Official Title: Bridging Pediatric and Adult Biomarkers in Graft-Versus-Host Disease

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Bridging Pediatric and Adult Biomarkers in Graft-Versus-Host Disease

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PROTOCOL SYNOPSIS

Title: Bridging Pediatric and Adult Biomarkers in Graft-Versus-Host Disease

Study Design

This study is designed to collect longitudinal biological samples from patients after hematopoietic cell transplantation (HCT) cared for at multiple bone marrow transplant centers to validate biomarkers of both acute and chronic GVHD as well as for use in future unspecified research.

After informed consent is signed, this study will involve 1) collection of basic HCT data and clinical data available in the medical record and 2) providing blood (and saliva in occasional cases) samples for processing, storage, DNA extraction, and analysis (including a seven biomarker protein panel as well as future unspecified research purposes)

Pediatric and adult patients will be included (all adult patients will be from the Fred Hutchinson Cancer Research Center, the Johns Hopkins Sidney Kimmel Comprehensive Cancer Center, and the Indiana University Simon Cancer Center).

Primary Objective:

1. To confirm that ST2 alone or the seven-biomarker panel measured at initiation of GVHD therapy predict a) D180 post-therapy non-relapse mortality; b) D28 post-therapy non-response, and c) GVHD grade 1-4 onset D180 post-therapy non-relapse mortality.

Secondary Objective:

1. To demonstrate that ST2 alone or the seven-biomarker panel measured at day 14 or day 21 post-HCT (or a combination of these time points) predicts D180 post-HCT non-relapse mortality.
2. To demonstrate that ST2 alone or the seven-biomarker panel measured at initiation of GVHD symptoms/therapy diagnose acute GVHD as compared to other complications presenting with similar symptoms (drug rash, CMV, Clostridium enteritis).
3. To demonstrate that ST2 alone or the seven-biomarker panel measured at initiation of GVHD symptoms/therapy diagnose the severity of acute GVHD at onset and maximum.
4. To develop a repository of biospecimens linked to clinical data for future unspecified research.

Eligibility:

All patients receiving an allogeneic hematopoietic stem cell transplant, cord blood transplant, bone marrow transplant, T cell depleted marrow, donor lymphocyte infusion (DLI), or donor cellular infusion (DCI) can be included.

Patients (or parent/guardian) must have signed the informed consent document and children who are at least 7 years of age must sign the assent document. For subjects who turn 18 years of age during participation in this study, participation in the future use portion of the study after their 18th birthday requires that they sign the informed consent statement following turning 18 years of age.

Accrual objective: Sample size: 430

Accrual period: The estimated accrual period is 24 months

Study duration: Patients will be followed for 12 months following initiation of GVHD therapy or 12 months post-HCT for non-GVHD patients.
This study will not require any extra clinic visits beyond those required for routine clinical patient care.
For subjects who consent to the future unspecified use of their biospecimens for research purposes, clinical data collected at any time post-transplant as part of routine clinical care may also be accessed.

Governance of samples stored for future research: Samples stored for future research can only be released for use by investigators once they have received 1) approval from the Pediatric HCT Bio-repository Network (PHCTBN) governance committee and 2) IRB approval for a hypothesis driven research question.

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1. BACKGROUND AND RATIONALE

1.1. Acute Graft-versus-Host Disease (GVHD)

Allogeneic hematopoietic cell transplantation (HCT) is a potential curative therapy for cancers of the blood and bone marrow, including leukemias that represent approximately one third of all cancer cases occurring among children and the leading cause of cancer deaths in children[1]. Use of HCT has increased as new techniques have allowed for transplantation in patients who previously would not have been considered HCT candidates. Approximately 30,000 allogeneic HCTs will be performed worldwide in 2020; 20% of allogeneic HCT are performed in the pediatrics population. However, the efficacy of this procedure has been impeded by frequent and severe graft versus host disease (GVHD). The development of GVHD is tightly linked to the beneficial graft versus tumor (GVT) effect, the most potent form of immunotherapy. Nowadays, acute GVHD is measured by clinical symptoms in three organ systems: the skin, liver and gastrointestinal (GI) tract[2-4]. Acute GVHD typically occurs between two and eight weeks after transplant, but may occur later[4], and is often clinically indistinguishable from other causes of clinical symptoms such as conditioning regimen toxicity, infection, or medication. Endoscopic biopsy is often used to confirm the diagnosis[2,5], but histologic severity on biopsy has not consistently correlated with clinical outcome[3,5-7]. A non-invasive, reliable blood biomarker specific for GVHD with strong prognosis information would thus significantly aid in the management of patients with this disorder.

1.2. Therapy resistant GVHD

In addition, there are currently no therapies that target GVHD biomarkers, and no effort has been made to develop drugs specific for GVHD treatment. Thus far, because of the potentially overlapping pathophysiology, all drugs used to treat GVHD were originally designed for other diseases, particularly autoimmune disorders or loss of tolerance in organ transplantation. Some examples include etanercept and infliximab, which are anti-tumor necrosis factor (TNF) drugs approved to treat rheumatoid and psoriatic arthritis. Another example, mycophenolate mofetil, is an immunosuppressant used extensively in organ transplantation and autoimmune diseases. Some targets have been proposed based on preclinical models, but few have been implemented clinically, and none are based on patient-specific biomarkers. Steroids, which are general immunosuppressors, remain the first line of treatment for patients presenting with GVHD symptoms. However, less than half of patients treated with the standard therapeutic regimen for acute GVHD (high dose steroids) have a sustained response[8], and more severe GVHD is even less likely to respond to treatment[9]. Only the clinical response on day 14 is highly predictive of the response on day 28, and the day 28 response most accurately estimates overall survival (OS)[10]. Once GVHD occurs, the most important predictor of long-term OS is the primary response to first-line treatment; patients who respond completely to treatment have low non-relapse mortality (NRM) equivalent to patients without GVHD, whereas patients with incomplete or no response have a NRM approaching 50%[11]. Thus, a major challenge for clinicians is to identify which patients will respond to current GVHD treatment and to design more efficient treatment regimens. The contribution of this study is expected to be significant, because

the identification of steroid-refractory GVHD biomarker panels at symptom onset has tremendous potential for impacting our ability to risk stratify patients before initiating GVHD treatment. It may also ultimately guide the intensity and duration of treatment and minimize the toxicity associated with chronic steroid administration. The ability to identify patients who will not respond to traditional treatment and who are at particularly high risk for morbidity and mortality could permit tailored treatment plans, such as additional immunosuppressive treatments for high-risk patients that may be more effective if introduced early. Equally important is the identification of low-risk patients who will respond well to treatment. These patients may tolerate a more rapid tapering of steroid regimens to reduce long-term toxicity, infections, and a loss of the graft versus leukemia effect. Follow-up marker monitoring in high-risk patients could also help decide whether to taper the treatment.

1.3. Acute GVHD biomarker studies

OVERVIEW. A major barrier to graft-versus-host disease (GVHD) research and treatment is that the diagnosis and prognosis of GVHD relies almost entirely on clinical symptoms (onset and maximum grade). No simple test for diagnosis and response to treatment of acute GVHD exists. We performed discovery and validation experiments to identify a panel of informative biomarkers with diagnostic and prognostic value for acute GVHD[12]. We recently identified a skin-specific GVHD marker, elafin with significant diagnostic and prognostic ability[13]. Using the same proteomics strategy, we also identified a gastro-intestinal specific GVHD marker, regenerating islet-derived 3-alpha with prognostic ability[14]. These studies have been among the first to apply proteomic technology to identify biomarkers for GVHD, yet blood tests were still not able to predict a patient's response to treatment. We, therefore, embarked on a novel proteomics experiment as a discovery engine and ELISA for validation, we discovered that among a panel of seven biomarkers, ST2 (suppression of tumorigenicity 2 or interleukin-33 receptor) measured at the initiation of GVHD treatment accurately predicted the day 28-non-response and the day 180-post treatment non-relapse mortality in a validation set of 381 patients, and outperformed all other biomarkers for discriminating responders from non-responders of GVHD therapy (Vander Lugt et al. ASH presentation). These seven biomarkers have not yet been validated prospectively in samples from different institutions. This study addresses 1) the use (at minimum) of the four-biomarker panel (interleukin-2-receptor-alpha, tumor necrosis-factor-receptor-1, interleukin-8, and hepatocyte growth factor)[12], and the two newly identified skin-specific marker, elafin[13], and gastro-intestinal specific marker, regenerating islet-derived 3-alpha[14] for a) diagnosis of acute GVHD, b) association with therapeutic responses and mortality, c) prediction of acute GVHD and 2) the use of ST2 for a) association with therapeutic responses and mortality, b) prediction of acute GVHD and non-response. Upon completion, these studies will likely result in novel biomarker panels that may facilitate the therapeutic decision making process for allogeneic HCT patients.

PRELIMINARY STUDIES.

Graft-versus-Host Disease Biomarkers. In our initial approach to the identification of GVHD biomarkers, we screened patient plasma samples by competitive hybridization to arrays of antibodies specific for 120 diverse proteins including acute phase reactants, cytokines, angiogenic factors, tumor markers, leukocyte adhesion molecules, metalloproteinases, and inhibitors of metalloproteinases. We used array technology during the discovery phase, followed by sandwich enzyme-linked immunosorbent assay (ELISA) to quantitate the concentrations of individual proteins. Due to the limited quantity of plasma available for each patient, we used a sequential ELISA protocol for eight potential biomarkers rather than multiplex techniques to improve detection and quantification of low abundance proteins. This approach identified and validated a four-protein biomarker panel with high specificity for GVHD diagnosis (Figure 1) and prognostic value that was published as a plenary paper in *Blood*[12]. All four of the biomarkers identified with the antibody array approach to biomarker screening have been associated with GVHD in previous studies[15-26]. However, due to the size of our study, we provided **the first demonstration that these biomarkers are associated with GVHD clinical outcome and prognosis.** While the antibody biomarker screen can be considered internally unbiased because each antibody is represented at an equal level, the inherent limitation of this approach is introduced by the availability and selection of antibodies. To overcome these limitations and create a broader, less biased approach, we utilized state-of-the-art proteomic technology that is straightforward, gel-free, and based on high-resolution mass spectrometry. This technique interrogates complex proteomes present in samples, such as plasma, by matching mass spectra to a sequence database for protein identification. To develop this approach, we collaborated with Samir Hanash at the University of Washington and embarked upon a second discovery study using Intact Protein Analysis System (IPAS). The primary goal of this new discovery phase was to find biomarkers present at the time of GVHD diagnosis that were specific for GVHD target organs. To

Fig 1. Individual Receiver Operating Characteristic curves (ROC) for IL-2Rα, TNFR1, HGF, IL-8 and the composite panel.

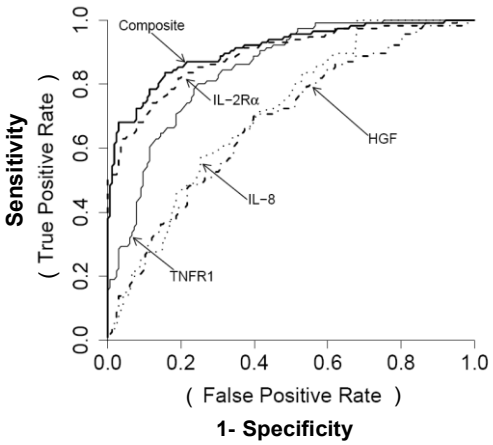
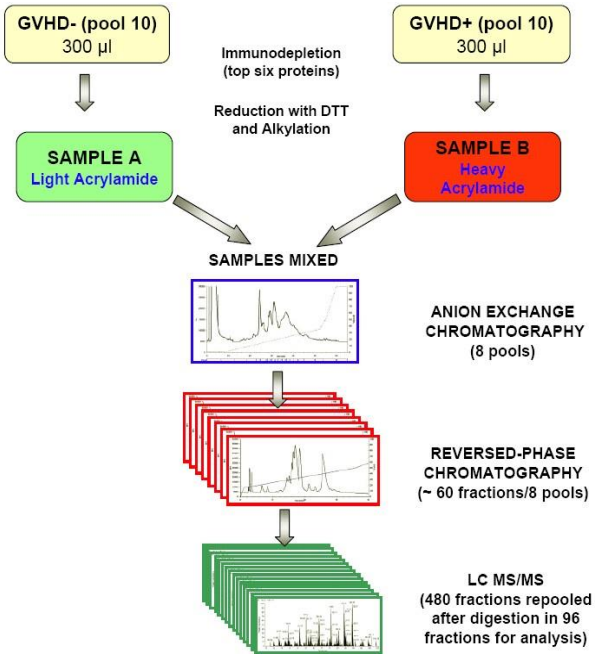


Fig 2. Intact Protein Analysis System workflow



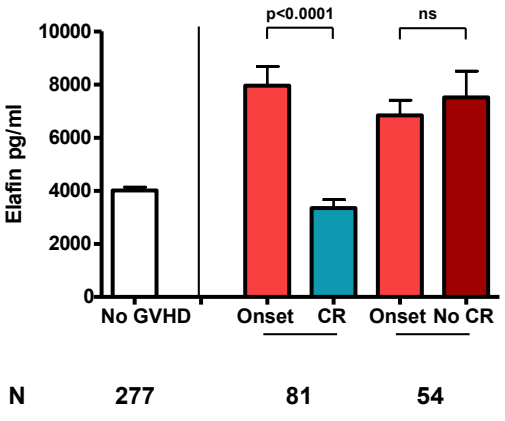
assess the validity of this approach, we limited our initial search to the two most frequent GVHD targets (skin and GI tract). We compared plasma pooled from 10 patients with skin-specific GVHD to 10 controls in the first IPAS run, and plasma pooled from 10 patients with GI tract-specific GVHD to 10 controls in a second IPAS run. The criteria for inclusion in the discovery samples were 1) patients conditioned with a myeloablative regimen and 2) patients who experienced development of acute GVHD (grade II-IV) without other severe complications. The controls included patients without GVHD who were matched for age, intensity of the conditioning regimen, donor source (related versus unrelated), HLA match (matched versus mismatched), and time of sample acquisition. The workflow of IPAS is shown in Figure 2 and described here. IPAS analysis compared plasma pooled from 10 patients with GVHD versus plasma pooled from 10 matched patients without GVHD. The pools were individually immunodepleted of the six most abundant plasma proteins (Albumin, IgG, IgA, transferrin, haptoglobin, and antitrypsin). Intact proteins were then labeled on cysteine residues with acrylamide. The GVHD– pool received the light acrylamide isotope (^{12}C acrylamide), and the GVHD+ pool received the heavy ^{13}C-acrylamide isotope. The two pools were mixed together for further analysis, and specimens were then subjected to a two-dimensional protein fractionation where anion-exchange chromatography represents the first dimension of the protein fractionation. A total of eight pools were collected from the anion exchange chromatography and then subjected to a second dimension of separation by reversed-phase chromatography. Sixty fractions were collected during the run, corresponding to a total of 480 fractions for each experiment. Then, the 480 individual digested fractions from each reversed-phase run were re- pooled for a total of 96 reverse-phase pools and analyzed on a LTQ-FT mass spectrometer. Because protein digestion is performed at the last step, just before liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis, the term “intact” protein analysis was used. The acquired spectra were automatically processed by the Computational Proteomics Analysis System for the identification of proteins with a false discovery rate of < 5%. The result was a reduced complexity of individual fractions subjected to analysis and identification of proteins with a range of concentrations spanning seven logs. This technique was therefore able to detect very low abundance proteins.

