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Phase I/II Trial of Intravenous Azacitidine in Patients Undergoing Matched Unrelated Stem Cell Transplantation

Washington University School of Medicine
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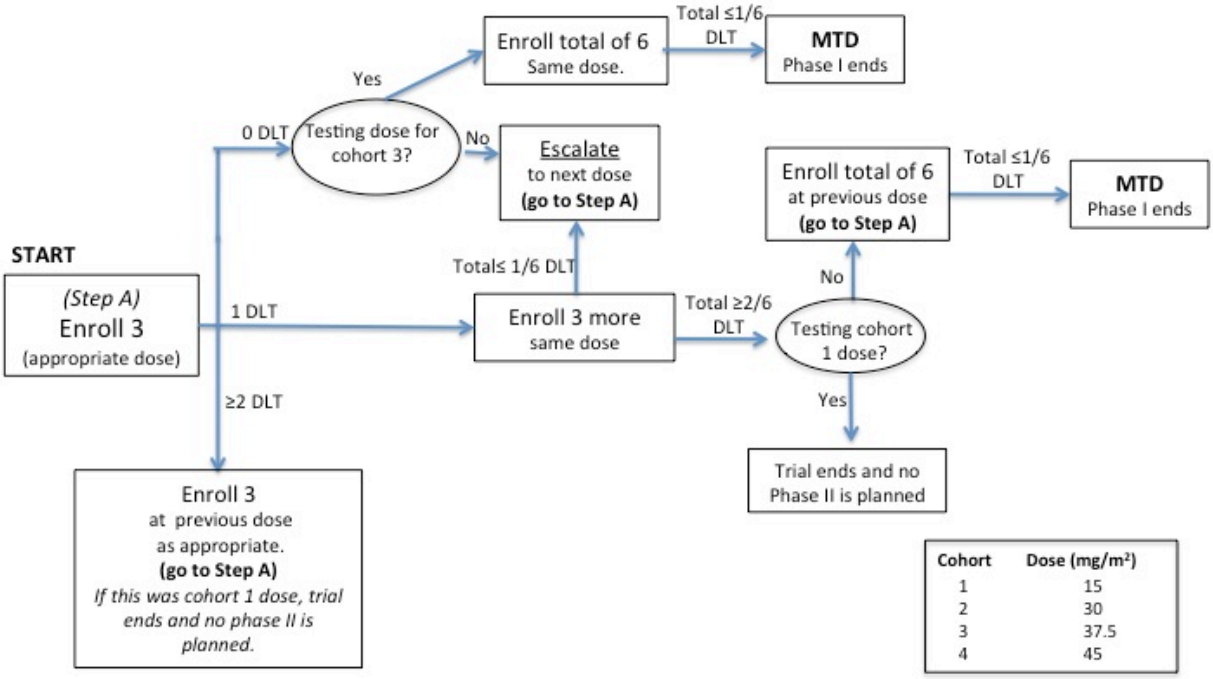
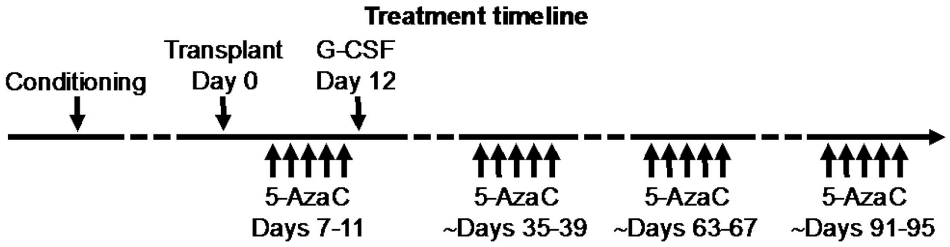
SCHEMA

Schema for 3+3 phase I dose escalation/de-escalation design



Azacitidine dosing cohorts

Cohort 1	Cohort 2	Cohort 3	Cohort 4
15 mg/m^2	30 mg/m^2	37.5 mg/m^2	45 mg/m^2



Glossary of Abbreviations

5-AzaC	5'-azacytidine (azacytidine, azacitidine)
AE	Adverse event
aGvHD	Acute Graft versus Host Disease
ALL	Acute Lymphoid Leukemia
ALLO-HSCT	Allogeneic hematopoietic stem cell transplant
ALT	Alanine transaminase
AML	Acute myeloid leukemia
ANC	Absolute neutrophil count
ASP	Aspirate
AST	Aspartate transaminase
AUC	Area under the curve
BJH	Barnes-Jewish Hospital
BM	Bone marrow
BMT	Bone marrow transplant
BUN	Blood urea nitrogen
CALGB	Cancer and Leukemia Group B
CBC	Complete blood count
CCR	Conventional care
CFR	Code of Federal Regulations
cGvHD	Chronic Graft vs. Host Disease
CI	Confidence interval
CIBMTR	Center for International Blood and Marrow Transplant Research
CML	Chronic Myelogenous Leukemia
CMML	Chronic myelomonocytic leukemia
CMP	Complete metabolic panel
CR	Complete remission
CRc	Cytogenetic complete remission
CRi	Complete remission incomplete
CRm	Morphologic complete remission
CRF	Case report form
CTCAE	Common Terminology Criteria for Adverse Events
CTEP	Cancer Therapy Evaluation Program
DC	Dendritic cell
DLCO	Diffusing capacity of the lung for carbon monoxide
DLI	Donor lymphocyte infusion
DLTs	Dose limiting toxicities
DMSO	Dimethyl sulfoxide
DNA	deoxyribonucleic acid
DOB	Date of birth
DSM	Data and safety monitoring
DSMC	Data and Safety Monitoring Committee
ECG	Electrocardiogram
ECOG	Eastern Cooperative Oncology Group
EDTA	ethylenediaminetetraacetic acid
FAB	French American British
FCBP	Females of child bearing potential
FDA	Food and Drug Administration
G-CSF	Granulocyte colony stimulating factor, filgrastim (NEUPOGEN)
GvHD	Graft vs. Host Disease
GvL	Graft vs. Leukemia
HCT	Hematopoietic cell transplant
HIV	Human Immunodeficiency Virus
HRPO	Human Research Protection Office (IRB)
HSCT	Hematopoietic stem cell transplant
HSV	Herpes simplex virus

HTLV	Human T-lymphotropic virus
IBMTR	International Bone Marrow Transplant Registry
IBW	Ideal body weight
ICH	International Conference on Harmonization
IND	Investigational New Drug
IPSS	International prognostic scoring system
IRB	Institutional Review Board
IV	Intravenous (i.v.)
IWG	International Working Group
LDH	Lactate dehydrogenase
LLN	Lower limit of normal
MA	Myeloablative
MDS	Myelodysplastic syndrome
MED	Minimum effective dose
MM	Multiple myeloma
MRI	Magnetic resonance imaging
MTD	Maximum tolerated dose
MUD	Matched unrelated donor
MUGA	Multiple Gated Acquistion scan
NCI	National Cancer Institute
NIH	National Institutes of Health
NMDP	National Marrow Donor Program
OHRP	Office of Human Research Protections
OS	Overall survival
PB	Peripheral blood
PBSC	Peripheral blood stem cell
PD	Progressive disease
PI	Principal investigator
PK	Pharmacokinetic
PR	Partial response (Partial remission)
QASMC	Quality Assurance and Safety Monitoring Committee
RAEB	Refractory anemia with excess blasts
RAEB-T	Refractory anemia with excess blasts in transformation
RFS	Relapse-free survival
RIC	Reduced intensity conditioning regimen
RNA	Ribonucleic acid
ROS	Review of Systems
RR	Response rate
SAE	Serious adverse event
SC	Subcutaneous
SCC	Siteman Cancer Center
SCT	Stem cell transplant
SD	Stable disease
SLCH	St. Louis Children's Hospital
TBI	Total body irradiation
TCD	T cell depleted
Tconv	Conventional T cell
TF	Treatment failure
TREC	T cell receptor excision circle
Treg	Regulatory T cell
TRM	Treatment-related mortality
ULN	Upper limit of normal
UPN	Unique patient number
WHO	World Health Organization
WU	Washington University

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1.0 BACKGROUND AND RATIONALE

1.1 Graft-versus-Host Disease after Allogeneic Stem Cell Transplantation for Acute Myeloid Leukemia and Myelodysplastic Syndrome

1.1.1 Myelodysplastic Syndrome and Acute Myeloid Leukemia

1.1.1.1 General Background

Myelodysplastic syndromes (MDS) are clonal hematologic disorders characterized by ineffective hematopoiesis in one or more hematopoietic lineages. MDS is estimated to affect 3/100,000 persons per year in the United States with peak incidence occurring at age >60[1,2] The three-year overall survival estimate based on registry data is approximately 35-45%[1,2] but median survival is less than six months in high-risk MDS.[3] Approximately half of all MDS cases will transform to acute myeloid leukemia (AML). The only available curative therapy for MDS remains allogeneic hematopoietic stem cell transplantation (allo-HSCT). However, few patients are good candidates for this intensive therapy and the incidence of relapse and mortality is increased in high-risk and older patients.[4-6] The hallmarks of MDS include peripheral blood cytopenias and bone marrow hyperplasia with dysplastic hematopoietic precursor morphology. MDS and AML are similar diseases in the elderly and likely represent a spectrum of neoplastic evolution in this patient population. Front-line therapy for MDS includes supportive care with transfusions and growth factors in low-risk disease.

In AML, the most frequently diagnosed form of adult leukemia, myeloid precursor cell differentiation is inhibited leading to an accumulation of immature and blast cells in the bone marrow, peripheral blood and/or other organs. Diagnosis of AML is based on cellular morphology, immunologic, cytogenetic, and molecular features and prompts rapid treatment since the median survival of untreated AML patients is less than 3 months.[7,8] The Leukemia and Lymphoma Society expected ~12,000 cases in the United States in 2010 and the incidence is anticipated to rise as the population ages and the number of cases swells exponentially from 0.8 per 100,000 people at age 45 to >15 per 100,000 people at age 75.[9]

When given standard induction chemotherapy for AML (cytosine arabinoside and an anthracycline), a complete remission is possible in 60-80% of patients younger than 60 years[7,8] and in 45-60% of patients older than 60 years.[10-13] Unfortunately nearly all of these patients relapse (median 4-8 months) due to minimal residual disease unless given additional consolidation therapy. Such treatment usually consists of additional cytotoxic chemotherapy with or without allo-HSCT. Those patients most apt to be helped by allo-HSCT can be identified by pre-treatment variables such as AML subtype, unfavorable cytogenetics, FLT-ITD mutational

status, and pre-existing hematological disorders. After a nonmyeloablative allo-HSCT, relapse remains the most common reason for treatment failure with a median relapse time of 3-6 months. If a myeloablative preparative regimen is used, median relapse time is somewhat extended.

The Center for International Blood and Marrow Transplant Research (CIBMTR) maintains a registry of hematopoietic cell transplantation (HCT) outcomes from more than 500 centers worldwide. Recently, the CIBMTR reported the registry outcomes of adults with AML undergoing a matched unrelated donor HCT.[14] Two-thirds of patients lack a suitable related donor. Of 2223 adult AML patients undergoing allogeneic HCT between 2002 and 2006 who were evaluated, 1193 had 8/8 HLA matched unrelated donor transplants. The Day 100 incidence of clinically significant acute graft-versus-host disease (GvHD) in this group was 51% (95% CI 48-54), with life threatening acute GvHD occurring in 25% (95% CI 23–28). The overall survival and relapse free survival at 1 year was 52% (95% CI 50–55) and 35% (95% CI 32–37) respectively.

Relapse is a major problem after allo-HSCT. The National Cancer Institute international workshop on the biology, prevention, and treatment of relapse after allo-HSCT summarized current data on the outcomes of patients with AML and MDS who relapse after transplant.[15] The incidence of relapse for AML in first remission is 10–40%. This incidence increases to >40–50% in advanced disease. The outcomes after relapse are poor and depend on patient age, remission duration, cytogenetics, disease stage at relapse, and other comorbidities. Standard treatment for relapse after allo-HSCT includes chemotherapy and donor leukocyte infusion(s), but this is rarely curative and most patients relapsing will end up dying from their disease. For patients with MDS, the risk of relapse post allo-HSCT is 20-60%. Those with low risk disease have a recurrence rate of 5–10%, and the rate for those with high-risk disease is 10–40% (similar to AML). Those relapsing with MDS after allo-HSCT also have poor long-term survival, which depends on factors similar to those for AML patients (disease activity at transplant, cytogenetics, age, therapy related MDS). Long-term survival after relapse of MDS post allo-HSCT is 0%-40%. In conclusion, the outcomes of relapse of MDS and AML after allo-HSCT are dismal and strategies to reduce this major cause of morbidity and mortality after transplant are needed.

We evaluated our own institutional data on relapse and GvHD after allo-HSCT at Washington University School of Medicine in Saint Louis. Our data collected over a recent two year period (2010 – 2011) suggests similar outcomes to that reported by the CIBMTR. Clinically significant GvHD occurred in 52–66% of recipients of unrelated donor transplants, similar to the 51% observed by the CIBMTR. Life-threatening GvHD occurred in 17–21% of recipients. The overall survival and relapse free survival at 1 year was 46–49% and 30–36%, respectively, dependent on the type of

conditioning regimen used. Table 1 summarizes these findings.

Table 1. Washington University Transplant Outcomes (2010 – 2011)

Donor	Preparative Regimen	n	aGvHD Gr II-IV	aGvHD Gr III-IV	1 yr NRM	1 yr OS	1 yr Relapse
MRD							
	Myeloablative	79	43%	9%	15%	57%	32%
	Reduced Intensity	33	56%	12%	18%	52%	30%
URD							
	Myeloablative	116	66%	17%	25%	49%	36%
	Reduced Intensity	47	52%	21%	13%	46%	30%

Incidence of acute GvHD, non-relapse mortality (NRM), overall survival (OS), and relapse after 8/8 HLA matched related (MRD) and unrelated donor (URD) allogeneic transplant in patients with AML or MDS.

In the event of a relapse, a remission may be re-established by salvage chemotherapy followed by a donor lymphocyte infusion (DLI) in which the patient receives additional lymphocytes from the same donor without prophylactic immunosuppression. A retrospective analysis[16] of ~400 AML patients in 41 centers indicates that such therapy doubled the estimated survival at 2 years from ~9% to ~21%. The drawback of providing a new infusion of allogeneic cells is that many patients suffered from acute GvHD (43%) and 80% of these had grade II to IV disease. In patients surviving at Day 100 post-DLI, 46% had chronic GvHD.

DLI recipients also experience pancytopenia in 18-50% of cases. Infusing more donor stem cells can often correct the marrow aplasia, especially if it was due to bone marrow failure. While it usually resolves without therapy and only 2-5% have sustained marrow aplasia, the cause of the pancytopenia is unclear. We retrospectively examined data from 20 relapsed AML patients at Barnes Jewish Hospital in St. Louis who had received a DLI after allo-HSCT (Table 2).

Table 2. Effect of time between chemotherapy and DLI on recovery from pancytopenia

Patient	Time interval (days)			GVHD 100 days after DLI (Y: 1, N: 2)	Mobilized: 1 Non-mobilized: 2	#CD3+ cells infused (x 10^6)	#CD34+ cells infused (x 10^6)
	Chemotherapy to DLI	Chemotherapy to neutrophil count recovery[1]	DLI to neutrophil count recovery				
1	15	25	10	2	1	10	1
2	13	28	15	1	1	10	0.8
3	37	28	0 (-9)	1	2	10	0.03
4	14	21	17	n/a[2]	1	10	0.39
5	55	28	0(-27)	1	2	10	0.01
6	17	28	11	2	1	10	0.44
7	n/a[3]	n/a	0	1	2	10	0.04
8	27	32	5	2	2	1	0
9	38	43	5	n/a	1	100	1.76
10	18	35	17	2	2	89	0.15
11	70	24	0(-46)	2	2	10	0.03
12	28	29	1	1	1	10	0.53
13	15	39	24	2	1	10	2
14	46	26	0(-20)	2	2	10	0.01
15	15	31	16	2	1	10	0.13
16	14	34	20	n/a	1	10	0.33
17	16	21	5	1	1	30	1.1
18	92	27	0(-65)	1	2	10	0.01
19	11	25	14	2	1	10	0.12
20	15	49	34	n/a	1	10	0.51
Mean (Range)	27.8 (11-92)	28.6 (21-49)	13.8 (1-34)	Yes: 7 (35%) No: 9 (45%) N/A: 4 (20%)	Mob: 12 (60%) Non-Mob: 8 (40%)	19 (1-100)	0.47 (0-2)
SD	22.2	9.5	9.5				
Median	16.5	28	(14.5)			10	0.24

[1]Defined as absolute neutrophil count >500/mm^3

[2]Not applicable.

[3]Patient 7 received no chemotherapy.

Nineteen patients had chemotherapy prior to DLI. Among these patients, there was a striking drop in the time required to recover absolute neutrophil counts (ANC) post-DLI in those who received the infusion ≥27 days after pre-DLI chemotherapy (median = 0 days, range = 0-5 days) compared to those who received an earlier infusion (median = 16 days, range 5-34 days). The DLI caused no new cytopenia in these patients.

In conclusion, the current treatments for relapse after allo-HSCT are unsatisfactory and novel approaches are needed to both prevent relapse and decrease the incidence of GvHD and its associated morbidity and mortality.

1.1.1.2 *Role of DNA Methylation in MDS and AML*

Epigenetic mechanisms, such as DNA methylation of CpG dinucleotides and deacetylation of histones, have evolved to allow genetically identical cells to express different phenotypes, especially during differentiation into the tissues and organs of multicellular organisms. Genes with heavily methylated promoter regions are generally silent, while those with hypomethylation tend to be active. Alteration of the methylation state may

lead to unintended expression or shut down of genes resulting in aberrant or pathological phenotypes, such as uncontrolled proliferation, angiogenesis, and altered cell adhesion. In addition altered methylation can lead to chromosome instability, a hallmark of many tumor cells. Virtually all cancers are characterized by changes in the methylation state, suggesting that this is a predisposing "hit" in Knudson's two-hit model of carcinogenesis (for a review see Lopez[17]). Because hypermethylation is associated with only rare genes in healthy individuals, the majority of methylation changes in cancer cells are specific to the disease. Loss of heterozygosity resulting from progressive DNA methylation has been reported as a critical event in MDS patients that progress to AML.[18] Recently, the hypomethylating agent azacitidine has been shown to reverse hypermethylation in patients with MDS and secondary AML.[19] Blocking the hypermethylation of tumor suppressor genes may halt or delay progression to AML.

Aberrant methylation has long been recognized as being associated with AML, prompting Figueroa, et al.[20] to perform a retrospective analysis of the methylation patterns of ~14,000 genes in 344 patients. Their work revealed 16 different AML groups with internally common clinical outcomes. Five of these established new AML subtypes. The majority of these groups featured distinct strong hypermethylation patterns, although a few were also characterized by hypomethylation. Some of the groups had normal genotypes, which emphasizes the importance that epigenetics has in the establishment of disease. A set of 15 genes was identified whose methylation status could be used to predict the clinical prognosis for the patients, and for some of the groups this status represents the only currently available diagnostic method.

Sudan et al.[21] retrospectively explored the use of azacitidine (5-AzaC), a drug that decreases DNA methylation, in AML outpatients (median age=66) using multiple cycles of 75mg aza/m^2/day for 7 days every 4 weeks. Those who responded to therapy (60%) survived with a median of over 15 months while those who did not had a median of only 2.5 months. The treatment was deemed well tolerated with only 8 out of 20 needing hospitalization for complications during any of the treatment cycles. In comparison the patients receiving traditional chemotherapy were hospitalized during infusion and during pancytopenia, if it occurred, for 2-4 more weeks. The FDA has approved a dosing of 525 mg aza/m^2 per treatment cycle.

Histocompatibility antigens[22] as well as some tumor specific antigens[23] become upregulated upon treatment of cells with hypomethylating agents. Thus Lübbert et al.[24] reasoned that pre-treating AML or chronic myeloid leukemia patients with a low dose of azacitidine (less than a third of the FDA approved dose per treatment cycle) prior to DLI might increase the anti-leukemia effect of the infused cells. Care was taken to allow a 7-day

wash-out period between the last injection of azacitidine and the DLI so as not to affect the function of the donated lymphocytes. Many of the patients were given one or more additional cycles of azacitidine approximately every 3 weeks after the DLI. Overall survival at 2 years was 16%. Notably, only 2 out of 19 patients had *de novo* cases of GvHD despite initial concerns that even low doses of a non-tissue specific demethylating agent might increase GvHD by upregulating target genes on normal tissues in addition to those on cancer cells.

