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2. Introduction

Graft versus host disease (GVHD) is a frequent complication of allogeneic hematopoietic cell transplantation (HCT) in adults and in children. Morbidity and mortality as a consequence of GVHD result from direct organ damage, and from infections and organ toxicity related to the use of immunosuppressive drugs to treat GVHD.[1]

Despite the administration of immunosuppressive drugs early post transplant to prevent GVHD, following myeloablative allogeneic bone marrow transplant from unrelated donors in children at the FHCRC and other centers the incidence of grade II – IV acute GVHD is 40-90% and the incidence of extensive chronic graft versus host disease is 20-60%.[2-7] In an analysis of data reported to the Center for International Bone Marrow Transplant Research (CIBMTR), Eapen and colleagues observed a rate of chronic GVHD of 32% amongst 116 children with acute leukemia who received bone marrow transplants without T cell depletion from 8/8 allele matched unrelated donors. [4] Similarly, amongst 51 children who received myeloablative bone marrow transplants from 10/10 allele matched unrelated donors (URD) at FHCRC the rate of clinically extensive chronic GVHD was 35%.[8] & personal communication AW and TG

Although bone marrow is currently the most frequently used donor stem cell source for pediatric HCT recipients there has been a trend toward increasing use of peripheral blood stem cells (PBSC) in pediatrics as well as adults. At FHCRC, of the transplants performed in pediatric recipients with HLA matched unrelated donors in the past ten years, 40% have employed PBSC and 60% have employed bone marrow as the stem cell source . PBSC has several advantages over BM including more rapid engraftment and a shorter duration of initial hospitalization, and is frequently preferred by the donor center or donor for feasibility reasons. However, a large randomized study demonstrated an increased rate of chronic GVHD was observed amongst predominantly adult patients who received PBSC (53%, CI 45 to 61) compared with bone marrow (41%, CI 34 to 48) from HLA matched (78%) or 1-2 antigen mismatched (22%) URD [9], although the rate of graft failure was lower amongst PBSC recipients (3% CI 1-5%) compared to bone marrow recipients (9% CI 6-13%). There was no significant difference in overall survival between the groups. There is a clear need to develop strategies to reduce the rate of chronic GHVD, particularly in PBSC transplants. An optimal approach would preserve the benefit of the rapid engraftment time and low rate of graft failure associated with the use of PBSC but substantially reduce the rate of chronic GVHD to lower than that observed with either PBSC or BM.

In HCT using HLA-identical donors, GVHD results from recognition of minor histocompatibility (H) antigens expressed on recipient tissues by donor T cells.[10] Complete depletion of T cells from the donor hematopoietic cell product is a highly effective alternative to pharmacologic immunosuppression for preventing GVHD, but is complicated by a profound delay in immune reconstitution, which contributes to life threatening infections, and has been associated with an increased risk of graft rejection and leukemia relapse.[11,12] Laboratory studies have suggested that donor T cells specific for recipient minor H antigens are found predominantly within the naïve subset of T cells that express the cell surface molecule CD45RA, and that the selective depletion of $CD45RA^+$ T cells from PBSC grafts can preserve the subset of pathogen-specific memory T cells that lack CD45RA.This protocol will evaluate the selective removal of $CD45RA^+$ cells from PBSC as a strategy to reduce chronic GVHD and provide rapid

recovery of T cell immunity to pathogens in children after HCT from HLA-identical unrelated donor transplants.

3. Background

3A. GVHD

The pathogenesis of GVHD involves multiple interacting factors including tissue damage resulting from the conditioning regimen, the release of inflammatory cytokines, and the activation and proliferation of mature donor T cells that express the αβ T cell receptor (TCR) and recognize recipient alloantigens presented as peptides by class I and II major histocompatibility complex (MHC or HLA in humans) molecules on antigen presenting cells (APCs) [1,13]. The mature T cell repertoire is "tolerant" to peptides derived from self-proteins due to thymic deletion and/or peripheral suppression of autoreactive T cells [14]. However, in the setting of allogeneic HCT between HLA-identical individuals, the repertoire of peptides displayed on recipient cells will include distinct species that differ from those on donor cells as a consequence of polymorphisms in genes outside of the MHC, and those peptides can be recognized as minor H antigens by donor T cells.[10] The activation of donor T cells to recipient minor H antigens is likely to occur after contact with specialized APCs and/or other cell types particularly in the gastrointestinal tract and leads to proliferation of T cells and their differentiation to effector cells that produce cytokines including IFN-γ and TNFα, and mediate cytotoxicity against recipient tissues [10]. The tissues that are most frequently damaged during acute GVHD include skin, gastrointestinal tract, and liver, although other organs can be involved, particularly when acute GVHD evolves into chronic GVHD. Chronic GVHD has a characteristic clinical presentation which often resembles autoimmune vascular diseases and is distinct from acute GVHD. Chronic GVHD can be highly debilitating and prolonged and has a 20-50% mortality rate due to immune dysregulation and opportunistic infections. [15]

The administration of immunosuppressive drugs that interfere with T cell activation or proliferation such as methotrexate, cyclosporine, FK506, prednisone, and mycophenolate mofetil, alone or in various combinations, are used at most transplant centers to prevent GVHD [16]. The development in the early 1980s of the combination of a short course of methotrexate with the calcineurin inhibitor cyclosporine represented a significant advance in the prophylaxis of GVHD, but major improvements in pharmacologic immunosuppression have not been achieved since that time [16,17]. At the FHCRC, a short course of methotrexate combined with either cyclosporine or tacrolimus continues to be a frequently used immunosuppressive regimen for GVHD prophylaxis after allogeneic HCT. With this regimen of GVHD prophylaxis, following myeloablative allogeneic bone marrow transplant from unrelated donors in children at the FHCRC and other centers the incidence of grade II – IV acute GVHD is 40-90% and the incidence of extensive chronic graft versus host disease is 20-60%.[2-7] Patients who develop GVHD typically require treatment with additional immunosuppressive drugs, which increases their risk of post transplant infections and may diminish the graft versus leukemia (GVL) effect [18]. Additionally, many patients with acute leukemia will relapse after HCT and those with GVHD are less likely to be eligible for, or benefit from, T cell immunotherapy designed to augment the GVL effect.

3B. T Cell Depletion to Prevent GVHD

The recognition of recipient minor H antigens by donor T cells is an essential requirement for the induction of GVHD after HCT from HLA-identical donors. Thus,

many centers have used partial or complete depletion of T cells from the donor bone marrow or PBSC graft as an alternative approach to pharmacologic immunosuppression for preventing GVHD [11,12,19-23]. A variety of methods have been used to remove T cells from bone marrow or PBSC grafts including soybean lectin agglutination, monoclonal antibodies, and positive selection of $CD34^+$ hematopoietic progenitors [11,12,19-22,24]. Depending on the approach, the extent of T cell depletion can vary from 2-5 $\log_{10}$. More complete T cell depletion is generally associated with less GVHD, although an absolute minimum threshold dose of donor T cells needed for development of GVHD has been difficult to define in humans.

T cell depletion is a highly effective strategy for preventing GVHD in both murine models and in humans [11,12,19-23] but recipients of T cell-depleted grafts have poor reconstitution of T cell immunity to pathogens, and some studies found T-cell depletion to be associated with an increased risk of graft rejection and relapse [11,12,19,25]. The Bone Marrow Transplant Clinical Trials Network recently published a multicenter trial of complete T cell depletion for AML patients undergoing HCT from HLA-identical sibling donors.[22,26]The BMT-CTN study 0303 used TBI (13.75 Gy), Cyclophosphamide, and ATG as a conditioning regimen, and the Miltenyi CliniMACS device to select $CD34^+$cells from G-CSF mobilized PBSC using anti-CD34-coated immunomagnetic beads. CD34 selection with the Miltenyi CliniMACS device provided a 4. 9 $\log_{10}$ removal of total T cells (3.2-5.9) including both the memory and naïve subsets. All patients engrafted with secondary graft failure occurring in only 1 of 44 patients. Low rates of acute GVHD (grade II-IV 22.7%, III-IV 4.5%) and extensive chronic GVHD (6.8%) were observed. The DFS and OS at three years after $CD34^+$ transplant were 53% and 56% respectively for the whole group, 58% and 60% for patients transplanted in CR1, and 29 and 38% for patients transplanted in CR2. [22,26]The OS and DFS figures are broadly comparable to those seen after T-replete transplant, at least for patients in CR1. [27-31] However, as expected the $CD34^+$ selected transplant was associated with a high risk of opportunistic viral infections (57% of patients). In particular high-level EBV reactivation requiring treatment was observed in 18% of patients and 1 of 44 patients died of EBV-PTLD. [31]

Two single center studies performed for MRD and URD recipients with hematologic malignancies at Memorial Sloan Kettering Cancer Center employed a similar conditioning regimen TBI (13.75 Gy), Thiotepa (10mg/kg over two days), Fludarabine (125mg/m2 over five days) and equine ATG (60mg/kg) and $CD34^+$ selection of stem cells from G-PBSC or BM from matched ($\geq$ 7/10) unrelated donors with the Isolex Device followed by sRBC-rosette depletion of T cells, achieving a 5 log depletion of T cells.[20,21] Amongst MUD recipients the rates of aGVHD II-IV and extensive chronic GVHD were reported to be 5.8% and 11% respectively. DFS and OS at 4 years were 57 and 59% respectively, whilst the relapse rate was surprisingly low at 6% at 4 years. Opportunistic infections were also frequent in this trial, with several clinically significant adenoviral infections (14.7%) including one death, and three (9%) EBV reactivations-including one death from EBV PTLD.

In children there is less published experience with $CD34^+$ TCD transplants from unrelated donors. The largest study was published by a European group in 2003.[32] Purified URD $CD34^+$ stem cells (median 8 x 10^6/kg, obtained using Miltenyi MACs devices) were infused following a range of conditioning regimens each including ATG.

All patients with an HLA matched URD achieved sustained primary engraftment. Acute GVHD was observed in 10% of the patients, and chronic GVHD was <10%. Patients with ALL in CR1 (N=14) mostly received TBI containing conditioning regimens and the overall survival was 63% amongst this group. Patients with AML were treated without TBI (BuMelCy) and had a poor survival (14%). A retrospective analysis of the MSKCC pediatric experience with $CD34^+$ TCD using the Isolex device followed by sRBC-rosette depletion of T cells was published more recently. Eleven patients with AML or advanced MDS received a standard conditioning of Busulphan, Melphalan, Fludarabine and ATG. All engrafted and achieved full donor chimerism. The overall survival of the Bu-Mel-Flu subgroup is 54.5% with patients dying of relapse (27%), GVHD (9%) or infection (9%).[33]

3C. Naive T cells (T_N) are primarily responsible for GVHD in rodent models

Transplant approaches that selectively deplete T cells that recognize minor H antigens and retain T cells specific for pathogens in the stem cell graft could reduce GVHD and improve reconstitution of T cell immunity. Studies in murine models of allogeneic stem cell transplantation have provided insights into how such selective manipulation of the T cell content of hematopoietic cell grafts might be achieved. Mature $CD3^+$ $CD8^+$ and $CD3^+$ $CD4^+$ T cells can be classified into naïve (T_N) and memory (T_M) subsets that differ in cell surface phenotype, prior exposure to cognate antigen, and functional activity [34,35]. In mice, the T_N subset is $CD44^-$ $CD62L^+$ and the T_M subset is $CD44^+$ and can be further subdivided into a $CD62L^+$ central memory (T_{CM}) population, and a $CD62L^-$ effector memory (T_{EM}) population [35]. Studies in murine bone marrow transplant models have evaluated the potential for T cells derived from T_N and T_M subsets to cause GVHD across both minor and major histocompatibility differences. The first study to examine this question employed a multiple minor H antigen mismatched $CD4^+$ dependent GVHD model [B10.D2 ($H\text{-}2^d$) → BALB/c ($H\text{-}2^d$)]. In this model, transplantation of irradiated BALB/c mice with T-cell depleted bone marrow combined with unfractionated splenocytes or with purified $CD4^+$ $CD44^-$ $CD62L^+$ T_N from B10.D2 donors caused severe GVHD. However, transplantation of T-cell depleted bone marrow with purified $CD4^+$ $CD44^+$ $CD62L^-$ T_M did not cause GVHD and provided for the transfer of T cell immunity to a model antigen [36].

The efficacy of removing T_N from the stem cell graft for preventing GVHD has been confirmed in other murine strain combinations including CD8 dependent minor H antigen-mismatched and MHC-mismatched models, and in rats [36-44][Table 1]. It should be noted that the intent in these studies was to deplete T_N, but the cell selection procedure that was utilized in some studies would also remove the T_{CM} subset of T_M from the graft. In other studies, the selection procedure targeting $CD44^+$ did not allow the effects of T_{CM} or T_{EM} to be delineated. In subsequent experiments by the Shlomchik group, purified T_N or T_{CM} obtained by cell sorting were transplanted with T cell depleted bone marrow in a CD8 dependent minor H antigen-mismatched murine model of GVHD in the absence of immunosuppression. Mice that received T_N developed severe GVHD, whereas mice that received T_{CM} developed only mild GVHD [37]. Similar results were obtained in an MHC-mismatched model. These results demonstrate that in murine allogeneic minor H antigen and MHC mismatched bone marrow transplantation, the T_N subset of T cells is primarily responsible for GVHD and bone marrow grafts containing only T_M exhibit limited capacity to cause GVHD.

Table 1. Rodent studies of T cell depleted BMT with selective addition of T cell subsets

Transplant Model	Study	T Cell Subsets	Experimental Design	Outcome
Multiple Minor H Antigen Mismatched, CD4 dependent	Anderson B et al. J Clin Invest, 112: 101, 2003[36]	$CD4^+$ $CD44^+$ $CD62L^-$ (T_M) vs $CD4^+$ $CD44^-$ $CD62L^+$ (T_N)	B10.D2 (H-2^d) →BALB/c (H-2^d)	No GVHD in mice receiving T_M, severe GVHD in mice receiving T_N
Multiple Minor H Antigen Mismatched, CD8 dependent	Zhang Y. et al. Blood 103; 3970 – 3978, 2004[37]	$CD8^+$ $CD44^+$(T_M) vs $CD8^+$ $CD44^-$ (T_N)	CH3.SW (H-2^b) →B6 (H-2^b)	Minimal GVHD in mice receiving T_M, severe GVHD in mice receiving T_N
Multiple Minor H Antigen Mismatched CD8 dependent	Zheng H et al. J. Immunology 182; 5938-5948 2009 [42]	$CD8^+CD44^+CD62L^+$(T_{CM}) vs $CD8^+$ $CD44^-$ $CD62L^+$(T_N)	CH3.SW (H-2^b) →B6 (H-2^b)	Mild GVHD in mice receiving T_{CM}, severe GVHD in mice receiving T_N
MHC Mismatched	Chen B et al. Blood 103: 1534-1541, 2004[38]	$CD4^+$ + $CD8^+$ $CD62L^-$ (T_{EM}) vs $CD4^+$ + $CD8^+$ $CD62L^+$ (T_N & T_{CM})	C57BL/6 (H-2^b) → BALB/c (H-2^d) or C3H/HeJ (H-2^k)	No GVHD in mice that received $CD62L^-$ T cells, lethal GVHD in recipients of $CD62L^+$ T cells
MHC Mismatched	Chen B et al Blood 109: 3115-3123 2007[39]	$CD4^+$&$CD8^+CD62L^+$ $CD44^-$(T_N) vs. $CD4^+$&$CD8^+$ T_M (all other), $CD4^+T_M$ (all other), $CD4^+$&$CD8^+$ $CD62L^+$ $CD44^-T_{CM}$	C57BL/6 (H-2 b) →BALB/c (H-2d) And C57BL/6 (H-2 b) to BALB/c (H-2d)	T_N Severe GVHD T_M total No GVHD $CD4^+T_M$No GVHD T_{CM} No GVHD
MHC Mismatched	Dutt S et al J. Immunology 179: 6547-6554 2007 [40]	$CD4^+CD62L^+$ $CD44^-$(T_N) $CD4^+CD62L^-CD44^+$(T_{EM})primed $CD4^+CD62L^-CD44^+$(T_{EM})unprimed	C57BL/6 (H-2 b) →BALB/c (H-2d	T_N Severe GVHD T_{EMP} Severe GVHD T_{EMUP} No/min GVHD
MHC Mismatched	Zheng et al, Blood, 111: 2476-2484.2008 [41]	$CD4^+CD44^+CD62L^-$(T_{EM}) vs CD4+CD44-CD62L-(T_N)	$B6^{bm12}$→B6	No GVHD in T_{EM} recipients; GVHD in T_N recipients.
MHC Mismatched	Zheng H et al. J. Immunology 182; 5938-5948 2009 [42]	$CD8^+CD44^+CD62L^+$(T_{CM}) vs $CD8^+$ $CD44^-$ $CD62L^+$(T_N)	C57BL/6 (H-2 b) →BALB/c (H-2d)	T_N Severe GVHD +GVL T_{CM} Milder GVHD +GVL
MHC Mismatched	Dutt S et al, Blood 117, 3230-3239 2011 [43]	$CD8^+CD44^-$ (T_N) vs $CD8^+CD44^+$(T_M)	C57BL/6 (H-2 b) to BALB/c (H-2d)	T_N Severe GVHD +GVL T_M total minimum GVHD+GVL
MHC Mismatched (Rat Model)	Xystrakis E et al. Eur J Immunol, 34: 408, 2004 [44]	$CD4^+$ $CD45RC^{high}$ (T_N) vs $CD4^+$ $CD45RC^{low}$ (T_M)	Parental →F1 (LEWxBN)	No GVHD in rats receiving T_M, lethal GVHD in rats receiving T_N