For quantitative proteome analysis, we labeled each GVHD+ pool and matched GVHD– pool with differential isotopic labeling. Heavy and light isotopes label approximately 35% of proteins, and 834 proteins were confidentially quantified based on differential stable isotope labeling in the skin GVHD run. Of the 834 proteins, 66 were increased by at least a factor of two in GVHD plasma. To sequentially refine the list of candidate proteins, we further selected the proteins based on their relationship to the tissue of origin (Human Protein Atlas [http://www.proteinatlas.org/]), information from published literature, pathway networks (MetaCore analytical suite version 4.7, GeneGO, Inc., St. Joseph, MI), and the availability of a sandwich ELISA. Fourteen candidates were expressed primarily in the skin. Elafin emerged as the lead biomarker candidate of skin GVHD at the time of clinical diagnosis because it is highly expressed in skin tissue and has been shown to be overexpressed in inflamed epidermis in diseases such as psoriasis. Furthermore, elafin is an epidermal proteinase inhibitor that is induced by TNF-α[27], an inflammatory cytokine that activates host antigen presenting cells (APCs),

which in turn activate the mature donor T cells that mediate GVHD pathophysiology [4]. Concentrations of elafin were measured by ELISA in 492 allogeneic HCT recipients. Elafin concentrations were significantly higher in plasma from patients with skin-specific GVHD than in control samples from patients without GVHD, with GI-specific GVHD, or with non-GVHD rash. In addition, elafin concentrations may have value not only as a noninvasive diagnostic test, but also as a prognostic marker as published recently in *Science Translational Medicine*[13]. This data provides a proof-of-principle demonstration that biomarkers of disease-related, tissue-specific changes can be detected in the plasma of patients. We used this approach as the basis for extension of the validation of plasma GI biomarkers. We identified Regenerating Islet-Derived 3-alpha (REG3α) as a biomarker of lower gastrointestinal GVHD and validated it in 2 independent sets totaling 1014 patients from three different centers. We showed that plasma REG3α concentration at the onset of GVHD diarrhea is the best biomarker (i) to differentiate GVHD diarrhea from non-GVHD diarrhea; and (ii) to provide important prognostic information, including eventual response to GVHD treatment and survival. We also showed that the addition of this marker to clinical stage and histologic grade leads to a more refined grading system and risk stratification of patients at the time of GVHD diagnosis. This was recently published in *Blood*[14].

Further, we first validated elafin as biomarker of resistance to GVHD treatment (unpublished data). As shown in Figure 3, we performed additional measurements at four weeks post-treatment and correlated the concentrations of elafin with the response to GVHD treatment. Patients with complete response (CR) at four weeks exhibited no rash symptoms and had concentrations of elafin in the range of patients without GVHD (white bar), whereas patients with persistent or progressive rash (No-CR) (30% of the population) had unchanged elafin concentrations. Likewise, measurements of TNFR1 concentrations declined as compared to the onset in patients who had a complete resolution of GVHD symptoms [28].

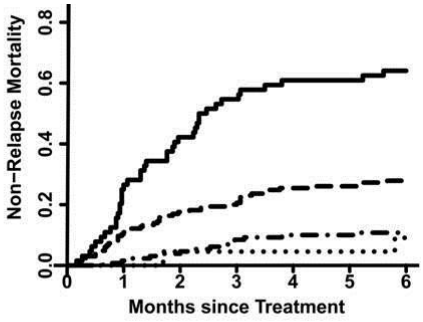
Fig 3. Elafin concentrations at onset and 4-week post-treatment in CR and No CR patients (paired t-test)



These studies have been among the first to apply proteomic technology to identify biomarkers for GVHD, yet blood tests were still not able to predict a patient's response to therapy, particularly at GVHD onset, when risk-stratification is most beneficial. We hypothesized that biomarkers discovered with an intact proteomic analysis system (IPAS) approach that we have described above will discriminate between therapy responsive (resolution or improvement of GVHD by day (D) 28 post-therapy initiation) and unresponsive (absence of complete or partial response) patients and predict survival in patients receiving GVHD therapy.

We first performed an IPAS comparing pooled plasma taken at D16 ± 5 post-therapy from 10 responders (R) and 10 non-responders (NR). Ten candidate biomarkers with an NR/R ratio of > 1.5 in the IPAS were measurable by ELISA. We therefore measured concentrations in the 20 individual plasma aliquots. Five were significantly increased in NR vs. R, with an area under the receiver operator characteristic curve of ≥ 0.85 (ST2, IL1sRII, MIF, LYVE, and Lipocalin). These were then measured at therapy initiation (D0), with 6 previously validated diagnostic biomarkers of GVHD (IL2Rα, TNFR1, HGF, IL8, Elafin, a skin-specific marker, and Reg3α, a gut-specific marker) in plasma samples from a validation set of 381 patients with acute GVHD grade 1-4 at onset and treated with systemic steroids at the University of Michigan from 2000 to 2011. Preliminary analyses (not shown) determined that D0 measurements predicted D28 non-response and D180 OS. HLA match (match vs. mismatched; Odds Ratio (OR) 1.5, p = 0.07), conditioning intensity (full vs. reduced; OR 1.7, p = 0.04), and GVHD onset grade (grade 3-4 vs. grade 1-2; OR 2.2, p = 0.001) predicted day 28 non-response in univariate analysis, while age at transplant (≥ 55 years vs. < 55 years), donor (unrelated vs. related), and stem cell source (peripheral blood vs. bone marrow/cord) did not. After adjustment for the 3 clinical characteristics which predicted D28 response, multivariate analysis of the 11 protein concentrations showed that 3 predicted D28 response (ST2, p = 0.001; IL1sRII, p = 0.07; and IL8, p = 0.03) and 7 predicted post-therapy D180 OS (ST2, p = 0.003; IL1sRII, p = 0.07; IL8, p = 0.05; Elafin, p = 0.06; MIF, p = 0.04; TNFR, p = 0.03; and Reg3α, p = 0.002 in gut-GVHD subset). Using logistic regression, we examined the ability of both the 7 biomarkers and ST2 alone to predict for D28 non- response, as ST2 was the most significant marker in all previous analyses. ST2 is the IL33 receptor, a member of the IL1/ Toll-like receptor superfamily, which promotes a Th2-type immune response in diseases such as arthritis and asthma [29]. A high biomarker value was defined as a plasma concentration greater than 50% above the median value of the responders' group. A high panel was defined as having at least 5 of 7 high biomarkers. Patients with high ST2 levels were 2.6 times more likely not to respond to therapy independent of the aforementioned significant clinical characteristics (p < 0.001) while patients with a high panel were only 1.9 times more likely not to respond (p = 0.004). Thus, only ST2 measurement was used for further analyses. Because ST2 concentrations correlated with response, we hypothesized that ST2 would predict D180 non-relapse mortality (NRM), independent of GVHD onset grade, the strongest clinical predictor of NRM (20% for GVHD grade 1-2 vs. 50% for GVHD grade 3-4, Hazard ratio (HR) 3.0, p

Fig 4. Risk stratification with ST2 and onset GVHD grade for day 180 NRM



Onset GVHD grade & D0 ST2	D180 post-therapy NRM	Hazard Ratio compared to (a)	p-value
(a) Low ST2 & Grade 1-2 (·—·— N=130)	11%		
(b) Low ST2 & Grade 3-4 (····· N=22)	9%	0.8	0.80
(c) High ST2 & Grade 1-2 (— — N=165)	28%	2.9	<0.001
(d) High ST2 & Grade 3-4 (——— N=64)	64%	9.0	<0.001

< 0.001). NRM cumulative incidence curves and HR for the 4 risk categories of low ST2 / grade 1-2, low ST2 / grade 3-4, high ST2 / grade 1-2, and high ST2 / grade 3-4 are shown in Figure 4. Interestingly, patients presenting with high clinical grade and low ST2 had a good prognosis, suggesting that ST2 provides important prognostic information at initiation of therapy above the clinical grade.

In conclusion, soluble ST2, the form measured by ELISA, is a decoy receptor that drives the Th2 phenotype toward Th1, a mechanism by which it may act in the pathophysiology of resistant GVHD. ST2 concentrations obtained at initiation of GVHD therapy significantly enhance the accuracy of outcome prediction independent of GVHD grade. Measurement of ST2 may allow for early identification of patients at risk for subsequent non-response and mortality, and may provide a promising target for novel therapeutic interventions.

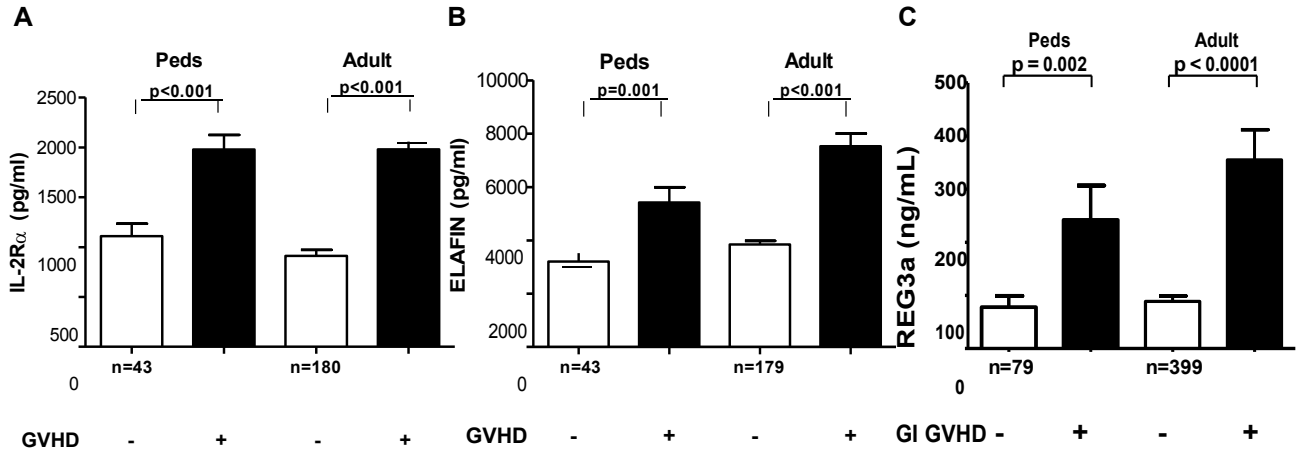
These six diagnostic biomarkers interleukin-2-receptor-alpha, tumor-necrosis-factor-receptor-1, interleukin-8, hepatocyte growth factor, elafin, regenerating islet-derived 3-alpha, and suppression of tumorigenicity 2 (ST2) have not been validated blindly in samples from different institutions. We therefore hypothesized that this seven GVHD biomarker panel, measured during the transplant course, would help diagnose GVHD from other post-HCT complications, discriminate between therapy-responsive and unresponsive patients and predict survival in samples prospectively obtained on a multicenter trial.

Pediatric biomarkers. As shown above, the majority of candidate biomarkers have been studied in adult population. The identification and adaptation of adult biomarkers, for those conditions that are similar in children and adults, is an efficient, expedient and economical way to advance knowledge in the use of biomarkers in pediatrics. Many biomarkers proposed or developed in pediatrics have not been subjected to the rigorous evaluation and validation process needed for their use in clinical trials. Cross-age studies of biomarkers, whether between children of different ages or between children or adolescents and adults, can provide new insights in disease, response to treatment and toxic effects. There are a number of factors that may affect the usefulness of potential biomarkers and surrogate endpoints in pediatrics. The marked difference in the natural history of some pediatric diseases when compared to adults must be taken into account when biomarkers that have been developed for the adult population are used in pediatrics. Following HCT, younger age has been associated with lower GVHD incidence and severity as well as better response to treatment and therefore better overall outcomes[30,31]. In addition, children are undergoing development and hence are not a homogeneous population. The amount or activity of many biomarkers used in pediatrics varies according to age and development; examples include beta-2 microglobulin, and host immunologic markers such as CD4 counts. Following HCT, one major difference is the developmental stage of the thymus. Furthermore, the usual small sample sizes characteristic of pediatric trials may also prevent the validation of surrogate endpoints
thus multicenter trials are necessary.

However, the majority of candidate biomarkers that are associated with a specific mechanism relevant to disease progression have been studied in adults. Advances in -omics technology provide a plethora of analytical tools to discover and analyze mechanism- and disease-specific biomarkers for risk stratification and potential drug development. Translation of innovative systems approaches such as genomics, proteomics and metabolomics into a developmental framework has not been exploited to understand mechanisms of GVHD treatment efficacy and toxicity in children.

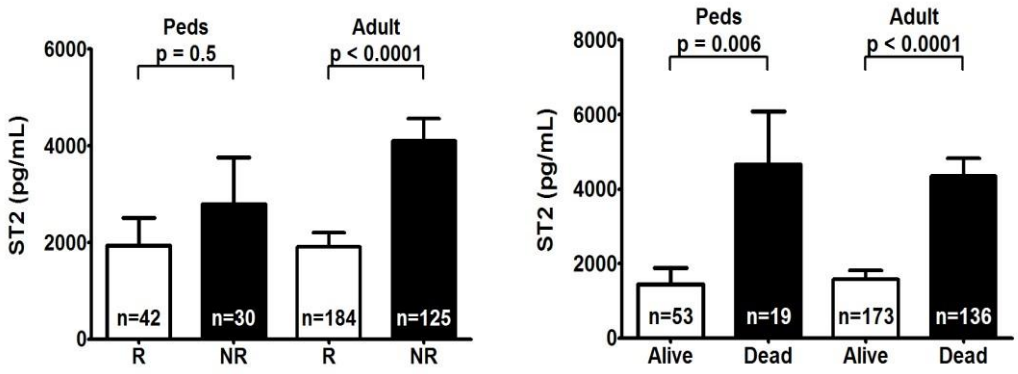
Thus, we have further analyzed our previous GVHD biomarkers focusing solely on pediatric patients as this population represents approximately 20% of our total allogeneic HCT population. These preliminary data are the ground for further validation in samples prospectively obtained on a multicenter trial. We determined that there is a statistically significant increase in all four proteins included in the panel (Figure 5A, IL-2Rα as a representative), elafin, a skin-specific GVHD marker (Figure 5B), and REG3α, a gastro-intestinal (GI) specific GVHD marker (Figure 5C), based on the presence or absence of GVHD in both pediatric (age ≤ 21 yr) and adult (age > 21 yr) HCT recipients.

Figure 5: A. IL-2Rα concentrations; B. Elafin concentrations; C. Reg3α concentrations, in pediatric and adult HCT patients with and without acute GVHD.



We also examined the novel biomarker of unresponsiveness to GVHD treatment: suppressor of suppression of tumorigenicity 2(ST2) in the pediatric population. Interestingly, the clinical response to GVHD treatment is different in children as compared to adults because 37% of children who are non-responders at day 28 post- initiation of therapy will be alive at day 180 post-therapy whereas all adults who are non- responders at day 28 post-initiation of therapy will be dead at day 180 post-therapy suggesting that, as opposed to the adult population, in the pediatric population day 28 post-initiation of therapy is not a good surrogate for day 180 post-therapy mortality. Not surprisingly, we therefore did not observe a significant difference in onset ST2 concentrations between responders and non-responders at day 28 post-initiation of therapy (**Figure 6A**). However, we still observe a significant higher onset ST2 concentrations in patients who will die at day 180 post-therapy (**Figure 6B**).

Figure 6: A. GVHD onset ST2 concentrations in pediatric and adult responders and non-responders at day 28 post-therapy; B. GVHD onset ST2 concentrations in pediatric and adult, alive or dead at day 180 post-therapy.



Therefore, we feel that analysis of biomarkers in pediatric patients and comparison with adults is not only appropriate, but essential in order to further our understanding of this disease in this vulnerable patient population. Upon completion, these studies will result in **novel biomarker panels that may facilitate the therapeutic decision-making process for allogeneic HCT patients**. The ability to identify patients at high risk for GVHD early in their transplant and treatment course has **important therapeutic consequences, including more stringent monitoring and/or preventative care**. This analysis may also offer new key insights into the **biology of GVHD and identify novel pathways and proteins with potential as therapeutic targets.**

2. STUDY DESIGN

2.1. Rationale for study design and objectives

For this study, blood samples will be collected on a calendar basis: Day -7, day 7, day 14, day 21, day 28, day 56, day 100, day 180 and day 360 post-HCT, as well as at appearance of clinical symptoms suggestive of acute GVHD and that trigger treatment with systemic steroids. A saliva sample will also be collected at the day-7 and day +56 time-points from subjects with a HCT white blood cell count of <400/μL to provide an additional specimen for DNA extraction. Samples will be processed at local sites and will then be shipped to the Indiana University Simon Cancer Center (the Data and Specimen Coordinating Center) from the other five participating Blood and Marrow Transplant Programs (Dana-Farber Cancer Institute and Boston Children's Hospital, Johns Hopkins Sidney Kimmel Comprehensive Cancer Center, Fred Hutchinson Cancer Research Center, Texas Children's Hospital, and Children's National Medical Center). The appropriate samples will be transported to Dr. Paczesny's laboratory for analysis of a seven-protein biomarker panel. The results from previous discovery and validation studies suggested that the panel of six biomarkers can confirm the diagnosis of acute GVHD in patients at onset of clinical symptoms of acute GVHD and provide prognostic information independent of acute GVHD severity. Furthermore, suppression of tumorigenicity 2 measured at initiation of therapy can predict day 28-non-response and the day 180-post treatment non-relapse mortality in patients with GVHD treated with at least systemic corticosteroids. ST2 outperforms all previously identified biomarkers for prognostic information. The plasma protein biomarker panel will include at minimum: interleukin-2-receptor-alpha, tumor-necrosis-factor-receptor-1, interleukin-8, hepatocyte growth factor, elafin, regenerating islet-derived 3-alpha and suppression of tumorigenicity 2.