1.1.2 Graft versus Host Disease

1.1.2.1 GvHD correlates with GvL

Allogeneic bone marrow transplant (BMT) cures leukemia by means of cytoreduction induced by the preparative regimen and by transfer of immunocompetent alloreactive donor T cells from the bone marrow allograft that exert an anti-leukemic effect called the Graft-versus-Leukemia (GVL) effect.[25,26] For many years, the evidence for this GvL effect was derived from experimental animal models and retrospective comparative analyses of leukemia relapse rates after allogeneic and syngeneic BMT in studies with patients experiencing the presence or absence of acute or chronic GvHD, and between patients receiving unmodified or T cell depleted BMT. More recently, direct evidence for this anti-leukemic effect was demonstrated by the infusion of unaltered donor peripheral blood leukocytes into patients who had relapsed after allogeneic BMT (for a review see Schleunig[27]).

The International Bone Marrow Transplant Registry (IBMTR) analyzed data from 2,254 patients who underwent HLA-identical sibling BMT for leukemia (Chronic Myeloid Leukemia [CML] in chronic phase, AML and Acute Lymphoblastic Leukemia [ALL] in first remission). After adjusting for other variables that affect relapse, they showed that there was a statistically significant reduction in the relapse risk for patients who developed GvHD.[25,26] For patients that received unaltered BMT and did not develop GvHD, the relative risk for relapse was defined as 1.0. Patients who developed only acute GvHD had a relative risk of relapse of 0.68 ($p=0.03$), and patients who developed only chronic GvHD had a relative risk of 0.43 ($p=0.01$), while patients who developed both acute and chronic GvHD had a relative risk of 0.33 ($p=0.0001$). The relative risk for relapse for patients who developed both acute and chronic GvHD was 0.34 for AML. *Thus, there is a strong association between anti-leukemic effect and GvHD, especially chronic GvHD.*

It was also shown that the anti-leukemic effect parallels the severity of GvHD. The removal of mature T cells from the bone marrow graft represents the most effective way to prevent the development of acute and

chronic GvHD. This benefit of T cell depletion is offset by increased graft failure and leukemia relapse, so that overall survival is not improved. The IBMTR compared HLA-identical sibling transplants in 731 recipients of T cell depleted transplants with 2,480 recipients of non-T cell depleted transplants. The relative risk of relapse after T cell depleted transplant was significantly increased compared to non-T cell depleted transplant for all types of leukemia (ALL, AML, CML) and all stages (early, intermediate, advanced) of disease with the exception of AML in or beyond second remission and CML in blast crisis.[28] Because T cell depletion decreases GvHD, which affects leukemia relapse, the analysis was repeated after adjusting for incidence and severity of acute and chronic GvHD to examine if T cell depletion independently affects leukemia relapse. After adjusting for GvHD, the relative relapse risks with T cell depletion for AML and ALL in first remission and CML in chronic phase were 1.66 (p=N5), 1.55 (p=N5) and 4.87 ($p < 0.0001$) respectively compared to non-T cell depleted transplants. Therefore it appears that T cell depletion increases leukemia relapse in AML and ALL via loss of GvHD. Thus, there is need for transplant strategies that effectively eliminate GvHD while maintaining the graft-versus-tumor effect.

Demonstration that the GVL effect exists in the absence of GvHD is best illustrated by transplant studies using identical-twin donors that are associated with no or minimal GvHD and a relatively high rate of leukemia relapse. In a matched-pair analysis of IBMTR data, leukemia relapse rates were compared between 103 patients transplanted from identical-twin donors and 1,030 patients transplanted from HLA-identical siblings.[28,29] The 3-year probability of relapse for AML was 52% after an identical twin transplant compared with 16% after an HLA-identical sibling transplant. After adjustment for acute and chronic GvHD the relative risks for relapse of syngeneic compared to allogeneic transplants was 3.1 (95% confidence interval, 1.9 to 5.1) for AML. Therefore, it appears that there is an allogeneic GvL effect for AML that is independent of GvHD, yet still dependent on T cells.

Despite the potential positive impact on reducing disease recurrence after transplant, the negative impact of acute GvHD on morbidity and mortality after allogeneic transplant is significant. According to a recent study by the CIBMTR[30], including 4224 patients with AML and 1517 patients with MDS, those patients that develop acute GvHD after transplant have an increased risk of treatment related mortality (HR 2.51 (95% CI 2.18 – 2.89)), and lower overall survival (HR 1.71 (95% CI 1.55 – 1.89)) based on a time dependent multivariate analysis. Our own institutional incidence of acute GvHD after allo-HSCT is similar to that reported by the CIBMTR registry (Table 1). Clinically significant acute GvHD occurred in 52–66% of recipients of unrelated donor transplants and life threatening acute GvHD occured in 17–21% of recipients. **Acute GvHD remains a significant**

problem in our study population; thus, finding a means to harness the GvL effect while reducing or eliminating GvHD is a major goal in designing novel transplant trials.

1.1.2.2 *Regulatory T cells and GvHD*

Since GvHD is a result of donor cells recognizing recipient cells as foreign entities, GvHD should be controlled by those cells that mediate self-tolerance and homeostasis: regulatory T cells (Tregs). These are the only cells known to express FOXP3, the forkhead transcription factor, which has a prominent role in maintaining self-tolerance. Defects in FOXP3 expression lead to severe immunosuppressive diseases both in humans and mice,[31-33] and depleting rodents of Tregs mimics these diseases. Ectopic expression of FOXP3 in conventional mouse CD4+ T cells leads to acquisition of suppressive activity and expression of molecules characteristic of Tregs, including CD25. These results suggest that FOXP3 is a master regulator that differentiates conventional CD4+ cells into Tregs.

In a recent prospective clinical trial Magenau et al[34] found that the ultimate severity of GvHD could be predicted by the relative frequency of Tregs at its onset. Patients who received autologous BMT had average ratios of Tregs to conventional T cells similar to those of healthy individuals (1.09 and 1.17 respectively), as did patients who received an allogeneic BMT, but did not acquire GvHD (~1.06). In contrast when patients first began to suffer from GvHD, their average ratio was ~0.75. The lower the frequency of CD4+CD25^{hi}FOXP3+ cells, the more severe the ultimate GvHD. Frequencies below 0.5% were associated with poor outcomes in terms of overall survival, non-relapse mortality, maximum GvHD grade, and treatment response at 4 weeks.

In an animal model Tregs controlled GvHD while maintaining effective GvL. Edinger et al.[35] showed that Treg cells (CD4+CD25+FOXP3+) coinfused into mice along with conventional T cells (CD4+CD25- and CD8+CD25-), inhibited growth of a luciferase-tagged leukemia cell line as determined by biolumenscence measurements. In addition the Tregs suppressed GvHD by limiting the normal increase in number of conventional T cells even though these cells still migrated to the skin, lymph nodes, and gut. Most animals (~67%) survived the entire 60d observation period.

Similar administration of Tregs to patients might thus be expected to have a therapeutic benefit. However the difficulty in developing a rapid antibody-based purification method has thwarted attempts to do this. FOXP3, the most reliable marker for Tregs, is an intracellular protein, thus precluding its use as a selectable marker. Although cell surface expression of CD25 closely correlates with FOXP3 expression, the suppressive Treg

effect is found only in those few cells with the highest CD25 expression. In addition there is a threshold level of FOXP3 and CD25 expression needed for fully developed Treg functions: CD4+CD25^{mid}FOXP3^{low} cells still retain a naïve T cell phenotype (CD45RA^{hi}CD45RO-). Selection for cells lacking CD127 has also been explored as a means to enrich for Tregs, but when used along with CD25+ selection, it results in co-purification of a subset of FOXP3^{low} cells.

In lieu of infusing purified Tregs into patients to achieve an antileukemic effect with decreased GvHD, post-transplant treatment strategies that increase the proportion or absolute numbers of Tregs could prove beneficial. In a kidney transplant setting it has been shown that switching patients from immunotherapy with multiple agents to one using only rapamycin (sirolimus) resulted in an increase in the number of CD4+CD25$^{bright+}$FoxP3+ Treg cells.[36] This increase did not occur when a switch was made to tacrolimus. Even haploidentical patients had a decreased incidence of GvHD when rapamycin was used post-bone marrow transplant.[37] Likewise a brief high dose of cyclophosphamide post-BMT tended to increase CD4+CD25+FoxP3+ numbers, and patients with the highest numbers had the least severe GvHD.[38-40] However relapse rates remained high with either rapamycin or hi dose cyclophosphamide therapy. When mice were given azacitidine post-transplant, we found an increase in the number of Tregs, decreased GvHD, and very effective tumor control (see section 1.1.2.3). In the trial described here we will begin to examine the safety and effectiveness of azacitidine treatment administered following allogeneic stem cell transplant as GvHD prophylaxis.

1.1.2.3 *DNA methylation, Tregs, and GvHD*

Just as DNA methylation plays a role in the phenotype of cancer cells, DNA methylation also affects the differentiation of immune cells with important consequences for controlling both tumor growth and GvHD. In both mice and humans a demethylated FOXP3 locus represents the most reliable criterion for natural Tregs.[33,41-43] The Foxp3 locus is heavily methylated and silenced in all cell types examined with the sole exception of Tregs, the only cell type in which it is known to be expressed.

Reasoning that hypomethylating agents such as azacitidine or decitabine might affect the expression of FOXP3, Sanchez-Arbaca et al.[44] treated CD4+CD25-FOXP3- T cells with 100 nM of the hypomethylating agent, azacitidine for 14 days. They found that this treatment decreased the fraction of the FOXP3 promoters existing in a methylated state. Simultaneously the percentage of cells with a CD4+CD25+FOXP3+ phenotype had increased from 6% before drug treatment to 27% afterwards. Unfortunately these cells neither proliferated nor became activated in vitro. Nonetheless with the hope of limiting GvHD, these investigators then

explored whether azacitidine could induce Tregs *in vivo* when provided after an allogeneic T cell infusion in mice. They saw the best overall survival and least GvHD when the drug was provided at 1 mg/kg in 2 cycles, the first at 60 and 80 hours post-transplant, and the second at 19, 21, and 23 days post-transplant. However they saw a significant increase in the number of Tregs only when the second cycle of azacitidine was given.

Recently in the laboratory of one of our sub-investigators (J.F. DiPersio), Choi, et al.[45] also demonstrated a dramatic increase in the percentage of human or mouse cells expressing FOXP3 in vitro increases after treatment with azacitidine or decitabine, another hypomethylating agent. We extended these observations to our allogeneic mouse model in which mice were irradiated and then infused the following day with T cell depleted (TCD) bone marrow (BM) to promote recovery from the irradiation-induced myelosuppression. Eleven days later the mice were given an infusion of mismatched conventional T cells. Only 3 out of 23 of these animals survived. In contrast every mouse (23/23) survived, became engrafted with donor cells, and had no GvHD-related symptoms (weight loss, ruffled fur, or diarrhea) when given a single course of four azacitidine injections at 2mg/kg, every other day beginning 4 days post-infusion of conventional T cells (Tconv, figure 1). When the hypomethylating agent decitabine was used instead, the majority of mice died from pancytopenia. Azacitidine-treated mice also had high levels of donor CD3+ T cells and B220+ B cells as has been seen clinically in patients who experience less severe GvHD.

While Sanchez-Abarca[44] had found that azacitidine inhibits the proliferation and activation of T cells in vitro, our dosing method permitted carboxyfluorescein succinimidyl ester-labeled allogeneic donor cells to proliferate vigorously in vivo. The proportion of FOXP3+ cells also increased (figure 1), suggesting that Tregs were at least partly responsible for mediating these effects. Notably this increase in Tregs after only 1 cycle of azacitidine treatment occurred even when the transplant was depleted of Tregs prior to infusion. This result suggests that azacitidine was not just inducing an expansion of Tregs already present in the infused population. Instead, Tconv cells were actually being converted into Tregs post-infusion as a result of treatment with azacitidine.

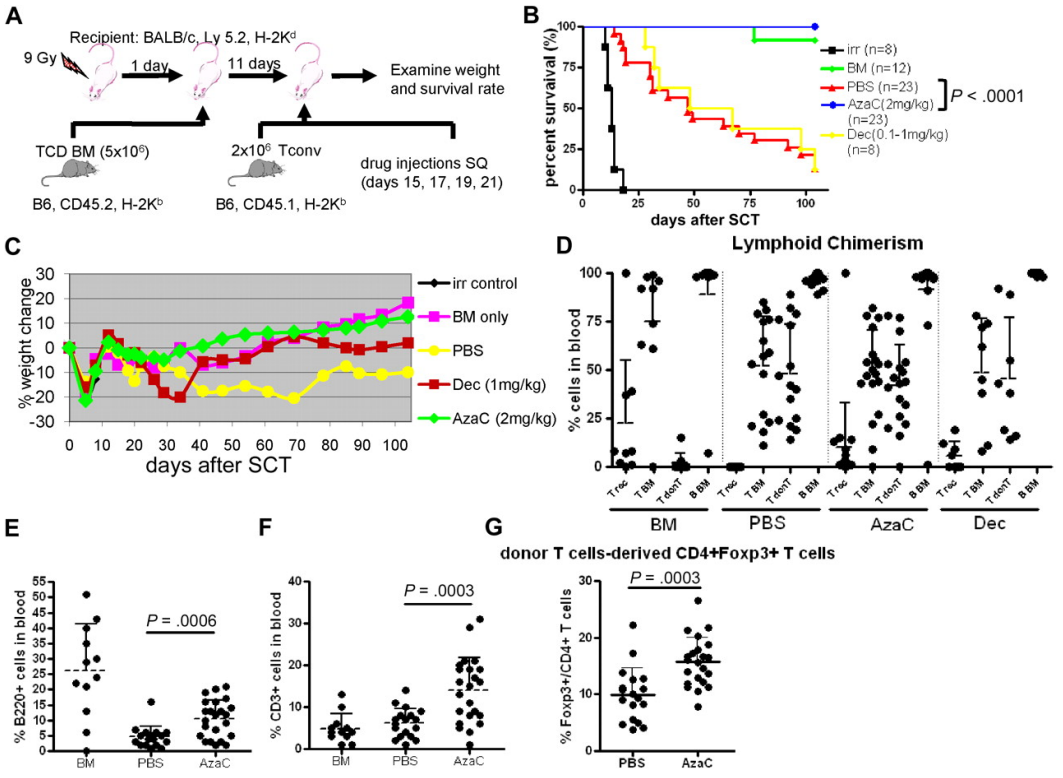


Figure 1. AzaC treatment of mice that underwent a transplantation with delayed allogeneic T cells mitigates GVHD. (A) Schema of the experiments. B6 mice (CD45.2) TCD BM (5 x 10^6 cells) were used as a stem cell source. To induce GVHD, 2 x 10^6 Tconv (B6, CD45.1) were given on day 11 after SCT followed by the treatment with AzaC/Dec/PBS (every other day; 4 doses) starting on day 15 after SCT. (B-F) Mice treated with AzaC (2 mg/kg) show significantly higher survival rate (B), less weight loss (C), and more B cells (E) and T cells (F) with donor engraftment (D) than mice infused with pbsTs. (G) AzaC group also show increased Treg population in peripheral blood. T rec: T cells from recipient; T BM: T cells from donor BM; T donT: T cells from donor T cells; B BM: B cells from donor BM. (D-G) Analyzed 1 month after transplantation. A pool of 4 independent experiments (Excerpted from Choi et al.,[45], figure 4).

As emphasized earlier (Section 1.1.2.1), finding a means to decrease GvHD while retaining a GvL effect is a major goal in this field. Consequently we next examined the effect of azacitidine in our well-established GvL model using luciferase-tagged tumor cells and bioluminescence imaging of the tumor-injected mice. Irradiated mice were infused as above with T cell depleted bone marrow, but this time along with an A20 leukemia cell line, and the tumor became well established during the 11 days allowed for bone marrow recovery. At this point conventional T cells were provided, followed again by the single low dose course of azacitidine described above. In the absence of T cells, this low dose of azacitidine did nothing to control tumor growth. Remarkably, mice treated with azacitidine after the T cell infusion had both a much lower leukemic burden and significantly better survival than tumor-injected mice given the T cells but no azacitidine (figure 2). These data suggest that our method not only preferentially suppresses GvHD, but that it also leaves the strong GvL effect intact. Such an effect is a major goal in the treatment of AML patients.

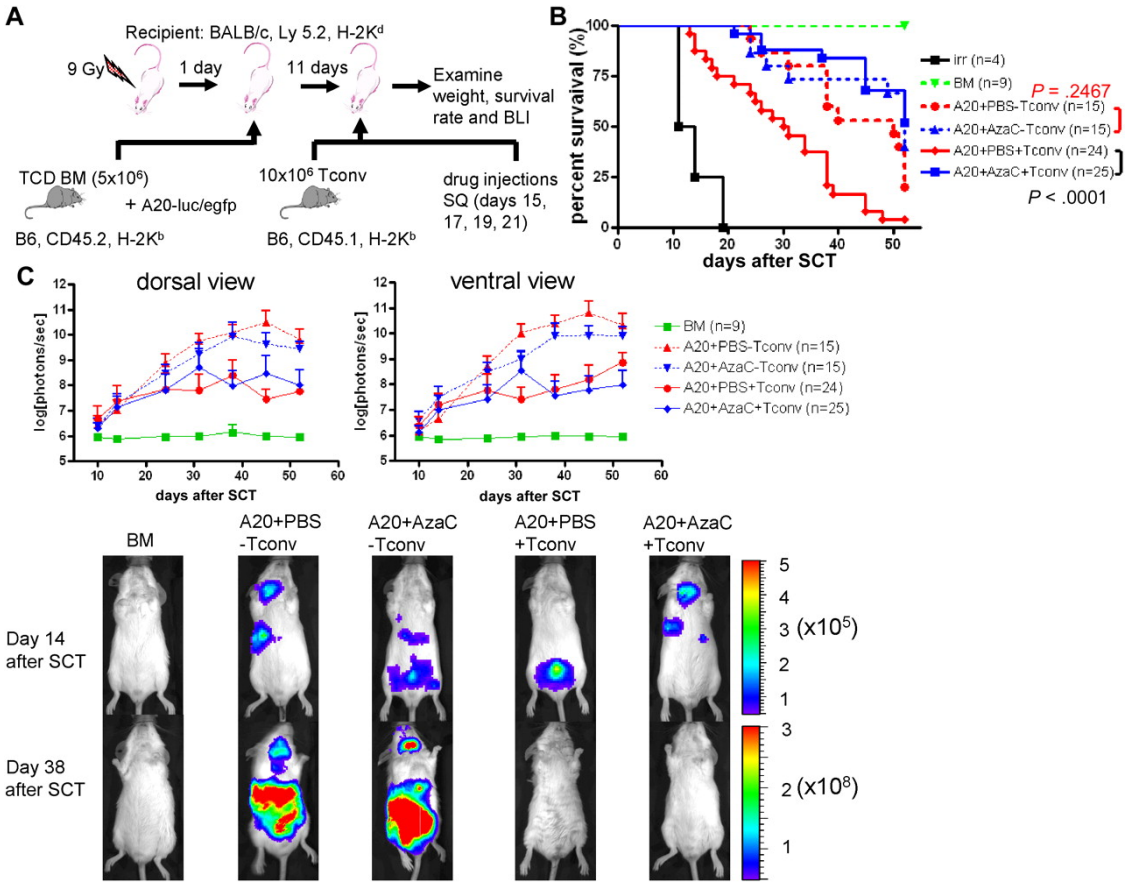


Figure 2. AzaC treatment of mice that underwent a transplantation with delayed allogeneic T cells mitigates GVHD while preserving GVL (A) Schema of the experiments. B6 mice (CD45.2) TCD BM (5 x 10^6 cells) were used as a stem cell source. To induce GVHD, 10 x 10^6 Tconv (B6, CD45.1) were given on day 11 after SCT followed by the treatment with AzaC or PBS (every other day; 4 doses) starting on day 15 after SCT. For examination of GVL effect, 1 x 10^4 A20-*luc/egfp* leukemic cells were given along with TCD BM. (B-C) Mice treated with AzaC show significantly higher survival rate (B) and lower leukemic burden (C). Y axis in top panels indicates photon flux (photons/sec) in log scale measured from the dorsal and the ventral view with a region of interest drawn over the entire body of each mouse. Actual images of 1 representative mouse from each group are shown in bottom panels (scale: photons/sec/cm^2/sr). A pool of 3 independent experiments (Excerpted from Choi et al.[45] Figure 6).

