NOD mice. Combination therapy resulted in markedly lower insulitis intensity scores when compared with islets from control animals after 8 weeks (Figure 8B). In addition, insulin staining revealed improved beta cell area in animals receiving combination therapy versus murine ATG monotherapy, while control animals demonstrated a decline in beta cell area over the 8 week period (Figure 8C).

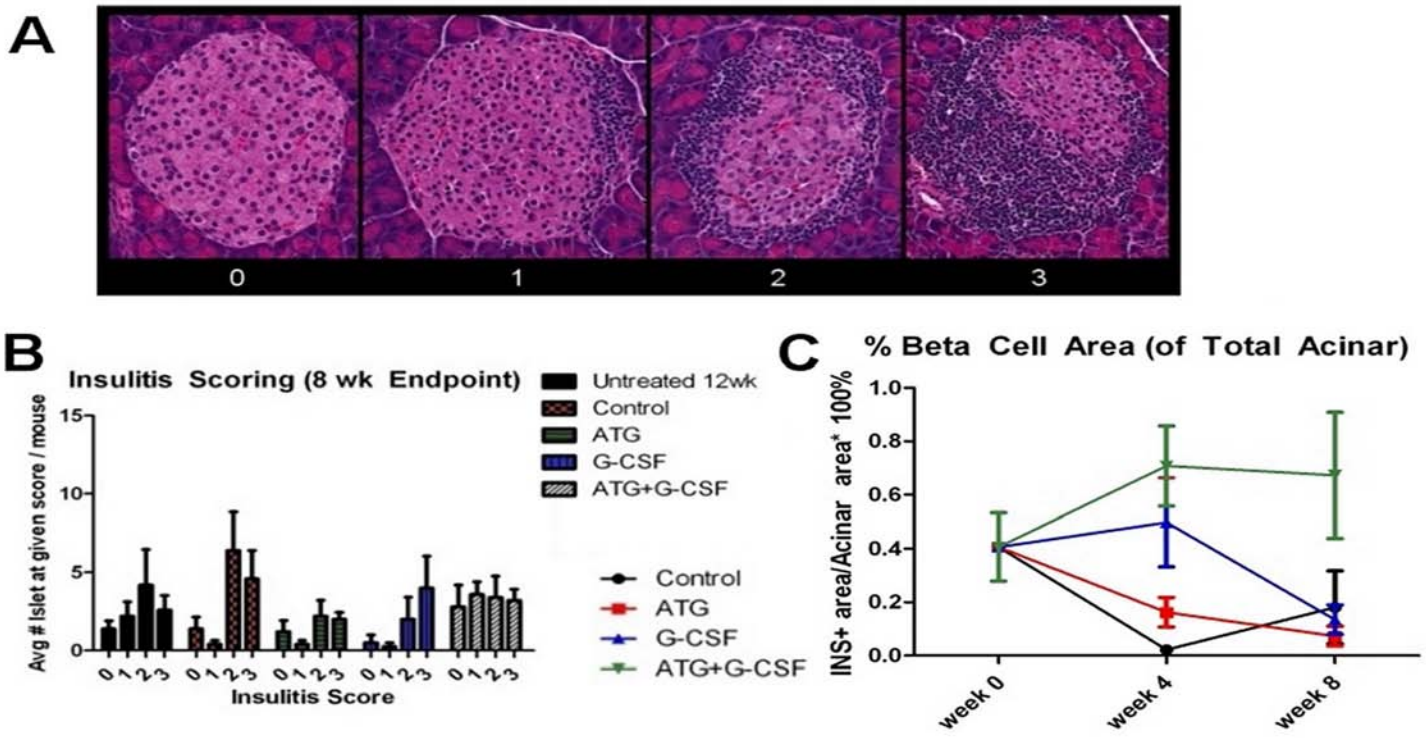


Figure 8. Murine ATG and G-CSF combination therapy in NOD mice protects pancreatic islets from autoimmune destruction. (A) Insulitis scoring of (B) islets 8 weeks after initiation of therapy indicated that combination therapy skewed scoring toward healthy islets, with significant improvement versus control in the number of islets with a score of 1 (P= 0.0055, unpaired t test). (C) Insulin staining at the 8 week time point revealed that combination therapy afforded the greatest b-cell/acinar area and was significantly improved versus murine ATG monotherapy (P = 0.0359 unpaired t test)

In summary, the apparent lack of beta cell durability in the murine ATG-treated prediabetic mice reflects a similar finding in a previous report in which 12 week-old pre-diabetic NOD mice exhibited only transient protection following anti-CD3 monoclonal antibody therapy (20). The transient protection seen with G-CSF monotherapy group reflects the reversal study in which G-CSF only led to a delayed return to hyperglycemia compared with control-treated mice. By combining these two monotherapies, however, the health of the islets was maintained relative to control as measured by insulitis scoring and beta cell area. These results indicate that combined treatment of murine ATG with G-CSF offers a highly effective means for reversal of T1D in NOD mice. This combination therapy provides for a series of beneficial mechanistic actions (e.g., increased Treg frequency, reduced islet inflammation, improved beta cell area, etc.) and dramatically extends the range of beta cell dysfunction allowable for effective and durable disease remission. These studies also provide strong support for the performance of human T1D trials with this combination of agents and suggest that this form of therapy may be amenable to treatment of other autoimmune disorders.

1.6 ATG and G-CSF Monotherapy Clinical Trials Update

1.6.1 G-CSF

In August of 2008, the University of Florida T1D research group embarked on a pilot (JDRF/NIAID supported) study of G-CSF monotherapy, in order to firmly establish the safety of pegylated-GSCF (Neulasta®) and demonstrate the potential for efficacy in mobilizing Treg in the T1D population. The study was created to follow the traditional TrialNet design using a 2:1 drug:placebo randomization scheme and using AUC C-peptide as the primary efficacy outcome measure. To date, we have randomized 13 recent onset T1D patients (all 12 years of age and older). Study participants complete a 12 week course of the study drug (Neulasta®, 6mg sq q 2 weeks versus placebo). While blinded, several study participants have reported bone pain and headache within the first 24-72 hours following study drug dosing. There have been no severe side effects..

1.6.2 ATG

In late 2006, the ITN approved ATG (Thymoglobulin®) monotherapy in new-onset T1D at a limited number of sites; the START trial (Study of Thymoglobulin to Arrest Type 1 Diabetes). After safety and tolerability of the drug was demonstrated, the age group was lowered to 12 years in Fall 2009. There are now a total of 11 participating sites. To date 32 subjects have been enrolled, 11 ages 12-18. Given our interest in this agent, we have been in continual communications with the PI of that effort (Stephen Gitelman, M.D.; UCSF). Also, of note, Dr. Gitelman will serve as the UCSF site PI for this combination ATG/GSCF protocol.

2. Objectives:

2.1 Conduct a randomized, placebo controlled study using a combination of Thymoglobulin® + Neulasta® in patients with established T1D in order to:

2.1.1 Determine the safety of combined Thymoglobulin® + Neulasta® therapy in this population.

2.1.2 Determine its efficacy in preserving endogenous insulin secretion by evaluating metabolic function (i.e., mixed-meal stimulated C-peptide production, blood glucose control, insulin use) in those receiving combination therapy versus controls.

2.2 Ascertain the immunological mechanisms leading to such protection by determining the influence of Thymoglobulin® + Neulasta® on humoral and cellular immune responses associated with the disease by:

2.2.1 Assessing whether the combination of Thymoglobulin® + Neulasta® therapy alters the frequency and/or functional capacity of Treg in peripheral blood.

2.2.2 Analyzing dendritic, B and T cell markers by flow cytometry.

2.3 Examining the course of anti-islet cell immunity as assessed by the production of T1D associated islet cell autoantibodies/antibodies (i.e., IA, GADA, ICA, ZnT8A, and IA-2A).

3. Study Design

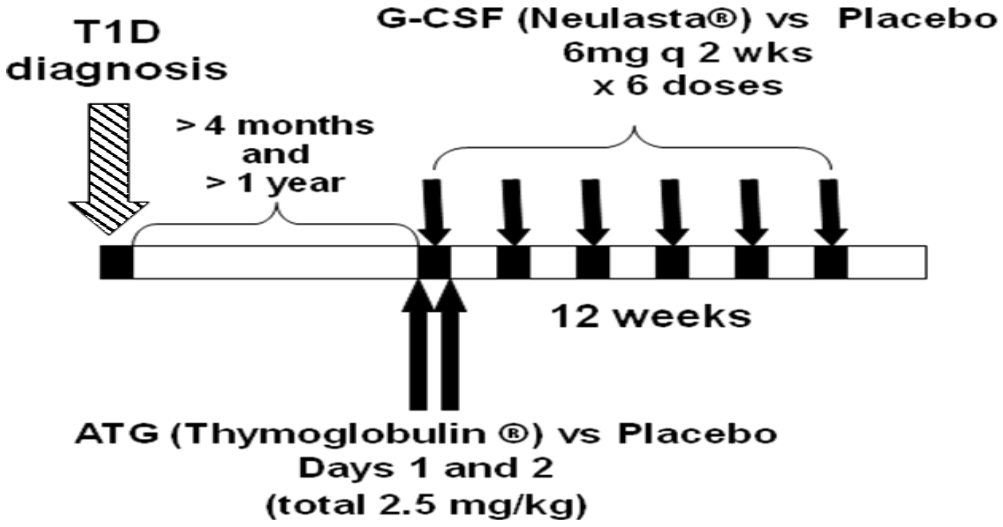
3.1 Overview

- Given the promising pre-clinical data described above, we will conduct a study using a combination of Thymoglobulin® and Neulasta® in patients with established T1D.

- 25 subjects with established T1D will be randomized, 2:1 (drug:placebo).
  - 17 will be assigned to receive a combination of Thymoglubulin® and Neulasta®.
  - 8 will receive placebo

- Those receiving active drug will receive a total of 2.5 mg/kg Thymoglobulin® and 6 doses of Neulasta® (6mg per dose)

- All subjects will be followed for at least 2 years (and up to 5 years if still producing C-peptide) with the primary statistical endpoint being collected at the 1 year.



ATG (Thymoglobulin®) and G-CSF (Neulasta®) Intervention Time Line. ATG will be given over 2 doses to total 2.5mg/kg and G-CSF will be given as 6mg SC q2 weeks for 10 weeks. No more than 12 subjects with a diagnosis of diabetes of less than one year will be randomized. All other subjects randomized will be at least 12 months from diagnosis in order to honor the interests of the Helmsley foundation in studying patients with established type 1 diabetes.

3.2 Inclusion and Exclusion Criteria:

Participants must meet all of the following inclusion criteria:

1. Must be ≥ 12 years < 45
2. Must have a diagnosis of T1D of greater than 4 months duration, with an upper limit of 2 years*
3. Must have at least one diabetes–related autoantibody present (e.g., ICA, GAD, ZnT8, or IA2 autoantibodies).
4. Must have stimulated C-peptide levels ≥ 0.1 pmol/ml (0.3ng/mL) when measured during a mixed meal tolerance test (MMTT), conducted at least 4 months from diagnosis of diabetes, and within 8 weeks of randomization
5. Must be EBV PCR negative within two weeks of randomization if EBV seronegative at screening
6. 6.Be at least 6 weeks from last live immunization
7. Be willing to forgo live vaccines for 3 months following last dose of study drug
8. Be willing to comply with intensive diabetes management
9. Normal screening values for CBC, renal function and electrolytes (complete metabolic profile).