3D. Human T cells specific for minor H antigens are predominantly in the T_N subset. Inferential data suggests that the results obtained in murine models using selective depletion of T_N to prevent GVHD might also apply to humans. The T_N subset in humans, which expresses both CD45RA and CD62L, has been shown to contain the greatest T cell receptor (TCR) diversity [45,46]. The T_M subset, which contains $CD45RO^+$ $CD62L^+$ T_{CM} and $CD62L^-$ T_{EM} populations, and a subset of $CD62L^-$ cells that re-express CD45RA (T_{EMRA}), is comprised of cells that have clonally expanded in response to antigen stimulation and contains relatively less TCR diversity compared to T_N. A major fraction of the T_M subset consists of T cells specific for latent herpes viruses including CMV, EBV, herpes simplex virus (HSV) and varicella zoster virus (VZV). Cross reactivity of virus-specific T_M for major alloantigens has been observed infrequently and in humans does not appear to cause GHVD [47,48] , and cross reactivity with minor H antigens has not been reported and is likely to be exceedingly rare. To evaluate the presence of minor H antigen-reactive T cells in T_N and T_M subsets, we purified $CD8^+$ T_N ($CD45RA^{bright}$, $CD62L^+$, $CD45RO^-$) and T_M ($CD45RO^+$, $CD62L^{+/-}$, $CD45RA^-$) from leukapheresis products obtained from HLA-identical sibling pairs and determined the frequency of alloreactive T cells in each subset by limiting dilution analysis. Individuals may be exposed to minor H antigens by pregnancy or blood transfusion and develop minor H antigen-specific T_M responses, therefore donors without a prior history of pregnancy or blood transfusion were selected for these experiments. In multiple assays of different HLA-matched pairs, the frequency of minor H antigen-reactive T-cells was significantly higher in the T_N subset than in the T_M subset. Indeed, only very rare cells (~1/2,000,000-1/6,000,000) in the T_M subset exhibited weak recognition of recipient cells in these assays, and this weak alloreactivity was not reproducible with repeated testing. Thus, in individuals without a history of pregnancy or blood transfusion, minor H antigen-specific $CD8^+$ T cells are predominantly in the T_N subset [49]. We attempted to evaluate the frequency of T cells specific for minor H antigens in $CD4^+$ T_N and T_M subsets using lymphoproliferation assays, but these assays were confounded by high background reactivity to autologous cells in both subsets.

3E. Depletion of T_N from human PBSC grafts as a strategy to reduce GVHD
The *in vivo* data in animal models and *in vitro* analysis of alloreactivity of $CD8^+$ T_M and T_N subsets in HLA-identical siblings suggested that selective depletion of T_N from human PBSC grafts is a rational strategy to investigate for reducing GVHD and for improving the tempo of immune reconstitution after allogeneic HCT. In preclinical studies, we examined methods for selectively depleting T_N from G-CSF mobilized PBSC products. We initially considered using anti-CD62L mAb to deplete T_N, based on the data from murine models. However, CD62L is subject to proteolytic cleavage by ADAM-17 and other proteases released during G-CSF mobilization [50], and we found that cell surface CD62L declined on T cells in G-CSF mobilized products. CD45RA is expressed stably on all $CD8^+$ and $CD4^+$ T_N in G-CSF mobilized PBSC but a minor subset of $CD34^+$ cells also express CD45RA. Thus, to remove $CD45RA^+$ T cells without interfering with $CD34^+$ progenitors, we used sequential positive selection of $CD34^+$ progenitor cells using Miltenyi CD34 immunomagnetic selection, followed by depletion of $CD45RA^+$ cells from the CD34-negative fraction. Our goal was to develop a cell selection procedure that would result in an engineered stem cell graft that contains $>2.0 \times 10^6$ $CD34^+$ cells/kg recipient body weight, $<7.5 \times 10^4$ T_N/kg and $1\text{-}10 \times 10^6$ T_M/kg. The dose of $1\text{-}10 \times 10^6$ $CD3^+$ cells/kg was selected for two reasons. First, this number is nearly equivalent to the T cell content of an unmanipulated bone marrow graft and is 10 - 100 fold greater than

the threshold dose of unselected T cells that causes GVHD after HLA-identical sibling HCT [51]. It would therefore allow us to test the hypothesis that CD45RA$^-$ T cells can safely be transferred with less GVHD. Second, it would provide a sufficient number of memory T cells such that we would reasonably anticipate improved immune reconstitution as compared to recipients of only a TCD allograft.

A GMP-grade murine αCD45RA monoclonal antibody that is directly conjugated to Miltenyi iron dextran beads was produced by Miltenyi Biotec under contract from the NIH RAID program. Using the new αCD45RA mAb-conjugated bead and the existing αCD34-conjugated bead we have now successfully completed more than 30 clinical scale cell selection procedures, including preclinical validation runs and procedures for twenty-four patients who have been treated on our first (adult) Phase II Study of Selective Depletion of CD45RA$^+$ T Cells from Allogeneic Peripheral Blood Stem Cell Grafts for the Prevention of GVHD using HLA-matched related donors (see section 3H).

3F. T cell responses to pathogens are retained after depletion of CD45RA$^+$cells from G-PBSC

We have evaluated CD45RA depleted hematopoietic cell products for retention of functional memory T cells specific for common pathogens including CMV, EBV, influenza, Candida albicans, and VZV. For persistent viruses such as CMV and EBV, a major fraction of the CD8$^+$ T_M response may reside in the CD45RA$^+$ (T_{EMRA}) subset of T_M, and the use of αCD45RA for depletion of T_N could compromise the transfer of T_M responses to these pathogens. Therefore, we compared the frequency of CD8$^+$ T-cells specific for known epitopes of CMV, EBV and adenovirus in PBSC before and after depletion of CD45RA$^+$ cells using an interferon gamma Elispot assay after a single *in vitro* stimulation. Interferon-producing virus-specific CD8$^+$ T-cells were present in both the CD45RA-depleted fraction and unselected PBSC at comparable frequencies. CD4$^+$ T_M cells are uniformly CD45RO$^+$ CD45RA$^-$ and should be retained in G-PBSC depleted of CD45RA$^+$ T-cells. We analyzed lymphoproliferative responses to CMV, HSV, VZV, Adenovirus, Influenza and Parainfluenza and Candida antigens before and after depletion of CD45RA$^+$ T-cells from G-PBSC products. To avoid measuring CD8$^+$ T-cells that might respond to soluble proteins that are taken up by APC in the culture and enter the class I pathway, CD4$^+$ T-cells from the CD45RA depleted product were enriched by negative selection prior to plating in the assay. CD4$^+$ T_M responses to all the antigen preparations were detected in the CD45RA depleted product. These results demonstrate that CD8$^+$ and CD4$^+$ memory T cells to common opportunistic pathogens are retained after depletion of CD45RA$^+$ cells.

3G. Preliminary analysis of the first phase II study to evaluate selective depletion of naïve T cells from PBSC grafts for preventing acute GVHD in (adult) patients with acute leukemia or MDS undergoing allogeneic HLA-identical HCT from a related donor.

We are conducting a phase II study to evaluate selective depletion of naïve T cells from PBSC grafts for preventing acute GVHD in (adult) patients with acute leukemia or MDS undergoing allogeneic HLA-identical HCT from a related donor. We employ a myeloablative preparative regimen consisting of fludarabine, thiotepa, and TBI that provides a high rate of engraftment. The product that is administered to each patient consists of an infusion of purified CD34$^+$ cells, obtained by Miltenyi CliniMACS selection of G-CSF mobilized cells collected from the donor by apheresis, and an infusion of CD45RA$^-$ cells obtained by depletion of CD45RA$^+$ cells from the flow-

through remaining after the $CD34^+$ cell selection.

As discussed above, our goal is to administer a $CD34^+$ cell dose of >2.0 $x10^6$/kg of recipient body weight and a total T cell dose of 1-10 x 10^6 $CD3^+$ cells/kg, of which <7.5$x10^4$/kg are $CD45RA^+$ RO^- naïve T cells. The cell selection goals were readily achieved in each of the first twenty-four patients treated on the trial. All patients received >5.0 $x10^6$/$CD34^+$cells/kg, with the median $CD34^+$ dose being 8.1 $x10^6$ $CD34^+$cells/kg. The median T cell dose administered was 10.00 x 10^6 $CD3^+$cells/kg (1.59-10.04) with most (22/24) patients receiving 9.9-10.04 x 10^6 $CD3^+$cells/kg. All patients received less than 7.5 $x10^4$ $CD45RA^+$ RO^- naïve T cells per kg with a median of 0.37 $x10^4$ $CD45RA^+$ RO^- naïve T cells per kg (range 0.09-7.46, all but one patient received ≤1 $x10^4$ naïve T cells/kg). Approximately two thirds of the residual $CD45RA^+$ RO^- naïve T cells were within the $CD34^+$ cell fraction (median 0.21 $x10^4$) and one third were from the $CD45RA^-$ depleted PBSC product (median 0.12 $x10^4$). In summary, cell selection process to retain $CD34^+$ stem cells and memory T cells whilst depleting naïve T cells is effective and reproducible.

To date we have treated twenty-four patients with acute leukemia or advanced MDS (ALL N=16, AML N=6, MDS N=2) with an age range of 19-55 years. Fourteen patients were in CR1 without minimal residual disease (MRD) prior to HCT, four patients had MRD but were in first morphological remission, and six patients were in CR2 or CR3. The median time of follow-up is 545 days (40-1134) (February 2013). The preliminary analysis of engraftment and survival is encouraging (Table 2).

Table 2: Engraftment and survival amongst first 24 patients treated with naïve T cell-depleted PBSC (FHCRC Protocol 2222)

Outcome	
Neutrophil engraftment (>500/μl)	24/24 patients
Time to neutrophil engraftment	Median 12 days (range 9-18days)
Platelet engraftment (>20,000/μl)	24/24 patients
Time to platelet engraftment	Median 12 days (range 9-111 days)
Overall survival	83%
Disease free survival	75%
Relapse	3/24 patients
Death from relapse	1/24 patients
Non-relapse mortality all patients	3/24 patients
Non-relapse mortality patients <40years	0/11
Non-relapse mortality all patients ≥40 years	3/13

a. Mild acute GVHD grade II-III, universally steroid-responsive, has been observed in 17/22 (77%) evaluable patients. Fifteen patients developed grade II GVHD and responded rapidly to corticosteroids. Two other patients were considered to have grade III GVHD on the basis of large volume diarrhea but also responded rapidly to prednisone with no subsequent recurrence. In each of the 17 cases GVHD within the gastrointestinal tract was observed, predominantly involving the upper GI without lower GI involvement (12/17). Skin involvement was also seen in 7/17 patients while liver GVHD has not been observed to date. A biopsy of the intestinal tract was obtained in all patients diagnosed with gastrointestinal GHVD. The histopathologic changes were considered minimal or mild. The high rate of mild upper gastrointestinal GVHD is similar to the historical experience for adult patients treated with myeloablative TBI containing conditioning with T replete BMT or PBSCT at FHCRC where there is a high diagnostic sensitivity and awareness of the potential for

mild gastrointestinal GVHD without skin involvement [52].

b. Chronic GVHD: Amongst the 22 patients have completed >100 days of follow-up three have been diagnosed with chronic GVHD fulfilling NIH criteria, including one mild classic chronic (steroid responsive skin rash and dry eyes) and two late acute gastrointestinal with subclinical oral involvement. The frequency of chronic GVHD appears to be lower than the historical experience of 45-50% for adult patients receiving unmanipulated PBSC transplants from HLA-identical sibling donors.[30]

c. Discontinuation of immune suppression. The median time to discontinuation of prednisone for patients treated on the study was 100 days and the median time to discontinuation of all immune suppression was 298 days. Of the 11 patients aged ≤40 years at the time of HCT the median time to discontinuation of prednisone for patients treated on the study was 67 days and the median time to discontinuation of all immune suppression was 211 days. All young patients (<40 years) who were alive without relapse at one year post HCT completed all immune suppression at or prior to that time point. The time to completion of all immunosuppression amongst patients treated on this naïve T cell depletion study appears to be considerably faster than in concurrent controls who received standard myeloablative PBSC transplants at FHCRC, amongst whom 50% of the living patients are still on immune suppression at two years after transplant.

d. Immune reconstitution: The median $CD3^+$ T cell/mm^3 of peripheral blood observed in recipients of naïve T cell depleted transplant is above 300 at 28 days after transplant and remains at this level for the first three months and increases to 700 at 1 year post transplant. $CD4^+$ T cells median values are above 100 $CD4^+$ T cells/mm3 of blood for the first 3 months and 250 at one year. The numbers of $CD4^+CD3^+$ and $CD4^+CD8^+$ T cells in peripheral blood after naïve T cell depleted HCT exceed those seen after TCD HCT over the first 3 months after HCT after which time the levels are similar.[22] EBV reactivation has been monitored weekly by quantitative PCR and no EBV reactivation has been detected in patients receiving naïve T cell depleted PBSC. In comparison a much higher 18% rate of EBV reactivation was seen after TCD HCT.[22] In summary, in the first three months after HCT overall immune reconstitution appears to be superior in recipients of naïve T cell depleted grafts compared to total TCD transplants, with a corresponding reduced level of viral reactivation.

3H. <u>New pediatric clinical trial</u>: A Phase II Study of Selective Depletion of $CD45RA^+$ T Cells from Allogeneic Peripheral Blood Stem Cell Grafts for the Prevention of GVHD in Children

The strategy of depleting naïve T cells from stem cell grafts is effective for preventing GVHD in murine models. In adult humans naïve T cell depletion of PBSC appears to reduce the frequency, severity and duration of chronic GVHD relative to unmanipulated PBSC, and to improve immune reconstitution relative to TCD HCT. The frequency of acute GVHD is not reduced using naïve T cell depletion and tacrolimus monotherapy compared to T cell replete HCT with conventional calcineurin inhibitor and methotrexate prophylaxis, but the acute GVHD observed is mild and steroid responsive.

Children are not eligible for the open ongoing trial of naïve T cell depletion as they mostly have pediatric donors and bone marrow is preferred to PBSC as a stem cell source from younger pediatric donors on research studies due to safety concerns primarily related to the insertion of central venous catheters required for the

collection of apheresis. Selective depletion of naïve T cells from bone marrow grafts would not provide a stem cell product with an optimal number of $CD34^+$ cells in many patients, as the available number of $CD34^+$ cells in the starting bone marrow product is substantially lower than PBSC (median of 2.75 x 10^6/kg $CD34^+$ cells in bone marrow in adults, range1.94-4.53 x 10^6/kg[9]; median of 6.2 x 10^6/kg $CD34^+$ cells in bone marrow in children [Heimfeld personal communication]) and there is typically a 20-30% loss of $CD34^+$ cells during $CD34^+$ enrichment using current CliniMACs technology.

We plan to study the naïve T cell depletion approach in 20 pediatric recipients of PBSC from HLA-identical unrelated donors and to employ tacrolimus and methotrexate as pharmacological prophylaxis of GVHD. The primary objective of the trial will be to estimate the time to discontinuation of systemic immunosuppression in pediatric recipients of $CD45RA^+$ T cell-depleted PBSCT. Unpublished data from our center shows that amongst 55 children with a history of leukemia who received a myeloablative bone marrow (N=34) or PBSC (N=21) transplant from an unrelated donor (HLA-A, B, C, DRB1, and DQB1 molecularly matched) following conditioning with TBI and cyclophosphamide between July 2002 and December 2010 the median time to discontinuation of all immunosuppression was 1076 days, with a median time to discontinuation of 608 days for bone marrow recipients, and median time to discontinuation not reached for recipients of PBSC. The goal is to increase the proportion of patients who discontinue all systemic immunosuppression by 608 days from the historical value of 50% to 80% in the current trial by depletion of naïve T cells from PBSC without observing an increased frequency of graft failure. A secondary objective will be to estimate the frequency of acute GVHD in pediatric recipients of T_N cell-depleted PBSC who receive tacrolimus and methotrexate pharmacological prophylaxis.

4. Objectives

4A. Primary objectives

1. Estimate the time to discontinuation of systemic immunosuppression in pediatric recipients of $CD45RA^+$ T cell-depleted PBSCT.
2. Estimate the probability of graft failure in pediatric recipients of $CD45RA^+$ T-cell-depleted PBSCT.