1.2 Investigational Agent: Azacitidine (5'-Azacytidine, Vidaza, Celgene)

In this trial we hope to extend to patients the observation from our mouse model that azacitidine treatment post-DLI mitigates GvHD while retaining GvL. This effect was not seen with another hypomethylating agent, decitabine, as mouse survival was compromised by pancytopenia.

1.2.1 Mechanism of Action

Azacitidine is a pro-drug that is metabolized *in vivo* to its more active substrate, 5-azacitidine, a pyrimidine nucleoside analog of cytidine. The FDA has approved its use in treating MDS and other leukemias, e.g. AML. Its cytotoxic effects are directly related to dose, and this is greatest in cells that are proliferating and metabolically active.[46-48]

Azacitidine incorporates primarily into RNA and is incorporated into DNA only after 5-aza-ribonucleotides are converted into 5-aza-deoxyribonucleotides by ribonucleotide reductase. Normally the mammalian enzymes DNMT1 and DNMT3b catalyze the covalent attachment of methyl groups to the C5 position of cytosine in CpG dinucleotides. DNMT3b is believed to establish *de novo* methylation patterns during mammalian development, while DNMT1 transfers and maintains the pattern in daughter strands of DNA. In treated cells 5-aza-deoxyribonucleotides can covalently trap DNMTl, thus depleting it from the cellular pool and resulting in global demethylation.[49,50] A third highly conserved enzyme, DNMT2, has limited DNA methytransferase activity, but is noteworthy for its recently discovered ability to specifically methylate cytosine 38 in $tRNA^{Asp}$.[51] DNMT2 is expressed predominantly in testis, but Schaefer et al.[52] discovered that it is also expressed in many human cancer cell lines, including three representing myeloid leukemias: HL-60, K562, and ML-1. In these cells 5-AzaC, but not decitabine, caused hypomethylation of $tRNA^{Asp}$, thus altering incorporation of this amino acid into proteins and consequently affecting cellular metabolism. Hypomethylation of $tRNA^{Asp}$ was greatest after one 3-day azacitidine treatment period.

1.2.2 Toxicology and Safety

1.2.2.1 *Preclinical studies*

Cytotoxic effects from azacitidine are directly related to dose and are greatest in cells that are proliferating and metabolically active.[46-48] Studies in mice, rats, dogs, and Rhesus monkeys identified the bone marrow, liver, kidneys, and lymphoid tissues (spleen, lymph nodes, and thymus) as the main target organs of toxicity for azacitidine.[53] The lethal single dose of intravenous azacitidine in mice, rats, and dogs was approximately 250 mg/m^2. Repeated daily dosing increases the toxicity.[53]

In studies on carcinogenicity, mice had an increase in hematopoietic tumors when given 3 injections/week for 52 weeks of 2.2mg/kg azacitidine (~6.6 mg/m^2 which corresponds to ~8% of the recommended human daily dose on a mg/m^2 basis). There was also an increase in the number of lymphoreticular system, lung, mammary gland, and skin tumors in those given 2.0 mg/kg once a week for 50 weeks. An increase in testicular tumors was seen in rats given twice weekly doses of 15-60 mg aza/m^2 (20-80% of the recommended human dose on a mg/m^2 basis). These tumors may in part

be due to the mutagenic potential demonstrated in mouse lymphoma cells and human lymphoblast cells and the clastogenic potential seen in L5178Y mouse cells and Syrian hamster embryo cells.

In studies on reproductive effects, male mice given 9.9 mg aza/m^2 daily (corresponding to ~9% of the recommended human daily dose) for 3 days prior to mating with untreated females had both decreased fertility and loss of offspring. Male rats had decreased testicular and epididymal weight, lower sperm counts, as well as decreased pregnancy weight and a loss of embryos in their untreated female mates when treated 3 times per week for 11 or 16 weeks at 15-30 mg/m^2 (~20-40%, the recommended human daily dose). A similar study showed an increase in abnormal embryos in mated females after 2 days gestation.[54]

1.2.2.2 *Clinical Toxicities*

The FDA approved starting dose of azacitidine for MDS is 75 mg/m^2/day for 7 days every 28 days. Azacitidine at a dose of 75 mg/m^2 single SC injection is well tolerated.[55] Three trials of azacitidine in MDS were updated using the IWG response criteria and showed overall response rates between 40% and 70% (10-17% CR, 23-36% hematologic improvement).[56] A large randomized Phase III trial in higher-risk MDS patients, classified by FAB as RAEB, RAEB-T, or CMML with 0-29% marrow blasts, and an IPSS of Intermediate -2 or High was performed using azacitidine (75 mg/m^2/day x 7days in 28 day cycles) versus conventional care regimens.[57] Three hundred fifty-eight patients were randomized to azacitidine (N=179) or conventional care (CCR) (N=179): best supportive care only (N=105, 59%), low-dose cytarabine (N=49, 27%), or standard chemotherapy (N=25, 14%). At baseline, 95% of patients were higher risk: RAEB (58%), RAEB-T/WHO AML (34%), CMML (3%), and other (5%). By IPSS, 87% were higher risk: Intermediate -2 (40%), High (47%), and 13% Indeterminate/other. Azacitidine was administered for a median of 9 cycles; low-dose cytarabine for 4 cycles. Median follow-up for the survival analysis was 21.1 months. Azacitidine demonstrated statistically superior overall survival compared to CCR, with a median overall survival of 24.4 months vs. 15 months for CCR (stratified log-rank p=0.0001, hazard ration 0.58). Two-year survival approximately doubled in the azacitidine arm compared to CCR: 51% vs. 26% ($p<0.0001$). Azacitidine was well tolerated with safety data consistent with previous reports. The efficacy and safety of azacitidine has been established by the subcutaneous and intravenous routes and is described further in the package insert and the investigator's brochure, which contains comprehensive pharmacology, toxicology, pharmacokinetics, pharmacodynamics, metabolism, preclinical, and clinical efficacy and safety data information.

In AML patients, Sudan et al.[21] retrospectively explored the use of azacitidine in outpatients (median age=66) using 1 or more cycles of 75

mg/m^2/day for 7 days every 4 weeks. Those who responded to therapy (60%) survived with a median of over 15 months while nonresponders had a median of only 2.5 months. Only 8 out of 20 needed hospitalization for complications, while all patients receiving traditional chemotherapy were hospitialized during infusion and for 2-4 more weeks during pancytopenia. Infection was the most common toxic event. In an analysis of 3 sequential Cancer and Leukemia Group B (CALGB) trials, AML patients receiving an azacitidine dosing schedule similar to the one above had a median survival of 19.3 months compared to 12.9 months in those with conventional treatment. There was no increase in infection or bleeding. Similar observations on the drug's safety come from a phase III trial reported by Fenaux, et al.[57] in which patients receiving azaciditine had significantly fewer morbidities and an overall survival of 24.5 months compared to 16.0 months for conventionally treated patients. More azacitidine-treated patients achieved transfusion independence, and although infections were a problem, fewer required intravenous antibiotics. The most frequent hematological problems were leukopenia, granulocytopenia, thrombocytopenia, neutropenia, and anemia. Such toxicity was usually transient, and patients recovered prior to their next cycle of azacitidine treatment.

Azacitidine has been used along with other drugs in AML patients. When combined with hydroxyurea and gemotuzumab treatment, there was a low overall toxicity (5% early death due to disease progression). A study combining azacitidine with all-trans retinoic acid and escalating doses of valproic acid eventually resulted in dose-limiting neurotoxicity. In a study of patients with a variety of advanced malignancies, Braiteh et al.[58] examined the safety of azacitidine in combination with valproic acid, a histone deacetylating agent that also causes epigenetic modifications. The initial dosing used 20 mg/m^2 and was escalated to a maximum tolerated dose of 75 mg/m^2. Grade 3 or 4 toxicities were neutropenic fever and thrombocytopenia. At the grade 1 or 2 level, the most common problems were somnolence and tremor.

1.2.3 Rationale for Dosing Regimen

The FDA determined that there is an acceptable safety profile using the approved dosing of azacitidine in multiple cycles of 75 mg/m^2 every 28 days for MDS. The study proposed here is designed as a traditional 3+3 phase I trial with the intention of defining the Maximum Tolerated Dose (MTD) of azacitidine when given intravenously daily on Days 7, 8, 9, 10, and 11 in conjunction with a matched (8/8) unrelated peripheral blood transplant. In order to optimally suppress relapse and GvHD, the same dose of azacitidine will be given daily for 5 days beginning on approximately Days 35, 63, and 91 post-transplant. Appropriate dose reductions of azacitidine in the last 3 cycles will be performed based on the guidelines in Section 5.4. Allowed conditioning regimens for transplant are shown in Section 5.1.1.

Standard mini-dose methotrexate and tacrolimus will be given for GvHD prophylaxis (Section 5.1.2). For the Phase I portion of this trial, patients with other hematological malignancies will be eligible. After determination of the MTD from the first cycle of azacitidine (days 7-11), we will use this dose to perform a phase II study in patients with AML in remission 1 or 2 or MDS (≤70 years old). The primary endpoint to this study will be grade II–IV acute GvHD. The study is powered based on the mean incidence of acute GvHD by Day +180 from registry data of unrelated allo-HSCT for AML CR1 or 2 (n = 404) provided by the CIBMTR which is 44% (95% CI 39% – 49%). (*The data presented here are preliminary and were obtained from the Statistical Center of the Center for International Blood and Marrow Transplant Research. The analysis has not been reviewed or approved by the Advisory or Scientific Committee of the CIBMTR.*). Of note is that the actuarial risk of aGvHD provided by the CIBMTR is slightly less than our own institutional data (see Table 1) perhaps because we are evaluating all diseases undergoing allo-HSCT. **We feel a reduction in the incidence of acute GvHD at Day +180 from 44% to ≤ 20% would be clinically significant.** The only change to the standard of care is the addition of four 5-day cycles of intravenous azacitidine beginning on Days 7, 35, 63, and 91 post-transplant (total of 20 doses per each subject). In our mouse studies (see Section 1.1.2.3), early dosing with azacitidine eliminated GvHD while retaining GvL.

To date no studies have administered azacitidine early after allogeneic stem cell transplant prior to neutrophil engraftment. Thus the dose selected for the phase I portion of this trial is based on our own limited institutional data obtained from an ongoing study of azacitidine given during marrow aplasia after chemotherapy and DLI for relapsed AML or MDS after allogeneic stem cell transplant. Data from the first 3 patients dosed at 45 mg/m^2 on Days 4, 6, 8, and 10 after DLI is summarized in Table 3. Only 1 of the 3 treated patients had delayed count recovery, defined as absence of ANC>500 cells/uL greater than 42 days post chemotherapy. This cohort is currently being expanded and if a second DLT (delayed count recovery) is observed, we will proceed to the dose minus 1 level of 30 mg/m^2. Additional data on azacitidine use after allo-HSCT is available from 2 other studies.[59,60] In the de Lima study, an adaptive Bayesian approach was used to determine the optimal dose based on time to toxicity. Azacitidine was given daily for 5 days starting around Day +40 after transplant at the following doses: 8, 16, 24, 32, and 40 mg/m^2 and 4 schedules: 1, 2, 3, or 4 cycles, each with 5 days of drug and 25 days of rest. They found that reversible thrombocytopenia was the dose limiting toxicity (DLT) and the best dose was 32 mg/m^2 given for 4 cycles. At a median follow up of 20.5 months, the 1 year overall survival was 77%. In another trial by Goodyear et al., a dose of 36 mg/m^2 starting on Day +42 in patients with stable engraftment was given after reduced intensity allogeneic transplant. Azacitidine was given daily for 5 days on a 28 day cycle for up to 10 cycles and a dose reduction was planned to 24 mg/m^2 if grade 3–4 hematologic toxicity occurred for >2 weeks. Of note, no grade 3–4 hematologic toxicities were observed. This dosing schedule resulted in limited acute GvHD (3/27 (11.1%) in the treatment group and 7/19 (37%) in the control group) and no significant reduction in blood counts or donor engraftment at Day

+90 post allo-HSCT (full donor peripheral blood chimerism: 19/24 treatment group, 19/19 control group). This was also associated with an increase in circulating Tregs up to 3 fold compared to time matched controls. This increase in Treg numbers was only observed early after transplant at cycle 3 of treatment and not when measured after 6 or 9 cycles of azacitidine treatment. **Based on this data, we believe that a dose between 30 and 45 mg/m^2 for 4 cycles of treatment will likely be the optimal dose to establish tolerance and efficacy but because aza has never been used after transplant we will start dosing at 50% of this dose (15 mg/m^2).**

Table 3: Azacitidine post DLI preliminary study outcomes

Subject	Azacitidine Dose	Adverse Events DLT for count recovery? (Y/N)	Outcome
001	None	-	
002	None	-	
003	None	-	
004	45 mg/m^2	N	
005	45 mg/m^2	Y	Delayed count recovery to day +56
006	45 mg/m^2	N	Grade III GI Acute GvHD (patient had grade IV GI GvHD at the time of original transplant)

In addition to these studies in humans our preliminary data in mice also supports the starting dose of 30 mg/m^2. We chose to use 2 mg/kg dose in our mouse studies because it approximates using 75 mg/m^2 in a 70 kg, 1.7 m person. Unpublished data from our mouse model indicate that using 1 mg/kg for each of the 4 injections prevented GvHD as effectively as using 2 mg/kg. Since 1 mg/kg is ~30 mg/m^2, we have chosen this as our starting dose in this trial.

1.2.4 Pharmacokinetics

Azacitidine is rapidly absorbed when given subcutaneously to humans, with maximum plasma concentrations found 0.5 to 2 hours after dosing. In patients with MDS, the peak plasma concentration was reached 11 min after intravenous administration (Cmax=12 μM [2750 ng/ml]).[55] Azacitidine and its metabolites are renally cleared. The effects of renal or hepatic impairment, gender, age, or race on the pharmacokinetics of azacitidine have not been studied. The bioavailability after subcutaneous administration is 89% (range 52% to 128%) with a mean half-life of 0.69 hours.[53] A much lower peak serum concentration of 3 μM azacitidine is reached after subcutaneous administration of a therapeutic dose of 75 mg/m^2.[55]

1.2.5 Route of elimination

Azacitidine and its metabolites are renally cleared.

1.2.6 Metabolism of drug in humans

An *in vitro* study suggests that azacitidine may be metabolized by the liver, however the effect on azacitidine metabolism by microsomal enzyme inhibitors or inducers has not been studied.[54]

1.2.7 Drug interactions

There have not been extensive drug interaction studies with azacitidine. Azacitidine neither inhibited nor induced cytochrome P450 (CYP) enzymes in cultured human hepatocytes at 70uM or lower.[61] A much lower peak serum concentration of 3 uM azacitidine is reached after subcutaneous administration of a therapeutic dose of 75 mg/m^2.[55]

1.3 Study Rationale

In this trial, we will begin to explore the possibility that, as in mice, azacitidine given after allo-HSCT may mitigate GvHD while retaining GvL (see Section 1.1.2.3). Lübbert et al[2424] (Section 1.1.1.2) observed a low rate of GvHD when they treated patients with azacitidine both prior to DLI and for multiple rounds after DLI. In our mouse study (Section 1.1.2.3), a single cycle of azacitidine post-DLI resulted in effective tumor control and a profound decrease in GvHD. This effect was hypothesized to be due to azacitidine-induced demethylation of the FOXP3 locus with subsequent conversion of conventional T cells into Tregs. It is noteworthy that in patients a higher relative frequency of Tregs after allogenic HSCT is associated with a lower rate and severity of GVHD, a lower rate of non-relapse mortality, and equivalent relapse mortality.[34] We hypothesize that providing a single cycle of azacitidine early after allogeneic stem cell infusion and continuing cycles after count recovery on ~Days 35, 63, and 91 will be safe and well tolerated and associated with reduced rates of GVHD without affecting the rate of complete remission (GVL). **The purpose of this phase I/II study is to define the maximum tolerated dose of 5-AzaC and the effect on grade II-IV GvHD when given after matched unrelated donor transplant (MUD).**

1.4 Correlative Studies Background

1.4.1 Pharmacokinetics

This will be the first time azacitidine has been used upfront after transplant and its pharmacokinetics (PK) in recipients remains unknown. In the phase I portion of the trial only, we will obtain serial blood samples prior to and after the first dose of azacitidine administered on Day +7. Plasma will be isolated and cryopreserved for later PK analyses to determine AUC, Cmax, and half-life of azacitidine in this setting. The PK data will be used to correlate and interpret clinical results and will help to guide future studies.

1.4.2 Immune Reconstitution after Transplantation

Immune reconstitution after transplantation is an important determinate for risk of infection after transplantation. To understand the kinetics of immune reconstitution after transplantation with a mobilized peripheral blood stem cell product and early administration of azacitidine after transplant, we will collect peripheral blood samples at pre-determined time points from the recipient. These samples will be analyzed by multiparameter flow cytometry for lymphocyte subsets (including T-regs, naïve/central memory/effector memory, Th1/Th2/Th17 and NKT cells) and circulating dendritic cells. To determine the number of bone marrow derived T-cells that are recent thymic emigrants, PCR TREC analysis will be used.

1.4.3 Regulatory T Cell Frequency and Number

As indicated in Section 1.1.2.3, we have seen that azacitidine treatment post-DLI in mice increases the frequency of Tregs resulting in a suppression of GvHD while the GvL effect is maintained. Our hypothesis is that a similar increase in Tregs will occur in AML/MDS patients who are given 4 cycles of azacitidine beginning on Days 7, ~35, ~63, and ~91 post allo-HSCT resulting in a corresponding decrease in GvHD, while the GvL affect from the MUD will be retained.

We will assess relative Treg numbers and percentages within the peripheral blood before (from the donor PBSC product) and after transplant (recipient peripheral blood). In addition, we will examine several subsets that may have functional significance. Miyara et al[62]. determined that Tregs can be separated into 3 subsets based on their expression of cell surface markers. These subsets differ in their proliferative capacity, effector functions, and ability to secrete specific cytokines. Upon stimulation resting Tregs (rTreg, CD4+CD25++CD45RA+) respond with more active and sustained proliferation than do effector or "activated" Tregs (aTreg, CD4+CD25+++CD45RA-), with the latter dying after only a few days. rTregs are able to differentiate into activated T cells in response to stimulus. These aTregs not only more effectively suppress proliferation of naïve T cells (CD4+CD25-CD45RA+), but also suppress rTregs as a form of negative feedback. A third subpopulation of Tregs (CD4+25++CD45-) is nonsuppressive, but they secrete abundant amounts of proinflammatory cytokines such as IL-17. The proportion of each subset varies with age and disease state. We will use flow cytometry to measure the proportion of rTregs and aTregs in the donor PBSC product pre-Allo-HSCT, and in the recipient peripheral blood at several times after subsequent 5-AzaC treatment to explore the effect of 5-AzaC on frequency of both rTregs and aTregs and whether there is a connection between these frequencies and GvHD. Importantly, as a control for these correlative studies we will utilize a biospecimen bank of cryopreserved peripheral blood mononuclear cells from patients with AML or MDS in CR1 or 2 undergoing a 8/8 MUD over the last 2 years, collected at serial time points after transplant (pre-transplant, Day +30, Day+100, and Day +365).

In addition to the Treg subsets, the differentiation state of Tregs within the peripheral blood will be assessed by flow cytometry (see review by Sakaguchi et al[63]). Naïve Tregs are phenotypically CD45RA+CD45RO-FOXP3^{low}, indicating

that they have not yet undergone T cell receptor-stimulated maturation in the thymus. As recent emigrants from the thymus, most naïve Tregs also express CD31. However because they are believed to require constant stimulation by their cognate antigen to remain in the periphery, they are not strictly "naïve" T cells. In humans, but not mice, these cells have potent suppressive activity and can also be found in cord blood. Cells that are actively proliferating express Ki67, thus providing a means to distinguish resting (naïve) Tregs from activated Tregs. Once naïve Tregs have undergone T cell receptor-stimulated maturation, they not only proliferate, but are also very resistant to apoptosis. When activated these cells convert to highly suppressive and proliferative CD45RO+CD25^{hi}FOXP3^{hi} "effector" Treg cells, with no memory functions despite the association of CD45RO with conventional memory T cells. Effector Tregs are more prevalent in adults and the elderly than in utero or in cord blood. Using flow cytometry for a variety of these markers, the proportion of Tregs and other T cell subsets (naïve/central memory/effector memory and Th1/Th2/Th17) within the blood will be determined.