**No more than 12 subjects with diabetes duration < 1 year will be randomized. This will ensure that the interests of the Helmsley Trust in performing studies in patients with established T1D are maintained.*

Participants must not have any of the following exclusion criteria:

1. Be immunodeficient or have clinically significant chronic lymphopenia: (Leukopenia (< 3,000 leukocytes /μL), neutropenia (<1,500 neutrophils/μL), lymphopenia (<800 lymphocytes/μL), or thrombocytopenia (<125,000 platelets/μL).
2. Have a chronic infection at time of randomization
3. Have a positive PPD
4. Be currently pregnant or lactating, or anticipate getting pregnant within the next two years
5. Require use of other immunosuppressive agents
6. Have serologic evidence of current or past HIV, Tuberculosis, Hepatitis B or Hepatitis C infection
7. Have any complicating medical issues or abnormal clinical laboratory results that interfere with study conduct, or cause increased risk to include pre-existing cardiac disease, COPD, sickle cell disease, neurological, or blood count abnormalities (e.g., lymphopenia, leukopenia, or thrombocytopenia)
8. Have a history of malignancies
9. Evidence of liver dysfunction with AST or ALT greater than 3 times the upper limits of normal
10. Evidence of renal dysfunction with creatinine greater than 1.5 times the upper limit of normal
11. Vaccination with a live virus within the last 6 weeks
12. Current use of non-insulin pharmaceuticals that affect glycemic control
13. Active participation in another T1D treatment study in the previous 30 days
14. Known allergy to G-CSF or ATG
15. Prior treatment with ATG or known allergy to rabbit derived products
16. Any condition that in the investigator's opinion, may adversely affect study participation or may compromise the study results

4. Study Medications

4.1 Thymoglobulin® (ATG)

Anti-thymocyte Globulin (ATG) (Thymoglobulin®) is indicated for the treatment of renal transplant acute rejection in conjunction with concomitant immunosuppression. Each 10 mL vial contains 25 mg anti-thymocyte globulin (rabbit) as well as 50 mg glycine, 50 mg mannitol, and 10 mg sodium chloride.

The dose of ATG for this protocol was chosen based on a combination of the demonstrated safety and efficacy in treating human autoimmune and alloimmune diseases in adults and children, and the suggestion from our animal data indicating that lower dose ATG could be used successfully when utilized in combination with G-CSF. Several autoimmune diseases (e.g., SLE, RA, Wegner's Granulomatosis, Systemic Sclerosis, and T1D) have been treated with some success with ATG based protocols utilizing doses from 2.5mg/kg to in excess of 10 mg/kg (68). In addition, induction therapy for kidney transplantation using low doses of ATG (2.5-5mg/kg) has demonstrated efficacy in protecting the graft and reducing acute rejection in both children and adults (69, 70).

In the ITN study, subjects receive ATG at a total dose of 6.5 mg/kg (0.5 mg/kg Day 1, then 2 mg/kg for next 3 doses). This dose has been effective and well tolerated in bone marrow and solid organ transplantation, and is well below the dose of 20 mg/kg that has been used for some transplantation protocols. Based on the observed efficacy in the mouse with combined low-dose ATG and G-CSF therapy, the dose of ATG was reduced, albeit somewhat empirically, to 2.5 mg/kg with the hopes of minimizing side effects while still providing for efficacy in preserving beta cell function. The dose and duration of pegylated G-CSF was chosen on the basis of its safety as demonstrated to date in the T1D study currently underway at the University of Florida, and on the basis of other 12-week G-CSF studies that have demonstrated potential for benefit in autoimmune disease (71).

4.1.1 Thymoglobulin® / Placebo Administration

All participants will be admitted to the hospital for the duration of the infusions and will be discharged no sooner than 24 hours after the last infusion. Body weight at baseline (Time 0 – admission for the Thymoglobulin®/placebo infusion) will be used in calculating the doses for all infusions.

For the Thymoglobulin® infusion, to minimize the risk for thrombophlebitis, 1000 units of heparin and 20 mg of hydrocortisone will be added to the Thymoglobulin® infusion bag for each dose given via peripheral intravenous administration. Since there is only a slight risk for thrombophlebitis for placebo infusion, the heparin and hydrocortisone will not be included in the infusion bag. If a central line is used, either peripherally or centrally inserted, the heparin and hydrocortisone will not be included in the infusion bag since the risk for thrombophlebitis is minimal.

The first dose will be infused over a minimum of 12 hours, and the second dose over a minimum of 8 hours. The second dose should be given no less than 12 and no more than 36 hours after the previous dose, unless problems arise that require drug modification or discontinuation. A maximum of 96 hours from the start of the first IV infusion will be allowed to complete all IV study drug. If a subject has not completed the two IV study drug infusions at the 96th hour, the infusion will be discontinued and they will not receive any additional study drug. Vital signs will be checked every 15 minutes for the first 4 hours of the study drug infusion and thereafter at 30-minute intervals. Blood for laboratory testing should be drawn at least 6 hours after completion of each infusion in order to obtain reliable and comparable results.

4.2 Neulasta

Pegfilgrastim (Neulasta®) is a c ovalent c onjugate o f r ecombinant m ethionyl hum an g ranulocyte c olony stimulating factor (filgrastim) and monomethoxypolyethylene glycol. Neulasta® is a Colony Stimulating Factor that acts on hem atopoietic cells by binding to specific cell surface receptors, thereby stimulating proliferation, differentiation, commitment, and end cell functional activation. Neulasta® is supplied in 0.6 mL prefilled syringes for subcutaneous injection. Each syringe contains 6 mg pegfilgrastim (based on protein weight), in a sterile, clear, colorless, preservative-free solution (pH 4.0) containing acetate (0.35 mg), sorbitol (30.0 mg), polysorbate 20 (0.02 mg), and sodium (0.02 mg) in water for injection, USP. For subjects who weigh less than 45 kg, the dose of pegfilgrastim will be adjusted.

4.2.1 Neulasta® / Placebo Administration

Neulasta®/Placebo treatment will begin no sooner than 12 hours but no greater than 36 hours after completion of the Thymoglobulin/Placebo. Neulasta® will be given subcutaneously every 2 weeks for a total of 6 doses. Normal saline will be used for the placebo injections.

4.3 Premedication:

To reduce the risk of adverse reactions to Thymoglobulin® infusion, methylprednisolone 0.25 mg/kg IV will be given no less than 30 minutes before each infusion and 0.25 mg/kg IV will be given 12 hours (±15 min) after the start of each infusion.

Placebo group: Because the risk for adverse reactions with placebo is only minimal, a placebo (saline) infusion similar in appearance will be g iven no l ess than 30 minutes before and 12 hours(±15 min) after the start of each infusion.

Both groups: Participants in both groups will be pre-medicated with an antihistamine and acetaminophen PO at least 30 minutes before each infusion and every 4–6 hours as needed during the infusion, as follows:

- Diphenhydramine 1.25 mg/kg/dose to a maximum of 50 mg.
- Acetaminophen 10–15 mg/kg/dose to a maximum of 650 mg.

If mild hypersensitivity or an infusion related event develops, the study infusion will be temporarily interrupted. Bronchodilators and saline i nfusions may be us ed if i ndicated for br onchospasm or m ild hypotension, respectively. The study infusion will continue or be resumed upon improvement of patient symptoms. Due to the potential side effect profile of ATG and G -CSF, all study subjects will be admitted for the ATG infusion portion of this protocol. All ATG infusions will take place within a CTSI with the principal investigator and/or study coordinator readily available. Specifically, the study PI or a study nurse will be i n the subject's room during the first hour of the infusion, will remain on the CTSI for the next 2 hours of the infusion, and will be readily available for the entire duration of all IV infusions. There will be in-house medical coverage during the infusion. Subjects will not be discharged from the hospital until at least 24 hou rs following the second ATG infusion. Subjects will be in the CTSI/GCRC until they can be s afely discharged in the event of an adv erse event.

4.4 Modification or Discontinuation of Thymoglobulin®

Thymoglobulin® dose may need to be reduced by 50% or more, temporarily held, or discontinued according to the guidelines listed below. However, Thymoglobulin® must be administered by the end of 96 hours. if any dose adjustments occur. If the repeat tests show acceptable values (WBC count >2,000 cells/mm3, neutrophil count >1,200 cells/mm3, and platelet count >75,000 cells/mm3), Thymoglobulin® will be resumed at the full dose.

4.4.1 Modification of Thymoglobulin® (50% dose reduction)

The Thymoglobulin®/plaebo dose will be reduced by 50% if a participant's platelet count falls between 50,000 and 75,000 cells/mm3, or if the neutrophil count falls to a value > 800 but < 1,200 cells/mm3. Although the Thymoglobulin® package insert recommends reducing the dose by 50% if a patient has a total white blood cell (WBC) count of between 2000 and 3000 cells/mm3, it has been decided not to reduce the dose in this protocol for the following reasons:

- Because the primary focus of this study is initial T-cell depletion, premature or unnecessary reduction of the Thymoglobulin® dose may result in reduced efficacy.
- If the WBC count falls to a significantly lower level, i.e. <2000 cells/mm3, then Thymoglobulin® will be held
- Participants will be closely monitored for infectious disease risk and offered prophylaxis as warranted to minimize their risk
- Neulasta® is expected to minimize the time/severity of neutropenia

4.4.2 Discontinuation of Thymoglobulin®

The Thymoglobulin®/placebo will be discontinued if any one of the following is observed:

- A total WBC count <2,000 cells/mm3 that persists up to 48 hours after the time of the planned infusion.
- A neutrophil count <800 cells/mm3 that persists up to 48 hours after the time of the planned infusion.
- A platelet count <50,000 cells/mm3 that persists up to 48 hours after the time of the planned infusion.
- The participant experiences exacerbation of Cytokine Release Syndrome (CRS) twice consecutively despite the use of glucocorticoids.
- If the investigator believes that the study treatment is no longer in the best interest of the participant

Participants who prematurely discontinue study treatment will remain in the study and undergo all efficacy and safety assessments.

4.5 Modification or Discontinuation of Neulasta®

Subjects will have a physical exam at each study visit and a CBC obtained every two weeks after the initiation of G CSF/placebo until they have completed study drug therapy. If at any time during the G CSF/placebo therapy the subject develops an ANC greater than 35 x 10^9/L, placebo doses will be given regardless of study randomization until the ANC is again lower than 35 x 10^9/L. In addition, if splenomegaly is noted on physical exam, placebo will be given for the remainder of the drug course regardless of randomization.

4.6 Management of Adverse Events

4.6.1 Cytokine Release Syndrome:

With Thymoglobulin® infusion, the subject may experience Cytokine Release Syndrome (CRS). The signs and symptoms can span a wide clinical spectrum.

