

Islet Cell and ST2 Axis Dysregulation in Post-Transplant Diabetes Mellitus

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Table of Contents

1.0 Rationale and Specific Aims		3
2.0 Introduction and Background		4
3.0 Preliminary Data		6
4.0 Inclusion/Exclusion Criteria		8
5.0 Enrollment		9
6.0 Study Procedures	10	
7.0 Risks and Toxicity Management		14
8.0 Reporting of Adverse Events or Unanticipated Problems		15
9.0 Study Withdrawal/Discontinuation	1	5
10.0 Statistical Considerations	16	
11.0 Privacy/Confidentiality Issues	1	8
12.0 Followup and Record Retention	18	
13.0 References	19	
14.0 Appendix		
A. Study Table		22

1.0 Rationale and Specific Aims:

The development of type 2 diabetes mellitus is a significant complication for long-term cancer survivors including patients treated with allogeneic hematopoietic stem cell transplantation (HCT). New onset, post-transplant diabetes mellitus (PTDM) occurs in up to 60% of HCT recipients and negatively impacts transplant survival (1). Currently 8,000 HCT's are performed each year in the United States primarily for patients with high risk hematological disorders. These individuals comprise a well defined group that is at significant risk for developing new metabolic abnormalities including PTDM.

We have found that PTDM resembles type 2 diabetes mellitus. Normally, type 2 diabetes mellitus results from a combination of insulin resistance and decreased insulin secretion. The transition from compensated insulin resistance to frank diabetes occurs as a result of β -cell failure mediated by metabolic overload and inflammation. In animal models, loss of IL-33 signaling through its receptor serum STimulation-2 (ST2) decreases immunosuppressive ST2+ regulatory T cells (Tregs) in visceral adipose tissue, increases Th1 inflammation, worsens insulin resistance, and promotes β -cell stress and diabetes conversion. We found that new-onset PTDM is characterized by: 1) elevated fasting C-peptide levels pre-HCT; 2) impaired islet response to hyperglycemia and GLP-1 after HCT with decreased β -cell insulin secretion and blunted α -cell suppression; and 3) increased post-transplant serum levels of soluble ST2 (sST2), a decoy receptor which sequesters IL-33. We hypothesize that in PTDM, initial β -cell compensation progresses to exhaustion during the course of HCT, which coincides with increased tissue demand for insulin due to changes in IL-33 signaling, inflammation, and/or hyperglucagonemia. We propose to test this hypothesis with the following aims in a prospective, observational study in HCT recipients.

Specific Aim 1. To determine if changes in islet cell physiology are detectable before or after matched related donor (MRD) HCT in patients developing new-onset PTDM.

Subaim 1A. To determine if a compensatory increase in glucose stimulated insulin secretion (GSIS) by β -cells precedes PTDM development in patients without diabetes undergoing MRD HCT. Based on our C-peptide and oral glucose tolerance test (OGTT) data, we hypothesize that PTDM-prone individuals maintain euglycemia pre-transplant via enhanced β -cell secretory capacity followed by progressive β -cell dysfunction and relative insulin insufficiency after transplant. In a prospective MRD HCT cohort without diabetes, β -cell function and secretory capacity will be directly measured with hyperglycemic clamps before and 90 days after transplant.

Subaim 1B. To determine if excess glucagon secretion and impaired α -cell response to glucose or GLP-1 contributes to the hyperglycemia of PTDM. We will test the hypothesis that PTDM patients have increased glucagon levels and that glucose and GLP-1 induced α -cell suppression are incomplete. To assess α -cell dysregulation, glucose-induced glucagon suppression will be measured during a hyperglycemic clamp and during 2 OGTTs either with or without GLP-1 infusion, before and 90 days after HCT.

Specific Aim 2. To determine if the IL-33/ST2 axis promotes immune/islet cell dysregulation during PTDM. We hypothesize that PTDM is characterized by decreased protective IL-33/ST2 signaling in adipose tissue at rest (*i.e.* pre-transplant)

followed by increased systemic, inflammatory signaling during stress (*i.e.* post-transplant), which leads to β -cell compensation and exhaustion, respectively. To investigate the ST2 pathway, we will measure adipose and plasma levels of IL-33 and sST2 and quantify ST2 expression on circulating Tregs and Th1 cells before and after HCT from our MRD cohort. IL-33 effects on T cells and on human islet function will be assessed *in vitro*.

2.0 Introduction and Background

Role of HCT

Over 30,000 allogeneic HCTs are performed yearly, with the primary indication being hematological malignancy (2). Although malignancy relapse is the most important cause for death after transplant, HCT recipients who are cured of cancer continue to be at increased risk of developing significant secondary medical conditions that can impact transplant survival negatively (3, 4). Of particular interest is PTDM, due to its high prevalence after transplant and its detrimental effects on cardiovascular health (5, 6).

Impaired glucose homeostasis in HCT recipients (PTDM)

HCT recipients are over 3 times more likely to report a diagnosis of diabetes than their sibling counterparts (7). Our prospective data indicates that early onset PTDM occurs in up to 60% of individuals with no history of diabetes, and it can persist long-term in about 10-30% of these patients with a negative impact on survival (1, 6-11). Risk factors for diabetes in HCT recipients have been described including: older age, nonwhite ethnicity, steroid exposure, and total body irradiation, however the actual mechanisms causing PTDM are unknown (1, 6, 7, 10-13). We have found that new-onset PTDM is characterized by: 1) elevated fasting C-peptide levels pre-HCT; 2) impaired islet response to hyperglycemia and GLP-1 after HCT; and 3) increased post-transplant serum levels of soluble serum STimulation-2 (sST2), a decoy receptor which sequesters IL-33 (1, 14). We hypothesize that in PTDM, initial β -cell compensation progresses to exhaustion during the course of HCT, which coincides with increased tissue demand for insulin due to changes in IL-33 signaling, inflammation, and/or hyperglucagonemia.