Primary Objective: The primary objective is to confirm that ST2 alone or the seven-biomarker panel measured at initiation of GVHD therapy predict a) D180 post-therapy non-relapse mortality; b) D28 post-therapy non-response, and c) GVHD grade 1-4 onset.

Secondary Objective:

1. To demonstrate that ST2 alone or the seven-biomarker panel measured at day 14 or day 21 post-HCT (or a combination of these time points) predict D180 post-HCT non-relapse mortality.
2. To demonstrate that ST2 alone or the seven-biomarker panel measured at initiation of GVHD symptoms/therapy diagnose acute GVHD as compared to other complications presenting with similar symptoms (drug rash, CMV, Clostridium enteritis).
3. To demonstrate that ST2 alone or the seven-biomarker panel measured at initiation of GVHD symptoms/therapy diagnose the severity of acute GVHD at onset and maximum.
4. To develop a repository of biospecimens linked to clinical data for future unspecified research.

2.2. Eligibility

- All patients receiving an allogeneic hematopoietic stem cell transplant, cord blood transplant, bone marrow transplant, T cell depleted marrow, donor lymphocyte infusion (DLI), or donor cellular infusion (DCI) can be included.
- Patients (or parent/guardian) must have signed the informed consent document

2.3. Accrual objective and period

- **Accrual objective**: Sample size 430

- **Accrual period:** The estimated accrual period is 24 months

2.4. Study duration: Patients will be followed for 12 months following initiation of GVHD therapy or 12 months post-HCT for non-GVHD patients.

- This study will not require any extra clinic visits beyond those required for routine clinical patient care.
- For subjects who consent to the future unspecified use of their biospecimens for research purposes, clinical data collected at any time post-transplant as part of routine clinical care may also be accessed.

3. STUDY ENDPOINTS

3.1. Primary endpoint: The primary objective is to confirm that ST2 alone or the seven-biomarker panel measured at initiation of GVHD therapy predict a) D180 post-therapy non-relapse mortality; b) D28 post-therapy non-response, and c) GVHD grade 1-4 onset.

3.2. Secondary endpoints:

1. To demonstrate that ST2 alone or the seven-biomarker panel measured at day 14 or day 21 post-HCT (or a combination of these time points) predict D180 post-HCT non-relapse mortality.
2. To demonstrate that ST2 alone or the seven-biomarker panel measured at initiation of GVHD symptoms/therapy diagnose acute GVHD as compared to other complications presenting with similar symptoms (drug rash, CMV, Clostridium enteritis).
3. To demonstrate that ST2 alone or the seven-biomarker panel measured at initiation of GVHD symptoms/therapy diagnose the severity of acute GVHD at onset and maximum.

3.3. Clinical and histological correlatives:

- Pre-transplant characteristics

Age at transplant, gender, race and ethnicity, Disease diagnosis, disease risk (CIBMTR classification), intensity of conditioning, conditioning regimen, donor source,

HLA-match, stem cell source, other significant pre-HCT diagnoses and clinical characteristics

- GVHD prophylaxis

- Infectious prophylaxis, if any

- Onset Acute GVHD: day of onset, skin stage, liver stage, gastrointestinal stage and overall grade.

- Maximum Acute GVHD: day max, skin stage, liver stage, gastrointestinal stage and max overall grade.

- Biopsies:
    - - yes - No,
    - location skin, upper GI, lower GI, liver
    - GVHD yes or no, other diagnostic, specify
    - This will help identify the incidence of other complications presenting with GVHD symptom that is not GVHD (e.g. infectious diarrhea, drug rash…)

- Systemic therapy
    - Start day
    - Steroids 2 mg/kg/day Yes No
    - Other first line

- Proportion of complete response (CR), Partial response (PR), mixed response, No response, and progression at day 28 post-therapy (among surviving patients)

- Incidence of additional therapy of GVHD given at day 28, and day 56 post-therapy (including for GVHD flare).

- Non-relapse mortality (NRM) at day 180 post-therapy for GVHD patients and NRM at day 180 post-HCT for patients without GVHD.

- Overall survival, GVHD-free survival at day 180 and 360 post-therapy for GVHD patients and NRM at day 180 and 360 post-HCT for patients without GVHD.

- Relapse at day 360 post-HCT.

- Incidence of systemic infections related to GVHD treatment.

- Incidence of chronic GVHD at day 180 and day 360.

- For subjects consenting to future unspecified research, data collected at any time-point post-transplant as part of routine clinical care may be accessed for future studies.

Staging and grading of acute GVHD

In this study we will classify patients by a modified Keystone grading schema (see below).

Table 1: Acute GVHD Staging				
Stage	**Skin**	**Liver (bilirubin)**	**Lower GI (stool output/day)**	**Upper GI**
0	No rash	≤ 2 mg/dl	Adult: < 500 ml/day Child: < 10 ml/kg/day	No protracted nausea and vomiting
1	Maculopapular rash < 25% BSA	2.1-3 mg/dl	Adult: 500–1000 ml/day Child: 10 -19.9 ml/kg/day	Persistent severe nausea, vomiting with a positive upper GI biopsy
2	Maculopapular rash 25 – 50% BSA	3.1-6 mg/dl	Adult: 1001-1500 ml/day Child: 20 – 30 ml/kg/day	
3	Generalized erythroderma (>50% BSA)	6.1-15 mg/dl	Adult: >1500 ml/day Child: > 30 ml/kg/day	
4	Generalized erythroderma (>50% BSA) plus bullous formation and desquamation > 5% BSA	>15 mg/dl	Severe abdominal pain with or without ileus, or grossly bloody stool or melena (regardless of stool volume)	

For GI staging:

- The "adult" stool output values should be used for patients > 50 kg in weight.
- Use 3 day averages for GI staging based on stool output. If stool and urine are mixed, stool output is estimated to be 50% of total stool/urine mix.

Table 2: Overall Acute GVHD Clinical Grade:

Grade 0: No stage 1-4 of any organ
Grade I: Stage 1-2 skin and no liver or gut involvement
Grade II: Stage 3 skin, or Stage 1 liver, or Stage 1 GI
Grade III: Stage 0-3 skin, with Stage 2-3 liver, or Stage 2-3 GI Grade IV: Stage 4 skin, liver or GI

Response
Definitions:

Complete Response (CR) is defined as a GVHD grade of 0 in all evaluable organs. For a response to be scored as CR at day 28, the patient must still be in CR on that day and have had no intervening additional therapy for an earlier progression, PR or NR.

Partial Response (PR) is defined as improvement in one or more organs involved with GVHD symptoms without progression in others. For a response to be scored as PR at day 28, the patient must still be in CR on that day and have had no intervening additional therapy for an earlier progression, PR or NR.

Mixed Response (MR) is defined as improvement in one or more organs with deterioration in another organ manifesting with GVHD symptoms or development of GVHD symptoms in a new organ.

Progression is defined as deterioration in at least one organ without any improvement in others.

No response (NR) is defined as absence of any improvement or progression as defined. Patients receiving secondary therapy (including need to re-escalate steroid dose to ≥ 2.5 mg/kg/day of prednisone or equivalent 2 mg/kg/day of methylprednisolone will be classified as non-responders.

Proportion of complete response (CR), Partial response (PR), mixed response (MR), progression, and no response (NR) at day 28 post-therapy (among surviving patients). Scoring of CR, PR, MR, progression, and NR are in comparison to the patient's acute GVHD status on day 0 of systemic therapy for GVHD.

Incidence of additional systemic GVHD therapy given at day 28, and day 56 post-therapy (including for GVHD flare).

Flares are defined as any progression of acute GVHD after an initial response (e.g. earlier CR or PR) that requires re-escalation of steroids or initiation of additional systemic therapy for GVHD.

Steroid dose at day 28 and day 56 post-GVHD therapy
Dose of methylprednisolone will be converted to prednisone by multiplying the methylprednisolone dose by 1.25. Prednisone dose for each patient will be converted to mg/kg. The cumulative prednisone dose for each patient at day 28 will be calculated by adding the doses (end of each week's dose) for each of the four weeks of treatment, divided by the number of days of survival during this interval. The prednisone dose for each patient at day 28 and day 56 will be recorded.

Chronic GVHD is defined per the BMT CTN manual of procedures chapter 2. Symptoms of chronic GVHD if present will be reported using the GVHD symptom record. The incidence of chronic GVHD at 6 and 12 months will be computed.

Non-relapse mortality (NRM) at day 180 post-therapy for GVHD patients and NRM at day 180 post-HCT for patients without GVHD will be computed. The events for NRM are death due to any cause other than relapse of underlying malignancy.

Overall survival, GVHD-free survival at day 180 and 360 post-therapy for GVHD patients and at day 180 and 360 post-HCT for patients without GVHD will be computed.

Relapse at day 360 post-HCT will be computed.

Incidence of systemic infections related to GVHD treatment through day 180 post-therapy for GVHD patients and day 180 post-HCT for patients without GVHD will be computed. All microbiologically documented infections (bacterial and fungal) or significant infections requiring antibiotic/antifungal therapy occurring within 6 months of initiation of therapy for GVHD patients or 6 months post-HCT for no-GVHD patients will be reported by site of disease, date of onset, and severity. For definitions, see the BMT CTN manual of procedures.

Incidence of severe viral infections related to GVHD treatment through day 180 post-therapy and in no-GVHD patients will be recorded (EBV-PTLD, CMV viremia, HHV6 encephalitis…).

3.4. Studies of GVHD Biomarkers

If consent for research samples is obtained, blood samples will be collected on Day -7, day 7, day 14, day 21, day 28, day 56, day 100, day 180, and day 360 post-HCT, as well as at appearance of clinical symptoms suggestive of acute GVHD and that triggers systemic steroid treatment. These samples will be analyzed for the seven-protein biomarker panel. Remaining plasma sample aliquots will be stored for future biomarker studies. Additional, whole blood collected from the recipient on day 56 post-HCT (representing the donor DNA) and day -7 whole blood or saliva DNA (if white blood cells count < 400/μL) (representing recipient DNA) will be collected, frozen and stored for future genomic studies. Donor chimerism results recorded at the time nearest to each sample collection will be noted. Please note all sample collection days are targets with collection of samples on the closest business day to the suggested day being acceptable.

4. PATIENT ENROLLMENT AND EVALUATION

4.1. Enrollment

Patients will be registered for this trial using a web-based database that will be utilized for collection of all clinical and laboratory data.

Follow this procedure:

1. An authorized user at the clinical center completes the initial screening by entering patients' demographics.
2. If the patient signs the informed consent and assent (if applicable), a study number is generated.
3. A visit schedule based on enrollment date is displayed for printing.

Subjects enrolled when they are less than 18 years of age will be re-consented at the first office visit following their 18th birthday. Data and specimens collected from these subjects prior to their 18th birthday may continue to be used without this additional consent; however, no clinical data or specimens from after the 18th birthday may be collected or utilized until the re-consenting process has been completed.

4.2. Study monitoring

The follow-up schedule is outlined in Table 3 (see below).

Table 3: Required assessments													
	All allogeneic HCT, day pre/post-HCT									GVHD +	Day post-GVHD systemic therapy		Chimerism
	-7	7	14	21	28	56	100	180	360	Onset (0)	28	56	If Standard of Care in Center
Biopsy of involved tissue (as part of routine clinical care, not research)										X			
Acute GVHD evaluation ^		X	X	X	X	X	X	X		X	X	X	
Chronic GVHD evaluation ^							X	X	X				
Steroid dose		X	X	X	X	X	X	X		X	X	X	
Other GVHD medications, doses, and drug levels collected as part of clinical care		X	X	X	X	X	X	X	X	X	X	X	
Blood Samples*	X	X	X	X	X	X	X	X	X	X	X		
Saliva DNA (if WBC < 400/µL)	X					X							
Chimerism (% donor) ‡ **													X

*Up to day 28, sample collection may be ±4 days from the target day to allow for scheduling flexibility, holidays, etc…
*Day 100,180, and 360, sample collection will be collected at the clinic visit closest to the scheduled time point to allow for scheduling flexibility, holidays, etc…

*GVHD onset sample will be collected within a 48 hour window of systemic treatment initiation if subject is inpatient

‡ Donor chimerism results recorded at the time nearest to each sample collection will be noted.

^ Refer to Appendix D for GVHD assessment forms
** Will only collect data if already being done as standard of care at center

The timing of follow-up visits is based on the date of BMT and then on the

date of onset of GVHD for post-GVHD assessments.

Assessments

- Baseline weight
- For all, complete acute GVHD staging and grading information including assessment of rash, diarrhea, nausea/vomiting, liver function and weight at day
  7, 14, 21, 28, 56, 100, and 180 post-HCT
- If GVHD or GVHD-like symptoms,
  - recommended biopsy of involved tissue (this is standard of care, not research)
  - Complete acute GVHD staging and grading information including assessment of rash, diarrhea, nausea/vomiting, liver function and weight at onset of symptoms and 28 and 56 days post-systemic GVHD therapy
  - Steroids dose weekly until day 56
  - Samples for laboratory studies at day -7, 7, 14, 21, 28, 56,100 and 180
    post-HCT and at onset of GVHD symptoms, and 28 days post-systemic
    GVHD therapy
- Chronic GVHD evaluation at day 100, 180 and 360
- Infections requiring therapy up to 360 days post-HCT

Sample Collection

Samples will be collected at a time when clinical samples are being collected as well if possible. That is, this protocol will NOT require an additional procedure or for a patient's central venous line to be accessed at a time when these things are not being done as part of routine clinical care.

Blood will be collected as part of this study for use in evaluation of biomarkers of HSCT-related toxicities and outcomes and future unspecified research.

- Blood will be collected using the schedule detailed above: day -7 and the following post-transplant days: 7, 14, 21, 28, 56, 100, 180, and 360. Please note that the above blood draw schedule is a target only. Subjects will never be required to come for a clinic visit specifically to have
  samples collected for the purposes of this study. As such, sample time- points are targets and samples will be collected at the time closest to the target time when collection is feasible. In addition, during inpatient periods, sample collection will take place on the business day closest to
  the day scheduled per protocol (to allow for flexibility around weekends and holidays).

- Blood will also be collected at onset of acute GVHD and at day 28 after initiation of steroid therapy.
- In addition, blood will be collected at the onset of other changes in status, which may be clinically significant (such as suspected sinusoidal obstruction syndrome, respiratory distress, relapse, etc.).
- Blood volume to be collected:

  Please note cumulative collection volumes at various points in time of participation are summarized in the table below and are detailed for patients >40 kg in text immediately below for clarity.

  - Patients > 40 kg: up to 15 mL per time point)
    - 9 scheduled collections in year one = 135 mL
    - 2 scheduled collections in patients who develop acute GVHD = 30 mL
    - Maximum additional blood draws at the time of developing other complications will be set at **four**.
      - 9 scheduled collections + 2 additional collections GVHD collections (if applicable) + maximum of four additional collections with other complications = 15 collections maximum = maximum blood draw of 225 mL.