Mild Reactions: For mild (grade 1) reactions per the NCI-CTCAE for CRS, the study medication will be continued. The investigator shall take one or more of the following actions, depending on the type of the reaction:

1. Administer additional doses of antihistamine and acetaminophen.
2. Reduce the rate of infusion by 50% or more.
3. For chills and rigors, meperidine may be considered.

Moderate Reactions: For moderate (grade 2) reactions per the NCI-CTCAE for CRS, the study medication may be interrupted. The investigator shall take the following actions, depending on the type of the reaction:

1. Interrupt infusion if any of the following occurs:
   a. Oral temperature of > 40.0 °C
   b. Symptomatic bronchospasm or pulmonary edema
   c. Allergy-related edema
   d. Hypotension
2. When the temperature is < 38.5 °C and signs and symptoms improve, restart Thymoglobulin.
3. Closely monitor the subject with pulse oximetry and a blood pressure monitoring; provide ongoing nursing evaluation until at least 2 hours after the infusion is completed.
4. If necessary, glucocorticoids can be given every 6 hours at a dose of 0.5 mg/kg of methylprednisolone or equivalent.
5. If a subsequent dose of Thymoglobulin® further exacerbates the signs and symptoms of CRS despite following the above guidelines, the study treatment must be permanent discontinued.
6. Additional supportive or resuscitative measures (such as the use of epinephrine) may be needed if clinically indicated.

Severe Reactions: For severe (grade 3) reactions or greater per the NCI-CTCAE for CRS , the study medication will be permanently discontinued.

4.6.2 Allergic Reactions

Hypersensitivity: In rare cases, patients may experience hypersensitivity, which refers to immediate allergic, IgE mediated reactions to Thymoglobulin. Such patients primarily develop skin rash and respiratory distress early in the course of the infusion (usually within the first hour). In very rare cases patient may develop a grade 4 allergic reaction (anaphylaxis). For such reactions, the investigator shall take one or more of the following actions:

1. Discontinue the infusion.
2. Apply appropriate resuscitation measures, including administration of 0.3–0.5 mL aqueous epinephrine (1:1000 dilution) subcutaneously.
3. Use other resuscitative measures, as clinically indicated, including oxygen, intravenous fluids, antihistamines, corticosteroids, pressor amines, and airway management.

Mild to Severe Reactions: For mild to severe (grade 3 or less) reactions per the NCI-CTCAE for allergic reactions, the study medication may be restarted at the discretion of the investigator. For those with severe (grade 3) reactions, any subsequent doses should be accompanied by pre-medication with additional corticosteroids.

Life-threatening Reactions: For life-threatening (grade 4) reactions per the NCI-CTCAE for allergic reactions, the study medication will be permanently discontinued.

4.6.3 Serum Sickness

Serum sickness from host immunization against rabbit protein may occur 7–15 days after the first dose of ATG. The patient may require glucocorticoid treatment for supportive care. The dose will depend on the severity of signs and symptoms. For severe symptoms, we recommend a maximum dose of 1.5 mg/kg prednisone PO per day for 3 days with rapid tapering such that the total treatment course is given over 7 days.

4.7 Concomitant Mediations

The use of concomitant medications will be assessed at each study visit and recorded on an appropriate source document and CRF. Participants are allowed to use preparations of insulin as advised by the investigator or the referring physician.

The following medications are contraindicated during participation in this study:

- Agents that influence insulin sensitivity or secretion (pramlintide, sulfonylureas, metformin, diphenylhydantoin, thiazide, or other potassium-depleting diuretics, beta-adrenergic blockers, niacin).
- Live vaccination 6 weeks before enrollment and throughout study participation. (Live vaccines include varicella, measles, mumps, rubella, cold-attenuated intranasal influenza vaccine, Bacillus Calmette-Guérin, and smallpox.)
- Any medication that may result in immunosuppression or immunomodulation, including
- Systemic glucocorticoids (unless required during Thymoglobulin administration or for the treatment of cytokine release syndrome or serum sickness).

If participants receive, or if the investigator believes that participants must receive, any of the above medications, the case must be immediately discussed with the medical monitor. The use of prohibited medications must be documented on the source document and CRF, and a protocol deviation must be requested. A decision regarding continuation of the participant in the trial will be made by the study PI, and the medical monitor.

4.7.1 Prophylaxis for PCP

Immediately after Thymoglobulin® treatment, the resultant T-cell depletion may render treated participants vulnerable to opportunistic infections. All participants receiving Thymoglobulin® will receive prophylaxis for *Pneumocystis carinii* pneumonia (PCP). The standard regimen is one single-strength trimethoprim-sulfamethoxazole tablet (80 mg400 mg) orally daily. For sulfa-allergic patients, inhaled pentamidine may be used for PCP-prophylaxis once per month: adults and children 5 years of age and older may be administered 300 mg every 4 weeks. Treatment should begin on the day following Thymoglobulin® infusion, at hospital discharge. The placebo group will receive placebo-trimethoprim-sulfamethoxazole or placebo-pentamidine inhalation.

<u>Treatment will continue for a minimum of 3 months or until the patient's CD4 count is greater than 200 cells/mm3, whichever comes later.</u>

If other changes in PCP prophylaxis are deemed necessary by the investigator because of adverse reactions, they should be discussed with the medical monitor.

4.7.2 CMV Monitoring and Treatment

No prophylaxis will be given for CMV. Both seropositive and seronegative subjects will undergo regularly scheduled monitoring for CMV viremia by quantitative PCR. Treatment will be given based on parameters described below.

The decision to use preemptive therapy rather than universal prophylaxis for CMV-seropositive subjects is based upon several factors:

- Therelatively transient nature of lymphopenia expected and the absence of additional immunosuppression

- Use of low dose Thymoglobulin
- The experience that many solid-organ transplant recipients spontaneously resolve low-level CMV reactivation
- Long-standing experience with the use of ATGAM plus cyclosporin in patients with aplastic anemia who rarely, if ever, experience significant CMV reactivation.

Therefore, the risk of serious CMV disease is thought to be low, and the potential benefits of prophylaxis in a small number of subjects may well be outweighed by adverse events with universal prophylaxis. However, if treatment is required for CMV for three or more of the first 10 CMV seropositive patients treated with Thymoglobulin, then universal prophylaxis will be reconsidered for subsequent seropositive enrollees.

4.7.3 CMV-seropositive Subjects

- If quantitative PCR for CMV is > 1,000 copies/mL, the subject will have weekly PCR performed.
- If quantitative PCR for CMV is > 1, 000 and < 10,000 copies/mL, the subject will be treated if concomitant symptoms, signs, or laboratory abnormalities consistent with CMV infection are present (e.g., fever [>100.4 ° F], malaise, weakness, lymphadenopathy, hepatomegaly, splenomegaly, leukopenia, thrombocytopenia, and elevated AST and ALT.)
- If quantitative PCR for CMV is > 10,000 copies/mL, the subject will be treated for CMV reactivation.

4.7.4 CMV-seronegative Subjects

A positive PCR on a second sample from a previously seronegative subject indicates primary infection. Because primary infection during Thymoglobulin-induced lymphopenia confers higher risk for tissue invasive disease than reactivation in seropositive subjects, the threshold for treatment of CMV in previously seronegative subjects is lower. Once the PCR is below the limit of detection, monthly monitoring may be resumed. These subjects should have repeat CMV serology performed to document seroconversion:

- If quantitative PCR is > 1,000 copies/mL, the subject will have weekly PCR performed.
- If quantitative PCR is two-fold compared with the previous measurement or >2,000 copies/mL
- will be treated for primary CMV infection

4.7.5 CMV Treatment

- *Induction* treatment: 900 mg valganciclovir PO 2 times daily, or adjusted for renal function for 2 weeks.
- Maintenance treatment: 900 mg valganciclovir PO per day, or adjusted for renal function for 2 weeks or until PCR is undetectable.

4.8 Epstein-Barr Virus Monitoring and Treatment

EBV-seronegative subjects will be monitored in two ways:

- At each visit post treatment, during which adverse events are reviewed, the subjects will be questioned regarding signs or symptoms suggestive of EBV reactivation, e.g., fever (>100.4°F), lymphadenopathy, pharyngitis or tonsillar swelling, skin rash, weight loss, malaise, or fatigue.
- EBV serology and quantitative PCR will be performed for EBV viremia beginning at week 2 visit

EBV-seropositive subjects will be monitored in two ways:

- At each visit post treatment, during which adverse events are reviewed, the subjects will be questioned regarding signs or symptoms suggestive of EBV reactivation, e.g., fever (>100.4°F), lymphadenopathy, pharyngitis or tonsillar swelling, skin rash, weight loss, malaise, or fatigue.
- Quantitative PCR will be performed for EBV viremia beginning at week 2 visit

In case of symptoms or signs suggestive of EBV reactivation at any time, a PCR will be performed:

- If quantitative PCR > 1,000 copies/mL on a screening test or on a test performed for symptoms will then have PCR performed one week later and weekly thereafter.
- If quantitative PCR is persistently positive or rising, subjects will be thoroughly examined for evidence of EBV-related disease, including fever, lymphadenopathy, pharyngitis, hepatomegaly, splenomegaly, liver function abnormalities, atypical lymphocytosis, or leukopenia.
- If quantitative PCR is > 10,000 copies/mL, strong consideration for treatment with valacyclovir (1 g PO tid) should be prompted. However, a recent study with an anti-CD3 monoclonal antibody showed that EBV reactivation was cleared spontaneously without intervention. Therefore, individual site investigators may choose to closely observe those with no or mild clinical signs and symptoms without treatment but will continue to monitor weekly PCR results and will maintain a low threshold for initiation of antiviral therapy.

Persons whose symptoms do not resolve spontaneously over 4–6 weeks or who display progression will prompt further evaluation. Persons with localized or diffuse lymphadenopathy should undergo lymph-node biopsy with appropriate histologic and immunohistochemical evaluation for EBV genomes and gene products. Further evaluation may include a CT scan of the chest, abdomen, and pelvis to investigate possible visceral lymphadenopathy, liver and spleen size and homogeneity, and masses in the lungs or abdominal cavity. Any such event is believed to be highly unlikely, but if it occurs, consultation with an oncologist should be obtained for consideration of further diagnostic and treatment options (e.g., liver biopsy, characterization of the clonality and origin of lymphoid proliferation, and B-cell ablation with rituximab).

4.9 Herpes Simplex 1,2 and Varicella Zoster Monitoring and Treatment

Subjects will be screened for serologic evidence of prior infection with herpes simplex virus (HSV1 and HSV2) and varicella zoster virus (VZV). Subjects treated with Thymoglobulin® who are seropositive for prior infection with HSV1, HSV2, or VZV will receive oral acyclovir prophylaxis for a minimum of 3 months or until their CD4 count is greater than 200 cells/mm3, whichever comes later. Subjects who weigh 50 kg or more should receive 400 mg acyclovir PO twice daily; those who weigh less than 50 kg should receive 200 mg PO twice daily. Treatment should begin on the day following completion of Thymoglobulin infusion, at hospital discharge.