4B. Secondary objectives

1. Estimate and compare to an appropriate historical cohort the probability of chronic GVHD (NIH criteria) requiring treatment with systemic pharmacological immunosuppression in pediatric patients who receive $CD45RA^+$ T cell depleted PBSC
2. Estimate the probability of acute GVHD grade II-IV
3. Estimate the probability of steroid refractory acute GVHD
4. Evaluate immune reconstitution
5. Estimate the probability of transplant-related mortality by day 100
6. Estimate the probability of relapse

5. Patient Selection

5A. Inclusions

1. Patients who are considered appropriate candidates for allogeneic hematopoietic stem cell transplantation and have one of the following diagnoses:

a. Acute lymphocytic leukemia in first or subsequent remission
b. Acute myeloid leukemia in first or subsequent remission
c. Acute lymphocytic leukemia in relapse or primary refractory disease with a circulating blast count of no more than 10,000/mm^3
d. Acute myeloid leukemia in relapse or primary refractory disease with a circulating blast count of no more than 10,000/mm^3
e. Refractory anemia with excess blasts (RAEB-1 and RAEB-2)
f. Chronic myelogenous leukemia with a history of accelerated phase or blast crisis
g. Other acute leukemia (including but not limited to 'biphenotypic', 'undifferentiated' or 'ambiguous lineage' acute leukemia)

2. Patient age <22 years old
3. Patient with a HLA-identical (HLA-A, B, C, and DRB1 molecularly matched) unrelated donor or related donor capable of donating PBSC.

5B. Exclusions

1. Patients with CNS involvement refractory to intrathecal chemotherapy and/or standard cranial-spinal radiation
2. Patients on other experimental protocols for prevention of acute GVHD
3. Patients who weigh ≥70kg must be discussed with the principal investigator prior to enrolling on the protocol.
4. Patients who are HIV+
5. Patients with uncontrolled infections for whom myeloablative HCT is considered contraindicated by the consulting infectious disease physician (Upper respiratory tract viral infection does not constitute an uncontrolled infection in this context)
6. Patients with organ dysfunction
   a. Renal insufficiency (creatinine >1.5 mg/dl)
   b. Cardiac ejection fraction < 45%
   c. Patients who can perform pulmonary function tests will be excluded if they have a DLCO (corrected for hemoglobin) of <60% predicted. Patients who are unable to perform pulmonary function tests (for example, due to young age and/or developmental status) will be excluded if the O2 saturation is < 92% on room air.
   d. Liver function abnormality. Patients who have LFTs (including total bilirubin, AST and ALT) ≥ twice the upper limit of normal should be evaluated by a GI physician unless there is a clear precipitating factor (such as an azole, methotrexate, bactrim or another drug) If the GI physician considers that HCT on protocol 2660 is contraindicated for that patient the patient will be excluded from the protocol. Patients with Gilbert's syndrome and no other known liver function abnormality and patients with reversible drug-related transaminitis do not necessarily require GI consultation and may be included on the protocol.
7. Patients with a life expectancy <3 months from co-existing disease other than the leukemia or RAEB
8. Patients who are pregnant or breast-feeding
9. Fertile patients of child bearing age unwilling to use contraception during and for 12 months post transplant
10. Patients with a significant other medical conditions that would make them unsuitable for transplant
11. Patients with a known hypersensitivity to tacrolimus

6. Donor Selection

6A. Inclusions

1. HLA-matched unrelated donors (HLA-A, B, C, and DRB1 matched based on high-resolution typing) capable and willing to donate PBSC.
2. HLA-matched related donors ≥18 years and capable and willing to donate PBSC.

6B. Exclusions

1. Donors who are HIV-1, HIV-2, HTLV-1, HTLV-2 seropositive or with active hepatitis B or hepatitis C virus infection
2. Donors who fail eligibility requirements for donation of cells or tissue per section 21 CFR 1271 for donation of a HCT/P will be excluded unless use of the cells complies with 21 CFR 1271.65(b)(iii) (urgent medical need) or with 21 CFR 1271.65(b)(i) (allogeneic use in a first-degree or second-degree relative)
3. Unrelated donors donating outside of the USA or Germany.

7. Evaluation and Counseling of Patient

Patients will be referred to the Fred Hutchinson Cancer Research Center (FHCRC) for consideration of a hematopoietic cell transplant. The protocol will be discussed thoroughly with patient, and other family members if appropriate, and all known risks to the patient will be described. The procedure and alternative forms of therapy will be presented as objectively as possible, and the risks and hazards of the procedure explained to the patient or, in the case of minors, to the patient's responsible legal guardian. Consent will be obtained using forms approved by the FHCRC Institutional Review Board. A conference summary detailing what was covered will be dictated for the medical record. Related donors will be evaluated separately at the FHCRC. The protocol will be discussed thoroughly with donor and other family members if appropriate. The procedure and alternative forms of therapy will be presented as objectively as possible, and the known risks and hazards of the procedure explained to the donor and in the case of minors, to the donor's responsible legal guardian. Consent will be obtained using forms approved by the FHCRC Institutional Review Board.
Unrelated donors will be managed by the NMDP (US donors) or DKMS (German donors).

8. Protocol Registration

Patients enrolled will be assigned to the protocol by the SCCA (Seattle Cancer Center Alliance) Clinical Coordinator. Once full consent has been obtained from patients and if applicable from related donor the SCCA Consent Coordinator will register the patient with the Registration Office (206) 667-4728, between 8:30 am and 4:00 pm, Monday through Friday. After hours, the Registration Office can be reached by paging (206) 995-7437. The FHCRC regulatory coordinator will then complete a patient registration form and fax it to the FHCRC Registration Office who will generate a unique patient identifier. This patient will then be entered into the **Protocol Patient Performance Program (**PPPP) database by FHCRC study staff. Informed consent must be signed by the subject or their legal guardian prior to the performance of any study related procedures or assessments.

9. Plan of Treatment

9A. Conditioning Regimen

The conditioning regimen will consist of fludarabine, thiotepa, and fractionated total body irradiation (TBI) and will be administered as outlined below and shown

schematically in Table 2. Note that for chemotherapy administered during conditioning and other drugs listed in this protocol, in keeping with institutional practice it is acceptable to administer a dose within 10% of the protocol specified dose.
TBI will be given as 165 cGy fractions twice per day x 4 days – total dose 1320cGy (days -10 to -7).

1. After completing the TBI, patients will be treated with thiotepa 5 mg/kg/day (adjusted body weight –see appendix I) administered intravenously over approximately 4 hours on each of two consecutive days (days -6 and -5). If actual weight is *less* than ideal body weight, actual body weight will be used. The total dose is 10 mg/kg (adjusted body weight - see appendix I).
2. Patients will receive fludarabine 25 mg/m^2/day (m^2 based on actual weight see appendix I) administered intravenously over approximately 30 minutes for 5 days beginning on the first day of thiotepa (days -6 to -2).
3. Day -1 will be a day of rest.
4. GCSF-mobilized CD34 enriched PBSC and CD45RA depleted cells will be infused on day 0.

Table 3a. Treatment Plan –patients with unrelated donors

Day of the week (***example only***)	**M**	**T**	**W**	**Th**	**F**	**S**	**S**	**M**	**T**	**W**	**Th**	**F**	**S**	**S**
Day relative to transplant of CD34$^+$ cells	**-10**	**-9**	**-8**	**-7**	**-6**	**-5**	**-4**	**-3**	**-2**	**-1**	**0**	**0/1**	**2**	**3**
TBI 165 cGy BID x 4 days	X	X	X	X										
Thiotepa 5 mg/kg/day x 2 days					X	X								
Fludarabine 25 mg/m^2/day x5 days					X	X	X	X	X					
Infusion of CD34$^+$ stem cells and CD45RA$^+$-depleted cells											X	+/-[2]	+/-[2]	+/-[2]
Donor GCSF						X	X	X	X	X	+/-[2]	+/-[2]		
Donor apheresis										X		+/-[2]	+/-[2]	
Cell selection											X			

[1] A single apheresis will be collected on day -1, and transported as soon as possible on day-1. The selected product will be infused on day 0.[2] On rare occasions a second apheresis could be required to make the cell selection goals and would be collected on day+1 or +2 and infused without cell selection on day+1, +2 or +3. (Also see section 9C, 9D &9E and Appendix A).

Table 3b. Treatment Plan –patients with related donors

Day of the week (***example only***)	**M**	**T**	**W**	**Th**	**F**	**S**	**S**	**M**	**T**	**W**	**Th**	**F**	**S**	**S**
Day relative to transplant of CD34$^+$ cells	**-10**	**-9**	**-8**	**-7**	**-6**	**-5**	**-4**	**-3**	**-2**	**-1**	**0**	**0/1**	**2**	**3**
TBI 165 cGy BID x 4 days	X	X	X	X										
Thiotepa 5 mg/kg/day x 2 days					X	X								
Fludarabine 25 mg/m^2/day x5 days					X	X	X	X	X					
Infusion of CD34$^+$ stem cells and											X	+/-[2]	+/-[2]	+/-[2]

$CD45RA^+$-depleted cells														
Donor GCSF						X	X	X	X	X	+/-[2]			
Donor apheresis										X[1]	+/-[1]	+/-[2]	+/-[2]	
Cell selection											X	+/-		

[1] An apheresis collection will be performed on day -1, and may also be performed on day 0. The selected product will be infused on day 0. (Also see section 9C, 9D &9E and Appendix A). [2]On rare occasions an additional collection of PBSC, or bone marrow could be required.

9B. Central Nervous System Prophylaxis and Testicular Irradiation

Patients will have a diagnostic lumbar puncture prior to the preparative regimen. Intrathecal chemotherapy will be given according to institutional standard practice if a. the CSF is positive for malignant cells; b. the patient has a prior history of CNS disease; c. the patient has a history of ALL; or d. the patient is otherwise deemed to be at significant risk of CNS disease. Intrathecal chemotherapy may be omitted if the attending physician considers that it is contraindicated for an individual patient based on prior toxicities or comorbidities. No dose of intrathecal therapy will be given within 72 hours of $CD34^+$ stem cell infusion. Male patients with ALL will receive a 400 cGy testicular irradiation boost pre-transplant during the conditioning regimen. For patients with a history of CNS leukemia a CNS irradiation boost may also performed if it is clinically indicated in the opinion of the pediatric BMT physician and radiation oncologist.

9C. Collection of GCSF mobilized PBSC (see also appendix A)

All donors will receive GCSF administered by subcutaneous injection for 5 consecutive days on days -5 thru day-1. Unrelated donors will receive a dose of 10 µg/kg/day of GCSF according to standard NMDP and DKMS practice for unrelated donors whilst related donors will receive a dose of 16 µg/kg/day of GCSF according to FHCRC standard practice. On infrequent occasions 1-2 additional doses of GCSF may be required for further PBSC collections if the cell infusion goals are not met initially. The schedule of G-CSF administration and PBSC collections will be determined when the schedule for the conditioning regimen and day 0 is established and should be confirmed with the personnel in the Cellular Therapy Laboratory. Day 0 should usually be fixed on a Monday-Friday. Typically, for URD transplant recipients the first apheresis would be performed on a Wednesday and transported to the SCCA Cellular Therapy Laboratory for cell selection on a Thursday. PBSC collection by apheresis: Unrelated donors will undergo vein to vein apheresis collections. Vein to vein apheresis is also preferred for related donors but insertion of a central venous catheter for apheresis collection is permitted for related donors if necessary. PBSCs will be collected on day -1 (unrelated donors and related donors) and sometimes also day 0 (some related donors).
On rare occasions $CD34^+$ cell target numbers may not be achieved according to the specified plans, and an additional PBSC collection would be requested and infused without cell selections.

9D. PBSC graft engineering - $CD34^+$ cell selection and depletion of $CD45RA^+$ cells (see also appendix A)

- Prior to cell selection, a sample of the PBSC products will be analyzed for the content of total nucleated cells, $CD34^+$ cells, $CD3^+$, $CD3^+$ $CD45RA^+$ RO^- and total $CD45RA^+$ cells and an aliquot of cells will be obtained for subsequent analysis of other cell subsets (see section 9D.3 below).

- Cell selections will *only* be performed when the PBSC product from day -1 or a pooled collection from day-1 and day 0 contains $\geq 5x10^6$ $CD34^+$ cells/kg of recipient body weight to ensure that the final $CD34^+$ cell dose will be $\geq 2x10^6$ $CD34^+$ cells/kg of recipient body weight. In *most* cases we expect that cell selections will be performed on a PBSC product containing $\geq 10x10^6$ $CD34^+$ cells/kg of recipient body weight which should provide a final $CD34^+$ cell dose of $\geq 5x10^6$ $CD34^+$ cells/kg of recipient body weight.
- If the available PBSC product contains $<5x10^6$ $CD34^+$ cells/kg of recipient body weight the PBSCs will be infused without any cell selection and the patients will not be included in the analysis of study efficacy end points. We expect that ≤5% of patients enrolled on protocol 2660 may receive a G-PBSC product that does not undergo any cell selection.
- For the patients in whom cell selection is performed we expect to achieve:
  - The CD34 goal of a minimum of 2.0×10^6 $CD34^+$ cells/kg of recipient body weight in all patients
  - A maximum of 5×10^4 $CD45RA^+RO^-CD3^+$ T cells/kg recipient body weight and a range of $1\text{-}10 \times 10^6$/kg total $CD3^+$ T cells in >95% of patients.
- Cell processing would commence ideally within 24 hours of the end of an apheresis collection, but may be delayed for up to 48 hours if necessary.
- Timing (for additional details please see Appendix A):
  - Unrelated donors
    - The apheresis will be performed on day -1, and transported as soon as possible on day -1.
    - Cell selections will be performed on day 0 using the day -1 apheresis
    - The cells will be infused as soon as possible after cell selection and release testing are completed.
  - Related donors
    - The first apheresis will be performed on day -1. A second apheresis may be performed on day 0, depending on the size of the recipient and the $CD34^+$ content of the day -1 apheresis product (Appendix A).
    - Cell selections will be performed on day 0 using the day -1 apheresis, or a pooled product from the combined day-1 and day 0 apheresis depending on the size of the recipient and the $CD34^+$ content of the day -1 apheresis product.
    - The cells will be infused as soon as possible after cell selection and release testing are completed.
  - On rare occasions $CD34^+$ cell target numbers may not be achieved according to the specified plans, and an additional PBSC collection would be requested and infused without cell selections.

The cell selection will be performed as outlined below and in the flow chart provided as Appendix A.

1. $CD34^+$ cell selection (Also see Appendix A)
   The apheresis products will be processed by $CD34^+$ cell selection using the clinical-grade Miltenyi anti $CD34^+$ conjugated iron dextran microbeads and magnetic selection with the CliniMACs device using the institution's Standard Operating Procedures. The goal is to obtain a target CD34 cell dose of $>5.0x10^6$/kg (minimum of $>2.0x10^6$/kg) of recipient body weight and this goal is expected to be achieved for

all products that undergo cell selection. The CliniMACS system of CD34 selection results in a 4-5 log_{10} depletion of $CD3^+$ cells and typically results in a median $CD3^+$ cell dose of 2 $x10^4$/kg of which ≤ 30% are $CD45RA^+RO^-$.

2. $CD45RA^+$ cell depletion to remove T_N cells (See also Appendix A)
   The CD34-depleted flow through fraction(s) will be collected and processed to deplete $CD45RA^+$ cells using clinical grade anti-CD45RA-conjugated iron dextran beads and magnetic selection with the CliniMACS device according to the institution's standard operating procedures. The phenotype and content of T cells that remain in the product after removal of $CD45RA^+$ cells will be determined by flow cytometry (see section 9D3, below). We will plan to administer a total $CD3^+$ T cell dose of 10 $x10^6$/kg to the patient including both the $CD34^+$ enriched fraction and the CD45RA depleted fraction. 10 $x10^6$/kg total T cells is highly likely to contain < 5 $x10^4$/kg of $CD3^+CD45RA^+RO^-$ cells/kg on the basis of our experience to date. If necessary the total T cell dose may be reduced to maintain a maximum infusion of 5 $x10^4$/kg of $CD3^+CD45RA^+RO^-$ cells/kg.
3. Analysis of the engineered cell products. Samples will be taken from the apheresis products before and after the CD34 selection and depletion of $CD45RA^+$ cells, and analyzed as follows (also see Appendix B "Product Testing"):
   a. Viability testing
   b. Sterility testing
   c. Total nucleated cell count
   d. Immunophenotyping by flow cytometry will be performed on the initial apheresis product(s) and cells that have completed the selection process:
      i. The following cell subsets will be enumerated to guide cell selection and to determine whether the goals for the composition of the product are achieved:
         - $CD34^+$ cells
         - $CD3^+$ cells
         - $CD3^+$ $CD45RA^+$ RO^- cells
      ii. Additional cell markers may be evaluated such as
         - $CD3^+$ $CD45RA^+$ $CD45RO^-$ $CCR7^+$ (T_N)
         - $CD8^+CD45RA^+$ $CD45RO^-$ $CCR7^+$ (CD8 T_N) and $CD4^+CD45RA^+$ $CD45RO^-$ $CCR7^+$ (CD4 T_N)
         - $CD3^+$ $CD45RO^+$ (T_M), $CD3^+CD8^+$ $CD45RO^+$ (CD8 T_M) and $CD3^+$ $CD4^+$ $CD45RO^+$ (CD4 T_M)
         - $CD3^+$ $CD45RO^+$ $CCR\text{-}7^+$ (T_{CM}) and $CD3^+$ $CD45RO^+$ $CCR\text{-}7^-$ (T_{EM})
         - $CD8^+$ $CD45RO^+$ $CCR\text{-}7^+$ (CD8 T_{CM}) and $CD8^+$ $CD45RO^+$ $CCR\text{-}7^-$ (CD8 T_{EM}), and $CD4^+$ $CD45RO^+$ $CCR\text{-}7^+$ (CD4 T_{CM}) and $CD4^+$ $CD45RO^+$ $CCR\text{-}7^-$ (CD4 T_{EM})
         - $CD3^{+/-}$ $CD56^+$, $CD3^{+/-}$ $CD16^+$, $CD14^+$, $CD19^+$ and $CD20^+$
         - $CD4^+$ $CD25^+FoxP3^+$.
   e. Specific T cell responses to pathogen-derived antigens may be performed on aliquots of the CD45RA depleted cell product and PBSC prior to CD45RA depletion using functional assays which may include ELISPOT, intracellular cytokine staining and/or tetramer analysis.