Table 4: Summary of maximum blood volume to be collected for this study

	Patient Size on Enrollment			
	> 40 kg	25-40 kg	10-25 kg	<10 kg
Maximum volume of each scheduled blood draw	15 mL	10 mL	7.5 mL	5 mL
Maximum volume of blood of each blood draw if develops acute GVHD	15 mL	10 mL	7.5 mL	5 mL
Maximum volume of blood with each complication	15 mL	10 mL	7.5 mL	5 mL
Maximum volume for scheduled blood draws (9 collections)	135 mL	90 mL	67.5 mL	45 mL
Maximum blood volume if develops acute GVHD (2 collections)	30 mL	20 mL	15 mL	10 mL
Maximum blood volume for other complications (4 collections)	60 mL	40mL	30 mL	20 mL
Maximum volume collected in year one (scheduled plus collections with complications)	225 mL	150 mL	112.5 mL	75mL

*Cumulative blood volume will be monitored for each subject and individual blood draws will be decreased as needed to maintain annual cumulative blood collection of <10% of total blood volume.

Also note, for pediatric patients, we expect patient weights to increase over time due to normal growth and as such, updated patient weights will be used to re-calculate permitted blood volume collections every six months.

GVHD scoring

Acute GVHD

A sample Acute GVHD data record for use in this trial is attached below.

Today's Clinical Acute GVHD Assessment

Today's date _____ Patient ID _____ Karnofsky/Lansky _____

	Codes					Differential diagnosis						
	0	1	2	3	4	GVHD	Drug Rxn	Cond Reg	Infect	TPN	VOD	Other
Skin	☐	☐	☐	☐	☐ % body rash: ____	☐	☐	☐	☐			_____
Liver	☐	☐	☐	☐	☐ Current bili: _____	☐	☐	☐	☐	☐	☐	_____ Lower
GI	☐	☐	☐	☐	☐ Vol: _____	☐	☐	☐	☐	☐		_____ Upper GI
☐	☐					☐	☐	☐	☐	☐		_____ **Systemic**

agents: ☐ CSA ☐ Tacrolimus ☐ Infliximab ☐ Daclizimab

☐ Pentostatin ☐ Sirolimus ☐ Etanercept ☐ MMF

☐ Prednisone ☐ Methylprednisolone ☐ Ontak ☐ Other _____

Current steroid dose: Prednisone _____mg/kg/day; Methylprednisolone _____ mg/kg/day

Has the steroid dose been increased to ≥ 2.5 mg/kg/day of prednisone (or 2 mg/kg/day methylpred)?
☐YES ☐ NO

If yes, date dose increased _____; reason for dose increase: ☐GVHD flare ☐ Other (specify) _____

Topical agents: Skin topical steroids**:** ☐ YES ☐ NO; Non-absorbed oral steroids (e.g. Budesonide, Entocort) ☐ YES ☐ NO

Chronic GVHD: Does the patient have evidence of chronic GVHD? ☐ YES ☐ NO

Codes	Skin	Liver (bilirubin)	Lower GI (stool output/day)	Upper GI
0	No rash	≤ 2 mg/dl	Adult: < 500 ml/day Child: < 10 ml/kg/day	No protracted nausea and vomiting
1	Maculopapular rash < 25% BSA	2.1-3 mg/dl	Adult: 500–1000 ml/day Child: 10 -19.9 ml/kg/day	Persistent severe nausea, vomiting with a positive upper GI biopsy
2	Maculopapular rash 25 – 50% BSA	3.1-6 mg/dl	Adult: 1001-1500 ml/day Child: 20 – 30 ml/kg/day	
3	Generalized erythroderma (>50% BSA)	6.1-15 mg/dl	Adult: >1500 ml/day Child: > 30 ml/kg/day	
4	Generalized erythroderma (>50% BSA) plus bullous formation and desquamation > 5% BSA	>15 mg/dl	Severe abdominal pain with or without ileus, or grossly bloody stool or melena (regardless of stool volume)	

Signature _____ Date _____

Chronic GVHD

Patients developing symptoms of chronic GVHD will have their symptoms recorded on the chronic GVHD scoring form (**Appendix D**) at the scheduled follow-up visits:

Table 4: Definite and Possible Manifestation of Chronic GVHD*		
Organ system	**Definite manifestations of chronic GVHD**	**Possible manifestations of chronic GVHD**
Skin	Scleroderma (superficial and fasciitis), lichen planus, vitiligo, scarring alopecia, hyperkeratosis pilaris, contractures from skin immobility, nail bed dysplasia	Eczematoid rash, dry skin, maculopapular rash, hyperpigmentation, hair loss
Mucous Membranes	Lichen planus, non-infectious ulcers, corneal erosions, non-infectious conjunctivitis	Xerostomia, keratoconjunctivitis sicca
GI tract	Esophageal strictures, steatorrhea	Anorexia, malabsorption, weight loss, diarrhea, abdominal pain
Liver	None	Elevation of alkaline phosphatase, transaminitis, cholangitis, hyperbilirubinemia
GU	Vaginal stricture, lichen planus	Non-infectious vaginitis, vaginal atrophy
Musculoskeletal/serosa	Non-septic arthritis, myositis, myasthenia, polyserositis, contractures from joint immobilization	Arthralgia
Hematologic	None	Thrombocytopenia, eosinophilia, autoimmune cytopenias
Lung	Bronchiolitis obliterans	Bronchiolitis obliterans with organizing pneumonia, interstitial pneumonitis

*From BMTCTN MOP chapter 2

Pediatric considerations

Children with chronic GVHD can present with skin, GI, eye or systemic disease leading to failure to thrive, persistent immunodeficiency, or pulmonary disease. Chronic “eczema’ or dry skin are common manifestations of mild chronic GVHD of skin. Liver disease can present as asymptomatic hyperbilirubinemia or elevated alkaline phosphatase. As children can have marked elevations in alkaline phosphatase during growth spurts, children with this laboratory abnormality should have a fractionated alkaline phosphatase to confirm that the band is from the liver and not from bone before a diagnosis of chronic GVHD is made. Children with chronic GVHD of the upper GI tract may have chronic anorexia or poor growth due to malabsorption. Rather than losing weight, children may “fall off” their growth curve with decreased gains in height or weight velocity. In children < 2 years of age, this may also affect growth of head circumference. Chronic GVHD may also cause persistent low or intermediate immunodeficiency. In addition to increasing the risk of opportunistic infection, this may cause GI dysfunction and lead to malabsorption, low IgG (due to protein losing enteropathy), lactose deficiency, and poor growth. Duodenal intubation with quantitation of pancreatic enzymes is useful in these children. If pancreatic enzymes are low, children may benefit from supplementation of pancreatic enzymes.

See Appendix D for a sample of chronic GVHD data record for use in this trial.

Summary of Required Assessments

- For all subjects
    - complete acute GVHD staging and grading information including assessment of rash, diarrhea, nausea/vomiting, liver function and weight at day 7, 14, 21, 28, 56, 100, and 180 post-HCT
    - Chronic GVHD evaluation at day 100, 180 and 360 post-HCT (please refer to Appendix D for GVHD assessment forms)
    - Infections requiring therapy up to 360 days post-HCT
    - Baseline weight
    - Blood samples for laboratory studies and future use at day -7, 7, 14, 21, 28, 56,100,180, and 360 post-HCT

- For subjects who develop GVHD or GVHD-like symptoms,
    - recommended biopsy of involved tissue (this is standard of care, not research)
    - Complete acute GVHD staging and grading information including assessment of rash, diarrhea, nausea/vomiting, liver function and weight at onset of symptoms and 28 and 56 days post-systemic GVHD therapy
    - Steroid dosing information weekly until day 56 after initiation of steroids
    - Blood samples for laboratory studies and future use at onset of GVHD symptoms and 28 days post-systemic GVHD therapy initiation

4.3. Clinical data submission

4.3.1. Source documents for all required evaluations and assessments should be stored at local sites for all GVHD evaluations (both acute and chronic). All clinical data required for this study will be input at local sites into the web-based database developed specifically for the Pediatric HCT Bio-repository Network. All clinical information should be updated on subjects a least monthly through 1-year post-HCT.

4.3.2. Source documents for 10-15% of required assessments and data will be requested for submission to IUSCC for data control evaluations.

4.4. Sample Processing

After collection, samples will be processed, aliquoted, and frozen utilizing the standard operating procedures developed by the Paczesny laboratory and the Indiana CTSI Specimen Storage Facility (described below in the section entitled “Storage”). SOPs for sample processing established by the Paczesny laboratory will be followed for all

processing procedures. A manual for sample processing for this study will be provided to all participating sites. These can also be provided when requested and during audits.

4.5. Sample Storage and Tracking

A global unique identifier is randomly assigned to each sample and derivative. Samples of the same person can only be linked by authorized persons who have access to the data in the database. All sample data will be organized and stored in the SpecimenTrack system from Remedy Informatics (http://www.remedyinformatics.com/product/specimentrack/). A series of biobanking electronic forms will be created to track the collection, inventory, and distribution of HSCT biosamples. Since SpecimenTrack can be readily integrated with the HSCT Registry, the samples will always be associated with their clinical, lab, and genotypic annotations. Both the HSCT Registry and SpecimenTrack will be deployed in a HIPAA aligned environment and supported by staff trained in HIPAA best practices. Both systems are enterprise level systems with powerful and granular control of user permissions, roles, and administration. The HSCT systems will be supported by members of the Indiana CTSI informatics team, who are experienced with deploying, maintaining, and providing user support for systems such as REDCap (https://en.wikipedia.org/wiki/Redcap_%28Research_Electronic_Data_Capture%29).
Both Remedy systems, MosaicBuilder and SpecimenTrack, are web applications accessible through a web browser using HTTPS, thus allowing both local and distant collaborators to use the system in an encrypted and manner.

The Pediatric HCT Bio-repository Network samples will be stored in the Indiana Biobank in affiliation with the Indiana CTSI Specimen Storage Facility (SSF). The Indiana CTSI manages a Specimen Storage Facility dedicated to sample storage in accord with International Society of Biologic and Environmental Repositories (ISBER) Best Practices and protocol defined sample parameters. The SSF is located in the new Wells Children's Clinical Research Center in Riley Children's Hospital.

The SSF is directed by Lilith Reeves, CSO, Indiana CTSI, and operates under the Indiana CTSI Specimen Storage Facility Advisory Committee, Ken Cornetta, Chair. Quality Compliance oversight is provided by Anne Kaiser, Cincinnati Children's Hospital Medical Center (CCHMC) Office of Research Compliance and Regulatory Affairs (ORCRA). The approaches and day-to-day activities of the SSF are managed by QA reviewed SOPs organized into the following chapters:

- Administration and Quality Oversight: SOP SF-1-1 through SOP SF-1-13. This collection of procedures defines maintenance of SOPs, Scope of Services, Organization of the SSF, Management of the SSF, Training, Controlled Document Management, Safety Quality Management, Deviation Management, Out-of-Specification Management, Audit Management, Commissioning/ Validation/ Revalidation policies and Housing for Good Laboratory Practices. The central

approach of the SSF is to provide the sample storage controls for PI owned collections rather than to manage the protocols under which the specimens are collected and released. These SOPs control all procedures associated with specimen intake and management while the specimens are located in the SSF.

- Facility Management: SOP SF-2-1 through SOP SF-2-4. The four facility systems within the SSF that are critical to ensuring storage compliance with ISBER and specimen quality are Mechanical Freezer Storage Room Controls, Liquid Nitrogen System and Freezer Room Controls, Controlled Access and Alarm Management and Response. These SOPs define procedures for monitoring and responding to situations within the facility critical to ensuring sample integrity.
- Equipment Management: SOP SF-3-1-SOP SF-3-9. All equipment in the SSF is maintained, monitored, and function verified according to strict specifications included in the SOPs for Refrigerators and Freezers, Liquid Nitrogen Freezers, Pipettes, Centrifuges, Biological Safety Cabinets, Timers, Thermometers, Personal O2 monitors, and Emergency Escape Breathing Apparatus to be used in the event of a large-scale liquid nitrogen release event. These SOPs are written to ensure that equipment is used, calibrated, and generally maintained to support quality specimen management.
- Specimen Processing: SOP SF Chapter 4. This group of SOPs defines the technical steps for supporting storage of the multiple collections according to the defined parameters of each. This chapter of SOPs is ever evolving with the over-arching SOPS within the chapter being SOP SF-4-1 Managing Requests for Sample Support and SF-4-4 Managing Sample Receipt, Log-in and Tracking.
- These SOPs are available on-line at IndianaCTSI.org/ictsissf/sops or can be provided for review by contacting the SSF Director (Lilith Reeves) or Chair of the SSF Advisory Committee (Kenneth Cornetta, MD). The Indiana CTSI SSF is a CTSI Designated Core Laboratory signifying that operating policies and charges are reviewed at least annually by the CTSI Translational Technology Resources (TTR) core oversight committee. All personnel in the SSF who have access to PHI have completed human subjects training.

4.6. Sample Shipment

Samples should be shipped Monday through Thursday via Fedex overnight delivery every six months. Samples must be shipped on dry ice to the following address:

Pediatric HCT Biorepository Network
Wells Children's Clinical Research Center
Attention: Lindsey Elmore or Jenny Then
705 Riley Hospital Dr. RI 2606, Room 2641
Indianapolis, IN 46202-5225

Bill sample shipment costs to the following Indiana University account number provided.

Contact the following individuals prior to all shipments:

Lindsey Elmore, Operations Manager: lhelvaty@iupui.edu

Jenny Then, Core Coordinator: jeglassb@iu.edu

4.7. HSCT Multi-Center Informatics Infrastructure

Data obtained during this clinical trial will be collected at six cancer centers: SKCCC, DFCI, FHCRC, IUSCC, TXCCC and CNMC. Since this proposal involves a very large amount of data to be collected from a multi-center clinical trial as well as a large amount of proteomic data, we have put procedures in place to ensure the integrity of the data transmitted and to allow consistent handling of the clinical data at all sites. The clinical and laboratory data collected will be organized and stored in the HSCT Registry, constructed using the MosaicBuilder platform from Remedy Informatics (http://www.remedyinformatics.com/mosaic-platform/mosaic-builder-edition-for-it-professionals/). Data will either be imported directly from the Cerner Electronic Medical Record system, exchanged using flat files, or entered manually into the system using electronic forms. The Mosaic enterprise level platform is deployed and maintained as a service by the Indiana Clinical Translational Sciences Institute, and enables the creation of biobanks that are integrated with clinical, lab, genotypic, and phenotypic data. This platform is engineered to support complex workflows, and is deployed in a HIPAA aligned environment such that the server, database, and application, are located at secured locations and supported by professional staff trained in HIPAA best practices. The Mosaic platform is also ontologically aware so that the semantic definition of the data can be standardized. This further enables meaningful data integration and querying. All of these factors ensure that the HSCT Registry will be a secure, stable, robust and extensible research informatics platform for the future.

4.8. Future Use Sample analysis

One objective of this protocol is sample and clinical data collection for future unspecified research. It is anticipated that some of the sample analyses may be carried out prior to specific research questions or projects using the advanced diagnostic techniques listed below; however, the data generated using these analyses will only be released for data analysis once there is IRB approval for the investigation of a specific hypothesis-driven research question.

The sample analyses that may be carried out as part of this protocol may include, but are not limited to:

- Genomics
- Proteomics
- Cytokine analyses
- Flow cytometry

- Metabolomics

These analyses will generally be performed at one of the six participating sites in the PHCTBN. On occasion, the analyses of research samples will involve collaborators at other institutions in order to have access to the most technologically advanced or cost effective techniques. All samples analyzed at another institution as part of this project will maintain the bar code assigned to the subject on enrollment in this study. This process allows for use of a unique identifier that the principal investigator of this study may use to link the data to other subject information but eliminates the possibility of transmission of privileged health information. Samples analyzed at another center will generally be entirely consumed by the research procedures. Any residual will be destroyed at the end of this project or returned to the Indiana University storage facility.

5. GOVERNANCE

The PHCTBN is managed by the PIs (Paczesny and Renbarger) and the PHCTBN Steering Committee (Duncan, Cooke, Carpenter, Jacobsohn, Bollard, Krance, and Leung). In order for samples/information to be shared with researchers, an IRB-approved protocol will need to be provided and the Steering Committee in conjunction with the PIs will make determinations about allocation of samples/information to researchers.