Subjects who are VZV seronegative will be excluded at centers without access to VariZIG®. For centers with access subjects will be cautioned to avoid any contact with individuals who have chickenpox or shingles during the 3 months following treatment with Thymoglobulin®. These subjects are at significant risk for severe varicella, particularly if they are not receiving acyclovir prophylaxis for HSV. Participants will be counseled to report any such contact immediately in order to receive the varicella immunoglobulin within 96 hours of exposure. The placebo group will receive placebo-acyclovir as described above.

5. Study Procedures

5.1 Informed Consent

The potential participant will sign an informed consent form, in which the research trial will be explained in layman's language, before undergoing any study-specific screening procedures. Both a screening and treatment consent will be used.

5.2 Screening

The screening visit cannot occur until the participant has signed the informed consent. At the screening visit, the participant will undergo the following assessments: medical history, physical examination with height and weight, blood draw for serum chemistries, CBC, and other clinical and immunologic tests (see Table of Study Assessments), and, if female and of childbearing potential, a pregnancy test. The subject will then undergo a standard 4 hour Mixed Meal Tolerance Test (MMTT). Subjects who meet all inclusion and exclusion criteria will be randomized after the potential participant has signed the written informed consent.

5.3 Randomization and Blinding

Participants who meet the inclusion and exclusion criteria will be randomly assigned in a 2:1 fashion to either the Thymoglobulin®/Neulasta® group or the placebo group. Participants must receive their first study drug dose within 56 days (8 weeks) from their screening MMTT. Each randomized participant will be given a unique participant number. Participants will be randomly assigned to their treatment groups stratified by study site.

5.4 Unblinding

Before emergency unblinding of individual participants, the medical monitor will be notified. The site investigator will inform the study PI and the IND sponsor of the unblinding event. All unblinding information will be recorded and reported to the DSMB. Any unblinding event will require a full written account including the name of the medical monitor who was notified, date, time, and reason for the unblinding of treatment for an individual participant. During site visits, the site monitor must verify that the medical monitor of the trial was notified and that a written account was completed. The reasons for unblinding each individual participant's treatment will be included in the final study report.

5.5 Clinical Procedures

A 4-hour MMTT will be administered to all participants at screening and at 6-month intervals throughout the 24-month study. Glucose and C-peptide levels for each test will be obtained at time -10, 0, and at 15, 30, 60, 90, 120, 150 , 180 , 210 , and 240 minutes after oral ingestion of Boost®(6 mL/kg, max 360mL). A1c will be assessed every 3 months throughout the study to optimize diabetes management.

Insulin use and hypoglycemic episodes will be recorded on appropriate case report forms (CRFs). Participants will periodically undergo blood draws for both clinical and immunologic tests. Blood volumes drawn will be based on participant body weight and may be modified to limit the total blood volume drawn at any one visit.

Participants are required to have a CBC performed after each infusion in a timely manner so that the results are available before the next infusion.

5.6 Intensive Diabetes Management

During the study period, all participants will be expected to maintain “intensive” management of their diabetes,. A1c will be assessed every 3 months to evaluate metabolic control. The goal of treatment will be to maintain the A1c level as close to normal as possible, without frequent occurrence of hypoglycemia. All individuals should strive for targets in accordance with current American Diabetes Association recommendations, with A1c levels of < 7% in adults and < 8% in adolescents (age 13-16 years), and with preprandial glucose levels of 90–130 mg/dL (plasma), postprandial levels of <180 mg/dL, and bed time levels of 110–150 mg/dL. During the hospitalization, more frequent glucose monitoring will occur, and insulin dosing will be adjusted as necessary in order to maintain glycemic control.

All participants will be expected to take a sufficient number of daily insulin injections to meet the glycemic targets. In general, the expectation is that all participants will receive at least three injections of insulin daily, including short- and long-acting insulin preparations, or will utilize continuous subcutaneous insulin infusion (CSII insulin pump). Glucose levels should be checked at least four times daily. After reviewing these records, the local diabetes management team will contact the treating physician about possible adjustments in the insulin regimen, referral to a registered dietitian, or other approaches that the diabetes management team believes would improve the glucose control if necessary. Records of glucose measurements and communication with the participant will be kept as source documentation. However, diabetes management will not be provided directly as a part of the study.

Insulin use and hypoglycemic events will be captured at each 3 and 6 month visit on the appropriate CRFs. Participants will be required to record the type and amount of insulin they have used during the 5-day period immediately preceding each study visit. Insulin use logs will be provided to participants at each study visit and collected at the next visit. These logs will serve as the source documents.
Participants who fail to achieve an A1c level within the stated goals will not be excluded from the study, but additional measures will be instituted to improve the glycemic control. Any episodes of severe hypoglycemia (i.e., unconsciousness, seizure, or needing the assistance of another individual to correct the hypoglycemia) will prompt a review of the cause of the episode and adjustment of insulin dosing, diet, and exercise as deemed appropriate.

5.7 Hypoglycemia

For the purposes of this study, hypoglycemic events will be recorded as follows:

- Minor hypoglycemia will be defined as a glucose concentration of <65–55 mg/dL.
- Major hypoglycemia will be defined as a glucose concentration <55 mg/dL (grades 2–5, NCICT CAE version 4.0), or clinically: involving seizure or involving loss of consciousness (coma), or requiring assistance from another individual in order to recover.

All episodes of hypoglycemia that require hospitalization and/or emergency care will be reported to the DSMB.

5.8 Discontinuing Insulin

Exogenous insulin will be continued for all participants unless they reach an insulin-free endpoint, as defined in the Immunology of Diabetes Society guidelines for new-onset trials; that is, normal A1c levels on two occasions 3 months apart. Subjects should then have documentation of a normal standard oral glucose tolerance test after omitting insulin for 3 consecutive days. The subjects will continue to be followed closely in the context of the study, with repeated A1C and further glucose tolerance tests at 3 month intervals, and

reinstitution of insulin if abnormalities in metabolism are present as per standard American Diabetes Association guidelines.

5.9 Study Visits

Participants will have biweekly contact with the study staff by means of scheduled study visits, unscheduled visits, telephone calls, or email. At a minimum, participants should be assessed for adverse events, insulin use, and concomitant medications during any unscheduled visit. An early termination visit will be required for those who withdraw consent and decide not to continue their participation in the study. All the assessments listed for month 12 will be performed for participants who withdraw from the study before month 12. All assessments listed for month 24 will be performed for participants who withdraw between months 12 and 24.

5.10 Visit Windows

Visits	Window
-1	-
0	-
D1	Infusion must begin < 56 days from date of screening
D2	At least 8 hours after day 1 infusion completed and no more than 48 hours after day 1 infusion started
D3	Neulasta/Placebo must be given > 12 hours after Thymoglobulin/Placebo is completed and no more than 36 hours after completion
1 Wk	+/- 2 days of Target
2 Wk	+/- 2 days of Target
4 wk	+/- 2 days of Target
6 weeks	+/- 2 days of Target
8 weeks	+/- 3 days of Target
10 weeks	+/- 3 days of Target
12 weeks	+/- 3 days of Target
6 to 24 months	+/- 7 days of Target
30 to 60 months	+/- 30 days of Target

5.11 Long Term Follow-Up

Participants who have peak residual c-peptide levels above 0.017 ng/mL at the 24 month visit will be invited to participate in long term follow up. These follow up visits will continue every 6 months until the 60 month time point is reached or the the peak residual c-peptide is less than 0.017 n.g/mL as measured in a two hour Mixed Meal Tolerance Test. Study assessments will be as noted on the Study Assessments – Follow up table.

Follow up between visits will be 2-3 times. Subjects will also continue to have reasonable travel provided and will be paid $100 per MMTT.

<u>6. Safety Monitoring:</u>

6.1 Data Safety Monitoring Board (DSMB)

A study-wide DSMB will be established using experts from the University of Florida and other academic centers in the United States. The DMSB members will include at least one pediatric endocrinologist (Dr. Arlan Rosenbloom, University of Florida), one infectious disease expert (Dr. Jay Taureen, University of California, San Francisco), and one hematologist/oncologist (Dr. William Slayton, University of Florida). In addition, data will be closely monitored by an independent medical monitor (Dr. Brett Lochelt, George Washington University)

and will be reported to all parties as required including the multisite IRBs. The DSMB will meet every 6 months to review interim analyses of efficacy and adverse events. All adverse events will be recorded on adverse event forms which will be provided to the local IRBs per local reporting requirements and to the University of Florida Coordinating Center. The DSMB will independently evaluate whether the adverse events constitute grounds to discontinue the study. After the last subject has reached the end of study treatment (Week 12), then the next time the DSMB will review adverse events will be at the end of the study. That is, when subjects have reached the last study visit.

DSMB reports will also include conditional power analyses conducted both under the study hypotheses and under the current trend of the data to allow early termination due to futility (i.e., lack of beneficial treatment effect). Additional analysis will assess potential adverse outcomes of treatment and will assess the incidence of all severe adverse events. The DSMB may require changes to the study protocol.

A planned futility evaluation will be per formed when the 12th subject has reached the 1 year follow up visit. Early termination will be based on the treatment effects on C-peptide values at 1 year of follow-up.

6.2 Independent Medical Monitor

In addition to the DSMB an independent medical monitor will review all AE reports and will perform data review. A pause in enrollment will be initiated after the first 3 subjects have completed their course of study drug in order to allow the independent medical monitor to assess study progress. With the monitor's approval, enrollment will resume. After the 12th subject has reached the 3 month post treatment visit a second safety review will be performed. Enrollment will again be temporarily suspended to allow for safety analysis and further enrollment will be predicated on approval from the independent medical monitor. If liver function fests (LFTs) are confirmed greater than 3 times the upper limit of normal (ULN) in more than one subject, then enrollment will be stopped for a safety evaluation and will not be restarted until approval is provided by the DSMB. Additional stoppages to evaluate safety will be at the discretion of the medical monitor and the DSMB.

6.3 Adverse Event Reporting

Study personnel will assess adverse events and the use of concomitant medications throughout the study. Adverse events will be reported to the University of Florida Coordinating Center via a web based data entry system, designed by the Clinical & Translational Research Informatics Program (CTRIP) at the University of Florida, that will immediately notify both the medical monitor and the study PI. Adverse events will be graded as to severity according to common toxicity criteria or study-specific criteria, and the investigator will make a determination as to the relation to therapy. Events will be assessed and reported in accordance with the ICH Guideline for Good Clinical Practice and per the guidance of the DHHS Office for Human Research Protections (OHRP).

In addition, Serious Adverse Events and Adverse Events will be reported to Genzyme Pharmacovigilance. The investigator shall, within twenty-four (24) hours (US) or one business day (EU) of first knowledge of such serious and related adverse event, will notify Genzyme via fax, attention Genzyme Pharmacovigilance (PV), 617-761-8506 (US) or +1-617-761-8506 or by e-mail to pharmacovigilancesafety@genzyme.com. Prior to or at the time of filing any such report with the governing regulatory authority, the Investigator will also transmit an information copy of the report as sent to the authorities to Genzyme PV. The Investigator shall make available to Genzyme promptly such records as may be necessary and pertinent to investigate any such expedited adverse event, if specifically requested by Genzyme.