9E. PBSC Infusion

The $CD34^+$ selected product and CD45RA-depleted product will be infused through a central venous catheter on day 0 or + 1 ('second day 0') of the transplant (see Appendix D). The CD34+ product will be infused first followed as soon as possible by the

CD45RA-depleted product. In the very unlikely event that additional $CD34^+$ cells are required (see section 9D.1 and Appendix A and D) unselected PBSC will be administered.

9F. Post-transplant immunosuppression

GVHD prophylaxis will consist of tacrolimus and methotrexate. Tacrolimus (FK506) will be started on day -1 and should be continued for 50 days followed by a standard taper if there is no GVHD (see section 9F.1b). Methotrexate should be administered on day +1, +3, +6, +11 (see section 9F.3).

1. Tacrolimus administration
   a. Tacrolimus will be administered beginning on day -1 at a dose of 0.03 mg/kg/day by continuous IV infusion per institutional standard practice guidelines. Tacrolimus doses are based on actual body weight. If actual weight is *more* than ideal body weight, it is recommended to use adjusted body weight. Conversion to the oral formulation of tacrolimus (IV: PO ratio of 1:4) may be made when oral feeding is established. Oral tacrolimus is recommended to be given in two divided daily doses every 12 hours on an empty stomach. Children < 6 years old may require oral dosing every eight hours to maintain target serum trough levels.
   b. If there is no evidence of grade II-IV acute GVHD on or prior to day 50, tacrolimus should then be tapered at the rate of approximately 5% of the day 50 dose each week for liquid, and 20% of the day 50 dose per month for capsules.
   c. If there is evidence of acute GVHD, then the standard recommendations for treatment of acute GVHD and tapering of immunosuppression should be followed according to institutional practice. Patients with GVHD may be considered for enrollment in investigational protocols for GVHD treatment after discussion with the principal investigator.
   d. If there is evidence of disease progression and no evidence of GVHD prior to day 50, patients should taper tacrolimus and all other immunosuppressive agents within 2 weeks and be observed for the development of GVHD. The taper may be accelerated or tacrolimus may be discontinued, as clinically indicated.

2. Monitoring of tacrolimus levels and dose adjustment
   a. It is recommended that tacrolimus levels are maintained in the range of 5-15 ng/ml. It is recommended that whole blood trough levels are obtained on approximately day 2, then approximately once each week or more often if clinically indicated. Dose adjustments are recommended if the levels are outside the therapeutic range, or if there is evidence of toxicity that may be related to tacrolimus.
   b. When initiating therapy with voriconazole or posaconazole in patients receiving tacrolimus, it is recommended that the tacrolimus dose be reduced according to institutional practice, and followed with frequent monitoring of the tacrolimus blood levels.
   c. Weekly tacrolimus levels can be discontinued during a tacrolimus taper when the dose has been reduced by 25% if the patient has an adequate oral intake, volume status, renal function and the absence of toxicities that might be attributed to tacrolimus.
   d. Blood pressure, renal function tests (creatinine, BUN), electrolytes and magnesium should be monitored regularly as per institutional standard practice

guidelines.

3. Methotrexate administration

In patients weighing >10kg methotrexate should be administered by IV push on days +1, +3, +6 and +11 at a dose of 15 mg/m^2 (on day +1) and a dose of 10 mg/m^2 (on day +3, +6, +11). Calculation of m^2 should be according to institutional standard practice guidelines. Children weighing 10kg or less will have methotrexate dosed per kg rather than per /m^2 and should receive 0.5mg/kg on day +1 and 0.33mg/kg on days +3,+6, and +11. The first dose of methotrexate should be given at approximately 24 hours, but no sooner than 24 hours after completion of the cell infusion. The day 11 methotrexate dose may be omitted if the patient develops severe mucositis, a pleural effusion, ascites, or another relative contraindication to methotrexate administration.

9.G. Use of hematopoietic growth factors

There will not be routine post-transplant use of growth factors in patients enrolled on this protocol. Growth factors may be recommended by the Attending Physician to manage slow engraftment or in the event of infectious complications as indicated in Section 12 E.

10. Evaluation

10A. Donor evaluation

Pre-donation evaluation for unrelated US donors should be conducted as per NMDP (National Marrow Donor Program) standard procedure. Pre-donation evaluation for unrelated German donors should also be conducted according to the same FDA required screening and eligibility tests. Infectious disease markers (IDM) testing for both NMDP and DKMS donors is performed in CLIA (Clinical Laboratory Improvement Amendments) certified laboratories using FDA licensed, cleared and approved test kits. IDM testing for DKMS donors is sent to the central NMDP contracted laboratory (currently Labs Inc, Centennial, Colarado).

Related donors evaluation: The following should be obtained for all related donors:

1. Screening for high-risk behavior and HepBsAg, antiHepB core antibody, HBV NAT, antiHep C antibody, HCV NAT, HTLV-1 and HTLV-2 antibodies, a serologic test for syphilis, and HIV (1 & 2) antibodies and HIV NAT, all performed within 30 days of donation and West Nile Virus NAT testing and Trypanosoma antibody testing according to institutional standard practice. These tests are performed using FDA licensed, cleared, and approved test kits in a CLIA-certified laboratory.
2. CMV and EBV serologies performed within 30 days of donation.
3. Serum pregnancy qualitative within 2 weeks of the start of conditioning
4. G-CSF and monitoring blood draws (including CBC and Hepatic Function Panel with LDH) should be conducted per institutional standard practice guidelines.
5. Research Tests: A 90 cc sample of related donor blood should be collected (preferably before the donor starts GCSF) in sodium heparin or ACD tubes for immunologic studies, specifically T cell responses to pathogens by functional assays which may include ELISPOT, lymphoproliferation assays, intracellular cytokine staining and/or tetramer analysis (SCCA patients send to the Bleakley lab and contact the protocol 2660 research technician at (206) 667-4804 or Marie Bleakley at (206) 469-4487).

10B. Patient pre-transplant evaluation

Patient pre-transplant evaluation should be conducted as per standard institutional practice. Results of tests and/or procedures conducted as part of that evalution may be used for eligibility determination. It is recommended that the following information is obtained for all patients:

1. History
    a. Possible antecedent causes for the development of leukemia or MDS including prior cytotoxic therapy.
    b. Hematologic, cytogenetic and flow cytometric findings at diagnosis and at the time of enrollment.
    c. Prior therapies and response to therapy

2. Laboratory evaluation
    a. Bone marrow aspirate for morphology with standard cytogenetics and where appropriate, molecular cytogenetics, within 30 days of the start of conditioning.
    b. Bone marrow flow cytometry for determination of blast counts within 30 days of the start of conditioning.
    c. Lumbar puncture with CSF evaluation within 30 days of the start of conditioning.
    d. Panel reactive antibody testing and, if indicated (e.g. if PRA>10% activity or as determined by clinical immunogenetics laboratory specialist recommendations) leukocytotoxic and/or fluorescence activated cell sorter cross match between recipient and donor.
    e. ABO and Rh typing and two way red cell cross match between recipient and donor as per standard practice.
    f. HepBsAg, antiHepB core antibody, antiHep C antibody, HTLV-1, and HTLV-2 antibodies, a serologic test for syphilis, and HIV (1 & 2) and HIV p24 antigen or HIV PCR quantitation within 30 days of the start of conditioning.
    g. CMV, EBV, HSV, VZV serologies within 30 days of the start of conditioning.
    h. CMV, EBV and adenovirus plasma PCR obtained and run within 2 weeks prior to starting conditioning.
    i. CBC within 2 weeks of the start of conditioning.
    j. LFTs, to include ALT, AST, and Bili T/D, within 2 weeks of the start of conditioning.
    k. Serum chemistry, to include Na, K, Cl, CO_2, BUN, Cr, Ca, Mg, and Phos, within 2 weeks of the start of conditioning
    l. Serum pregnancy qualitative testing within 2 weeks of the start of conditioning in females of childbearing age.

3. Other evaluations:
    a. Echocardiogram within one month of the start of conditioning.
    b. Pulmonary Function test (patients > 6 years if considered developmentally capable of PFTs by attending physician and PFT lab technician) or documented O_2 saturation on room air.

10C. <u>Patient post transplant evaluation guidelines</u>

See Standard Practice Manual for standard evaluation procedures during the first 100 days post transplant, evaluation prior to departure and long-term follow-up. Post transplant evaluation prior to day 80 will in most cases be performed whilst the patient is under the care of the SCCA pediatric BMT team. After day 80 evaluations may be performed under the supervision of the SCCA pediatric BMT team and/or the patient's

local physician. Specific recommendations (which may be modified by the clinical team as deemed clinically appropriate) include:

1. It is recommended that bone marrow aspiration is performed at baseline and on approximately days +28 and between approximately day +80 and + 100. Bone marrow aspiration may also be performed at approximately one year after transplant. Additional bone marrow aspirations and/or biopsies may be performed at the discretion of the treating physicians.
2. It is recommended that donor and recipient chimerism of $CD3^+$ and $CD33^+$ subsets in the peripheral blood is evaluated at approximately day +28 +56, and +80 by STR polymorphism. Peripheral blood chimerism studies are also recommended to be performed at approximately 6, 9 and 12 months post transplant.
3. It is recommended that quantitative immunoglobulins (to include IgG, IgA and IgM) are assessed at approximately days +28, +56 and +80 and if possible on approximately day +180, +270 and +360.
4. CMV, EBV and Adenovirus monitoring is recommended for all patients. Specifically it is recommended that:
   a. CMV PCR should be performed at least once every week until day 180 according to institutional standard practice. Preemptive antiviral therapy should be instituted for a positive PCR according to institutional guidelines for recipients of T cell depleted allogeneic HCT (or an IRB approved research protocol for CMV management). CMV PCR monitoring is suggested to continue for at least one year beyond transplant for patients who have had CMV reactivation in the first 100 days and/or have active GVHD requiring steroids or other agents.
   b. Surveillance for EBV reactivation should be performed using a quantitative EBV DNA PCR assay approximately weekly until day 180. Infectious disease (ID) consultation should be obtained for patients who develop EBV DNA levels of >1000 copies/ml plasma on any test.
   c. Surveillance for Adenovirus reactivation should be performed using a quantitative PCR assay (including adenovirus AF, and BCDE serogroups) approximately weekly until day 180. ID consultation should be obtained for patients who develop Adenoviral DNA levels of >300 copies/ml plasma on any test.
   d. HHV6 monitoring by PCR may also be performed in the first 1-3 months after transplant at a frequency and for a duration determined by the patients attending physician as clinically indicated.
5. Research Tests (Also see appendix K for pediatric research blood draw guidelines)
   a. It is recommended that a 4ml peripheral blood sample is obtained (sent to SCCA/UWMC Hematopathology lab) for research flow cytometry for lymphocyte subset evaluation on approximately days +28, +56 and + 80 and if possible on approximately day +180, +270 and +360. Specifically, it is recommended that the following subsets are enumerated: $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, $CD4^+T_N$ ($CD3^+/CD4/CD45RA^+/ CD45RO^-/CD62L^+$), $CD8^+T_N$ ($CD3^+/CD8^+/CD45RA^+/CD45RO^-/CD62L^+$), NK ($CD3^-CD56^+CD16^+$), B cells ($CD19^+$), Tregs ($CD4^+/CD25^+/CD127^-$).
   b. It is recommended that peripheral blood (≤1ml/kg, maximum 50ml for a single draw) is obtained on days +28, +56, +80-100, +180, +270 and +360 for research tests that may include
      i. T cell responses to EBV and possibly to other pathogens performed using functional assays which may include ELISPOT, lymphoproliferation assays, intracellular cytokine staining, and/or tetramer analysis.

ii. T cell receptor excision circle (TREC) analysis
iii. TCR Vβ spectratyping or deep sequencing
iv. Additional lymphocyte subset analysis which may include detailed analysis of T regulatory cell subsets.

In smaller patients it may not be possible to perform all of the tests specified above due to the smaller recipient blood volume and the need to avoid contributing to anemia or hypovolemia. Blood samples for research testing will not exceed the volumes specified in Appendix K. Research samples may also be deferred or declined by the patient, their guardian or attending physician depending on the patient's clinical status and preferences. The research tests performed will be prioritized by the PI depending on the number of available cells and the patient's clinical course.

A summary of recommended patient evaluations post-transplant is provided in Table 4.

Table 4. Patient evaluations over the course of the study

Approximate days post transplant

Study Assessments/ Testing	Baseline	7	14	21	28	35	42	49	56	63	70	77	Between 80-100	180	270	360
History, physical exam, height[1] and weight	X				X				X				X	X	X	X
Karnofsky/Lansky performance status	X												X			X
Automated CBC with differential, platelet count	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Serum chemistry [2]	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
CMV PCR	X	X	X	X	X	X	X	X	X	X	X	X	Weekly to day 180			
EBV PCR	X	X	X	X	X	X	X	X	X	X	X	X	Weekly to day 180			
Adenoviral PCR	X	X	X	X	X	X	X	X	X	X	X	X	Weekly to day 180			
Quantitative Immunoglobulins for IgG, IgA and IgM					X				X				X	X	X	X
Quantitative blood lymphocyte subset research evaluation					X				X				X	X	X	X
Peripheral blood T cell & myeloid chimerism ($CD3^+$ & $CD33^+$)	X Baseline DNA				X				X				X	X	X	X
Bone marrow aspirate/biopsy[3]	X				X								X			X
Acute GVHD		X	X	X	X	X	X	X	X	X	X	X	X			
Chronic GVHD[4]													X	X	X	X
Skin biopsy[5]													X			
Oral medicine[5] consult													X			
Pulmonary function test including DLCO[5]	X												X			X
Research Tests[6]					X				X				X	X	X	X

Post transplant time points represent guidelines for performance of required evaluations. Due to numerous factors influencing scheduling (pt and provider availability, testing services limitations etc), variation in evaluation performance dates is anticipated and acceptable to the protocol (e.g., within +/- 7 days of time points < day 100; +/- 30 days for time points > day 100). There may also be variation in the content of the evaluation performed particularly beyond day 100 depending on the patient's health status, size, geographical location, physician and/or PI recommendations and unanticipated variables. In particular the timing of blood draws may be spaced out or some tests not obtained to ensure that excessive blood is not drawn from small children at any given time, or over a given period.

1. Height on baseline only
2. Serum Chemistry should include the following: Na, K, Cl, CO2, BUN, Cr, Ca, Mg, Phos, ALT, AST, and BILIT/D
3. Bone marrow aspiration; evaluation with **a.** histopathology **b**. flow cytometry **c.** cytogenetics and/or molecular testing where clinically relevant (FISH and PCR informative for disease status eg BCR-ABL for Ph+ ALL)
4. Chronic GVHD evaluation should be performed between approximately day 80 and 100, and on day 180, 270, 360 where possible. Day 180, 270, 360 evaluations should be performed at the SCCA if possible, or patients may have a 'home town evaluation' conducted by their regular medical provider with guidance by SCCA Pediatric long term follow-up (LTFU) as appropriate. Patients with symptoms or signs compatible with chronic GVHD between day 100 and day 360, based on report by the PMD and/or patient, should evaluated by the pediatric LTFU team at the SCCA or using telemedicine and/or medical photography in consultation with the patients regular medical provider as appropriate.
5. Optional test: May or may not be performed depending on the age and developmental stage of the child and the recommendation of the pediatric attending physician.
6. Research tests: In smaller patients it may not be possible to perform all of the tests specified in section 10C.6.due to the smaller recipient blood volume and the need to avoid contributing to anemia or hypovolemia. Blood samples for research testing will not exceed the volumes specified in Appendix K. Research samples may also be deferred or declined by the patient, their guardian or attending physician depending on the patient's clinical status and preferences. The research tests will be prioritized by the PI depending on the number of available cells and the patient's clinical course.

11. Drugs, Irradiation and PBSC Administration - Toxicities and Complications.
Note that for chemotherapy administered during conditioning and other drugs listed in this protocol, in keeping with institutional practice, it is acceptable to administer a dose within 10% of the protocol specified dose.

11A. Total Body Irradiation (TBI)

1. TBI will be given as 165 cGy fractions twice per day x 4 days (days -10 to -7) to all patients using the linear accelerator at a rate of 6-7 cGy/min. Dosimetry calculations will be performed by the radiation oncologist.
2. Toxicity:
   a. Myelosuppression is the major dose limiting toxicity.
   b. Erythema may occur in the first 24 hours.
   c. Hyperpigmentation may occur in the first month following TBI.
   d. Oral ulceration, anorexia, nausea, vomiting and diarrhea, fatigue and alopecia occur frequently.
   e. Parotitis
   f. Decreased production of saliva and tears
   g. Hepatic dysfunction and rarely liver failure.
   h. Late effects include cataracts, growth failure, gonadal failure and sterility, hypothyroidism, pulmonary dysfunction and secondary malignancies.