Type 2 diabetes mellitus results from a combination of impaired tissue response to insulin action and decreased insulin secretion. Due to compensatory changes in β -cell function, frank hyperglycemia occurs when pancreatic β -cells can no longer meet the insulin demands needed for glycemic control, an event termed β -cell failure. Distinct to transplantation is the use of diabetogenic immunosuppressive medications. There are at least 3 lines of evidence indicating that PTDM is not just an iatrogenic complication of immunosuppressive drugs. 1) Despite near universal treatment of HCT recipients with calcineurin inhibitors, not all patients develop PTDM. 2) The diagnosis of PTDM generally precedes corticosteroid treatment (1, 8, 14). 3) Rates of diabetes among HCT survivors off of immunosuppression are still higher compared to their sibling controls. Therefore the assumption that PTDM is merely a manifestation of diabetogenic medications is flawed (7). Since islet cells are major regulators of glucose homeostasis, their physiology during PTDM needs to be investigated with validated techniques including the oral glucose tolerance test (OGTT) and hyperglycemic clamp.

IL-33/ST2 axis and immune/metabolic dysregulation

Type 2 diabetes mellitus is an inflammatory disease characterized by the accumulation of IFN- γ secreting T cells (Th1 cells) and depletion of immunosuppressive Foxp3+ regulatory T cells (Tregs) in adipose tissue (15-18). The balance between Th1 cells and Tregs is a critical determinant of insulin sensitivity/demand which in turn influences β -cell function and stress. IL-33 is a dual cytokine with both inflammatory and regulatory functions. Normally, adipose tissue expresses IL-33, however cytokine levels are lower during obesity and insulin resistance (19). The IL-33 receptor, serum STimulation-2 (ST2) can exist as a membrane bound protein on immune cells including Tregs but not usually Th1 cells, or as a soluble form (sST2) where it functions as a decoy receptor sequestering excess IL-33 (19, 20). In pre-clinical obesity models, the administration of IL-33 increases ST2+Tregs in adipose tissue and improves glucose tolerance compared to PBS-injected control animals (21, 22). Resolution of visceral adipose tissue inflammation by ST2+Tregs decreases insulin requirements and improves fasting blood glucose levels (17, 18). Similarly, our collaborators have shown in a murine model of GVHD, that blockade of sST2 with a neutralizing antibody increased IL-33, expanded ST2+Tregs, decreased inflammation, and improved transplant survival (20). However IL-33 functions as a double-edged sword. In the setting of HCT where multiple noxious stimuli are present, Th1 cells which usually do not express ST2, can acquire the receptor and become responsive to IL-33 released from damaged tissue resulting in lethal alloreactivity (23-25). Theoretically, this ST2+Th1 response could exacerbate inflammation leading to impaired insulin action, increased insulin demand, and increased β -cell work after HCT. IL-33 also directly induces β -cell endoplasmic reticulum stress which accompanies the insulin secretory defect of type 2 diabetes (26). Thus, the IL-33/ST2 axis could affect PTDM conversion through several different mechanisms depending on the differential expression of ST2 by Tregs or Th1 cells, or whether IL-33 exerts its action predominantly in the periphery by determining insulin needs or by affecting β -cell function directly. Although IL-33's influence on glucose homeostasis during periods of quiescence have been described, IL-33's metabolic affects following transplant of an allogeneic immune system and in the presence of ST2+Th1 cells during HCT is unknown.

Rationale and clinical application

HCT recipients are at significant risk for developing new-onset PTDM, which in turn confers inferior survival. The initiating events and mechanisms that culminate in PTDM development remain understudied, and formal recommendations for screening and treatment are lacking. In this proposal, we will use OGTT and hyperglycemic clamps to characterize the onset and role of pancreatic β - and α -cell dysfunction in metabolic complications after HCT. These results will be adapted into future screening tools for PTDM and translated into therapeutic clinical trials targeting either insulin insufficiency or glucagon excess. By analyzing IL-33 signaling and ST2 expression on Tregs and Th1 cells sequentially before and after transplant, we will 1) investigate the relationship between islet function and ST2 immune regulation during HCT, 2) establish an immune timeline for the development of PTDM, and 3) develop a biological rationale for why treatment/ prevention of metabolic complications could alter ST2 signaling and improve HCT outcomes. We propose that understanding the immunology and the metabolic abnormalities generating PTDM will promote rapid improvements in the care of HCT recipients.

3.0 Preliminary Data

Incidence and risk factors for early PTDM

We prospectively analyzed PTDM incidence (defined as a weekly fasting glucose \geq 126 mg/dL or random glucose \geq 200 mg/dL) during the first 100 days of HCT in 84 patients without diabetes (1). New-onset PTDM was diagnosed in 50 (60%) patients at a median of 23 days after HCT and prior to the start of steroids in the majority. PTDM was associated with inferior survival when analyzed in a time-dependent manner [hazard ratio (HR), 3.27; 95% confidence interval (CI), 1.3-8.2; $P=0.01$]. In multivariate analysis, elevated, pre-transplant fasting C-peptide level independently predicted both new-onset PTDM [odds ratio (OR), 5.9; 95% CI, 1.77-20.22; $P=0.004$] and increased mortality (HR, 1.05; 95%CI, 1.01-1.09; $P=0.013$) (1). This prospective study indicates that compensatory β -cell changes, as evidenced by increased fasting C-peptide level, could be present prior to HCT (and before exposure to immunosuppression) and that changes in glucose metabolism could lead to inferior HCT outcomes.