Furthermore, the Investigator will inform Genzyme of the following:

- ☐ Any events that result in protocol amendments for safety reasons, as well as any safety related regulatory action such as a clinical hold of the Research.
- ☐ Any pregnancies occurring in patients who are exposed to the Product in connection with the Research.
- ☐ In addition, the Investigator shall notify Genzyme within twenty-four (24) hours (US) or one business day (EU) of first knowledge of any Product complaints (communication of dissatisfaction that alleges deficiencies related to the identity, quality, durability, effectiveness, safety, labeling, purity, stability, and appearance) by email to GEMG@genzyme.com or via fax to 508-661-8771 (US) or Genzyme Customer Services Europe, +31 (0)35 699 1222.
- ☐ The Investigator will also inform Genzyme within one (1) business day of becoming aware of any actions from any authority that may affect the performance of the Research

The adverse event case report form for the protocol will be completed for all adverse events (AE) of Grade 2 or greater severity regardless of relationship to therapy. For reporting serious adverse events (SAE), a MedWatch Form will also be completed and faxed to the University of Florida within 24 hours of when the site was notified of the event. This will be reviewed by the Medical Monitor and the DSMB as appropriate. Deaths will be reported immediately. Event outcome and other follow-up information regarding the treatment and resolution of the event will be obtained and reported when available, if not known at the time the event is reported. The follow-up information will contain sufficient detail to allow for a complete medical assessment of the case and an independent determination of possible causality. All adverse events will be assessed by the Medical Monitor. The DSMB will conduct regular safety reviews approximately every six months (and as needed) of adverse events by treatment group assignment. Serious adverse events as well as adverse events leading to treatment discontinuation will be reviewed by the DSMB.

6.3.1 Grading and Attribution

The study site will grade the severity of adverse events experienced by study participant according to the criteria set forth in the National Cancer Institute's Common Terminology Criteria for Adverse Events Version 4.0. 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.

Adverse events will be graded on a scale from 1 to 5 according to the following standards in the NCI CTCAE manual:

Grade 1 = mild adverse event.
Grade 2 = moderate adverse event.
Grade 3 = severe and undesirable adverse event.
Grade 4 = life threatening or disabling adverse event.
Grade 5 = death.

All adverse events will be reported and graded whether they are or are not related to disease progression or treatment.

6.3.2 Definition of Attribution

The relationship, or attribution, of an adverse event to an investigational product will be determined by the site investigator. The site investigator will also record the determination of attribution on the appropriate CRF and/or SAE report form. The relationship of an adverse event to the study treatment will be defined as noted below.

<u>Unrelated Category:</u>

1 Unrelated : The adverse event is clearly not related to the investigational agent(s).

Related Categories

2 Unlikely : The adverse event is doubtfully related to the investigational agent(s).
3 Possible : The adverse event may be related to the investigational agent(s).
4 Probable: The adverse event is likely related to the investigational agent(s).
5 Definite : The adverse event is clearly related to the investigational agent(s).

6.3.3 Serious Adverse Events

Serious adverse events will be collected from the time the participant begins study treatment until 30 days after he or she completes study treatment or until 30 days after he or she prematurely withdraws from the study.
Recording Procedure: Serious adverse events will be recorded on the adverse event CRF and on the SAE form.

Reporting Procedure: The following process for reporting a serious adverse event ensures compliance with the ICH guidelines and U.S. FDA regulations.

Reporting Criteria: After the SAE has been assessed, the event will be reported to the appropriate health authorities in the required manner based on the following criteria:

Standard reporting (i.e., should be included in the IND annual report to the health authorities). This requirement applies if the adverse event is classified as any of the following:

- Serious, expected, and drug-related.
- Serious, expected, and not drug-related.
- Serious, unexpected, and not drug-related.

Expedited reporting. This requirement applies if the adverse event is considered serious unexpected, and drug-related. This type of SAE must be reported by the sponsor to the appropriate health authorities within 15 days; fatal or life-threatening events must be reported within 7 days. All serious adverse events and unexpected adverse events will be reported to local IRBs within 24 hours, or sooner if mandated by the IRB, from the time the investigator learns of such events.

Reporting Timeline: When an investigator identifies an SAE, he or she must complete the study specific AE/SAE CRF and submit to the University of Florida Coordinating Center within 24 hours of discovering the event via the secure web based data reporting system. A confirmation e-mail will be sent to the investigator within 1 business day. The investigator should contact Dr. Michael Haller if this confirmation is not received. This process and timeline applies to both initial and follow-up SAEs.

Notifying the Institutional Review Board and Ethics Committee: In addition to reporting adverse events to the University of Florida, each site will be responsible for reporting to their local IRB per institutional guidelines.

.

6.4 Stopping Rules for Premature Termination of the Study

Enrollment will be suspended if any one of the following occurs:

1. The Data and Safety Monitoring Board (DSMB) requests termination of the study upon review of safety data.
2. Any death related to study therapy.

3. Two or more of the first 10 treated participants experience an adverse event resulting in the permanent discontinuation of study treatment.
4. A case of significant EBV-associated lymphoproliferative disease defined as lymphadenopathy (focal or diffuse), or hepatosplenomegaly, or organ infiltration, with or without fever, of 1 month or greater duration, and confirmation of EBV by tissue biopsy or viral load.
5. The medical monitor will be immediately notified of any such event. Resumption of enrollment is contingent upon a favorable medical monitor review.

7. Statistical Analysis:

This is a pilot and feasibility study where monitoring of safety for this combination, in this population, is a key outcome. While our hope is that the metabolic outcomes will be present to the degree that statistical significance will be achieved, it is possible that this will not occur. In order to account for potential dropouts and protect the limited statistical power of this pilot study, we will seek to randomize 25 subjects but base our power calculations on following only 21 subjects.

Presumptive power calculations for the 21 subject sample size demonstrates that the study would be sensitive to a difference of 1.23 SD in the AUC ratio, at P=0.10 (two-sided) and 80% power to reject the primary null hypothesis in favor of ATG & GCSF. For normally distributed variables, this corresponds to the median of one group (50^{th} percentile) being the 11^{th} or 89^{th} percentile of the other. In comparison to data from Dr. Herold's anti-CD3 studies, a conservative SD for these ratios will be about 40%, and hence the study will be sensitive to a difference in responses of approximately 50% (Herold, 2003).

7.1 Primary Outcome

The primary outcome of each participant will be the stimulated C-peptide AUC over the first 2 hours of a 4-hour mixed meal glucose tolerance test, conducted at the 1 year visit. The AUC is computed using the trapezoidal rule that is a weighted sum of the C-peptide values over the 120 minutes. By the mean value theorem of integral calculus, the weighted mean C-peptide in pmol/mL is simply AUC/120. We shall carry the last observation forward for those completing at least six months.

The primary statistical hypothesis to be assessed in the study is whether the mean C-peptide value for study subjects receiving Thymoglobulin® and Neulasta® differs significantly from the mean value for placebo subjects.

The primary analysis will employ the AUC defined above for the 2 hour AUC for each participant. This will be transformed to limit the role of outliers as *log[AUC/120)+1]*. The comparison between the two treatment arms will be based on a t-test of treatment effect in an ANCOVA model adjusting for gender, baseline age and baseline *log(C-peptide+1)* (72).

7.2 Secondary Metabolic Outcomes

1. A log rank test of the difference in the hazard function between groups in the incidence of the loss of the 2 hour peak C-peptide < 0.2 pmol/ml on a semi-annual MMTT
2. Longitudinal analyses using mixed effects models with a random intercept and slope of the C-peptide values over the post-treatment period, adjusted for the baseline level of C-peptide. The average intercept and slope will be compared between groups adjusting for age, gender and the baseline *log(C-peptide+1)*.
3. Mean area under the stimulated C-peptide AUC over 4 hours at 24 months
4. A1c levels over time
5. Insulin dose (units/kg) over time
6. Number of severe hypoglycemic events

The mean levels of quantitative variables (e.g., A1c and insulin dose) over all follow-up values will be compared between groups using a normal errors longitudinal analysis. The prevalence of a binary characteristic (e.g., yes/no or positive/negative) at a single visit (e.g., a history of hypoglycemia during follow-up) will be assessed using a logistic regression model (76). The prevalence of a binary characteristic over time will be assessed using generalized estimating equations. The rates of severe hypoglycemic events and severe adverse events will be computed (total number of events divided by total patient years of follow-up) and the rates compared using a Poisson regression model, allowing for over-dispersion using a quasi-likelihood model as appropriate. Tests of significance will employ a robust estimate of the variance (73). These analyses will also be conducted to adjust for age, gender, baseline *log(C-peptide+1)* and baseline A1c; and by race/ethnicity, as appropriate. Analyses will also be conducted to examine the effect of HLA or other genotype. Analyses will also be conducted to assess heterogeneity of the effect of treatment group (the group difference) as a function of age, gender, baseline *log(C-peptide+1)* and baseline HbA1c; and by race/ethnicity and HLA or other genotype. Heterogeneity will be assessed using a test of treatment group by covariate interaction in an appropriate regression model.

7.3 Secondary Immunological Outcomes

1. Assessing whether the combination of Thymoglobulin® and Neulasta® therapy alters the frequency and/or functional capacity of Treg in peripheral blood.
2. Examining the course of anti-islet cell immunity as assessed by the production of T1D associated islet cell autoantibodies (i.e., islet cell cytoplasmic, glutamic acid decarboxylase, and IA-2 autoantibodies).
3. Monitoring the effect of this therapeutic intervention on the humoral and cellular immune response against immunization based antigens (e.g.,tetanus), environmental antigens e.g., Candida), as well as islet antigens including insulin and GAD.
4. Analyzing dendritic, B and T cell markers by flow cytometry and serum cytokines.

7.4 Safety Outcomes:

The rate of the following adverse events in patients receiving Thymoglobulin® and Neulasta®
1. Infusion reactions
2. Cytokine release syndrome (CRS)
3. Opportunistic infections
4. Lymphopenia
5. CD4/CD8 ratio
6. Neutropenia
7. Thrombocytopenia
8. Serum sickness
9. Frequency and severity of all adverse events in participants receiving Thymoglobulin/Neulasta or placebo
10. Adverse event frequencies in the Thymoglobulin® and Neulasta® group will be compared to those in the control group.

Technical Support

Statistical support will be provided by Dr. Jonathan Shuster in his role as biostatistician for the University of Florida GCRC/CTSI. In addition to analyzing the data, Dr. Shuster will provide a study-wide randomization schema. Data collection and data management will be provided by CTRIP through their creation of a web-based data entry portalThe costs of this support has already been obtained via a CTSI major initiative award to Dr. Michael Haller and CTRIP faculty.