6. DATA AND SAFETY MONITORING PLAN

A meeting of the Data and Safety Monitoring Board was held on June 1, 2015. The Data Safety Monitoring Board consisted of DSMB Chair, Stella Davies, MD, Wade Clapp, MD, Scott Baker, MD, and Paul Szabolcs, MD. The board reviewed and discussed the protocol. It was decided that since this is a low-risk, non-interventional study, it does not require a DSMB according to the NIH guidelines. Therefore, the DSMB has been eliminated.

Data and Safety will continue to be monitored by the study Principal Investigators.

7. DATA SHARING

Indiana University School of Medicine (IUSM) as well as all the participating institutions are committed to supporting the NIH Statements on Sharing Research Data and on Availability of Research Results: Publications, Intellectual Property Rights, and Sharing Research Resources. These Universities have used a variety of means as appropriate and expeditious to share data resulting from sponsored projects with colleagues, such as depositing data on a secure web-accessible data warehouses or arranging distribution of data, reagents, targets, and protocols to other researchers using established mechanisms and repositories. IUSM, and all participating institutions are signatory to the Uniform Biological Materials Transfer Agreement and will use the simple letter agreement to distribute appropriate research materials to the research community. The availability of data sharing will be publicized by individual investigators in publications and presentations.

The Universities will assure the timely release and sharing of data no later than the acceptance for publication of the main findings from final datasets, and will protect the rights and privacy of human subjects who participate in NIH-sponsored research by redacting all identifiers, and adopting other strategies to minimize risks of unauthorized disclosure of personal identifiers in accordance with authorization and consent documents. The Universities agree that data sharing is essential for expedited translation of research results into knowledge, products, and procedures to improve human health.

In addition, for this proposal, data from future research studies that may include, but are not limited to Genomics, Proteomics, and Metabolomics will also be shared through the same mechanism. These analyses will generally be performed at one of the six participating sites in the Pediatric HCT Bio-repository Network (PHCTBN). On occasion, the analyses of research samples will involve collaborators at other institutions in order to have access to the most technologically advanced or cost effective techniques. All samples analyzed at another institution as part of this project will be maintain the bar code assigned to the subject on enrollment in this study. This process allows for use of a unique identifier that the principal investigators of this study may use to link the data to other subject information but eliminates the possibility of transmission of privileged health information. Samples analyzed at another center will generally be entirely consumed by the research procedures. Any residual will be destroyed at the end of this project or returned to the Indiana University storage facility.

With respect to licensing patented inventions developed through this project, the Universities expect to elect title to inventions made with federal funds by their investigators, as appropriate under the Bayh-Dole Act, and in accordance with the NIH policy. The Universities understand that the NIH encourages the filing of patent applications on unique research resources if doing so will aid in the prompt commercialization of diagnostic, prognostic or therapeutic products. Since institutional ownership of such inventions may be of concern to collaborators, especially those who are the source of proprietary biomarkers, reagents, and/or technologies, the Universities will develop agreements with third party collaborators that assure them both adequate patent coverage and opportunities to license such patent rights, as appropriate. This agreement will be implemented in a manner that does not restrict research use by the scientific community, both nonprofit and for profit, but promotes and facilitates their active involvement in this project.

Genomic Studies

DNA from patients might be used in high-throughput genomic studies (including potentially genome-wide association studies or sequencing) for a future project.

We plan to deposit both the phenotype and genotype data obtained from the work proposed in this project into the NIH genotype and Phenotype Database that is managed by the National Center for Biotechnology Information (NCBI). The NIH genotype and

Phenotype Database collects well curate data (clinical and genetics). All the data planned for submission will be deidentified/HIPAA compliant and has been properly consented with IRB approvals.

We also plan to release the data to qualified researchers who wish to collaborate with the investigators involved in this proposal. The data will be available through a secure FTP site maintained by the University's IT department, or sent by encrypted physical media via trackable mail.

Sharing Model Organisms

N/A

8. STATISTICAL CONSIDERATION

7.1. Accrual

Accrual will be monitored within each center with the expectation that the enrolled patient population is representative of the transplanted patient population at each center. Representation will be examined by comparing gender, race, ethnicity and age distributions. Accrual of minority patients will be expected to be in proportion to the number of minorities patients transplanted at each center.

7.2. Primary and Secondary Endpoints

Appendix C will detail sample size and statistical analysis to be performed

9. APPENDIX A: HUMAN SUBJECTS

Subject consent: Candidates for the study will be defined as patients receiving an allogeneic hematopoietic stem cell, cord blood, or bone marrow transplant, or T cell depleted marrow can be included. The principal investigator or his/her designee at each transplant center will contact the candidates and enroll them onto the study. The study coordinator at each center will provide the patient with information about the purpose of the study and obtain consent. The PIs of the study will provide a template of the consent form to each center that can be customized according to their local requirements and submit it for review by the local Internal Review Board (IRB). Each center must provide evidence of IRB approval.

Confidentiality: Confidentiality will be maintained by individual names being masked and assigned a patient identifier code. The code relaying the patient's identity with the ID code will be kept separately at the center. The ID code will be transmitted to the center that keeps all the clinical data (separate from the center that keeps the biological data).

Participation of women, children, minorities and other populations: Women, children and ethnic minorities will be included in this study.

Accrual will be monitored within each center with the expectation that the enrolled patient population is representative of the transplanted patient population at each center. Representation will be examined by comparing gender, race, ethnicity and age distributions. Accrual of minority patients will be expected to be in proportion to the number of minorities patients transplanted at each center.

10. APPENDIX B: INFORMED CONSENT TO PARTICIPATE IN RESEARCH

Informed Consent to Participate in Research

Study Title: Bridging Pediatric and Adult Biomarkers in Graft-Versus-Host Disease

The word "you" in this consent form, may mean either you or your child; "we" means the doctors and other staff.

WHAT IS THE PURPOSE OF THIS STUDY?

You are invited to participate in a research study called "**Bridging Pediatric and Adult Biomarkers in Graft-Versus-Host Disease**." It is a study to collect specimens and information about patients who receive a bone marrow or stem cell transplant or a donor lymphocyte infusion (DLI) to help us to better understand graft-versus-host disease and to do future research aimed at understanding more about your disease, stem cell transplant and complications that people have after their transplants. You are about to undergo a treatment with chemotherapy and/ or radiotherapy followed by infusion of bone marrow-replenishing stem cells or donor lymphocyte cells for the treatment of your disease. The purpose of this study is to collect data and blood from patients undergoing this type of treatment. We will code data by assigning a number to each individual patient and then store the data by that number. No mention of patient identities is made in analyses of the data or in any publications, which result from such research.

If you decide to join the study, your blood will be collected and stored. These samples will be used for measurement of proteins in blood that might help us predict which patients will develop GVHD and for future research.

We will enroll a total of 430 children and adults to this study and biobank.

This consent form will tell you about the purpose of the research, its benefits and minimal risks, and your right as a participant in the study. Please take your time to make your decision.

Everyone who takes part in research at [insert your institution] should know that:

- Being is any research study is voluntary.
- You may or may not benefit from being in the study. Knowledge we gain from this study may benefit others.
- If you join the study, you can quit at any time.
- If you decide to quit the study, it will not affect your care.
- Please ask the study staff questions about anything that you do not understand, or if you would like to have more information.

- You can ask questions now or anytime time during the study.
- Please take the time you need to talk about the study with your doctor, study staff, and your family and friends. It is your decision to be in the study. If you decide to join, please sign and date the end of the consent form.

This study does not change the therapeutic standard of care and therefore has no more than minimal risk for you.

WHAT WILL PARTICIPATING IN THIS STUDY INVOLVE?

You are being asked to allow collection of data regarding your transplant to be entered into our database. We may collect information from your hospital chart on multiple occasions. As we collect this database of information, it can become a valuable resource to help us in improving the health and outcomes of subjects receiving a transplant. One way to do this is to use results that are in the past medical record (such as your clinical lab tests, physical exams, vital signs, for example) to investigate how those results may be associated with better or worse outcomes or what complications develop. At this time, we do not know which results may hold the key to gaining a better understanding. This may change as we gain more knowledge. The study doctors will also collect information related to your treatment. This can include any information about your disease, your past medical history, your stem cell transplant course, and how you do long term. This information can be collected from your medical records or from the NAME OF YOUR INSTITUTION database. The investigators will keep track of what medicines you receive and how well those medicines work for you and whether you have side effects from the medicines.

In addition, you are being asked to allow collection of 15 ml (3 tsp or 1 tablespoons) of blood (less for children or adults under 40 kg of body weight—see table below) on multiple occasions. This blood will be collected at 9 different times before and after your transplant. You will have a total of approximately 9 tablespoons collected throughout the study. Blood will be collected specifically for this study to test biomarkers of GVHD and to use for future research. We may use this blood to run certain tests on the blood to use for future research aimed at developing a better understanding of stem cell transplant complications and outcomes that certain patients have. Doctors involved in this study may use some of these samples to do certain tests on the blood in order to learn more about complications and outcomes that people have after getting stem cell transplants, how to best predict which patients are at risk for a complication before it happens, and how to diagnose certain complications that are sometimes hard to diagnose. Samples and information will only be released to someone doing a study like this if they have approval from the Institutional Review Board (IRB) to do the study.

The specific times of the blood draws will be prior to your transplant and at the following times after your hematopoietic stem cell transplant:

- Day 7

- Day 14
- Day 21
- Day 28
- Day 56
- Day 100
- Day 180
- Day 360

All of these planned time-points are approximate and will always be coordinated with a time when you are having blood drawn as part of your routine clinical care. Also blood samples will be obtained on the occasion of major complications (or events), or of other important clinical findings and their follow up after transplant. Examples of major complications might be onset of blood stream infection, onset of veno-occlusive disease of the liver, onset of graft-versus-host disease (GVHD), placement of a tube into the airway to help breathing, or at the time of relapse. If you develop GVHD, we will collect two additional blood samples: at the onset of symptoms that require treatment initiation and 28 days after treatment begins. The reason that it is important to collect blood samples at times like that are that sometimes certain things may be changing in your body around these times that could cause changes in the levels of certain chemicals, cells, or other things that might help us to better understand how to predict or prevent complications from happening. In this study, we can collect samples like this up to six times (including the two additional samples for GVHD). After collecting the samples, we can do any of the following:

- Process and freeze the samples to use for GVHD biomarker test and for future unspecified use
- Process and do certain tests on the samples. We would save this information for future research questions about stem cell transplant that investigators ask to do and have approval to do in the future. It is sometimes important to do tests like this and save the information because some important tests have to be done on fresh samples and cannot use samples that have been frozen

The amount of blood you will have collected depends on how much you weigh. The table below summarizes the volume of blood we plan to collect depending on your size. If you have a central line or port a cath in place as part of your regular care, blood will be drawn from your central line or port a cath. A central line (central venous catheter) is a special type of tubing inserted into a large vein in the chest by a surgeon during a short operation. The central line is used to give chemotherapy drugs and to withdraw small amounts of blood for testing during treatment.

We will collect a sample of your saliva by asking you to spit into an ORAGENE collection kit on day -7 and/or day +56 of your transplant if your total white blood counts

< 400/microliter so that we can use the saliva to extract DNA for genetic tests in the future if needed.

Table 5: Summary of maximum blood volume to be collected for this study

	Patient Size on Enrollment			
	> 40 kg	25-40 kg	10-25 kg	<10 kg
Maximum volume of each blood draw	3 tsp	2 tsp	1.5 tsp	1 tsp
Maximum volume of scheduled blood draws (9 collections)	27 tsp	18 tsp	14 tsp	9 tsp
Maximum volume of blood if develops acute GVHD (2 collections)	6 tsp	4 tsp	3 tsp	2 tsp
Maximum volume of blood with complication (4 collections)	12 tsp	8 tsp	6 tsp	4 tsp
Maximum volume in year 1 for scheduled blood draws (maximum of 15 collections)	45 tsp	30 tsp	22.5 tsp	15 tsp

Additionally, information about you and your disease and stem cell transplant course and complications will continue to be collected forever. It will be stored indefinitely and only the investigators for this study will have access to it.

You have the choice of allowing us to draw and store your blood samples and to use them for future transplant-related research. The samples may be used up or destroyed without your consent and the samples may be used after your death without further consent of your estate or legal representative. Future use of your stored sample for genetic studies will always require the review and approval of the Institutional Review Board.

I have read the description of this part of the study and willingly give my consent to participate in the collection of blood samples from me for use in the future to study my disease, hematopoietic stem cell transplant, complications and outcomes of hematopoietic stem cell transplant, and other complications of treatment.

Yes _____ or No _____ _____Initials

Weekly Health Evaluation

We will evaluate your health every week for 8 weeks after your transplant. If you develop acute GVHD, we will also evaluate your health every week for 8 weeks once you start your GVHD treatment. Each health evaluation will include tests to evaluate your:

- GVHD stage and grade
- Ability to do daily activities (performance status)

- Blood counts, liver and kidney function, and level of GVHD prevention medications in your blood (if it applies to you)
- Development of any infection

These tests and how often they are scheduled are standard care for patients receiving an allogeneic HCT and/or with acute GVHD and would be done even if you were not part of this study.

Monthly Health Evaluation

We will evaluate your health at approximately 3, 6 and 12 months after your transplant. Each health evaluation will include tests to evaluate your:

- GVHD stage and grade
- Ability to do daily activities (performance status)
- Blood counts, liver and kidney function, and level of GVHD prevention medications in your blood (if it applies to you)

These evaluations must be done at the transplant center. Other exams and tests may be done at the transplant centers or your local doctor's office at the discretion of your transplant doctor.

These tests and how often they are scheduled are standard care for patients receiving an allogeneic HCT and/or with acute GVHD and would be done even if you were not part of this study.

WHAT BENEFITS MAY YOU EXPERIENCE FROM THIS STUDY?

This study will not directly benefit you. By taking part in this study you may help us to improve treatment for hematopoietic stem cell transplant patients in the future.

WHAT RISKS OR DISCOMFORTS ARE INVOLVED?

Blood Draw Side Effects:

Risks associated are slight, but some possible risks include: excessive bleeding, fainting or feeling lightheaded, bruising, and infection (a slight risk any time the skin is broken). To decrease the risk, blood will be drawn by trained personnel and will always be taken at a time when blood is being drawn for other tests your study doctor has ordered. You will not have to have an IV placed specifically for this study.

Loss of Confidentiality (personal information):

There is a risk of loss of privacy. All the genetic information gathered in this study will be used together with information from other subjects. Your name or other personal health information will not be used in the information collected from the study or in publications. All your personal and medical data will be considered confidential. All of the information collected on you will be coded (codes used to be unable to identify the personal health information). Only research staff will have access to the samples. Only the study doctor in charge of this study will be able to access the coded information.

This research follows the Genetic Information Nondiscrimination Act (GINA), a federal law which generally makes it illegal for health insurance companies, group health plans, and most employers to request the genetic information we get from this research and discriminate against you based on your genetic information.

WHAT FINANCIAL COSTS ARE INVOLVED IN PARTICIPATING?

Costs associated with your care, such as laboratory tests, x-rays, scans, clinic visits, medications, and hospitalizations, are all considered a part of standard care for someone with your illness; therefore you will be responsible for these costs.

Lab tests or procedures required for the research, which are not considered a part of standard care for your illness, and the costs for these specified tests will be paid for by the study.

ARE THERE ALTERNATIVES TO PARTICIPATION?

You have the option to choose not to participate in this study.

Refusal to participate in this study or stopping participation will not change the medical care you will receive.

WHAT COMPENSATION IS AVAILABLE IF YOU HAVE A COMPLICATION OR ARE INJURED BY PARTICIPATION?

In the event of physical injury resulting from your participation in this research, necessary medical treatment will be provided to you and billed as part of your medical expenses. Costs not covered by your health care insurer will be your responsibility. Also, it is your responsibility to determine the extent of your health care coverage. There is no program in place for other monetary compensation for such injuries. However, you are not giving up any legal rights or benefits to which you are otherwise entitled.

WHAT COMPENSATION IS AVAILABLE FOR PARTICIPATION IN THIS STUDY?

There will be no monetary compensation for participation in this study.

WHO MAY YOU CALL WITH QUESTIONS ABOUT YOUR PARTICIPATION IN THIS STUDY?