Implications for β -cell failure and α -cell dysregulation during PTDM development

Glucose homeostasis is maintained by the coordinated secretion of insulin by pancreatic β -cells and glucagon by α -cells (27, 28). In addition to blood glucose, this process is regulated by the gut-derived incretin hormones glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) which are secreted postprandially, promoting insulin release and glucagon suppression (29). In a prospective observational cohort study funded by the NHLBI a standard OGTT was performed on HCT recipients without established diabetes before and 90 days after transplant (Figure 1). Although insulin levels appear similar before and after transplant in the 2 groups, only in patients maintaining post-transplant euglycemia are β -cells able to prevent significant glucose excursions during the challenge (bottom panel B). Compared to pre-transplant values, blood sugars were elevated at all time points after HCT with no corresponding increase in insulin secretion by the PTDM group (top panel A). These data are most consistent with the development of a new β -cell defect after HCT.

Glucagon is secreted in response to hypoglycemia where it promotes hepatic glycogen breakdown and gluconeogenesis. Our preliminary data suggests that PTDM patients have elevated fasting and post-prandial glucagon levels when compared to their pre-transplant values and when compared to individuals maintaining euglycemia after HCT. This inappropriate glucagon elevation in PTDM patients exists despite factors known to inhibit α -cells including elevations in blood glucose, insulin, and GLP-1 (Figure 1). Taken together these data indicate a role for hyperglucagonemia and impaired α -cell regulation in PTDM

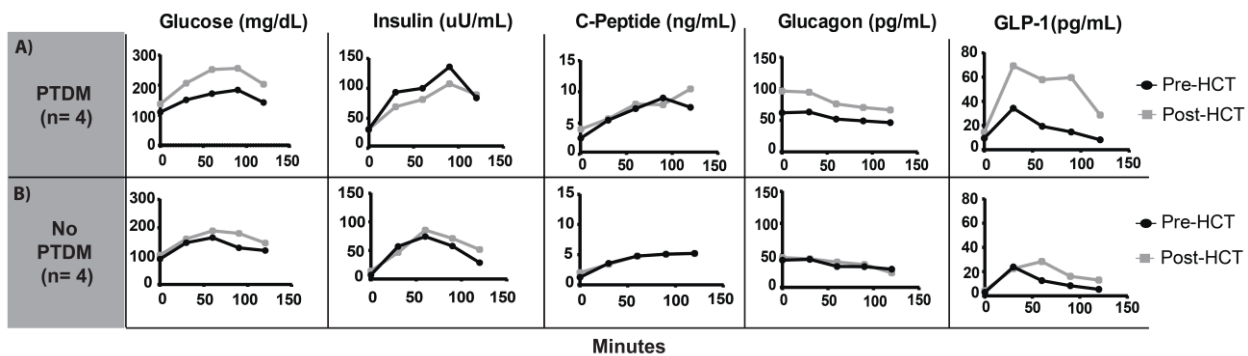


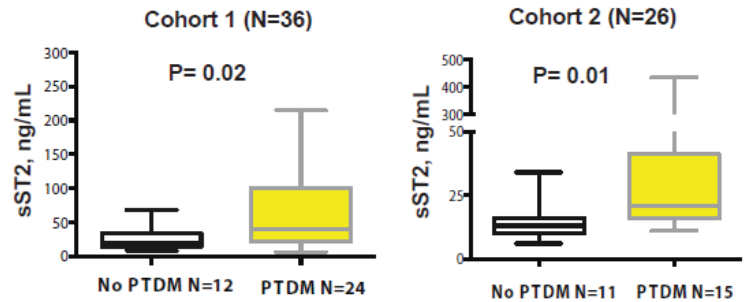
Figure 1. OGTT suggests β -cell failure and impaired glucagon suppression during PTDM after HCT. Representative data from 8 HCT recipients without a pre-transplant diagnosis of diabetes is shown. Blood glucose, insulin, C-peptide, glucagon, and GLP-1 were measured every 30 minutes for 2 hours during a standard OGTT performed before and 90 days after HCT. Median values are depicted and data is stratified for the development of new-onset PTDM (A) or maintenance of euglycemia (B).

GLP-1 is an important regulator of glucagon suppression. As expected, after an oral glucose load, GLP-1 is secreted with a corresponding increase in insulin and decline in glucagon (Figure 1). We hypothesize that the exaggerated GLP-1 secretion in PTDM patients after transplant represents an incomplete compensatory mechanism meant to restore homeostasis which will be explored further in Aim 1B with possible therapeutic ramifications.

PTDM is Th-1 skewed and characterized by altered ST2 signaling

Elevated fasting C-peptide levels pre-transplant and PTDM development after HCT both predict inferior survival (1, 8). Our collaborators have demonstrated that pre-transplant elevations in the decoy receptor sST2 are seen with obesity and that high sST2 after HCT is a validated biomarker for non-relapse mortality due to immune dysregulation (30-32). Published literature indicates that low IL-33 levels are involved in insulin resistance in non-transplant animal models of diabetes (21, 22). Therefore we sought to examine sST2 as a predictor of PTDM independent of GVHD. In a group of HCT recipients without preexisting diabetes, serum sST2 was elevated at neutrophil engraftment in patients who were prospectively diagnosed with new-onset PTDM after HCT (Figure 2). This increase in engraftment sST2 was confirmed in a separate cohort of HCT recipients who were retrospectively diagnosed with PTDM (14). We also confirmed that the majority of PTDM diagnoses occurred before steroids exposure (67% in cohort 1 and 93% in cohort 2). Since PTDM generally occurs before steroids then steroids should not influence the development of PTDM for the majority. After adjustment for myeloablative chemotherapy and grade 3-4 acute GVHD (factors known to influence sST2 and survival), high engraftment sST2 ($\geq 75^{\text{th}}$ percentile of combined cohorts 1 and 2) predicted increased PTDM (HR, 2.25; 95%CI, 1.07-4.75; P= 0.03) and non-relapse mortality risk (HR, 8.54; 95%CI, 1.62-45.15; P = 0.01) (14). Thus, the data suggests that dysregulation of the IL-33/ST2 axis may influence metabolic complications after HCT which in turn presages clinical GVHD, steroid exposure, and mortality.