8. Study Assessments:

	--[1]	0	D1	D2	D3	1 wk	2	4	6	8	10	12	6 m	9	12	18	24[4]
ATG/Placebo: D1 and 2			X	X													
GCSF/Placebo q 2 wk6					X		X	X	X	X	X						
History	X	X															
Physical exam	X	X				X	X	X	X	X	X	X	X	X	X	X	X
PPD Test (read in 48-72 hrs)	X																
CBC with differential	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
CD4/CD8 Ratio		X				X		X		X		X	X[5]	X[5]	X[5]	X[5]	X[5]
Chemistries	X	X				X	X	X	X	X	X	X	X	X	X	X	X
Urine pregnancy test (if female)	X	X					X	X	X	X	X	X	X	X	X	X	X
Urinalysis	X	X				X	X	X		X							
HIV, Hep B, Hep C, Varicella, HSV	X																
IgG		X															
EBV serology/PCR[2]	X[2]						X[2]	X[2]		X[2]		X[2]	X[2]	X[2]	X[2]	X[2]	X[2]
CMV serology and PCR[3]	X[3]						X[3]	X[3]		X[3]		X[3]	X[3]	X[3]	X[3]	X[3]	X[3]
HLA determination		X															
Serum for autoantibodies	X							X				X	X	X	X	X	X
Mechanistic studies		X				X	X	X				X	X		X	X	X
Adverse Events Assessments			X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Hemoglobin A1c	X											X	X	X	X	X	X
Mixed Meal Tolerance Test (4 hr)	X														X		X
Mixed Meal Tolerance Test (2 hr)												X	X			X	
Insulin Dosing Assessment		X				X	X	X				X	X	X	X	X	X
Hypoglycemia assessment		X				X	X	X				X	X	X	X	X	X
Repository		X				X	X	X				X	X		X		X
Rubella titers		X						X					X		X	X	X
Viral flu titers		X						X					X		X	X	X

--[1]: must be done before to ensure eligibility
EBV[2]: this testing is completed as scheduled for all study entrants who are **EBV seropositive** at randomization, (samples drawn will be for PCR analysis only). If study entrants are **EBV seronegative** at the screening visit, then an EBV PCR must be done within 2 weeks of enrollment. Thereafter, both EBV serology and EBV PCR will be drawn for **EBV seronegative** study entrants..
CMV[3]: this testing is completed as scheduled (i.e. including both serology and PCR) for all study entrants who are CMV seronegative at randomization. If seropositive at randomization, then samples drawn will be for PCR analysis only
[4]Month > 24: Post-treatment follow-up visits will continue every 6 months for up to 5 years total follow up for participants with residual c-peptide at the 24 month visit.
[5] The CD4/CD8 ratio will continue to be monitored until the CD4 count is above **500.**

8. Study Assessments – Long Term Follow up

Follow Up Study Assessments/ # months after Treatment	30 m	36 m	42 m	48 m	54 m	60 m
History	X	X	X	X	X	X
Physical exam	X	X	X	X	X	X
CBC with differential	X	X	X	X	X	X
CD4/CD8 Ratio[1]	X[1]	X[1]	X[1]	X[1]	X[1]	X[1]
Chemistries	X	X	X	X	X	X
Urine pregnancy test (if female)	X	X	X	X	X	X
Serum for autoantibodies	X	X	X	X	X	X
Mechanistic studies	X	X	X	X	X	X
Adverse Events Assessments	X	X	X	X	X	X
Hemoglobin A1c	X	X	X	X	X	X
Mixed Meal Tolerance Test (2 hr)	X[2]	X[2]	X[2]	X[2]	X[2]	X[2]
Insulin Dosing Assessment	X	X	X	X	X	X
Hypoglycemia assessment	X	X	X	X	X	X
Repository	X	X	X	X	X	X
Concomitant Medication Review	X	X	X	X	X	X

[1]The CD4/CD8 ratio will continue to be monitored until the CD4 count is above **500**.
[2]Post-treatment follow-up visits will continue until the c-peptide is less than 0.017.

9. Protection of Human Subjects:

9.1 Human Subjects Involvement and Characteristics

All patients with T1D, regardless of race or gender and greater than 16 years of age who meet eligibility criteria will initially be considered for randomized. The carcinogenic effects and the effect of ATG and G-CSF on fertility have not been established. Pregnant and lactating women will not be included in the study. Sexually active females must have a negative pregnancy test prior to enrolling in the study and will be required to use effective birth control during the study. At every study visit the sexual activity of female participants of reproductive age will be re-assessed. If a subject who was previously sexually inactive becomes sexually active, she will be counseled about the need to use a reliable form of birth control. Subjects will be requested to avoid pregnancy for 2 years after the last therapy and instructed to use birth control.

9.2 Sources of Materials

Blood and urine specimens will be collected per protocol. Study related data required per protocol will be captured on source documents for the study. Data related to current health, blood glucose monitoring and diabetes control will be collected on source documents.

9.3 Potential Risks

9.3.1 ATG

ATG has been widely used in transplantation and autoimmune disorders (42). With the low dose, the absence of other immunosuppressive medications, and the lack of severe medical conditions other than T1D we expect minimal side effects. In the ITN study, subjects receive ATG at a total dose of 6.5 mg/kg, a dose which has been effective and well tolerated in bone marrow and solid organ transplantation, and well below the dose of 20 mg/kg that has been used for some transplantation protocols. The doses will be administered as 0.5 mg/kg on day 1, and then at 2 mg/kg on day 2 to reduce reactions. ATG will be given slowly by continuous IV infusion over at least a 12 hour period on day 1, and at least an 8 hour period on day 2. Subjects will receive pre-medication with methylprednisolone, acetaminophen and diphenhydramine to minimize side effects from possible cytokine release syndrome (CRS), and will be closely observed in the hospital during the treatment period. Symptoms of CRS may include fever, chills, rigors, headache, tremor, nausea, vomiting, diarrhea, abdominal pain, muscle and joint pain, and malaise. Anaphylactic reactions have been rarely reported with ATG, but delayed allergic reactions with serum sickness are occasionally observed.

ATG contains a variety of antibodies that may cross-react with cell-surface markers. ATG causes lymphocyte depletion with marked depletion of lymphocytes occurring acutely. The circulating number of T cells gradually increases with cessation of therapy, usually reaching pre-treatment levels by 2 months. Subjects are at risk for possible opportunistic infection during this window of recovery, and will require close surveillance as well as antimicrobial prophylaxis for Pneumocystis carinii pneumonia. We will follow the guidelines established for the HIV and transplantation populations with T cell depletion (76, 77).

ATG may also lead to leucopenia and thrombocytopenia. Effects are dose-dependent and are mainly encountered with overdosage. In up to 3% severe thrombocytopenia may occur but this is invariably seen in doses 4-5 times that which we are proposing. Furthermore, thrombocytopenia in these subjects often occurred in a postoperative transplant setting and with other immunosuppressants.

Transient abnormalities in liver function tests have been described in patients with aplastic anemia treated with ATG preparations. Such adverse events have not been noted in numerous other clinical settings in which ATG has been used, and it is not clear if this is related to the underlying disease or to associated medications used.

Conflicting data exists about the risk of EBV-related lymphoproliferative disease. Furthermore, in renal transplant patients, the overall risk in this transplant population is low (0.25%-0.85%), and patients received several immunosuppressive therapies and were on continuous immunosupression (42). There were no reported cases of EBV-associated lymphoproliferative disease in over 1,675 treated subjects (including children) treated for aplastic anemia.

The long term safety profile of ATG is clouded in many instances by the drug's co-administration with other agents, as in transplant populations, and by the nature of the underlying disease of subjects entering the studies. We anticipate that the reported side effects will be less in our proposed study than in previous ones because of the low ATG dose to be employed. Nonetheless, because of the potential side effects from this therapy, the initial study will be conducted in those 12 years of age or older.

9.3.2 G-CSF

There are some potential risks associated with pegylated G-CSF therapy. Although the risk is entirely theoretical at this time, there is a possibility that the G-CSF injections could worsen and not improve the autoimmunity associated with T1D. The side effect profile of pegylated G-CSF injection will be monitored by close clinical observation of the subjects for 1 hour after the first G-CSF injection as well as with daily phone calls to the subjects during the first 5 days of G-CSF/placebo therapy and weekly thereafter.

Pegylated G-CSF carries a risk (10-20% of patients) of bone pain and a risk (less than 10%) of fever, pain or redness at the sight of injection, headache, and dizziness. There is also a risk that the subjects' WBC could become elevated while taking G-CSF. This will be monitored by the study team at each center and the dose of study drug will be dispensed by the pharmacy as placebo regardless of randomization (to ensure blinding is protected) if the ANC is > 35 x 10^9/L. There are also now a small number of reports in which recipients of G-CSF have developed splenomegaly and/or splenic rupture. This risk will be explicitly discussed in the consent form and subjects will have close physical examination and phone follow-up to screen for any signs or symptoms. In addition, weekly phone calls for the month following therapy will be used to document any adverse reactions. The CBC and complete metabolic progile at each blood draw will be carefully reviewed for any clinically relevant abnormalities. Any potential deleterious effects of GSCF/placebo therapy on the subjects' glycemic control will be monitored by close observation of their HbA1c, insulin requirements, and stimulated C-peptide. There are no other known long-term risks from a 12 week course of G-CSF.

9.4 Adequacy of Protection from Risks

9.4.1 Recruitment and Informed Consent

There will be two consents for this study. One for the screening process and another used after eligibility is confirmed and prior to the randomization visit. Written material will include instructions for the MMTT as well as a study participation card which will have the name of the site principal investigator, the name of the study, and the study treatment and written consent forms. The consent form will be reviewed with participants and the participant will be given time to review the written consent form and ask questions. The consent process will be conducted by qualified study personnel (Study Coordinator, and/or Investigator or other designee). All participants are asked to read, then sign and date a consent form prior to participation in the study, and/or undergoing any study-specific procedures.

It is anticipated that enrollment will be completed within the first year. The follow up of subjects is expected to continue for 2 years after the last subject is randomized to treatment. Subjects will not be enrolled who have other active serious medical problems. Frequent monitoring of patients with history, physical and neurological examination, and laboratory studies will allow for early identification of adverse events. Every attempt will be made to minimize the number of venipunctures. All study drugs will be delivered in a facility that has

resuscitation capabilities, and subjects will be closely monitored during and after the injections.

Subjects will be counseled to report any change in health status between or at the time of visits. Directed questioning about concurrent illness will occur. Subjects will be counseled by study personnel and requested to avoid pregnancy for 2 years. As described below, in addition to the physician investigators, an independent medical monitor assesses all safety and data integrity as well as a DSMB. In addition, all adverse events are will be reported to the local IRB.

9.4.2 Protections Against Release of Health Information

Study records with the study subject's information for internal use at the clinical sites will be secured at the study site during the study. At the end of the study, all records will continue to be kept in a secure location. There are no plans to destroy the records. Study subject data will be stored at the University of Florida. Case report forms (CRF) will be filled out electronically via the University of Florida site maintained by the University of Florida CTSI. Participants will be identifiable by a unique Identification Number. The data entry system is a secure, web based, password protected system. In the event that the electronic web site is down or inaccessible, paper CRFs will be available to ensure that data is collected in a timely manner. For the other two sites, the completed CRFs will be faxed to the University of Florida Coordinating Center.