11B. Thiotepa

1. Dosage. Thiotepa will be administered in a dose of 5 mg/kg/day (adjusted body weight) IV over approximately 4 hours for 2 consecutive days (day -6 and day -5). The total dose is 10 mg/kg (adjusted body weight). If actual weight is *less* than ideal body weight, actual body weight will be used. Thiotepa is available in 15 mg vials and is reconstituted with sterile water resulting in an isotonic solution with 10 mg/ml of thiotepa.
2. Toxicity
   a. The major dose limiting toxicity of thiotepa is myelosuppression.
   b. Oral ulceration, anorexia, nausea, vomiting and diarrhea, fatigue and alopecia occur frequently.
   c. Occasionally patients develop a skin rash that involves darkening of the skin and peeling, particularly in the axillary and inguinal folds. Thiotepa is secreted in sweat, therefore the axillary and inguinal areas should be washed twice daily during administration and for 2 days after administration.
   d. Dizziness and headache
   e. Hepatic toxicity including elevation in bilirubin and transaminases, and hepatic damage can occur.
   f. Rarely reported toxicities include CNS toxicity with somnolence, confusion, seizures, forgetfulness and inappropriate behavior.
   g. Allergic reactions during infusion occur rarely.
   h. Late effects include sterility and secondary malignancies.

11C. Fludarabine

1. Dosage: Fludarabine will be administered in a dose of 25 mg/m^2/day (m^2 always based on actual body weight) IV over approximately 30 minutes for 5 consecutive days (day -6 to -2). The total dose of fludarabine will be 125 mg/m^2.
2. Toxicity

a. Immunosuppression is the major toxicity of fludarabine.
b. Oral ulceration, anorexia, nausea, vomiting and diarrhea, fatigue and alopecia occur frequently.
c. Myelosuppression (lymphopenia, granulocytopenia, and anemia) is common.
d. Numbness and tingling in hands or feet and visual changes occur and rarely somnolence, mental state changes, cortical blindness, coma and other neurotoxicity
e. Other reported toxicities include rash, hepatocellular toxicity, hemolytic anemia and interstitial pneumonitis.

11D. PBSC infusions

Refer to Appendix D for infusion of selected cells.

11E. Tacrolimus administration

1. Also see section 9F for information about tacrolimus administration and dosage adjustments.
2. Administration and dosage
   a. Intravenous dosing - The standard mode of IV administration is by continuous infusion over 22-24 hours. Tacrolimus should be initiated as an IV continuous infusion on day –1 at a dose of 0.03 mg/kg/day based on actual body weight. If actual weight is greater than ideal body weight, it is recommended to use adjusted body weight.
   b. Oral dosing – The oral formulation of tacrolimus is supplied as 0.5 mg, 1 mg or 5 mg capsules or as oral syrup (0.5 mg/ml).
   c. Conversion from IV to PO dosing of Tacrolimus. Patients should be converted to an oral dose at 4 times the IV dose to be given in divided (Q 12 hour) doses. Children < 6 years old may require oral dosing every eight hours to maintain target serum trough levels.
3. Toxicity

   Side effects are generally reversible and may include:
   a. Rise in serum creatinine, electrolyte wasting, hemolytic uremic syndrome, and renal failure.
   b. Nausea and vomiting and hepatic dysfunction.
   c. Hypertension,
   d. Increases in cholesterol and triglycerides
   e. Paresthesia, tremors, seizures, headache, insomnia, dizziness, depression, confusion, hallucinations, psychosis, myoclonus, neuropathy, agitation.
   f. Blurred vision, photophobia.
   g. Hirsutism

11F. Methotrexate administration

1. Also see section 9F for information about methotrexate administration and dosage adjustments.
2. Administration and dosage
   a. In patients weighing >10kg methotrexate should be administered by IV push on days +1, +3, +6 and +11 at a dose of 15 mg/m^2 (on day +1) and a dose of 10 mg/m^2 (on day +3, +6, +11). Calculation of m^2 should be according to institutional standard practice guidelines.
   b. Children weighing 10kg or less will have methotrexate dosed per kg rather than

per /m^2 and should receive 0.5mg/kg on day +1 and 0.33mg/kg on days +3,+6, and +11.

c. The first dose of methotrexate should be given at approximately 24 hours, but no sooner than 24 hours after completion of the cell infusion.
d. If clinically indicated methotrexate levels may be obtained and/or methotrexate dose adjusted according to institutional standard practice guidelines and/or leucovorin rescue may be considered. In the case of severe mucositis or in the presence of effusions, ascites, or another relative contraindication to methotrexate administration, the day 11 methotrexate may be withheld at the discretion of the attending physician.

3. Toxicity
   a. In this setting of HCT, mucositis is the primary toxicity related to methotrexate.
   b. If there is also renal dysfunction methotrexate clearance may be delayed.
   c. Methotrexate may cause an elevation in serum transaminases.
   d. Severe skin reactions after single or multiple doses of methotrexate have occasionally been reported.

12. Protocol Enrollment and Special Considerations

12A.

Projected Target Accrual
ETHNIC AND GENDER DISTRIBUTION CHART

TARGETED / PLANNED ENROLLMENT: Number of Subjects			
Ethnic Category	**Sex / Gender**		
	Females	**Males**	**Total**
Hispanic or Latino	0	0	0
Not Hispanic or Latino	8	12	20
Ethnic Category Total of All Subjects*	8	12	20
Racial Categories			
American Indian / Alaska Native	0	0	0
Asian	0	0	0
Native Hawaiian or Other Pacific Islander	0	0	0
Black or African American	0	0	0
White	8	12	20
Racial Categories: Total of All Subjects*	8	12	20

12B. Infection Prophylaxis

1) Antibiotics
   (a) Prophylactic antibacterial antibiotics should be used for all patients that develop neutropenia (ANC <500/ml) according to institutional standard practice guidelines or according to currently active protocols.
   (b) In order to prevent early *Streptococcus viridians* bacteremia all patients should receive additional prophylactic therapy. Patients should be started on oral penicillin daily beginning at day -2 until they are unable to take PO, when they should be moved to penicillin IV through day +10. Patients with a known allergy to penicillin should receive Vancomycin IV from day -2 through day +10. The recommended standard regimens are:
      (i) ***Standard:*** Penicillin VK 25mg/kg PO BID (maximum 1 gram PO BID) starting at day -2 until not able to take oral therapy and then IV Penicillin G 25,000U/kg IV every 6 hours (maximum one million units IV every six hours, 4 million units total/day) until day +10.
      (ii) ***If Penicllin Allergic***: IV Vancomycin 15 mg/kg IV every 12 hours from day -2 through day +10
   (c) Neutropenic fever: In patients with any active mucositis and neutropenia, IV Vancomycin (15 mg/kg IV at least every 12 hours) or an alternative antibiotic regimen covering streptococcus viridans should be given in addition to the other empiric neutropenic fever antibiotic therapy specified by institutional standard practice guidelines (e.g. Cefazidime). Prophylactic penicillin may be discontinued when vancomycin or an alternative anti-streptococcus viridans agent is initiated.
2) Prophylactic fluconazole or an alternative antifungal medication should be used from the first day of conditioning or sooner until day +75 post transplant in accordance with standard practice (or according to an IRB approved anti-fungal research protocol)
3) Preemptive therapy for CMV reactivation should be administered according to institutional standard practice guidelines for a T cell depleted allogeneic HCT (or according to an IRB approved CMV research protocol).
4) Infectious disease consultation should be obtained for patients who develop EBV DNA levels of >1000 copies/ml plasma on any test.
5) Infectious disease consultation should be obtained for patients who develop Adenovirus DNA levels of >300 copies/ml plasma on any test.
6) Patients should receive acyclovir or valacyclovir according to institutional standard practice guidelines as prophylaxis for HSV and VZV. Acyclovir or valacyclovir can be withheld during periods that the patient is receiving ganciclovir, foscarnet or other effective antiviral drug for management of CMV.
7) After engraftment and ≥20-30 days after HCT, patients may receive prophylaxis for pneumocystis carinii according to institutional standard practice guidelines.

12C. Ursodeoxycholic acid (UDCA) prophylaxis of hepatic complications of transplant

1. UDCA is recommended to prevent hepatic complications according to standard practice guidelines.

12D. Management of acute and chronic GVHD

1. Patients who develop ≥ grade II acute GVHD should be treated with systemic

corticosteroids and/or with beclomethasone or budesonide if clinically indicated. Patients failing initial therapy will be eligible for second line therapy. Second line immunosuppressant therapy includes but is not limited to sirolimus, mycophenolate mofetil, monoclonal antibodies, pentostatin, denileukin difitox, thalidomide and extracorporeal photopheresis, given on or off study protocols.

2. Patients who develop chronic GVHD and require systemic immunosuppressant therapy should be treated with immunosuppressive therapy according to institutional guidelines or may be treated on research protocols.

12E. Management of Delayed Engraftment

1. If the ANC has not reached 100 by Day 21, a bone marrow examination may be performed to ascertain cellularity. If the ANC is <100 on Day 21, G-CSF, GM-CSF or other appropriate cytokines may be utilized and chimerism studies should be performed. Care of patients that have continued poor graft function after cytokine administration or who reject their grafts after initial engraftment is at the discretion of the attending physician.

12F. Management of Relapse

1. Patients who are at high risk of relapse, have persistent leukemia or who relapse after transplant may be treated according to institutional practice which can include but are not limited to chemotherapy, small molecule inhibitors and/or donor lymphocyte infusions (DLI), or may be eligible for research protocols including immunotherapy with antigen-specific T cells.

13. Records

The medical record containing information regarding treatment of the patient will be maintained as a confidential document, within the guidelines of the Fred Hutchinson Cancer Research Center, Seattle Children's, the University of Washington Medical Center, the Seattle Cancer Care Alliance.
Each patient is assigned a unique patient number to assure patient confidentiality. Patients should not be referred to by this number, by name, or by any other individual identifier in any publication or external presentation. The Clinical Statistics Department maintains a patient database at FHCRC to allow storage and retrieval of patient data collected from a wide variety of sources. The licensed medical records departments, affiliated with the institution where the patient receives medical care, maintains all original inpatient and outpatient chart documents.

The primary research records will be contained and accessed through CORE, an encrypted, password-protected web site maintained by the FHCRC Clinical Research Data Systems division. Access is restricted to personnel authorized in writing by the FHCRC principal investigator.

Information gathered from this study regarding patient outcomes and adverse events will be made available to the Federal Drug Administration. All precautions to maintain confidentiality of medical records will be taken.

14. Evaluation and statistical considerations

14A. Type of study

This is a prospective phase II study of allogeneic stem cell transplantation in pediatric patients using PBSC that are selectively depleted of $CD45RA^+$ T cells.

14B. Definition of endpoints

1. Estimate the time to discontinuation of systemic immunosuppression in pediatric recipients of $CD45RA^+$ T cell-depleted PBSCT (a. including and b. excluding calcineurin inhibitors)
   a. Definitions:
      i. Time from transplant to the final discontinuation of all systemic immune suppression.
      ii. Time from transplant to the discontinuation of all systemic immune suppression with the exception of calcineurin inhibitors.
   b. Evaluation: The research team will follow the study participants via the database of the FHCRC long term follow-up program (LTFU) and the patient's primary care physician and/or oncologist as required to determine the final date of completion of immunosuppression as defined.
2. Graft failure
   a. Definition: Graft failure is defined operationally as:
      i. Failure to reach an ANC of >500/µl for 3 consecutive days by day 28
      ii. Irreversible decrease in ANC to <100 after an established donor graft: If the reduction in ANC is the result of relapse, as determined by histopathology, flow cytometry or molecular studies, this will not be considered graft failure. If there is a reasonable explanation, such as viral infection or drug effect that may be responsible for a reversible decrease in ANC, this will be not necessarily be considered graft failure.
   b. Evaluation: Engraftment endpoints will include:
      i. Time to ANC of >500/uL on the first of three consecutive days.
      ii. Time to ANC of >1,000/uL on the first of three consecutive test results
      iii. Time to platelet count >20,000/µL for 3 days without transfusion.
      iv. Time to platelet count >50,000/µL for 3 days without transfusion.
      v. It is recommended that chimerism analysis of CD3 and CD33 cells in peripheral blood be performed on or around day 28, 56, 80-100, 180, 270 and 360. Additional peripheral blood or marrow chimerism studies may be performed as clinically indicated.
3. Chronic GVHD
   a. Definition: Chronic GVHD will be diagnosed using NIH criteria outlined in Appendix F. Chronic GVHD will be defined operationally as the occurrence of compatible symptoms fulfilling NIH criteria and requiring treatment with systemic immunosuppression.
   b. Evaluation will be conducted by the FHCRC pediatric LTFU team at day 80-100 and where possible on approximately day 180, 270, 360 and yearly up to 5 years after HCT. The patient's primary care physician and/or pediatric oncologist will conduct clinical assessments between these time points as required.
      i. The primary chronic GVHD endpoint will be the occurrence of chronic GHVD meeting NIH criteria and requiring systemic pharmacological immunosuppression (systemic corticosteroids and/or other systemic agents, excluding calcineurin inhibitors which are generally not considered adequate as sole agents for the treatment of chronic GVHD)
      ii. A secondary chronic GVHD endpoint will be use of additional immune

suppressive agents other than first line therapy (first line therapy is considered prednisone and tacrolimus/cyclosporin).

c. If patients do not develop chronic GVHD after transplant but do relapse and then receive donor lymphocyte infusion or antigen specific T cells as treatment of relapse they will not be considered evaluable for the chronic GVHD endpoint.

4. Acute GVHD
   a. Definition:
      i. Acute GVHD will be diagnosed and graded using the clinical and laboratory criteria in Appendix E. Acute GVHD is defined operationally as the occurrence of compatible symptoms or signs in the skin, gastrointestinal tract, or liver prior to Day +100. In most cases histology of biopsy material of at least one involved organ will be used to confirm acute GVHD, but biopsy is not absolutely required for the diagnosis.
      ii. Steroid refractory GVHD will be defined by the decision of the attending physician to initiate secondary systemic therapy for acute GVHD due to concerns that corticosteroids are providing inadequate control of acute GVHD manifestations.
   b. Evaluation: During the inpatient stay, clinical evaluations will occur daily. During the outpatient stay, each patient should be evaluated at least weekly until departure. Clinical evaluations will be performed by an attending physician. Cutaneous, hepatic and gastrointestinal GVHD should be confirmed by biopsy except where medically contraindicated or considered medically unnecessary. An attending pathologist will interpret the biopsy material. The decision to initiate GVHD therapy will be made by the attending pediatric BMT physician.
      At the conclusion of the study, two pathologists with experience in GVHD will independently review the histology of biopsy specimens. An acute GVHD grade will be assigned for each patient by three experienced transplant physicians.
   c. Endpoints for acute GVHD that will be collected include:
      i. Presence of acute GVHD grades II-IV
      ii. Presence of acute GVHD grade III-IV
      iii. Presence of steroid refractory acute GVHD
5. Relapse
   a. Definition: Relapse is defined by the presence of malignant cells in marrow, peripheral blood, or extramedullary sites by histopathology. Minimal residual disease is defined as the presence of malignant cells in the marrow, peripheral blood, or extramedullary sites detectable only by molecular methods, cytogenetics, or flow cytometry, but not observed by histopathology.
   b. Evaluation: Testing for recurrent malignancy in the blood and bone marrow will be performed by monitoring the CBC and bone marrow as outlined in Table 3. Suspected extramedullary sites of recurrent disease may be evaluated by biopsy and/or lumbar puncture if clinically indicated.
6. Transplant related mortality (TRM). TRM is defined as mortality in any patient for whom there has not been a diagnosis of relapse.

14C. Statistical Analysis and Stopping Rules

1. Duration of immunosuppression
   The first primary objective is to estimate the time-to-discontinuation of systemic immunosuppression in pediatric recipients of $CD45RA^+$ T cell-depleted PBSCT. The

goal is to observe a statistically significant reduction in the time-to-discontinuation of systemic immunosuppression compared to the historical experience without an accompanying increase in the probability of graft failure.
Unpublished data from FHCRC shows that amongst 55 children with a history of leukemia who received a myeloablative bone marrow (N=34) or PBSC (N=21) transplant from an unrelated donor (HLA-A, B, C, DRB1, and DQB1 molecularly matched) following conditioning with TBI and cyclophosphamide between July 2002 and December 2010 the median time to discontinuation of all immunosuppression was 1076 days, with a median time to discontinuation of 608 days for bone marrow recipients, and median time to discontinuation not reached for recipients of PBSC. Based on these data, we shall use a median time to discontinuation of systemic immunosuppression of 608 days as our benchmark for comparison in the current trial. The goal is to increase the proportion of patients who discontinue all systemic immunosuppression from the historical value of 50% to 80% in the current trial. Evaluation of 20 patients will provide 90% power to observe a statistically significant difference between (the fixed probability of) 50% and 80% of patients discontinuing by 608 days (with a 1-sided significance level of 0.05).
Pediatric patients with HLA-identical related donors ≥18 years will also be eligible for enrollment on the protocol but only a few such patients are likely to be enrolled and GVHD may be less frequent and less prolonged in these patients. Therefore the time to discontinuation of immunosuppression data for the subgroup of pediatric patients with related donors will be analyzed separately.