Figure 2. Increased sST2 after HCT is a validated PTDM predictor. Serum sST2 was measured by ELISA at engraftment in 36 euglycemic HCT recipients followed prospectively for PTDM (cohort 1). Results were confirmed in a second cohort of 26 patients without pre-existing diabetes retrospectively analyzed for PTDM (cohort 2).



Type 2 diabetes mellitus is characterized by systemic inflammation (15-18). Using established cytometric assays, we found that by day+10 after HCT inflammatory IFN- γ + Th1 cells were increased in patients prospectively followed for PTDM (Figure 3). This inflammatory response in PTDM could be driven by IL-33/ST2 disequilibrium and upregulation of ST2 by Th1 cells following HCT (Figure 4). The net effect of this IL-33/ST2 signaling after HCT could be Th1 inflammation which overwhelms immune regulation and causes β -cell stress

Figure 3. PTDM is characterized by an inflammatory / Th-1 skewed environment. A cohort of 18 HCT recipients were prospectively followed for PTDM. The frequency of circulating Th1 cells were determined by flow cytometry.

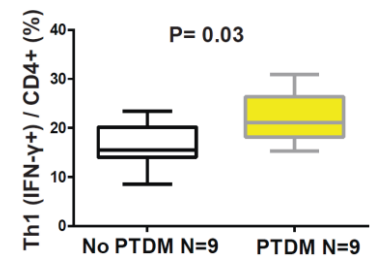
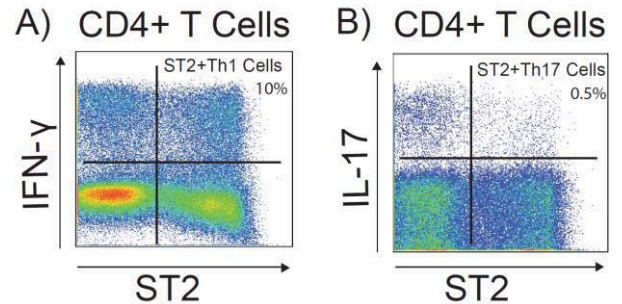


Figure 4. ST2 is expressed by a significant population of Th1 cells but not Th17 cells. HCT recipient PBMCs were stimulated for 5 hours with PMA/ionomycin/monensin. Cells were surface-stained, fixed and permeabilized and then intracellularly stained with an assay containing: CD14, CD3, CD4, ST2, IL-17, IFN- γ , and amine viability dye. Viable CD3+CD4+ T cells were identified followed by gating on ST2 vs. IFN- γ or IL-17 to determine the frequency of ST2+Th1 (A) or ST2+Th17 cells (B), respectively.



In summary, our data suggests that immune dysregulation and changes in IL-33/ST2 signaling contributes to the pathogenesis of PTDM which begins before transplant with compensatory β -cell changes followed by islet dysfunction manifesting as insulin insufficiency and hyperglucagonemia after HCT.

4.0 Inclusion/Exclusion Criteria

Inclusion Criteria for Patients:

- Age \geq 18 years
- Patients undergoing MRD allogeneic HCT

Exclusion Criteria for Patients:

- Patients who have not received an allogeneic HCT
- Recent or current history of diabetes mellitus, defined as: 1) diabetes therapy within 6 months of enrollment, or 2) fasting blood glucose at “pre-admit” (screening) visit \geq 126mg/dL
- Pregnancy or breastfeeding
- Unrelated donor, umbilical cord blood, mismatched, or haploidentical transplants
- Patients receiving T cell depletion or thymoglobulin as part of their transplant
- Patients on established, chronic corticosteroid therapy (> 10 mg /day of prednisone or prednisone equivalent) prior to transplant. Established, chronic corticosteroid therapy is defined as daily dosing of > 10 mg / day of prednisone or prednisone equivalent for at least 2 weeks prior to the start of conditioning/chemotherapy or plans to continue pre-transplant corticosteroids (> 10 mg /day of prednisone or prednisone equivalent) indefinitely after transplantation.
- Inability to give informed consent
- Any condition which, in the opinion of the investigator, might interfere with study objective
- Any reason which, in the opinion of the investigator, adds additional risk to the patient

Additional exclusion criteria (Aim 1 only):

- Diagnosis of diabetes by standard oral glucose tolerance testing prior to transplant (2-hour plasma glucose value ≥ 200 mg/dL).

Inclusion Criteria for Donors:

- Age ≥ 18 years
- Donors undergoing stem cell collection for match related allogeneic stem cell transplant

Exclusion Criteria for Donors:

- Individuals not donating stem cells
- Pregnancy or breastfeeding
- Inability to give informed consent
- Any condition which, in the opinion of the investigator, might interfere with study objective

5.0 Enrollment

Potential study candidates will be recruited from the Hematology-Oncology Clinics at the Vanderbilt Ingram Cancer Center (VICC) by a member of the research team prior to their allogeneic stem cell transplant. Potential participants including patients and donors will be identified at the time of acceptance for transplant by their treating physicians. Patients and donors who may qualify for participation will be provided a copy of the IRB-approved Informed Consent document to read. Subjects who express interest will then meet with a member of our research team during their regularly scheduled clinic visit. Interested subjects will be screened for inclusion and exclusion criteria. Standard of care laboratories (*i.e.* pregnancy test if indicated and fasting blood glucose) will be reviewed.

Those subjects who sign consent and who fulfill all inclusion and exclusion criteria will then be enrolled in the study.