Finally as indicated in section 1.1.2.2, Magenau et al[34] have found that the frequency of Tregs within the blood at onset of GvHD has predictive value for the ultimate severity of GvHD symptoms and patient outcome. In our trial we will use flow cytometry to measure the proportion of naïve (resting) Tregs (rTregs) and activated (effector) Tregs (aTregs) in patients pre-Allo-HSCT, immediately prior to each cycle, and at ~14 days and ~28 days after each cycle of 5-AzaC to explore the effect of 5-AzaC on frequency of both rTregs and aTregs and whether there is an association between these frequencies and GvHD. Based on the observations above, we will define Tregs subtypes as follows:

Naive (resting) Tregs (CD4^{+}CD25^{++}FoxP3^{low}CD45RA^{+}CD31^{+})
Activated (effector) Tregs (CD4^{+}CD25^{+++}FoxP3^{high}CD45RA^{-}CD31^{-})

Based on these correlative studies we hope to have a better understanding of the kinetics of Treg and other T cell subsets (naïve/central memory/effector memory and Th1/Th2/Th17) recovery after Allo-HSCT and determine the relative frequency of peripheral blood Tregs from patients treated with 5-AzaC after allo-HSCT compared with matched controls and correlate their levels with the development of aGvHD.

2.0 OBJECTIVES

2.1 Primary Objective

Phase I: to determine the maximum tolerated dose (MTD) of azacitidine in patients undergoing matched (8 out of 8 HLA matched) unrelated donor transplant for any hematological malignancy (excluding myelofibrosis) in remission or with stable minimal residual disease.

Phase II: to determine the effect of azacitidine on grade II-IV GvHD in patients undergoing matched (8 out of 8 HLA matched) unrelated donor transplant for AML in remission 1 or 2 or MDS.

2.2 Secondary Objectives

1. To determine the rate of Grades III-IV aGVHD at Day +180.

2. To determine the rate of cGVHD at one year after transplant.

3. To determine overall survival at one year after transplant.

4. To determine the effects of azacitidine on frequency and absolute number of Tregs by comparing to a standard care group of matched historical controls.

5. To determine treatment-related mortality at Day 100.

6. To determine the rate of relapse-free survival at one year after transplant.

3.0 PATIENT SELECTION

3.1 Inclusion Criteria

Patients must meet the following criteria within 30 days prior to Day 0 unless otherwise noted.

1. Phase I: Diagnosis of a hematological malignancy listed below (excluding myelofibrosis) in remission or with stable minimal residual disease
   a. Acute myelogenous leukemia (AML) in 1st or subsequent remission or in relapse after any remission.
   b. Acute lymphoblastic leukemia (ALL) in 1st or subsequent remission or in relapse after any remission
   c. Myelodysplastic syndrome either intermediate 1 or 2, or high risk by the International Prognostic Scoring System,
   d. Chronic myelogenous leukemia (CML) in accelerated or second chronic phase,
   e. Non-Hodgkin's lymphoma (NHL) or Hodgkin's disease (HD) in 2nd or greater complete remission, partial remission, or refractory relapse,
   f. Chronic lymphocytic leukemia (CLL), Rai Stage 2-4, failing at least 2 prior regimens,
   g. Multiple myeloma (MM), Stage 2-3,
   h. Myeloproliferative disorder or neoplasm

   Phase II: Diagnosis of AML in remission 1 or 2 or a diagnosis of Myelodysplastic syndrome either intermediate 1 or 2, or high risk by the International Prognostic Scoring System.

2. Patients with MDS must be transplant candidates by current clinical standards.

3. Patients who have been treated with hypomethylating agents prior to entering the study are eligible.

4. Must have matched unrelated donor (8 of 8 HLA match at A, B, C, and DR loci) by high resolution DNA typing.

5. Must have donor peripheral blood stem cells mobilized by NMDP standards. No bone marrow donors.

6. Must have 4-8 x 10^6 CD34+ cells/kg (recipient weight) infused on Day 0.

7. Must have at least one additional aliquot of ≥1 x 10^6 CD34/kg cryopreserved cells stored at the time of transplant.

8. Must receive a myeloablative or reduced intensity conditioning regimen for SCT as defined by the CIBMTR[64]
   a. Cyclophosphamide and single dose total body irradiation
   b. Fludarabine and busulfan
   c. Fractionated TBI and cyclophosphamide
   d. Busulfan and cyclophosphamide

9. Must be able to receive GVHD prophylaxis with tacrolimus and methotrexate as outlined in Section 6.2.

10. Must be ≥ 18 years old and ≤ 70 years old. Azacitidine is not approved by the FDA for use in children.

11. Must have an Eastern Cooperative Oncology Group (ECOG) performance status ≤ 2 (see Appendix 1).

12. Must have laboratory results indicating:
    a. Total bilirubin < 2.0 mg/dl, unless a diagnosis of Gilbert's disease
    b. AST/ALT ≤ 3 X the upper limit of institutional normal
    c. Serum creatinine ≤ 2.0 mg/dl

13. Patient must have ability to understand and willingness to provide written informed consent prior to participation in the study and any related procedures being performed.

14. The effects of azacitidine on the developing human fetus at the recommended therapeutic dose are unknown. For this reason and because category D agents as well as other therapeutic agents used in this trial are known to be teratogenic, women of childbearing age must have a negative serum pregnancy test (ß-human chorionic gonadotropin) within 72 hours prior to initiating the conditioning regimen and be

willing to not become pregnant by using effective contraception while undergoing treatment and for at least 3 months after the last dose of azacitidine.

15. Men must be willing not to father a new child while receiving therapy. They must use an effective barrier method of contraception during the study and for 3 months following the last dose.

3.2 Exclusion Criteria

1. Must not have myelofibrosis or other disease known to prolong neutrophil engraftment to > 28 days after transplant.

2. Must not be receiving any other investigational agents within 14 days of first dose of azacitidine (Day 7).

3. Must not have nonmyeloablative conditioning as defined below[64]:
   a. TBI </= 2 Gy ± purine analog
   b. Flu + Cy ± ATG
   c. Flu +AraC + Ida
   d. Cladribine + AraC
   e. Total Lymphoid Irradiation + ATG

4. Must not recieve antithymocyte globulin as part of pre-transplant conditioning regimens. Antithymocyte globulin is excluded due to its potential impact on modulating the incidence of GvHD or GvL.

5. Must not have uncontrolled intercurrent illness including ongoing or active infection, symptomatic congestive heart failure, unstable angina pectoris, or psychiatric illness/social situations that would limit compliance with study requirements.

6. Must not be pregnant or breastfeeding. Pregnant women are excluded from this study because azacitidine is a Category D agent with the potential for teratogenic or abortifacient effects. Because there is an unknown but potential risk for adverse events in nursing infants secondary to treatment of the mother with azacitidine, breastfeeding should be discontinued if the mother is treated azacitidine. These potential risks may also apply to other agents used in this study.

7. Must not have a known or suspected hypersensitivity to azacitidine, mannitol, or compounds of similar composition to azacitidine.

8. Must not have an advanced malignant hepatic tumor.

9. Must not be HIV, HBV or HCV positive.

10. Must not have undergone a prior allogeneic donor (related, unrelated, or cord) transplant. Prior autologous transplant is not exclusionary.

3.3 Donor Selection

Donors will not receive any study procedures on this protocol. Donors will sign the standard NMDP donor consent and undergo standard mobilization prior to leukopheresis according to NMDP guidelines.

Donors will be tested for relevant communicable diseases and only donors free from risk factors for, and no clinical evidence of, infection due to relevant communicable disease agents and diseases including: Human immunodeficiency virus; Hepatitis B virus; Hepatitis C virus; Human transmissible spongiform encephalopathy, including Creutzfeldt-Jakob disease; *Treponema pallidum* and HTLV 1 and 2 will be eligible to donate.

Donor must be HIV-1&2 antibody, HTLV-I&II antibody, HBV and HCV sero-negative, by FDA licensed test.

3.4 Eligibility of Women and Minorities

Both men and women and members of all races and ethnic groups are eligible for this trial.

4.0 REGISTRATION PROCEDURES

Patients must not start any protocol intervention prior to registration through the Siteman Cancer Center.

The following steps must be taken before registering patients to this study:

1. Confirmation of patient eligibility
2. Registration of patient in the Siteman Cancer Center OnCore database
3. Assignment of unique patient number (UPN)

4.1 Confirmation of Patient Eligibility

Confirm patient eligibility by collecting the information listed below and completeing registration form (Appendix 2):

1. Registering MD's name
2. Patient's race, sex, and DOB
3. Three letters (or two letters and a dash) for the patient's initials
4. Copy of signed consent form
5. Completed eligibility checklist, signed and dated by a member of the study team
6. Copy of appropriate source documentation confirming patient eligibility

4.2 Patient Registration in the Siteman Cancer Center OnCore Database

All patients must be registered through the Siteman Cancer Center OnCore database.

4.3 Assignment of UPN

Each patient will be identified with a unique patient number (UPN) for this study. All data will be recorded with this identification number on the appropriate CRFs.

5.0 TREATMENT PLAN

5.1 Overall Treatment Plan

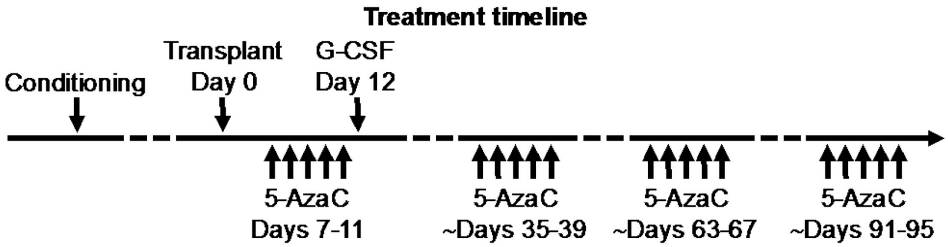
Patients with advanced hematological malignancies (except myelofibrosis) who are eligible for hematopoietic cell transplantation and who have a matched (8/8 HLA antigen matched) unrelated donor will be eligible for phase I of this study. Cohorts of patients in the phase I portion of the trial will receive escalating doses of azacitidine (as described in Section 5.3.3) to determine the maximum tolerated dose (MTD). To ensure engraftment, the treatment of the first 3 subjects will be staggered by an interval of 4 weeks. After the first 3 subjects are treated and engraftment confirmed, enrollment and treatment can occur weekly.

Phase I Azacitidine dosing cohorts

Cohort 1 (Starting Dose)	Cohort 2	Cohort 3	Cohort 4
15 mg/m^2	30 mg/m^2	37.5 mg/m^2	45 mg/m^2

Forty-six additional patients with AML in 1st or 2nd remission or MDS who are eligible for hematopoietic cell transplantation and who have a matched (8/8 HLA antigen matched) unrelated donor will be enrolled in the phase II portion of this study to be treated with the dose of azacitidine determined to be the MTD during phase I.

5.2 Treatment Schema



5.3 Definition of MTD, DLT, Dose Escalation Criteria, and Toxicity, Response, and DLT Evaluations

5.3.1 Definition of MTD (Maximum Tolerated Dose)

The maximum tolerated dose (MTD) is defined as the dose level immediately below the dose level at which 2 patients of a cohort (of 2 to 6 patients) experience dose-limiting toxicity as descrbed in Section 5.3.2. To ensure engraftment, the treatment of the first 3 subjects will be staggered by an interval of 4 weeks. After the first 3 subjects are treated and engrafted enrollment and treatment can occur weekly. Dose escalations will proceed until the MTD has been reached.

5.3.2 DLTs (Dose Limiting Toxicities) Cycle 1

Dose limiting toxicities (DLTs) are:

- failure to have neutrophil engraftment (ANC >500 x 3 days) by Day 28 post-stem cell infusion
- grade 4 or higher organ toxicities (neurologic, pulmonary, cardiac, gastrointestinal, genitourinary, renal, hepatic, cutaneous) not attributable to any other causes that occur through Day 28 (not due to primary malignancy, or infection)

5.3.3 DLTs (Dose Limiting Toxicities) Cycles 2-4

- Grade 3 or higher organ toxicity at least possibly related to azacitidine
- Failure to administer at least 75% of the azacitidine dose due to hematologic toxicity.

5.3.4 Dose Escalation Criteria

All subjects receiving a single dose of azacitidine are evaluable for toxicity. Dose escalation is determined by DLTs in the first cycle regardless of future ability to receive azacitidine or the development of non-hematologic DLTs in Cycles 2-4. If a subject is only able to receive the first cycle of azacitidine, this is adequate to escalate dosing to a subsequent cohort.

Dose escalations will proceed as follows after the occurrence of dose-limiting toxicity (DLT):

Number of Patients with DLT at a Given Dose Level	**Escalation Decision Rule**
0 out of 3	Enter 3 patients at the next dose level.
$\geq$2	Dose escalation will be stopped. This dose level will be declared the maximally administered dose (highest dose administered). Three (3) additional patients will be entered at the next lowest dose level if only 3 patients were treated previously at that dose.
1 out of 3	Enter at least 3 more patients at this dose level. If 0 of these 3 patients experience DLT, proceed to the next dose level. If 1 or more of this group suffer DLT, then dose escalation is stopped, and this dose is declared the maximally administered dose. Three (3) additional patients will be entered at the next lowest dose level if only 3 patients were treated previously at that dose.
$\leq$1 out of 6 at highest dose level below the maximally administered dose	This is generally the recommended phase 2 dose. At least 6 patients must be entered at the recommended phase 2 dose.

If >1 patient in the de-escalation cohort (Cohort -1) has a DLT, then the trial will be stopped and no phase II portion of the trial will be performed.

If 0 of 3 in Cohort 3 experience a DLT then 3 additional subjects will be enrolled. If $\leq$1 out of 6 at this highest dose level experience a DLT then 45 mg/m^2 will be confirmed as MTD.

5.3.5 Toxicity, Response, and DLT Evaluations

All patients who receive any dose of azacitidine are evaluable for toxicity. Patients are evaluated for toxicity from the first dose of azacitidine (Day 7) until Day +140 (or 30 days after the last dose of azacitidine for patients who fail to complete all 4 cycles).

All patients are evaluable for effects of azacitidine on acute GvHD after receiving at least 1 dose of azacitidine unless they die or are removed from the study prior to Day 100 without acute GvHD.

All patients are evaluable for response of the hematologic malignancy to the stem cell transplant unless they are discontinued from the study prior to Day 100 and have not had any disease assessment.

A patient in phase I is evaluable for DLT assessment only through Day 28. Whether phase I subjects have toxicity from study drug in subsequent cycles or are unable to receive subsequent cycles of azacitidine will not have a bearing on the determination of the MTD and phase II dosing.

5.4 Replacement of Ineligible Patients

Should a patient be enrolled and sunsequently be unable to start treatment with azacitidine on Day +7 as per protocol (due to declining performance status, unacceptable lab results, inadequate stem cell product infusion, or other reason), the patient will be removed from the study and will be replaced.

6.0 ADMINISTRATION OF PROTOCOL TREATMENT

6.1 Conditioning Regimens for Transplant

Although including myelo-ablative (MA) and reduced-intensity conditioning (RIC) regimens may result in added variability, retrospective studies comparing MA and RIC in AML/MDS show similar outcomes of survival, treatment related mortality and GvHD.[65,66] Both types of regimens are being allowed in this protocol to tailor conditioning at the discretion of the treating physician and to enhance patient enrollment. Historically about 50% of our transplants for AML and MDS are performed using RIC.

Recipients will undergo institutionally standard myeloablative or reduced intensity chemotherapy or chemoradiotherapy using one of the following conditioning regimens, which, will be administered at the discretion of the treating physician:

- cyclophosphamide and single dose total body irradiation (TBI)
- fludarabine and busulfan
- fractionated TBI and cyclophosphamide
- busulfan and cyclophosphamide

Antithymocyte globulin will **not** be permitted as part of pre-transplant conditioning regimens in this protocol due to its potential impact on modulating the incidence of GvHD or GvL. Non-myeloablative conditioning as defined by the CIBMTR working group will not be permitted on this trial.

6.2 GvHD Prophylaxis

Tacrolimus + mini-methotrexate is the only prophylactic GvHD regimen allowed on this trial.

Tacrolimus will be administered beginning on Day -2 and should continue for a minimum of 100 days prior to tapering. Dosing and adjustments are at the discretion of the treating physician with goal trough between 5 and 10.

Methotrexate will be administered as an intravenous dose of 10mg/m^2 on Day +1 post-transplant and 7.5mg/m^2 on Days +3 and +6 (mini-methotrexate). Dose adjustments per institutional guidelines are allowed for renal and liver dysfunction and mucositis.

6.3 Stem Cell Transplantation (Day 0)

On Day 0 the allograft will be infused per standard institutional practice. The maximum CD34+ cell dose to be given is 8.0x10^6. The reason for this is because of concern for a higher risk of both acute and chronic GVHD that has been seen with G-CSF mobilized peripheral blood transplants at higher CD34 cell doses.[67,68]

In the event that ≤ 4.0 x 10^6 CD34+ cells/kg are infused, the recipient will be removed from the trial and no study drug will be administered at Day +7.

In addition, at least one aliquot of ≥1 x 10^6 CD34+ cells/kg must be stored per institutional guidelines at the time of transplant and reserved to treat potential graft failure. **If this is not possible, the subject will be excluded from the trial and not receive study drug.** At the discretion of the transplant physician, additional cells may be cryopreserved for use at a later time.

6.4 Azacitidine Administration

Azacitidine will be administered for 5 consecutive days each cycle. Cycle 1 begins on Day +7. Patients will receive up to 3 more cycles of treatment starting on Days +35, +63, and +91 (+/- 4 days). It is recommended that cycles 2 through 4 be started on a Monday.

The starting dose will be 15 mg/m^2 during phase I; the phase II dose will be the MTD as determined in phase I. The dose escalation schedule for Phase I may be found in the table below:

Phase I Azacitidine dosing cohorts

Cohort 1	Cohort 2	Cohort 3	Cohort 4
15 mg/m^2	30 mg/m^2	37.5 mg/m^2	45 mg/m^2

Azacitidine dose should be calculated based on adjusted ideal body weight. Adjusted body weight = [0.4 (Actual weight – IBW)] + IBW

Ondansetron 4 mg IV will be administered prior to all azacitidine doses.

Azacitidine solution will be administered intravenously. Azacitidine reconstituted for intravenous administration may be stored at 25°C (77°F), but administration must be

completed within 1 hour of reconstitution.[54] Administer the total dose over a period of 15 minutes.

Subjects should have a dedicated line for azacitidine administration. Before and after administration, the line must be flushed with a minimum of 20 mL of normal saline. If a dedicated line is not possible, the existing line must be flushed before and after drug administration.

Azacitidine is incompatible with 5% Dextrose solutions, Hespan, or solutions that contain bicarbonate. These solutions have the potential to increase the rate of degradation of azacitidine and should therefore be avoided.[54]

6.4.1 Azacitidine Dose Modifications

Azacitidine should not be administered at any time if creatinine > 2.0mg/dl, or if total bilirubin > 2.0mg/dl, or AST or ALT > 3.0 x ULN with an albumin <3.0 g/dl.

Any patient that develops grade 3 or 4 non-hematologic toxicity (excluding nausea/vomiting, electrolyte abnormalities, or infection) related to azacitidine (definitely, probably, or possibly) will have study treatment discontinued.