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 study. As part of the quality assurance and legal responsibilities, however, the investigational site must permit authorized representatives of the sponsor(s) and regulatory agencies to examine (and, when required by applicable law, to copy) 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 (obscuring any personally identifying information). Authorized representatives as noted above are bound to maintain the strict confidentiality of medical and research information that may be linked to identify individuals. The investigational site will normally be notified in advance of auditing visits.

With the subject's approval, remaining de-identified samples will be stored at the University of Florida. Samples could be used to learn more about causes of T1D, its complications and other conditions for which individuals with diabetes are at increased risk and how to improve treatment. The results of these future analyses will not be made known to the participant.

9.5 Potential Benefits of the Proposed Research to the Subjects and Others

This study will examine whether the combination of ATG and G-CSF injections will preserve beta cell function, but there is no guarantee that this will occur. There is the prospect of direct benefit to the individual subjects for their participation in the study. These potential benefits include the recognized benefits of being in a clinical study, including close monitoring and additional resources available to maintain tight glycemic control offered to all subjects, regardless of group assignment. These additional resources include frequent in-person and other contact with study physicians and coordinators throughout the duration of the study. Further, the intervention has the prospect of direct benefit to a given subject and is likely to yield general knowledge about T1D which is of importance for the understanding and amelioration of T1D.

9.6 Inclusion of Women and Minorities

Both male and female, and members of all racial and ethnic populations will be screened. Enrollment is expected to reflect the known difference in frequency of T1D amongst different racial and ethnic groups. In the United States, non-Hispanic whites comprise the majority of cases of T1D, accounting for 86% of all cases, with blacks (9%) and Hispanics (5%) comprising most of the remainder (80). The disease is exceedingly rare in Asians (0.25%) and American Indians (0.048%). The low frequency of T1D in Asians is also noted when comparing disease frequency in different countries. It has been shown that the differences in disease frequency are, in large part, accounted for by differences in frequency of the HLA Class II (DR/DQ) genes that confer both risk of T1D and protection from T1D. Amongst the different ethnic groups, in the context of susceptible HLA genes, the pathogenesis of T1D appears to be the same. It is anticipated that approximately half of the study population will be males and approximately half will be females. Special efforts will be made to recruit amongst minority groups in order to attain enrollment proportional to the frequency of the disease in the population of the United States. If the study population does not reflect the recruitment targets, corrective action will be taken. Should this occur, attempts will be expended to determine why particular groups are under-represented, and strategies devised to overcome obstacles. Such efforts might include attempts to secure funds to provide for reimbursement of costs incurred by potential minority volunteers for subject participation (e.g., costs for travel, lost wages, etc.).

10. REFERENCES

1. Atkinson MA. Thirty Years of Investigating the Autoimmune Basis for Type 1 Diabetes: Why Can't We Prevent or Reverse This Disease? Diabetes 2005;54(5):1253-63.

2. Schatz D, Gale EA, Atkinson MA. Why can't we prevent type 1 diabetes?: maybe it's time to try a different combination. Diabetes Care 2003;26(12):3326-8.

3. Haller MJ, Gottlieb PA, Schatz DA. Interventio n trials 2007: where are we and where are we going? Current Opinion in Endocrinology, Diabetes and Obesity 14:283-287, 2007.

4. Herold KC, Gitelman SE, Masharani U, Hagopian W, Bisikirska B, Donaldson D, Rother K, Diamond B, Harlan DM, Bluestone JA. A single course of anti-CD3 monoclonal antibody hOKT3gamma1(Ala-Ala) results in improvement in C-peptide responses and clinical parameters for at least 2 years after onset of type 1 diabetes. Diabetes 2005 54: 1763-69

5. Keymeulen B, Vandemeulebroucke E, Ziegler AG, Mathieu C, Kaufman L, Hale G, Gorus F, Goldman M, Walter M, Candon S, Schandene L, Crenier L, De Block C, Seigneurin JM, De Pauw P, Pierard D, Weets I, Rebello P, Bird P, Berrie E, Frewin M, Waldmann H, Bach JF, Pipeleers D, Chatenoud L. Insulin needs after CD3-antibody therapy in new-onset type 1 diabetes. N Engl J Med. 2005 ;352(25):2598-608

6. Xue S, Wasserfall CH, Parker M, Brusko TM, McGrail S, McGrail K, Moore M, Campbell-Thompson M, Schatz DA, Atkinson MA, Haller MJ. Exendin-4 therapy in new onset NOD mice increases regulatory T cell frequency. *Ann NY Acad Sci* 1150:152-158, 2008

7.Casteels KM, Mathieu C, Waer M, Valckx D, Overbergh L, Laureys JM, et al. Prevention of type I diabetes in nonobese diabetic mice by late intervention with nonhypercalcemic analogs of 1, 25-dihydroxyvitamin D 3 in combination with a short induction course of cyclosporin A. Endocrinology 1998;139(1):95-102

8. Ludvigsson J, Faresjö M, Hjorth M, Axelsson S, Chéramy M, Pihl M, Vaarala O, Forsander G, Ivarsson S, Johansson C, Lindh A, Nilsson NO, Aman J, Ortqvist E, Zerhouni P, Casas R. GAD treatment and insulin secretion in recent-onset type 1 diabetes. N Engl J Med. 2008 Oct 30;359(18):1909-20. Epub 2008 Oct 8

9.Silverstein J, Maclaren N, Riley W *et al.* Immunosuppression with azathioprine and prednisone in recent-onset insulin-dependent diabetes mellitus. N Engl J Med 1988; 319:599-604.

10.Mahon JL, Dupre J, Stiller CR.Lessons learned from use of cyclosporine for insulin-dependent diabetes mellitus. The case for immunotherapy for insulin-dependent diabetics having residual insulin secretion. Ann N Y Acad Sci. 1993 Nov 30;696:351-63

11. Eisenbarth GS, Srikanta S, Jackson R *et al.* Anti-thymocyte globulin and prednisone immunotherapy of recent onset type 1 diabetes mellitus. Diabetes Res 1985; 2:271-6.

12.Casteels KM, Mathieu C, Waer M, Valckx D, Overbergh L, Laureys JM, et al. Prevention of type I diabetes in nonobese diabetic mice by late intervention with nonhypercalcemic analogs of 1,25-dihydroxyvitamin D3 in combination with a short induction course of cyclosporin A. Endocrinology 1998;

13. Baecher-Allan C, Viglietta V, Hafler DA. Human CD4+CD25+ regulatory T cells. Semin Immunol 2004;16(2):89-98.

14.Sakaguchi S. Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses. Annu Rev Immunol 2004;22:531-62.

15.Takahashi T, Kuniyasu Y, Toda M, Sakaguchi N, Itoh M, Iwata M, et al. Immunologic self-tolerance maintained by CD25+CD4+ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. Int Immunol 1998;10(12):1969-80

16. Parker M, Xue S, Alexander J, Wasserfall C, Campbell-Thompson M, Battaglia M ,Gregori S, Mathews C, Song S, Troutt M, Eisenbeis S, Williams J, Schatz D, Haller M, Atkinson M. Immune depletion with cellular mobilization imparts immunoregulation and reverses autoimmune diabetes in NOD mice (PNAS; submitted 2009)

17. Ogawa N, List JF, Habener JF, Maki T.Cure of overt diabetes in NOD mice by transient treatment with anti-lymphocyte serum and exendin-4. Diabetes. 2004 Jul;53(7):1700-5

18. Maki T, Ichikawa T, Blanco R, Porter J. Long-term abrogation of autoimmune diabetes in nonobese diabetic mice by immunotherapy with anti-lymphocyte serum. Proc Natl Acad Sci U S A 1992;89(8):3434-8.

19. Like AA, Rossini AA, Guberski DL, Appel MC, Williams RM. Spontaneous diabetes mellitus: reversal and prevention in the BB/W rat with antiserum to rat lymphocytes. Science 1979; 206:1421-3.

20. Chatenoud L, Thervet E, Primo J, Bach J F. Anti-CD3 antibody induces long-term remission of overt autoimmunity in nonobese diabetic mice. *Proc Natl Acad Sci USA* 1994; 91(1):123-127.

21. Makhlouf L, Grey ST, Dong V, Csizmadia E, Arvelo MB et al. Depleting anti-CD4 monoclonal antibody cures new-onset diabetes, prevents recurrent autoimmune diabetes, and delays allograft rejection in nonobese diabetic mice. *Transplantation*. 2004; 77(7):990-997.

22. Egido EM, Silvestre RA, Hernandez R, Marco J. Exendin-4 dose-dependently stimulates somatostatin and insulin secretion in perfused rat pancreas. Horm Metab Res 2004;36(9):595-600.

23 Dupre J. Glycaemic effects of incretins in Type 1 diabetes mellitus: a concise review, with emphasis on studies in humans. Regul Pept 2005;128(2):149-57.

24.Fehse F, Trautmann M, Holst JJ, Halseth AE, Nanayakkara N, Nielsen LL, et al. Exenatide augments first- and second-phase insulin secretion in response to intravenous glucose in subjects with type 2 diabetes. J Clin Endocrinol Metab 2005;90(11):5991-7.

25. Swanson SJ, Hale DA, Mannon RB, et al. Kidney transplantation with rabbit antithymocyte globulin induction and sirolimus monotherapy. Lancet 2002; 360:1662-4.

26. Starzl TE, Murase N, Abu-Elmagd K, et al. Toleragenic immunosuppression for organ transplantation. Lancet 2003; 361:1502-1510.

27. Fassas A, Anagnostopoulos A, Kazis A, Kapinas K, Sakellari I et al. Peripheral blood stem cell transplantation in the treatment of progressive multiple sclerosis: first results of a pilot study. *Bone Marrow Transplant*. 1997; 20(8):631-638.

28. Frickhofen N, Heimpel H, Kaltwasser J P, Schrezenmeier H, Antithymocyte globulin with or without cyclosporine A: 11-year follow-up of a randomized trial comparing treatments of aplastic anemia. *Blood*. 2003; 101(4):1236-1242.

29.Kool J, de Keizer RJ, Seigert CE. Antithymocyte globulin treatment of orbital Wegner granulomatosis: a follow-up study. *Am J Ophthalmol*. 1999; 127(6):738-739.

30.McSweeney PA, Nash RA, Sullivan KM, Storek J, Crofford LJ et al. High-dose immunosuppressive therapy for severe systemic sclerosis: initial outcomes. *Blood*. 2002; 100(5):1602-1610.

31. Molldrem JJ, Leifer E, Bahceci E, Saunthararajah Y, Rivera M et al. Antithymocyte globulin for treatment of the bone marrow failure associated with myelodysplastic syndromes. *Ann Inter med*. 2002; 137(3): 156-163.

32.Musso M, porretto F, Crescimanno A, Bondi F, Polizzi V et al. Intense immunosuppressive therapy followed by autologous peripheral blood selected progenitor cell reinfusion for severe autoimmune disease. *Am J hematol*. 2001; 66(2):75-79.

33. Rosenfeld S, Follmann D, Nunez O, Young N S. Antithymocyte globulin and cyclosporine for severe aplastic anemia: association between hematologic response and lon-term outcome. *JAMA*. 2003; 289(9):1130-1135.