6.0 Study Procedures

Sources of materials- Donors

Donors who have given informed consent will undergo standard of care evaluations or study visits at either the Outpatient Transplant Unit or the inpatient Myelosuppression Unit at Vanderbilt University Medical Center. Study blood will be obtained once prior to beginning the stem cell mobilization procedure. A single blood draw of 50-60 ml will be collected once. Venous blood will be obtained via peripheral venipuncture and preferably will be collected during routine laboratory analysis. Samples will be coded with a unique alphanumeric identifier. Clinical information and research data will be obtained by donor interview, medical chart review, and from laboratory testing. Information will be stored in a password-protected electronic database. After the blood draw no further testing will be performed on the donor.

A single sample of 50-60 mL of blood will be collected from the donor at one time point during this study.

Sources of materials- Patients

Study participants who have given informed consent will undergo standard of care evaluations or study visits at either the Outpatient Transplant Unit, the inpatient Myelosuppression Unit, or the Clinical Research Center (CRC) at Vanderbilt University Medical Center. Two oral glucose tolerance tests and 1 hyperglycemic clamp will be performed on separate days prior to transplant and then each procedure will be repeated once between day+80 to day+100 (+/- 10 days) after transplant (Figure 5). In addition to the 6 research visits, study blood will be obtained at the following timepoints: pre-transplant (prior to beginning chemotherapy), Day+10, Day+20, Day+30, and Day+90 (all +/- 7 days) (the day of stem cell infusion is considered Day 0). A total of 550 mL of blood will be collected from the patient over approximately 110 days (over 3 to 4 months). Blood will be drawn for research purposes at the same time as routine blood sampling when feasible. Venous blood will be obtained by accessing the central venous catheter using sterile technique or via peripheral venipuncture. Samples will be coded with a unique alphanumeric identifier at the time of collection so that patient identity will be available only to study investigators and research staff. Clinical information, outcomes, and research data will be obtained by patient interview, medical chart review, and from laboratory testing. Information will be stored in a password-protected electronic database.

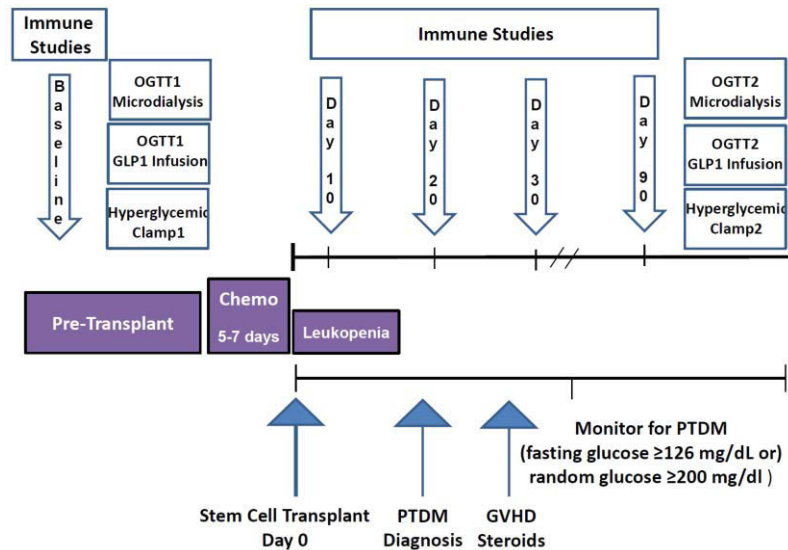


Figure 5. Study schema and timeline

OGTT

Two OGTTs (without and with GLP-1 infusion) will be performed prior to HCT and after an 8 hour overnight fast (water and medications allowed) in patients with a screening blood glucose < 126 mg/dL. Plasma samples will be analyzed for glucose, insulin, glucagon, C-peptide, GLP-1 every 30 minutes from Time -30 minutes to Time 120 minutes (6 time points) as per physician discretion. Remaining aliquots will be stored. At time 0, participants will ingest 75 g of water-free glucose dissolved in 300 mL of water. Pre-HCT patients will be categorized per the 2-hour OGTT plasma glucose value as normal (< 140 mg/dL), impaired glucose tolerance (140-199mg/dL), or diabetic (≥ 200 mg/dL) (33, 34). Study patients diagnosed with diabetes by standard OGTT prior to HCT will not be monitored for *de novo* PTDM. Study patients diagnosed with diabetes by standard OGTT prior to HCT will not undergo further testing as outlined in aim 1 after the diagnosis is made, however the immune / metabolic studies as outlined for aim 2 will be performed in these individuals.

The second OGTT procedure will be repeated on a different day with exogenous GLP-1 infusion using similar methods as described above. At Time -30 minutes, a 2.0 pmol/kg/min infusion of GLP-1 will be given IV and continued for the remainder of the procedure (150 minutes). This concentration of GLP-1 is well tolerated (35). GLP-1 (7-36) amide acetate will be purchased from Clinalfa, Bachem (Weil am Rhein, Germany). Vanderbilt Investigational Drug Service (IDS) has experience preparing this medication for use in clinical investigation (36). Biologically active GLP-1 is rapidly metabolized by the proteolytic enzyme DPP-4 with a half-life < 2 minutes in the circulation. Thus, the physiologic effects of GLP-1 should be negligible shortly after the infusion is stopped at time = 120 minutes (29).

A total of about 90 ml of blood will be drawn for the 2 OGTTs. The 2 OGTTs will be repeated after transplant between day +80 to day +100 +/- 10 days.

Adipose tissue microdialysis

During the OGTT without GLP-1 infusion, a microdialysis catheter will be inserted into abdominal subcutaneous adipose tissue using local anesthesia and sterile technique. At the start of the OGTT, the microdialysis pump containing T1 perfusion fluid (M Dialysis) will be infused at 1 μ l/min. After 60 minutes the microdialysate will be discarded and a fresh microdialysate sample will be obtained over 90 minutes. Samples will be collected on ice and stored at -80° C until analysis.