<table>
<tr><th>Cycle</th><th colspan="3"></th></tr>
<tr><td>1</td><td colspan="3">No dose modification.</td></tr>
<tr><td rowspan="2">2</td><td colspan="3">No dose modification, but cycle may be held for up to 2 weeks if ANC <500/mm^3 and/or Platelets <20,000/mm^3. If hold exceeds 2 weeks, the cycle is to be skipped.</td></tr>
<tr><td colspan="3">If platelet count recovers to >20,000/mm^3 and ANC>500/mm^3 at subsequent cycles, azacitidine treatment may be resumed.</td></tr>
<tr><td rowspan="5">3 & 4</td><td colspan="3">Depending on counts from preceding cycle and current counts:</td></tr>
<tr><td>ANC</td><td>Platelets</td><td>Dose Modification</td></tr>
<tr><td>≥1500/mm^3</td><td>≥100,000/mm^3</td><td>Full dose</td></tr>
<tr><td><1500/mm^3 - ≥500/mm^3</td><td><100,000/mm^3 - ≥20,000/mm^3</td><td>If ANC or platelets do not recover within 75% of start of prior cycles then reduce dose of azacitidine by 25%. *</td></tr>
<tr><td><500/mm^3</td><td><20,000/mm^3</td><td>Hold dose</td></tr>
<tr><td colspan="3">If platelet count recovers to ≥20,000/mm^3 and ANC to ≥500/mm^3 at subsequent cycles, azacitidine treatment may be resumed.</td></tr>
</table>

*Previous dose level may be resumed at subsequent cycles if the platelet and ANC have recovered to 75% of peak levels after transplant.

Treatment may be delayed by up to 2 weeks to allow resolution of grade 3 toxicity at least possibly related to azacitidine. If hold exceeds 2 weeks, the cycle is to be skipped.

Azacitidine should not be administered during Cycles 2 – 4 if patient is receiving G-CSF to support ANC>500

Additional dose modifications may be allowed for other hematologic or non-hematologic toxicities in Cycles 2-4 with consultation of the PI.

6.5 Growth Factor Administration

G-CSF will be administered starting on Day + 12 at a dose of 5 mcg/kg/day(actual recipient weight) by subcutaneous injection until the absolute neutrophil count is > 1500/ul for 2 consecutive days or > 5000/ul for one day. Dose will be rounded to the nearest vial size.

6.6 General Concomitant Medication and Supportive Care Guidelines

Concomitant medications and supportive care measures will be given per institutional guidelines and at the discretion of the treating physician whenever medically necessary with the exceptions of the prohibited medications listed in Section 6.7.

6.7 Prohibited Medications

The following medications are prohibited for the duration of the patient's study participation. Patients who require treatment with any prohibited medication will be removed from the protocol. The PI should be notified immediately, prior to their administration when possible.

- Agents or therapies with the intent to treat the patient's malignancy
- Experimental/investigational medications
- Conditioning regimen agents other than those specified in this protocol.
- All prophylactic medications for GVHD other than those specified in this protocol. The use of corticosteroids for reasons other than GVHD prophylaxis will be permitted with documentation of the rationale for usage.
- Any medication to accelerate platelet engraftment.

6.8 Women of Childbearing Potential

Women of childbearing potential (defined as women with regular menses, women with amenorrhea, women with irregular cycles, women using a contraceptive method that

precludes withdrawal bleeding, and women who have had a tubal ligation) are required to have a negative pregnancy test prior to initiating the conditioning regimen.

Female and male patients (along with their female partners) are required to use two forms of acceptable contraception, including one barrier method, during participation in the study and for 3 months following the last dose of azacitidine.

If a patient is suspected to be pregnant, azacitidine should be immediately discontinued. In addition a positive urine test must be confirmed by a serum pregnancy test. If it is confirmed that the patient is not pregnant, the patient may resume dosing. If a female patient or female partner of a male patient becomes pregnant during therapy or within 4 weeks after the last dose of azacitidine, the investigator must be notified in order to facilitate outcome follow-up.

6.9 Treatment of Acute GvHD

In the event that a subject develops acute GvHD the following steps will be taken:

1. Acute GvHD will be treated per the standard of care with high dose steroids.
2. Subsequent cycles of azacitidine will be continued unless treatment is contra-indicated by cytopenias (see Section 6.4.7.1) or if the subject develops steroid refractory GvHD. Steroid refractory acute GvHD will be defined as no improvement in GvHD within 7 days of starting high dose system steroids (2mg/kg) or progression of GvHD while on 2mg/kg steroids. Subjects who develop steroid refractory acute GvHD will still be followed for chronic GvHD and survival.
3. If steroid refractory GvHD develops, the subject will be taken off study and further treatment is at the discretion of the treating physician.

6.10 Failure to Engraft

If any patient transplanted with an allograft on study does not have evidence of an absolute neutrophil count (ANC) of at least 500/ul by Day 28, this is considered failure to engraft. At this time, the cryopreserved back-up product will be infused per institutional guidelines and the donor will be contacted to plan re-collection immediately.

6.11 Duration of Therapy

Study treatment may continue for up to 4 cycles or until one of the following criteria applies:

- Death
- Failure to engraft at Day 28
- Relapse of the patient's primary diagnosis
- Development of steroid refractory acute GvHD
- Adverse event(s) that, in the judgment of the Investigator, may cause severe or permanent harm or which rule out continuation of study drug.

- Any grade 4 organ toxicity that is at least possibly related to azacitidine
- General or specific changes in the recipient's condition rendering him/her unacceptable for further treatment in the judgment of the investigator
- Suspected pregnancy
- Serious noncompliance with the study protocol
- Lost to follow-up
- Patient withdraws consent
- The PI decides to remove the recipient from study
- The Siteman Cancer Center decides to close the study

6.12 Post-Treatment Follow-Up

Patients will be followed for 2 years after first dose of study drug. Patients who prematurely discontinue treatment should still complete the remaining study visits (if willing and able).

6.13 End of Study Definition

The end of study is defined as any one of the following (whichever occurs first):

- The date of the two-year follow-up
- The date of death
- Lost to follow-up
- Patient withdraws consent

6.14 Definition of Completed Patients

A completed patient is one who receives treatment and completes 2-year follow-up visit.

7.0 PHARMACEUTICAL INFORMATION

7.1 Azacitidine

[Information in this section was directly excerpted or adapted from the investigator brochure.[54]]

7.1.1 Mechanism of Action/Classification

Azacitidine is a pyrimidine nucleoside analog of cytidine. Azacitidine causes hypomethylation of DNA and direct cytotoxicity. Hypomethylation may restore normal function to genes that are critical for differentiation and proliferation. The cytotoxic effects are cell cycle dependent and cause the death of rapidly dividing cells. For additional information, please see Section 1.2.1.

7.1.2 Pharmacodynamics/kinetics

Azacitidine is rapidly absorbed when given subcutaneously to humans, with maximum plasma concentrations found 0.5 to 2 hours after dosing. In patients with MDS, the peak plasma concentration was reached 11 min after intravenous administration (Cmax=12 μM [2750 ng/ml]).[55] Azacitidine and its metabolites are renally cleared. The effects of renal or hepatic impairment, gender, age, or race on the pharmacokinetics of azacitidine have not been studied. The bioavailability after subcutaneous administration is 89% (range 52% to 128%) with a mean half-life of 0.69 hours.[53] A much lower peak serum concentration of 3 μM azacitidine is reached after subcutaneous administration of a therapeutic dose of 75 mg/m^2.[55]

The pharmacokinetics of azacitidine have been studied in 6 MDS patients following a single 75 mg/m^2 subcutaneous (SC) dose and a single 75 mg/m^2 intravenous (IV) dose. Azacitidine is rapidly absorbed after SC administration; the peak plasma azacitidine concentration of 750 ± 403 ng/ml occurred in 0.5 hour. The bioavailability of SC azacitidine relative to IV azacitidine is approximately 89%, based on area under the curve. Mean volume of distribution following IV dosing is 76 ± 26 L. Mean apparent SC clearance is 167 ± 49 L/hour and mean half-life after SC administration is 41 ± 8 minutes. Urinary excretion is the primary route of elimination of azacitidine and its metabolites (50-85%). The mean elimination half-lives of azacitidine and its metabolites were similar after IV and SC administrations, about 4 hours.

7.1.3 Formulation

VIDAZA (azacitidine for injection) is supplied as a lyophilized powder in 100 mg single-use vials packaged in cartons of 1 vial.

7.1.4 Availability

Azacitidine (VIDAZA) is commercially available through Celgene Corporation who will be providing azacitidine for this study.

7.1.5 Preparation

Reconstitute the appropriate number of VIDAZA vials to achieve the desired dose. Reconstitute each vial with 10 mL sterile water for injection. Vigorously shake or roll the vial until all solids are dissolved. The resulting solution will contain azacitidine 10 mg/mL. The solution should be clear. Parenteral drug product should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit. Withdraw the required amount of VIDAZA solution to deliver the desired dose and inject into a 100 mL infusion bag of 0.9% Sodium Chloride.[54]

VIDAZA is incompatible with 5% Dextrose solutions, Hespan, or solutions that contain bicarbonate. These solutions have the potential to increase the rate of degradation of VIDAZA and should therefore be avoided.[54]

7.1.6 Storage and Stability

VIDAZA is a cytotoxic drug and, as with other potentially toxic compounds, caution should be exercised when handling and preparing VIDAZA suspensions. The VIDAZA vial is single-use and does not contain any preservatives. Unused portions of each vial should be discarded properly. Store unreconstituted vials at 25º C (77º F); excursions permitted to 15º-30º C (59º-86º F). Procedures for proper handling and disposal of anticancer drugs should be applied. If reconstituted VIDAZA comes into contact with the skin, immediately and thoroughly wash with soap and water. If it comes into contact with mucous membranes, flush thoroughly with water [54].

7.1.7 Administration

See Section 6.4 for Azacitidine administration instructions.

7.1.8 Nursing Implications

Azacitidine will be administered at a facility capable of managing hypersensitivity reactions.

7.2 Methotrexate

7.2.1 Supply, Storage, and Preparation

Commercially available in 2 mL, 4 mL, 8 mL, 10 mL vials for injection at a concentration of 25 mg/mL. In addition, methotrexate is available in 1000 mg vials for injection. Please refer to the package insert for additional information. Stability and compatibility of methotrexate sodium solutions depend on several factors including the formulation of methotrexate sodium used, presence of preservatives, concentration of drug, specific diluents used, resulting pH, and temperature; the manufacturer's labeling and specialized references should be consulted for specific information. Methotrexate sodium solutions should be inspected visually for particulate matter and discoloration whenever solution or container permits. Administer via slow IV push.

7.3 Tacrolimus

7.3.1 Supply, Preparation, and Administration

Tacrolimus is a commercially available macrolide compound with potent immunosuppressant properties. Tacrolimus is available for oral administration as capsules containing the equivalent of 0.5 mg, 1 mg, or 5 mg of anhydrous tacrolimus. For IV use, tacrolimus is available as a sterile solution in 1mL ampuls

containing the equivalent of 5 mg of anhydrous tacrolimus per mL. Store tacrolimus at controlled room temperature (15-30˚C). Diluted solutions should be stored in glass or polyethylene containers and should be discarded after 24 hours. Tacrolimus for injection must be diluted prior to IV infusion. For IV infusion, the concentrate is diluted with 0.9% sodium chloride or 5% dextrose injection to a concentration of 4-20 mcg/mL. Diluted solutions should be stored in glass or polyethylene containers and should be discarded after 24 hours. A plasticized polyvinyl chloride (PVC) container should not be used because stability of the solution is decreased and polyoxyl 60 hydrogenated castor oil contained in the formulation may leach phthalates from PVC containers. In addition, PVC-free tubing should be utilized to minimize the potential for significant drug absorption onto the tubing. Tacrolimus concentrate for injection and diluted solutions of the drug should be inspected visually for particulate matter and discoloration prior to administration whenever solution and container permit. Tacrolimus is to be initiated on Day –2. Begin tapering between Day +90 to +120 in the absence of GVHD as tolerated with a goal of stopping by Day +150 to +180. Serum levels should be frequently checked according to institutional guidelines.

8.0 SCHEDULE OF ASSESSMENTS AND STUDY CALENDERS

8.1 Study Calendar – Screening and Treatment

	Screening[1]	Cycle 1[2]		Cycle 2[2]		Cycle 3[2]		Cycle 4[2]		D100 (-3/+7)
		D7	Daily D8-11	D35[8]	Daily D36-39[8]	D63[8]	Daily D64-67[8]	D91[8]	Daily D92-95[8]	
Treatment										
Azacitidine infusion[3]		X	X	X	X	X	X	X	X	
Labs/Procedures										
CBC & CMP	X	X[4]	X[9]	X[4]		X[4]		X[4]		X
Pregnancy test	X									
Virology screen	X									
Bone marrow biopsy	X			X		X		X		
Disease staging[5]	X			X		X		X		X
Treg analysis	X[6]			X		X		X		X
TREC analysis		X		X		X		X		X
Lymph 13 / DC subset analysis	X[6]	X		X		X		X		X
Chimerism analysis	X			X		X		X		X
Pharmacokinetics[7]		X								
Assessments										
Physical exam and ROS	X	X	X[9]	X		X		X		X
ECOG PS	X	X		X		X		X		X
Acute GVHD assessment[10]		X		X		X		X		X
Immunosupressive Medication Questionnaire		X		X		X		X		X
Quality of Life assessments	X									

1: Screening assessments should occur within 30 days prior to registration, except as noted in Section 8.2.1
2: It is recommended that the first day of the cycle take place on a Monday
3: All required labs/procedures and assessments should be completed prior to azacitidine dose
4: Results must be reviewed prior to azacitidine dosing
5: Clinical assessment only unless standard practice calls for a diagnostic procedure at these time points post-transplant
6: From recipient and infused product
7: Only for phase I patients; required timepoints: pre-dose, 2 hours, 4 hours, 6 hours, 12 hours and 24 hours post-dose
8: +/- 4 days
9: At least weekly review of systems, exam and laboratory testing until resolution of regimen-related toxicity and neutrophil recovery
10: Assessment every 1-2 weeks through Day 100. After Day 100, every 4 - 6 weeks (+/- 3 days) through Day 180. Patients will continue to be followed after relapse and/or after starting alternate therapy through Day 180.

8.2 Schedule of Assessments – Screening and Treatment

8.2.1 Screening

Within 30 days prior to registration (except where noted) the patient will undergo screening assessments as follows:

- Physical exam and review of systems (ROS)
- ECOG performance status
- Quality of Life assessments
- Complete blood count (CBC) with differential and platelet count
- Complete metabolic panel (CMP): sodium, potassium, chloride, bicarbonate, blood urea nitrogen (BUN), creatinine, glucose, calcium, total protein, albumin, total bilirubin, AST, ALT, alkaline phosphatase
- Pregnancy test for female patients of childbearing potential (serum or urine)
- Virology screen (HIV 1 and 2 and hepatitis B and C) (within 60 days)
- Bone marrow aspirate and biopsy (within 60 days)
- Disease-specific staging (within 60 days)
- Correlative Studies
    - Treg analysis (from recipient and infused product)
    - Lymph 13/DC subset analysis (from recipient and infused product)
    - Chimerism analysis

8.2.2 Treatment Schedule and Assessments

Up to four cycles of azacitidine treatment will be administered. If a patient does not receive treatment during a cycle, all other labs/procedures and assessments should still be completed.

Cycle 1 (Day +7 to Day +35)

Azacitidine will be administered on Days +7 to +11. On Day +7, the following assessments must take place prior to azacitidine infusion:

- Physical exam and ROS
- ECOG performance status
- Acute GVHD assessment every 1 – 2 weeks through day +100. After Day 100, Acute GvHD assessment every 2 weeks (+/- 3 days) through Day 180.
- Immunosupressive Medication Questionnaire
- CBC with differential and platelet count (results must be reviewed prior to azacitidine dosing)
- CMP (results must be reviewed prior to azacitidine dosing)
- Correlative Studies
    - TREC analysis
    - Lymph 13/DC subset analysis
    - Pharmacokinetics (phase I patients only):
        - Pre-dose
        - 2 hours
        - 4 hours

- 6 hours
- 12 hours
- 24 hours

Day +11 to Day +35

At least weekly review of systems, exam and laboratory testing (CBC and CMP) until resolution of regimen-related toxicity and neutrophil recovery

Cycle 2 (Day +35 to Day +39) [+/- 4 days]

Azacitidine will be administered on Days +35 to +39 (+/- 4 days). On Day +35 (+/- 4 days), the following assessments must take place prior to azacitidine infusion:

- Physical exam and ROS
- ECOG performance status
- Acute GVHD assessment every 1 – 2 weeks through day +100. After Day 100, Acute GvHD assessment every 2 weeks (+/- 3 days) through Day 180.
- Immunosupressive Medication Questionnaire
- CBC with differential and platelet count (results must be reviewed prior to azacitidine dosing)
- CMP (results must be reviewed prior to azacitidine dosing)
- Bone marrow biopsy and aspirate
- Disease-specfic staging
- Correlative Studies
    - Treg analysis
    - TREC analysis
    - Lymph 13/DC subset analysis
    - Chimerism analysis

Cycle 3 (Day +63 to Day +67) [+/- 4 days]

Azacitidine will be administered on Days +63 to +67 (+/- 4 days). On Day +63 (+/- 4 days), the following assessments must take place prior to azacitidine infusion:

- Physical exam and ROS
- ECOG performance status
- Acute GVHD assessment every 1 – 2 weeks through day +100. After Day 100, Acute GvHD assessment every 2 weeks (+/- 3 days) through Day 180.
- Immunosupressive Medication Questionnaire
- CBC with differential and platelet count (results must be reviewed prior to azacitidine dosing)
- CMP (results must be reviewed prior to azacitidine dosing)
- Bone marrow biopsy and aspirate
- Disease-specfic staging
- Correlative Studies
    - Treg analysis
    - TREC analysis
    - Lymph 13/DC subset analysis
    - Chimerism analysis

Cycle 4 (Day +91 to Day +95) [+/- 4 days]

Azacitidine will be administered on Days +91 to +95 (+/- 4 days). On Day +91 (+/- 4 days), the following assessments must take place prior to azacitidine infusion:

- Physical exam and ROS
- ECOG performance status
- Acute GVHD assessment every 1 – 2 weeks through Day +100. After Day 100, Acute GvHD assessment every 2 weeks (+/- 3 days) through Day 180.
- Immunosupressive Medication Questionnaire
- CBC with differential and platelet count (results must be reviewed prior to azacitidine dosing)
- CMP (results must be reviewed prior to azacitidine dosing)
- Bone marrow biopsy and aspirate
- Disease-specfic staging
- Correlative Studies
    - Treg analysis
    - TREC analysis
    - Lymph 13/DC subset analysis
    - Chimerism analysis

End of Treatment Day +100 [- 3/+7 days]

- Physical exam and ROS
- ECOG performance status
- Acute GvHD assessment every 1 – 2 weeks through Day 100. After Day 100, Acute GvHD assessment every 4 - 6 weeks (+/- 3 days) through Day 180.
- Immunosupressive Medication Questionnaire
- CBC with differential and platelet count
- CMP
- Disease-specfic staging
- Correlative Studies
    - Treg analysis
    - TREC analysis
    - Lymph 13/DC subset analysis
    - Chimerism analysis

8.3 Study Calendar – Post-Treatment Follow-Up

	D140 (±7)	D180 (±14)	D270 (±14)	D365 (±14)	Mos. 15, 18, 21, 24 (±28)
Labs/Procedures					
CBC & CMP	X	X	X	X	
Bone marrow biopsy		X		X	
Disease staging[1]	X	X	X	X	
Treg analysis	X	X	X	X	
TREC analysis	X	X	X	X	
Lymph 13/DC subset analysis	X	X	X	X	
Chimerism analysis	X	X	X	X	
Assessments					
Physical exam and ROS	X	X	X	X	X
ECOG PS	X	X	X	X	
Acute GVHD assessment[2]	X	X			
Chronic GVHD assessment[4]	X	X	X	X	X[3]
Immunosupressive Medication Questionnaire	X	X			
Quality of Life assessments		X		X	

1: Clinical assessment only unless standard practice calls for a diagnostic procedure at these time points post transplant
2: Assessment every 4 - 6 weeks through day +180. Patients will continue to be followed after relapse and/or after starting alternate therapy through Day 180.
3: Only for patients who are in remission.
4: Patients will continue to be followed after relapse and/or starting alternate therapy through Day 365.

8.4 Schedule of Assessments – Post-Treatment Follow-Up

Patients will be followed for 2 years after first dose of study drug. Patients who prematurely discontinue treatment should still complete post-treatment follow-up visits (if willing and able).