For questions about the study or a research-related injury, contact the researcher XXXX, MD at XXXX. If you cannot reach the researcher during regular business hours (i.e. 8:00AM-5:00PM), please call the Human Subjects office at XXXXX. After business hours, please call XXXX and ask the operator for the Stem Cell Transplant physician on call.

In the event of an emergency, please contact your local medical emergency response team by dialing 911. If you need assistance that is not an emergency, you can always reach the study doctor by calling the hospital operator at XXXX or ask for the Stem Cell Transplant physician on call.

For questions about your rights as a research participant or to discuss problems, complaints or concerns about a research study, or to obtain information, or offer input, contact the Human Subjects office at XXXX.

Additional information is available at the National Cancer Institute's Cancer Information Service at 1-800-422-6237 or visit the NCI's web sites with a computer:

- Cancer Trials: comprehensive clinical trials information

http://cancertrials.nci.nih.gov

- Cancer Net: accurate cancer information

http://cancernet.nci.nih.gov

MAY YOU WITHDRAW FROM THE STUDY?

Furthermore, if you decide you no longer want your blood to be used as part of the research, you will always have the right to request that samples no longer be used and that any remaining blood be destroyed. If you choose to have your blood destroyed please contact your study doctor in writing **insert name and address of site PI**.

Your participation in this study is entirely voluntary. If you choose not to participate in this study, this will in no way interfere with your care. The same high quality medical care will be given to you no matter what your decision might be. Likewise, you may discontinue participation in this study at any time without altering the quality of care given to you. You may also seek treatment from another physician of your choice. New information that may relate to your willingness to participate will be provided to you.

Your participation in this study may be terminated by the investigator for any of the following reasons:

A. If you fail to follow the investigator's instructions.

B. If you experiences a serious and unexpected side effect that may require evaluation.

C. If the investigator feels it is in the best interest of your health and welfare.

WILL YOUR STUDY RECORDS BE KEPT CONFIDENTIAL?

Efforts will be made to keep your personal information confidential. We cannot guarantee absolute confidentiality. Your personal information may be disclosed if required by law. Your identity will be held in confidence in reports in which the study may be published.

Organizations that may inspect and/or copy your research records for quality assurance and data analysis include groups such as the study doctor and his/her research associates, the Institutional Review Board or its designees, and (as allowed by law) state or federal agencies, specifically the Office for Human Research Protections (OHRP) and the National Institutes of Health (NIH).

SUBJECT'S STATEMENT OF CONSENT

I/My child have read the description of this study and have also talked it over with my/my child's study doctor to my satisfaction. I willingly give my consent (to allow my child) to participate in this study. I/My child will receive a copy of the consent to keep for future reference.

If the subject is 18 years of age or older:

Subject's Printed Name: _____

Subject's Signature: _____ **Date:** _____

(must be dated by the subject)

If the subject is 17 years of age or younger:

Printed Name of Parent: _____

Signature of Parent: _____ **Date:** _____

This must be completed:

Printed Name of Person Obtaining Consent: _____

Signature of Person

Obtaining Consent: _____ **Date:** _____

Health Insurance Portability and Accountability Act 1 (HIPAA1) Authorization to use and disclose individual health information for research purposes

A. Purpose

As a research participant, I authorized the principal investigators and the researcher's staff to use and disclose my individual health information for the purpose of conducting the research study:
Title: Bridging Pediatric and Adult Biomarkers in Graft-Versus-Host Disease

B. Individual health information to be used or disclosed

My individual health information that may be used or disclosed to do this research includes:

- Demographic information (for example: date of birth, sex, weight)
- Medical history (for example: diagnosis)
- Findings from physical exams
- Laboratory test results obtained at the time of the work up and after treatment (for example: blood tests, biopsy results)

C. Parties who may disclose my individual health information

The researcher and the researcher's staff may collect my individual health information from: (list hospitals, clinics and providers from which health care information can be requested).

D. Parties who may receive or use my individual health information

The individual health information disclosed by parties listed in item C and information disclosed by me during the course of the research may be received and used by the following parties:

Principalinvestigatorsandresearcher'sstaff
Dr. Sophie Paczesny

Dr. Jamie Renbarger

Study sponsors

U.S. government agencies that are responsible for overseeing research such as the Office of Human Research Protection (OHRP)

U.S. government agencies that are responsible for overseeing public health concerns such as the Center for Disease Control (CDC) and federal, state and local health departments.

E. Right to refuse to sign this authorization

I do not have to sign this authorization. My decision not to sign this authorization will not affect my treatment, payment, enrollment in health plans or eligibility for benefits.

F. Right to revoke

I can change my mind and withdraw this authorization at any time by sending a written notice to [the principal investigator at your transplant center] to inform the researcher of my decision. If I withdraw this authorization, the researcher may only use and disclose the protected health information already collected for this research study. No further health information already about me will be collected or disclosed to the researcher for this study. Any unused blood will be destroyed if you withdraw your permission.

G. Potential for Re-disclosure

My individual health information disclosed under this authorization may be subject to re-disclosure outside the research study and no longer protected. Examples include potential disclosures for law enforcement purposes, mandated reporting or abuse or neglect, judicial proceedings, health oversight activities and public health measures.

H. This authorization does not have an expiration date.

Title: Bridging Pediatric and Adult Biomarkers in Graft-Versus-Host Disease

I have read and understood this consent form. The nature and purpose of the research study has been explained to me. I understand that I will be given a copy of this signed Consent Form.

_____

Participant Name

_____ _____

Participant Signature *Date*

I certify that I have provided a verbal explanation of the details of the research study, including the procedures and risks. I believe the participant has understood the information provided.

Counseling Physician

Counseling Physician Signature *Date*

Assent to participate in research

XXXX University Assent To Participate in Research

Bridging Pediatric and Adult Biomarkers in Graft-Versus-Host Disease

We are doing a research study. A research study is a special way to learn about something. We are doing this research study because we are trying to find out more about patients who receive a bone marrow or stem cell transplant or donor lymphocyte infusion (DLI) and complications that people have after their transplants. We would like to ask you to be in this research study.

Why am I being asked to be in this research study?

You are being asked to be in this research study because you are about to have chemotherapy and/or radiation followed by a bone marrow or stem cell transplant or DLI. One thing we want to try to do is find a blood test we can use to diagnose acute Graft-Versus-Host Disease (GVHD). You are invited to join this research because you might develop acute GVHD. We want to learn
if a blood test will predict patients who will have worse acute GVHD following stem cell transplant. Acute GVHD is common in patients who have stem cell transplants. It happens when the new cells attack and damage your tissues such as the skin, gut and liver.

What will happen during this research study?

We want to tell you about some things that might happen if you are in the study. This study will take place at XX Hospital. Blood will be collected specifically for this study to use for future research about your disease, stem cell transplant, and problems people have after stem cell transplant. The study doctors will also collect information related to your treatment.

Blood will be collected specifically for this study at up to 15 different times before and after your transplant. You will have up to three tablespoons of blood drawn for this study over a 1-year period. Blood samples will always be coordinated with a time when you are having blood drawn as part of your routine clinical care. Also blood samples will be obtained on the occasion of major health complications (or events), like a blood infection or liver disease, for example.

We will also get information about you and your disease and what happens to you before and after your stem cell transplant from your medical record, which is information that your doctor's office and the hospital have about you.

Are there any bad things that might happen during the research study?

Sometimes bad things happen to people who are in research studies. These bad things are called "risks." The risks of being in this study might be loss of your personal information. Blood draw risks also include bleeding, fainting or feeling lightheaded,

bruising, and infection.
Not all of these things may happen to you. None of them may happen.

Are there any good things that might happen during the research study?

Sometimes good things happen to people who are in research studies. These good things are called "benefits." You will not receive a benefit from being in this study. However, we hope the information helps to collect information that may benefit future stem cell transplant treatments.

Will I get money or payment for being in this research study?

You will not get money for being in this research.

Who can I ask if I have any questions?

If you have any questions about this study, you can ask your parents or guardians or your doctor. Also, if you have any questions that you didn't think of now, you can ask later. You can call Dr. XXX at XXX.

What if I don't want to be in the study?

If you don't want to be in this study, you don't have to. It's up to you. If you say you want to be in it and then change your mind, that's OK. All you have to do is tell us that you don't want to be in it anymore. No one will be mad at you or upset with you if you don't want to be in it.

My choice:

If I write my name on the line below, it means that I agree to be in this research study.

_____ _____

Subject's Signature Date

_____

Subject's Name

_____ _____

Signature of person obtaining assent Date

_____

Name of person obtaining assent

11. APPENDIX C: STATISTICS

Sample Size and Power Calculation. Three clinical endpoints are chosen as the primary hypotheses: D180 Post Therapy non-relapse mortality, D28 post-therapy non-response, and GVHD grade 1-4 onset. Seven biomarkers will be tested at either onset of GVHD or at day 14 post-HCT (median 14 days prior GVHD) in predicting these clinical endpoints. Therefore, the overall type-I error is justified by a fact of 7*3*2 = 42, and the per comparison type I error is controlled at 0.05/42 = 0.0012. The effect size is pre- specified by the expected sensitivity and specificity of a biomarker, which ranges from 0.60 to 0.70. The following table present the power of our study (N = 350) for three clinical endpoints. It shows that we shall have more than 91% power to test our hypotheses. Given the fact that our preliminary data show that one biomarker has a 85% sensitivity and specificity, our proposed sample size will have sufficient power to test our hypothesis.

Response Type and Rate	Sensitivity and Specificity		
	0.60	0.65	0.70
D180 Post Therapy non-relapse mortality (0.26)	0.91	>0.99	>0.99
D28 post-therapy non-response (0.41)	0.96	>0.99	>0.99
GVHD grade 1-4 onset (0.50)	0.98	>0.99	>0.99

Our 'pre-specified' expected sensitivity and specificity are based on our previous work (taking the median as cutoff) (see Table below, Vander Lugt et al. NEJM, 2013)

Sensitivity and specificity of ST2 concentrations at D14 to predict six-month post-HCT NRM

Cutoff	Sensitivity (95% CI)	Specificity (95% CI)
75thpercentile	54% (50-59%)	83% (77-89%)
Median	75% (71-79%)	56% (48-64%)
25thpercentile	92% (89-94%)	29% (22-36%)

Biomarker Panel. We will construct the most informative and clinically useful biomarker panel to predict D180 post-therapy non-relapse mortality, D28 post-therapy non- response, and GVHD grade 1-4 onset. To this end, we will use standard logistic regression models to integrate the seven biomarkers into a multi-protein biomarker panel. This statistical step is a stepwise regression analysis to identify an optimal linear combination of biomarkers for the highest predictive value for predicting the three clinical outcomes mentioned above. We will first use Univariate and multivariate models similar to those performed in the preliminary data. We will also adjust the models for the clinical characteristics at GVHD onset: intensity of conditioning regimen, donor HLA match, age, and GVHD onset grade.

After we define the optimized biomarker panel, we will use logistic regression to examine the ability of either the biomarker panel or ST2 alone (because ST2 was the most significant marker in all previous analyses) to predict D28 non responders. A high biomarker value will be defined by a plasma concentration greater than 50% above the median value in the responder group. A high panel must have at least five high biomarkers. We will then evaluate whether the odds ratio (OR) of the high panel vs. low panel and the high ST2 vs. low ST2 are significantly greater, meaning that patients are more likely to not respond to therapy independent of the significant clinical characteristics described above. If ST2 presents a more similar OR than the panel, ST2 measurement will be used for further analyses. We will then hypothesize that the high panel (or high ST2) provides more important prognostic information at therapy initiation than the clinical grade. Similar to the preliminary data, we will evaluate if the high panel (or high ST2) at initiation of treatment can predict the D180 NRM independent of GVHD onset grade. NRM cumulative incidence curves and HR for the four risk categories will be evaluated: low panel/grade 1-2, low panel/grade 3-4, high panel/grade 1-2, and high panel/grade 3-4. We will evaluate the NRM and OS, as described in our previous biomarker studies[12,13]. Briefly, NRM will be modeled using the cumulative incidence regression methods described in Fine et al.[32], and the OS will be modeled using the Cox proportional-hazards model.

Predictive Model. If we validate the proteins as diagnostic biomarkers, we will in this next part of the project ask whether this biomarker can be used to predict the future development of GVHD before its clinical signs become manifest. This statistical step will evaluate the value of the composite panel as a screening test for the risk of developing GVHD by computing the positive and negative predictive values. In order to be clinically useful, we expect that both values will be > 75%. In this case, we would expect that the biomarkers will correlate with subclinical disease. This possibility is likely, given that our preliminary data showed that TNFR1 levels in day 7 post- allogeneic transplant plasma correlates with eventual severity and incidence of GVHD, and with overall survival[33,34]. We propose to use our novel repository of samples and the clinical data from SA 1 to determine if any of these biomarkers have predictive ability when measured earlier in the patient's course. We are in a unique position to perform this analysis because our bank will contain samples that are collected from patients on a weekly basis during the first month after transplant. We will measure concentrations of the biomarkers at D7, D14, and D21 post- HCT (7 to 21 days prior to the onset of GVHD) and from similar time points in control patients, which will permit the generation of a kinetic curve for the individual biomarker.

We will develop a predictive model using Cox proportional hazard model with time dependent covariates. Time to GVHD development is a time to event outcome, protein biomarkers measured at D7, D14, and D21 post HCT are time dependent covariates, and treatment/control data and other clinical and demographic variables will be time independent covariates. To perform these analyses GVHD onset will be used as the event outcome. The control group includes the patients accrued on this protocol who do not develop GVHD. Two strategies will be used to model the time dependent biomarkers. One strategy is a simple linear interpolation and extrapolation of biomarker measurement to days other than day 7, 14, and 21. The second strategy is to model biomarker with either the linear trend or quadratic trend.

Although these two strategies will pose different Cox regression models with time

dependent covariates, they can be implemented into the likelihood model framework proposed by Wulfsohn and Tsiatis[35]. We will follow this modeling strategy, and implement the model fitting in R. Given Dr. Li's extensive experiences in modeling longitudinal data with time dependent covariates[36,37], we are confident that we will be able to generate this time-dependent biomarker signature in predicting time to GVHD development.

12. APPENDIX D: GVHD scoring

A sample Acute GVHD data record suggested for use in this trial is attached below.

Today's Clinical Acute GVHD Assessment

Today's date _____ Patient ID _____ Karnofsky/Lansky _____

	Codes					Differential diagnosis						
	0	1	2	3	4	GVHD	Drug Rxn	Cond Reg	Infect	TPN	VOD	Other
Skin	☐	☐	☐	☐	☐ % body rash: _____	☐	☐	☐	☐			_____
Liver	☐	☐	☐	☐	☐ Current bili: _____	☐	☐	☐	☐	☐	☐	_____ Lower
GI	☐	☐	☐	☐	☐ Vol: _____	☐	☐	☐	☐	☐		_____ Upper GI
☐ ☐						☐	☐	☐	☐	☐		_____ **Systemic**

agents: ☐ CSA ☐ Tacrolimus ☐ Infliximab ☐ Daclizimab

☐ Pentostatin ☐ Sirolimus ☐ Etanercept ☐ MMF

☐ Prednisone ☐ Methylprednisolone ☐ Ontak ☐ Other _____

Current steroid dose: Prednisone _____mg/kg/day; Methylprednisolone _____ mg/kg/day

Has the steroid dose been increased to ≥ 2.5 mg/kg/day of prednisone (or 2 mg/kg/day methylpred)? ☐YES ☐ NO

If yes, date dose increased _____; reason for dose increase: ☐GVHD flare ☐ Other (specify) _____

Topical agents: Skin topical steroids**:** ☐ YES ☐ NO; Non-absorbed oral steroids (e.g. Budesonide, Entocort) ☐ YES ☐ NO

Chronic GVHD: Does the patient have evidence of chronic GVHD? ☐ YES ☐ NO

Codes	Skin	Liver (bilirubin)	Lower GI (stool output/day)	Upper GI
0	No rash	≤ 2 mg/dl	Adult: < 500 ml/day Child: < 10 ml/kg/day	No protracted nausea and vomiting
1	Maculopapular rash < 25% BSA	2.1-3 mg/dl	Adult: 500–1000 ml/day Child: 10 -19.9 ml/kg/day	Persistent severe nausea, vomiting with a positive upper GI biopsy
2	Maculopapular rash 25 – 50% BSA	3.1-6 mg/dl	Adult: 1001-1500 ml/day Child: 20 – 30 ml/kg/day	
3	Generalized erythroderma (>50% BSA)	6.1-15 mg/dl	Adult: >1500 ml/day Child: > 30 ml/kg/day	
4	Generalized erythroderma (>50% BSA) plus bullous formation and desquamation > 5% BSA	>15 mg/dl	Severe abdominal pain with or without ileus, or grossly bloody stool or melena (regardless of stool volume)	

Signature _____ Date _____

A sample chronic GVHD data record suggested for use in this trial is attached below.