34. Stratton RJ, Wilson H, Black CM. Pilot study of anti-thymocyte globulin plus mycophenolate mofetil in recent-onset diffuse scleroderma. *Rheumatology (Oxford).* 2001; 40(1):84-88.

35. Tarkowski A, Andersson-Gare B, Aurell M. Use of anti-thymocyte globulin in the management of refractory systemic autoimmune diseases. *Scand J Rheumatol*. 1993; 22(6):261-266.

36. Tyndall A. Immunoablation and haemopoietic stem cell transplantation for severe autoimmune disease with special reference to systemic lupus crythematosus. *Lupus*. 2001; 10(3):214-215.

37.van der Woude FJ, Schmitt WH, Birck R, Nowack R, Gobel U et al. Immunosuppression in ANCA-associated vasculitis. *Transplant Proc*. 2001; 33(3):2225-2226.

38.Van Laar JM, Tyndall A. Intense immunosuppression and stem-cell transplantation for patients with severe rheumatic autoimmune disease: a review. *Cancer Control*. 2003; 10(1):57-65.

39.Preville X, Flacher M, LeMauff B, Beauchard S, Davelu P et al. Mechanisms involved in antithymocyte globulin immunosuppressive activity in nonhuman primate model. *Transplantation.* 2001; 71(3):460-468.

40.Schatz D A, Riley WJ, Silverstein J H, Barrett D J. Long-term immunoregulatory effects of therapy with corticosteroids and anti-thymocyte globulin. *Immunopharmacol Immunotoxicol*. 1989; 11(2-3):269-287.

41. Saudek F. Polyclonal anti-T-cell therapy for type 1 diabetes mellitus of recent onset. *Rev Diabetic Studies*. 2004.

42. *Thymoglobulin® (Antithymocyte Globulin [Rabbit]) Investigator's Brochure*. SangState Medical Corp., April 2003

43.Genestier L, Fournel S, Flacher M, Assossou O, Revillard J P et al. Induction of Fas (Apo-1, CD95)-mediated apoptosis of activated lymophocytes by polyclonal antithymoctye globulins. *Blood*. 1998; 91(7):2360-2368

44. Halwani F, Guttmann RD, Ste-Croix H, Prud'homme GJ. Identification of natural suppressor cells in long-term renal allograft recipients. *Transplantation*. 1992; 54(6):973-9

45. Bas J, Mestre M, Grinyo JM, Massip E, Alsina J et al. Peripheral blood lymphoid subsets and long-term clinical course of kidney recipients: a longitudinal study. Cytometry. 1998; 34(2):103-112

46. Muller T F. Phenotypic changes with immunosuppression in human recipients. *Frontiers in Bioscience*. 2004; 8: 1254-1274.

47. Fagnoni FF, Lozza L, Zibera C, Zambelli A, Ponchio L et al. T-cell dynamics after high-dose chemotherapy in adults: elucidation of the elusive CD8+subset revels multiple homeostatic T-cell compartments with distinct implications for immune competence. *Immunology*. 2002; 106(1):27-37.

48.Davison G M, Novitzky N, Kline A, Thomas V, Abrahams L et al. Immune reconstitution after allogeneic bone marrow transplantation depleted of T cells. *Transplantation*, 2000; 69(7):1341-1347.

49. Belghith M, Bluestone JA, Barriot S, Megret J, Bach JF et al. TGF-beta-dependent mechanisms mediate restoration of self-tolerance induced by antibodies to CD3 in overt autoimmune diabetes. *Nat Med*. 2003; 9(9):1202-1208.

50. Herold KC, Burton JB, Francois F, Poumian-Ruiz E, Glandt M et al. Activation of human T cells by FeR nonbinding anti-CD3 mAb, hOKT3gamma1 (Ala-Ala). *J Clin Invest.* 2003; 11(3):409-418.

51. Muller T F, Grebe S O, Neumann M C, Heymanns J, Radsak K et al. Persistent long-term changes in lymphocyte subsets induced by polyclonal antibodies. *Transplantation*. 1997; 64(10):1432-1437.

52.Reipert B, Scheuch C, Lukowsky A, Reinke P, Fietze E et al. CD3+CD57+lymphocytes are not likely to be involved in antigen-specific rejection processes in long-term allograft recipients. Clin Exp immunol. 1992; 89(1):143-147

53.Autran B, Leblond V, Sadat-Sowti B, Lefranc E, Got P et al. Asoluble factor released by CD8+CD57+lymphocytes from bone marrow transplanted patients inhibits cell-mediated cytolysis. *Blood*. 1991; 77(10):2237-2241.

53. Smith JA, Tang Q, Bluestone JA. Partial TCR signals delivered by FcR-nonbinding anti-CD3 monoclonal antibodies differentially regulate individual Th subsets. *J Immunol.* 1998; 160(10):4841-4849

54.Merion R M, Howell T, Bromberg JS. Partial T-cell activation and anergy induction by polyclonal antithymoctye globulin. *Transplantation*. 1998; 65(11);1481-1489.

55. Monti P, Allavena P, Di C, V, Piemonti L. Effects of anti-lymphocytes and anti-thymoctyes globulin on human dendritic cells. *Int Immunopharmacol*. 2003; 3(2):189-196

56.Bonnefoy-Berard N, Genestier L, Flacher M, Rouault JP, Lizard G et al. Apoptosis induces by polyclonal antilymphocyte globulins in human B-cell lines. *Blood*. 1994;83(4):1051-1059.

57.Zand MS, Vo T, Huggins J, Felgar R, Liesveld J et al. Polyclonal rabbit antithymocyte globulin triggers B-cell and plasma cell apoptosis by multiple pathways. *Transplantation*. 2005; 79(11):1507-1515.

58.Serreze DV, Silveira PA. The role of B lymphocytes as key antigen-presenting cells in the development of T cell-mediated autoimmune type diabetes. *Curr Dir Autoimmun*. 2003;6:212-227.

59. Kared H, Masson A, Adle-Biassette H, Bach JF, Chatenoud L, Zavala F: Treatment with granulocyte colony-stimulating factor prevents diabetes in NOD mice by recruiting plasmacytoid dendritic cells and functional CD4(+)CD25(+) regulatory T-cells. *Diabetes* 54:78-84, 2005

60. Hadaya K, Kared H, Masson A, Chatenoud L, Zavala F. G-CSF treatment prevents cyclophosphamide acceleration of autoimmune diabetes in the NOD mouse. J Autoimmun 2005;24(2):125-34.

61. Dejaco C, Lichtenberger C, Miehsler W, Oberhuber G, Herbst F, Vogelsang H, Gangl A, Reinisch W: An open-label pilot study of granulocyte colony-stimulating factor for the treatment of severe endoscopic postoperative recurrence in Crohn's disease. *Digestion* 68:63-70, 2003

62. Korzenik JR, Dieckgraefe BK: An open-labelled study of granulocyte colony-stimulating factor in the treatment of active Crohn's disease doi:10.1111/j.1365-2036.2005.02287.x. *Alimentary Pharmacology and Therapeutics* 21:391-400, 2005

63. Vasiliu IM, Petri MA, Baer AN: Therapy with granulocyte colony-stimulating factor in systemic lupus erythematosus may be associated with severe flares. *J Rheumatol* 33:1878-1880, 2006

64. Snir O, Lavie G, Achiron A, Bank I, Ben-Aharon T, Sredni B, Cohen IR, Mandel M: G-CSF enhances the adhesion of encephalitogenic T cells to extracellular matrix components: A possible mechanism for exacerbation of multiple sclerosis. *Journal of Neuroimmunology* 172:145-155, 2006

65. Rutella S, Zavala F, Danese S, Kared H, Leone G: Granulocyte colony-stimulating factor: a novel mediator of T cell tolerance. *J Immunol* 175:7085-7091, 2005

66. Franzke A: The role of G-CSF in adaptive immunity. *Cytokine & Growth Factor Reviews* 17:235-244, 2006

67. Sheng J R, Li L, Ganesh B B, Vasu C, Prabhakar B S, Meriggioli MN: Suppression of Experimental Autoimmune Myasthenia Gravis by Granulocyte-Macrophage Colony-Stimulating Factor Is Associated with an Expansion of FoxP3+ Regulatory T Cells. *J Immunol* 177:5296-5306, 2006

68. Lytton SD, Denton CP, Nutzenberger AM. Treatment of autoimmune disease with rabbit anti-T lymphocyte globulin: clinical efficacy and potential mechanisms of action. Ann N Y Acad Sci. 2007 Sep;1110:285-96

69. Gavela Martínez E, Sancho Calabuig A, Escudero Quesada V et al. Induction treatment with low-dose thymoglobulin or basiliximab in renal transplants from older donors. Transplant Proc. 2008 Nov;40(9):2900-2

70. Baron PW, Ojogho ON, Yorgin P, Sahney S, Cutler D, Ben-Youssef R, Baqai W, Weissman J, Franco E, Zuppan C, Concepcion W. Comparison of outcomes with low-dose anti-thymocyte globulin, basiliximab or no induction therapy in pediatric kidney transplant recipients: a retrospective study. Pediatr Transplant. 2008 Feb;12(1):32-9

71. Rutella S. Granulocyte colony-stimulating factor for the induction of T-cell tolerance.. Transplantation. 2007 Jul 15;84(1 Suppl):S26-30.

72. Diggle PJ, Liang KY and Zeger SL (1994). *Analysis of Longitudinal Data*. Clarendon Press, Oxford, Oxford UK.

73. Lachin JM (2000). *Biostatistical Methods: The Assessment of Relative Risks*. John Wiley and Sons, New York.

74. Lan KKG, DeMets DL (1983). Discrete sequential boundaries for clinical trials. *Biometrika,* 70: 659-663.

75. Lachin JM. A review of methods for futility stopping based on conditional power. *Statistics in Medicine*, 24, 2747-2764, 2005.

76. Kaplan JE, Masur H, Holmes KK. Guidelines for preventing opportunistic infections among HIV-infected persons--2002. Recommendations of the U.S. Public Health Service and the Infectious Diseases Society of America. MMWR Recomm Rep 2002; 51:1-52.

77. Dykewicz CA. Guidelines for preventing opportunistic infections among hematopoietic stem cell transplant recipients: focus on community respiratory virus infections. Biol Blood Marrow Transplant 2001; 7:19S-22S.

78. Killick SB, Marsh JC, Booth JC, Gordon-Smith EC. Liver function abnormality following treatment with antithymocyte globulin for aplastic anemia. *Bone Marrow Transplant*. 1997; 19(3):249-251.

79. deMedeiros CR, Ribeiro RC, Bittencourt MA, Zanis-Neto J, Pasquini R. Long-term outcome of 25 children and adolescents with severe aplastic anemia treated with antithymocyte globulin. *Braz J Med Biol Res*. 2000; 33(5):553-558.

80. Karvonen M, Viik-Kajander M, Moltchanova E, Libman I, LaPorte R, Tuomilehto J. Incidence of childhood type 1 diabetes worldwide. Diabetes Mondiale (DiaMond) Project Group. Diabetes Care. 2000 Oct;23(10):1516-26