Day +140 [+/- 7 days]

- Physical exam and ROS
- ECOG performance status
- Acute GVHD assessment every 4 – 6 weeks (+/- 3 days) through day +180.
- Chronic GVHD assessment
- Immunosupressive Medication Questionnaire
- CBC with differential and platelet count
- CMP
- Disease-specfic staging
- Correlative Studies
  - Treg analysis
  - TREC analysis
  - Lymph 13/DC subset analysis

- Chimerism analysis

Day +180 [+/- 14 days]

- Physical exam and ROS
- ECOG performance status
- Acute GVHD assessment (+/-3 days)
- Chronic GVHD assessment
- Immunosupressive Medication Questionnaire
- QOL assessments
- CBC with differential and platelet count
- CMP
- Bone marrow biopsy and aspirate
- Disease-specfic staging
- Correlative Studies
  - Treg analysis
  - TREC analysis
  - Lymph 13/DC subset analysis
  - Chimerism analysis
  - TRM analysis

Day +270 [+/- 14 days]

- Physical exam and ROS
- ECOG performance status
- Chronic GVHD assessment
- CBC with differential and platelet count
- CMP
- Disease-specfic staging
- Correlative Studies
  - Treg analysis
  - TREC analysis
  - Lymph 13/DC subset analysis
  - Chimerism analysis

Day +365 [+/- 14 days]

- Physical exam and ROS
- ECOG performance status
- Chronic GVHD assessment
- QOL assessments
- (CBC with differential and platelet count
- CMP
- Bone marrow biopsy and aspirate
- Disease-specfic staging
- Correlative Studies
  - Treg analysis

- TREC analysis
- Lymph 13/DC subset analysis
- Chimerism analysis

Months 15, 18, 21, and 24 [+/- 28 days]

- Physical exam
- Chronic GVHD assessment (only for patients who are in remission)

9.0 CORRELATIVE STUDIES

9.1 Treg Analysis

As discussed in Section 1.1.2.3, we have evidence that providing azacitidine to mice post-DLI increases Treg numbers and frequencies. A higher Treg number is associated with a lower rate of GVHD in both animal and human studies (see Section 1.4). We will explore the correlation between azacitidine administration to patients and the frequency and absolute number of resting and activated Tregs in peripheral blood. Ten mL of blood in sodium heparin tube(s) will be collected at the following time points:

- Baseline sample from the recipient
- Peripheral blood stem cell product (not to exceed 1% of the total volume, 0.5 – 1 mL typically)
- Prior to beginning Cycle 2 (~Day 35 (pre-dose))
- Prior to beginning Cycle 3 (~Day 63 (pre-dose))
- Prior to beginning Cycle 4 (~Day 91 (pre-dose))
- Day 100
- Day 140
- Day 180
- Day 270
- Day 365

Note that no Day 7 sample will be obtained because of significant cytopenias at this time after transplant.

9.2 Recipient Immune-Reconstitution Analysis

9.2.1 TREC Analysis

The purpose of the TREC (T-cell receptor excision circle) assay is to evaluate de novo synthesis of T cells using a real-time quantitative polymerase chain reaction. Ten mL of peripheral blood in EDTA tube(s) will be collected at the following time points:

- Prior to beginning cycle 1 (Day 7(pre-dose))*
- Prior to beginning Cycle 2 (~Day 35 (pre-dose))
- Prior to beginning Cycle 3 (~Day 63 (pre-dose))
- Prior to beginning Cycle 4 (~Day 91 (pre-dose))
- Day 100

- Day 140
- Day 180
- Day 270
- Day 365

*Forty mL of peripheral blood in EDTA tube(s) will be collected on Day 7

9.2.2 Lymphocyte Subset-13 ("Lymph 13") and DC Subset Analysis

Lymphocyte subset analysis requires a 10mL blood sample collected in EDTA tube(s). DC subset analysis will be performed on a separate 10mL blood sample collected in sodium heparin tube(s). Blood for both of these analyses will be collected at the following time points:

- Baseline sample from the recipient
- Peripheral blood stem cell product (not to exceed 1% of the total volume, 0.5 – 1 mL typically)
- Prior to beginning cycle 1 (Day 7(pre-dose))
- Prior to beginning Cycle 2 (~Day 35 (pre-dose))
- Prior to beginning Cycle 3 (~Day 63 (pre-dose))
- Prior to beginning Cycle 4 (~Day 91 (pre-dose))
- Day 100
- Day 140
- Day 180
- Day 270
- Day 365

9.2.3 Chimerism Analysis

Chimerism analysis requires 30 mL EDTA tube(s) of peripheral blood will be collected at each the following time points:

- Baseline
- Prior to beginning Cycle 2 (~Day 35 (pre-dose))
- Prior to beginning Cycle 3 (~Day 63 (pre-dose))
- Prior to beginning Cycle 4 (~Day 91 (pre-dose))
- Day 100
- Day 180
- Day 270
- Day 365

9.2.4 TRM Analysis

TRM analysis on day 180 of the study will be planned to access for TRM.

9.3 Pharmacokinetic (PK) Studies

Pharmacokinetic studies of azacitidine will be performed during the first cycle on the first day of azacitidine treatment (Day 7). Four ml of blood will be drawn into EDTA tube(s) at the following time points:

- Pre-dose
- 2 hours post-dose
- 4 hours post-dose
- 6 hours post-dose
- 12 hours post-dose
- 24 hours post-dose

9.3.1 Sample Delivery

Samples must be maintained at room temperature and delivered as soon as possible to the DiPersio laboratory, room 626 P Southwest Tower, Washington University. Specimens will be identified by initials and study number.

9.3.2 Sample Processing

Peripheral blood samples will be processed for plasma if needed and then red cell lysed or purified by ficoll gradient. Cells may be analyzed by FACS or used in functional studies, or will be cryopreserved viably at 10^7/vial in 90% fetal bovine serum with 10% DMSO. Fresh peripheral blood may also be used for correlative studies at the discretion of the Principal Investigator. Plasma will be divided into 1-2 mL aliquots and frozen at -80ºC.

Samples delivered to the DiPersio lab will be analyzed by multiparameter flow cytometry to delineate the major classes of B, T (including Treg subsets, naïve/central memory/effector memory and Th1/Th2/Th17), and NK cells, monocytes and DCs using the flow cytometry panels recently described by the Human Immunophenotyping Consortium.[69] Intracellular staining of FOXP3 will be performed after fixation and permeabilization according to the manufacturer's recommendation.

The percentage and absolute number of the following cell types will be determined by FACS analysis:

- Resting (naïve) Tregs: $CD4^+CD25^{++}FoxP3^{low}CD45RA^+CD31^+$
- Activated (effector) Tregs: $CD4^+CD25^{+++}FoxP3^{high}CD45RA^-CD31^-$
- Total T cells (CD3+ cells)
- Total CD4+ and CD8+ cells
- Naïve (CCR7+CD45RA+), central memory (CCR7+CD45RA-), effector memory (CCR7-CD45RA-) and effector (CCR7-CD45RA+) CD3+CD4+ and CD3+CD8+ T cells
- Th1 (CXCR3+CCR6-), Th2 (CXCR3-CCR6-) and Th17 (CXCR3-CCR6+) CD3+CD4+ T cells

- naïve (CD19+CD27-) and memory (CD19+CD27+) B cells
- classical (CD14+CD16-) and non-classical (CD14+CD16+) monocytes
- myeloid (CD14-HLA-DR+CD11c+) and plasmacytoid (CD14-HLA-DR+CD123+) dendritic cells
- NKT cells (CD3+CD56+)
- NK cells (CD56+CD16- and CD56+CD16+).

Absolute Treg numbers will be calculated by multiplying Treg frequency by the absolute lymphocyte count (ALC) obtained by an automated differential assessment.

Lymph 13 processing to be done locally by the BJH flow lab and DC subset analysis will be performed by the DiPersio Lab.

9.4 Questionnaires

9.4.1 GVHD Assessments

Acute GVHD will be accessed using modified Glucksberg criteria (Appendix 3). Chronic GVHD will be accessed using the NIH concensus criteria (Appendix 4).

9.4.2 Quality of Life Assessments

Quality of Life (QOL) will be assessed by a patient self report questionnaire FACT-BMT and Human Activity Profile (HAP) (Appendices 5 and 6).

9.4.3 Immunosupressive Medication Questionnaire

The use of immunsupressive medications will be captured using the Immunosuppressive Medication Questionnaire (Appendix 7).

10.0 DATA SUBMISSION SCHEDULE

Case report forms will be completed according to the schedule listed in this section. There is a window of up to 14 days allowed between the time point listed in the submission schedule and the date when the form must be completed.

Case Report Form	Submission Schedule
Consent form	Prior to starting treatment
On Study Medical History Treatment History	Baseline
Safety Labs Correlative Studies	Baseline Cycle 1 Cycle 2 Cycle 3 Cycle 4 Day +100 Day +140 Day +180 Day +270 Day +365
Bone Marrow Response Assessment	Baseline (Bone Marrow only) Cycle 2 Cycle 3 Cycle 4 Day +180 Day +365
FACT-BMT Human Activity Profile 1 of 2 Human Activity Profile 2 of 2	Baseline Day +180 Day +365
Azacitidine Dosing	Cycle 1 Cycle 2 Cycle 3 Cycle 4
DLT Evaluation ANC Engraftment	Cycle 1
Platelet Engraftment	Day +100
Follow-Up	Month 15 Month 18 Month 21 Month 24
End of Study Survival	End of study
Acute GVHD Assessment	Every 1-2 weeks through Day +100 Every 2 weeks through Day +180
ChronicGVHD-Skin-Nails-Scalp-Body Hair Chronic GVHD-Mouth-Eyes	Day +140 Day +180 Day +270

Chronic GVHD-GI-Liver-Lungs Chronic GVHD-Muscle-Fascia-Joints -Genitalia Chronic GVHD-General	Day +365 Month 15 Month 18 Month 21 Month 24
Immunosuppressive Medications	Continuous
Adverse Events	Continuous

11.0 REGULATORY AND REPORTING REQUIREMENTS

The entities providing oversight of safety and compliance with the protocol require reporting as outline below.

The Washington University Human Research Protection Office (HRPO) requires that all events meeting the definition of unanticipated problem or serious noncompliance be reported as outlined in Section 11.7.

The FDA requires that all serious and unexpected adverse events be reported as outlined in Section 11.8. In addition, any fatal or life-threatening adverse experiences where there is a reasonable possibility of relationship to study intervention must be reported.

Celegene requires that all AEs be reported as outlined in Section 11.9.

11.1 Definitions

11.1.1 Adverse Events (AEs)

Definition: any unfavorable medical occurrence in a human subject including any abnormal sign, symptom, or disease.

Grading: the descriptions and grading scales found in the revised NCI Common Terminology Criteria for Adverse Events (CTCAE) version 4.0 will be utilized for all toxicity reporting. A copy of the CTCAE version 4.0 can be downloaded from the CTEP website.

Attribution (relatedness), Expectedness, and Seriousness: the definitions for the terms listed that should be used are those provided by the Department of Health and Human Services' Office for Human Research Protections (OHRP). A copy of this guidance can be found on OHRP's website: (http://www.hhs.gov/ohrp/policy/advevntguid.html).

11.1.2 Serious Adverse Event (SAE)

Definition: any adverse drug experience occurring at any dose that results in any of the following outcomes:

- Death

- A life-threatening adverse drug experience
- Inpatient hospitalization or prolongation of existing hospitalization
- A persistent or significant disability/incapacity (i.e., a substantial disruption of a person's ability to conduct normal life functions)
- A congenital anomaly/birth defect
- Any other experience which, based upon appropriate medical judgment, may jeopardize the subject and may require medical or surgical intervention to prevent one of the outcomes listed above

All unexpected SAEs must be reported to the FDA.

11.1.3 Unexpected Adverse Experience

Definition: any adverse drug experience, the specificity or severity of which is not consistent with the current investigator brochure (or risk information, if an IB is not required or available).
Events that are both serious AND unexpected must be reported to the FDA.

11.1.4 Life-Threatening Adverse Experience

Definition: any adverse drug experience that places the subject (in the view of the investigator) at immediate risk of death from the reaction as it occurred, i.e., it does not include a reaction that, had it occurred in a more severe form, might have caused death.
Life-threatening adverse experiences must be reported to the FDA.

11.2 Reportable Events

All AEs will be collected and reviewed for phase I patients regardless of grade. Only selected AEs as described below will be collected and reviewed for phase II patients:

- All grade 3, 4, or 5 AEs
- Any AE that requires azacitidine treatment to be delayed, held, or discontinued
- Any AE that requires modification of GVHD prophylaxis medications
- Other AEs of interest as determined following completion of the phase I portion of this study

11.3 Unanticipated Problems

Definition:

- unexpected (in terms of nature, severity, or frequency) given (a) the research procedures that are described in the protocol-related documents, such as the IRB-approved research protocol and informed consent document; and (b) the characteristics of the subject population being studied;

- related or possibly related to participation in the research (in this guidance document, possibly related means there is a reasonable possibility that the incident, experience, or outcome may have been caused by the procedures involved in the research); and
- suggests that the research places subjects or others at a greater risk of harm (including physical, psychological, economic, or social harm) than was previously known or recognized.

11.4 Noncompliance

Definition: failure to follow any applicable regulation or institutional policies that govern human subjects research or failure to follow the determinations of the IRB. Noncompliance may occur due to lack of knowledge or due to deliberate choice to ignore regulations, institutional policies, or determinations of the IRB.

11.5 Serious Noncompliance

Definition: noncompliance that materially increases risks, that results in substantial harm to subjects or others, or that materially compromises the rights or welfare of participants.

11.6 Protocol Exceptions

Protocol exceptions are not allowed.

11.7 Reporting to the Human Research Protection Office (HRPO) at Washington University

The PI is required to promptly notify the IRB of the following events:

- Any unanticipated problems involving risks to participants or others which occur at WU, any BJH or SLCH institution, or that impacts participants or the conduct of the study.
- Noncompliance with federal regulations or the requirements or determinations of the IRB.
- Receipt of new information that may impact the willingness of participants to participate or continue participation in the research study.

These events must be reported to the IRB within **10 working days** of the occurrence of the event or notification to the PI of the event. The death of a research participant that qualifies as a reportable event should be reported within **1 working day** of the occurrence of the event or notification to the PI of the event.

11.8 Reporting to the Quality Assurance and Safety Monitoring Committee (QASMC) at Washington University

The PI is required to notify the QASMC of any unanticipated problem occurring at WU or any BJH or SLCH institution that has been reported to and acknowledged by HRPO as reportable. (Unanticipated problems reported to HRPO and withdrawn during the review process need not be reported to QASMC.)

QASMC must be notified within 10 days of receipt of IRB acknowledgment via email to a QASMC auditor.

11.9 Reporting to the FDA

The conduct of the study will comply with all FDA safety reporting requirements. **PLEASE NOTE THAT REPORTIING REQUIREMENTS FOR THE FDA DIFFER FROM REPORTING REQUIREMENTS FOR HRPO/QASMC.** It is the responsibility of the investigator to report any unanticipated problem to the FDA as follows:

Report any unexpected fatal or life-threatening adverse experiences (Section 11.1.4) associated with use of the drug by telephone or fax no later than **7 calendar days** after initial receipt of the information. Report any serious, unexpected adverse experiences (Section 11.1.3), as well as results from animal studies that suggest significant clinical risk within **15 calendar days** after initial receipt of this information.

All MedWatch forms will be sent by the investigator or investigator's team to the FDA at the following address or by fax:

Food and Drug Administration
Center for Drug Evaluation and Research
Division of Oncology Drug Products
5901-B Ammendale Rd.
Beltsville, MD 20705-1266
FAX: 1-800-FDA-0178

11.10 Reports to Celgene

The investigator will also provide Celgene Corporation (as a supplier of azacitidine (VIDAZA) for this study) a copy of the Investigator IND annual report at the time of the investigators submission to the FDA. Submit reports to Celgene at:

Celgene Corporation / Attn: Medical Affairs Operations
Connell Corporate Park
400 Connell Drive, Suite 700
Berkeley Heights, NJ 07922
Tel: (908) 673-9000

All AE reports must include the patient number, age, sex, weight, severity of reaction (mild, moderate, severe), relationship to drug, date and time of administration of test medications and all concomitant medications, and medical treatment provided. The investigator is responsible for evaluating all adverse events to determine whether criteria for "serious" and as defined above are present**.** The investigator is responsible for reporting adverse events to Celgene as described below.

11.10.1 Expedited Reporting by Investigator to Celgene

Serious adverse events/unanticipated problems (SAE) are defined above. The investigator must inform Celgene of all SAEs within 24 hours of being aware of the event (regardless of of whether the SAE is expected or not). This must be documented on Celgene SAE form or a FDA 3500 or MEDWATCH form. This form must be completed and supplied to Celgene within 24 hours/1 business day. The initial report must be as complete as possible, including details of the current illness and (serious) adverse event, and an assessment of the causal relationship between the event and the investigational product(s). Information not available at the time of the initial report (e.g., an end date for the adverse event or laboratory values received after the report) must be documented on a follow-up MEDWATCH. A final report to document resolution of the SAE is required. The Celgene tracking number (VZ-CL-AML-PI-002423) and the institutional protocol number should be included on SAE reports (or on the fax cover letter) sent to Celgene. A copy of the fax transmission confirmation of the SAE report to Celgene should be attached retained with the patient records. Celgene drug safety contact information is as follows:

Celgene Corporation
Global Drug Safety and Risk Management
Connell Corporate Park
300 Connell Dr., Suite 6000
Berkley Heights, NJ 07922
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11.11 Pregnancies

Pregnancy of a female subject or the female partner of a male subject occurring while the subject is on azacitidine or within 4 weeks after the subject's last dose of azacitidine are considered expedited reportable events. If the subject is on azacitidine, it is to be discontinued immediately. The pregnancy must be reported to Celgene Drug Safety within 24 hours of the Investigator's knowledge of the pregnancy by phone and facsimile using the SAE Form.

The Investigator will follow the pregnant female until completion of the pregnancy, and must notify Celgene Drug Safety of the outcome as specified below. The Investigator will provide this information as a follow-up to the initial SAE.

If the outcome of the pregnancy meets the criteria for immediate classification as a SAE (i.e., spontaneous abortion [any congenital anomaly detected in an aborted fetus is to be documented], stillbirth, neonatal death, or congenital anomaly), the Investigator should follow the procedures for reporting SAEs (i.e., report the event to Celgene Drug Safety by facsimile within 24 hours of the Investigator's knowledge of the event).

Any suspected fetal exposure to azacitidine must be reported to Celgene within 24 hours of being made aware of the event. The pregnant female should be referred to an obstetrician/gynecologist experienced in reproductive toxicity for further evaluation and counseling. All neonatal deaths that occur within 30 days of birth should be reported, without regard to causality, as SAEs. In addition, any infant death after 30 days that the Investigator suspects is related to the *in utero* exposure to the study drug should also be reported. In the case of a live "normal" birth, Celgene Drug Safety should be advised as soon as the information is available.

11.12 Timeframe for Reporting Required Events

AEs will be collected from the time of the first dose of azacitidine (Day +7) through the Day 140 visit or 30 days after last dose of azacitidine (for patients that do no complete all four cycles of treatment).

12.0 DATA AND SAFETY MONITORING

In compliance with the Washington University Institutional Data and Safety Monitoring Plan, the Data and Safety Monitoring Committee (DSMC) will meet to review toxicity data at least every 6 months. The report will be prepared by the statistician with assistance from the study team and will be submitted to the Washington University Quality Assurance and Safety Monitoring Committee (QASMC).

Additionally, during the phase I dose escalation, the Principal Investigator will review all patient data at least monthly (or before each dose-escalation if occurring sooner than monthly), and provide a semi-annual report to the Quality Assurance and Safety Monitoring Committee (QASMC). In both instances, this report will include:

- HRPO protocol number, protocol title, Principal Investigator name, data coordinator name, regulatory coordinator name, and statistician
- Date of initial HRPO approval, date of most recent consent HRPO approval/revision, date of HRPO expiration, date of most recent QA audit, study status, and phase of study
- History of study including summary of substantive amendments; summary of accrual suspensions including start/stop dates and reason; and summary of protocol error(s), or breach of confidentiality including start/stop dates and reason

- Study-wide target accrual and study-wide actual accrual
- Protocol activation date
- Average rate of accrual observed in year 1, year 2, and subsequent years
- Expected accrual end date and accrual by cohort
- Objectives of protocol with supporting data and list the number of participants who have met each objective
- Measures of efficacy
- Early stopping rules with supporting data and list the number of participants who have met the early stopping rules
- Summary of toxicities separated by cohorts with the number of dose-limiting toxicities indicated
- Abstract submissions/publications
- Summary of any recent literature that may affect the safety or ethics of the study

The study principal investigator and Research Patient Coordinator will monitor for serious toxicities on an ongoing basis during both the phase I (as outlined above) and every 6 months during the phase II portion of the trial. Once the principal investigator or Research Patient Coordinator becomes aware of an adverse event, the AE will be reported to the HRPO and QASMC according to institutional guidelines.