Today's Clinical Chronic GVHD Assessment

Today's date _____ Patient ID _____

	0	1	2	3
Skin	□ No changes	□ < 18% BSA lichenoid, sclerodermatous, or ichthyotic involvement □ Eczema hypo or hyperpigmentation	□ 18-50% BSA lichenoid, sclerodermatous, or ichthyotic involvement	□ 50% BSA involved □ Interference with ADLs due to impaired mobility □ Skin ulceration
Hair Loss	□ None	□ Mild (< 50%)	□ > 50%	
Joints	□ No contractures □ Arthralgias □ Migratory arthritis	□ Persistent arthritis in 1 or 2 joints	□ Mild joint contractures (do not affect ADL) □ Polyarticular arthritis	□ Severe joints contractures (interfere with ADLs)
Oral	□ No changes	□ Symptomatic but no change in diet	□ Able to eat most foods, although some dietary changes due to oral chronic GVHD	□ Unable to eat most foods due to oral chronic GVHD
Ocular	□ No changes	□ Dry Eyes but not requiring therapy □ Keratoconjuctivitis, asymptomatic	□ Dryness of eyes requiring artificial tears, lachrymal plugging, or Schirmer's < 5 □ Keratoconjuctivitis, symptomatic	□ Pseudomembranes □ Corneal ulcerations □ Loss of vision
Esophagus	□ No changes	□ Symptomatic but can eat regular diet	□ Dysphagia or odynophagia requiring dietary changes	□ Need for parenteral nutrition □ Web/stricture formation
Pulmonary	□ Asymptomatic	□ 75-90% FEV1/FVC □ Asthma	□ Dyspnea with exertion □ 50-74% FEV1/FVC □ Desaturation with exercise	□ Dyspnea with normal activities □ <50% FEV1/FVC □ Requires supplemental oxygen
KPS/Lansky	□ Asymptomatic and fully active (KPS 100%, ECOG 0) □ Lansky = 90-100%	□ Symptomatic and fully ambulatory; restricted in physical strenuous activity (KPS 80-90%, ECOG 1) □ Lansky = 70-80%	□ Symptomatic; ambulatory; capable of self-care, > 50% of waking hours are spent out of bed (KPS 60-70%, ECOG 2) □ Lansky = 50-60%	□ Symptomatic; limited self-care, spends > 50% of waking hours in bed, but not bedridden (KPS 40-50%, ECOG 3) □ Lansky = < 50%
GI manifestations	□ None	□ Anorexia or malabsorption ± < 5% weight loss, or in children, growth deviation of < 5% of pre-transplant percentile after 1 year post-transplant	□ Anorexia or malabsorption ± 5-10% weight loss, or in children, growth deviation of < 5% of pre-transplant percentile after 1 year post-transplant	□ Anorexia or malabsorption ± > 20% weight loss, or in children, growth deviation of < 5% of pre-transplant percentile after 1 year post-transplant
Hematologic	□ Thrombocytopenia not attributed to other causes > 100K	□ Thrombocytopenia not attributed to other causes > 75K	□ Thrombocytopenia not attributed to other causes > 50K	□ Thrombocytopenia not attributed to other causes < 50K

Autoimmune (e.g. ITP, AHA)	□ None	□ Positive laboratory tests, clinically not requiring therapy	□ Require < 6 months additional immunosuppressive therapy to control symptoms	□ Require transfusions (AHA) or splenectomy (ITP) or > 6 months of increased immunosuppressive therapy to control symptoms
Laboratory and clinical variables (Please complete for this visit date)	Platelets _____ X 10^9 /L Total Bilirubin _____._____mg/dl Alkaline Phosphatase _____ Units/L Weight _____._____ kg			
Specific manifestations (Please circle Yes, No, or Not applicable)	Serositis Y / N Myositis Y / N Fasciitis Y / N	Scleroderma Y / N Steatorrhea Y / N Vaginitis/Vaginal stricture Y / N / NA		

*ADL = activities of daily living

Signature _____ Date _____

13. APPENDIX E: REFERENCES

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14. APPENDIX F: LABORATORY PROCEDURES

Research specimens

Research samples collected in this protocol will be used for both protocol-defined testing as well as future, undefined research supporting the protocol. All research sample aliquots will be given unique bar code designations that cannot be linked back to the recipient.

Patient samples will be collected as specified below.

- Peripheral Blood sample on day -7, pre-transplant: Whole blood, Plasma
- Peripheral Blood samples on day 56 post-transplant: Whole blood (donor DNA)
- Donor chimerism results will be recorded at the time nearest to the DNA sample collection
- Peripheral Blood samples at day -7, 7, 14, 21, 28, 56, 100, 180 post-transplant, and if the patient develops GVHD, two additional blood samples at the onset of symptoms that need treatment initiation and 28 days after treatment begins: Plasma

Samples processed into frozen cryovial aliquots will be shipped to the central Laboratory at IU every 6 months on dry ice by Fedex or UPS overnight priority.

Table 6: Collection, processing, and shipping procedures for patient blood samples for protocol-defined biomarker testing and for future undefined research

Time points	Collection of sample	Type of processing and storage	Specimen	Purpose of sample	Shipping specifications
Day -7 pre-transplant	12 mL peripheral blood (two 6 ml fill EDTA purple-top tube)	Centrifuge the EDTA containing whole blood tube at 1000-1300 g or 2100 rpm for 10 minutes within 60 minutes of collection. Remove the separated plasma. Aliquot to 1-4 cryovials and freeze at - 80º C in a scientific grade freezer.	Plasma	Future Biomarker studies	Batch ship every 6 months, frozen on dry ice in a shipping container, Fedex overnight priority to the Well's CCRC Laboratory at IU.
	3 mL peripheral blood (one 3 ml fill EDTA lavender-top tube) Source of patient DNA	Gently mix the tube by gently inverting the tube for 60 seconds and freeze at - 80º C in a scientific grade freezer.	Whole Blood. Until extracted for DNA	Future Biomarker studies	Batch ship every 6 months, frozen on dry ice in a shipping container, Fedex overnight priority to the Well's CCRC Laboratory at IU.
Day 7 post-transplant	12 mL peripheral blood (two 6 ml fill EDTA purple-top tube)	Centrifuge the EDTA containing whole blood tube at 1000-1300 g or 2100 rpm for 10 minutes within 60 minutes of collection. Remove the separated plasma. Aliquot to 1-4 cryovials and freeze at - 80º C in a scientific grade freezer.	Plasma	Seven protein biomarker panel/ Future Biomarker studies	Batch ship every 6 months, frozen on dry ice in a shipping container, Fedex overnight priority to the Well's CCRC Laboratory at IU.
Day 14 post-transplant	12 mL peripheral blood (two 6 ml fill EDTA	Ficoll (without diluting the blood). Centifuge at 2000 rpm at room temperature for 30 minutes, without brake. Remove the separated plasma. Aliquot	Plasma/ PBMCs	Seven protein biomarker panel/ Future	Batch ship every 6 months, frozen on dry ice in a shipping container, Fedex overnight priority to

	purple-top tube)	to 1-4 cryovials and freeze at -80º C in a scientific grade freezer. Then collect the "buffy coat" (PBMCs), Wash three times with cold sterile PBS 1X to remove Ficoll (1400 rpm 4C. 10 min.). Count cells. Freeze cells in 1 ml aliquots using 90% FBS and 10% DMSO: 1-5M/vial, at least 2 cryotubes up to 4 cryotubes. Gradual freezing: PBMCs are stored 24 hours (not more) in -80°C in the ethanol blue box (Cryo-safe cooler , to achieve a -1°C /min. rate of cooling) and then transfer in liquid nitrogen (liquid phase being better than gas phase). See details of the protocol below		Biomarker studies	the Well's CCRC Laboratory at IU.
Day 21 post-transplant	12 mL peripheral blood (two 6 ml fill EDTA purple-top tube)	Ficoll; see protocol below.	Plasma/ PBMCs	Seven protein biomarker panel/ Future Biomarker studies	Batch ship every 6 months, frozen on dry ice in a shipping container, Fedex overnight priority to the Well's CCRC Laboratory at IU.
Day 28 post-transplant	12 mL peripheral blood (two 6 ml fill EDTA purple-top tube)	Ficoll; see protocol below.	Plasma/ PBMCs	Seven protein biomarker panel/ Future Biomarker studies	Batch ship every 6 months, frozen on dry ice in a shipping container, Fedex overnight priority to the Well's CCRC Laboratory at IU.
Onset GVHD treated with systemic steroids	12 mL peripheral blood (two 6 ml fill EDTA purple-top tube)	Ficoll; see protocol below.	Plasma/ PBMCs	Seven protein biomarker panel/ Future Biomarker studies	Batch ship every 6 months, frozen on dry ice in a shipping container, Fedex overnight priority to the Well's CCRC Laboratory at IU.
28 days post-treatment for GVHD	12 mL peripheral blood (two 6 ml fill EDTA purple-top tube)	Ficoll; see protocol below.	Plasma/ PBMCs	Future biomarker studies	Batch ship every 6 months, frozen on dry ice in a shipping container, Fedex overnight priority to the Well's CCRC Laboratory at IU.
Day 56-60 post-transplant	12 mL peripheral blood (two 6 ml fill EDTA purple-top tube)	Ficoll; see protocol below.	Plasma/ PBMCs	Future Biomarker studies	Batch ship every 6 months, frozen on dry ice in a shipping container, Fedex overnight priority to the Well's CCRC Laboratory at IU.
	3 mL peripheral blood (one 3 ml fill EDTA lavender-top tube) Source of patient DNA	Gently mix the tube by gently inverting the tube for 60 seconds and freeze at - 80º C in a scientific grade freezer.	Whole Blood. Until extracted for DNA	Future Biomarker studies	Batch ship every 6 months, frozen on dry ice in a shipping container, Fedex overnight priority to the Well's CCRC Laboratory at IU.

Day 100 post-transplant	12 mL peripheral blood (two 6 ml fill EDTA purple-top tube)	Ficoll; see protocol below.	Plasma/ PBMCs	Future Biomarker studies	Batch ship every 6 months, frozen on dry ice in a shipping container, Fedex overnight priority to the Well's CCRC Laboratory at IU.
Day 180 post-transplant	12 mL peripheral blood (two 6 ml fill EDTA purple-top tube)	Ficoll; see protocol below.	Plasma/ PBMCs	Future Biomarker studies	Batch ship every 6 months, frozen on dry ice in a provided shipping container, Fedex overnight priority to the Well's CCRC Laboratory at IU.
Day 360 post-transplant	12 mL peripheral blood (two 6 ml fill EDTA purple-top tube)	Ficoll; see protocol below.	Plasma/ PBMCs	Future Biomarker studies	Batch ship every 6 months, frozen on dry ice in a shipping container, Fedex overnight priority to the Well's CCRC Laboratory at IU.

Isolation of Human Peripheral Blood Mononuclear Cells (PBMCs) and Plasma by Ficoll separation and cryopreservation Protocol

Purpose

This Standard Operating Procedure (SOP) describes procedures for the isolation and cryopreservation of Plasma and Peripheral Blood Mononuclear Cells (PBMC) from whole blood.

Scope

2.1 This procedure is to be utilized for processing blood samples for the isolation, cryopreservation, and storage of Plasma and PBMC samples.

Materials and Reagents

No.	Name	Description	Concentration
1.0	Purple top vacutainers	BD # 367863	
2.0	Ficoll or lymphoprep (sterile)	Amersham #17544202 (GMP grade)	Use undiluted.
3.0	PBS 1X (-calcium, -magnesium) (sterile)	Hyclone SH30256.02	
4.0	Cryotubes	Orange top for cells, Corning	
5.0	Cryotubes	Clear top for plasma	

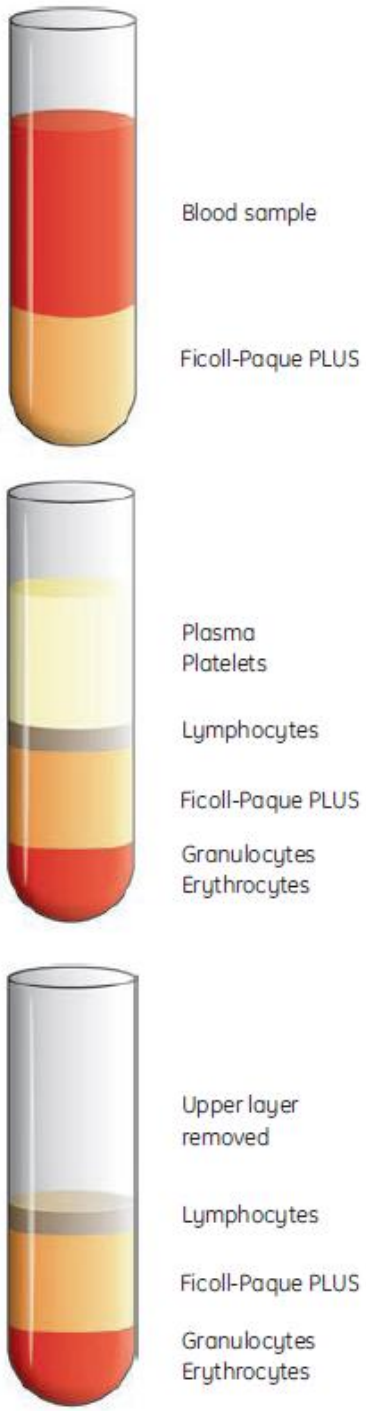
Procedure

- Do not dilute the blood
- For blood samples of up to 11 ml, add 5 ml of ficoll to a 15 ml tube. For blood samples over 11 ml, use 10 ml of ficoll in a 50 ml tube.
  - *Layer cells dropwise onto Ficoll.*
  - *Let the drops hit the side of the tube and settle slowly to avoid disturbing the meniscus.*

- Centifuge – see table below

Multi-Center GVHD Biomarkers and Biobank Centrifuge Instructions	
Parameter	**Setting**
RPM	2000
Time	30 minutes
Temperature	Room Temperature (20-24°C)
Brake	Off
Acceleration	Off

-



- Carefully observe the cloudy “buffy coat” at the interface between the layers.
- Collect first the plasma and distribute to 1-4 clear top tubes (1.5 to 1.7 ml in each of the tubes). Plasma samples are aliquoted without additives into cryovials and stored at -80°C.
- Then collect the cloudy “buffy coat” (= PBMCs), avoiding the Ficoll layer, using a 10 mL pipet (for 15 mL conical tube) or 25 mL pipet (for 50 mL Conical tube). Place into a clean 15 ml tube.
- Wash once with 15 ml sterile PBS 1X to remove trace of Ficoll (1400 rpm 4C. 10 min.)
- Wash PBMCs again with PBS 1X @ 1200 rpm, 4C. 5 min.
- Resuspend pelleted cells in PBS and count.

- Only if necessary (most of the time you don't need to), lyse residual erythrocytes.
- The last wash should be @900 rpm 10 min, to get rid of the platelets and trash).
- Freeze cells in 1 ml aliquots using 90% FBS and 10% DMSO: up to 30M/vial (see table below), at least 2 orange top cryotubes up to 4 orange top cryotubes. Gradual freezing: PBMCs are stored 24 hours (not more) in -80°C in the ethanol blue box (Cryo-safe cooler , to achieve a -1°C /min. rate of cooling) and then transfer in liquid nitrogen (liquid phase being better than gas phase).