2. Graft Failure
   Graft failure will be closely monitored throughout the study. A true probability of graft failure of 10% will be considered excessive. If there is sufficient evidence to suggest that the true probability of graft failure exceeds 10%, the study will be stopped. Sufficient evidence will be taken to be an observed rate whose lower one-sided 90% confidence limit exceeds 10%. Operationally this will occur if any of the following observed ratios occur: 2 of the first 5 or fewer, 3 of the first 11 or fewer, 4 of the first 18 or fewer, or 5 of the first 20 of fewer patients develop graft failure. If the true probability of graft failure is 0.05, the probability of stopping after 10 or 20 patients is approximately 0.03 and 0.04, respectively. If the true probability is 0.3, then the probability of stopping is approximately 0.67 and 0.88, respectively (probabilities estimated from 5,000 simulations).

3. Failure to deliver therapy
   If the $CD34^+$ stem cells cannot be delivered as planned for the first 2 patients enrolled, or for any other two consecutive patients, the study will be suspended for a period of time in order to optimize the procedure. This is defined as failure to deliver a minimum of 2.0×10^6 $CD34^+$ cells/kg recipient body weight. If an improved procedure is achieved, the study may be resumed.

4. Patient withdrawal from treatment or study
   All patients who receive infusion of PBSC with or without cell selection will be considered evaluable for study safety endpoints. If they received PBSC with cell selection they will be evaluable for study efficacy endpoints.

   When a patient withdraws consent from P2660 they will no longer have research

samples taken and the research team will no longer have access to clinical records to follow them for study endpoints unless they have signed the general FHCRC consent and authorization form allowing their leftover specimens and medical records to be used for research. If they have signed that consent then they will still be evaluable for clinical endpoints although research blood draws would be discontinued. If the patient refused to sign this general consent or withdrew general consent to the use of clinical data for research purposes then no further follow-up would be done.

15. Guidelines for Reporting and Tracking Events

15A. Toxicity Grading

Toxicities will be graded according to the current version of the NCI Common Terminology Criteria for Adverse Events (CTCAE) Version 4. The full text of the NCI CTCAE is available online at:

http://evs.nci.nih.gov/ftp1/CTCAE/About.html

15B. Definitions

Definitions associated with reportable events can be found on the FHCRC's Institutional Review Office (IRO) extranet website.

According to ICH guidelines (Federal Register. 1997; 62(90):25691-25709) and 21 CFR 312.32, IND Safety Reports, and ICH E2A, *Definitions and Standards for Expedited Reporting*, an adverse event is defined as follows:

An adverse event is any untoward medical occurrence in a clinical investigation subject administered a medicinal product and which does not necessarily have a causal relationship with this treatment. An AE can therefore be any unfavorable and unintended sign (including an abnormal laboratory finding), symptom, or disease temporally associated with the use of a medicinal product, whether or not considered related to the medicinal product.

Abnormal laboratory values for laboratory parameters specified in the study should not be recorded as an adverse event unless an intervention is required (repeat testing to confirm the abnormality is not considered intervention), the laboratory abnormality results in a serious adverse event or the adverse event results in study termination or interruption/discontinuation of study treatment.

Medical conditions present at screening (i.e., before the study treatment is administered) are not adverse events and should not be recorded on adverse event pages of the CRFs. These medical conditions should be adequately documented on the subject chart. However, medical conditions present at baseline that worsen in intensity or frequency during the treatment or post-treatment periods should be reported and recorded as adverse events.

15C. Tracking and reporting of events

Patients enrolled in this study are receiving treatments that are generally associated with high rates of "expected" adverse events (outlined in Appendix G as well as Section 11 of the protocol). The following events will be tracked and reported:

1. Non-hematologic adverse events assessed as Grade 3-5 per NCI CTCAE, expected or unexpected, from the start of study treatment (pre-transplant conditioning) through day 100 will be collected, with the exception of some abnormal lab values and medical conditions present at screening (as noted in section 15B).
2. Grade 3-5 Blood/Bone Marrow adverse events occurring between day 60 and day 100 will be collected.
3. Graft versus host disease assessment done as part of routine care will be reviewed approximately once weekly through day 100. Details about GVHD symptoms, diagnosis, treatment, and outcome will be collected. After day +100, GVHD data will be captured at day +180, +270, 1 year, and then yearly through 5 years after day 0 whenever possible.
4. Relapse, graft failure, and death data will be captured as they occur in the first 200 days post transplant for all patients, and from 200 days up to 5 years whenever possible.
5. All treatment related mortality attributed to the investigational cell product occurring in the first 100 days will be reported to the IRB and the FDA as stated in sections 15.D and E.
6. Grade 3-5 infusion reactions will be reported to the IRB and FDA as stated in sections 15.D and E.

15D. Reporting Requirement to FHCRC IRB

The Principal Investigator, study nurse, or coordinator shall submit to the FHCRC IRB reportable events according to current reporting policies as outlined in FHCRC IRB Policies for Reportable Events.

The FHCRC PI and research nurse will meet regularly and will together review all reported events that could potentially meeting reporting requirements. If the event meets FHCRC IRB current reporting obligations it will be sent to them.

All reportable events should be submitted on the relevant FHCRC Forms (URLs linking to the FHCRC IRO website are found in Table 5 FHCRC IRB Forms for Reporting).

Table 5. FHCRC IRB Policies for Reportable Events.

(Relevant FHCRC policies include, but are not limited to the following documents. Please also refer to the FHCRC IRO website.)

IRB Policy 2.6	Adverse Events and Other Unanticipated Problems Involving Risks to Subjects or Others	http://extranet.fhcrc.org/EN/sections/iro/irb/ae.html
IRB Policy 1.9	Noncompliance with the Office of the Director's Human Research Protection Program Policy	http://extranet.fhcrc.org/EN/sections/iro/irb/ae.html
IRB Policy 1.1	Reporting Obligations for Principal Investigators	http://extranet.fhcrc.org/EN/sections/iro/irb/policy/index.html
IRB Policy 2.2	Continuing Review	http://extranet.fhcrc.org/EN/sections/iro/irb/policy/index.html
IRB Policy 1.13	Investigational New Drugs (IND), Biologics and Investigational Device Exemptions (IDE)	http://extranet.fhcrc.org/EN/sections/iro/irb/policy/index.html

Table 6 FHCRC IRB Forms for Reporting

Adverse Event Reporting Form	http://extranet.fhcrc.org/EN/sections/iro/irb/forms/index.html
Expedited Reporting Form for Unanticipated Problems or Noncompliance	http://extranet.fhcrc.org/EN/sections/iro/irb/forms/index.html

15E. Reporting to the FDA

As a study conducted under IND (Investigational New Drug) regulations we will comply with the FDA regulations regarding safety reporting 21CFR312.32 including the following requirements:

1. A sponsor must promptly review all information relevant to the safety of the drug 21CFR312.32 (b).

2. A sponsor must notify FDA in an IND safety report of potential serious risks, as soon as possible but in no case later than 15 calendar days after the sponsor determines that the information qualifies for reporting under 21 CFR312.32 (c)(1). Information that is required to be reported includes, but is not limited to, a. Serious and unexpected adverse reactions and b. An increased rate of occurrence of serious suspected adverse reactions.

3. The IND safety report must be completed and sent to the FDA in a narrative format, on FDA Form 3500A, or an electronic format.

4. A sponsor must also notify FDA of any unexpected fatal or life-threatening suspected adverse reaction as soon as possible but in no case later than 7 calendar days after the sponsor's initial receipt of the information 21CFR312.32 (c)(2).

16. Data safety monitoring plan

16A. Monitoring the progress of trials and the safety of participants

The FHCRC PI/Sponsor is responsible for monitoring this clinical trial, with oversight by a Data and Safety and Monitoring Board (DSMB), the Data Safety Monitoring Committee (DSMC) and the IRB at the FHCRC. This is a Phase II study and the assessment of risk is considered above minimal. The PI reviews outcome data for each individual patient at approximately 3 and 12 months after HCT, at a minimum.

A DSMB will be in place to meet approximately every 6 months to review the data particularly as it relates to engraftment, grades III-IV GVHD and relapse. The DSMB confirms that the trial has not met any stopping rules and reviews any patient safety problems necessitating discontinuation of the trial. A report from the DSMB is submitted to the FHCRC IRB and the PI. The DSMB will discontinue the review of outcomes when all subjects on this trial have completed all protocol-specified follow-up.

P2660 DSMB Members

Name	Affiliation	Position
Eneida Nemecek, MD	Oregon Health & Sciences University	DSMB Chair
Mary Elizabeth Percival, MD, MS	Fred Hutch	DSMB Clinical Investigator
Rebecca Gardner, MD	Seattle Children's Research Institute	DSMB Clinical Investigator
Soheil Mesenchi, MD	Fred Hutch	DSMB Clinical Investigator

Vicky Wu, PhD	Fred Hutch	DSMB Biostatistician

All members will have experience in the management of patients with leukemia and myelodysplasia and in the conduct and monitoring of clinical trials. At least some of the members of the DSMB will have experience in the management of pediatric patients with leukemia and myelodysplasia.

The DSMC at FHCRC will review the progress of the protocol with respect to the monitoring plan at the time of each annual renewal. As with initial review, annual FHCRC IRB review and approval is also required.

A protocol monitor (from the FHCRC Clinical Research Support Office or an external monitor) will be retained to monitor study progress. The scope of monitoring will be based on the FHCRC/UW Data and Safety Monitoring Plan: http://centernet.fhcrc.org/CN/depts/iro/irb/dsm/ Per the DSMP subjects will be randomly selected for verification. An initial monitoring visit is expected within six months of enrollment of the first subject and preferred prior to enrollment exceeding 4 subjects as 100% verification is expected during an initial visit. Monitoring reports will be forwarded to the DSMB, and the Principal Investigator/Sponsor at FHCRC.

Flow of information concerning clinical trial participants originates with the clinicians and nurses in the clinic and is transmitted to the FHCRC Research Nurse. At the FHCRC health care providers and rotating attending physicians assess patients and record their observations regarding toxicity and response outcomes in the medical record. Thus, multiple health care providers provide independent observations and participate in monitoring this trial. The PI may be a clinician for some patients entered on this trial. However, assessments are the sum total of the multiple clinicians involved with the patient averting possible conflict of interest having the PI as the attending clinician for protocol patients. If determination of adverse events is controversial, co-investigators will convene on an ad hoc basis as necessary to review the primary data and render a decision.

16B. Plans for assuring data accuracy and protocol compliance

The study has a research nurse that follows patients to confirm eligibility, reporting of adverse events, reporting of events which are part of the safety-monitoring plan, and protocol adherence. The PI and research nurse are responsible for review and maintenance of all patient research records to ensure data integrity and protocol adherence.

Health care providers and rotating attending physicians assess patients and record their observations in the medical record. This documentation is extracted by the site's research staff by approximately day 100 (and no later than day +130) and again at approximately 1 year (no later than 15 months) after HCT via chart review and entered into electronic protocol specific Case Report Forms (CRFs). The principal investigator will review the CRFs and the primary source documents verifying by signature (electronic) their data accuracy.

The study is monitored under the FHCRC Monitoring Plan. The FHCRC Data and Safety Monitoring Plan details the full scope and extent of monitoring and provides for immediate action in the event of the discovery of major deviations.

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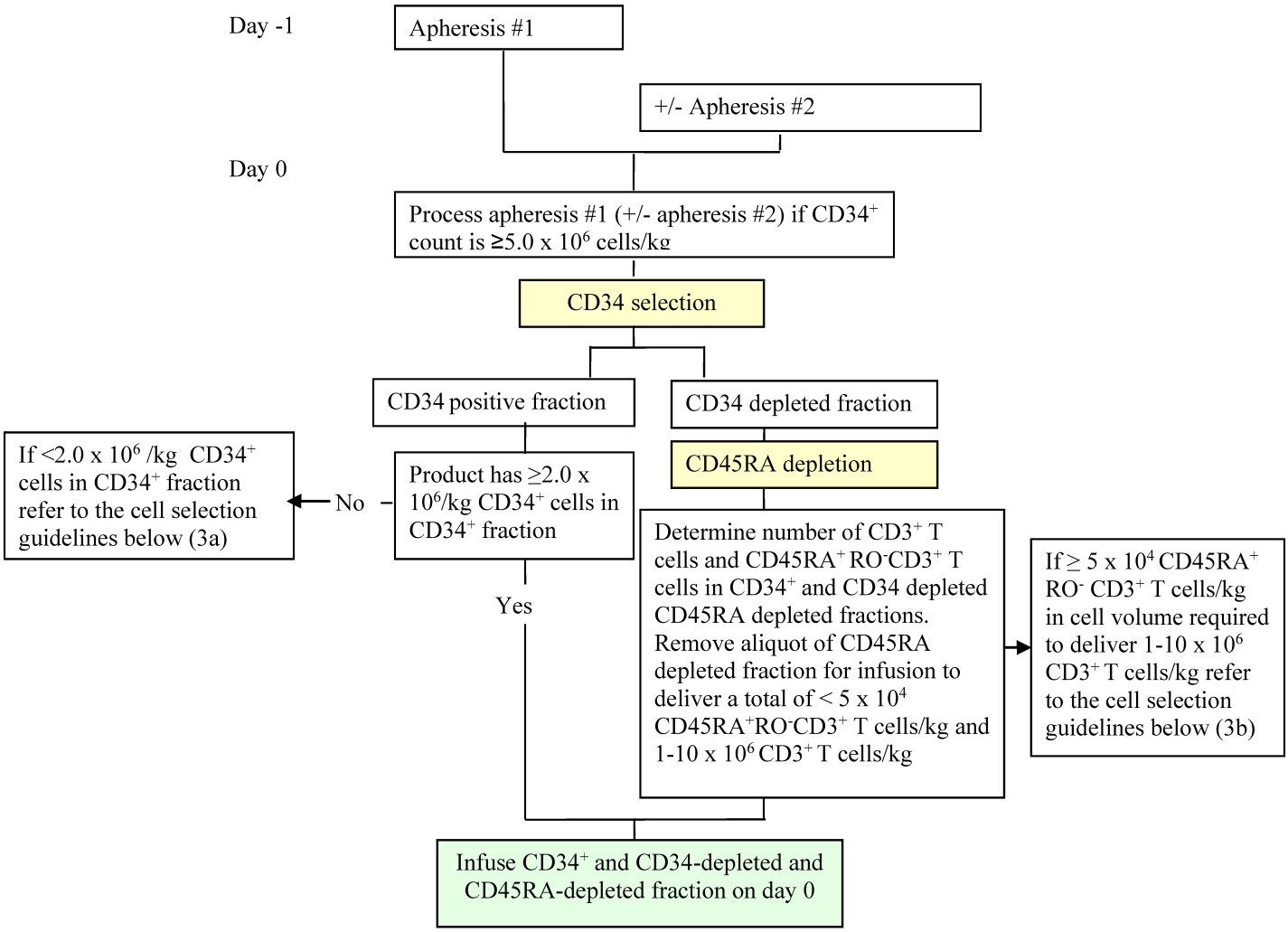
APPENDIX A
Cell Selection Flow Sheet



Based on prior experience with cell selection of PBSC, we expect the following.

- A starting total CD34 cell dose of ≥10x10^6 cells/kg of recipient body weight will be available in ≥ 90% of donors and a total CD34$^+$ cell dose of >2.0x10^6 cells/kg of recipient body weight (and in most cases >5.0x10^6 cells/kg) will be obtained following CD34 positive selection from an apheresis product containing 10 x10^6 CD34$^+$cells/kg. We expect to infuse 2-10 million CD34$^+$ cells/kg in most cases, and plan to limit the infused CD34$^+$ cell dose to no more than approximately 10 million CD34$^+$ cells/kg in most cases.
- The number of residual donor CD45RA$^+$RO$^-$ CD3$^+$ T cells in the CD34 selected product that will contribute to the overall dose of CD45RA$^+$RO$^-$ CD3$^+$ T cells will be <1 x10^4/kg of recipient body weight.
- Depletion of CD45RA$^+$ cells from the CD34 negative fraction will yield sufficient T cells to administer 10 x10^6 CD3$^+$ cells/kg and <1x10^4 CD45RA$^+$ RO$^-$ CD3$^+$ cells/kg of recipient body weight for >95% of patients.
- The *maximum* number of CD45RA$^+$ RO$^-$ CD3$^+$ cells anticipated in the sum of the CD34 selected product and the CD45RA depleted product will be 5 x 10^4 CD45RA$^+$RO$^-$CD3$^+$ T cells/kg and the number infused is very likely to be <2x10^4 CD45RA$^+$ RO$^-$ CD3$^+$ cells/kg.

<u>Cell Selection Guidelines (also see apheresis collection guidelines, below)</u>

1. We will perform cell selections only when the PBSC product from days -1 or day -1 and 0 combined contains ≥5x10^6 CD34$^+$ cells/kg of recipient body weight. If the PBSC product from days -1 and 0 combined contains <5x10^6 CD34$^+$cells/kg of recipient body weight the PBSCs will be infused without any cell selection and the patients will not be included in the analysis of study efficacy end points.