A DSMC will consist of no fewer than 3 members including 2 clinical investigators and a biostatistician. Like investigators, DSMC members are subject to the Washington University School of Medicine policies regarding standards of conduct. Individuals invited to serve on the DSMC will disclose any potential conflicts of interest to the trial principal investigator and/or appropriate university officials, in accordance with institution policies. Potential conflicts that develop during a trial or a member's tenure on a DSMC must also be disclosed.

Refer to the Washington University Quality Assurance and Data Safety Monitoring Committee Policies and Procedures for full details on the responsibilities of the DSMC at http://www.siteman.wustl.edu/uploadedFiles/Research_Programs/Clinical_Research_Resources/Protocol_Review_and_Monitoring_Committee/QASMCQualityAssurcance.pdf

13.0 STUDY EFFICACY AND DISCONTINUATION

13.1 Criteria to Measure Efficacy in Mitigation of GvHD

GVHD rate and severity will be assessed based on modified Glucksberg criteria.[70] Grade II-IV and III-IV aGVHD in first100 days after transplant will be assessed.

13.2 Definitions for Safety and Efficacy Assessments

13.2.1 Neutrophil Engraftment

Time to neutrophil engraftment is measured by determining the first of 3 consecutive measurements of neutrophil count ≥ 500/ul following conditioning regimen-induced nadir.

13.2.2 Platelet Engraftment

Time to platelet engraftment is measured by determining the first of 3 consecutive measurements of platelet count ≥ 20,000/ul without platelet transfusion support for 7 days.

13.2.3 Primary Graft Failure

Failure of neutrophil engraftment by Day 28.

13.2.4 Secondary Graft Failure

Primary engraftment followed by a drop in the neutrophil count to less than 500/ml for more than 3 consecutive days without any apparent cause such as drugs or opportunistic infection.

13.2.5 Full Donor Chimerism

Greater than or equal to 95% donor cells within the bone marrow.

13.2.6 Acute GVHD

Incidence and severity of acute GVHD will be assessed based on the modified Glucksberg criteria[70] and Seattle criteria.[71] Attempts should be made to confirm the diagnosis pathologically by biopsy of target organ(s).

13.2.7 Steroid Refractory Acute GVHD

Steroid refractory acute GvHD will be defined as no improvement in GvHD within 7 days of starting high dose system steroids (2mg/kg) or progression of GvHD while on 2mg/kg steroids.

13.2.8 Chronic GVHD

Incidence and severity of chronic GVHD will be assessed based on the NIH consensus criteria and global severity scoring system.[72] Attempts should be made to confirm the diagnosis pathologically by biopsy of target organ(s).

13.2.9 Treatment-Related Mortality

Death that results from a transplant procedure-related complication (e.g. infection, organ failure, hemorrhage, GVHD) rather than from relapse of the underlying disease or an unrelated cause.

13.2.10 Adverse Events

Adverse events will be assessed and graded according to NCI Common Toxicity Criteria version 4 as outlined in Section 10.2.

13.2.11 Determination of Relapse

A patient will be considered relapsed when there is a recurrence of the original malignant disease after transplantation. The time to relapse is the time from the date of transplant to date of the first observation of hematologic, radiographic, or cytogenetic changes, which result in characterization as relapse.

13.2.12 Determination of Survival

Survival will be measured by assessing if the patient remains alive by visual observation, telephone call or notification of death medical record or death certificate.

13.2.13 Evaluable for Toxicity

All subjects will be evaluable for toxicity from the time of their first dose of azacitidine.

13.2.14 Overall Survival

Defined as the date of transplant to the date of death from any cause.

13.2.15 Duration of Remission

Defined as the interval from the date complete remission is documented to the date of recurrence.

13.2.16 Disease-Free Survival

Defined as the interval from the date of first documentation of a CR to date of relapse.

13.3 Response Review

At the end of the study all responses will be reviewed by an expert independent of the study.

14.0 STATISTICAL CONSIDERATIONS

14.1 Study Design

The primary objectives of this study are: (1) to determine the maximum tolerated dose of azacitidine in patients undergoing matched (8 out of 8) unrelated donor transplant for any hematological malignancy in remission or with stable disease (phase I) and (2) to determine the effect of azacitidine on grade II-IV GvHD in patients undergoing matched (8 out of 8) unrelated donor transplant for AML in remission 1 or 2 or MDS (phase II).

14.1.1 Phase I

A 3+3 phase I clinical trial will be conducted to assess the toxicity profile and establish the MTD of azacitidine after allo-HSCT in patients with any hematological malignancy in remission or with stable disease. This is a dose escalating phase I, single arm, single institute, open label, non-randomized study. Three cohorts are planned; in addition, there exists one dose de-escalation cohort. A minimum of 12 and maximum of 24 patients will be treated in the phase I portion.

14.1.2 Stopping Rules for Phase I

If >1 patient in cohort 1 has a DLT, then the trial will be stopped and no phase II portion of the trial will be performed.

If graft failure with donor engraftment or loss of donor engraftment by Day 100 occurs in ≥20% of subjects, then trial will be stopped.

If grade III – IV acute GvHD occurs in ≥50% of subjects, then the trial will be stopped.

14.1.3 Phase II

Provided there are no reasons for stopping the trial in phase I, an open label single arm phase II trial will be conducted based on the MTD determined from the phase I portion. A total of 46 patients will be treated in the phase II portion of the trial. Efficacy of acute GvHD prevention will be compared to matched historical controls (matched for age, sex and disease) from the CIBMTR registry.

Toxicity will be assessed on an ongoing basis every 6 months during the phase II portion. The interim analyses will be performed as per Section 14.4.4.

14.2 Sample Size Calculation

14.2.1 Phase I

Given the 3+3 design of the phase I portion of this trial, a minimum of 12 patients and a maximum of 24 will be enrolled in the phase I portion of the trial.

14.2.2 Phase II

Given the exploratory nature of this study we are willing to accept an alpha of 0.1. Using a two-sided alpha of 0.1, 80% power and a Fisher's exact test, we will need a sample size of 46 per group to detect a statistical difference if the incidence of grade II-IV acute GvHD in the azacitidine group is 20%, assuming the incidence of 44% in the standard care group (matched historical controls from the CIBMTR registry).

14.2.3 Correlative Studies

Our pilot data shows that the mean change in Tregs in patients undergoing peripheral blood stem cell transplant is 4%, and if we assume that the mean change in the azacitidine group will be at least 2 fold (8%) (based on Goodyear et al.[59]), and assuming the standard deviation is 2.5%, the effect size is calculated as (8%-4%)/2.5%=1.6. Therefore we need 8 subjects in each group. (See effect size table below).

Effect size	Sample size per group
0.6	45
0.7	34
0.8	26
0.9	21
1	17
1.2	12
1.6	8

14.3 Accrual

We perform over 100 unrelated allogeneic HSCT per year at Washington University, approximately 50% of which are for AML. We estimate around 30 patients/year may be eligible for this trial. We anticipate enrollment of approximately 2-3 patients per month. Historically, around 50% of transplants are performed using reduced intensity conditioning.

14.4 Statistical Analyses

14.4.1 Descriptive Analysis

14.4.1.1 Patient Disposition

The number of patients discontinued, the reasons for discontinuation, and the amount of therapy administered will be summarized by patient and by reason for discontinuation.

14.4.1.2 Protocol Deviations

All significant deviations will be summarized by patient and by type of deviation.

14.4.1.3 Demographics and Baseline Characteristics

Descriptive summary statistics will be provided for demographic and important baseline characteristics including gender, age, performance status, FAB/WHO AML subtype, and cytogenetic abnormalities. For continuous variables, the number of patients, mean, standard deviation, median, minimum and maximum will be provided. For categorical variables the number and percentage of patients in each category will be summarized.

14.4.2 Primary and Secondary Endpoint Analysis

MTD will be determined as described in Section 5.3.1.

Type and grade of GVHD will be documented based on modified Glucksberg criteria.[70]
GVHD rate, and survival at 100, 180, and 365 days after transplant will be summarized using descriptive statistics. An exploratory descriptive stratified analysis by transplant conditioning regimen, and acute GvHD at day 180 and at one year after transplant will be performed, but the study is not powered to detect a difference between conditioning groups given its exploratory nature and large retrospective studies showing similar outcomes.[65,66]

Anyone dying prior to Day 100 without acute GvHD will be censored in assessing the incidence of Grade II-IV acute GvHD in the first 100 days after transplant in patients with MDS and AML. Subjects dying prior to study drug will be excluded and replaced.

Overall survival (OS) is defined as the date from transplant to death or last follow-up. Relapse-free survival (RFS) is defined as the time from the date of transplant to the date of relapse or last follow-up. Kaplan-Meier (KM) curves for OS and RFS will be generated.

Treg frequency and number will be assessed by paired analysis with control banked samples at pre-transplant (donor PBSC product), Day 30, and Day 100. Paired t-

test or Wilcoxon signed rank test will be used to compare Day 30 (or 100) change from the pre-transplant between azacitidine and standard care group. Repeated analysis which accounts for the correlation across the three time points (pre-transplant, Day 30, and Day 100) per subject will be considered as appropriate.

14.4.3 Safety Analysis

Subjects who receive at least one dose of azacitidine will be monitored for safety. AEs will be coded according to CTCAE v 4.0. The results will be tabulated to examine their frequency, organ systems affected, and relationship to study treatment.

The results of laboratory assessments will be evaluated similarly. Interim safety data will be examined on an ongoing basis to ensure subject safety. In addition the Quality Assurance and Safety Monitoring Committee (QASMC) will review the DSM semi-annually.

Applicable laboratory parameters will be graded according to NCI CTCAE v4.0. The incidence of maximum grade (number and percent of patients experiencing the maximum grade during the study) will be summarized for each laboratory parameter. Graphical display of selected hematological parameters will also be provided.

Vital signs, physical examination findings, ECOG performance status scores, and weights will be presented in listing format.

14.4.4 Interim Analysis

During the phase II portion of the study three interim analyses will be planned for TRM and hematologic toxicity as defined in sections **13.2.1-13.2.3**. A four-stage group sequential design with an O'Brien-Fleming method will be used to stop the trial early.

Assuming TRM in the azacitidine group is 30% and TRM in the standard care group is 10%, the design uses a one-sided alternative hypothesis with early stopping to reject the null hypothesis - TRM in the azacitidine group is same as TRM in the standard care group. The following table displays the required sample size and boundary values at each interim analysis. At each interim stage, if the standardized test statistics is larger than the corresponding boundary, then trial is stopped and we reject the null hypothesis. Otherwise, the process continues to next interim. At the final analysis, 4^{th} interim, the trial stops and TRM in the azacitidine group is statistically larger than TRM in the standard care group if the standardized statistics >=0.13705. Otherwise, TRM in the azacitidine group is statistically same from TRM in the standard care group.

Interim	N (Azacitidine)	N (Standard care)	**Boundary**
1	12	12	0.54819
2	24	24	0.27409
3	36	36	0.18273
4	48	48	0.13705

Assuming hematologic toxicity in the azacitidine group is 20% and hematologic toxicity in the standard care group is 5%, the design uses a one-sided alternative hypothesis with early stopping to reject the null hypothesis - hematologic toxicity in the azacitidine group is same as in the standard care group.

Interim	N (Azacitidine)	N (Standard care)	**Boundary**
1	15	15	0.41114
2	30	30	0.20557
3	45	45	0.13705
4	59	59	0.10278

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APPENDIX 1: ECOG Performance Status Scale

Grade	Description
0	Normal activity. Fully active, able to carry on all pre-disease performance without restriction.
1	Symptoms, but ambulatory. Restricted in physically strenuous activity, but ambulatory and able to carry out work of a light or sedentary nature (e.g., light housework, office work).
2	In bed <50% of the time. Ambulatory and capable of all self-care, but unable to carry out any work activities. Up and about more than 50% of waking hours.
3	In bed >50% of the time. Capable of only limited self-care, confined to bed or chair more than 50% of waking hours.
4	100% bedridden. Completely disabled. Cannot carry on any self-care. Totally confined to bed or chair.
5	Dead.

APPENDIX 2: Registration Form

Patient Initials ____-____-____	Date of Birth: ____/____/____ Age: _____yr
Sex: ☐ Male ☐ Female	Race: ☐ White ☐ Black or African American ☐ Native Hawaiian or Other Pacific Islander ☐ Asian ☐ American Indian or Alaska Native ☐ Unknown
Diagnosis: ☐ AML ☐ MDS ☐ Other Specify Other: _____	Ethnicity: ☐ Hispanic or Latino ☐ Non-Hispanic ☐ Unknown
Informed Consent Date: ____/____/____	Proposed Registration Date: ____/____/____
Current Zip Code:_____	ECOG at Baseline: ☐ 0 ☐ 1 ☐ 2
Does the participant satisfy all of the eligibility criteria? ☐ Yes ☐ No	
Were any waivers granted? ☐ Yes ☐ No	

Person completing this form

_____

Printed name Signature Date

Registering MD

_____

Printed name Signature Date

Please include:

1. Copy of signed consent form
2. Completed eligibility checklist, signed and dated by a member of the study team
3. Copy of appropriate source documentation confirming patient eligibility

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To be completed by PI or designee

Assigned UPN _____ Dose Level: ☐ 1 ☐ 2 ☐ 3 ☐ 4

Signature _____ Date_____

APPENDIX 3: Acute GVHD Assessment

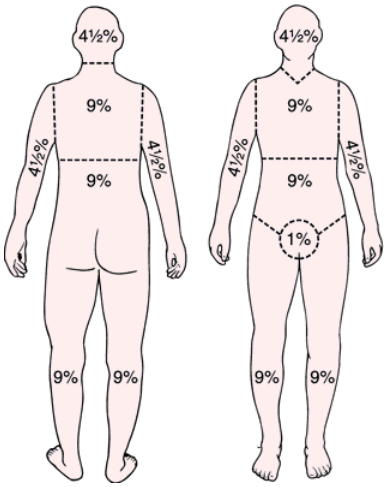
Date of Assessment: _____

Date of GvHD Onset: _____

Acute GVHD Stage			
Skin[1]	Stage 1	Rash on < 25% of skin	
	Stage 2	Rash on 25-50% of skin	
	Stage 3	Rash on > 50% of skin	
	Stage 4	Generalized erythoderma with bollous formation	
Liver[2]	Stage 1	Bilirubin 2-3 mg/dl	
	Stage 2	Bilirubin 3-6 mg/dl	
	Stage 3	Bilirubin 6-15 mg/dl	
	Stage 4	Bilirubin >15 mg/dl	
Gut	Stage 1	Diarrhea >500 ml/day or persistent nausea[3]	
	Stage 2	Diarrhea >1000 ml/day	
	Stage 3	Diarrhea >1500 ml/day	
	Stage 4	Severe abdominal pain with or without ileus	

Acute GVHD Grade			
	Skin	Liver	Gut
[ ] Grade 1	Stage 1-2	None	None
[ ] Grade 2	Stage 3 or	Stage 1 or	Stage 1
[ ] Grade 3	--	Stage 2-3 or	Stage 2-4
[ ] Grade 4	Stage 4	Stage 4	--

1. Use 'Rule of Nines' to determine extent of rash



2. Based on peak total blirubin. Decrease by one stage if another cause of hyperbilrubinemia has been documented
3. Persistent nausea with histologic confirmation of GVHD in the stomach or duodenum

Reference: D Przepiorka, D Weisdorf, P Martin et al. Consensus conference on acute GVHD grading. Bone marrow transplantation. 1995;15:825-828

APPENDIX 4: Chronic GVHD Assessment

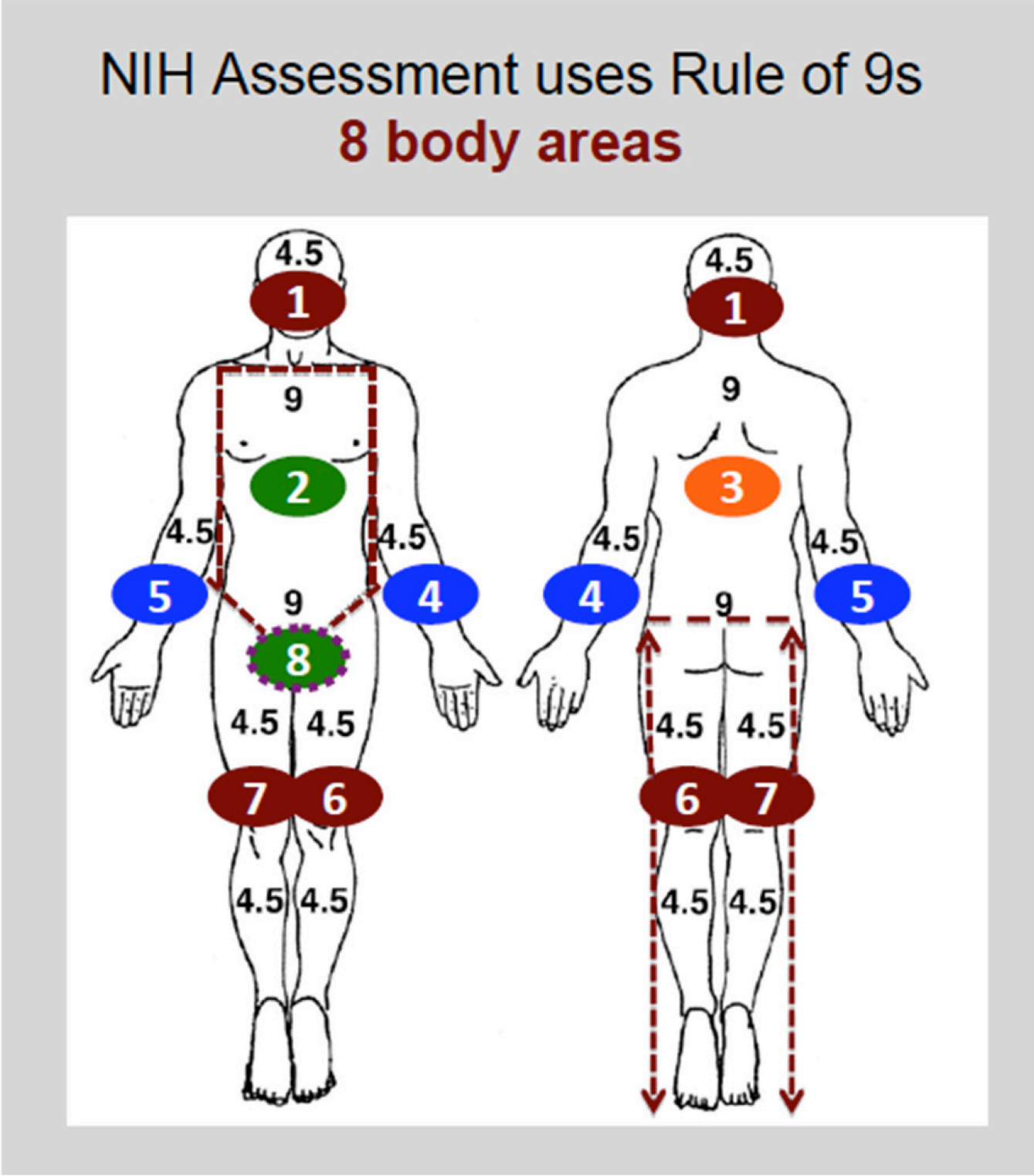
Date of Assessment: _____

SKIN (Patient History and Exam)
Score 0 ☐ Score 1 ☐ Score 2 ☐ Score 3 ☐

	0	1	2	3
Skin Score	No Symptoms	< 18% BSA with disease signs but **NO** sclerotic features	19-50% BSA **OR** involvement with superficial sclerotic features "not hidebound" (able to pinch	>50% BSA **OR** deep sclerotic features "hidebound" (unable to pinch) **OR** impaired mobility, ulceration or severe pruritus