PBMC Aliquot Instructions	
Number of Cells	**Number of cryotubes**
1 -10 million	Distribute in 2
10 - 20 million	Distribute in 3
20 - 35 million	Distribute in 3
35 million and over	Distribute in 4

References

- University of Michigan BMT laboratory SOP
- Baylor Institute for Immunology laboratory SOP
- HIV/AIDS Network Coordination SOP

15. APPENDIX G- CELLULAR PROTEIN BIOMARKER PANEL FOR VIRUS AND LEUKEMIA SPECIFICT CELL IMMUNE RECONSTITUTION PROTEIN BIOMARKER PANEL

1. SPECIFIC AIMS

Cellular populations in the peripheral blood may also serve as biomarkers for GVHD (e.g. Tregs). Therefore, we anticipate improving the panel by adding biomarkers of virus and leukemia specific T cell immune reconstitution that will be generated in collaboration with Dr. Bollard. This integrated proteomic/cellular approach will provide not only candidate biomarkers but also insights in the complex pathology of GVHD.

Accumulating evidence suggests that low levels of circulating Tregs following allo-HSCT in humans are associated with a higher incidence of GVHD in adults, and a causal link between these phenomena is suggested by murine models that show infusion of donor Tregs at the time of transplant or shortly thereafter prevents GVHD while maintaining the graft-versus-leukemia (GVL) effect. ***We hypothesize that increased donor-derived Tregs detected in the peripheral blood of pediatric patients is associated with a decreased risk of GVHD while sparing the cell mediated immune response to virus and tumor antigens after allo-HSCT.*** These hypotheses are formulated from extensive preclinical and clinical data and will be tested as follows:

Aim 1: To determine whether increased functional Tregs post allogeneic HSCT is associated with decreased GVHD without affecting cellular immune responses against leukemia and viruses.

We will evaluate patient peripheral blood samples. We will measure recovery of $CD4^+$ $CD25^+$ $FoxP3^+$ T cells as well as other major T-cell subsets using flow cytometry and confirm whether these cells are of donor or recipient origin. We will evaluate the ability of the $CD4^+$ $CD25^+$ Tregs to suppress alloreactive responses and analyze the mechanism should such suppression occur. We will also measure T-cell immune reconstitution to leukemia antigens (WT1, PR1, PRAME) and viruses (EBV, CMV and Adenovirus) after allo-HSCT. We will measure the frequency of virus and leukemia reactive T cells and test their functionality in cytokine secretion assays.

2. BACKGROUND AND SIGNIFICANCE

2.1 Regulatory T cells. A subpopulation of T cells that are $CD4^+$ $CD25^+$ plays a crucial regulatory role in the immune system. These Tregs control autoimmune processes[1] and modulate post-transplant alloreactive responses. Tregs constitutively express the transcription factor FoxP3, which is a key regulator of Treg development in humans and mice.[2-4] Intracellular expression of FoxP3 is one of the most specific markers for Tregs,[5] although other markers such as CD4, $CD25^{hi}$ and $CD127^{lo}$, may also define the most potent subpopulations.[6] In short-term *in vitro* assays, Tregs suppress proliferation of both $CD4^+$

and CD8$^+$ T cells after polyclonal or antigenic stimulation in a cell contact-dependent and cytokine-independent manner.[7] Moreover, interleukin 2 (IL-2) is essential for the generation of Tregs and crucial in maintaining their suppressive function.[8] Murine models show that high numbers of Tregs infused at the time of allo-HSCT can prevent GVHD while preserving the GVL effect.[9;10,11] Indeed, if Tregs present in the transplant product are depleted prior to infusion into irradiated allogeneic recipient mice, GVHD is significantly accelerated.[7] Additional studies have shown that Tregs infused post-transplant were able to control established GVHD.[4]

Human studies have demonstrated that poor Treg reconstitution post HSCT correlate with occurrence of acute or chronic GVHD.[12,13] Phenotypic and PCR analyses of FoxP3$^+$ regulatory T cells in 57 transplant recipients (27 with active, chronic GVHD and 30 without active, chronic GvHD) showed a decreased frequency of Tregs in patients with chronic GVHD compared to patients without ($p<0.0001$), and FoxP3 expression was reduced in chronic GVHD patients compared to those without or to controls ($p=0.009$ and 0.01, respectively).[14] Furthermore, there is an association between high donor levels of FoxP3$^+$ Tregs and a reduced risk of GVHD after HLA-matched allo-HSCT.[15] The above data support efforts to determine in the pediatric setting whether rapid Treg immune reconstitution after allo-HSCT will prevent GVHD.

2.2 Recovery of cellular immunity after allo-HSCT. Normal immune reconstitution following HSCT extends over many months and even years, rendering the patient highly susceptible to bacterial, viral, and fungal infections as well as leukemia relapse. Viral infections are one of the leading causes of morbidity and mortality among allo-HSCT recipients. Reactivation of latent viruses such as Epstein-Barr Virus (EBV) and cytomegalovirus (CMV), and infection with community viruses such as adenovirus and RSV are frequent and significant causes of morbidity and mortality.[16] Virus-specific T-cells are a critical protective element after allogeneic transplantation,[17] and the most prolonged delays in virus-specific immune responses are associated with protocols specifying high levels of T-cell depletion and post-transplant immune suppression to prevent GVHD.

Similarly, several groups have looked at the immune reconstitution of tumor-specific T cells and their effect on relapse. Along with viral infection, we and others have shown that relapse is one of the main causes of failure after allogeneic stem cell transplant. T-cells specific for leukemia associated antigens such as the Wilms tumor antigen (WT1) have been shown to contribute to a graft-versus-leukemia (GVL) effect after allogeneic stem-cell transplantation (HSCT) for acute leukemia. Furthermore, loss of these leukemia specific CD8+ T-cell responses has been shown to be associated with relapse.[18] ***We hypothesize that a balanced regulatory and virus and leukemia specific T cell recovery prevents GVHD without delaying antiviral immune reconstitution.***

3 . PRELIMINARY DATA

3.1. Preliminary data. To demonstrate our ability to measure the recovery of Tregs, NK cells and antigen-specific T cells, we isolated PBMC from transplant recipients immediately before and early (2-6 months) and late (>6 months) after transplantation (**Fig. 1A-C**). We plated the PBMC in a standard IFN-γ ELISPOT at a concentration of $2x10^5$cells/well, and stimulated them with peptide mixtures (pepmixes) spanning the adenovirus hexon and penton antigens, CMV IE1 and pp65 and the EBV antigens BZLF1, EBNA1, EBNA3A, EBNA3B and EBNA3C. To measure general T-cell function, we stimulated PBMC with *Staphylococcus aureus* (Staph) as a positive control, while unstimulated PBMC served as a negative control. Virus specific responses are shown in **Fig. 1B**. While we were able to detect robust specific T-cell reactivity to Staph early, we saw little evidence of virus-specific T-cell function in samples collected early (2 months) post-transplant. However, by 6 months after allo-HSCT, specific reactivity is detectable against viral antigens, indicating that patients who do not have GVHD can restore viral specific immunity within 3-6 months.[19,20] To detect tumor-specific T-cells, we stained the PBMC with HLA A2-restricted tetramers as shown in **Fig. 1C and D**. Detection of WT-1- and PRAME-specific T cells in the peripheral blood of patients with lymphoid and myeloid malignancies after allo-HSCT strongly correlated with a low relapse rate after transplant.[18] These preliminary data support our proposal to detect antigen-specific T cells as well as NK cells, post-transplant in the peripheral blood of patients after allo-HSCT. Hence, these results demonstrate the feasibility of tracking the effects of BMSC infusions on post-transplant immune reconstitution in patients with GVHD.

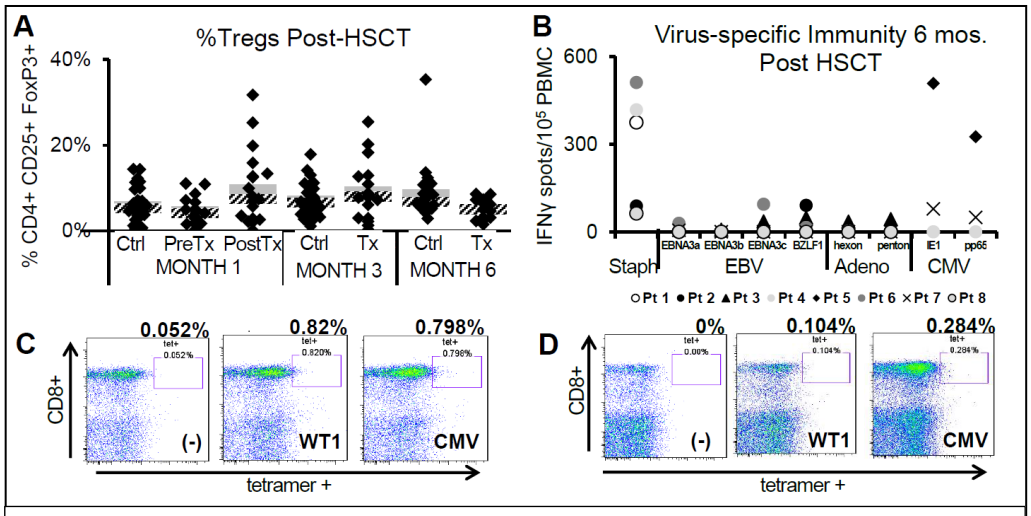


Figure 1. *Immunological studies of patients post HSCT. A.) T regs can be detected and analyzed post HSCT B.) T cell immunity to viral antigens as determined by IFNg ELISPOT in 8 patients. C and D. Identifying CMV- and WT-1 specific T cells from peripheral blood of 2 patients by tetramer analysis*

4. RESEARCH PLAN

4.1. Immune reconstitution of T-cells and NK cells Blood will be obtained at the designated timepoints (Day +14, +21, +28, +56, +100, +180 and +360 post HSCT as well

as at time of complications such as acute and chronic GVHD onset, start of systemic steroids, or other complications). Peripheral Blood Mononuclear Cells (PBMC) will be obtained by Ficoll separation. Flow cytometric analyses will be used to quantify T cells (CD3+ CD4+ CD8) and T-cell subsets, including naïve and memory T cells, and NK cells. Once the T cells and NK cells have recovered to a predefined level (≥ 500 $CD3^+$ cells/μL), samples will be frozen for batch analysis in the functional assays described below (4.2 to 4.5).

4.2. Are functional Tregs increased in pediatric patients without GVHD? To determine Treg phenotype, we will use the intracellular FoxP3 Treg kit (eBioscience) as well as CD4 and CD25 fluorescent antibodies (**Fig. 1A**). Treg induced suppression will be demonstrated by effects on thymidine uptake and CSFE assays in mixed lymphocyte reactions. Specifically, we will examine the response of T cells to a standard APC (Allogeneic PBMC or K562 cells expressing the high affinity IgG receptor CD64) loaded with OKT3. We will determine the mechanism for the suppression by measuring cytokine secretion (IL-10 and TGF-β). For thymidine uptake assays, PBMC will be separated into $CD4^+$ $CD25^+$ and $CD4^+$ $CD25^-$ fractions using fluorescent cell-sorting or magnetic bead separation. The $CD4^+$ $CD25^-$ fraction (responder cells) will be plated into a round-bottom 96-well plate coated with anti-CD3 (OKT3) or allogeneic PBMC. The $CD4^+$ $CD25^+$ Treg fraction will be added at a 1:1 ratio and incubated at 37^0C. On day 3, each well will be pulsed with 1 μCi of ^{3}H-thymidine. After 18 hours of additional incubation, plates are harvested and processed for counting.[20]

4.3. Do pediatric patients with GVHD have decreased pathogen-specific immune reconstitution compared to those without? Ten to 40 mL of blood (depending on the patient's size) will be taken at designated intervals post-HSCT to measure the frequencies of virus-specific T cells as well as NK cells. Absolute lymphocyte counts (ALC) will be measured to determine both absolute and relative frequencies of NK cells and virus-specific T-cells. Functional assays will determine the reconstitution of immunity to pathogens frequently detected in patients after allo-HSCT, including EBV, CMV, Aspergillus and adenoviruses.

(i) Capturing viral reactivation. Peripheral blood collected post-HSCT will be used for measurement of viral load (adenovirus, EBV and CMV), using PCR. These data will capture at each sample timepoint.

(ii) Immunity to common pathogens. To evaluate the immune response to EBV, CMV, Aspergillus and adenovirus, we will measure the precursor frequency of cytotoxic T lymphocytes (CTL) specific for these pathogens in ELISPOT assays.

4.4 What is the antileukemic T-cell activity in patients who develop GVHD? All

patients will be regularly monitored for disease status by our standard clinical management techniques, which include (i) morphologic analysis of bone marrow samples to assess "conventional" remission status and (ii) minimal residual disease analyses of marrow and peripheral blood samples using chromosomal markers. As an additional measure of disease recurrence, recipients will be regularly monitored for (iii) the level of donor chimerism in myeloid and lymphoid cells in the blood and marrow, as per our standard institutional protocols. Hence, in patients with myeloid and lymphoid malignancies, we will measure the precursor frequency of CTL specific for the leukemia-associated antigens PR3, PRAME and WT-1 using (i) A2-restricted tetramer assays, (ii) ELISPOT and/or intracellular cytokine assays using pepmix-pulsed PBMC.[20]

4.5. Do patients with GVHD have impaired NK cell persistence and function? To evaluate NK cell persistence and function post-HSCT, we propose to undertake immunophenotypic analyses of clinical samples obtained post-HSCT. NK-cell persistence will be defined as an absolute circulating NK cell count of >100 cells/μL after HSCT. To address the issue of possible dysfunction of NK cells in patients with GVHD, we propose to perform immunophenotypic analyses of clinical PBMC samples at the timepoints indicated above. Multiparameter (12-color) flow cytometry will be performed for NK cell receptors to evaluate the expression of activating and inhibitory receptors, NK activation and maturation, exhaustion (e.g. CD57 and T-bet)[21,22] and homing (e.g. CD62L, CCR7, CLA). Effector function will be assessed by the ^{51}chromium release assay, CD107a degranulation[23,24] and cytokine release (by intracellular cytokine assay [IFNγ]), using K562 and primary leukemia cells collected and stored from recipients pre-transplant. Plasma samples obtained from the recipient before and after HSCT will be assayed for cytokine levels using the BD Cytometric Bead Array for Th1/Th2 cytokines and ELISA assays.

4.6. Statistical analysis. The change in general immune reconstitution in patients with versus without GVHD will be summarized with descriptive statistics. After logarithmic transformation, if deemed appropriate, we will analyze the difference in changes by a two-sample t-test. In addition to descriptive statistics, Treg and antigen-specific T-cell immune reconstitution over time, as measured by phenotyping assays and functional assays will be plotted over time and represented as means ± SD.

4.7. Expected outcomes/potential problems. GVHD is known to be immune suppressive and we predict that there will be an increased Treg response in patients without GVHD with preservation of their cell mediated immune response. Given recent reports in the literature and our own preliminary data, we predict that if there is an increase in Tregs in patients without GVHD, that this will not dampen T-cell immune reconstitution against viral or

leukemia antigens. We also expect the immune reconstitution of NK cells to be unaffected in patients who do not have GVHD but that NK cells in patients with GVHD are either decreased and/or dysfunctional. Although unlikely, we cannot exclude the possibility that NK cells will not be detected in the periphery. This could reflect a complete loss of the NK cells or perhaps their limited expansion in vivo. It is also possible that T-cells and NK cells will home to sites of leukemia, prompting us to look for their presence in the bone marrow as well as blood. We do not anticipate any major obstacles with the proposed immune reconstitution studies, owing to our expertise at the CNMC in multiparameter flow cytometry and T-cell and NK cell functional immunoassays.[24-26] However, it is entirely possible that smaller patients will have too few lymphocytes in their peripheral blood, especially in the early post-HSCT period, to accommodate the proposed testing scheme. If so, we will prioritize our tests, requesting additional samples if necessary. Phenotypic analyses, specifically for biomarker assays and $CD4^+$ $CD25^+$ $FoxP3^+$ Tregs, will be performed first, followed by general phenotyping assays (T-cell subsets, NK cells and B cells), functional Treg assays and frequencies of CMV, then EBV and then adenovirus-specific CTL. In patients with evidence of viral infection, evaluation of the T-cell response specific to the infecting virus will be given priority.

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