2. We will also perform cell selection only when the T cells in the PBSC product include a distinct $CD45RO^+$, $CD45RA^-$ population observed by flow cytometry. ≤5% of donor products are expected to have a CD45 variant in which all the T cells express CD45RA and there is therefore no $CD45RO^+$, $CD45RA^-$ population. If there is no $CD45RO^+$, $CD45RA^-$ population the PBSCs will be infused without any cell selection and the patients will not be included in the analysis of study efficacy end points.
3. Situations may arise where the goals for cell selection are not achieved with cell processing. These very unusual situations will be discussed with the protocol PI and a recommendation made. General guidelines are as follows:
a) *If* a total CD34 cell dose of $\geq 2.0 \times 10^6$ cells/kg is *not* achieved after the processing the apheresis collection(s) all available $CD34^+$ cells will be administered to the patient as soon as possible and an additional apheresis collection will be requested and the un-manipulated G-PBSC product will be administered to the patient without any cell selection procedure. These patients will not be included in the analysis of study efficacy end points.
b) *If* $< 5 \times 10^4 CD3^+$ $CD45RA^+$ RO^- cells/kg in the sum of the $CD34^+$ fraction and CD45RA depleted fraction cannot be achieved by simply reducing the number of $CD3^+$ and/or $CD34^+$ cells within the target range (minimum of 2.0×10^6/kg $CD34^+$ cells and a range of 1-10 x 10^6/kg total $CD3^+$ T cells) as described above, the following steps may be required:
   i. Repeat the CD45RA depletion of the CD34 depleted fraction using a second magnetic column.
   ii. If 1×10^6 $CD3^+$ cells/kg and $<5 \times 10^4 CD3^+CD45RA^+$ RO^- cell/kg cannot be achieved by i. then the patients should receive the CD34 selected product (minimum of 2.0×10^6 $CD34^+$ cells/kg) and may receive an aliquot of the $CD3^+$ cells (i.e. less than 1×10^6 $CD3^+$ cells/kg) containing $<5 \times 10^4 CD3^+CD45RA^+$ RO^- cell/kg. These patients who do not meet the goals for cell selection will not be included in the analysis of study end points. Patients will receive immunosuppression as described in the protocol unless they receive only the $CD34^+$ selected product and $< 1 \times 10^5$ $CD3^+$ cells/kg in which case they will receive no immunosuppression.
3. In a typical case we expect to a. perform cell selections on the day -1 apheresis collection (unrelated donors, some related donors), or a product pooled from the day -1 and day 0 collection (some related donors), b. to use a portion of the apheresis collection(s) containing at least 8-10 x 10^6 $CD34^+$ cells/kg for the cell selections, c. to reserve at least 3-5 x 10^6 $CD34^+$ cells /kg as a back-up for infusion of unselected PBSC into the patient if unanticipated problems arise in the cell selection procedure, and d. (for URD recipients) to cryopreserve at least 1×10^7 $CD3^+$ cells/kg for potential future donor lymphocyte infusion (DLI). We also plan to cryopreserve any available additional unmanipulated PBSC left over from the cell processing requirements as a back-up in the unlikely event of graft failure. In the unlikely event that there are insufficient unmanipulated PBSC to cryopreserve as DLI for URD recipients we may instead cryopreserve aliquots of the CD34-depleted fraction to be used as DLI.

<u>Apheresis Collection Guidelines</u>
Collection of unrelated donor GCSF mobilized apheresis products will be managed by the NMDP (US donors) or DKMS (German donors). Collection of related donor GCSF mobilized apheresis products will be managed by the SCCA apheresis center. The following represents a general guideline
Unrelated donors
1. A large volume apheresis collection (processing approximately 24L of blood) will be performed on day -1 and transported as soon as possible on day -1.
Related donors
1. A large volume apheresis collection (processing approximately 18L of blood) will be performed on day -1. The CD34 content will be evaluated on day-1.
   a) If ≥ 15 million CD34 cells/kg have been obtained from the day -1 no further apheresis is required. Cell processing can commence on day 0 using 10 million CD34 cells/kg with 5 million CD34 cells/kg held in the event of cell processing problems, and cryopreserved if not required.
   b) If <15 million CD34 cells/kg have been obtained from the day -1 collection a second collection will be obtained on day 0 to a maximum of 18L.
      i. The second collection may be totaled with the day -1 apheresis for a pooled or two separate cell selection(s) on day 0 and/or day +1 (e.g. if the day -1 apheresis was ≤ 8 x 10^6 CD34/kg and the total of the day-1 and 0 apheresis is ≥ 5 x 10^6/kg)
      ii. The second collection may also be held as a back-up in case of unanticipated problems in the cell selection procedure of the first apheresis and subsequently cryopreserved if not required for cell selection.

On very rare occasions the minimum $CD34^+$ cell target number of 2×10^6/kg may not be achieved before or after cell processing and an additional donor apheresis collection may be requested and infused without cell selections.

APPENDIX B
Product Testing

The CD34-enriched and CD45RA-depleted cells, because they are derived solely as the result of a cell selection process with no culturing required, will be considered as having undergone "minimal manipulation". Each of the CD34-enriched and CD45RA-depleted cell batches will be tested for safety, purity and potency, identity and stability as indicated below. In general, once myeloablative therapy has been initiated, the patient must receive the CD34-selected PBSC product, to reconstitute his/her hematopoietic system.

a) Safety: Samples from the final products ($CD34^+$ and $CD45RA^-$) will be sent for sterility testing for bacterial and/or fungal contamination of products according to methods specified in 21 CFR 610.12. The tests will be performed in clinical laboratories. If positive cultures are found organism species identification will take place. FHCRC has defined procedures and action plans in place to notify appropriate personnel and take appropriate measures if positive cultures are detected after infusion has taken place.

b) Purity and potency:
$CD34^+$ product: Viability will be determined by flow cytometry using exclusion of propidium iodide or 7-AAD with a notification specification set of <70% viable cells (i.e. If there are <70% viable cells in the $CD34^+$ product the PI will be notified). Quantitation of $CD34^+$ cells will be performed by flow cytometry, with a notification specification of <70% final purity and $CD34^+$ cell dose of <= 2.0 x 10^6 $CD34^+$ cells/kg. Quantitation of residual naïve T-cells ($CD3^+CD45RA^+CD45RO^-$) and total T cells ($CD3^+$) will be performed by multi-color flow cytometry, however no specification will be set for these cell subsets other than the total dose indicated below.

$CD45RA^-$ product: Viability will be determined by flow cytometry using exclusion of propidium iodide or 7-AAD, with a notification specification set of <70% viable cells. Quantitation of residual naïve T-cells ($CD3^+CD45RA^+CD45RO^-$), and total T cells ($CD3^+$) will also be performed by multi-color flow cytometry, The aim is to infuse a maximum of 5 x 10^4 $CD3^+CD45RA^+RO^-$/kg and a range of 1-10 x 10^6 $CD3^+$ cells/kg in the sum of the CD45RA-depleted and $CD34^+$ cell products and a minimum of 2.0 x 10^6 $CD34^+$ cells/kg.

c) Identity: Proper identification of all the intermediate and final cell products will be assured by defined process and label controls as specified in standard operating procedures within the cell processing facilities to ensure the recipient is receiving the correct cell products.

d) Stability: The first leukapheresis collection will be transported and stored for a total of up to 48 hours from the end of apheresis collection at 4C prior to CD34 selection and CD45RA depletion. Unmanipulated leukapheresis products are routinely stored this duration and longer, particularly in matched unrelated donor transplantation, and we therefore do not anticipate that stability will be a major concern for this study. Viability, which is an important measure of stability, will be assessed immediately after all processing is completed, and infusion of the $CD34^+$ cells and CD45RA-depleted cells will take place within 6 hours after completion and release of the cell products.

APPENDIX C
Positive Culture from Donor Product Action Plan

In the event that a sterility-testing culture sample turns positive (after the infusion has taken place), the following actions will take place immediately:

1. The director or designee of the Cell Therapeutics Laboratory will notify the recipient's attending physician and the Principal Investigator or the designated study nurse of the positive culture result.
2. Identification of the organism and sensitivity testing will be completed.
3. The FHCRC PI /Sponsor will notify the FHCRC IRB, as soon as possible, but no later than 10 calendar days of finding out of the event and will notify the FDA as outlined in section 15.
4. After notification of the attending physician, the following actions will take place.
    a. The attending physician will notify the patient (recipient) of the positive culture.
    b. The attending physician or primary care provider will perform a thorough examination of the patient (recipient).
    c. Blood samples will be obtained from the patient (recipient) for cultures (bacterial, fungal, and viral cultures).
    d. Samples of other fluids will be obtained for cultures if clinically indicated.
    e. Assays for identification of the organism and sensitivity testing will be completed and results reported to the attending physician.
5. The patient will be treated with empiric antimicrobial agents until the following endpoints are reached:
    a. Assays for identification and sensitivity of the organism have been completed.
    b. Results of the patient (recipient) blood cultures are available.
    c. The absolute neutrophil count exceeds 500 cells/µl.
    d. Unexplained clinical signs or symptoms of systemic infection have resolved.
    e. Exceptions to the administration of antibiotics must be based upon lack of criteria 5 a-d. These exceptions must be discussed by the attending physician with the PI
6. The patient will be monitored daily for signs of systemic infection.
7. Quality Assurance measures will be enacted.

APPENDIX D
Infusion of Selected Cells

General Guidelines
The selected cells ($CD34^+$ and CD34 depleted CD45RA depleted fractions) will be infused through a blood administration set filter. The cells will be suspended in clinical grade Normosol-R (Hospira) plus HSA (Baxter) for infusion. Central venous access is preferred for infusion. A peripheral IV line may be used only if central access is not available. *Under no circumstances will these cells be irradiated*. The staff caring for the patient must be familiar with the practices and complications of HPC infusion. Staff should be prepared to treat the recipient for an acute hemolytic transfusion reaction.

Questions regarding ABO mismatches should be directed to the SCCA Transfusion Services Office or the SCCA Transfusion Medicine attending on call. Other questions regarding handling of selected cells should be directed to the Medical Director of the Cellular Therapy Laboratory.

Timing
After completion of the conditioning regimen cells should not be infused earlier than 36 hrs after the last dose of chemotherapy. Selected cells will be infused as soon as they are available. The $CD34^+$ fraction will be given first followed as soon as possible, by the CD34-depleted CD45RA-depleted fraction. If a delay is anticipated because of timing of conditioning regimen or patient medical status as determined by the attending physician, cells may be stored in the refrigerator (4°C). It is aimed to commence administration of each fraction within 6 hours from the end of processing of that fraction.

Volume
The $CD34^+$ cell volume will be approximately 50ml or less, and the CD34-depleted, CD45RA-depleted cells will also be approximately 50ml or less.

Pre-medication
No pre-medication will be given unless patient has previously reacted to blood or platelet transfusions, then premedication will be administered as per patient's transfusion guidelines for allergic or febrile reactions.

Filtration
Products should be filtered through a blood administration set.

Product Infusion Rate
Begin at 0.5 times maintenance rate for 15 minutes, then increase to 1.5 times maintenance rate as tolerated.

Monitoring
Vital signs before infusion, after 15 minutes, then hourly during infusion, and at completion of infusion. If any reaction occurs, notify primary care provider.

Concomitant Infusions:
No medications or fluids may be given "piggy-back" with the selected cells, although they may be given through the other lumen of a double lumen catheter. Amphotericin, antibodies, investigational medications or blood products should not be given concomitantly because of difficulty in evaluating reactions. Cells must not be infused during plasmapheresis or dialysis.

Reactions:
Volume Overload: Generally, the $CD34^+$ fraction and CD34 depleted CD45RA depleted cell volumes will be 50 ml each so volume overload is not expected. Volume reduction may be required if the cell volume of either individual infusion exceeds 15 ml/kg recipient weight (highly unlikely), or if the patient is volume overloaded by clinical criteria. Request volume reduction by contacting the Cellular Therapy Laboratory.

Transfusion Reaction: Recipients who have clinically significant antibodies to AB or other antigens found on donor red cells may be at risk for an acute hemolytic transfusion reaction (i.e. major mismatch). Red cell depletion (or recipient plasma exchange) may be indicated in some cases. Patients also may experience hemoglobinuria from damaged cells in the cell inoculum. Delayed hemolysis may occur in the setting of major mismatch and rebound of recipient antibodies after plasmapheresis (if performed). **Refer to the Clinical Coordinator's Patient Information Sheet for instructions regarding red cell reduction of product (or plasma exchange of recipient). The SCCA Transfusion Services Offices may also be consulted as a resource for management of specific patients.**

Patients with minor mismatch (i.e. donor antibodies against patient red cells) may experience hemolytic transfusion reactions. Circulating donor antibodies will be removed during processing of the manipulated cells by the Cellular Therapy Laboratory so early hemolytic transfusion reactions are unlikely. Delayed reactions can occur about 5-14 days after marrow infusion. Delayed reactions may occur from formation of antibodies by donor lymphocytes against either recipient or incompatible transfused red cells (see Red Blood Cell Infusion Guidelines).**The SCCA Transfusion Services Office may be consulted as a resource for management of specific patients.**

Allergic Reaction: Recipients may have allergic reactions (chills, fever, hives) to the selected cell product. Please note, these products have been manipulated in the laboratory and may contain foreign proteins or reagents. Treatment is the same (diphenhydramine, meperidine, hydrocortisone) as for reactions to platelet transfusions. For anaphylaxis, treat per Institutional standard practice Guidelines, “Anaphylaxis Emergency / Drug Chart Reference”.

Pulmonary Micro-Embolism: Fat and particulates may result in micro-emboli. Patients may complain of chest pain, dyspnea, or coughing. Excessive fat is highly unlikely in the selected cell products but can be removed by centrifugation by the Cellular Therapy Laboratory after discussion with the Medical Director. Slowing of infusion and administration of oxygen may alleviate mild dyspnea during infusion.

Excessive Anti-coagulation: Selected cells will not be anticoagulated with heparin and/or citrate solutions. When heparin is used, rapid or large volume infusions may result in transient anti-coagulation of the recipient.

APPENDIX E
Acute GVHD Staging and Grading Assessment
GRADING OF ACUTE GRAFT-VERSUS-HOST DISEASE[a]

Severity of Individual Organ Involvement		
Skin	+1	a maculopapular eruption involving less than 25% of the body surface
	+2	a maculopapular eruption involving 25-50% of the body surface
	+3	generalized erythroderma involving >50% of the body surface
	+4	generalized erythroderma with bullous formation and often with desquamation
Liver	+1	bilirubin (2.0-2.9 mg/100ml)
	+2	bilirubin (3-5.9mg/100ml)
	+3	bilirubin (6-14.9mg/100ml)
	+4	bilirubin > 15mg/100ml
Gut	Diarrhea is graded +1 to +4 in severity. Nausea and vomiting and/or anorexia caused by GVHD is assigned as +1 in severity. The severity of gut involvement is assigned to the most severe involvement noted. Patients with visible bloody diarrhea are at least stage +2 gut and grade +3 overall	
Diarrhea	+1	≤ 1000 ml of liquid stool/day* (≤ 15ml of stool/kg/day)†
	+2	>1,000 ml of stool/day* (> 15ml of stool/kg/day)†
	+3	>1,500 ml of stool/day* (> 20ml of stool/kg/day)†
	+4	2,000 ml of stool/day* (≥ 25ml of stool/kg/day)†

**In the absence of infectious/medical cause*
†For pediatric patients

Severity of GVHD	
Grade I	+1 to +2 skin rash
	No gut or liver involvement
Grade II	+1 to +3 skin rash and/or
	+1 gastrointestinal involvement and/or +1 liver involvement
Grade III	+4 skin involvement and/or
	+2 to +4 gastrointestinal involvement and/or
	+2 to +4 liver involvement with or without a rash
Grade IV	Pattern and severity of GVHD similar to grade 3 with extreme constitutional symptoms or death

a. From "Graft-vs-host disease" Sullivan, Keith M. *Hematopoietic Cell Transplantation* Ed: D. Thomas, K. Blume, S. Forman, Blackwell Sciences; 1999, pages 518-519.

APPENDIX F
GRADING OF CHRONIC GRAFT-VERSUS-HOST DISEASE


Chronic GVHD
Guidelines


Chronic GVHD
Appendix D

APPENDIX G
Potential Adverse Events Associated or Expected with Hematopoietic Cell Transplantation

1. Opportunistic infections, including viral and fungal infections, can results in severe pulmonary, neurologic, hepatic and other organ dysfunction, and possible death.

2. Gastrointestinal toxicity. Nausea and vomiting can be anticipated during the entire course of ablative therapy. Mucositis and diarrhea should be expected. Prednisone can cause GI bleeding.

3. Cardiac toxicity. Cardiotoxicity (congestive heart failure, pericardial effusion, EKG changes) is uncommonly associated with chemotherapy agents and TBI and these sequelae may prove lethal.

4. Pulmonary toxicity. Diffuse interstitial pneumonitis and diffuse alveolar hemorrhage of unknown etiology occurs with some regularity after BMT. Interstitial fibrosis occurs less frequently. Each are well-described complications of intensive chemotherapy and TBI regimens and may prove lethal.

5. Hepatic toxicity. Veno-occlusive disease of the liver is a common toxicity of high-dose chemoradiotherapy and may result in death. Tacrolimus may cause elevation of ALT/AST.