NIH item that scores maximum severity based on:
- → Percent body surface area involved by erythema ***OR***
- → Degree of sclerotic features ***OR***
- → Impaired mobility ***OR***
- → Ulceration ***OR***
- → Severe pruritus

Maculopapular rash	☐	Erythroderma	☐
Lichen planus-like features	☐	Poikiloderma	☐
Papulosquamous lesions or ichthyosis	☐	Sclerotic features	☐
Hyperpigmentation	☐	Pruritus	☐
Hypopigmentation	☐	Hair Involvement	☐
Keratosis pilaris	☐	Nail Involvement	☐
Erythema	☐	% BSA involved: _____	☐



***% BSA Reference**

cGVHD signs and symptoms seen with NAILS, SCALP, BODY HAIR

Score 0 ☐ Score 1 ☐ Score 2 ☐ Score 3 ☐

Check all that apply:

Distinctive (seen in Chronic GVHD, but insufficient alone to establish diagnosis of Chronic GVHD)

Dystrophy ☐
Longitudinal ridging, splitting, or brittle features ☐
Onycholysis ☐
Pterygium unguis ☐
Nail loss (usually symmetic; affects most nails) ☐
New onset of scarring or nonscarring scalp alopecia (after recovery from chemoradiotherapy) ☐
Scaling, papulosquamous lesions ☐

Other features/common (seen with both Acute and Chronic GVHD)

Thinning scalp hair, typically patchy, course or dull (not explained by endocrine or other causes) ☐
Premature gray hair ☐

Please describe:
Patient timeline, extent of finding, duration/rapidity of onset, relationship to immunosuppression and likely attributability to GVHD

_____
_____
_____

cGVHD signs and symptoms seen with MOUTH
Score 0 ☐ Score 1 ☐ Score 2 ☐ Score 3 ☐

	0	1	2	3
Oral Score	No Symptoms	Mild symptoms with disease signs but not limiting oral intake significantly	Moderate symptoms with disease signs with partial limitation of oral intake	Severe symptoms with disease signs on examination with major limitation of oral intake

Oral Exam
Check all that apply:

Diagnostic (sufficient to establish Chronic GVHD)
Lichen-type features ☐
Hyperkeratotic plaques ☐
Restriction of mouth opening from sclerosis ☐

Distinctive (seen in Chronic GVHD, but insufficient alone to establish diagnosis of Chronic GVHD)
Xerostomia ☐
Mucocele ☐
Muscosal atrophy ☐
Pseudomembranes ☐
Ulcers ☐

Other features/common (seen with both Acute and Chronic GVHD)
Gingivitis ☐
Mucositis ☐
Erythema ☐
Pain ☐

Please describe:
Patient timeline, extent of finding, duration/rapidity of onset, relationship to immunosuppression and likely attributability to GVHD

_____
_____
_____

cGVHD signs and symptoms seen with EYES

Score 0 ☐ Score 1 ☐ Score 2 ☐ Score 3 ☐

	0	1	2	3
Eye Score	No Symptoms	Mild dry eye symptoms not affecting ADL (requiring eye drops <3x per day) OR asymptomatic signs of keratoconjunctivitis sicca	Moderate dry eye symptoms partially affecting ADL (requiring eye drops >3x per day or punctal plugs) without vision impairment	Severe dry eye symptoms significantly affecting ADL (special eyewear to relieve pain) **OR** unable to work because of ocular symptoms **OR** loss of vision cause by keratoconjunctivitis sicca

Nuances for a patient using drops < 3 x per day:

- But wearing a Boston Scleral lens → Score 3
- But had punctal plugs placed 7 days → Score 2
- But had plugs placed a month ago →Score 1

Check all that apply:

Distinctive (seen in Chronic GVHD, but insufficient alone to establish diagnosis of Chronic GVHD)

New onset dry, gritty, or painful eyes ☐
Cicatricial conjunctivitis ☐
Keratoconjunctivitis sicca ☐
Confluent areas of punctate keratopathy ☐

Other features/common (seen with both Acute and Chronic GVHD)

Photophobia ☐
Periorbital hyperpigmentation ☐
Blepharitis (erythema of the eyelids with edema) ☐

Please describe:
Patient timeline, extent of finding, duration/rapidity of onset, relationship to immunosuppression and likely attributability to GVHD

_____
_____
_____

cGVHD signs and symptoms seen with GI TRACT
Score 0 ☐ Score 1 ☐ Score 2 ☐ Score 3 ☐

	0	1	2	3
GI Tract Score	No Symptoms	Symptoms such as dysphagia, anorexia, nausea, abdominal pain or diarrhea without significant weight loss (<5%)	Symptoms with mild to moderate weight loss (5-15%)	Symptoms associated with significant weight loss >15%, requires nutritional supplement for most calorie needs OR esophageal dilation

Check all that apply:

Diagnostic (sufficient to establish Chronic GVHD)
Esophageal web ☐
Strictures or stenosis in the upper to mid third of the esophagus ☐

Other features/common (seen with both Acute and Chronic GVHD)
Exocrine – pancreatic insufficiency ☐
Anorexia ☐
Nausea ☐
Vomiting ☐
Diarrhea ☐
Weight loss ☐
Failure to thrive (infants and children) ☐

Please describe:
Patient timeline, extent of finding, duration/rapidity of onset, relationship to immunosuppression and likely attributability to GVHD

_____
_____
_____

cGVHD signs and symptoms seen with LIVER

Score 0 ☐ Score 1 ☐ Score 2 ☐ Score 3 ☐

Liver Score	0	1	2	3
	Normal LFT	Elevated Bilirubin, AP, AST or ALT <2x ULN	Bilirubin >3 mg/dl or Bilirubin, enzymes 2-5 x ULN	Bilirubin or enzymes >5 x ULN

Check all that apply:

Other features/common (seen with both Acute and Chronic GVHD)

Total Bilirubin, alkaline phosphatase
>2x upper limit of normal ☐
ALT or AST > 2 x upper limit of normal ☐

Please describe:
Patient timeline, extent of finding, duration/rapidity of onset, relationship to immunosuppression and likely attributability to GVHD

_____

_____

_____

cGVHD signs and symptoms seen with LUNGS
Score 0 ☐ Score 1 ☐ Score 2 ☐ Score 3 ☐

	0	1	2	3
Lung Score	No Symptoms FEV1 > 80% **OR** LFS = 2	Mild symptoms (shortness of breath after climbing 1 flight of steps) FEV1 60-79% **OR** LFS 3-5	Moderate symptoms (shortness of breath after walking on flat ground) FEV1 40-59% **OR** LFS 6-9	Severe symptoms (shortness at rest requiring O_2) FEV1 $\geq$39% **OR** LFS 10-12

FEV1:_____ Not done☐ NA☐
DLCO: _____ Not done☐ NA☐
LFS: _____ Not done☐ NA☐

Check all that apply:

Diagnostic (sufficient to establish Chronic GVHD)
Bronchiolitis obliterans diagnosed with lung biopsy ☐

Distinctive (seen in Chronic GVHD, but insufficient alone to establish diagnosis of Chronic GVHD)
Bronchiolitis obliterans diagnosed with PFTs and radiology ☐

Other features/common (seen with both Acute and Chronic GVHD)
Bronchiolitis obliterans with organizing pneumonia (BOOP) ☐

Please describe:
Patient timeline, extent of finding, duration/rapidity of onset, relationship to immunosuppression and likely attributability to GVHD

_____
_____
_____

cGVHD signs and symptoms seen with MUSCLE, FASCIA, JOINTS
Score 0 ☐ Score 1 ☐ Score 2 ☐ Score 3 ☐

	0	1	2	3
Muscles, Fascia & Joints Score	No Symptoms	Mild tightness of arms or legs, normal or mild decreased range of motion (ROM) **AND** not affecting ADL	Tightness of arms or legs **OR** joint contractures, erythema thought due to fasciitis, moderate decrease ROM **AND** mild to moderate limitation to ADL	Contractures **WITH** significant decrease of ROM **AND** significant limitation of ADL (unable to tie shoes, button shirt, dress self etc.)

Check all that apply:

Diagnostic (sufficient to establish Chronic GVHD)

Fasciitis ☐
Joint stiffness or contracture secondary to sclerosis ☐

Distinctive (seen in Chronic GVHD, but insufficient alone to establish diagnosis of Chronic GVHD)
Myositis or polymyositis ☐

Other features/common (seen with both Acute and Chronic GVHD)
Edema ☐
Muscle cramps ☐
Arthralgia or arthritis ☐

Please describe:
Patient timeline, extent of finding, duration/rapidity of onset, relationship to immunosuppression and likely attributability to GVHD

_____
_____
_____

cGVHD signs and symptoms seen with GENITALIA

Score 0 ☐ Score 1 ☐ Score 2 ☐ Score 3 ☐

	0	1	2	3
Genitalia Score	No Symptoms	Symptomatic with mild signs on exam **AND** no effect on coitus and minimal discomfort with gynecologic exam	Symptomatic with moderate signs on exam **AND** with mild dyspareunia **OR** with discomfort with gynecologic exam	Symptomatic **WITH** advanced sign (stricture, labial, agglutination, or severe ulceration) **AND** severe pain with coitus or inability to insert vaginal speculum

Check all that apply:

Diagnostic (sufficient to establish Chronic GVHD)

Lichen planus-like features ☐

Vaginal scarring or stenosis ☐

Distinctive (seen in Chronic GVHD, but insufficient alone to establish diagnosis of Chronic GVHD)

Erosion ☐

Fissures ☐

Ulcers ☐

Other Indicators	None	Mild	Moderate	Severe	Not Assessed
Ascites (serositis)					
Myasthenia Gravis					
Polymyositis					
Platelets less than 100,000/ul					
Pericardial effusion					
Nephrotic syndrome					
Cardiomyopathy					
Cardiac conduction defects					
Progressive onset					
Pleural Effusion(s)					
Peripheral Neuropathy					
Eosinophilia >500ul					
Coronary artery					

Other, specify:_____

Does the patient have chronic GVHD? Yes ☐ No ☐
If yes, specify Severity: Mild ☐ Moderate ☐ Severe ☐

NIH CGVHD Global Severity Category reflects overall disability

SEVERITY	ORGAN SCORE	NO. OF ORGANS
Mild	**All 1 (0 for Lung)**	**1-2**
Moderate	**All 1 (0 for Lung)**	**3 or more**
	At least one 2 (1 for Lung)	**1-2**
Severe	**At least one 3 (2 for Lung)**	**1 or more**

Were there additional exams performed? Yes ☐ No ☐
If yes, check all that apply:

Procedure	Date	Result
☐Biopsy		
☐CT Scan		
☐MRI		
☐Photo		
☐Schirmer Eye Test		
☐ PFT		
☐ LFT		
☐Ultrasound/Echocardiogram		
☐X-Ray		
☐Other:_____		

Signature: _____ **Date:** _____

APPENDIX 5: FACT-BMT (Version 4)

Below is a list of statements that other people with your illness have said are important**. Please circle or mark one number per line to indicate your response as it applies to the <u>past 7 days</u>.**

	<u>PHYSICAL WELL-BEING</u>	**Not at all**	**A little bit**	**Some-what**	**Quite a bit**	**Very much**
GP1	I have a lack of energy	0	1	2	3	4
GP2	I have nausea	0	1	2	3	4
GP3	Because of my physical condition, I have trouble meeting the needs of my family	0	1	2	3	4
GP4	I have pain	0	1	2	3	4
GP5	I am bothered by side effects of treatment	0	1	2	3	4
GP6	I feel ill	0	1	2	3	4
GP7	I am forced to spend time in bed	0	1	2	3	4

	<u>SOCIAL/FAMILY WELL-BEING</u>	**Not at all**	**A little bit**	**Some-what**	**Quite a bit**	**Very much**
GS1	I feel close to my friends	0	1	2	3	4

GS2	I get emotional support from my family	0	1	2	3	4
GS3	I get support from my friends	0	1	2	3	4
GS4	My family has accepted my illness	0	1	2	3	4
GS5	I am satisfied with family communication about my illness	0	1	2	3	4
GS6	I feel close to my partner (or the person who is my main support)	0	1	2	3	4
Q1	*Regardless of your current level of sexual activity, please answer the following question. If you prefer not to answer it, please mark this box* ☐ *and go to the next section.*					
GS7	I am satisfied with my sex life	0	1	2	3	4

Please circle or mark one number per line to indicate your response as it applies to the <u>past 7 days</u>.

	<u>EMOTIONAL WELL-BEING</u>	**Not at all**	**A little bit**	**Some-what**	**Quite a bit**	**Very much**
GE1	I feel sad	0	1	2	3	4
GE2	I am satisfied with how I am coping with my illness	0	1	2	3	4
GE3	I am losing hope in the fight against my illness	0	1	2	3	4

GE4	I feel nervous	0	1	2	3	4
GE5	I worry about dying	0	1	2	3	4
GE6	I worry that my condition will get	0	1	2	3	4

	FUNCTIONAL WELL-BEING	**Not at all**	**A little bit**	**Some-what**	**Quite a bit**	**Very much**
GF1	I am able to work (include work at home)	0	1	2	3	4
GF2	My work (include work at home) is fulfilling	0	1	2	3	4
GF3	I am able to enjoy life	0	1	2	3	4
GF4	I have accepted my illness	0	1	2	3	4
GF5	I am sleeping well	0	1	2	3	4
GF6	I am enjoying the things I usually do for fun	0	1	2	3	4
GF7	I am content with the quality of my life right	0	1	2	3	4

Please circle or mark one number per line to indicate your response as it applies to the <u>past 7 days</u>.

	ADDITIONAL CONCERNS	**Not at all**	**A little bit**	**Some-what**	**Quite a bit**	**Very much**
BMT1	I am concerned about keeping my job (include work at home)	0	1	2	3	4
BMT2	I feel distant from other people	0	1	2	3	4
BMT3	I worry that the transplant will not work	0	1	2	3	4
BMT4	The effects of treatment are worse than I had imagined	0	1	2	3	4
C6	I have a good appetite	0	1	2	3	4
C7	I like the appearance of my body	0	1	2	3	4
BMT5	I am able to get around by myself	0	1	2	3	4
BMT6	I get tired easily	0	1	2	3	4
BL4	I am interested in sex	0	1	2	3	4
BMT7	I have concerns about my ability to have children	0	1	2	3	4
BMT8	I have confidence in my nurse(s)	0	1	2	3	4
BMT9	I regret having the bone marrow transplant	0	1	2	3	4
BMT10	I can remember things	0	1	2	3	4
Br1	I am able to concentrate	0	1	2	3	4
BMT11	I have frequent colds/infections	0	1	2	3	4
BMT12	My eyesight is blurry	0	1	2	3	4
BMT13	I am bothered by a change in the way food tastes	0	1	2	3	4
BMT14	I have tremors	0	1	2	3	4
B1	I have been short of breath	0	1	2	3	4
BMT15	I am bothered by skin problems (e.g., rash, itching)	0	1	2	3	4
BMT16	I have trouble with my bowels	0	1	2	3	4
BMT17	My illness is a personal hardship for my close family members	0	1	2	3	4
BMT18	The cost of my treatment is a burden on me or my family	0	1	2	3	4

APPENDIX 6: Human Activity Profile

This is a list of common physical activities. For each activity, check whether you currently engage in it, no longer engage in it, or have never engaged in it. The best way to decide is to ask yourself whether you would engage in the activity *today* if you had the opportunity. Please read the instructions and then complete the items as accurately as you can.

Patient Initials: _____ **Study ID#:** _____

	Still Doing	Stopped Doing	Never Did
1. Getting in or out of chairs or bed (without assistance)			
2. Listening to the radio			
3. Reading books, magazines, or newspapers			
4. Writing (letters, notes)			
5. Working at a desk or table			
6. Standing (for more than 1 minute)			
7. Standing (for more than 5 minutes)			
8. Dressing or undressing (without assistance)			
9. Getting clothes from drawers or closets			
10. Getting in or out of a car (without assistance)			
11. Dining at a restaurant			
12. Playing cards/table games			
13. Taking a bath (no assistance needed)			
14. Putting on shoes, stockings, or socks (no rest or break needed)			
15. Attending a movie, play, church event, or sports activity			
16. Walking 30 yards (27 meters)			
17. Walking 30 yards (nonstop)			
18. Dressing/undressing (no rest or break needed)			
19. Using public transportation or driving a car (99 miles or less)			
20. Using public transportation or driving a car (100 miles or more)			
21. Cooking your own meals			
22. Washing or drying dishes			
23. Putting groceries on shelves			
24. Ironing or folding clothes			
25. Dusting/polishing furniture or polishing a car			
26. Showering			
27. Climbing 6 steps			
28. Climbing 6 steps (nonstop)			
29. Climbing 9 steps			
30. Climbing 12 steps			
31. Walking ½ block on level ground			
32. Walking ½ block on level ground (nonstop)			
33. Making a bed (not changing sheets)			
34. Cleaning windows			
35. Kneeling, squatting to do light work			
36. Carrying a light load of groceries			
37. Climbing 9 steps (nonstop)			
38. Climbing 12 steps (nonstop)			
39. Walking ½ block uphill			
40. Walking ½ block uphill (nonstop)			
41. Shopping (by yourself)			
42. Washing clothes (by yourself)			
43. Walking 1 block on level ground			
44. Walking 2 blocks on level ground			

	Still Doing	Stopped Doing	Never Did
45. Walking 1 block on level ground (nonstop)			
46. Walking 2 blocks on level ground (nonstop)			
47. Scrubbing (floors, walls, or cars)			
48. Making a bed (changing sheets)			
49. Sweeping			
50. Sweeping (5 minutes nonstop)			
51. Carrying a large suitcase or bowling (one game)			
52. Vacuuming carpets			
53. Vacuuming carpets (5 minutes nonstop)			
54. Painting (interior/exterior)			
55. Walking 6 blocks on level ground			
56. Walking 6 blocks on level ground (nonstop)			
57. Carrying out the garbage			
58. Carrying a heavy load of groceries			
59. Climbing 24 steps			
60. Climbing 36 steps			
61. Climbing 24 steps (nonstop)			
62. Climbing 36 steps (nonstop)			
63. Walking 1 mile			
64. Walking 1 mile (nonstop)			
65. Running 110 yards (100 meters) or playing softball/baseball			
66. Dancing (social)			
67. Doing calisthenics or aerobic dancing (5 minutes nonstop)			
68. Mowing the lawn (power mower but not a riding mower)			
69. Walking 2 miles			
70. Walking 2 miles (nonstop)			
71. Climbing 50 steps (2 ½ floors)			
72. Shoveling, digging, or spading			
73. Shoveling, digging, or spading (5 minutes nonstop)			
74. Climbing 50 steps (nonstop)			
75. Walking 3 miles or golfing 18 holes without a riding cart			
76. Walking 3 miles (nonstop)			
77. Swimming 25 yards			
78. Swimming 25 yards (nonstop)			
79. Bicycling 1 mile			
80. Bicycling 2 miles			
81. Bicycling 1 mile (nonstop)			
82. Bicycling 2 miles (nonstop)			
83. Running or jogging ¼ mile			
84. Running or jogging ½ mile			
85. Playing tennis or racquetball			
86. Playing basketball/soccer (game play)			
87. Running or jogging ¼ mile (nonstop)			
88. Running or jogging ½ mile (nonstop)			
89. Running or jogging 1 mile			
90. Running or jogging 2 miles			
91. Running or jogging 3 miles			
92. Running or jogging 1 mile in 12 minutes or less			
93. Running or jogging 2 miles in 20 minutes or less			
94. Running or jogging 3 miles in 30 minutes or less			

Fix, A.J., and D.M. Daughton, *Human Activity Profile Professional Manual*. Odessa, Fla: Pyscholollgical Assessment Resources, Inc; 1988.

APPENDIX 7: Immunosuppressive Medication Questionnaire

This form should be completed by the patients RN, NP, or MD

Date of Assessment: _____

Current Daily Tacrolimus Dose_____mg

List any other medication administered for immunosupression within the past 30 days

Drug_____ Daily Dose_____

Start Date_____ Stop Date_____

Drug_____ Daily Dose_____

Start Date_____ Stop Date_____

Drug_____ Daily Dose_____

Start Date_____ Stop Date_____

Add additional lines below as needed

Printed Name_____ Signature_____