Hyperglycemic clamp

After an overnight fast, pre-HCT patients will undergo a hyperglycemic clamp procedure, as described by DeFronzo *et. al.* and by our collaborators (37, 38). Arterialized blood will be collected by using a heating pad after insertion of a venous catheter (39). Baseline values for glucose, insulin, C-peptide, and glucagon will be calculated by averaging the measurements obtained at -20, -10, and -1 minute before initiation of the glucose infusion. At time 0, a 200 mg/kg priming dose of 20% dextrose (Hospira) will be given for 10 minutes. Thereafter, blood glucose will be measured approximately every 5 minutes and plasma glucose will be maintained at 200 mg/dL with a variable infusion of 20% dextrose solution for 150 minutes (37, 38). Insulin, C-peptide, and glucagon will be measured 3 times during the first 10 minutes, and then every 10 minutes from 90 to 120 minutes. At time 120 minutes, 5 gm of the secretagogue L-arginine will be injected over 1 minute while the patient is hyperglycemic (40). Insulin, C-peptide, and glucagon levels will be measured 3 times from time 120-130 minutes and then every 10 minutes from 130 to 150 minutes. At 150 minutes the glucose infusion and the procedure will be stopped.

The clamp, 2 OGTTs, and microdialysis procedure will be repeated after HCT between day+80 to +100 (+/- 10 days). Patients diagnosed with PTDM requiring treatment after HCT will be maintained on short-acting insulin which will be held the morning of the studies. Oral antidiabetes medications are not routinely used at our HCT center, however if prescribed, these agents will be washed out for 5 days prior to the procedure and the patient will be maintained on short-acting insulin as above.

Calculations

First-phase GSIS will be calculated in 2 ways: 1) the difference between the maximum plasma insulin level during the first 10 minutes and the average of the 3 baseline insulin values (Δ Insulin₀₋₁₀) or 2) as the AUC for insulin above baseline value from time= 0 to 10 minutes (AUC _{Δ Insulin0-10}). Second or late-phase GSIS will be calculated in the same manner but from time 90-120 minutes (37). These analyses will be repeated using C-peptide and glucagon measurements. Maximum hormone secretion will be determined by the acute insulin, C-peptide, and glucagon response to L-arginine (AIR_{arg}, ACR_{arg}, and AGR_{arg}, respectively). These values will be calculated as the mean hormone level for the first 10 minutes after arginine stimulation minus their average baseline levels (37).

Immune / Metabolic Studies

In addition to the 6 study visits, this protocol will involve 5 separate blood draws for the patient [pre-transplant, Day +10, Day +20, Day +30, and Day +90 (all +/- 7 days)]. During each blood draw, no more than 50 ml of blood will be drawn for serum and lymphocyte analysis. Aliquots will be analyzed using standard immunologic techniques and by flow cytometry as outlined in the preliminary data section. Blood will be drawn for research purposes at the same time as routine blood sampling. Venous blood will be obtained by accessing the central venous catheter using sterile technique or via peripheral venipuncture. Samples will be coded with a unique alphanumeric identifier at the time of collection so that patient identity will be available only to study investigators and research staff. Codes will be sequential numbers and letters.

Blood samples and database will be maintained indefinitely by the study investigators or the laboratory of Crowe/Engelhardt and the research staff. A total of 250 ml of blood will be drawn on 5 occasions for lymphocyte and serum analysis.

Static islet incubation

Human islets will be obtained from the NIDDK supported, Integrated Islet Distribution Program (<http://iidp.coh.org/>). In this program, pancreatic islets are prepared from cadaver donors by islet isolation centers around the USA. This human tissue can be sent to more than 100 investigators without identifying personal health information. Investigators are unable to track any information related to the pancreas donor. The only information received is gender, age, BMI, and whether or not the donor had diabetes. Because of these factors, this research is generally considered non-human research. After receipt of the de-identified tissue, islet set viability and secretory function will be assessed during exposure to various cytokines including IL-33. Vanderbilt Islet Procurement and Analysis Core will perform islet experiments using standard techniques.

Diagnosis of PTDM

PTDM is defined by the occurrence of a fasting blood glucose level ≥ 126 mg/dL or a random blood glucose level ≥ 200 mg/dL at any point from day +1 (day after stem cell infusion) to day +100 after transplant. Fasting blood glucose level will be assessed weekly during routine outpatient visits from day 1 to day 100 after transplant. Patients will be evaluated following an 8 hour overnight fast (water and medications allowed) during standard of care visits. Random blood glucose levels from day +1 to day +100 after transplant will be assessed during standard of care visits or during hospitalization. For hospitalized patients and patients receiving total parenteral nutrition (TPN), all blood glucose determinants will be considered random blood glucose levels for purposes of analysis.

Data Management and Safety

All data will be collected prospectively until day 100 post-transplant. Information will be stored in a password protected electronic database. After day 100, the database will be updated when the patient is seen in the clinic for their annual visit (*i.e.* 1 year after transplant). In addition, the standard of care in the transplant program is to discuss complex inpatients and outpatients (within the first 100 days) on a weekly basis. Detailed minutes of these meetings are routinely maintained. Major events (*e.g.*: unexpected

toxicity and all deaths) are routinely reviewed in a Quality Assurance Meeting and detailed minutes are kept. The above measures will ensure that study patients are monitored closely after transplant and that data will be accurate.

A total of 550 mL of blood will be collected from the patient over approximately 110 days (over 3 to 4 months) for this study.