6. Renal dysfunction. Chemoradiotherapy may uncommonly cause renal dysfunction. More commonly, nephrotoxicity results from tacrolimus and generally responds to dose reduction. Rarely, idiopathic or calcineurin inhibitor-associated hemolytic-uremic syndrome may occur and may be progressive and fatal. A syndrome of moderate renal insufficiency and hemolysis has been seen 5-7 months post HSCT after intensive conditioning plus TBI.

7. Hemorrhagic cystitis, manifested either as gross or microscopic hematuria, is a common toxicity after high-dose chemoradiotherapy, but usually associated with regimens that include cyclophosphamide. Hemorrhagic cystitis may predispose to a long-term increased risk of bladder cancer.

8. Central nervous system toxicity. Radiation and chemotherapy can cause CNS toxicity, including seizures, depressed mental status, or leukoencephalopathy. Calcineurin inhibitors can cause seizures or other CNS toxicity.

9. Marrow aplasia. Severe neutropenia, thrombocytopenia, and anemia, is expected to occur for a period of 7 to 42 days following infusion of marrow. Transfusion of platelets and red blood cells is expected as supportive care. Transfusion of blood products may be associated with acquisition of HIV or a hepatitis virus. Neutropenia may increase the risk for acquiring serious infection. Thrombocytopenia may increase the risk of life-threatening hemorrhage. Hemorrhagic or infectious complications during the expected period of aplasia may result in death. Primary and secondary graft failure occur uncommonly.

10. Miscellaneous. Alopecia and sterility are expected complications of the program as a whole. Cataract development is possible after TBI and/or steroids. Deficiencies of growth hormone, thyroid hormone, and sex hormones are possible after TBI. Calcineurin inhibitors can cause transient gingival hyperplasia, tremor, seizure, hypertension, headache, dysesthesia, metabolic complications and hirsutism. Steroid therapy can also contribute to fluid retention, easy bruising, hypertension, aseptic necrosis of bone, metabolic complications including diabetes mellitus and increased susceptibility to infection. Hospitalization during conditioning and recovery period is expected to be 5-9 weeks in duration.

APPENDIX H
Karnofsky/Lansky Performance Status Scale

KARNOFSKY PERFORMANCE STATUS SCALE (RECIPIENT ≥16 YEARS)

Percentage	
100	Normal, no complaints, no evidence of disease
90	Able to carry on normal activity; minor signs or symptoms of disease
80	Normal activity with effort; some signs or symptoms of disease
70	Cares for self; unable to carry on normal activity or do active work
60	Requires occasional assistance, but is able to care for most of his/her needs
50	Requires considerable assistance and frequent medical care
40	Disabled; requires special care and assistance
30	Severely disabled, hospitalization indicated. Death not imminent
20	Very sick, hospitalization necessary, active supportive treatment necessary
10	Moribund, fatal processes, progressing rapidly
0	Dead

LANSKY PERFORMANCE STATUS SCALE (RECIPIENT <16 YEARS)

Percentage	
100	Fully active
90	Minor restriction in physically strenuous play
80	Restricted in strenuous play, tires more easily, otherwise active
70	Both restrictions of and less time spent in active play
60	Ambulatory up to 50% of the time, limited active play with assistance/supervision
50	Considerable assistance required for any active play, fully able to engage in quiet play
40	Able to initiate quiet play
30	Needs considerable assistance for quiet activity
20	Limited to very passive activity initiated by others (e.g. TV)
10	Completely disabled, not even passive play
0	Dead

APPENDIX I
Weight / Adjusted Body Weight for Drug Dosing

Drug Dosing By Body Size:
Drug dosing will be based on either body surface area (BSA) or body weight.
1. BSA is calculated in M^2. The formula by definition adjusts for both under and over-weight individuals.
The formula for this calculation is:

$$\frac{\sqrt{\textbf{actual*}\text{ weight in kg x height in cm}}}{60}$$

BSA calculations used in this protocol are based on **actual** body weight for all individuals, not on adjusted weight.

2. Body weight is measured in kg. Body weight may be adjusted in patients who are above ideal body weight.

Calculating adjusted weight. Calculating adjusted weight requires determining an ideal body weight first.

The ideal body weight (IBW) will be calculated in the following ways

*For Pediatric Patients**:***
Pre-pubertal children (females < 12 years old and males < 14 years old):
Ideal weight will be calculated by matching weight at the 50th percentile for length-for-age or height-for-age at the 50th percentile.

Post pubertal adolescents (females > 12 years old and males > 14 years old):
Ideal weight will be assessed using the body mass index (BMI) [weight in kg/height in meters squared)]. If the child's normal BMI is between the 25-75th percentile, the child may be considered at IBW.

Adolescents whose BMI exceeds the 75th percentile: Ideal weight will be the 75th percentile BMI weight.

Adolescents whose BMI is below the 25th percentile: Ideal weight will be the 25th percentile BMI weight

Note: When deviating from these age ranges (based on early or late maturity), the dietitian will document the rationale in the nutrition assessment.

Weight / Adjusted Body Weight for Drug Dosing

Adjusted Body Weight will be calculated as follows:
Ideal Weight + 0.25 (actual weight – ideal weight)

Weight Shifts After Initial Evaluation:
Individuals with significant weight shifts after the initial evaluation will have the adjusted body weight reassessed by the Clinical Nutrition Staff as appropriate.

APPENDIX J
Radiotherapy Treatment Guidelines

1.1 Total Body Irradiation

1.1.1 Patients
Every patient will receive total body irradiation as part of the preparatory regimen for stem cell transplantation. The treatment dose regimen will be 165 cGy BID over 4 treatment days to a total dose of 1320 cGy.
Lung shielding will be used as described below.
All male patients having ALL will receive a testicular boost as part of the transplant regimen. 400 cGy for one fraction will be delivered by an en fosse electron field. For patients with a history of CNS leukemia a CNS irradiation boost may also performed if it is clinically indicated in the opinion of the pediatric BMT physician and radiation oncologist.

1.1.2 Equipment

1.1.2.1 Modality:
High-energy photons with energy ≥ 6MV photons should be utilized. Although there is no upper limit on the energy as long as the skin dose requirements can be met, it is recommended that 18MV or lower be used.
The selection of energy is determined by the dose uniformity criterion.

1.1.3 Target Volume
The total body will be treated, including the head and feet, in one field (except in certain circumstances). Care should be taken to insure that the patient is entirely within the 90% isodose decrement line of the beam (i.e., not in the penumbra region of the beam).

1.1.4 Target Dose
The prescription point is defined as the point along the longitudinal axis of the patient at the midline at the level of the umbilicus (see **Point 5**, section 1.5.4.1). No tissue inhomogeneity correction will be made in the calculation of dose to the prescription point. The absorbed dose along the patient's head to toe axis (line formed by the intersection of the midsagittal plane and the midcoronal plane) shall be within 10% of the prescribed dose. The dose at selected anatomical points shall be calculated and these calculations are to be submitted as part of the quality assurance. Measurements of patient dimensions needed for the calculation of the prescription dose will be made at the time of the simulation for lung blocks. Measurement and calculations of required monitor units necessary for each treatment will be performed in the patient treatment position for AP-PA fields: either upright position or the reclining, lateral decubitus position . In the event the patient is intended to be treated in an upright position, but proves too ill for treatment, dose calculation will have been pre-calculated to permit treatment in the lateral decubitus position).

1.1.4.1 Prescription Point:
The following reference points will be determined:

1. **Head (Point 1)**: this reference point is defined along the longitudinal axis of the skull at the greatest mid-separation (immediately superior to the nasal bridge). The depth should be taken as midway between the entrance and exit points of the opposed radiation beams.
2. **Neck (Point 2)**: this reference point is defined along the patient's longitudinal axis at the level of C3/C4 (approximate mid-neck, but chosen for the thinnest mid-separation of the neck). The point is taken to be midway between the entrance and exit point of the beam.
3. **Upper Mediastinum (Point 3)**: this reference point is defined along the patient's longitudinal axis at the level of the angle of Louis. The reference point is midway between the entrance and the exit points of the opposed beams.
4. **Lower Mediastinum (Point 4)**: this reference point is defined along the patient's longitudinal axis at the level of the xiphisternal notch. The reference point is midway between the entrance and exit points of the opposed

beams
5. **Umbilicus (Point 5)**: THE PRESCRIPTION POINT is defined along the patient's longitudinal axis at the level of the umbilicus. The prescription point is midway between the entrance and exit points of the opposed beams.
6. **Knee (Point 6):** this reference point is defined along the midline in the midplane of the knee at the level of the patella.
7. **Ankle (Point 7)**: this reference point is defined along the midline at the midplane of the ankle at the level of the lateral malleolus.
8. **Shielded Lung Dose (Point 8)**: this reference point is located on the right chest wall under the lung block. It is centered (both medial/lateral and cephalocaudad) under the lung block as projected on the patient's skin. The depth should be taken as midway between the entrance and exit points of the opposed radiation beams. Dose measurements at this location will be taken during a fraction with lung shielding in place.
9. **Unshielded Lung Dose (Point 9)**: This reference point is the same as point 8. Dose measurements at this location will be taken during a fraction without lung shielding in place. The depth should be taken as midway between the entrance and exit points of the opposed radiation beams.

1.1.4.2 Dose definition:
The absorbed dose is specified as centigray (cGy)-to-muscle.

1.1.4.3 Prescribed Dose, and Fractionation, and Timing:
165 cGy will be delivered in one fraction.

1.1.4.4 Dose Rate:
A mid-plane dose rate of between 6 and 15 cGy per minute is required.

1.1.4.5 Dose Uniformity:
The objective is to keep the dose throughout the body, defined to extend to within 2 mm of the skin surface, to at least 90% of the prescription dose. In addition, the brain dose shall not exceed 107% of the prescription dose.

For AP/PA treatments, partial transmission lung blocks will be used to limit the overall total lung dose. The dose at the midpoint of the thickest part of the body, while in the treatment position, should be determined and if necessary, modifications made to the treatment to raise the dose in this region to at least 90% of the prescription dose.

In order to satisfy the requirement that the skin dose at a depth of 2 mm is within at least 90% of the prescription dose, beam spoilers or other equally effective devices should be used. The field size shall be such that no part of the patient extends into the portion of the penumbra region where the dose is less than 90% of the central axis dose.

1.1.5 Treatment Technique
Patients will be treated using AP/PA fields in an upright seated or standing position in a TBI positioning device. Treatment will be delivered with equally weighted parallel opposed portals, with each treatment including both AP and PA fields. An acceptable alternate arrangement will include equally weighted AP-PA parallel opposed fields delivered to the patient in a lateral decubitus position on a treatment couch or gurney.

Young patients requiring anesthesia will be treated in an AP/PA configuration at extended distance. If more than a single field is needed to accomplish treatment, the field junction should be at the level of the thighs.

1.1.5.1 Dose calculation for the Prescription Point
The calculation of the treatment time or the monitor units for the prescribed dose can be carried out using standard techniques. However, TBI presents special problems relative to the routine treatment situation in that the field sizes are much larger and the treatment distances much longer. The TBI percent depth dose (PDD) or Tissue Maximum Ratio (TMR) and output factors should be measured under TBI treatment conditions for a range of phantom sizes to establish the database for TBI beam-on time calculations or to validate the calculation methodology.

Typically, a calculation methodology will be adopted which uses PDD or TMR and output factors measured under standard conditions but then modified to account for the larger treatment distance. For example, modified values for inverse square corrected percentage depth dose or tissue-air ratios and tissue-phantom ratios are necessary for some treatment units when the patient is positioned at a long distance from the photon source and near the floor or one wall of the room. Also, some deviation from an exact inverse square decrease with distance has been demonstrated for certain geometries.

Measurements of dose at the center of a phantom about the size of the typical patient should be performed and compared to the calculated dose. If differences are found, additional correction factors should be introduced to the calculation method.

1.1.5.2 Critical Organ Dose Points
The required dose calculations should be performed for the 9 points referenced above (1.5.4.1). The midline dose at these locations should be recorded on the TBI Summary Form.

1) The dose can be calculated based on the thickness at each location and factors appropriate to the TBI treatment conditions.

It is recommended that entrance and exit TLDs or diodes be placed on the patient at each required dose assessment location. The midline dose can be calculated from these measurements making the appropriate corrections to the readings and then averaging the corrected values.

In younger patients it is also recommended that TLDs or diodes be placed underneath the lung blocks to document the transmission dose and scatter dose.

1.2 Lung Shielding
Lung shielding shall be used in all patients

1.2.1 Lung Block design
Lung blocks will confirm to the following guidelines: The lateral edges will be 1.0 – 1.5 cm from the inner border of the ribs; the inferior edges will be 1.0 – 1.5 cm from the dome of the apex of the diaphragm; the superior borders will be 1.0 – 1.5 cm below the clavicles; the medial border 2.0 – 2.5 cm from the lateral edges of the thoracic vertebral bodies. No contouring of the lung shields will be done around the hilum unless there is a residual abnormal hilar adenopathy, in which case the margins around the hilar mass will be 1.0 – 1.5 cm.

1.2.2 Timing
Lung blocks will be employed for sequential treatments starting with the first treatment. Should patient infirmity preclude upright positioning during a fraction when lung shielding is prescribed, that patient may be treated in the lateral decubitus position without lung shielding, and lung shielding can be deferred until the next treatment fraction. Alternatively, lung blocks may be used in the lateral decubitus position for patients being treated exclusively in that position.

For children receiving TBI under anesthesia, treatments will be performed in a modified supine and prone position, with the appropriate lung shielding as specified in the protocol.

1.2.2.1 Fractionation Schema
For a fractionation scheme of 165 cGy bid x 4 days; lung shielding will be used for the first 4 fractions. This schema is calculated to deliver a nominal dose of approximately 825 cGy to both lungs, without correction for lung homogeneity (660 cGy x 0.25 = 165 cGy + 660 cGy = 825 cGy).

1.2.3 Electron boost
No compensatory electron boost of that portion of the chest wall shielded by the lung blocks is required.

The lung dose will be reported on the TBI Summary form.

1.3 Testes Boost

1.3.1 Patients
All patients with biopsy-proven testicular leukemia at relapse will receive a testicular radiation boost (400 cGy in 1 fraction) prior to TBI. It is recognized that there will some patients who previously received radiotherapy prior to relapse. These patients will be treated similarly.

1.3.2 Equipment
1.3.2.1 Modality
Electron beams. The selection of energy is determined by the dose uniformity criterion, to cover the testes with no less than the 80% isodose curve.

1.3.3 Target Volume
The target volume consists of the testes in the scrotal sac. (Note: Cremasteric reflex may move testes high up in the inguinal canal.)

1.3.4 Target Dose
400 cGy

1.3.4.1 Prescription Point
The prescription point is to Dmax

1.3.4.2 Dose definition
The absorbed dose is specified as centigray (cGy)-to-muscle.

1.3.4.3 Prescribed Dose and Fractionation
The total dose to the prescription point shall be 400 cGy in 1 fraction.

1.3.4.4 Timing
Testicular irradiation should start within 3 treatment days of the beginning of the TBI preparative regimen if they have not already received testicular irradiation as part of their induction therapy.

1.3.4.5 Dose Uniformity:
The variations of dose within the planning target volume shall be within +20%, -20% of the dose to the prescription point. The uniformity requirement can in general be met with an electron beam of appropriate energy provided bolus is used, which is the simplest technique.

1.3.5 Treatment Technique

1.3.5.1 Patient Position
The patient shall be treated in the supine position.

1.3.5.2 Field-shaping
Field shaping can be done with electron cutout blocks of the appropriate thickness.

1.3.6 Normal Tissue Sparing

1.3.6.1 Perineum:
The testes shall be supported posteriorly and, if possible, extended caudally in order to minimize perineal irradiation. The field shall not be angled towards the perineum.

1.3.6.2 Penis:
The penis shall be excluded from the field by fixing it to the skin over the symphysis pubis.

1.4 Questions

Questions regarding the radiotherapy section of this protocol should be directed to the study radiation oncologist coordinators, Dr. Ralph Ermoian (206)598-4100.

1.5 Definitions of Deviation in Protocol Performance

Prescription Dose

Minor Deviation:

The dose to the prescription point differs from that in the protocol by between 6% and 10%.

Major Deviation:

The dose to the prescription point differs from that in the protocol by more than 10%.

Dose Uniformity

Minor Deviation:

The dose to any of the reference points in Section 16.5.5.1 differs from the protocol dose by more than 10% but less than 20%.

Major Deviation:

The dose to any of the reference points in Section 16.5.5.1 differs from the protocol dose by more than 20%.

Appendix K: Guidelines for Pediatric Research Blood Draws

Maximum recommended blood draws

A single research blood draw will be ≤1ml/kg to a maximum of 50ml.

The research blood draw volume will not exceed the lesser of 100 ml or 3 ml per kg in an 8-week period.

Weight in kilograms	**Maximum total amount at a single blood draw (ml) (≤1ml/kg or <50ml whichever is less)**	**Maximum total amount in 8-week period (ml) (≤3ml/kg or <100ml whichever is less)**
5-6.9	**5**	**15**
7-8.9	**7**	**20**
9-9.9	**9**	**25**
10-14.9	**10**	**30**
15-19.9	**15**	**45**
20-24.9	**20**	**60**
25-29.9	**25**	**75**
30-34.9	**30**	**90**
35-39.9	**35**	**100**
40-44.9	**40**	**100**
45-49.5	**45**	**100**
≥50	**50**	**100**