7.0 Risks and Toxicity Management

Risks

- 1) Study visits at Vanderbilt can be inconvenient
- 2) Venipuncture, blood sampling, and catheter insertion can cause mild discomfort, bruising, and rarely infection
- 3) Repeated blood sampling can worsen anemia, which is monitored frequently after transplant
- 4) Symptoms of hyperglycemia including: thirst, frequent urination, and dry mouth may develop during the hyperglycemic clamp. Symptoms will be monitored closely and minimized by keeping blood glucose levels at 200 mg/dL by adjusting the glucose infusion.
- 5) Arginine is a potent stimulator of insulin and glucagon secretion and will be used during the hyperglycemic clamp. Arginine is commonly employed as a diagnostic test for growth hormone deficiency. It may cause flushing, nausea, or vomiting in about 3% of subjects using doses 6 times the amount used in this study. Since arginine can cause vein irritation, it will be administered by a central venous catheter or large vein to minimize these symptoms.
- 6) GLP-1 [Cinalfa, Bachem (Weil am Rhein, Germany)] will be infused intravenously during the OGTT. GLP-1 can cause nausea, vomiting, headache, and hypoglycemia (usually in patients on combination therapy for diabetes). The dose of GLP-1 in this study is well tolerated and the hormone is rapidly metabolized with a half-life < 2 minutes in the circulation. Thus the effects of GLP-1 should be negligible shortly after stopping the infusion. Based on previous experience, GLP-1 should have no significant side effects at the dose given in this study.

For toxicity attributed to the study, the NCI Common Terminology Criteria for Adverse Events (CTCAE) version 5.0 will be used to characterize the severity.

Protection against risk

- 1) To minimize patient discomfort and venipuncture, blood draws for research purposes will be coordinated with routine clinical blood sampling when possible and will use existing central venous catheters when available
- 2) Nurse or physician will be present during study days

3) Anemia is common after HCT and patients are intensively followed with complete blood counts. Patients routinely receive blood transfusions for a hematocrit <24. Blood for research purposes will not be drawn if hematocrit is known to be <24 and the patient has not yet received transfusion. Once transfusion has been performed, study blood will be drawn.

4) Patients will be monitored closely during study visits and glucose infusions will be adjusted to prevent symptomatic hyperglycemia during hyperglycemic clamps.

5) A unique alphanumeric code will be assigned to each study participant and their study samples to protect their confidentiality. Clinical information and laboratory data will be kept in a password protected database maintained by and accessible to the study investigators and research staff. The key to decode patient identity will be kept in a password-protected database. Only the investigators and research staff will have access to the database.

8.0 Reporting of Adverse Events or Unanticipated Problems

Any untoward medical event will be classified as an adverse event (AE), regardless of its causal relationship with the study. An adverse event will be classified as serious (SAE) if it a) results in death, b) is life-threatening, c) requires inpatient hospitalization or prolongation of existing hospitalization, d) results in persistent or significant disability or incapacity, or e) is a congenital anomaly or birth defect.

Patients undergoing HCT are at high risk for AE's and often require multiple admissions to the hospital during the first 3-4 months after transplantation. For the purposes of this study, only the following SAE's will be reported to the IRB:

-Failure of neutrophil engraftment by day 35 after transplant. Neutrophil engraftment is defined as the first of 3 consecutive days of absolute neutrophil count $\geq 0.5 \times 10^9/L$.

-Any death not related to chemotherapy/conditioning/immunosuppression regimen, progression of malignancy, or GVHD (only until day +100 post transplant)

-Any grade 4 or higher non-hematologic toxicity (not related to chemotherapy/conditioning/immunosuppression regimen, progression of malignancy, or GVHD) (Only until day +100 post transplant).

Deaths not related to chemotherapy/conditioning regimen/immunosuppression regimen, progression of malignancy, or GVHD be reported to the IRB within 24 hours of the Investigators knowledge of the occurrence and all other toxicities will be reported within 7 days of the Investigator's knowledge of occurrence.

9.0 Study Withdrawal/Discontinuation

Patients will be followed after transplantation. Individuals may withdraw from the study at any time by notifying one of the study investigators. Withdrawal from the study will be noted in the database. Following withdrawal, no subsequent blood collections for study

purposes will be obtained, and no further clinical information will be collected for this study.

In addition, the investigator has the right to withdraw a patient from the trial for any of the following reasons:

-protocol violation

-non-compliance

-any condition which, in the opinion of the investigator, might interfere with study objective

-any reason which, in the opinion of the investigator, adds additional risk to the patient

10.0 Statistical Considerations

10.1 Power Analysis and Sample Size Calculation

D.3.c. Sample size calculation for Aims 1 and 2.

β -cell function, glucagon suppression, and IL-33 signaling are the primary outcomes for the aims. Sample size was calculated using a 2-group t-test with PS Software (version 3.0, Dupont&Plummer, 2009) at a 0.05 two-sided significance level. We estimate a 50% incidence of PTDM by day+100 (1, 8, 14). The sample size provides at least 80% power to detect a biologically meaningful difference in mean between patients with and without PTDM for each aim. The same patient cohort will be monitored for all aims. We will enroll 34 patients to ensure at least 80% power for each aim. Assuming the dropout rate is about 10% (early death or pre-HCT diagnosis of diabetes by OGTT), an additional 6 subjects will be recruited (41-43). Forty subjects are feasible to accrue over 4 years, given that 151 patients underwent allogeneic HCT at Vanderbilt in 2015.

Subaim 1A primary outcome: second-phase GSIS. A mean difference of 7.5 μ U/mL represents a significant difference in second-phase insulin release between healthy controls and individuals at risk for type 2 diabetes (44-46). A sample size of 17 patients in each group (either with or without PTDM) has 80% power to detect at least a 7.5 μ U/mL difference in insulin secretion between groups using a standard deviation of 7.5 μ U/mL.

Subaim 1B primary outcome: glucagon secretion during OGTT. AUC was calculated for each patient to present as total glucagon secretion during OGTT. Our preliminary data observed 8.83 ± 0.73 pg/mL \cdot min (mean \pm SD) in log(AUC) scale for patients with PTDM (Figure 4). With a sample size of 9 each group, there is 80% power to detect a log(AUC) change of from 8.83 pg/mL \cdot min to 7.83 pg/mL \cdot min.

Aim 2 primary outcome: IL-33 signaling Extrapolating our preliminary and pre-clinical data, a mean difference of 70 pg/mL is likely to represent a significant difference in IL-33 signaling between groups (14, 20). With a sample size of 17 in each group, there is 80% power to detect a 70 pg/mL difference in IL-33 production between groups using a standard deviation of about 70 pg/ml.

10.2 Statistical Analysis Plan

Primary analysis of Subaim 1A

We will compare pre-transplant insulin secretion (first- and second-phase GSIS) among patients who do or do not go on to develop PTDM. Wilcoxon rank sum test will be applied to compare the population mean difference between these two groups. Multivariable logistic regression will evaluate whether GSIS is an independent predictor of PTDM after adjusting for the following covariates: gender, conditioning (ablative vs. reduced intensity), or acute GVHD requiring steroids. The estimated OR and 95% CI of the OR will be provided to measure the effect of the association. We will take a similar approach for the primary analysis of Aims 1B and 2 which will compare post-transplant total glucagon secretion during the OGTT ($AUC_{\text{Glucagon}0-120}$) and serum IL-33 levels among patients with or without PTDM, respectively.

Secondary analyses for Subaims 1A and 1B

1) determining if glucagon secretion and OGTT results pre-transplant predict PTDM development (herein OGTT results refers to 2 hour blood glucose and peak/nadir/total hormone levels during OGTT), 2) determining if GLP-1 infusion increases insulin secretion and glucagon suppression compared to OGTT values without GLP-1, 3) comparing GSIS, glucagon suppression, and OGTT results at day +90 among patients with or without established PTDM, and 4) comparing the change in GSIS, glucagon suppression, and OGTT results at baseline to those determined at day +90 after transplant in the entire cohort or among groups stratified based on PTDM, conditioning regimen (ablative vs. reduced intensity) or for the presence or absence of acute GVHD treated with steroids.

Secondary analyses for Aim 2

1) determining if PTDM is predicted by pre- or post-transplant levels of IL-33/sST2 from serum/tissue microdialysate, or by the frequency of circulating ST2+Th1 cells and ST2+Tregs, 2) analyzing whether IL-33/sST2 levels and ST2+Th1 cells and ST2+Tregs correlate with insulin/glucagon secretion during OGTT and hyperglycemic clamps, 3) test whether *in vitro* exposure of HCT recipient CD4+ T cells to IL-33 promotes proliferation and inflammatory Th1 immunity, and 4) test if *in vitro* exposure of human islets to IL-33 impairs insulin secretion and decreases islet cell viability in culture.

General statistical plan

Data will be presented using means and standard deviations for continuous variables, as well as percentage and frequency for categorical variables. Investigations for outliers and assumptions for statistical analysis, e.g., normality and homoscedasticity will be made for the parametric test such as t-test and mixed model. If necessary, data will be transformed utilizing appropriate transformations such as the log or square root. Comparison between two independent groups, e.g., with or without PTDM, will be carried out using either two-sample t-test or Wilcoxon rank sum test for continuous variables and Chi-square test or Fisher's exact test for categorical variables. A Spearman correlation will be used to test the relationship between 2 continuous variables. For matched analyses and for the comparison of the changes between baseline and day+90 after transplant in the entire cohort, the mean difference will be compared with paired Student's t-test or Wilcoxon signed rank test for continuous

variables and a McNemar's test for categorical variables. To compare the differences between baseline (pre-transplant) and day +90 test results among various groups we will apply linear mixed model to continuous outcomes and generalized linear mixed model to binary/ordinal outcomes. Groups of interest will be created by stratifying patients based on PTDM, conditioning regimen (ablative vs. reduced intensity) or acute GVHD treated with steroids. All data will be stored in a computerized database and analyzed with R version 3.3.1 (R Foundation for Statistical Computing, Vienna, Austria). *P*-values will be 2-tailed and considered significant at $P < 0.05$.

11.0 Privacy/Confidentiality Issues

A unique alphanumeric code will be assigned to each study participant to protect their confidentiality. Clinical information and laboratory data will be kept in a password protected database maintained by and accessible to the study investigators and research staff. The key to decode patient identity will be kept in a password protected database. Only the investigators and research staff will have access to the database. All communications with non-investigators of this study and any publication of results will identify patients by code only to protect patient identity.

12.0 Followup and Record Retention

All research records including study results will be retained for at least 6 years after the study is finished. At that time, the research data that has not been put in a participant's medical record and all study samples will be kept for an unknown length of time. Any research data that has been put into a participant's medical record will be kept for an unknown length of time.

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Appendix A

Study Table - Donor

	Baseline	Pre-Collection
Study Blood collection		X
Informed Consent	X	
Review of Eligibility	X	
Data Collection	X	X

Study Table - Patient

	Baseline	Pre-Transplant	Day 10 post-transplant	Day 20 post-transplant	Day 30 post-transplant	Day 80-100 post-transplant	D365 post-transplant	D730 post-transplant
Study Blood collection	X		X	X	X	X		
OGTT / Microdialysis - CRC visit		X				X		
OGTT / GLP-1 Infusion - CRC visit		X				X		
Hyperglycemic Clamp - CRC visit		X				X		
Informed Consent	X							
Review of Eligibility	X							
Data Collection	X	X	X	X	X	X	X	X

All dates for blood collection +/- 7 days, all dates for procedures / study visits +/- 10 days

Oral glucose tolerance testing and the hyperglycemic clamps will be performed on separate days as scheduling allows